Proceedings of the Ninth International Symposium
June 3-7, 2007, Tradewinds Resort & Conference Center
St. Pete Beach, Florida

Recent Advances in Otitis Media

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International Symposium on Otitis Media, Inc.
University of Pittsburgh School of Medicine, Center for Continuing Education in the Health Sciences and
Department of Otolaryngology

Chronology of the Symposia

International Symposium on Recent Advances in Otitis Media with Effusion

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1st Symposium   **May 29-31, 1975, Columbus, Ohio**
Program Committee: Ben H. Senturia, MD
        Charles D. Bluestone, MD
        David J. Lim, MD
Guest of Honor: Ben H. Senturia, MD

2nd Symposium   **May 9-11, 1979, Columbus, Ohio**
Program Committee: Charles D. Bluestone, MD
        David J. Lim, MD
        Ben H. Senturia, MD
Guest of Honor: Sven Inglested, MD

3rd Symposium   **May 17-20, 1983, Bahia Mar, Ft. Lauderdale, Florida**
Program Committee: Charles D. Bluestone, MD
        Jerome O. Klein, MD
        David J. Lim, MD
        John D. Nelson, MD
Guest of Honor: Gunnar D. Proud, MD

1st Extraordinary International Symposium on Recent Advances in Otitis Media with Effusion

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        January 13-15, 1985, Kyoto, Japan
Symposium Directors: Tadami Kumazawa, MD
        Kazutomo Kawamoto, MD
Program Committee
Chairman: Goro Mogi, MD
Co-Chairman: Iwao Honjo, MD
        Tetsuo Ishii, MD

International Symposium on Recent Advances in Otitis Media

4th Symposium   **June 1-4, 1987, Bal Harbour, Florida**
Program Committee: David J. Lim, MD
        Charles D. Bluestone, MD
        Jerome O. Klein, MD
        John D. Nelson, MD
Guest of Honor: David J. Lim, MD

International Symposium on Recent Advances in Otitis Media

5th Symposium   **May 20-24, 1991, Ft. Lauderdale, Florida**
Program Committee: David J. Lim, MD
Charles D. Bluestone, MD
Jerome O. Klein, MD
John D. Nelson, MD
Pearay L. Ogra, MD
Guest of Honor: Tadami Kumazawa, MD

2nd Extraordinary International Symposium on Recent Advances in Otitis Media with Effusion

        March 31-April 3, 1993, Oita, Japan
Symposium Director: Goro Mogi, MD
Program Committee: Iwao Honjo, MD
Tetsuo Ishii, MD
Tomonori Takasaka, MD
Guest of Honor: David J. Lim, MD

International Symposium on Recent Advances in Otitis Media

6th Symposium   **June 4-8, 1995, Ft. Lauderdale, Florida**
Program Committee: David J. Lim, MD
Charles D. Bluestone, MD
Margaretha Casselbrant, MD, PhD
Jerome O. Klein, MD
Pearay L. Ogra, MD
Guest of Honor: Goro Mogi, MD

3nd Extraordinary International Symposium on Recent Advances in Otitis Media with Effusion

        June 1-5, 1997, Copenhagen, Denmark
Symposium Director: Mirko Tos, MD
Program Committee: Viggo Balle, MD
Jens Thomspon, MD
Mirko Tos, MD
Guest of Honor: Charles D. Bluestone, MD

International Symposium on Recent Advances in Otitis Media

7th Symposium	**June 1-5, 1999, Ft. Lauderdale, Florida**

7th **Symposium**   **June 1-5, 1999, Ft. Lauderdale, Florida**

Symposium Director: David J. Lim, MD
Symposium Co-Directors: Charles D. Bluestone, MD
Program Chair: Margaretha Casselbrant, MD, PhD
Program Committee: Lauren O. Bakaletz, PhD
Thomas F. DeMaria, MD
William J. Doyle, MD
G. Scott Giebink, MD
Jerome O. Klein, MD
Pearay L. Ogra, MD
Guest of Honor: Mirko Tos, MD, DMSc
Distinguished Service Marion P. Downs, DHS
Award:

4th Extraordinary International Symposium on Recent Advances in Otitis Media with Effusion

April 16-20, 2001, Sendai, Japan

President: Tomonori Takasaka, MD, PhD
Vice President: Ryo Yuasa, MD
Honorary Presidents: Kazutomo Kawamoto, MD
Tadami Kumazawa, MD
Goro Mogi, MD
Mirko Tos, MD
Secretary General: Koji Hozawa, MD
Guest of Honor: Michael M. Paparella, MD

International Symposium on Recent Advances in Otitis Media

8th Symposium   **June 3-7, 2003, Ft. Lauderdale, Florida**

Symposium Director: David J. Lim, MD
Symposium Co-Directors: Charles D. Bluestone, MD
Margaretha Casselbrant, MD, PhD
Program Committee: Lauren O. Bakaletz, PhD
G. Scott Giebink, MD
Jerome O. Klein, MD
Pearay L. Ogra, MD

5th Extraordinary International Symposium on Recent Advances in Otitis Media with Effusion

April 24-27, 2005, Amsterdam, The Netherlands

President: Anne Schilder
Vice President: Gerhard Zielhuis
Organizing Committee: Frank van Balen
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                               Jo Curfs
                               Erwin Dunnebier
                               Nelly van Eden
                               Kees Graamans
                               Niels van Herbeek
                               Gerrit Jan Hordijk
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Preface
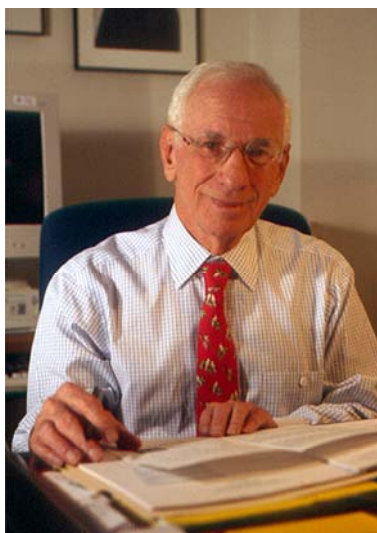
David J. Lim   Charles D. Bluestone   Margaretha L. Casselbrant

It is hard to believe that it is already the 9th International Symposium on Recent Advances in Otitis Media. We held our first Symposium in 1975 in Columbus, Ohio, and since then have had another in Columbus and six more quadrennial Symposia in Florida. In addition, we have held five outstanding Extraordinary Symposia, three in Japan and one each in Denmark and The Netherlands, held in between the ones in the United States. We are also pleased to inform you that the 6th Extraordinary Symposia will be held in Seoul, South Korea, in 2009 organized by Keehyun Park.

We are especially gratified with the present Symposium: we have many faithful attendees who have been at the nine meetings, a host of other committed clinicians and scientists who have attended several of our meetings, and still others who are with us for the first time. We are especially delighted to have attracted many young investigators to this Symposium who have made otitis media one of their major research interests; many have presented their findings at this Symposium. At this Symposium, we had over 255 participants from 25 countries and over 200 clinicians and scientists are participating in 109 oral presentations and 81 posters. In this symposium, we had special lectures from leaders in the field, lunchtime presentations, and several mini-symposia. Also, we had experts in the field discussing some of the most important controversial aspects in the "Great Debates." Subjects covered included areas of epidemiology, diagnosis, pathogenesis, as well as the microbiologic, immunologic, and biochemical aspects of middle-ear disease. In addition, we covered genetics, molecular biology, management (including the latest in vaccines for prevention), and the complications and sequelae of otitis media. We also discussed how expanding animal models of otitis media, particularly mice, became an important laboratory research tool.

We acknowledge the contributions made by our Organizing Committee, Lauren O. Bakaletz, Jerome O. Klein, Jian-Dong Li, and Pearay Ogra. We are also indebted to our Program Committee, chaired by the indispensable Richard M. Rosenfeld and his able committee members Cuneyt Alper, Lauren Bakaletz, Steve Barenkamp, Tasnee Chonmaitree, Kathy Daly, Joseph Kerschner, Jian-Dong Li, Steve Pelton, and Allen Ryan; each helped make this Symposium the state-of-knowledge for otitis media and brought us the leading experts in the field to present their cutting-edge research. We also want to acknowledge our International Advisors (listed in this Proceedings Book) who helped ensure the success of this Symposium.

David J. Lim, Symposium Director
Charles D. Bluestone, Symposium Co-Director
Margaretha L. Casselbrant, Symposium Co-Director

Special Welcome

I am very pleased to be part of your symposium and I want to thank Dr. Bluestone, Dr. Casselbrant, and Dr. Lim for their untiring efforts and leadership in making this 9th Symposium on Recent Advances in Otitis Media a reality. To further the importance of this meeting, I would be remiss not to acknowledge the attendance of Dr. Jim Battey, Director of the National Institutes on Deafness and Other Communication Disorders, National Institutes of Health, at your symposium. The first symposium was held 32 years ago! Since that time there have been nine symposiums, four extraordinary symposia, and a symposium planned for 2009 in Korea. That is simply a remarkable achievement attested by the fact that more than 255 participants from 25 countries are here for the symposium and more than 200 clinicians and scientists are participating in 109 oral presentations and 81 posters. Probably of equal importance is getting together over coffee and in the hallway to discuss work that is being done to advance many aspects of the science of otitis media including prevention and evidence-based treatment of otitis media. The House Ear Institute is honored to be part of your symposium.

James D. Boswell, Ph.D.
Chief Executive Officer
House Ear Institute

Special Remarks

Thank you for the kind introduction. I would like to begin by thanking the organizers for all their efforts; meetings like this do not happen by accident and they take a lot of effort. I have been involved in the planning of several meetings myself and I know how much work it is. We owe a debt of gratitude to the House Ear Institute and the University of Pittsburgh for their institutional support to have this meeting. Without such support, it is not easy, if not impossible, to have such a meeting.

Everyone in the audience well knows that otitis media is the most common reason that a young sick child will come to see a physician. It is estimated to be a five billion dollar a year health problem. For this reason, it is a high priority for research support by the National Institute on Deafness and Other Communication Disorders, and why it will go on being a very high in priority for the Institute.

I am looking forward to seeing the wonderful showcase of scientific progress that will take place over the next four days in this beautiful venue here in South Florida, where I have come so many times for the ARO meeting. It is great to be here and I am looking forward to seeing the remarkable progress you have made in the last four years.

Again, I thank you all for being here.

Jim Battey, MD, PhD
Director, National Institute on Deafness and Other Communications Disorders
National Institutes of Health
Bethesda, Maryland

In Memoriam
Lars-Eric Stenfors, MD, PhD

Professor Lars-Eric Stenfors**,** Department of Otolaryngology of Tromsø University, died of melanoma on December 10, 2004, in Kokkola at the age of 63. He was born on October 3, 1941, in Närpiö.

Lars-Eric completed undergraduate study in 1961, and completed his medical education at the University of Aarhus in Denmark in 1971. He obtained medical licensure in Norway in 1971 and for Denmark and Finland in 1972. He received ear, nose, and throat specialist qualifications in 1977.

In 1980, Lars-Eric defended his thesis on "Structure and pattern of the motion of the tympanic membrane with special reference to the experimental perforations and subsequent healing processes" at Umeå University.

His lifelong research has been the pathogenesis of otitis media, particularly the bacteriology of otitis media, and he has written extensively on this topic. He became a well-recognized world leader in this field of science. He was a member of the St. Petersburg Academy of Sciences.

As a lifelong friend of the Otitis Media Symposium, he made many important contributions, serving as an international advisor and as a member of many post-symposium research conferences.

After he had become Umeå University associate professor of ear disease in 1982, he served as an ENT specialist in the Central Hospital of Osterbothnia at Kokkola, and became the medical superintendent. In 1989, he became a professor at the University of Tromsø, a position he held until his death.

Lars-Eric's scientific activity is reflected in the fact that he organized a series of joint Nordic Tromsø scientific ear, nose, and throat disease seminars attended by many distinguished scientists and physicians. One of these seminars was arranged at the exotic Svalbard.

Lars-Eric was an easily approachable consensus builder and scientific leader who successfully combined basic and clinical research. He was a mentor to several doctoral students in Umeå University and the University of Tromsø. He was an energetic and popular teacher among students. His humor was often accompanied by scathing satire.

He is survived by his beloved wife Asta and their sons Alexis, Nicolai, and Anton and their families.

Original obituary written in Swedish by Sten Hellström, Ove Söderberg, and Herman Diaman, and modified by David Lim and Sten Hellström.

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Molecular basis of *Streptococcus pneumoniae* and *Haemophilus influenzae* infections

Jeffrey Weiser, M.D.

Both *Streptococcus pneumoniae* and *Haemophilus influenzae* colonize the mucosal surface of the upper respiratory tract of their human host. Although otherwise diverse species, they share many common mechanisms to promote their colonization and, as a result, are often associated with a similar pattern of disease. This talk will discuss some aspects of their pathogenesis from *in vitro* studies and the use of animal models. Both species, for example, express a protease with specificity for human IgA1--the most abundant immunoglobulin type on the mucosal surface. Both species put phosphorylcholine on their surface, a molecule that allows for binding to a common receptor that also makes the organisms susceptible to innate immunity through recognition by C-reactive protein. Both species are also recognized by the same pattern recognition receptors, TLR2 and TLR4, and cause the activation of p38 MAP kinase. Despite their similarities, these species appear to compete *in vivo* through the differences in the responses they induce in their host.

Role of antimicrobial innate immune molecules in otitis media pathogenesis

David J. Lim, M.D.

The middle ear cavity of normal human and laboratory animals is believed to be sterile. How the middle ear cavity, lined only with a thin mucous membrane and with a poorly developed immune system can so effectively ward off infection and remain sterile is not clearly understood. The protection of the nasopharyngeal tract, E-tube and the middle ear is maintained in part by anti-microbial innate immune molecules (AIIMs) whose function is to defend mucosal surfaces against microbial invasion. Among these molecules are the defensins, lysozyme (Lz), lactoferrin and members of the collectin family, including the surfactant proteins.

We demonstrated that the AIIMs such as beta-defensins 1 and 2 and a novel member of beta defensin EP2E, and Lz are expressed in the tubotympanum and they were effective against NTHi, *M. catarrhalis* and *S. pneumoniae* (Lim et al, 2000; Lee et al., 2004; Cha et al. 2007, Abstract #326). We further demonstrated that Lz and β -defensin2 could act synergistically against *S. pneumoniae*. Using lysozyme-M knockout mice, we were able to demonstrate the Lz-M deficient animals showed greater susceptibility to S. pneumonia-induced otitis media (Shimada et al., 2007, Abstract #302).

We also showed that beta-defensin 2 was up-regulated during OM and that the transcriptional up-regulation of this molecule by IL-1a was mediated mainly via the Src-dependent Raf-MEK1/2-ERK signaling pathway (Moon et al., 2002). NTHi up-regulates beta-defensin 2 via activation of the p38, but not MAPK ERK signaling pathway. Moreover, we have demonstrated that IL-1 and NTHi act synergistically to up-regulate beta-defensin 2 expression (Moon et al, 2006). We also demonstrated that induction of beta-defensin 2 by NTHi requires the TLR2 mediated MyD88-IRAK1-TRAF6-MKK3/6-p38MAPK signaling pathway, which is different from the IL-1a-induced beta-defensin 2 pathway (Lee et al., 2007, Abstract #188). In addition, recent evidence from our laboratory indicates that NOD2-dependent NF-kB activation is involved in NITHi-induced beta-defensin 2 (DEFB4) upregulation via the TLR2-independent signaling pathway (Moon et al., 2007, Abstract #295).

Advances in our understanding of role of innate immunity in otitis media and the cell signaling pathways involved in the regulation of antimicrobial innate immune molecules will help in the identification of molecules effective against the three OM pathogens and open up new avenues for the treatment of OM based on the use of innate immune molecules.

Regulation of mucin in respiratory tract

Reen Wu, Ph.D., Daphne C. Y. Wu, Artem Loukoianov, M.D., Yin Chen, Ph.D., Philip Thai, Ph.D., Mary M. J. Chang, Ph.D.

Airway mucus overproduction and epithelial mucous cell hyperplasia/metaplasia are major hallmarks associated with various airway diseases, such as chronic obstructive pulmonary disease, cystic fibrosis, and asthma. Gel-forming mucin genes, MUC5B and MUC5AC, are important components of the lung lining fluid that participate in maintaining the visco-elasticity of airway mucus. The expression of these gel-forming mucin genes, including the recently found MUC19 gene, are elevated in diseased airways. The nature of the regulation is still incompletely understood.

In the normal human airway, MUC5AC is mainly expressed by surface goblet epithelial cells, while MUC5B and MUC19 are predominantly expressed by the mucous cells of submucosal glands. However, in contrast to a restricted MUC5AC expression, MUC5B products could be demonstrated in the surface epithelium of diseased airways. Our lab has shown IL-17, rather than the Th2 cytokines, is the most potent inducer for MUC5AC and MUC5B gene expression, while MUC19 expression was stimulated by Th2 cytokines. In addition, we also showed different signaling pathways are involved in the regulation of MUC5AC and MUC5B expression by phorbol 12-mystate 13-acetate (PMA), a PKC activator. Further studies have identified both cis- and trans-activating elements in the regulation of the promoter activity of these mucin genes. Lastly, we have shown a persistent elevation of mucin production in smokers' and ex-smokers' airways. This persistence is perhaps through an epigenetic mechanism relevant to DNA methylation. These studies have shown that the expression of airway mucin genes is regulated by various cellular responses to the environmental pollutants and inflammatory stimuli.

Regulation of inflammation in otitis media

Jian-Dong Li, M.D., Ph.D.

Nontypeable *Haemophilus influenzae* (NTHi), a Gram-negative bacterium, is an important human pathogen in both adults and children.[1] In adults, it exacerbates chronic obstructive pulmonary diseases, and in children, it causes otitis media, the most common childhood infection and the leading cause of conductive hearing loss.[2-4] Despite the need for prophylactic measures, development of a vaccine for preventing NTHi infections has been difficult and still remains a great challenge. Moreover, inappropriate antibiotic treatment contributes to the worldwide emergence of antibiotic-resistant strains of NTHi. Therefore, there is an urgent need for developing alternative therapeutic strategies for the treatment of NTHi infections based on understanding the molecular pathogenesis of NTHi infections. Like most other bacterial infections, NTHi infection is characterized by inflammation, which is mainly mediated by nuclear factor kappaB (NF-κB)-dependent production of pro-inflammatory mediators.[5-8] We have previously shown that NTHi induces Toll-like receptor (TLR) 2-dependent activation of NF-κB via an IKKβ-IκBα- and p38 mitogen-activated protein kinase (MAPK)-dependent signaling pathway.[6,7,10] However, the key signaling adaptors that link TLR2 with IKK and MAPK in mediating NTHi-induced inflammation remain unknown.

Although the inflammatory response triggered by bacteria is essential for eradicating bacterial pathogens, excessive inflammatory response is clearly detrimental to the host, due to severe tissue damage.[11,12] To avoid an overactive and detrimental inflammatory response in infectious disease, the bacteria-induced inflammatory response must be tightly regulated. During evolution, the host has developed a variety of strategies to prevent a detrimental inflammatory response during bacterial infections. Among all strategies, bacteria-induced negative feedback regulation is thought to play a critical role in preventing an overactive inflammatory response by tightly regulating the activity of the key receptor-dependent signaling adaptors specifically activated by bacterial pathogens. In the pathogenesis of chronic obstructive pulmonary diseases and otitis media, to avoid detrimental inflammatory responses in NTHi infection, TLR2 signaling must be tightly regulated. However, despite the importance of tight regulation in preventing an overactive inflammatory

response, the molecular mechanisms underlying the negative feedback regulation of inflammation in the pathogenesis of NTHi infection remain unknown.

CYLD was originally identified as a tumor suppressor, loss of which causes a benign human syndrome called cylindromatosis.[13-15] In vitro studies have indicated that CYLD is a member of the deubiquitinating enzyme family that specifically digests polyubiquitin chains. Transfection studies showed that CYLD deubiquitinates TRAF2 and TRAF6 and acts as a negative regulator for activation of NF-κB by tumor necrosis factor receptor (TNFR) and TLR.[16-18] Recently, we, together with others, showed that CYLD also negatively regulates activation of MAPKs, including p38 MAPK.[19-22] Moreover, the expression of CYLD is itself under the control of NF-κB[20,23], suggesting that CYLD is involved in a negative feedback regulation of NF-κB activation and NF-κB-dependent gene expression. Given the important role that NF-κB plays in host immune and inflammatory response in bacteria infection, it is logical to hypothesize that CYLD may act as a negative regulator for immune and inflammatory response against invading bacteria such as NTHi in vivo. However, despite recent studies demonstrating the role of CYLD in regulating T-cell receptor signaling and tumor cell proliferation in vivo, the biological role of CYLD, especially its negative role in inflammation in vivo, still remains unknown.

In the present study, by using an NTHi-induced otitis media and pneumonia model in wild type (wt) and CYLD-deficient mice, we provide *in vivo* evidence that NTHi induces pro-inflammatory response through TLR2-dependent MyD88-TRAF6/7-NF-κB signaling pathway, and CYLD negatively regulates NF-κB-dependent inflammatory response by NTHi via deubiquitination of TRAF6 and TRAF7. These studies may lead to development of novel therapeutic strategies for controlling an overactive inflammatory response in respiratory bacterial infections.

References

1. Foxwell AR, Kyd JM, Cripps AW. Nontypeable Haemophilus influenzae: pathogenesis and prevention. Microbiol Mol Biol Rev 1998; 62:294–308.
2. Rao VK, Krasan GP, Hendrixson DR, Dawid S, St Geme JW 3rd. Molecular determinants of the pathogenesis of disease due to non-typable Haemophilus influenzae. FEMS Microbiol Rev 1999; 23:99–129.
3. Sethi S, Murphy TF. Chronic obstructive pulmonary disease. N Engl J Med 2000; 343: 1969–1970.
4. Murphy TF. Haemophilus influenzae in chronic bronchitis. Semin Respir Infect 2000; 15: 41–51.
5. Murphy TF. The role of bacteria in airway inflammation in exacerbations of chronic obstructive pulmonary disease. Curr Opin Infect Dis 2006; 19:225–230.
6. Watanabe T, Jono H, Han J, Lim DJ, Li JD. Synergistic activation of NF-kappaB by nontypeable Haemophilus influenzae and tumor necrosis factor alpha. Proc Natl Acad Sci USA 2004; 101:3563–3568.
7. Hayden MS, Ghosh S. Signaling to NF-kappaB. Genes Dev 2004; 18:2195–2224.
8. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. Cell 2004; 109:SupplS81–96.
9. Shuto T, Xu H, Wang B, Han J, Kai H, et al. Activation of NF-kappa B by nontypeable Hemophilus influenzae is mediated by toll-like receptor 2-TAK1-dependent NIK-IKK alpha/beta-I kappa B alpha and MKK3/6-p38 MAP kinase signaling pathways in epithelial cells. Proc Natl Acad Sci USA 2001; 98:8774–8779.
10. Imasato A, Desbois-Mouthon C, Han J, et al. Inhibition of p38 MAPK by glucocorticoids via induction of MAPK phosphatase-1 enhances nontypeable Haemophilus influenzae-induced expression of toll-like receptor 2. J Biol Chem 2002; 277:47444–47450.
11. Ulevitch RJ, Tobias PS. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu Rev Immunol 1995; 13:437–457.
12. Kurt-Jones EA, Chan M, Zhou S, et al. Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. Proc Natl Acad Sci USA 2004; 101:1315–1320.
13. Bignell GR, Warren W, Seal S, et al. Identification of the familial cylindromatosis tumour-suppressor gene. Nat Genet 2000; 25:160–165.
14. Leonard N, Chaggar R, Jones C, et al. Loss of heterozygosity at cylindromatosis gene locus, CYLD, in sporadic skin adnexal tumours. J Clin Pathol 2001; 54:689–692.
15. Poblete Gutierrez P, Eggermann T, Holler D, et al. Phenotype diversity in familial cylindromatosis: a frameshift mutation in the tumor suppressor gene CYLD underlies different tumors of skin appendages. J Invest Dermatol 2002; 119:527–531.
16. Trompouki E, Hatzivassiliou E, Tsichritzis T, et al. CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. Nature 2003; 424:793–796.
17. Brummelkamp TR, Nijman SM, Dirac AM, Bernards R. Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. Nature 2003; 424:797–801.
18. Kovalenko A, Chable-Bessia C, Cantarella G, et al. The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. Nature 2003; 424:801–805.
19. Reiley W, Zhang M, Sun SC. Negative regulation of JNK signaling by the tumor suppressor CYLD. J Biol Chem 2004; 279:55161–55167.
20. Yoshida H, Jono H, Kai H, Li JD. The tumor suppressor cylindromatosis (CYLD) acts as a negative regulator for toll-like receptor 2 signaling via negative cross-talk with TRAF6 AND TRAF7. J Biol Chem 2004; 280:41111–41121.
21. Zhang J, Stirling B, Temmerman ST, et al. Impaired regulation of NF-kappaB and increased susceptibility to colitis-associated tumorigenesis in CYLD-deficient mice. J Clin Invest 2006; 116:3042–3049.
22. Reiley WW, Jin W, Lee AJ, et al. Deubiquitinating enzyme CYLD negatively regulates the ubiquitin-dependent kinase Tak1 and prevents abnormal T cell responses. J Exp Med 2007; 204:1475–1485.
23. Jono H, Lim JH, Chen LF, et al. NF-kappaB is essential for induction of CYLD, the negative regulator of NF-kappaB: evidence for a novel inducible autoregulatory feedback pathway. J Biol Chem 2004; 279:36171–36174.

Middle ear mucosal hyperplasia and recovery in otitis media

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Introduction

Mucosal hyperplasia is a hallmark of otitis media (OM) pathogenesis, and is a unique property of the middle-ear (ME) mucosa. The mucosa can rapidly transform from a simple squamous epithelium with vestigial stroma to a full-thickness, respiratory epithelium featuring ciliated and secretory cells, and an active, well-vascularized, subepithelial connective tissue layer.[1, 2, 3] The mechanisms that regulate ME tissue growth and differentiation during OM are not well understood, but the inhibition of mucosal hyperplasia could help to limit the extent of this disease.

The mucosa of the ME also recovers to its resting state after the resolution of OM, often with no, or only a few, residual changes. This recovery process is orderly, without extensive necrosis or sloughing of mucosal cells into the ME lumen. It therefore presumably involves active processes of cell death and resorption of apoptotic cells. The details of how this occurs are also unknown. However, enhancement of recovery represents another potential treatment for OM.

Before either mucosal hyperplasia or recovery can be utilized as therapeutic targets, we must understand how they occur. In particular, the signal transduction pathways that link extracellular ligands and their cellular receptors to intracellular events should be identified.

Materials and methods

OM was induced in rats or mice by ME inoculation of 0.5×10^5 nontypeable *Haemophilus influenzae* (NTHi) into the tympanic bulla, using a ventral approach. Animals were allowed to survive for periods ranging from hours to weeks. The extent of mucosal hyperplasia was then evaluated histologically. Whole-genome gene arrays, quantitative polymerase chain reaction (qPCR) and protein blotting were used to document the expression of ligands, receptors, and signaling genes during the course of OM. The activation of signaling pathways was also documented via protein kinase assays. Inhibition and activation of these pathways was used *in vitro* and *in vivo*, to explore their influence on the ME mucosa. Finally, mice with defined genetic lesions were used to further evaluate the contributions of receptors and signaling molecules that might contribute to mucosal growth and recovery.

Results

Gene array and qPCR expression data indicated that mRNAs encoding a variety of receptors and endogenous ligands are upregulated in OM. This included a number of Toll-like receptors (TLRs) and Nod-like receptors (NLRs) that mediate innate immune responses to pathogens, as well as growth factors (GFs) and GF receptors (GFRs) that can influence cell proliferation. Moreover, activation of GFRs *in vitro* and *in vivo* led to proliferation of the mucosa, implicating these systems in hyperplasia. Application of GFs that influence stromal cells, such as fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF) resulted in the proliferation of subepithelial fibroblasts and blood vessels, respectively. As expected, stromal growth factors had no effect on ME epithelial cells. In contrast, some epithelial growth factors, including epidermal growth factor (EGF) and heregulin, increased the growth of mucosal epithelial cells.

These and other receptors in turn activate signaling cascades that depend upon the binding or post-translational modification of signaling proteins, leading to cellular responses. Substantial expression in gene networks related to such cell signaling cascades, as well as to downstream events including cell division and differentiation, occurred early in OM, with a time course related to mucosal hyperplasia. Moreover, a family of signaling molecules, the mitogen-activated protein kinases (MAPKs) was found to be strongly activated during OM, as evidenced by phosphorylation. This included the ERK, P38, and JNK MAPKs. However, only activation of the JNK MAPK was kinetically related to the growth and recovery of the ME mucosa. In addition, only JNK inhibition reduced mucosal growth *in vivo*. Interestingly, mice deficient in TLRs 2 or 4, or the TLR signaling adaptor MyD88, displayed the same

initial mucosal hyperplasia seen in wild-type mice, suggesting that hyperplasia is not dependent upon these innate immune responses.

With respect to recovery from mucosal hyperplasia, gene array and PCR data indicated that genes encoding death ligands and receptors were upregulated late in OM, as were apoptosis signaling and effector molecules such as caspases. Activation of caspase 3 was also observed during experimental OM, and a mutation in the Fas death receptor delayed recovery from OM, further supporting the involvement of apoptosis in recovery from this disease.

Discussion

The data reviewed above suggest that activation of TLRs probably does not mediate the initial ME mucosal growth and differentiation induced by NTHi. Other sources of inflammation must trigger this response. On the other hand, binding of GFs to their receptors and activation of downstream signaling appears to be very important to mucosal hyperplasia, with separate factors participating in epithelial and stromal growth.[4,5,6] In particular, the JNK MAPK may play a critical role in regulating mucosal proliferation, as it does in several forms of cancer.[3]

TLR signaling is required for recovery from hyperplasia and OM, but this seems more likely to be due to its role in the resolution of infection rather than to a direct effect. The clearance of mucosal cells during OM recovery appears to involve apoptosis,[7] which presumably reduces the release of inflammatory products during the cell degradation process.

Acknowledgements

References

1. Lim DJ. Functional morphology of the lining membrane of the middle ear and Eustachian tube. Ann Otol Rhinol Laryngol 1974; 83:1-22.
2. Lim, DJ, Birck H. Ultrastructural pathology of the middle ear mucosa in serous otitis media. Ann Otol Rhinol Laryngol 1971; 80:838-853.
3. Furukawa M, Ebmeyer J, Pak K, Austin DA, Webster NJG, Ryan AF. Regulation of bacterial otitis media-induced mucosal hyperplasia in rat middle ear epithelium by JNK. Infect Immun 2007; 75:2562-2571.
4. Palacios SD, Kayali AG, Rivkin AZ, et al. Participation of Ras and extracellular regulated kinase in the hyperplastic response of middle-ear mucosa during bacterial otitis media. J Infect Dis 2002; 186:1761-1769.
5. Palacios SD, Pak K, Rivkin AZ, Bennett T, Ryan AF. Growth factors and their receptors in the middle ear mucosa during otitis media. Laryngoscope 2002; 112:420-423.
6. Palacios SD, Pak K, Rivkin AZ, et al. Role of p38-Mitogen-Activated Protein Kinase in middle ear mucosa hyperplasia during bacterial otitis media. Infect Immun 2004; 72: 4662-4667.
7. Palacios S, Rivkin S, Pak K, Ryan AF. The role of *Fas*–mediated apoptosis in otitis media: observations in the *lpr/lpr* mouse. Hearing Res 2005; 207:110-116.

Top-down bottom-up phylogenic approach for early stage vaccine development

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Diversifying immunological selection by the host is an impediment to the development of protein-based vaccines. A consequence of such selective pressure is intra-species epitope variability commonly found for major outer membrane proteins (OMPs) of *Haemophilus influenzae* and surface-exposed proteins (SEPs) of *Streptococcus pneumoniae*. Recombination-based lateral (horizontal) gene transfer (Fig. 1) is the primary genetic mechanism responsible for this phenomenon.[1-4] As epitope conservation is a requisite of a vaccine candidate, variability renders such proteins unsuitable for vaccine development. The top-down bottom-up phylogenic strategy described here provides a means to directly screen for this problem, eliminating such unsuitable candidates at an early stage in preclinical vaccine development.

New approach to an old problem

The preclinical vaccine development literature of the past 35 years is filled with numerous reports of promising vaccine candidates protective in homologous challenge animal trials that subsequently fail in heterologous challenge due to variability of the target antigen across species diversity. We directly address this question at an early stage in preclinical vaccine development. This involves a new top-down bottom-up strategy, with initial focus on acquisition of clinical isolate collections followed by their phylogenic organization (see Fig. 2).

Once so organized, population structure correlations relevant to vaccine development become immediately apparent, e.g., as depicted in Fig. 3 for the case of *Haemophilus influenza*. This is true whether strains causing a particular disease are clonal or distributed throughout the population structure, or whether strains causing a particular disease are associated with unique geographical niche(s) or distributed throughout the world.

When is enough, enough?

Constructing a phylogenic dendrogram such as that shown in Fig. 3 has become routine.[2, 5-8] However, for application to vaccine development, we need to understand how many independent isolates must be included in the analysis to be confident that the depicted population structure represents the actual evolutionary diversity of the involved microbial species. Non-linear regression analysis of genetic polymorphisms used to construct such a dendrogram provides an answer to this question. Here, for the case of two of the major pathogens associated with otitis media (*H. influenzae* and *S. pneumoniae)*, a considerable difference in the number of independent isolates required is revealed with such an analysis (see Fig. 4). For these two species, the non-linear regression analysis is in accord with their previously established degree of clonality/panmixia: significantly more polymorphic profiles are predicted for the panmictic *S. pneumoniae* than for the relatively clonal *H. influenzae*.

Top-down bottom-up strategy based on microbial population structure

Dendrogram depiction of population structure allows us to proceed 'top-down' by choosing a manageable phylogenically representative subset to work with from the large dendrogram [1, 2, 5-7, 9, 10] prior to returning to the full collection ('bottom-up') for confirmatory purposes. The representative subset can be used to select new candidate antigens and/or the initial screening of pre-selected candidate antigens. The former is accomplished using microarray and/or q-RT PCR analysis to identify surface-exposed proteins expressed in common in vivo, and by a phylogenically representative subset during nasopharyngeal (NP) colonization and the course of middle-ear (ME) infection. Microarray and qRT-PCR are likewise used to determine whether pre-selected targets are expressed in common by a phylogenically representative subset during animal model studies. In both cases, necessary ex vivo bacteria, e.g., *H. influenzae* or *S. pneumoniae*, can be directly obtained from the animal model NP and ME using multiple consecutive lavage methods.[7] Numbers of isolates in the representative subset used for screening is determined on a practical basis. Where the question involves universality of a target within a

species' evolutionary diversity, rapid inexpensive hybridization allows the complete collection to be analyzed. Where sequence conservation of target-encoding genes is the question, analysis of a relatively large representative subset of isolates is feasible (Fig. 5), as the cost for DNA sequencing is not extravagant.[1, 9] The time-intensive and costly nature of microarray analysis of ex vivo isolates from animal models limits this particular screen for in vivo expression to a significantly smaller representative subset. If such an analysis was based on limited qRT-PCR for select candidates rather than broader microarray analysis, a smaller representative subset would likely also be employed because of the time and expense involved with the critical animal model from which ex vivo isolates are acquired.

Those vaccine candidates passing the phylogenic screening tests for universality, conservation of encoding gene, and expression in vivo are then isolated and used to verify surface accessibility and evaluate which antigens and adjuvants elicit functional antibodies before proceeding to large-scale animal studies to fulfill proof of concept. We are currently applying this top-down bottom-up phylogenic approach for early stage vaccine development against both nontypable *H. influenzae* and *S. pneumoniae*.[1, 2, 5-7, 9, 10]

Salient examples of the top-down strategy

(i) Rapidly eliminating unsuitable vaccine candidates subject to diversifying antigenic selection

Multiple studies have suggested that OMPs of *H. influenzae* might be effective vaccine candidates.[11-15] In particular, many reports demonstrated that both polyclonal antibodies and a monoclonal antibody against OMP P1 were protective in passive immunization in the infant rat model of bacteremia caused by serotype b *H. influenzae*[16-18], while active immunization with P1 was also found to induce partial protection against bacteremia in the model.[19]

Rather than resorting to time-consuming, expensive animal studies, our initial strategy for characterization of P1 as an immunogen was based on DNA sequence variability analysis of the encoding gene *ompP1* across the diversity of a phylogenically organized collection of ~500 typeable and nontypable *H. influenzae* isolates (Fig. 5).[1, 9] This DNA-based analysis revealed the precise degree of *ompP1* variability among 42 evolutionarily divergent clinical

isolates of *H. influenzae*. Percent identities at the DNA level were 87.8-100% and at the amino acid level 82.5-100%. Using a 95% cutoff point, when grouped by percentage similarity at the amino acid level, some 23 OMP P1 variants could be resolved. The greatest amount of variability proved to be associated with the surface-exposed regions of OMP P1, suggesting that this protein would not make for an efficacious vaccine candidate (see Fig. 6). Proof-in-principle studies involving homologous and heterologous challenge in the chinchilla model of experimental otitis media were then carried out to demonstrate this.[1, 9]

This population-, biology-based DNA-sequence survey was particularly revealing. It allowed for unique heterologous challenge studies using bacterial strains with known minor and major differences in surface-exposed amino acid residues of the native antigen, and thus the means to test the range of specificity of the P1 immunogen. It also unambiguously demonstrated that: 1) the phylogenic screening of a candidate immunogen provides a rational approach to rapidly eliminating OMP vaccine candidates subject to diversifying antigenic selection, and 2) this can be determined a priori without the necessity for animal testing.

(ii) Ascertaining the universality of a virulence factor and the role it plays in pathogenicity

While a dominant virulence factor of *H. influenzae* type b is its capsule, it remained unknown whether an equivalent essential virulence factor existed for the case of capsule-deficient nontypable *H. influenzae* (NTHi) associated with otitis media. One such candidate was sialic acid, a terminal residue of the lipopolysaccharide core sugars of NTHi. To test this hypothesis for the NTHi per se, we used a phylogenically representative subset of NTHi isolates spanning the evolutionary diversity of strains causing otitis media (Fig. 3). All were obtained by tympanocentesis.[20]

For each epidemiologically distinct isolate, an otherwise isogenic sialic acid-deficient mutant (disrupted sialyltransferase or CMP acetylneuraminic acid synthetase genes) was constructed to test both the wild-type (wt) and sialic acid-deficient mutant using the chinchilla model of experimental otitis media (EOM). All animals infected with wt strains developed acute otitis media while isogenic sialic acid-deficient mutants proved to be profoundly attenuated.[5] As such, these studies demonstrated that sialylated glycoforms play an intrinsic role in the pathogenicity of otitis media-associated NTHi per se. As wt incocula were deliberately grown in the absence of sialic acid, and ex

vivo wt NTHi recovered from the ME were determined by sensitive CE-ESI-MS analysis to be sialylated, these studies also revealed that otitis media-associated NTHi scavenge the essential precursors from the host during the course of infection.[5]

Such species-wide conclusions would not have been possible in the absence of the phylogenically representative subset selection depicted in the top-down strategy shown in Fig. 5. This broad, species-wide perspective on the essential nature of sialylated glycoforms laid the foundation for subsequent follow-up studies revealing a central role for complement in innate immune defenses against NTHi-associated EOM.[10]

Conclusions

The described top-down bottom-up screening strategy (Fig. 5) represents the means to overcome seemingly inherent ambiguities associated with microbes for which there exist vast, constantly evolving populations. For the case of pathogenic species, consequent genetic diversity is further amplified under the pressure of diversifying antigenic selection.

While the foundation for this strategy obviously rests on a phylogenically organized collection of isolates for the species of interest (Fig. 2), it cannot be too strongly emphasized that such a collection must be representative of actual species diversity (Fig. 4). If not, the strategy is likely to fail.

We have constructed relatively large-scale models of the natural population structures of the two major otitis media pathogens, NTHi[1, 2, 5-7, 21] (Fig. 3) and *Streptococcus pneumoniae* (Fig. 7).[22-24] Associated, organized isolate collections are currently being used to carry out screening of potential immunogen vaccine candidates for the essential nature of their encoding genes and for their degree of conservation. Those immunogens encoded by genes demonstrated to be essential for both viability and virulence, while also exhibiting a high degree of conservation across the natural population structure,

are then to be used for animal testing. This thus eliminates a major factor, i.e., antigenic variability, that has typically confounded vaccine development. Involved candidate vaccine targets are all encoded by genes uniquely mapped to regions of the bacterial chromosome that are significantly less subject to the primary force responsible for diversifying antigenic selection[6], i.e., recombinational mutation.[1-4]

Acknowledgements

Figure 1. Recombination-based lateral transfer across a species. Lateral gene transfer under the pressure of diversifying immunogenic selection is depicted by the horizontal connections between vertical lineage branches of the prototypic evolutionary tree.

Figure 2. Phylogenic organization of a bacterial species collection[1, 2, 5-8, 21, 25-27]. Small colored rings at bottom of figure depict a collection of epidemiologically independent clinical bacterial isolates. Upper right portion of figure shows actual ribotype polymorphism for Hi and NTHi isolates. Upper left side of figure shows a dendrogram generated from the ribotype polymorphisms[2, 5-7].
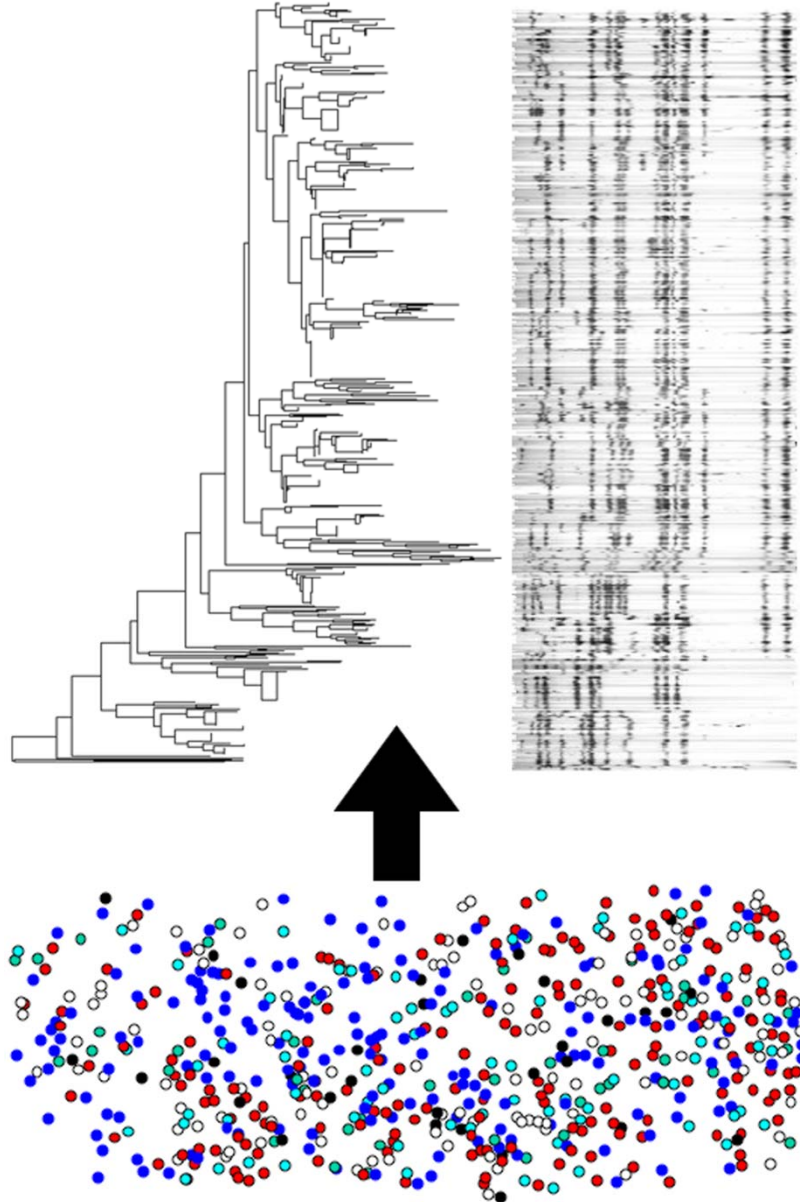
Figure 3. Phylogenic organization of ~600 Hi and NTHi isolates. Adapted from Bouchet et al[5]. Bar code columns reveal correlations of isolate lineage with capsule serotype, disease type, and geographic origin. Independent confirmation of the ribotype-based dendrogram was obtained based on capsular operon gene polymorphism analysis[28] and congruence of a ribotype-based phylogenic tree of a subset of the type a-f isolates with results from multilocus enzyme electrophoresis analysis[29]. Further validation was obtained by comparative gene sequencing of *recA* and 16S ribosomal RNA genes among a representative subset of 50 of the isolates and multilocus sequence typing analysis of a representative set involving 51 of the nontypable isolates.
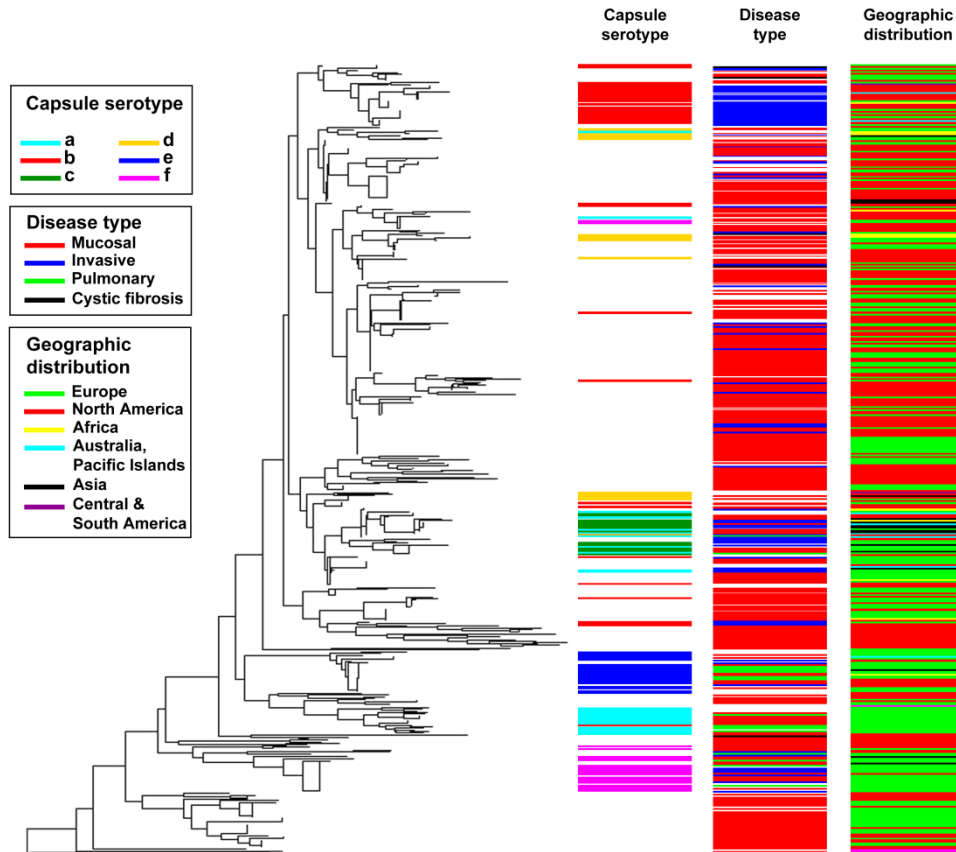
Figure 4. Non-linear regression-based estimation of the number of isolates necessary to analyze phylogenically in order to reach species diversity, modified from[6]. For the case of both *H. influenzae* and *S. pneumoniae*, a randomized list of ribotyped isolates was generated. For each isolate (x-axis), y-value increases by one when the isolate is of a new ribotype profile not previously identified in the randomized set. The resulting plot is depicted in a dark color, with fitted non-linear regression in lighter color. The model used for the non-linear regression is the one phase exponential association function: $y = y_{max}(1-e^{-Kx})$. These actual results reveal that as the data set increases and becomes more representative of species diversity, fewer new clusters appear, and the tangent to the regression curve tends toward slope = 0, at which point it becomes possible to estimate both the number of possible ribotype profiles attainable and the approximate number of isolates required in order to achieve this maximal representation of the diversity of the species. For these two otitis media-associated pathogens, the non-linear regression analysis is in accord with their known population structures, with considerably more polymorphic profiles predicted for the panmictic *S. pneumoniae* than for the relatively clonal *H. influenzae*.
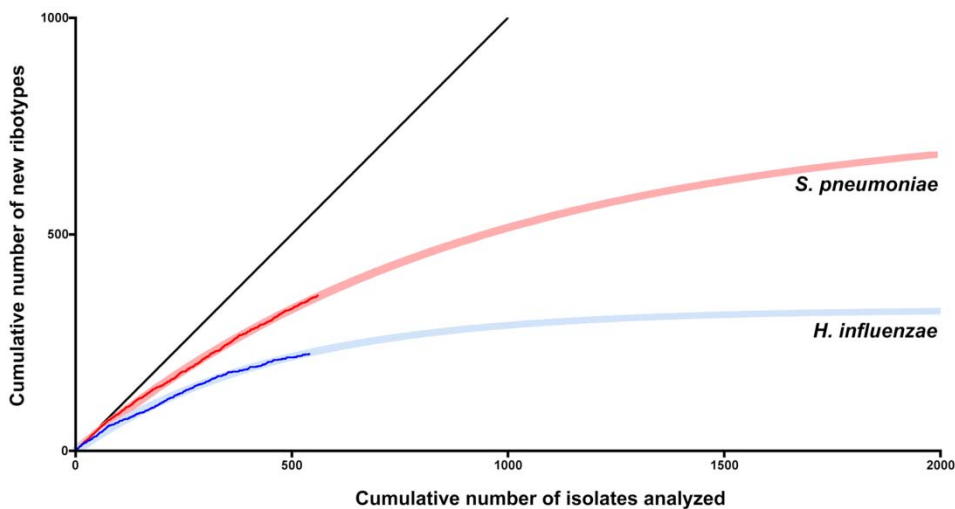
Figure 5. Top-down analysis[21]. Step 1, acquisition of independent isolates from human subjects. Step 2, phylogenic organization of clinical isolates based on ribotyping or MLST. Step 3, selection of a subset of isolates representative of species evolutionary diversity. Step 4. Rapid, PCR-based amplification of gene encoding pre-selected vaccine candidate target from each member of the subset followed by DNA sequencing-based conservation analysis. Any candidates fulfilling requisite conservation across species diversity of the subset is then moved on to steps 5 and 6 as depicted[1, 21].
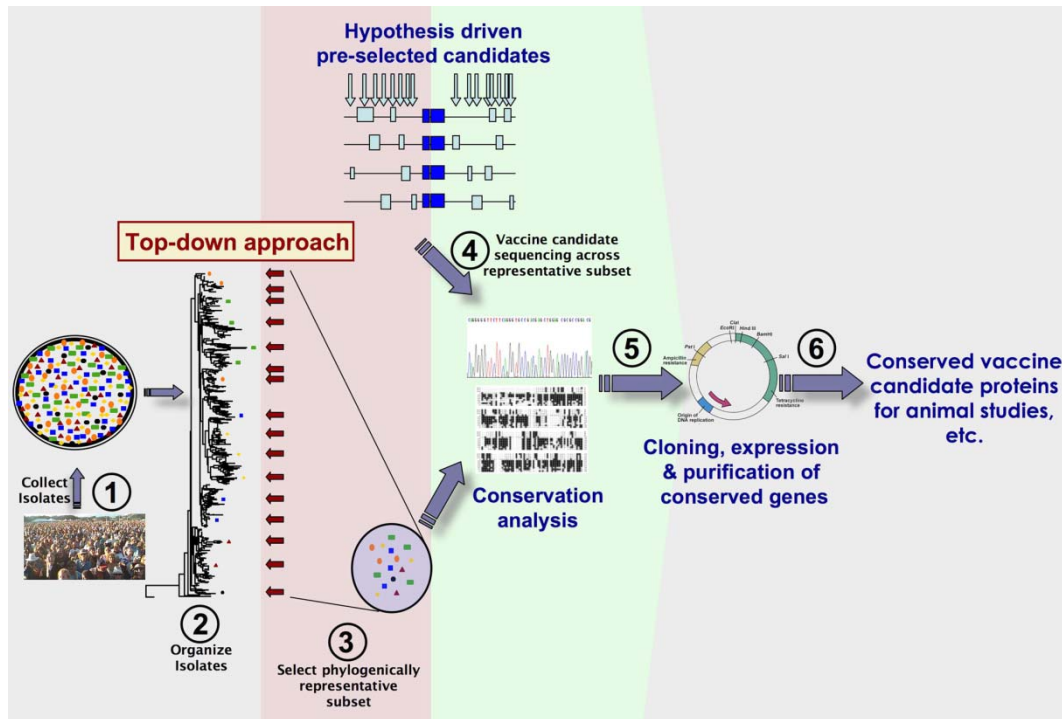
Figure 6a. Dendrogram of ~500 Hi and NTHi isolates. Arrows indicate lineage branches from which strains were used for the sequencing of *ompP1*[1]. Different colors behind the dendrogram correlate with primary evolutionary lineages.
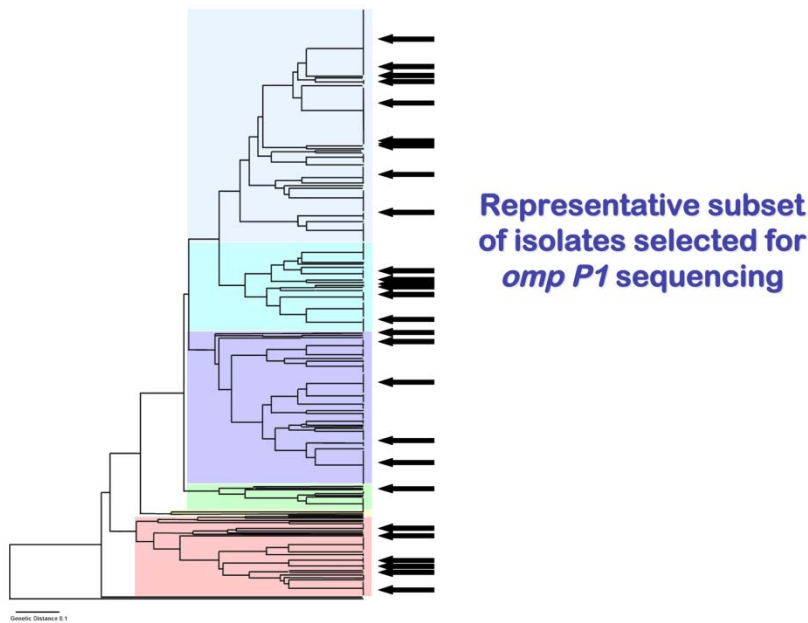
Figure 6b. OMP P1 amino acid sequence-based dendrogram. Colors at the end of each lineage branch correlate with primary evolutionary lineages of the strain sequenced for *ompP1* in the *H. influenzae* dendrogram depicted in Fig. 6a. As such, intermingling of primary lineage colors in the gene trees shown in this figure makes evident the degree of lateral (horizontal) transfer of *ompP1*. Dendrogram to the left, based on highly variable OMP P1 amino acid sequences associated with four of the surface-exposed regions of protein OMP P1 (variable region I, loop 2; variable region II, loop 4; variable region III, loop 5; variable region IV, loop 8)[1]. Dendrogram to the right is based on OMP P1 amino acid sequences omitting the four highly variable regions of protein OMP P1.

15
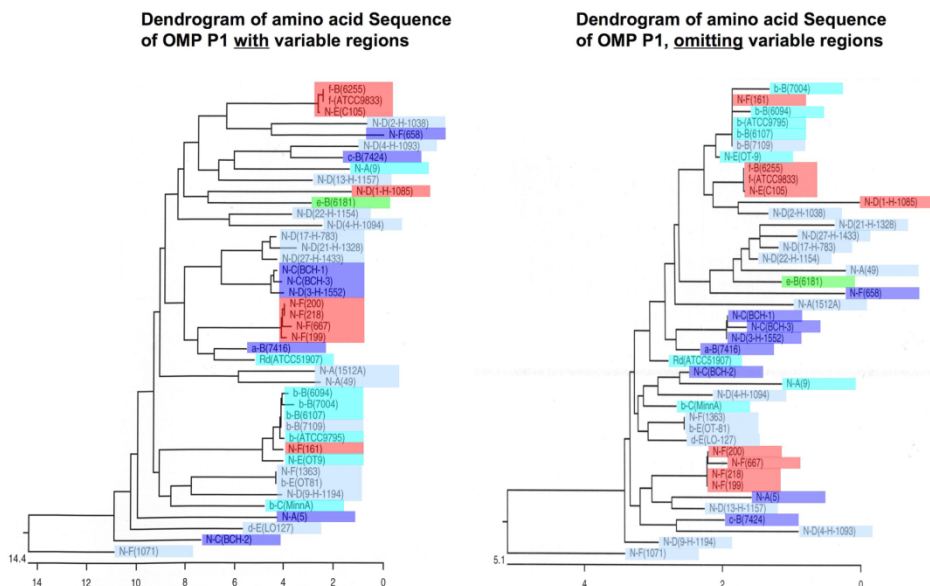
Figure 7. Phylogenic organization of ~900 *S. pneumoniae* isolates. Adapted from Huot et al[22, 23]. Colors behind dendrogram correlate with major evolutionary lineages numbered 1-13. Red arrows to the right depict choice of representative subset of otitis, carriage, and invasive isolates used in a top-down approach shown in Fig. 5. Displayed ribotype-based dendrogram was confirmed based on MLST analysis of ~500 or the 900 isolates.
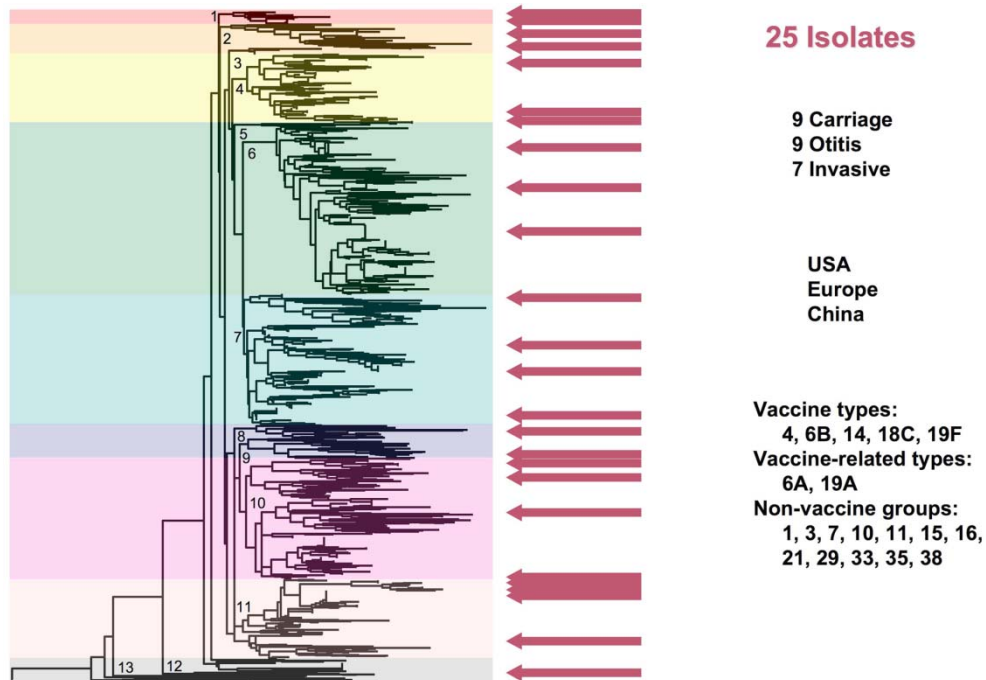
References

1. Bolduc GR, Bouchet V, Jiang RZ, et al. Variability of outer membrane protein P1 and its evaluation as a vaccine candidate against experimental otitis media due to nontypeable Haemophilus influenzae: an unambiguous, multifaceted approach. Infect Immun 2000; 68:4505-4517.
2. Cody AJ, Field D, Feil EJ, et al. High rates of recombination in otitis media isolates of non-typeable Haemophilus influenzae. Infect Genet Evol 2003: 3:57-66.
3. Feil EJ, Holmes EC, Bessen DE, et al. Recombination within natural populations of pathogenic bacteria: short-term empirical estimates and long-term phylogenetic consequences. Proc Natl Acad Sci U S A 2001; 98:182-187.
4. Meats E, Feil EJ, Stringer S, et al. Characterization of encapsulated and noncapsulated Haemophilus influenzae and determination of phylogenetic relationships by multilocus sequence typing. J Clin Microbiol 2003; 41:1623-1636.
5. Bouchet V, Hood DW, Li J, et al. Host-derived sialic acid is incorporated into Haemophilus influenzae lipopolysaccharide and is a major virulence factor in experimental otitis media. Proc Natl Acad Sci U S A 2003: 100:8898-8903.
6. Bouchet V, Huot H, Goldstein R. Molecular genetic basis of ribotyping. Clin Micro Reviews 2008; 21:262-273.
7. Leroy MO, Cabral H, Figueira M. Multiple consecutive lavage samplings reveal greater burden of disease and provide direct access to the nontypeable Haemophilus influenzae biofilm in experimental otitis media. Infect Immun 2007; 75:4158-4172.
8. Sun L, Jiang RZ, Steinbach S, et al. The emergence of a highly transmissible lineage of cbl+ Pseudomonas (Burkholderia) cepacia causing CF centre epidemics in North America and Britain. Nat Med 1995; 1:661-666.
9. Bolduc GR, Bouchet V, Jiang RZ, et al. Variability of outer membrane protein P1 and its evaluation as a vaccine candidate against

experimental otitis media due to nontypeable Haemophilus influenzae: an unambiguous, multifaceted approach. Infect Immun 2000; 68:4504-4517.

10. Figueira MA, Ram S, Goldstein R, Hood DW, Moxon ET, Pelton SI. Role of complement in defense of the middle ear revealed by restoring the virulence of nontypeable Haemophilus influenzae siaB mutants. Infect Immun 2007; 75:325-333.

11. Barenkamp SJ. Protection by serum antibodies in experimental nontypable Haemophilus influenzae otitis media. Infect Immun 1986: 52:572-578.

12. Gnehm HE, Pelton, SI, Gulati S, Rice PA. Characterization of antigens from nontypable Haemophilus influenzae recognized by human bactericidal antibodies. Role of Haemophilus outer membrane proteins. J Clin Invest 1985; 75:1645-1658.

13. Karasic RB, Trumpp CE, Gnehm HE, Rice PA, Pelton SI. Modification of otitis media in chinchillas rechallenged with nontypable Haemophilus influenzae and serological response to outer membrane antigens. J Infect Dis 1985; 151:273-279.

14. Shurin PA, Pelton SE, Tager IB, Kasper DL. Bactericidal antibody and susceptibility to otitis media caused by nontypable strains of Haemophilus influenzae. J Pediatr 1980; 97:364-369.

15. Sloyer JL Jr., Cate CC, Howie VM, Ploussard JH, Johnston RB Jr. The immune response to acute otitis media in children. II. Serum and middle ear fluid antibody in otitis media due to Haemophilus influenza. J Infect Dis 1975: 132:685-688.

16. Gonzales FR, Leachman D, Norgard MV, Radolf JD, McCracken GH Jr., Evans C, Hansen EJ. Cloning and expression in Escherichia coli of the gene encoding the heat-modifiable major outer membrane protein of Haemophilus influenzae type b. Infect Immun 1987; 55:2993-3000.

17. Hanson MS, Cope LD, Hansen EJ. I Expression of the heat-modifiable major outer membrane protein of Haemophilus influenzae type b is unrelated to virulence. Infect Immun 1989; 57:1639-1646.

18. Munson R Jr., Hunt A. Isolation and characterization of a mutant of Haemophilus influenzae type b deficient in outer membrane protein P1. Infect Immun 1989; 57:1002-1004.

19. Loeb MR. Protection of infant rats from Haemophilus influenzae type b infection by antiserum to purified outer membrane protein a. Infect Immun 1987; 55:2612-2618.

20. Hood DW, Makepeace K, Deadman ME, et al. Sialic acid in the lipopolysaccharide of Haemophilus influenzae: strain distribution, influence on serum resistance and structural characterization. Mol Microbiol 1999; 33:679-692.

21. Leroy M, Huot H, Bouchet V, et al. Microbial population genetics as the missing link to functional genomics-based vaccine development (abstract). ASM Conference on Functional Genomics and Bioinformatics Approaches to Infectious Disease Research. Portland, Oregon, 2004:36-37.

22. Huot H, Bouchet V, Leroy M, Pelton S, Goldstein R. Implications for vaccine development based on pneumococcal population structure (abstract). 4th International Symposium on Pneumococci and Pneumococcal Diseases. Helsinki, Finland, 2004:153.

23. Huot H, Bouchet V, Leroy M, et al. Congruence of pneumococcal population structure resolved by ribotyping and MLST in a PO5.20 defined community (abstract). 5th International Symposium on Pneumococci and Pneumococcal Diseases. Alice Springs, Australia, 2006:205.

24. Lipsitch M, O'Neill K, Cordy D, et al. Pneumococcal Population Biology of Vaccine Effectiveness and Serotype Replacement in PO10.19 *S. pneumoniae* (SP) Isolated During the American Indian Clinical Trial of the 7-Valent Pneumococcal Conjugate Vaccine (PCV)(abstract). 5th International Symposium on Pneumococci and Pneumococcal Diseases. Alice Springs, Australia, 2006:254.

25. Arbeit RD, Arthur M, Dunn R, Kim C, Selander RK, Goldstein R. Resolution of recent evolutionary divergence among Escherichia coli from related lineages: the application of pulsed field electrophoresis to molecular epidemiology. J Infect Dis 1990; 161:230-235.

26. Arthur M, Arbeit RD, Kim C, et al. Restriction fragment length polymorphisms among uropathogenic Escherichia coli isolates: pap-related sequences compared with rrn operons. Infect Immun 1990: 58:471-479.

27. Holmes A, Nolan R, Taylor R, Finley R, Riley M, Jiang RZ, Steinbach S, Goldstein R. An epidemic of burkholderia cepacia transmitted between patients with and without cystic fibrosis. J Infect Dis 1999; 4: 221-227.

28. Kroll JS, Ely S, Moxon ER. Capsular typing of Haemophilus influenzae with a DNA probe. Mol Cell Probes 1991; 5:375-379.

29. Musser JM, Barenkamp SJ, Granoff DM, Selander RK. Genetic relationships of serologically nontypable and serotype b strains of Haemophilus influenzae. Infect Immun 1986; 52:183-191.

Protein D of *Haemophilus influenzae*–the antigenic potential of a carrier protein

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To deceive the human host, *Haemophilus influenzae* has outer membrane proteins that display extensive sequence variation. However Protein D (PD) is a highly conserved 42 kDa surface lipoprotein in all *H. influenzae,* including nontypeable *H. influenzae* (NTHi).[1-5] PD is an important pathogenicity factor in acute otitis media (AOM) in rats, and has been shown to impair ciliary function in a human nasopharyngeal tissue culture model.[6,7] When PD recently was introduced as an antigenically active carrier protein in an 11-valent pneumococcal conjugate investigational vaccine, a significant protection was achieved against AOM caused by pneumococci and/or NTHi.[8] PD also has the potential to protect children against *H. influenzae* as was shown in a randomized, double-blind efficacy study where PD was conjugated with pneumococcal capsular polysaccharides.[7] This study confirmed that using *H. influenzae*-derived PD as a carrier protein for pneumococcal polysaccharides not only allowed protection against pneumococcal otitis media (OM), but also against AOM due to NTHi. The present paper reviews the properties of PD and its potential applications as an antigenically active carrier protein for conjugate vaccines.

PD was originally defined as a surface-exposed outer membrane protein of *H. influenzae*, which was detected by its ability to bind to human immunoglobuldin D (IgD) myeloma protein 4490.[1] It was later shown by Sasaki and Munson[9] that PD did not bind to IgD but was rather a target of the unique IgD myeloma protein. PD with an apparent molecular weight of 42kDa was found in all *H. influenzae* strains when outer membrane protein preparations of 127 *H. influenzae* strains, including encapsulated serotypes a-f and NTHi strains, were analyzed by Western blot using three different mouse anti-PD monoclonal antibodies and the human IgD myeloma 4490.[2] The number of PD molecules was estimated to be 2,800 per bacterial cell. These studies thus suggested that PD is antigenically conserved and surface-located, and is present amongst most, if not all, *H. influenzae*, making it an atttractive vaccine candidate.

DNA sequence analysis of the region-encoding PD revealed an open reading frame of 1,092 base pairs (bp), encoding a putative protein of 346 amino acids with a calculated molecular weight of 41,821 Dalton.[3] A very limited diversity of PD among 14 *H. influenzae* strains (Table 1) was found.[4-5, 10-13] Moreover, there seems to be little drift in the PD gene as shown by sequencing the PD gene of *H. influenzae* isolated from the lungs of patients with persisting bronchitis.[15]

PD is a virulence factor involved in the pathogenesis of respiratory tract infections. Approximately 100 times more colony-forming units of a PD mutant were required to cause infection in the middle ears of rats in an OM model.[6] In a human nasopharyngeal tissue culture model, the impairment of ciliary function caused by the PD-expressing strain was significantly greater than that caused by the PD-negative mutant (p <0.01).[7] After 48 hours of incubation, the PD-expressing strain caused a significant loss of cilia. Viable PD-deficient *H. influenzae* were isolated from the middle ears of rats, with or without diagnosed AOM, 4 days after challenge at frequencies similar to that of the PD-expressing wild type strain 772 (unpublished results). These results indicated that PD was not essential for survival in the middle ear of rats but that it clearly enhanced the severity of the disease, and that PD is involved in the pathogenesis of upper respiratory tract infections due to NTHi, probably by enhancing functional and morphological damage to ciliated epithelial cells. It is becoming increasingly accepted that NTHi, which is usually regarded as a non-invasive pathogen, can enter non-ciliated epithelial cells.[14,15] It was therefore interesting to observe that PD expression was shown to promote adherance and internalization of NTHi into human epithelial and monocytic cells.[7, 16]

PD is an important pathogenicity factor for *H. influenzae,* and choline by PD, because of its glycerophosphodiester phosphodiesterase (GlpQ) activity, facilitates the acquisition of choline directly from host epithelial cells.[17] The ChoP-decorated LOS serves as ligand for the platelet-activating factor (PAF) receptor of bronchial epithelial cells. More recent data demonstrate that the binding of the PAF receptor by NTHi initiates receptor coupling to a pertussis toxin-sensitive heterotrimeric G protein complex, resulting in a multifactorial host cell signal cascade and bacterial invasion.[18, 19] Phosphorylcholine also promotes the establishment of stable biofilm communities of NTHi in a chinchilla model of AOM.[20] Moreover, phosphorylcholine is a well-known constituent of

teichoic acid in *Streptococcus pneumoniae* and is a critical determinant of the inflammatory activity of this particular species.[21]

PD is protective in animal models. A rat middle-ear clearance model developed by Kyd and colleagues was used to evaluate the potential of PD as a protective antigen against NTHi.[22] Rats were immunized on day 0 via intestinal Peyer's patches followed by an intratracheal boost on day 14. An intrabullar challenge with live NTHi was performed on day 21, and bacteria were counted after 4 hours. Rats immunized with PD tended to clear NTHi more efficiently as compared to rats that received $A1PO_4$-(p=0.083). The pulmonary clearance of NTHi observed after immunization with PD was highly significant compared with the non-immunized group (p <0.001). Differences of more than one log in NTHi colony-forming units recovered 4 hours post-challenge were observed either in bronchoalveolar lavage or in lung homogenates.[22]

A pronounced activity for PD antibodies was shown in the chinchilla AOM model[23, 24], a highly reproducible viral-bacterial superinfection animal model. A dual infection step was developed to mimic human NTHi infection, where *H. influenzae* is an opportunistic pathogen. Juvenile chinchillas are first challenged intranasally with adenovirus (serotype 1) and subsequently with NTHi to coincide with maximal viral damage of the eustachian tube mucosa. AOM begins to develop 7 days after NTHi challenge. The AOM induced in chinchillas according to this model shows many similarities with the polymicrobial nature and natural disease in children.

To evaluate the protective capacity of anti-PD antibodies, chinchillas were injected intracardially with chinchilla anti-PD serum 1 day before challenge with NTHi. Compared with sham-immunized animals, delivery of anti-PD serum by passive transfer reduced the incidence of middle-ear effusion-(p≤ 0.001).[24] In another viral-bacterial co-infection study, passive transfer of a pediatric human serum pool generated against an 11-valent investigational vaccine comprised of pneumococcal capsular polysaccharide conjugated to PD-conferred ≈ 34% (p ≤ 0.001) protection against development of ascending NTHi-induced AOM.[25]

Human serum antibodies to nonlipidated PD were measured by ELISA. The mean IgG level in infants younger than 6 months was slightly above the detection limit, then showed a drop to almost undetectable levels between 6 months and 1 year of age, before increasing steadily up to age 5 years. After a plateau in the 5- to 10-year age group, the IgG level increased steadily up to 20 years, before slowly declining in the subsequent age groups. The IgA antibody concentrations were a background levels until the age of 1 year. The levels were slightly higher in the 1- to 10-year old groups and increased further thoughout life.[26]

PD is an antigenic carrier protein in a conjugated pneumococcal vaccine. The two leading bacterial pathogens causing AOM are *S. pneumoniae* and NTHi. Both are also recognized as a major cause of lower respiratory tract infections. Vaccines containing plain capsular polysaccharides of *S. pneumoniae* have been used for decades, but they show poor immunogenicity in children. However, a vaccine (Prevnar™, Wyeth, Philadelphia, PA) containing polysaccharides from 7 serotypes each conjugated to CRM197, a non-toxic mutant of diphteria toxin that is an immunogenically inactive carrier, has shown high efficacy in young children against invasive pneumococcal disease and intermediate efficacy against AOM caused by vaccine pneumococcal serotypes.[27] An intrinsic disadvantage of the CRM197-based vaccine is that the same carrier, CRM197, is used in Hib and meningococcal conjugate vaccines, which might have a negative impact on the immunogenicity of the other conjugate vaccines injected concomitantly and on the pneumococcal conjugate vaccine itself.[28] For those reasons and due its properties, such as surface localization, high degree of conservation, wide distribution, pathogenicity, as well as the promising preclinical results with PD, it was decided to use PD, in a non-acylated form, as the antigenically active carrier protein in a new 11-valent pneumococcal conjugate vaccine. Thus, PD is expected to function as a carrier protein with antigenic potential resulting in dual protection against *S. pneumoniae* and NTHi.

The immunogenicity and safety of the new 11-valent pneumococcal vaccine with PD as a carrier was tested in a randomized, single blinded, controlled phase II study enrolling 154 healthy Finnish infants.[29] Vaccine was given at 2, 4, 6, and 12-15 months of age. Pneumococcal antibody concentrations after the first three doses varied between 1.26 and 4.94 µg/ml depending on the serotype and study group. It was concluded that the PD conjugate pneumococcal vaccine was immunogenic and safe in infants.

In a randomized, double-blind efficacy study, Prymula et al[8] assessed the efficacy of the new pneumococcal polysaccharide vaccine for prevention of AOM caused by both *S. pneumoniae* and NTHi. The primary endpoint was protective efficacy against the first episode of AOM caused by vaccine serotypes. Analysis showed that the PD conjugate vaccine provided a significant reduction (33.6%) in the overall incidence of AOM. Vaccine efficacy for AOM caused by pneumococcal vaccine serotypes was 52.6–57.6%

and 35.3% for NTHi. The vaccine also reduced the frequency of infection from all vaccine-related cross-reactive serotypes (including 6A and 19A) by 65.5%, but did not significantly affect AOM caused by other serotypes or other bacterial species. No increase in pneumococcal AOM caused by other non-vaccine serotypes or other bacterial pathogens was recorded over the study period. Interestingly, the PD-based vaccine also reduced the carriage rate of both pneumococci and NTHi. After the fourth immunization, vaccine serotype pneumococci were isolated from 6% of the infants in the PD-conjugate group versus 11% of controls, and *H. influenzae* was isolated in 10% of the infants in the PD-conjugate group versus 18% in the control group. This is important as the success of current conjugate vaccination programs against Hib, pneumococci, and meningococci is due in part to the lowered transmission of the bacteria.

The overall vaccine efficacy for AOM episodes caused by pneumococcal vaccine serotypes was remarkably similar for the 11-valent PD conjugate vaccine and the 7-valent conjugate vaccines from a Finnish trial[30, 31], where a decrease in episodes linked to the vaccine strains of 56-57% was observed. However, after vaccination with the 7-valent CRM 197 conjugate vaccine, an increase in AOM due to non-vaccine pneumococcal or other pathogens has been recorded. There was no evidence of such replacement phenomenon in the patient population included in the 11-valent pneumococcal PD-conjugate study.[8]

Table 1. Protein D is highly conserved[4-5, 10-13]

Strain	Serotype	Accession number	Identity (%) with consensus sequence of PD	
			DNA	Protein
772	NT	M37487	99.7	100
86-028NP	NT	CP000057	99.6	100
3639	NT	Z35656	99.2	99.7
3640	NT	Z35657	99.5	99.7
6-7626	NT	Z35658	98.1	98.1
A850079	NT	X90495	99.7	100
A850053	NT	X90493	98.9	99.1
A850047	NT	X90489	98.9	99.1
A850048	NT	X90491	97.2	98.2
MinnA	b	L15200	99.6	99.4
HK695	b	Z35660	99.6	99.4
Eagan	b	Z35659	99.6	99.4
NCTC8468	b	Z35661	97.5	98.1
Rd KW20	d	L42023	99.1	98.9

References

1. Ruan M, Akkoyunlu M, Grubb A, Forsgren A. Protein D of Haemophilus influenzae. A novel bacterial surface protein with affinity for human IgD. J Immunol 1990; 145:3379-3384.
2. Akkoyunlu M, Ruan M, Forsgren A. Distribution of protein D, an immunoglobulin D-binding protein, in Haemophilus strains. Infect Immun 1991; 59:1231-1238.
3. Janson H, Hedén LO, Grubb A, Ruan M, Forsgren A. Protein D, an immunoglobulin D-binding protein of Haemophilus influenzae: cloning, nucleotide sequence, and expression in Escherichia coli. Infect Immun 1991; 59:119-125.
4. Janson H, Ruan M, Forsgren A. Limited diversity of the protein D gene (hpd) among encapsulated and nonencapsulated Haemophilus influenzae strains. Infect Immun 1993; 61:4546-4552.
5. Duim B, Ruiter P, Bowler LD, Dankert J, van Alphen L. Sequence variation in the hpd gene of nonencapsulated Haemophilus influenzae isolated from patients with chronic bronchitis. Gene 1997; 191:57-60.
6. Janson H, Melhus Å, Hermansson A, Forsgren A. Protein D, the glycerophosphodiester phosphodiesterase from Haemophilus influenzae with affinity for human immunoglobulin D, influences virulence in a rat otitis model. Infect Immun 1994; 62:4848-4854.
7. Janson H, Carlén B, Cervin A et al. Effects on the ciliated epithelium of protein D-producing and -nonproducing nontypeable Haemophilus influenzae in nasopharyngeal tissue cultures. J Infect Dis 1999; 180:737-746.
8. Prymula R, Peeters P, Chrobok V et al. Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both Streptococcus pneumoniae and non-typable Haemophilus influenzae: a randomised double-blind efficacy study. Lancet 2006; 367:740-748.
9. Sasaki K, Munson RS, Jr. Protein D of Haemophilus influenzae is not a universal immunoglobulin D-binding protein. Infect Immun 1993; 61:3026-3031.
10. Munson RS, Jr., Sasaki K. Protein D, a putative immunoglobulin D-binding protein produced by Haemophilus influenzae, is glycerophosphodiester phosphodiesterase. J Bacteriol 1993; 175:4569-4571.

11. Song XM, Forsgren A, Janson H. The gene encoding protein D (hpd) is highly conserved among Haemophilus influenzae type b and nontypeable strains. Infect Immun 1995; 63:696-699.

12. Fleischmann RD, Adams MD, White O et al. Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. Science 1995; 269:496-512.

13. Harrison A, Dyer DW, Gillaspy A et al. Genomic sequence of an otitis media isolate of nontypeable Haemophilus influenzae: comparative study with H. influenzae serotype d, strain KW20. J Bacteriol 2005; 187:4627-4636.

14. St.Geme III JW, Falkow S. Haemophilus influenzae adheres to and enters cultured human epithelial cells. Infect Immun 1990; 58:4036-4044.

15. Ketterer MR, Shao JQ, Hornick DB et al. Infection of primary human bronchial epithelial cells by Haemophilus influenzae: macropinocytosis as a mechanism of airway epithelial cell entry. Infect Immun 1999; 67:4161-4170.

16. Ahrén IL, Janson H, Forsgren A, Riesbeck K. Protein D expression promotes the adherence and internalization of non-typeable Haemophilus influenzae into human monocytic cells. Microb Pathog 2001; 31:151-158.

17. Fan X, Goldfine H, Lysenko E, Weiser JN. The transfer of choline from the host to the bacterial cell surface requires glpQ in Haemophilus influenzae. Mol Microbiol 2001; 41:1029-1036.

18. Swords WE, Buscher BA, Ver Steeg K et al. Non-typeable Haemophilus influenzae adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. Mol Microbiol 2000; 37:13-27.

19. Swords WE, Ketterer MR, Shao J et al. Binding of the non-typeable Haemophilus influenzae lipooligosaccharide to the PAF receptor initiates host cell signalling. Cell Microbiol 2001; 3:525-536.

20. Hong W, Mason K, Jurcisek J et al. Phosphorylcholine decreases early inflammation and promotes the establishment of stable biofilm communities of nontypeable Haemophilus influenzae strain 86-028NP in a chinchilla model of otitis media. Infect Immun 2007; 75:958-965.

21. Cabellos C, MacIntyre DE, Forrest M et al. Differing roles for platelet-activating factor during inflammation of the lung and subarachnoid space. The special case of Streptococcus pneumoniae. J Clin Invest 1992; 90:612-618.

22. Poolman JT, Bakaletz L, Cripps A et al. Developing a nontypeable Haemophilus influenzae (NTHi) vaccine. Vaccine 2000; 19 Suppl 1:S108-S115.

23. Bakaletz LO, Kennedy BJ, Novotny LA et al. Protection against development of otitis media induced by nontypeable Haemophilus influenzae by both active and passive immunization in a chinchilla model of virus-bacterium superinfection. Infect Immun 1999; 67:2746-2762.

24. Bakaletz LO. Peptide and recombinant antigens for protection against bacterial middle ear infection. Vaccine 2001; 19:2323-2328.

25. Novotny LA, Jurcisek JA, Godfroid F et al. Passive immunization with human anti-protein D antibodies induced by polysaccharide protein D conjugates protects chinchillas against otitis media after intranasal challenge with Haemophilus influenzae. Vaccine 2006; 24:4804-4811.

26. Akkoyunlu M, Janson H, Ruan M, Forsgren A. Biological activity of serum antibodies to a nonacylated form of lipoprotein D of Haemophilus influenzae. Infect Immun 1996; 64:4586-4592.

27. Black S, Shinefield H, Fireman B et al. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Pediatr Infect Dis J 2000; 19:187-195.

28. Burrage M, Robinson A, Borrow R et al. Effect of vaccination with carrier protein on response to meningococcal C conjugate vaccines and value of different immunoassays as predictors of protection. Infect Immun 2002; 70:4946-4954.

29. Nurkka A, Joensuu J, Henckaerts I et al. Immunogenicity and safety of the eleven valent pneumococcal polysaccharide-protein D conjugate vaccine in infants. Pediatr Infect Dis J 2004; 23:1008-1014.

30. Eskola J, Kilpi T, Palmu A et al. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N Engl J Med 2001; 344:403-409.

31. Kilpi T, Åhman H, Jokinen J et al. Protective efficacy of a second pneumococcal conjugate vaccine against pneumococcal acute otitis media in infants and children: randomized, controlled trial of a 7-valent pneumococcal polysaccharide-meningococcal outer membrane protein complex conjugate vaccine in 1666 children. Clin Infect Dis 2003; 37:1155-1164.

Pneumococcal proteins as vaccine candidates

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Streptococcus pneumoniae, pneumococcus, is a leading cause of morbidity and mortality worldwide and remains the leading cause of acute otitis media (AOM).[1] The availability of the conjugate vaccine has reduced the incidence of invasive pneumococcal disease in children younger than 2 years of age in the United States.[2] The currently available conjugate vaccine is composed of purified capsular polysaccharide from seven different serotypes commonly associated with disease in young children in the United States.[3] The conjugate vaccine has been reported to have an impact on pneumococcal otitis media (OM).[4] Some studies have reported as much as a 57% reduction in the rate of AOM caused by vaccine-specific serotypes following immunization.[5] However, the same study reported a 33% increase in AOM caused by vaccine-replacement serotypes. Therefore, a vaccine that is effective against a wide range of serotypes, is cost-effective, and reduces the level of AOM would be highly advantageous, and a protein-based vaccine has the potential to meet these requirements.

Historically, there has been great interest in developing an effective pneumococcal vaccine. A whole cell vaccine was among the first to be tried in the prevention of pneumococcal disease. However, due to a lack of knowledge regarding capsular serotypes, this vaccine was not very efficacious and soon abandoned. Interestingly, studies have once again focused on using intact pneumococci to elicit protective immune responses.[6] Also, it was recently demonstrated that colonization of mice with live attenuated pneumococci elicits significant cross-protection against intranasal challenge with distantly related strains in mice.[7]

It is very likely that a vaccine containing one or more pneumococcal proteins will be approved for use. Such a vaccine has the potential to block both carriage in the nasopharynx and systemic disease. If a protein-based vaccine is effective at preventing carriage in children less than 2 years of age, the likely result would be a reduction in the incidence of AOM.

There are more than 100 proteins that have been identified that have the potential to elicit protection against pneumococcal challenge.[8] As more pneumococcal genome sequences become available, there will be a refinement in the potential vaccine candidates, making it easier to focus on those proteins that are most likely to be effective.

Most studies of pneumococcal proteins have used mouse models of infection. While it has limitations, the mouse has proven to be an important system in which to study pneumococcal disease. This is largely due to the fact that pneumococcal disease in the mouse closely mirrors the disease seen in humans. Depending on the mouse strain used and the pneumococcal isolate, one can independently study colonization, pneumonia, or systemic infection.[9, 10] Animal studies of AOM have used primarily the chinchilla[11] or rat.[12] There have been recent efforts to develop an effective model of OM in the mouse[13], but it remains to be seen how useful this model will be in determining the vaccine potential of pneumococcal proteins against AOM.

There are several classes of pneumococcal proteins, and each class contains proteins that are being actively studied as vaccine candidates. The different classes of pneumococcal proteins are composed of cytoplasmic proteins, secreted proteins, and cell-surface anchored proteins (Table 1). The pneumococcus employs several means of localizing proteins to the bacterial surface. This includes the classic Gram-positive anchor motif of LPxTG, which is dependent on the action of sortase to covalently attach the protein to the pneumococcal surface. There are lipoproteins as well as a family of proteins known as choline-binding proteins (CBPs), which are non-covalently attached to the surface through interaction with choline.[14] Some pneumococcal proteins appear to localize to the bacterial surface by mechanisms that are not fully understood.[15]

There is convincing data that some pneumococcal proteins can elicit a strong specific antibody response in the mouse and that these antibodies can protect mice from subsequent pneumococcal challenge. We and others have demonstrated that pneumococcal surface protein A (PspA) is such a protein.[16, 17] Not only is a specific serum antibody elicited to PspA (Table 2), but depending upon the challenge strain, the antibody protects mice from a lethal challenge of at least three logs greater than the LD_{50} as compared to controls.

PspA is the most highly characterized pneumococcal protein with vaccine potential. PspA is immunogenically variable among different

pneumococcal isolates. While this may introduce problems in designing an effective vaccine, it implies that there is selective pressure from the host immune system against PspA. This may indicate that a vaccine containing PspA that elicits the proper response in the host could be very effective against the pneumococcus. Efforts to elicit immune responses with PspA have focused on the surface-exposed α-helical domain of PspA, which has been demonstrated to contain protection-eliciting epitopes.[18] Sequence analysis of this domain has allowed PspA to be organized into six clades that are further grouped into three family types.[19] Such groupings may help in selecting the specific PspAs to include in a vaccine that would result in the broadest coverage. There have been several studies showing that PspA family 1 and family 2 isolates make up the significant portion of pneumococci causing disease from different geographic regions and among different age populations.[20-22] We have seen that PspA family 1 and family 2 cover 100% of the pneumococcal isolates causing OM in the state of Mississippi.[23]

It is known that PspA plays a role in pneumococcal virulence[24], but the exact mechanism of PspA that aids the pneumococcus in survival in the host is not fully understood. One function of PspA is to inhibit complement activation by preventing the binding of C3 to the pneumococcal surface.[25, 26] PspA has also been shown to bind apolactoferrin[27], the precursor for lactoferrin, which can inhibit bacterial growth.[28] When bound to the pneumococcus by PspA, apolactoferrin is prevented from converting to the active form lactoferrin, thus increasing pneumococcal survival.[29]

Studies have demonstrated that passive administration of antibodies against PspA can protect mice against pneumococcal challenge by promoting clearance of the bacteria.[30-32] Interestingly, it has not been possible to demonstrate in vitro opsonophagocytosis with anti-PspA antibodies. It has been suggested that the in vivo clearance occurs because the anti-PspA antibodies block the binding of apolactoferrin by PspA and increases the amount of C3 deposited on the pneumococcal surface.[29, 33]

PspC is another member of the CBP family. PspC was first identified using a DNA probe constructed from PspA.[34] This cross-hybridization reaction and subsequent sequence analysis identified significant homology between the proline-rich and choline-binding domains of PspA and PspC.[35] Additional sequence analysis revealed that PspC is highly variable among pneumococcal isolates, and that some variants are surface-attached via the LPxTG motif instead of the choline-binding domain.[36]

PspC is a multi-functional protein. It has been shown to bind both secretory IgA and complement factor H (FH).[37] Studies have shown that recruitment of FH to the pneumococcal surface by PspC increases the ability of the pneumococcus to resist complement-mediated clearance.[38] While PspC plays a role in nasopharyngeal carriage[39], it also increases pneumococcal binding to lung epithelial cells by interaction with FH.[40]

The potential usefulness of PspC in a pneumococcal vaccine remains unresolved. There is evidence that PspC can elicit protective immunity in mice[39], but it may be that the protection results from cross-reactive antibodies to PspA.[35] Nonetheless, PspC should be considered as a candidate protein for a vaccine against AOM because of its potential to elicit responses that block pneumococcal carriage.

Another important pneumococcal protein is pneumolysin (Ply), which is a member of a family of proteins known as thiol-activated, cholesterol-dependent cytolysins (CDCs). Other members of this family include streptolysin O, listeriolysin O, perfringolysin O, and anthrolysin O.[41, 42] The CDCs exhibit extensive serum cross-reactivity and have in common the ability to lyse mammalian cells.[43] Beyond cell lysis, Ply has a range of effects on mammalian cells[44, 45] and helps the pneumococcus to overcome host immune responses.

In addition to toxic effects, Ply can activate complement.[10] Therefore, a main consideration in using Ply in a pneumococcal vaccine has been the potential for adverse reactions. Genetically detoxified Ply has been produced and demonstrated to still elicit immune responses.[46] Immunization of mice with the mutant Ply produced specific serum antibodies against Ply and increased the survival time of mice when systemically challenged with pneumococci.[46] Another issue with Ply is that it lacks a signal peptide and is thought to be an intracellular protein.[47] However, some pneumococcal strains release significant amounts of Ply during exponential growth.[48] It is likely that protection by antibodies against Ply results from a neutralization of the toxic effects of Ply and would not be expected to produce a sterilizing immunity. Such protection might be of benefit in localized areas like the inner ear where inflammatory processes can cause damage.

Neuraminidase A (NanA) is a pneumococcal cell surface protein anchored to the cell wall by the LPxTG motif.[49] Recent studies have demonstrated that NanA is required for pneumococcal growth in vitro when mucin is provided as the sole carbon source.[50] NanA seems to play a role in the ability of the pneumococcus to colonize the nasopharynx and cause

AOM.[51] NanA appears to remodel mucin-containing surfaces and thus exposing host receptors for adherence, which aids pneumococcal colonization.[52] Additionally, the protein has been implicated in destruction of host defense proteins[53], modification of the surface of bacteria that compete with the pneumococcus in the nasopharynx [54], and in the development of sepsis in the mouse.[55] Immunization of chinchilla with NanA has been shown to elicit protection against both pneumococcal carriage and OM.[11, 56]

PiaA and PiaU are lipoproteins that allow the pneumococcus to utilize heme as an iron source under iron-limiting conditions. Unlike the proteins already described here, PiaA and PiaU are genetically organized into operons.[57] The proteins are encoded by ABC transporter operons consisting of genes for a substrate-binding protein, a membrane permease, and an ATPase. Studies have demonstrated that PiaA and PiaU are accessible to antibody on the pneumococcal surface[58], and the proteins appear to be highly conserved among pneumococcal isolates.[59] Additionally, there is evidence that humans recovering from pneumococcal septicemia have serotype-independent antibodies against these two proteins.[60]

Initial vaccine studies with PiaA and PiaU were carried out in mice using a systemic challenge model and indicated that immunization with the two proteins in combination was effective in protecting mice from challenge.[61] Subsequent studies demonstrated that mucosal immunization with PiaA and PiaU elicited specific antibody responses, and the mice were protected from pneumococcal intranasal challenge.[62]

There are a number of secreted proteins that do not have any apparent classical means of associating with the pneumococcal surface. One such protein is PotD of the *potABCD* operon. The genes encoded by this operon are involved in the transport of polyamines and similar molecules by the pneumococcus.[63] Studies have shown that PotD is localized to the pneumococcal surface by what appears to be a novel mechanism.[64] A mutant that failed to express PotD was significantly attenuated in both systemic and respiratory mouse infection models.[65] Immunization with recombinant PotD protected more than 90% of mice compared to 0% survival of controls in a systemic model.[66] The ability of PotD to elicit mucosal responses remains to be proven.

There are a number of pneumococcal proteins under investigation as vaccine candidates that can protect against invasive disease, block carriage, and potentially reduce AOM. Additional proteins are being added to the list all the time. The question becomes is there anyone protein that will likely be highly efficacious against the pneumococcus? The accumulating data suggests that a mixture of pneumococcal proteins will be more effective at preventing disease than any single vaccine candidate.[67-69] There is an apparent synergism when combinations of pneumococcal proteins are used in immunization studies resulting in increased protection. Not only is there enhanced protection against the individual pneumococcal challenge strain, but the protection also extends over a broader range of strains. Additionally, the enhanced protection covers more routes of challenge. Importantly, some proteins that do not appear to be effective immunogens when used by themselves, elicit increased immune responses by administering other pneumococcal proteins with the less immunogenic protein.[69] Increasingly it seems that the emphasis should be not just on what pneumococcal protein to use in a vaccine, but what combination of proteins will be most effective.

It has long been recognized that antibodies play an important role in protection against pneumococcal infection. Passive protection studies have shown that antibodies against pneumococcal proteins can protect mice against pneumococcal challenge.[30] However, we observed that protection elicited by pneumococcal proteins did not always correlate with the presence of detectable specific antibody[70] suggesting that T-cell responses could have a role in protection elicited by pneumococcal proteins. Subsequent studies have suggested that CD4+ T cells can provide protection against pneumococcal colonization in mice.[71, 72] Recent studies have shown that immunization with purified pneumococcal proteins can protect mice against colonization in an antibody independent, CD4+ T-cell–dependent process.[73] These data indicate that T-cell responses against pneumococcal proteins need to be investigated further. The responses appear to play a role in preventing pneumococcal colonization and may therefore impact AOM.

Another important aspect of in the development of a pneumococcal protein-based vaccine that is effective against AOM is delivery of the antigen to the appropriate target site that is cost-effective and elicits the most robust immune response. Studies have examined ways to enhance responses to pneumococcal proteins and augment immunization on mucosal surfaces. This includes incorporating pneumococcal proteins into polymers that can modulate the immune response.[17] Additionally, pneumococcal proteins have been expressed in other bacteria as a means of stimulating mucosal responses.[74, 75] Recently, Gram-positive Enhancer Matrix (GEM) systems have been

used to deliver pneumococcal proteins to mucosal surfaces and enhance the response.[76, 77]

Vaccine prevention of AOM that results from the pneumococcus is likely more achievable than ever before. Based on what we have learned from the conjugate vaccine, it is clear that a protein-based vaccine will need to have broad coverage against the pneumococcus. A combination of pneumococcal proteins will be more effective, and the response needs to be tailored to preventing the pneumococcus from gaining access to the inner ear. To aid the development of an effective vaccine, end points for outcome of AOM in animal models need to be further defined. Also, examination of T-cell responses against pneumococcal protein antigens and understanding the role of the responses in AOM will further assist in the development of an effective vaccine.

Table 1. Classes of Pneumococcal Proteins with Vaccine Potential

Class	Example	Advantage	Disadvantage
Choline Binding Protein	PspA	Highly immunogenic	Highly variable
Cytoplasmic	Pneumolysin	Present in many strains	Toxic
Cell Wall Anchored	NanA	Important in colonization	Needs further study
Lipoprotein	PiaA/PiaU	Highly conserved	Combination more effective
Non-Classical Cell Wall Associated	Pot D	Conserved	Needs further study

Table 2. Immunization with PspA*

	PspA	No PspA
Antibody response (ug/ml)	630.0 ± 8.6	<0.1
LD_{100} (CFU's)	$> 5 \times 10^4$	< 80

*Mice were immunized subcutaneously with 10 μg of purified recombinant PspA on alum, boosted at 14 days, bleed for antibody titer and challenged with *S. pneumoniae* WU2 (serotype 3) intravenously on day 21.

References

1. McDaniel LS, Swiatlo E. Pneumococcal disease: Pathogenesis, treatment, and prevention. Infect Dis Clin Pract 2004; 12:93-98.
2. Grijalva CG, Nuorti JP, Arbogast PG, Martin SW, Edwards KM, Griffin MR. Decline in pneumonia admissions after routine childhood immunisation with pneumococcal conjugate vaccine in the USA: a time-series analysis. Lancet 2007; 369:1179-1186.
3. Black S, Shinefield H, Baxter R, et al. Postlicensure surveillance for pneumococcal invasive disease after use of heptavalent pneumococcal conjugate vaccine in Northern California Kaiser Permanente. Pediatr Infect Dis J 2004; 23:485-489.
4. O'Brien KL, Santosham M. Potential impact of conjugate pneumococcal vaccines on pediatric pneumococcal diseases. Am J Epidemiol 2004; 159:634-644.
5. Eskola J, Kilpi T, Palmu A, et al. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N Engl J Med 2001; 344:403-409.
6. Hvalbye BK, Aaberge IS, Lovik M, Haneberg B. Intranasal immunization with heat-inactivated *Streptococcus pneumoniae* protects mice against systemic pneumococcal infection. Infect Immun 1999; 67:4320-4325.
7. Roche AM, King SJ, Weiser JN. Live attenuated *Streptococcus pneumoniae* strains induce serotype-independent mucosal and systemic protection in mice. Infect Immun 2007; 75:2469-2475.
8. Wizemann TM, Heinrichs JH, Adamou JE, et al. Use of a whole genome approach to identify vaccine molecules affording protection against *Streptococcus pneumoniae* infection. Infect Immun 2001; 69:1593-1598.
9. Ogunniyi AD, LeMessurier KS, Graham RMA, Watt JM, Briles DE, Stroeher UH, Paton JC. Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of *Streptococcus pneumoniae* D39 in a mouse model. Infect Immun 2007; 75:1843-1851.
10. Quin LR, Moore QC, McDaniel LS. Pneumolysin, PspA, and PspC contribute to pneumococcal evasion of early innate immune responses during bacteremia in mice. Infect Immun 2007; 75:2067-2070.
11. Long JP, Tong HH, DeMaria TF. Immunization with native or recombinant *Streptococcus pneumoniae* neuraminidase affords protection in the chinchilla otitis media model. Infect Immun 2004; 72:4309-4313.
12. Cripps AW, Kyd JM. Comparison of mucosal and parenteral immunisation in two animal models of pneumococcal infection: otitis media and acute pneumonia. Vaccine 2007; 25:2471-2477.
13. McCullers JA, Karlstrom A, Iverson AR, Loeffler JM, Fischetti VA. Novel strategy to prevent otitis media caused by colonizing *Streptococcus pneumoniae*. PLoS Pathog 2007; 3:e28.
14. Swiatlo E, McDaniel LS, Briles DE. Choline-binding proteins. In: Tuomanen EI, Mitchell TJ, Morrison DA, Spratt BG, eds. The Pneumococcus. Washington, DC: American Society for Microbiology; 2004:49-60.
15. Mills MF, Marquart ME, McDaniel LS. Localization of PcsB of *Streptococcus pneumoniae* and its differential expression in response to stress. J Bacteriol 2007; 189:4544-4546.
16. Darrieux M, Miyaji EN, Ferreira DM, et al. Fusion proteins containing family 1 and family 2 PspA fragments elicit protection against *Streptococcus pneumoniae* that correlates with antibody-mediated enhancement of complement deposition. Infect Immun 2007; 75:5930-5938.
17. Moore QC, 3rd, Johnson L, Repka M, McDaniel LS. Immunization with PspA incorporated into a poly(ethylene oxide) matrix elicits protective immunity against *Streptococcus pneumoniae*. Clin Vaccine Immunol 2007; 14:789-791.
18. McDaniel LS, Ralph BA, McDaniel DO, Briles DE. Localization of protection-eliciting epitopes on PspA of *Streptococcus pneumoniae* between amino acid residues 192 and 260. Microb Pathogen 1994; 17:323-337.
19. Hollingshead SK, Becker R, Briles DE. Diversity of PspA: Mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. Infect Immun 2000; 68:5889-5900.
20. Ito Y, Osawa M, Isozumi R, et al. Pneumococcal surface protein A family types of *Streptococcus pneumoniae* from community-acquired pneumonia patients in Japan. Eur J Clin Microbiol Infect Dis 2007; 26:739-742.
21. Pimenta FC, Ribeiro-Dias F, Brandileone MC, et al. Genetic diversity of PspA types among nasopharyngeal isolates collected during an ongoing surveillance study of children in Brazil. J Clin Microbiol 2006; 44:2838-2843.
22. Simell B, Melin M, Lahdenkari M, Briles DE, Hollingshead SK, Kilpi TM, Kayhty H. Antibodies to pneumococcal surface protein A families 1 and 2 in serum and saliva of children

and the risk of pneumococcal acute otitis media. J Infect Dis 2007; 196:1528-1536.

23. Onwubiko C, Shires C, Quin LR, Swiatlo E, McDaniel LS. Characterization of *Streptococcus pneumoniae* isolated from children with otitis media. FEMS Immunol Med Microbiol 2007; 50:119-125.

24. McDaniel LS, Yother J, Vijayakumar M, McGarry L, Guild WR, Briles DE. Use of insertional inactivation to facilitate studies of biological properties of pneumococcal surface protein A (PspA). J Exp Med 1987; 165:381-394.

25. Ren B, McCrory MA, Pass C, et al. The virulence function of *Streptococcus pneumoniae* surface protein A involves inhibition of complement activation and impairment of complement receptor-mediated protection. J Immunol 2004; 173:7506-7512.

26. Tu AT, Fulgham RL, McCrory MA, Briles DE, Szalai AJ. Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. Infect Immun 1999; 67:4720-4724.

27. Håkansson A, Roche H, Mirza S, McDaniel LS, Brooks-Walter A, Briles DE. Characterization of Binding of Human Lactoferrin to Pneumococcal Surface Protein A. Infect Immun 2001; 69:3372-3381.

28. Farnaud S, Evans RW. Lactoferrin--a multifunctional protein with antimicrobial properties. Mol Immunol 2003; 40:395-405.

29. Shaper M, Hollingshead SK, Benjamin WH, Jr., Briles DE. PspA protects *Streptococcus pneumoniae* from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin [corrected]. Infect Immun 2004; 72:5031-5040.

30. Bosarge JR, Watt JM, McDaniel DO, Swiatlo E, McDaniel LS. Genetic Immunization with the Region Encoding the α-Helical Domain of PspA Elicits Protective Immunity against *Streptococcus pneumoniae*. Infect Immun 2001; 69:5456-5463.

31. McDaniel LS, Scott G, Kearney JF, Briles DE. Monoclonal antibodies against protease sensitive pneumococcal antigens can protect mice from fatal infection with *Streptococcus pneumoniae*. J Exp Med 1984; 160:386-397.

32. Swiatlo E, King J, Nabors GS, Mathews B, Briles DE. Pneumococcal surface protein A is expressed in vivo, and antibodies to PspA are effective for therapy in a murine model of pneumococcal sepsis. Infect Immun 2003; 71:7149-7153.

33. Ren B, Szalai AJ, Hollingshead SK, Briles DE. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. Infect Immun 2004; 72:114-122.

34. Swiatlo E, Brooks-Walter A, Briles DE, McDaniel LS. Oligonucleotides identify conserved and variable regions of *pspA* and *pspA*-like sequences of *Streptococcus pneumoniae*. Gene 1997; 188:279-294.

35. Brooks-Walter A, Briles DE, Hollingshead SK. The *pspC* gene of *Streptococcus pneumoniae* encodes a polymorphic protein, PspC, which elicits cross-reactive antibodies to PspA and provides immunity to pneumococcal bacteremia. Infect Immun 1999; 67:6533-6542.

36. Iannelli F, Oggioni MR, Pozzi G. Allelic variation in the highly polymorphic locus pspC of *Streptococcus pneumoniae*. Gene 2002; 284:63-71.

37. Dave S, Carmicle S, Hammerschmidt S, Pangburn MK, McDaniel LS. Dual roles of PspC, a surface protein of *Streptococcus pneumoniae*, in binding sIgA and factor H. J Immunol 2004; 173:471-477.

38. Quin LR, Carmicle S, Dave S, Pangburn MK, Evenhuis JP, McDaniel LS. In vivo binding of complement regulator factor H by *Streptococcus pneumoniae*. J Infect Dis 2005; 192:1996-2003.

39. Balachandran P, Brooks-Walter A, Virolainen-Julkunen A, Hollingshead SK, Briles DE. Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of *Streptococcus pneumoniae*. Infect Immun 2002; 70:2526-2534.

40. Quin LR, Onwubiko C, Moore QC, Mills MF, McDaniel LS, Carmicle S. Factor H binding to PspC of *Streptococcus pneumoniae* increases adherence to human cell lines in vitro and enhances invasion of mouse lungs in vivo. Infect Immun 2007; 75:4082-4087.

41. Cowan GJ, Atkins HS, Johnson LK, Titball RW, Mitchell TJ. Immunisation with anthrolysin O or a genetic toxoid protects against challenge with the toxin but not against Bacillus anthracis. Vaccine 2007; 25:7197-7205.

42. Palmer M. The family of thiol-activated, cholesterol-binding cytolysins. Toxicon 2001; 39:1681-1689.

43. Boulnois GJ, Paton JC, Mitchell TJ, Andrew PW. Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of *Streptococcus pneumoniae*. Mol Microbiol 1991; 5:2611-2616.

44. Rogers PD, Thornton J, Barker KS, McDaniel DO, Sachs GS, Swiatlo E, McDaniel LS. Pneumolysin-dependent and -independent gene expression identified by cDNA microarray

analysis in THP-1 human mononuclear cells stimulated by *Streptococcus pneumoniae*. Infect Immun 2003; 71:2087-2094.

45. Thornton J, McDaniel LS. THP-1 monocytes up-regulate intercellular adhesion molecule 1 in response to pneumolysin from *Streptococcus pneumoniae*. Infect Immun 2005; 73:6493-6498.

46. Kirkham LA, Kerr AR, Douce GR, Paterson GK, Dilts DA, Liu DR, Mitchell TJ. Construction and immunological characterization of a novel nontoxic protective pneumolysin mutant for use in future pneumococcal vaccines. Infect Immun 2006; 74:586-593.

47. Walker JA, Allen RL, Falmagne P, Johnson MK, Boulnois GJ. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. Infect Immun 1987; 55:1184-1189.

48. Balachandran P, Hollingshead SK, Paton JC, Briles DE. The Autolytic Enzyme LytA of *Streptococcus pneumoniae* Is Not Responsible for Releasing Pneumolysin. J Bacteriol 2001; 183:3108-3116.

49. Camara M, Boulnois GJ, Andrew PW, Mitchell TJ. A neuraminidase from *Streptococcus pneumoniae* has the features of a surface protein. Infect Immun 1994; 62:3688-3695.

50. Yesilkaya H, Manco S, Kadioglu A, Terra VS, Andrew PW. The ability to utilize mucin affects the regulation of virulence gene expression in *Streptococcus pneumoniae*. FEMS Microbiol Lett 2008; 278:231-235.

51. Simell B, Jaakkola T, Lahdenkari M, Briles DE, Hollingshead S, Kilpi TM, Kayhty H. Serum antibodies to pneumococcal neuraminidase NanA in relation to pneumococcal carriage and acute otitis media. Clin Vaccine Immunol 2006; 13:1177-1179.

52. Tong HH, James M, Grants I, Liu X, Shi G, DeMaria TF. Comparison of structural changes of cell surface carbohydrates in the eustachian tube epithelium of chinchillas infected with a *Streptococcus pneumoniae* neuraminidase-deficient mutant or its isogenic parent strain. Microb Pathog 2001; 31:309-317.

53. King SJ, Hippe KR, Gould JM, et al. Phase variable desialylation of host proteins that bind to *Streptococcus pneumoniae* in vivo and protect the airway. Mol Microbiol 2004; 54:159-171.

54. Shakhnovich EA, King SJ, Weiser JN. Neuraminidase expressed by *Streptococcus pneumoniae* desialylates the lipopolysaccharide of *Neisseria meningitidis* and *Haemophilus influenzae*: a paradigm for interbacterial

competition among pathogens of the human respiratory tract. Infect Immun 2002; 70:7161-7164.

55. Manco S, Hernon F, Yesilkaya H, Paton JC, Andrew PW, Kadioglu A. Pneumococcal neuraminidases A and B both have essential roles during infection of the respiratory tract and sepsis. Infect Immun 2006; 74:4014-4020.

56. Tong HH, Li D, Chen S, Long JP, DeMaria TF. Immunization with recombinant *Streptococcus pneumoniae* neuraminidase NanA protects chinchillas against nasopharyngeal colonization. Infect Immun 2005; 73:7775-7778.

57. Brown JS, Gilliland SM, Holden DW. A *Streptococcus pneumoniae* pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. Mol Microbiol 2001; 40:572-585.

58. Jomaa M, Yuste J, Paton JC, Jones C, Dougan G, Brown JS. Antibodies to the iron uptake ABC transporter lipoproteins PiaA and PiuA promote opsonophagocytosis of *Streptococcus pneumoniae*. Infect Immun 2005; 73:6852-6859.

59. Whalan RH, Funnell SG, Bowler LD, Hudson MJ, Robinson A, Dowson CG. Distribution and genetic diversity of the ABC transporter lipoproteins PiuA and PiaA within *Streptococcus pneumoniae* and related streptococci. J Bacteriol 2006; 188:1031-1038.

60. Whalan RH, Funnell SG, Bowler LD, Hudson MJ, Robinson A, Dowson CG. PiuA and PiaA, iron uptake lipoproteins of *Streptococcus pneumoniae*, elicit serotype independent antibody responses following human pneumococcal septicaemia. FEMS Immunol Med Microbiol 2005; 43:73-80.

61. Brown JS, Ogunniyi AD, Woodrow MC, Holden DW, Paton JC. Immunization with Components of Two Iron Uptake ABC Transporters Protects Mice against Systemic *Streptococcus pneumoniae* Infection. Infect Immun 2001; 69:6702-6706.

62. Jomaa M, Terry S, Hale C, Jones C, Dougan G, Brown J. Immunization with the iron uptake ABC transporter proteins PiaA and PiuA prevents respiratory infection with *Streptococcus pneumoniae*. Vaccine 2006; 24:5133-5139.

63. Ware D, Watt J, Swiatlo E. Utilization of putrescine by *Streptococcus pneumoniae* during growth in choline-limited medium. J Microbiol 2005; 43:398-405.

64. Shah P, Marquart M, Quin LR, Swiatlo E. Cellular location of polyamine transport protein PotD in *Streptococcus pneumoniae*. FEMS Microbiol Lett 2006; 261:235-237.

65. Ware D, Jiang Y, Lin W, Swiatlo E. Involvement of potD in *Streptococcus pneumoniae* polyamine

transport and pathogenesis. Infect Immun 2006; 74:352-361.

66. Shah P, Swiatlo E. Immunization with polyamine transport protein PotD protects mice against systemic infection with *Streptococcus pneumoniae*. Infect Immun 2006; 74:5888-5892.

67. Briles DE, Hollingshead SK, Paton JC, Ades EW, Novak L, vanGinkel FW, Benjamin Jr WH. Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with *Streptococcus pneumoniae*. J Infect Dis 2003; 188:339-348.

68. Cao J, Chen D, Xu W, et al. Enhanced protection against pneumococcal infection elicited by immunization with the combination of PspA, PspC, and ClpP. Vaccine 2007; 25:4996-5005.

69. Ogunniyi AD, Grabowicz M, Briles DE, Cook J, Paton JC. Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. Infect Immun 2007; 75:350-357.

70. McDaniel LS, Loechel F, Benedict C, Greenway T, Briles DE, Conry RM, Curiel DT. Immunization with a plasmid expressing pneumococcal surface protein A (PspA) can elicit protection against fatal infection with *Streptococcus pneumoniae*. Gene Therapy 1997; 4:375-377.

71. Malley R, Trzcinski K, Srivastava A, Thompson CM, Anderson PW, Lipsitch M. CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. Proc Natl Acad Sci U S A 2005; 102:4848-4853.

72. McCool TL, Weiser JN. Limited role of antibody in clearance of *Streptococcus pneumoniae* in a murine model of colonization. Infect Immun 2004; 72:5807-5813.

73. Basset A, Thompson CM, Hollingshead SK, Briles DE, Ades EW, Lipsitch M, Malley R. Antibody-independent, CD4+ T cell-dependent protection against pneumococcal colonization elicited by intranasal immunization with purified pneumococcal proteins. Infect Immun 2007; 75:5460-5464.

74. Hanniffy SB, Carter AT, Hitchin E, Wells JM. Mucosal delivery of a pneumococcal vaccine using *Lactococcus lactis* affords protection against respiratory infection. J Infect Dis 2007; 195:185-193.

75. Nayak AR, Tinge SA, Tart RC, McDaniel LS, Briles DE, Curtiss III R. A live recombinant avirulent oral salmonella vaccine expressing pneumococcal surface protein A (PspA) induced protective responses against *Streptococcus pneumoniae*. Infect Immun 1998; 66:3744-3751.

76. Audouy SA, van Roosmalen ML, Neef J, Kanninga R, Post E, van Deemter M, Metselaar H, van Selm S, Robillard GT, Leenhouts KJ, Hermans PW. *Lactococcus lactis* GEM particles displaying pneumococcal antigens induce local and systemic immune responses following intranasal immunization. Vaccine 2006; 24:5434-5441.

77. Audouy SA, van Selm S, van Roosmalen ML, et al. Development of lactococcal GEM-based pneumococcal vaccines. Vaccine 2007; 25:2497-2506.

The role of viruses in otitis media

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Introduction

In this review the following topics will be addressed: 1) our understanding of the pathogenesis of viral otitis media (OM); 2) the viruses implicated in OM, 3) current and future viral vaccines for the prevention of OM; and 4) an estimation of what the impact of such vaccines might be on this incredibly common and debilitating problem.

Pathogenesis of OM

A logical assumption could be that viruses play little role in OM. Classically, the pathogenesis of OM is thought to be obstruction of the eustachian tube leading to a vacuum and subsequent collection of fluid in the middle ear into which bacteria that colonize the nasopharynx migrate and multiply. Bacteria can be recovered from the middle-ear space in a substantial number of cases of OM, and the appearance of the fluid in the acutely inflamed ear suggests a bacterial infection. Furthermore, a response to antibiotics is commonly seen, and though some have argued for watchful waiting in approaching the care of acute OM, the widespread use of antibiotics has limited the occurrence of serious complications of OM, such as mastoiditis.

There is evidence that bacterial vaccines, notably pneumococcal vaccines, decrease the occurrence of OM. However, this is most strikingly seen when the results are limited to proven pneumococcal OM, and the overall decrease in OM in one major study was only 6%, with confidence limits that overlapped zero.[1] The complexity of analyzing prevention of OM by vaccines was nicely documented in a paper in Vaccine.[2] These considerations of diagnostic method, prevention of primary versus recurrent disease, duration of follow-up, and statistical methods applied will equally apply to assessment of viral vaccines.

An equally strong argument can be made for a primary role for viruses in the causation of OM based on the frequency of preceding viral upper respiratory infection (30-50% of viral infections with respiratory syncytial virus (RSV), parainfluenza virus (PIV), and influenza infections are accompanied by OM) and the frequent identification of viruses, particularly by PCR, from middle-ear fluid (see discussion below).

We, and most investigators in the field, now consider OM to be a combined viral and bacterial infection. One of the clearer demonstrations of the interactions of viruses and bacteria in OM came out of our collaboration with Dr. Giebink using his chinchilla model.[3,4] From this work emerged the following concepts:

- In the chinchilla, bacterial OM results only with direct inoculation of pneumococci into the middle ear through the tympanic membrane.
- In contrast, antecedent establishment of influenza A/H3N2 nasal infection leads to OM after intranasal inoculation of pneumococci.
- Influenza caused negative middle-ear pressure as a result of mucosal edema and cellular sloughing that extended into the eustachian tube, suggesting the role of viruses in creating the middle-ear environment for bacterial growth.

The effect of a type 7F specific pneumococcal vaccine and both live and inactivated influenza A/Texas/77 H3N2 vaccines on influenza virus replication were compared in the chinchilla model (Fig. 1). The pattern of influenza virus shedding reflected the importance of local immunity induced by the live intranasal vaccine in rapidly terminating influenza infection, although the inactivated influenza vaccine reduced virus shedding on day 6. These studies were not expanded to a full study of the role of the respective vaccines on limiting the induction of OM, but suggest the potential importance of viral vaccines in altering the frequency of OM.

We have studied influenza and RSV replication in primary cells of respiratory tract origin derived from adenoids.[5] In these systems, the effect of viruses on cell morphology and function can be examined (Fig. 2). We have tried to demonstrate an interaction between pneumococci and influenza or RSV. Although we can clearly show adherence and limited invasion of epithelial cells by pneumococci, there does not seem to be an enhancement of either viral or bacterial growth and only rarely a co-localization of viruses and bacteria with co-infection.

Interesting work suggests a possible enhancement of influenza viral infection by virtue of proteases produced by some bacteria.[6]

Viruses implicated in OM

The literature on viral involvement in OM is confounded by a number of variables. The first is the site and quality of the specimen collection. Most specimens are taken from the respiratory tract, often using a nasopharyngeal swab. This may not reflect what is present in the middle-ear fluid. Because OM may be a secondary phenomenon occurring late in the course of the infection, the sample may be taken after the optimal time for virus recovery.

A second variable is the technique used for virus identification. The gold standard has been viral culture. Although effective for some viruses, for others—rhinovirus, human metapneumovirus, or coronavirus as examples—it is insensitive. The most current technique to be considered is polymerase chain reaction (PCR). Exciting results are emerging when this technique is applied to middle-ear fluid, with 48% of aspirates being positive for rhinovirus, RSV, or human coronavirus.[7] There is little question that PCR will prove to be our most sensitive technique. However, we have recently shown the high rate of recovery of rhinovirus PCR product in well individuals of all age groups, indicating that specificity and or duration of virus replication after infection needs further examination. Rapid viral diagnosis by detection of viral antigen is a technique that has practical utility because it can be applied in physicians' offices. However, such rapid techniques are neither sensitive nor specific enough for research studies on the role of viruses in OM.

A third variable is that few studies have looked comprehensively at all of the viruses implicated in OM. Some studies have focused on influenza, RSV, and parainfluenza.[8-10] Others, particularly those that are PCR-based, have selectively looked for rhinovirus and coronavirus. A recent paper overcomes some of these problems with a comprehensive look at nasopharyngeal carriage of most of the common viruses by PCR (the most recently identified viruses human metapneumovirus, BoCa virus, and the newer corona viruses are not included).[11] An extraction of the data for total viral-associated illness is shown in Table 1. Cold-like respiratory illness was strongly associated with viral identification as was OM even if unaccompanied by a discrete cold-like illness. Of note, no virus had a particular association with OM, but by far the most commonly identified virus was rhinovirus–the most difficult target for a vaccine because of the multiple serotypes.

Current viral vaccines for OM

Currently, two influenza viral vaccines may provide protection for OM. Trivalent inactivated influenza vaccines are licensed and recommended for use from the age of 6 months: "Because children aged 6-23 months are at substantially increased risk for influenza-related hospitalizations, ACIP [Advisory Committee on Immunization Practices], AAP [American Academy of Pediatrics], and AAFP [American Academy of Family Physicians] continue to encourage vaccination when feasible."[8] Two studies have examined the prevention of OM by inactivated influenza vaccine.[9,10] The results of these studies are summarized in Table 2.

A live, attenuated, intranasal vaccine similar to the vaccine used in the chinchilla study described above is now licensed for use in people ages 5 to 50 years.[11] Unfortunately, the current licensure does not provide an indication in the age group most susceptible to influenza-associated OM. In trials in younger children, the incidence of febrile OM was 30% (95% CI 18–45) lower among vaccine recipients (14 cases per 100 vaccine recipients compared with 20 cases per 100 placebo recipients) ($p < .001$) from the time of vaccination (summer/fall 1996 to 1997).[15] A recent study has shown greater effectiveness of live, attenuated vaccine than inactivated influenza vaccines in the prevention of influenzal illness. With OM, this difference was particularly apparent when the vaccine and circulating virus were not perfectly matched. Overall, there were twice as many cases of OM diagnosed in recipients of inactivated vaccine as live vaccine.[16]

Viral vaccines under development

For the parainfluenza viruses and RSV, there are active programs in vaccine development using reverse genetics to define and stabilize attenuating mutations, optimize gene expression, and/or create chimeric viruses that may take advantage of host range restriction. With parainfluenza type III, bovine-human chimeric viruses and cold-passaged virus, cp45, have undergone human trials.[17,18] As live, attenuated, intranasally administered viruses, the possibility that the vaccines themselves might initiate OM has been examined carefully with no evidence of such an association. Neither vaccine has been evaluated in a formal efficacy trial. Live, attenuated RSV vaccines have been developed that are safe and immunogenic in

the 6- to 24-month-old age range.[15] They have undergone phase I and phase II trials alone and in combination with cp45 PIV 3 vaccine. The challenge of deriving a candidate suitable for use in 1- to 2-month-old infants has so far blocked their broader assessment for efficacy against OM.

Future viral vaccines for OM

The remaining viruses in the pantheon of viruses implicated in OM each have their own problems. A capsule summary would be that:

- Coronaviruses have not, to date, been the target for any significant vaccine effort; however, they will get a boost through research for a severe acute respiratory syndrome (SARS) vaccine.
- Rhinoviruses have a daunting number of serotypes (more than 100), which has discouraged development of immunoprophylaxis.
- Successful adenovirus vaccines have been delivered enterically and used to prevent adenovirus-associated acute respiratory illness in the military. At present, there is widespread use of adenoviruses as vectors for vaccination or gene therapy. It is possible that this may stimulate the field.
- Human metapneumovirus have recently been discovered, and there is already a vaccine effort underway.

Conclusions

Viral vaccines, particularly those that limit viral replication in the upper respiratory tract by stimulating virus-specific immunoglobulin A antibody, may have a dramatic effect on the incidence of OM and subsequent use of antimicrobials in this disease. This hypothesis will have to be addressed in carefully designed prospective trials with OM as a specific primary endpoint.

Figure 1. Influence of prior vaccination with live attenuated influenza vaccine (CR29), inactivated influenza vaccine (Killed A/Tex), and pneumococcal vaccine (Pneumovax) on shedding of influenza virus from the chinchilla respiratory tract.

Days after challenge with wild type virus
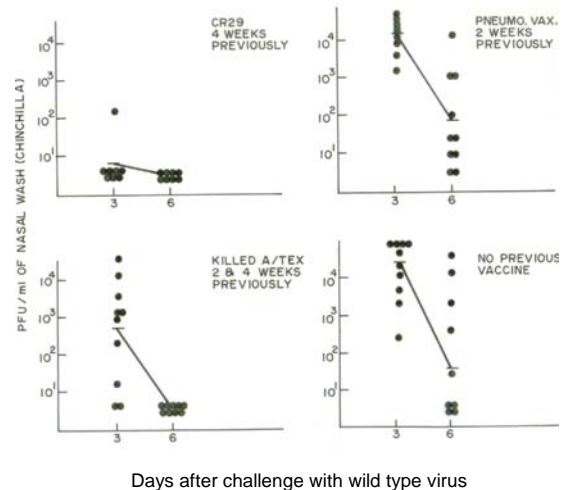
Figure 2. Immunoperoxidase staining for influenza-specific proteins in primary epithelial cells growing as an adenoidal organ culture.

A/Beijing AOC 24 hr 40x
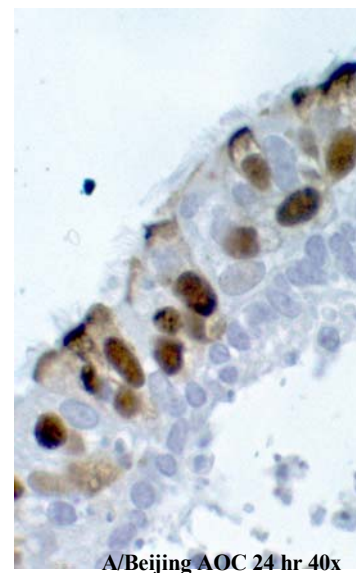
Table 1 Association of viruses with cold-like illness (CLI) and otitis media (OM) (adapted from reference 11)

Clinical setting at time of Viral Sample (#Positive/total sampled (% pos.))				
CLI	No CLI	OM withCLI	OM without CLI	Well
94/128 (73%)	54/297 (18%)	43/55 (78%)	18/24 (75%)	36/273 (13%)

Table 2 Protection against otitis media afforded by inactivated influenza vaccines

Reference	Group	No.	AOM	AOM with documented influenza
[13]	Vaccine	187	36	3
	Control	187	60	9
[14]	Vaccine	94	20	NA
	Control	92	34	NA

AOM = acute otitis media; NA = not available.

*$p < .01$ (6-week study period during influenza epidemic).

†$p = .02$ (6-week study period during influenza epidemic).

References

1. Eskola J, Kilpi T, Palmu A, et al. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. NEJM 2001; 344:403-409.
2. Jahn-Eimermacher A, du Prel J-B, Schmitt H-J. Assessing vaccine efficacy for the prevention of acute otitis media by pneumococcal vaccination in children: A methodological overview of statistical practice in randomized controlled clinical trials Vaccine 2007; 25:6237-6244.
3. Giebink GS, Wright PF. The pathogenesis of experimental otitis during respiratory viral infection 1984. In: Lim DJ, Bluestone CD, Klein JO, Nelson JD, eds. *Recent advances in otitis media with effusion: proceedings of the Third International Symposium*. Philadelphia: BC Decker Inc; 1984:107–111.
4. Giebink GS, Wright PF. Different virulence of influenza A virus strains and susceptibility to pneumococcal otitis media in chinchillas. Infect Immun 1983; 41:913–920.
5. Endo Y, Carroll KN, Ikizler MR, Wright PF. Growth of influenza A virus in primary, differentiated epithelial cells derived from adenoids. J Virol 1996; 70:2055–2058.
6. Scheiblauer H, Reinacher M, Tashiro M, Rott R. Interactions between bacteria and influenza A virus in the development of influenza pneumonia. J Infect Dis 1992; 166:783-791.
7. Pitkäranta A, Virolainen A, Jero J, et al. Detection of rhinovirus, respiratory syncytial virus, and coronavirus infections in acute otitis media by reverse transcriptase polymerase chain reaction. Pediatrics 1998**;** 102**:**291–295.
8. Neuzil KM, Mellen BG, Wright PF, et al. The effect of influenza on hospitalizations, outpatient visits, and courses of antibiotics in children. N Engl J Med 2000; 342:225–231.
9. Fisher RG, Gruber WC, Edwards KM, et al. Twenty years of outpatient respiratory syncytial virus infection: a framework for vaccine efficacy trials. Pediatrics 1997; 99:E7.
10. Reed G, Jewett PH, Thompson J, et al. Epidemiology and clinical impact of parainfluenza virus infections in otherwise healthy infants and young children < 5 years old. J Infect Dis 1997; 175:807–813.
11. Winther B, Alper CM, Mandel EM, Doyle WJ, Hendley JO. Temporal relationships between colds, upper respiratory viruses detected by polymerase chain reaction, and otitis media in young children followed through a typical cold season. Pediatrics 2007; 119:1069-1075.
12. Fiore AE, Shay DK, Haber P, et al. Prevention and control of influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP), 2007. MMWR Morb Mortal Wkly Rep 2007; 56/RR-6:1–54.
13. Heikkinen T, Ruuskanen O, Waris M, et al. Influenza vaccination in the prevention of acute otitis media in children. Am J Dis Child 1991; 145:445–448.
14. Clements DA, Langdon L, Bland C, Walter E. Influenza A vaccine decreases the incidence of otitis media in 6- to 30-month-old children in day care. Arch Pediatr Adolesc Med 1995; 149:1113–1117.
15. Belshe RB, Gruber WC. Prevention of otitis media in children with live attenuated influenza vaccine

given intranasally. Pediatr Infect Dis J 2000; 19:S66–71.

16. Belshe RB, Edwards KM, Vesikari T. Live Attenuated versus inactivated influenza vaccine in infants and young children. NEJM 2007; 356:685-696.

17. Karron RA, Wright PF, Murphy BR, et al. A live attenuated bovine parainfluenza type 3 virus vaccine is safe, infectious, immunogenic and phenotypically stable in healthy infants and children. J Infect Dis 1995; 171:1107–1114.

18. Karron RA, Wright PF, Newman FK, et al. A live human parainfluenza type 3 virus vaccine is attenuated and immunogenic in healthy infants and children. J Infect Dis 1995; 172:1445–1450.

19. Wright PF, Karron RA, Belshe RB, et al. Evaluation of a live, cold-passaged, temperature sensitive, respiratory syncytial virus vaccine in infancy.

20. J Infect Dis 2000; 182:1331–1342.

Mini-Symposium 3: Relevance of Biofilms in Managing and Preventing Otitis Media

The biofilm paradigm and the distributed genome hypothesis provide a useful rubric for the study of chronic bacterial infections such as otitis media with effusion

Garth Ehrlich, Ph.D.

The biofilm paradigm of chronic infectious mucosal diseases, which we first promulgated more than a decade ago, has proven to be highly informative and instructive with respect to modeling and understanding chronic otitis media with effusion and recurrent otitis media. In today's session we have a mix of the old guard in this field as well as the young scientists who are pushing this discipline forward.

We will begin with an update on the latest in bacterial communication and signaling, which is important for the structure and dynamics of biofilm communities. Next we will hear convincing evidence that OME and ROM are indeed mucosal biofilm diseases. These presentations will be followed by a series of talks providing the evidence that supports the Distributed Genome Hypothesis (DGH). The DGH provides a framework for understanding bacterial genotypic heterogeneity in much the same way that the biofilm paradigm provides a framework for understanding phenotypic heterogeneity. The first of these talks documents that natural bacterial colonizations/infections are polyclonal in nature, thereby providing the fuel for genetic recombination. The next talk elegantly demonstrates that the biofilm matrix contains large amounts of DNA, thus demonstrating that these naturally competent bacteria have DNA available for transformation. The final talk in this series provides evidence of bacterial supragenomes and demonstrates the enormous genomic plasticity present among clinical isolates of otitis media with effusion pathogens. The session will then conclude with a look at emergent anti-biofilm methods that are designed to exploit our new knowledge of chronic infections to effect a cure.

The ying and yang of quorum sensing in *Pseudomonas aeruginosa* biofilms

Barbara Iglewski, Ph.D.

Pseudomonas aeruginosa is a common cause of chronic otitis media (OM) following the insertion of ear tubes. Under these conditions, P. aeruginosa forms a biofilm and as such becomes highly resistant to conventional antibiotics. To prevent or treat such infections, it is imperative that we have a better understanding of how P. aeruginosa forms these biofilms and identify what genes or gene products are essential for biofilm development and maintenance.

Previous studies have shown that P. aeruginosa utilizes cell-to-cell communication systems called quorum sensing (QS) to respond in a cell density-dependent manner. Two distinct QS systems have been identified in P. aeruginosa, the lasR and the rhlR system. The lasR system has been shown to be the dominant system in that it is required for maximum transcription of the rhlR system. Together these two QS systems have been shown to regulate expression of more than 600 genes, including some thought to play a role in biofilm development and maintenance.

Our research has focused on trying to elucidate the QS regulon in P. aeruginosa to determine which genes contribute the most to biofilm development and maintenance. The results suggest that QS is much more complicated than originally thought. Clearly, there are numerous levels of regulation in this global regulon. Research from a number of laboratories has now shown that interference with the las QS system impedes normal biofilm development, whereas interference with the rhlR system prevents normal biofilm dispersal. Importantly, interference with normal biofilm architecture and development renders P. aeruginosa more susceptible to antimicrobials. These observations suggest a possible approach to both prevention of P. aeruginosa biofilms as well as one that may be useful in rendering established P. aeruginosa biofilms more susceptible to conventional therapeutic agents.

Chronic otitis media as a biofilm disease

Joseph Kerschner, M.D., J. Christopher Post, M.D., Ph.D., Paul Stoodley, Ph.D., Laura Nisticio, Ph.D., Luann Stoodley, Ph.D., Fen Ze Hu, Ph.D., Garth Ehrlich, Ph.D.

Background

Chronic otitis media (OM) is a common pediatric infectious disease. Intervention for OM has generally involved the use of antimicrobial agents to treat episodes of acute infection, with myringotomy and tympanostomy tube insertion for children with chronic conditions. However, the use of antimicrobials has become increasingly complicated by the development of antimicrobial resistance by each of these microbes. This has necessitated higher dosing of antimicrobial agents and attempts to develop new compounds. Given these difficulties, there has been significant interest in obtaining a greater understanding of the pathophysiology of OM on a cellular and molecular level to obtain new insights and to potentially develop novel treatment strategies and interventions.

Previous studies demonstrate that metabolically active bacteria exist in culture-negative pediatric middle-ear effusions. Further, experimental infection with *Haemophilus influenzae* in the chinchilla model of OM results in the formation of adherent mucosal biofilms, suggesting that chronic OM may result from a mucosal biofilm infection.

Although the concept that biofilms are the primary source of bacterial infection in chronic forms of OM is novel, the association of biofilms with other chronic forms of infection is certainly not. Other examples of chronic bacterial biofilm infection in humans include infections of orthopedic implants, endocarditis, indwelling catheter infections, and periodontal infections.[1-4]

Most bacteria in nature exist in a biofilm state and not the more commonly understood planktonic state. The planktonic state, which is the form of bacteria usually considered in human infectious diseases, consists of bacteria floating in suspension, which then attack host cells and cause cellular damage. In contrast, a biofilm forms when an organized collection of bacteria become anchored to a surface and form a microcolony surrounded by a complex matrix that is constantly being remodeled and includes polysaccharides, nucleic acids, and proteins that are secreted by the bacteria themselves. The characteristics of the biofilm state of bacteria include: 1) anchored to a surface, 2) surrounded by polymeric matrix, 3) low metabolic rate, 4) ability to escape host immune surveillance, 5) reliance on complex intracellular communication system that provides for organized growth characteristics called quorum sensing, 6) resistance to standard culture techniques because of altered metabolism, 7) altered genetic expression and ability to rapidly share genetic information, and 8) periodic showering of planktonic bacteria that result in episodes of acute infection: fever, inflammation, and cellular injury.

Evidence that chronic OM is the result of a biofilm infection has been based on several well-designed studies. Studies using an animal model demonstrated that clinical isolates of *H. influenzae* formed biofilms on the middle-ear mucosa (MEM) of the chinchilla.[5] This study was the first to demonstrate direct evidence of biofilm formation in an in vivo animal model. However, these studies were performed under controlled conditions and did not conclusively demonstrate that biofilms were present or important in the pathogenesis of OM in children.

Objective

The objective is to discuss recent data demonstrating that chronic OM in humans is biofilm-related and to integrate these findings into current understanding of OM management. These studies have demonstrated that biofilms exist in the middle-ear space in association with OM and that these biofilms represent an underlying causative factor in the development of chronic OM.

Results

Recent human studies have also demonstrate that an overwhelming percentage, (>90%) of children that have OM severity that requires tympanostomy tube placement, also have middle-ear mucosal (MEM) biofilms.[6] In this study, the existence of biofilms on the MEM of children with chronic OM was demonstrated using multiple specific staining and visualization techniques. Perhaps as importantly, control specimens from patients without evidence of OM demonstrated no evidence of biofilm formation.

Study group

Children undergoing tympanostomy tube placement for chronic otitis media with effusion (COME) or recurrent otitis media (RecOM).

Definitions

1)	COME: Any patient with persistent middle-ear effusion for greater than three months' duration.
2)	RecOM: Any patient with three or more episodes of acute OM over a 6-month period with resolution between episodes and middle-ear fluid that does not persist greater than 3 months' duration.
3)	Absence of history of O (Control Group): One or fewer episodes of OM requiring a visit to a health professional per 12-month period of life.

In this study, from a tertiary referral otolaryngology practice, MEM biopsy specimens were obtained from 26 children (mean age, 2.5 years) undergoing tympanostomy tube placement for treatment of chronic OM. Thirteen of these children had a diagnosis of OME and 20 had a diagnosis of recurrent OM with seven having both diagnoses. Middle-ear fluid and the MEM biopsies were analyzed using microbiological culture, polymerase chain reaction (PCR)-based diagnostics, direct microscopic examination, fluorescence in situ hybridization, and immunostaining. Uninfected (control) MEM specimens were obtained from patients undergoing cochlear implantation.

Using these specific bacteriologic identification strategies, molecular techniques, and microscopy, both *H. influenzae* and *S. pneumoniae* biofilms were identified in these clinical specimens at a high rate (92%) by two or more of these methods. Patients without a history of OM did not demonstrate MEM biofilms.

Conclusion

Direct detection of biofilms on MEM biopsy specimens from children with OME and recurrent OM supports the hypothesis that these chronic middle-ear disorders are biofilm-related. These findings, along with other controlled experiments, support the hypothesis that biofilms play a role in the pathogenesis and chronicity of OME and also provide evidence that the MEM in patients with recurrent OM harbor bacterial biofilms. It has been postulated that each of these recurrent infections represented a new episode caused by a unique bacterial strain ascending through the nasopharynx and eustachian tube into the middle-ear space. However, these findings would suggest that viable bacteria are capable of residing as biofilms within the middle-ear space in between acute exacerbations of bacterial infection and that, at least in part, may be responsible for the recrudescence of these acute infections.

The findings from these studies do not exclude other potential pathogenic factors associated with chronic OM, and the new findings can be incorporated with the earlier concepts to provide more robust explanations for both OM pathogenesis. Factors such as viral upper respiratory infection, eustachian tube dysfunction, or a genetically predisposed host, could each be incorporated into the concept of facilitating biofilm formation. However, the ubiquitous presence of biofilms in clinical specimens would argue that investigations aimed at linking these factors to biofilm formation may provide significant scientific advances toward understanding chronic OM and developing novel treatment regimens.

In summary, there is a growing body of evidence demonstrating that chronic infections in humans are the result of biofilm formation, and recent research would suggest that OM should also be included in this category. An understanding of the concepts of biofilm formation will likely be important to advance our knowledge of the pathogenesis of OM.

References

1.	Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999; 284:1318-1322.
2.	Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as complex differentiated communities. Annu Rev Microbiol 2002; 56:187-209.
3.	Costerton W, Veeh R, Shirtliff M, Pasmore M, Post C, Ehrlich G. The application of biofilm science to the study and control of chronic bacterial infections. J Clin Invest 2003; 112:1466-1477.
4.	Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2004; 2:95-108.
5.	Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 2002; 15:167-193.
6.	Hall-Stoodley L, Hu FZ, Gieseke A, et al. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. JAMA 2006; 296:202-211.

Pharyngeal colonization dynamics of *Haemophilus influenzae*

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Bacterial colonization of the human respiratory mucosal surface represents a dynamic process in which bacteria are acquired, replaced, and reacquired many times in a lifetime. Past studies have demonstrated that nontypeable *Haemophilus influenzae* (NTHi) colonization of the pharynx is characterized by rapid bacterial turnover[1-4] and carriage of multiple strains at any one time.[4-8] In addition to *H. influenzae,* the phylogenetically closely related, non-pathogenic species *Haemophilus haemolyticus* is also found in the pharynx of healthy adults.[9] This organism is classically differentiated from NTHi in the laboratory by its ability to produce a clear hemolytic zone on horse blood agar. The hemolytic activity of *H. haemolyticus*, however, may be lost on subculture resulting in difficulty differentiating it from *H. influenzae*. Since discriminating *H. haemolyticus* from *H. influenzae* is important to fully understand the pathogenesis of *H. influenzae* infection, in this study the non-hemolytic *H. influenzae*-like isolates were further tested for the presence of a specific epitope on the outer membrane protein P6 and the presence of *iga*, both of which have been shown to be associated with *H. influenzae* and not *H. haemolyticus.*[10-11]

Although *H. influenzae* pharyngeal colonization in the context of disease has been relatively well studied, its carriage and the dynamics of colonization have not been well-characterized in healthy adults. This study used pulsed field gel electrophoresis (PFGE) to describe the genetic diversity and dynamics of colonization of *H. haemolyticus* and *H. influenzae* over 7 months in four healthy adults.

Materials and methods

Study design. Throat culture samples for NTHi were obtained from 16 healthy adults after informed consent as approved by the University of Michigan Institutional Review Board. Repeated throat culture samples from four of the participants shown to carry NTHi were obtained, 5 work days a week for the first month followed by once a week for the next 6 months.

Bacteriologic methods. The throat culture swabs were streaked on selective chocolate agar plates as previously described.[12] Up to 30 isolates per throat sample consistent with *Haemophilus* morphology were

selected for further analysis. *H. influenzae* isolates were defined as X and V factor dependent, porphyrin negative, *iga* (which encodes IgA protease) positive[13], reactive with monoclonal antibody 7F3, which recognizes an epitope on P6 that is highly specific for *H. influenzae* strains[14], and unable to hemolyze horse erythrocytes. *H. haemolyticus* isolates were defined as X and V factor dependent, porphyrin negative, *iga* negative, non-reactive with monoclonal antibody 7F3, and either negative or positive for hemolysis of horse erythrocytes.

Capsular typing. All suspected *H. influenzae* isolates were analyzed by slide agglutination for capsular serotype using *H. influenzae* capsular serotyping sera (Difco, Detroit MI). Ambiguous results were confirmed by PCR using the method described by Falla et al.[15] Strains that carried the type b specific gene region but did not have the *bexA* gene required for export of the capsule to the cell surface were designated as b⁻ mutants.

Genotyping. The bacterial strains were initially genotyped using Uptake Signal Sequence (USS)-PCR, a repetitive-element genotyping method recently developed in our laboratory that takes advantage of the multiple copies of the 9 base-pair DNA segment (5'-AAGTGCGGT-3') that promotes dsDNA uptake by the naturally competent *Haemophilus* bacteria. Confirmatory genotyping of representative USS-PCR genotypes in each carrier (468 strains) used PFGE[16-17], employing first *SmaI* digestion and then *EagI* digestion for *SmaI* undigestible isolates. Cluster analysis was performed separately for the PFGE band-patterns generated by *Sma*I and *Eag*I using BioNumerics software from Applied Math (Kortrijk, Belgium) and the patterns were confirmed by visual inspection. Band patterns were designated unique on the basis of ≥7 band differences, in accordance with Tenover's criteria[18] for determining genotypic differences between possible epidemic bacterial strains.

Results

The *Haemophilus* isolates from the four carriers showed significant genotypic diversity within each carrier and the genotypes of the recovered strains varied from day-to-day and from week-to-week. Carrier 2 was colonized solely with non-typeable NTHi (15 different genotypes over seven months) and

the other three carriers were colonized with non-typeable NTHi as well as hemolytic and non-hemolytic *H. haemolyticus*. Carrier 4 carried *H. haemolyticus* as unencapsulated *H. influenzae* type b. Tables 1-4 show the patterns of colonization over the sampling period.

Discussion

Widespread genetic diversity is well-recognized[13, 16, 17] among NTHi strains. The results of this study in healthy adults confirm previous studies in children[17] that demonstrated genotypic diversity and apparent frequent bacterial turnover. We hypothesize that the day-to-day and week-to-week turnover reflects a large sampling bias, as each sample likely contained only a few of the NTHi actually present in each subject's respiratory tract; each sample most likely captured the most predominant strains at that sampling site at that time.

In addition, these results appear to reflect evolutionary processes that occur among *H. influenzae* during asymptomatic colonization. For example, genotype S1 in carrier 4 contains both hemolytic and non-hemolytic *H. hemolyticus*, suggesting that some of the isolates of that genotype have lost their hemolysin genes. Similarly, genotype S3 in carrier 1 contains two colonies of non-hemolytic *H. haemolyticus* and one colony of *H. influenzae*, suggesting that a parent *H. influenzae* clone lost both the 7F3 epitope of P6 and iga or that a parent *H. haemolyticus* clone gained both. Genotype S6 of carrier 3, which contains both NTHi and Hib- isolates is difficult to explain. It is possible that both genotype S6 of carrier 3 and S3 of carrier 1 actually represent two distinct genotypes that weren't detected by *Sma*I digestion. Furthermore, our results (not shown) demonstrated a variation in patterns that did not meet the criteria for a unique genotype and likely reflect evolutionary changes that occurred over time in the restriction sites cut by *Sma*I or *Eag*I.

Table 1

Day	S1‡	S2	S3	S4	S5	S6	E7†	E8	E9	E10	E11
			Carrier 1: Major PFGE types								
0										1NTHi	
1										2NTHi	
2							1nhHh	1nhHh			
3										7NTHi	
8		1nhHh							2NTHi		
14	2NTHi										
15	1NTHi										
16	1NTHi					1NTHi					
17	1NTHi										
22						1NTHi					
23	1NTHi										
60	1NTHi			2Hh						1NTHi	1Hh
64	1NTHi										
71										1NTHi	
81	3NTHi										
108	2NTHi										
116	1NTHi				2Hh						
122	1NTHi										
128	2NTHi										
141	4NTHi		1NTHi+2nhHh								
150		1nhHh									
161										1NTHi	
168	1NTHi										

‡ S genotypes based on digestion of genomic DNA with *Sma*I

† E genotypes based on failure to digest with *Sma*I and successful digestion with *Eag*I

NTHi = nontypeable *Haemophilus influenzae*

Hh = *Haemophilus haemolyticus*

nhHh = nonhemolytic *Haemophilus haemolyticus*

Table 2

Day	S1‡	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	E12†	E13	E14
					Carrier 2: Major PFGE types									
1				3Hi										
2											4Hi			
3											3Hi			
4				6Hi										
7											5Hi			
8			3Hi	5Hi										
9				9Hi										2Hi
10		1Hi		7Hi										
11				10Hi										1Hi
15				8Hi		1Hi								
16				2Hi										
17				5Hi			1Hi							
18			1Hi	18Hi										
21				1Hi										
22	3Hi	1Hi			1Hi									
23				5Hi										
24													13Hi	
25													6Hi	
28													11Hi	
59												7Hi	2Hi	
67												4Hi	9Hi	
72					1Hi							8Hi	2Hi	
88												2Hi		
102												4Hi		
114												6Hi		
123					1Hi	2Hi					1Hi	4Hi		
129				1Hi	1Hi	3Hi			2Hi	1Hi				
135									2Hi					
143									2Hi					

‡ S genotypes based on digestion of genomic DNA with *Sma*I

† E genotypes based on failure to digest with *Sma*I and successful digestion with *Eag*I

Hi= non-typeable *Haemophilus influenzae*

Table 3

Days	S1‡	S2	S3	S4	S5	S6	S7
		Carrier 3: Major PFGE types					
0	1Hib⁻		1Hib⁻				
1	2Hib⁻						
2	2Hib⁻						
3	1Hib⁻			1Hib⁻	1nhHh		
4	3Hib⁻						
8						1NTHi	
11	2Hib⁻			1Hib⁻			
15	2Hib⁻						
16	1Hib⁻		.				
17							1NTHi
18							1NTHi
21							1NTHi
22							3NTHi
23	1Hib⁻					1Hib⁻	5NTHi
25	1Hib⁻						3NTHi
31							2NTHi
51						1NTHi	
57	3Hib⁻					1Hib⁻	
71	1Hib⁻	1Hib⁻					1NTHi
106	2Hib⁻						
148	7Hib⁻	1Hib⁻					
155	3Hib⁻	1Hib⁻					
172	1Hib⁻	1Hib⁻					
197		1Hib⁻					

‡ S genotypes based on digestion of genomic DNA with *Sma*I
NTHi = nontypeable *Haemophilus influenzae*
Hb- = nonencapulated *Haemophilus influenzae* type b
nhHh = nonhemolytic *Haemophilus haemolyticus*

Table 4

Day	S1‡	S2	S3	S4	S5	S6	S7	S8	S9	S10
		Carrier 4: Major PFGE types								
0	1nhHh			5 Hh						
2	1Hh	1Hh	1Hh	1Hh						
3				1Hh						
8	1nhHh									
9	1nhHh									
23				2 Hh				2 Hh		
25									2 Hh	
28										1Hh
56							1Hh			
129	1nhHh									
137		3 Hh								
144		2 Hh								
148		4 Hh			1Hh					
157		1Hh								
172		2 Hh				1Hh				
185		1 Hh								
197				1Hh	1Hh					

‡ S genotypes based on digestion of genomic DNA with *Sma*I
Hh = *Haemophilus haemolyticus*
nhHh = nonhemolytic *Haemophilus haemolyticus*

References

1. Dhooge IM, Vaneechoutte G, Claeys G, Verschraegen, VanCauwengerge P. Turnover of *Haemophilus influenzae* isolates in otitis-prone children. Int J Ped Otorhinol 2000; 54:7-12.
2. Faden HL, Duffy A, Williams D, Krystofik A, Wolf J. Epidemiology of nasopharyngeal colonization with nontypeable *Haemophilus influenzae* in the first 2 years of life. J Infect Dis 1995; 172:132-135.
3. Samuelson A, Freijd A, Jonasson J, Lindberg AA. Turnover of nonencapsulated *Haemophilus influenzae* in the nasopharynges of otitis-prone children. J Clin Microbiol 1995; 33:2027-2031.
4. Trottier S, Stenberg K, Svanborg-Eden C. Turnover of nontypable *Haemophilus influenzae* in the nasopharynges of healthy children. J Clin Microbiol 1989; 27:2175-2179.
5. Gratten MJ, Montgomery G, Gerega H, Gratten H, Siwi H, Poli A, Koki G. Multiple colonization of the upper respiratory tract of Papua New Guinea children with *Haemophilus influenzae* and *Streptococcus pneumonia*e. Southeast Asian J Trop Med Public Health 1989; 20:501-509.
6. Moller LV, Regelink AG, Grasselier H, Dankert-Roelse JE, Dankert J, van Alphen L. Multiple *Haemophilus influenzae* strains and strain variants coexist in the respiratory tract of patients with cystic fibrosis. J Infect Dis 1995; 172:1388-1392.
7. Murphy TF, Sethi S, Klingman KL, Brueggemann AB, Doern GV. Simultaneous respiratory tract colonization by multiple strains of nontypeable *Haemophilus influenzae* in chronic obstructive pulmonary disease: implications for antibiotic therapy. J Infect Dis 1999; 180:404-409.
8. Smith-Vaughan HC, Leach AJ, Shelby-James TM, Kemp K, Kemp DJ, Mathews JD. Carriage of multiple ribotypes of non-encapsulated *Haemophilus influenzae* in aboriginal infants with otitis media. Epidemiol Infect 1996; 116:177-183.
9. Kuklinska D, Kilian M. 1984. Relative proportions of *Haemophilus* species in the throat of healthy children and adults. Eur J Clin Microbiol 1984; 3:249-352.
10. Fung WW, O'Dwyer CA, Sinha A, Brauer AL, Murphy TF, Kroll JS, Langford PR. Presence of copper- and zinc-containing superoxide dismutase in commensal *Haemophilus haemolyticus* isolates can be used as a marker to discriminate them from nontypeable *H. influenzae* isolates. J Clin Microbiol 2006; 44:4222-4226.
11. Murphy TF, Brauer AL, Sethi S, Kilian M, Cai X, Lesse AJ. *Haemophilus haemolyticus*: a human respiratory tract commensal to be distinguished from *Haemophilus influenzae*. J Infect Dis 2007; 195:81-89.
12. Farjo RS, Foxman B, Patel MJ, et al. Diversity and sharing of *Haemophilus influenzae* strains colonizing healthy children attending day-care centers. Pediatr Infect Dis J 2004; 23:41-46.
13. Ecevit IZ, McCrea KW, Pettigrew MM, Sen A, Marrs CF, Gilsdorf JR. Prevalence of the *hifBC, hmw1A, hmw2A, hmwC,* and *hia* Genes in *Haemophilus influenzae* Isolates. J Clin Microbiol 2004; 42:3065-3072.
14. Murphy TF, Kirkham C, Sikkema DJ. Neonatal, urogenital isolates of biotype 4 nontypeable *Haemophilus influenzae* express a variant P6 outer membrane protein molecule. Infect Immun 1992; 60:2016-2022.
15. Falla TJ, Crook DW, Brophy LN, Maskell D, Kroll JS, Moxon ER. PCR for capsular typing of *Haemophilus influenzae*. J Clin Microbiol 1994; 32:2382-2386.
16. Pettigrew MM, Foxman B, Ecevit Z, Marrs CF, Gilsdorf J. Use of pulsed-field gel electrophoresis, enterobacterial repetitive intergenic consensus typing, and automated ribotyping to assess genomic variability among strains of nontypeable *Haemophilus influenzae*. J Clin Microbiol 2002; 40:660-662.
17. St. Sauver J, Marrs CF, Foxman B, Somsel P, Madera R, Gilsdorf JR. Risk factors for otitis media and carriage of multiple strains of *Haemophilus influenzae* and *Streptococcus pneumoniae*. Emerg Infect Dis 2000; 6:622-630.
18. Tenover FC, Arbeit RD, Goering RV, Mickelsen AP, Murray BE, Persing DH, Swaminathan B. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995; 33:2233-2239.

The biofilm formed by nontypeable *Haemophilus influenzae* in the middle ear of the chinchilla during experimental otitis media contains dsDNA and type iv pilin protein

Joseph Jurcisek, B.S., Lauren Bakaletz, Ph.D.

Nontypeable *Haemophilus influenzae* (NTHi) is a member of the normal human nasopharyngeal flora, as well as a frequent opportunistic pathogen of the upper respiratory tract. NTHi accounts for a large portion of cases of acute otitis media (OM) and is a major leading causative agent of chronic and recurrent OM. Recently, it's been shown that NTHi can form a biofilm both *in vitro* and *in vivo*. The ability to form a biofilm in the mammalian airway is likely a contributing factor to the recurrent and/or chronic nature of NTHi-induced diseases of the respiratory tract. DNA has been identified as a key component of the biofilm matrix formed *in vitro* by several bacterial pathogens. Herein, we have employed immunofluorescent labeling of cryo-preserved biofilms recovered from the middle ears of chinchillas, to demonstrate that both immature as well as mature NTHi-induced biofilms contained type IV pilin protein, and a significant amount of dsDNA *in vivo*. The DNA assembled into a network of closely spaced interwoven strands, as well as extending as sparse but thicker rope-like strands, across water channels. Type IV pilin protein was evident throughout the biofilm matrix as both small aggregates as well as tracking along thin dsDNA strands. Due to the intricate arrangement and density of the dsDNA strands, we hypothesize that this abundant DNA likely plays a key role in providing structural stability to the biofilm. A better understanding of the biochemistry of the NTHi-induced biofilm matrix will be important for the development of new and/or improved strategies for the treatment or prevention of chronic and recurrent OM.

Biofilms as bacterial breeding grounds: A counterpoint to the adaptive host response

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The Distributed Genome Hypothesis states that chronic bacterial pathogens utilize a strategy of polyclonal infection and reassortment of genes to ensure persistence in the face of adaptive host defenses. We show by genomic sequencing of large numbers of strains (>20), gene clustering, and mathematical modeling that both the nontypeable *Haemophilus influenzae* (NTHi) and pneumococcus possess a species-level supragenome that is many times larger than the core genome common to all strains of the species.

The number of genes/NTHi strain ranged from 1711:1916 and for pneumococcus from 1850:2157, thus the genes/genome varied by 12% and 17%, respectively. Exhaustive pairwise genic comparisons were made among the strains, which revealed divergence levels of between 16 and 575 genes per pair (mean = 389) for the NTHi and 103 and 572 (mean = 368) for the pneumococcal strains. Genome comparisons between 12 NTHi strains and Rd show between 107-158 insertions and between 100-213 deletions per genome. The mean insertion and deletion sizes were 1356 and 1020 bp, respectively, with mean maximums of 26977 and 37299 bp. The fact that these pathogens possess natural competence and transformation mechanisms, taken together with the size of their supragenomes, the extensive differences in total gene content among strains, the high mean number of genic differences among all strain pairs, and the very high numbers of insertions and deletions in each genome relative to all other genomes suggests that these pathogens use horizontal gene transfer mechanisms to generate diversity as a counterpoint to the host's adaptive immune response.

Integration of basic research findings into a clinical model that explains the persistence of biofilms

J. Christopher Post, M.D., Ph.D.

It has been established that both otitis media with effusion (OME) and recurrent otitis media (OM) are mucosal biofilm diseases. While biofilms have been well-known to be refractory to antimicrobial and host defenses, recent advances have demonstrated even greater challenges in successfully treating biofilms. These advances include: 1) the recognition that each strain produces a unique biofilm phenotype and disease phenotype; 2) natural infecting populations of the OM bacterial pathogens are polyclonal; 3) the biofilm matrices contain large amounts of DNA as a potential source of material for recombination; and 4) sequencing studies have shown that there is a tremendous degree of genomic plasticity among strains. These data demand that multiple novel complementary anti-biofilm strategies be developed.

Anti-biofilm strategies can broadly be divided into anti-matrix, dispersive, anti-bacterial, and vaccine. Anti-matrix strategies include disruptive agents specifically directed against DNA or exopolysaccharides and antimucolytic agents. Anti-bacterial strategies include bacterial-lytic strategies that target non-dividing bacteria, employing a bifunctional synthetic peptide for species-specific recognition and killing, thus providing a 'magic bullet' that spares normal microbial flora. Another anti-bacterial strategy includes high-charge density approaches, which disrupt bacterial membranes. While traditional vaccine development pursued strain-specific capsular antigen-based strategies, these approaches provide insufficient coverage. Thus, novel peptide-based vaccine strategies that target antigens universally possessed by all strains of the species would obviate the challenges associated with serotype replacement. These strategies have the potential to not only treat recurrent OM and OME, but are generically applicable to other biofilm-associated diseases as well.

Bacterial genomics and the pathogenesis of *Haemophilus influenzae* otitis media

Richard Moxon, F.R.C.P., F.R.C.P.C.H.

The availability of the whole genome sequence of *Haemophilus influenzae* (Hi) strain Rd in 1995 enormously facilitated the progress on the genetics of lipopolysaccharide (LPS) biosynthesis of this commensal pathogen.[1] LPS is a critical determinant of colonization, evasion of host clearance, and tissue injury in the pathogenesis of Hi diseases, including otitis media (OM). The majority of episodes of OM are caused by unencapsulated, nontypeable strains. The genome sequence of strain Rd allowed identification of core biosynthetic genes, for example those required for making lipid A and conserved core sugars (LPS inner core) as well as the glycosyl transferases responsible for the highly variable outer core sugars and the several non-sugar additions such as phosphoethanolamine, phosphorylcholine, glycine, and acetate.

The sequenced genome of strain Rd indicated that the content and organization of the LPS genes of Hi are sporadically distributed compared to the more discrete clustering of LPS biosynthetic genes typical of the enterobacteraceae. The legacy of LPS biosynthetic gene discovery, together with the construction of defined mutants, enabled detailed elucidation of the LPS structures of the highly variable glycoforms that are a hallmark of Hi LPS. The heterogeneity of the LPS glycoforms[2] derived from a single progenitor strain of Hi is remarkable and is in part the result of the variable expression of a subset of LPS biosynthetic genes that is mediated through slippage of simple sequence (tetranucleotide) repeats.

Studies of an epidemiologically diverse collection of encapsulated and NT strains of Hi, both carriage and disease isolates, have also revealed extraordinary diversity between the strains that constitute the natural population of the Hi species, largely but not exclusively owing to the heterogeneity of LPS outer core sugars and other additions. For example, there are several distinct sialyl transferases, allelic variants of glycosyl transferases, non-stoichiometric addition, and allelic variation in the location of phosphoryl choline. A particular challenge lies in determining how this abundant structural diversity determines biological variation in the adaptive biology of Hi and its implications for its commensal and pathogenic behavior. An example of

this is afforded by studies on the role of sialylated glycoforms in the pathogenesis of Hi OM. Mutations

in genes required for sialylation of LPS resulted in drastic attenuation of Hi following direct inoculation into the middle ear of chinchillas.[3] Subsequent studies have indicated that sialylation of LPS impedes complement-dependent host clearance of Hi.

Sialylation of Hi LPS is apparently dependent upon scavenging the required N-acetyl neuraminic acid precursors from the host as the genes for the early steps in sialic acid biosynthesis are absent from the several Hi genomes that have been sequenced to date. Indeed, the recent completion of the entire sequences of several other genomes of Hi strains and the genomic data from related species such as *H. somnus, H. ducreyi,* and *H. parainfluenzae* will afford further opportunities to mine data relevant to understanding the biological role of LPS in OM and the many other diseases of the genus Haemophilus.

References

1. Hood DW, Deadman ME, Allen T, et al. Use of the complete genome sequence information of Haemophilus influenzae strain Rd to investigate lipopolysaccharide biosynthesis. Mol Microbiol 1996; 22:951-965.
2. Schweda EKH, Richards JC, Hood DW, Moxon ER. Expression and structural diversity in the lipopolysaccharide of Haemophilus influenzae; implication in virulence. Int J Med Microbiol 2007; 5:297-306.
3. Bouchet V, Hood DW, Li J, et al. Host-derived sialic acid is incorporated into Haemophilus influenzae lipopolysaccharide and is a major virulence factor in experimental otitis media. Proc Natl Acad Sci U S A 2003; 100:8898-8903.

Clinical trials in evaluating antimicrobials in acute otitis media

John Powers, M.D.

For clinical trials to provide useful information for clinical practice (i.e., external validity), investigators should design trials to minimize random error, bias, and confounding and thereby maximize internal validity. To address internal validity as well as conform to regulatory requirements, trials should address seven basic concepts in evaluating effectiveness of interventions: 1) specify a clear objective, 2) make a quantitative comparison with a control group, 3) select patients with the disease, 4) ensure baseline comparability of study subjects, 5) minimize potential sources of bias, 6) measure well-defined and reliable endpoints that are clinically important to patients, and 7) utilize appropriate analyses.

Once there is evidence of effectiveness, one needs to determine the balance of benefits compared to potential harms. The goal of administering antimicrobials in acute otitis media is to provide net benefits to patients in terms of increasing the proportion of patients who experience cure of their symptoms. Pre-clinical and early clinical and microbiological data may aid in developing hypotheses to test in confirmatory clinical trials. We will discuss the current lack of data to support active controlled noninferiority trials and the need for superiority trials in studying this disease entity. We will also discuss the issues with selecting patients with bacterial disease, issues with surrogate and clinical endpoints including patient or caregiver-reported outcomes, issues with the timing of endpoints, and issues with appropriate analysis of clinical trials. Finally, we will discuss the issues regarding the ethics of clinical trials and the need for scientifically valid study designs to justify the potential risks to research subjects.

Computer modeling in otitis media

Samir Ghadiali, Ph.D.

The use of computers to simulate and model complex physiological processes is now ubiquitous in the biomedical sciences. These computational techniques are providing clinicians with new insights into the mechanisms responsible for a variety of medical disorders. In this presentation, I will provide an overview of how our laboratory is using computational and mathematical models to elucidate the basic etiology of Eustachian tube (ET) dysfunction and Otitis Media (OM). I will focus primarily on the development of sophisticated computational models which simulate active ET opening phenomena as well as the resulting gas and fluid transport between the middle ear and nasopharynx. These multi-scale models account for normal and pathological tissue anatomies, patient-specific tissue mechanics, asynchronous muscle contractions, surface tension forces and molecular adhesion dynamics. These models were used to ascertain how specific biophysical properties influence ET function in different patient populations (i.e. adults, cleft palate infants and young children). In addition, computational simulations were used to analyze and interpret experimental measurements of ET function. Specifically, statistically significant correlations were obtained between computational results and experimental data from both normal adults and OM patients. These correlations indicate that although contraction of the tensor veli palatini muscle (TVPM) is responsible for ET opening, the asynchronous and extended contraction of the levator veli palatini muscle (LVPM) promotes closure of the ET (i.e. lumen constriction). Correlations between experiments and computational results also indicate that the up-regulation and expression of mucus glycoproteins on luminal epithelial cells may be responsible for the irreversible closure of the ET lumen observed in OM patients. The computational models are therefore providing important new insights into the specific biophysical parameters responsible for ET dysfunction in different patient populations. Our long-term goal is to apply these modeling techniques in a clinical setting in order to identify patient-specific mechanisms of ET dysfunction and novel treatments for OM.

The role of viruses in otitis media

Terho Heikkinen, M.D., Ph.D.

Acute otitis media (AOM) is conventionally considered a bacterial infection that is treatable with antibiotics. For three main reasons, however, investigators have been interested in the potential role of viruses in the etiology and pathogenesis of AOM. First, clinical experience clearly indicates that AOM is closely associated with viral upper respiratory tract infections. Second, bacterial pathogens are usually cultured from the middle-ear fluid (MEF) in only approximately 70% of AOM cases. Third, the clinical response to antibiotic treatment in children with AOM is often poor. During the past 25 years, increasing research into the role of viruses in AOM has provided strong evidence for the crucial role of viruses in the etiopathogenesis of this condition.[1]

Epidemiology

The occurrence of AOM shows an extensive seasonal variation. The incidence rates of AOM are highest in the wintertime, which parallels the incidence of viral upper respiratory tract infections. The incidence of AOM also peaks in the youngest children in whom viral respiratory infections are most prevalent. In a study of 363 children with newly diagnosed AOM, symptoms of viral upper respiratory infection were observed in 94% of the patients at the time of diagnosis.[2] Further, most signs and symptoms that are traditionally associated with AOM have been shown to be unspecific to AOM but to be caused by the underlying viral infection.[3, 4] An extensive hospital-based study demonstrated a significant correlation between AOM and laboratory-documented epidemics of respiratory viruses.[5] In the majority of cases in infants and children, AOM can be clearly regarded as a complication of a preceding or concomitant viral upper respiratory infection.

Two studies, including a total of more than 1,200 children, have specifically assessed the temporal development of AOM during the course of the upper respiratory infection.[6, 7] In these studies, the highest incidence of AOM was observed 3-4 days after the onset of upper respiratory symptoms. In children in whom two separate episodes of AOM could be analyzed, the time to development of AOM during the first occurrence did not correlate with the time lag during the second episode.[7] This suggests that the type of virus may have a greater impact on the temporal development of AOM than any host-related individual factor.

In the 1980s and early 1990s when the detection of respiratory viruses in clinical specimens was mostly based on viral culture or various antigen detection techniques, the rates of viral detection in the nasopharynges of children with AOM ranged between 30-50%.[2, 8, 9] However, the increasing availability and use of polymerase chain reaction (PCR)-based assays has substantially increased the yield of respiratory viruses in the specimens. This is especially true for rhinoviruses that are the most frequent viruses causing respiratory infections and for which antigen detection methods cannot be routinely used. In a PCR-based study searching for rhinovirus, respiratory syncytial virus (RSV), and coronavirus only, one or more of these viruses was found in nasopharyngeal aspirates from 62% of children with AOM.[10] In our study, in which we searched for viruses using antigen detection for RSV, influenza A and B viruses, parainfluenza type 1, 2, and 3 viruses, and adenovirus, and PCR for rhinovirus, enterovirus, coronavirus, and influenza C virus, we identified one or more of these viruses in the nasopharyngeal specimens of 91% of children with AOM (Heikkinen T, Ruohola A, Waris M, Ruuskanen O. The 4th Extraordinary International Symposium on Recent Advances in Otitis Media, Sendai, Japan, 2001; Abstract 133).

Pathogenesis

Dysfunction of the eustachian tube is considered the most important factor in the pathogenesis of AOM.[11] Viral infection of the upper respiratory tract results in congestion of the nasal and nasopharyngeal mucosa, and congestion in and around the nasopharyngeal orifice of the eustachian tube leads to dysfunction of the tube. Numerous experimental studies in animals have demonstrated that intranasal inoculation of viruses results in negative middle-ear pressure, marked damage to and disappearance of ciliated epithelial cells in the eustachian tube, and increased accumulation of mucus and cellular debris in the tubal lumen.[12, 13] Animal studies have also shown a clear synergistic effect between many viruses and bacteria in causing AOM.[14, 15]

The causal role of respiratory viruses in the disruption of normal eustachian tube function has been

demonstrated in several clinical studies in both children and adults.[16, 17] In preschool children, significant negative middle-ear pressures were observed in 13% of asymptomatic children, but the rate of abnormal tympanograms increased to 75% during upper respiratory infections.[17] More than 80% of the abnormalities were already detectable by the second day of illness, and 97% after 4 days. These findings are in concordance with the clinical studies demonstrating that the peak incidence of AOM occurs 3-4 days after the onset of respiratory symptoms in children.[6, 7] In experimental viral challenge studies in adults, substantial deterioration of eustachian tube function has been observed in 50-80% of volunteers following intranasal challenge with rhinoviruses or influenza A viruses, and significant negative middle-ear pressures have developed in 30-80% of the subjects.[18-20]

Viruses in the middle-ear fluid

The most important piece of evidence for the role of viruses in AOM has been obtained by searching for them in MEF directly. In the 1950s and 1960s, when detection of viruses was based on viral culture only, the detection rates remained lower than 5%, and in many studies viruses could not be found at all. Since the 1980s, improved viral culture techniques and the development of various antigen detection methods have allowed demonstration of viruses or viral antigens in the MEF in approximately 20% of children with AOM.[1] In about one-third of virus-positive cases, the virus has been the sole pathogen found in the MEF.

During the past decade, the increased use of PCR techniques has dramatically increased the detection rates of viruses in the MEF. In one of the first studies, the use of PCR for the detection of RSV yielded positive findings in 53% of children with AOM during an RSV outbreak.[21] In another study, the use of PCR for rhinovirus, RSV, and coronavirus revealed one or more of these viruses in the MEF from 48% of children.[10] Recently, Chonmaitree and Henrickson studied MEF samples in which viruses had been previously searched for by culture.[22] The use of multiplex PCR for the detection of RSV, influenza A and B viruses, and parainfluenza type 1, 2, and 3 viruses increased the rate of viral detection in the MEF from 29% to 72%. Two-thirds of specimens that had remained negative after virus culture turned out to be virus-positive by PCR.

Despite the identification of viruses in the MEF in a substantial proportion of children with AOM, it has been speculated that the true prevalence of viruses could be even greater than reported. One major problem related to viral detection is the limited volume of MEF that can be obtained by tympanocentesis. The small volume of specimen effectively hinders diagnostic testing for a broad spectrum of microbial pathogens. Another reason is that most previous studies have searched for only a limited number of viruses, and PCR techniques have not been used extensively to evaluate most of the bacterial and viral causes of AOM concomitantly. Recently, we studied the microbiological etiology of AOM in 79 young children (median age, 21 months) with acute-onset otorrhea through a tympanostomy tube.[23] In 73% of the children, the otorrhea had started within 24 hours before clinical examination, and in all children it had lasted less than 48 hours. All children except one had a concomitant viral-type respiratory infection. Bacteria in the MEFs were identified by conventional bacterial culture and multiplex and broad-range PCR techniques. Viruses were detected by culture, antigen detection for RSV, adenovirus, influenza A and B viruses, and parainfluenza type 1, 2, and 3 viruses, and PCR-based assays for rhinovirus, enterovirus, coronaviruses 229E, OC-40, and NL63, RSV, influenza A and B viruses, human metapneumovirus, adenovirus, parainfluenza viruses type 1, 2, 3, and 4, and human bocavirus. Overall, bacteria were identified in the MEF in 92% of the children and viruses in 70% of the children. In 66% of the children, both bacteria and viruses were found, indicating that most cases of AOM in children are combined viral-bacterial infections. Rhinoviruses and enteroviruses accounted for 60% of all viral findings in the MEF.

High rates of viral detection in the MEF by sensitive PCR methods have raised the question of the true clinical significance of viruses in the middle ear. Our recent study of the relative prevalence of different viruses in the MEF of children with AOM provided additional evidence for the active role of at least some viruses in the pathogenesis of AOM.[24] In children with AOM during laboratory-confirmed RSV infection, the same virus could be found in the MEF in 74% of the cases, whose rate was significantly higher than the corresponding rates for parainfluenza viruses (52%) or influenza viruses (42%). The relative prevalences of all these viruses, in turn, were higher than those observed for enteroviruses (11%) or adenoviruses (4%). These findings suggest that at least some viruses actively invade the middle-ear cavity. If all viruses detected in the MEF were the result of passive influx only, they should be detected in the MEF at roughly similar rates.

Viruses and the outcome of AOM

The recent finding that most cases of AOM in children are combined viral-bacterial infections [23] seems to provide an excellent explanation to the question why the clinical response to antibiotic treatment is often poor despite the use of wide-spectrum antibiotics that are known to be effective against the causative bacteria. If the middle-ear mucosa is infected with both bacteria and viruses simultaneously, eradication of the bacteria with antibiotics may not be sufficient to suppress the inflammatory cascades in the middle ear, and the continuing viral infection may lead to more prolonged disease.

Although the full impact of different viruses on the inflammatory processes in the middle ear remains to be determined, some studies indicate that viruses play an important role in the resolution of otitis media. Chonmaitree et al studied 58 children with AOM and obtained MEF samples by tympanocentesis both before starting the antibiotic treatment and 2-4 days into the treatment.[25] At the second tympanocentesis, bacteriologic failure was observed in 33% of the children who had both bacteria and virus in the initial MEF but only in 3% of children who had only bacteria in the initial MEF. Further, the prolongation of otitis media in children with mixed viral-bacterial infections has been shown in some studies.[26, 27] The detailed mechanisms by which viruses in the middle ear might interfere with the resolution of otitis media are still unclear. However, one probable explanation is virus-induced production of inflammatory mediators in the middle ear. Chonmaitree et al. measured the concentrations of interleukin-8 and leukotriene B4 in MEF specimens from children with AOM.[28] The concentrations of these mediators were higher in bacterial than in viral AOM, but the highest concentrations of both mediators were measured in MEFs that contained both bacteria and virus. Antibiotic treatment had no effect on the concentrations of the mediators.

Summary

There is ample evidence to support a crucial role for respiratory viruses in the etiology and pathogenesis of AOM. Viral infection of the upper respiratory mucosa initiates the cascade of events that finally leads to the development of AOM as a complication. Viruses have a central role in the disruption of normal eustachian tube function. In most children with AOM, both bacteria and viruses can be found in the MEF, indicating a combined viral-bacterial infection. Viruses

also seem to enhance the inflammatory process in the middle ear, which may significantly impair the resolution of otitis media. The expanding role of viruses in AOM is of crucial importance in the development of effective strategies for prevention and better management of this illness.

References

1. Heikkinen T, Chonmaitree T. Importance of respiratory viruses in acute otitis media. Clin Microb Rev 2003; 16:230-241.
2. Arola M., Ruuskanen O, Ziegler T, et al. Clinical role of respiratory virus infection in acute otitis media. Pediatrics 1990; 86:848-855.
3. Heikkinen T, Ruuskanen O. Signs and symptoms predicting acute otitis media. Arch Pediatr Adolesc Med 1995; 149:26-29.
4. Niemelä M., Uhari M, Jounio-Ervasti K, Luotonen J, Alho OP, Vierimaa E. Lack of specific symptomatology in children with acute otitis media. Pediatr Infect Dis J 1994; 13:765-768.
5. Ruuskanen O, Arola M, Putto-Laurila A, Mertsola J, Meurman O, Viljanen MK, Halonen P. Acute otitis media and respiratory virus infections. Pediatr Infect Dis J 1989; 8:94-99.
6. Heikkinen T, Ruuskanen O. Temporal development of acute otitis media during upper respiratory tract infection. Pediatr Infect Dis J 1994; 13:659-661.
7. Koivunen P, Kontiokari T, Niemelä M, Pokka T, Uhari M. Time to development of acute otitis media during an upper respiratory tract infection in children. Pediatr Infect Dis J 1999; 18:303-305.
8. Sarkkinen H, Ruuskanen O, Meurman O, Puhakka H, Virolainen E, Eskola J. Identification of respiratory virus antigens in middle ear fluids of children with acute otitis media. J Infect Dis 1985; 151:444-448.
9. Chonmaitree T, Howie VM, Truant AL. Presence of respiratory viruses in middle ear fluids and nasal wash specimens from children with acute otitis media. Pediatrics 1986; 77:698-702.
10. Pitkäranta A, Virolainen A, Jero J, Arruda E, Hayden FG. Detection of rhinovirus, respiratory syncytial virus, and coronavirus infections in acute otitis media by reverse transcriptase polymerase chain reaction. Pediatrics 1998; 102:291-295.
11. Bluestone CD. Pathogenesis of otitis media: role of Eustachian tube. Pediatr Infect Dis J 1996; 15:281-291.
12. Giebink GS, Ripley ML, Wright PF. Eustachian tube histopathology during experimental influenza

A virus infection in the chinchilla. Ann Otol Rhinol Laryngol 1987; 96:199-206.

13. Chung MH, Griffith SR, Park KH, Lim DJ, DeMaria TF. Cytological and histological changes in the middle ear after inoculation of influenza A virus. Acta Otolaryngol 1993;1 13:81-87.

14. Giebink GS, Berzins IK, Marker SC, Schiffman G. Experimental otitis media after nasal inoculation of *Streptococcus pneumoniae* and influenza A virus in chinchillas. Infect Immun 1980; 30:445-450.

15. Suzuki K, Bakaletz LO. Synergistic effect of adenovirus type 1 and nontypeable *Haemophilus influenzae* in a chinchilla model of experimental otitis media. Infect Immun 1994; 62:1710-1718.

16. Bylander A. Upper respiratory tract infection and Eustachian tube function in children. Acta Otolaryngol 1984; 97:343-349.

17. Sanyal MA, Henderson FW, Stempel EC, Collier AM, Denny FW. Effect of upper respiratory tract infection on Eustachian tube ventilatory function in the preschool child. J Pediatr 1980; 97:11-15.

18. Doyle WJ, McBride TP, Skoner DP, Maddern BR, Gwaltney JM Jr, Uhrin M. A double-blind, placebo-controlled clinical trial of the effect of chlorpheniramine on the response of the nasal airway, middle ear, and Eustachian tube to provocative rhinovirus challenge. Pediatr Infect Dis J 1988; 7:229-238.

19. McBride TP, Doyle WJ, Hayden FG, Gwaltney JM Jr. Alterations of the Eustachian tube, middle ear, and nose in rhinovirus infection. Arch Otolaryngol Head Neck Surg 1989; 115:1054-1059.

20. Buchman CA, Doyle WJ, Skoner DP, Post JC, Alper CM, Seroky JT, Anderson K, Preston RA, Hayden FG, Fireman P, Ehrlich GD. Influenza A virus-induced acute otitis media. J Infect Dis 1995; 172:1348-1351.

21. Okamoto Y, Kudo K, Ishikawa K, Ito E, Togawa K, Saito I, Moro I, Patel JA, Ogra PL. Presence of respiratory syncytial virus genomic sequences in middle ear fluid and its relationship to expression of cytokines and cell adhesion molecules. J Infect Dis 1993; 168:1277-1281.

22. Chonmaitree T, Henrickson K. Detection of respiratory viruses in the middle ear fluids of children with acute otitis media by multiplex reverse transcription-polymerase chain reaction assay. Pediatr Infect Dis J 2000; 19:258-260.

23. Ruohola A, Meurman O, Nikkari S, Skottman T, Salmi A, Waris M, Österback R, Eerola E, Allander T, Niesters H, Heikkinen T, Ruuskanen O. Microbiology of acute otitis media in children with tympanostomy tubes: prevalences of bacteria and viruses. Clin Infect Dis 2006; 43:1417-1422.

24. Heikkinen T, Thint M, Chonmaitree T. Prevalence of various respiratory viruses in the middle ear during acute otitis media. N Engl J Med 1999; 340:260-264.

25. Chonmaitree T, Owen MJ, Howie VM. Respiratory viruses interfere with bacteriologic response to antibiotic in children with acute otitis media. J Infect Dis 1990; 162:546-549.

26. Chonmaitree T, Owen MJ, Patel JA, Hedgpeth D, Horlick D, Howie VM. Effect of viral respiratory tract infection on outcome of acute otitis media. J Pediatr 1992; 120:856-862.

27. Arola M, Ziegler T, Ruuskanen O. Respiratory virus infection as a cause of prolonged symptoms in acute otitis media. J Pediatr 1990; 116:697-701.

28. Chonmaitree T, Patel JA, Garofalo R, Uchida T, Sim T, Owen MJ, Howie VM. Role of leukotriene B4 and interleukin-8 in acute bacterial and viral otitis media. Ann Otol Rhinol Laryngol 1996; 105:968-974.

History of serous otitis media

Robert Ruben, M.D.

Serous otitis media (SOM) is a condition that appears to have always affected humankind but, in general, it was not conceptualized as a separate entity until the last third of the 19th century. There is some evidence that it may have been recognized by the Romans[1] and by Arabic physicians[2] (Fig 1.)

Du Verney's[4] 17th century (Fig. 2) and Valsalva's[5] 18th century studies of the eustachian tube led to the first attempt by Guyot[6] to care for probable SOM by means of eustachian tube catheterization.

> *"Though it is, the anatomists do not (believe) that the horn of Eustachian can be syringed by the mouth, however Mr. Guyot, postmaster in Versailles, found for this use an instrument that the Academy considered to be very ingenious. The principal part is a bent pipe, which one insinuates into the bottom of the mouth behind and into the top of the palate, intentionally to apply it to the House of the Horn that one wants to inject. One washes at least the mouth of it what can be useful in certain case."[6]*

This was followed by several reports of myringotomy in the late 18th Century. Cooper[7] in 1801, brought myringotomy into the accepted medical/surgical armamentarium, but it was subsequently identified as infective and dangerous (Fig. 3).

By the middle of the 19th century, Deleau[8] (Fig. 4) and then Toynbee[9] developed diagnostic and therapeutic techniques dependent upon eustachian tube function. Meyer's[10] discovery of the adenoid and adenoidectomy to treat middle ear catarrh, a.k.a. SOM, was rapidly adopted. Politzer[11] describes catarrh as SOM, and uses a combination of modalities—eustachian tube insufflation (Politzerization)(Fig. 5), adenoidectomy, and myringotomy—to treat SOM.

The increased awareness of SOM and its sequelae came about in the latter half of the 20th century though a combination reports, including Eagle's 1946 epidemiological report (Fig. 6).[13] Then, Armstrong, in 1954[14], reported five cases in which the insertion of a vinyl tube through a myringotomy was successful in treating chronic secretory otitis media. He gives a second report three years later[15] concerning his data from 1,016 patients with SOM, of which 69

(6.8%) of the ears were treated with the vinyl tube. Seventy-eight percent of his patients were younger than 8 years. His diagnostic techniques still depended upon otoscopy and a variation of the Toynbee test. He stated that he had no recurrence in 60/69 after an initial insertion, and no recurrence after treating one or more recurrences in 8 ears. Infection was considered the cause of the fluid as bacteria were found in the middle-ear fluids in many of the experimental and clinical studies. Proud[16], in 1970, showed that the fluid would accumulate without an infection. His data (Fig. 7) showed that a patent myringotomy prevented the accumulation of the fluid. These and other studies laid the foundation for the widespread use of the tympanostomy tube in patients with chronic SOM.

Zwislocki's[17] development, in 1957, of a physical method to measure the impedance of the tympanic membrane in a living human allowed for its clinical application. This was developed into an instrument by Masden (http://www.gnotometrics.com/about_us/story_of_gn_ otometrics/story_of_gn_otometrics_backtround/story_ of_gn_otometrics_backtround_poul_madsen.htm; accessed July 20, 2006) in 1960. Jerger brought one of the instruments to the United States, studied patients, and reported his results in 1970[18]; his suggested classification has been widely accepted (Fig. 8). Three years later, Bluestone et al.[19] published the clinical correlation of the findings in the middle ear with tympanometry and audiology. The diagnosis of SOM could now be made with excellent sensitivity and specificity.

The ubiquity of the disorder and the seemingly little morbidity other than those ears with severe retraction of the tympanic membrane, raised the question of why to treat. The cognitive and social effects of SOM were occasionally mentioned, but not until a 1967 report of Holm and Kunze[20] did this become a major medical concern. They found that children with fluctuating hearing losses from otitis media performed significantly worse than controls in 8 of 12 cognitive/linguistic measures, which required the receiving or processing of auditory stimuli or the production of verbal response. The four in which there was no differences were all dependent on visual and motor skills.

There was now an accurate way to diagnosis SOM, an effective treatment, and an apparent linguistic morbidity. These factors caused widespread

concern and stimulated research. The first meeting, and prototype for subsequent special meetings, took place at the 10th International Congress of Otolaryngology held in Venice in 1973 and report in1974.[21] This was followed by the 1st International symposium on Recent Advances in Otitis Media with Effusion held in1975. The program committee was made up of Ben Senturia (Fig. 9), Charles Bluestone, and David Lim. At the end of the meeting, there was a research meeting to examine which areas needed further investigation. Since then, there have been seven additional symposia with a name change in 1987 to "Recent Advances in Otitis Media" and a number of Extraordinary International Symposia. The 9th International Symposium on Recent Advances in Otitis Media took place in 2007.

During the latter part of the 20th century, in industrialized nations, many children were treated for SOM. This became and still is a significant medical care expenditure. As there were and are still critical questions concerning SOM, including etiology, why and who to care for, what is the most effective form of therapy and for whom, etc., governments and other health funding agencies implemented two strategies. The first was to use available data to establish guidelines. Two such sets of guidelines have been published by United States Public Health Service in 1994[22] and 2003.[23] The second was to establish and support research directed toward SOM. The National Institutes of Health funded 365 research grants in the area of SOM from 1972 to 2006. Large prospective clinical studies were initiated, two examples of which are Haggard's TARGET study in the United Kingdom and Rover et al.'s KNOOP-3 study in the Netherlands.[24]

Conclusion

The historical study of SOM exemplifies the way in which disease is recognized, how prevalence changes, and the role it plays in human health. Physicians developed technology, including otoscopy and impedance to facilitate the accurate diagnosis of SOM. The observations and documentation in the 1950s[25,26] clearly show that the prevalence of SOM greatly increased, probably due to antibiotics, and, as we are now learning, by the formation biofilms.[27,28] The assumption[29] that SOM was always as prevalent as it is now and that the care of SOM is a 'fashion' is not sustained. This is a disease that appears to be made more prevalent by the change in the bacterial environment and from the use and overuse of antibiotics.

A disease is a condition that renders a person less fit. During most of human history, fitness for almost all was physical. The 20th and 21st centuries have changed that paradigm, and now fitness for most is dependent upon communication--hearing, voice, and speech and language skills.[30] SOM causes communication disorders, especially in susceptible subjects. Thus, SOM became an important disease to be prevented and cared for so as to optimize the fitness of the person and society.

Figures

Figure 1. Possible myringotomy knife of Albucasis as pictured in the Marsh manuscript (1271 -1272) and the Huntington manuscript (1465-1466) in Bodleian library, Oxford.[3]
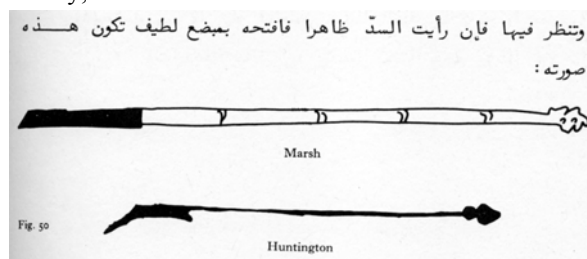
Figure 2. Plate IX from Du Verney, Traite de l'organ de l'ouie; contenant la Structure, les Usages et les Maladies de toutes les parties del' Oreille, 1683 showing the tympanic and pharyngeal orifices of the eustachian tube.[4]
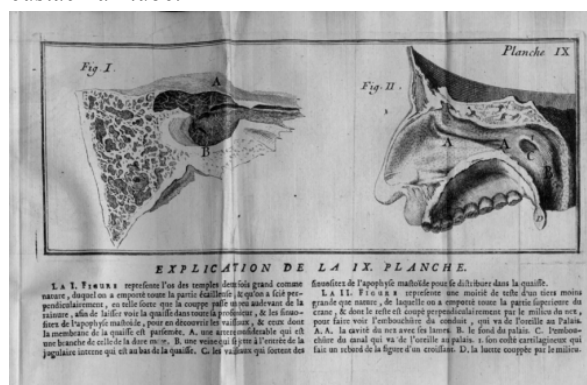
Figure 3. Illustrations 1-10 from Sir Astley Paston Cooper's Farther Observations on the Effects which take Place from the Destruction of the Membrana Tympani of the Ear: an Account of an Operation for the Removal of a particular Species of Deafness illustrating the technique of myringotomy (#2) and tympanic membranes (#3-10): #3--perforation of long standing from infection, #4--from head trauma, #5--laceration from an attempt to extract a pin, #6 and #7--fungus infection, #8--from infection, #9--normal, and #10--after a myringotomy.[7]
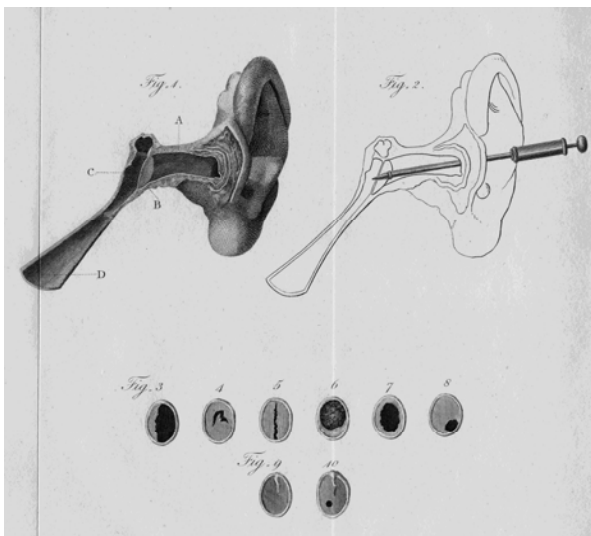
Figure 4. From Deleau, showing his device for eustachian tube catechization and insufflation.[8]
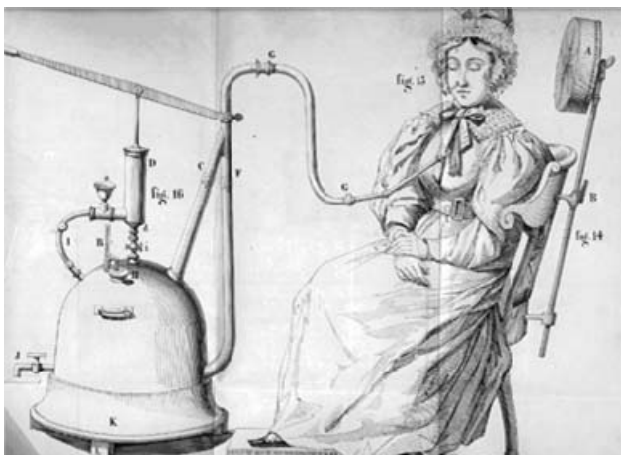
Figure 5. Politzer's air bag and eustachian tube catheterization[12]

Fig. 61.

Die Luftdouche mit dem Gummiballon.

Figure 6. After Eagle showing the dramatic increase in the prevalence of SOM.[13]

After Eagle* showing the dramatic increase in the prevalence of SOM.
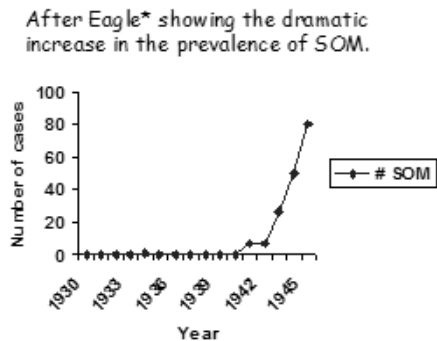
* Eagle WW, Secretory Otitis Media. Annals of Otology, Rhinology and Laryngology 55. 1946

Figure 7. The results of aseptic ligation of the eustachian tube in cats[16]

TABLE I

Procedure	Number of Ears	Effusion	Mucosal Abnormality
Ligation alone	11	11	11
Ligation and patent myringotomy	5	0	0
Ligation and healed myringotomy	4	4	4
Sham operation	4	0	0

Figure 8. Jerger's initial description of the now commonly used "A", "B", and "C" tympanometric curves. These are 'inverted' as the peak pressures are negative.[18]

Air pressure in mm (water)
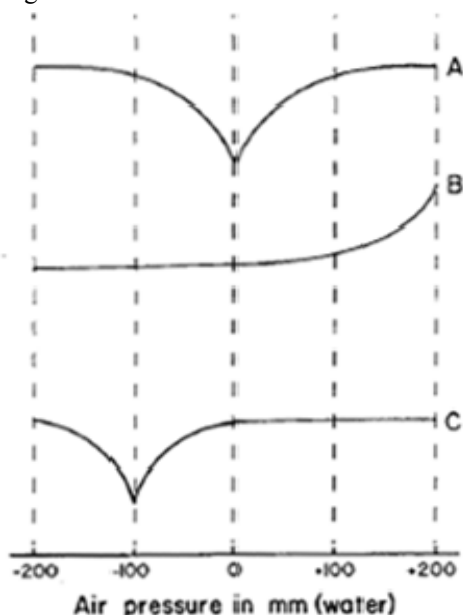
Figure 9. Ben Harlan Senturia, M.D., 1910-1982.

References

1. Celsus, AC, Spencer, WG Translator. De medicina. Cambridge: Harvard University Press; 1935.
2. Mansour AM. Surgery of Abul-Qasim Al-Zahrawi [Muslim scientists website]. Accessed at http://www.islamset.com/isc/zahrawi/mansour.html Accessed. Jan. 21, 2006.
3. Spink MS, Lewis GL. Albucasis. On Surgery and Instruments: A definitive edition of the Arabic text with English translation and commentary. Berkeley: University of California Press; 1973.
4. Du Verney, GJ. Traite de l'organ de l'ouie; contenant la Structure, les Usages et les Maladies de toutes les parties del' Oreille. Paris: Estienne Michallet; 1683.
5. Valsalva, AM. De Aura Humana Tractatus. Trajecti ad Rhenum, Utrecht, 1704.
6. Guyot EG. Divsese anatomail Obsevations VI. Histoire de l'Academie Royale des Sciences, 1724; 37.
7. Cooper A. Farther observations on the effects which take place from the destruction of the Membrana Tympani of the ear; with an account of the operation for the removal of a particular species of deafness. Philosophical Transactions of the Royal Society, Part 1:435-451; 1801.
8. Deleau N. Sur le cathétérisme de la trompe d'Eustache, et sur les expériences de M. Itard, mémoire qui démontre l'utilité de l'air atmosphérique dans le traitement de diverses espèces de surdité. Paris: Chez l'Auteur; 1828.
9. Toynbee J. The Diseases of the ear: their nature, diagnosis and treatment. London: John Churchill; 1860.
10. Meyer HW. Om adenoide Vegetationer Nasesvaelgrummet. Hospitalstidende 1868; 11:177-181.
11. Politzer A. A Text-book of the Diseases of the Ear and Adjacent Organs. (Translation by Cassells JP.) Philadelphia: Lea, 1883.
12. Politzer A. Lehrbuch der Ohrenheilkunde für practische Aerzte und Studirende. Stuttgart: F. Enke; 1878.
13. Eagle WW. Secretory otitis media. Ann Otol Rhinol Laryngol 1946:55; 55-67.
14. Armstrong B. A new treatment for chronic secretory otitis media. AMA Arch Otolaryngol 1954; 59:653-654.
15. Armstrong B. Chronic secretory otitis media: diagnosis and treatment. South Med J 1957; 50:540-546.

16. Proud GO, Odoi H. Effects of Eustachian tube ligation. Ann Otol Rhinol Laryngol 1970; 79:30-32.

17. Zwislocki J. Some measurements of the impedance at the eardrum. J Acoust Soc Am 1957; 29:349-356.

18. Jerger J. Clinical experience with impedance audiometry. Arch Otolaryngol 1970; 92:311-324.

19. Bluestone CD, Beery QC, Paradise JL. Audiometry and tympanometry in relation to middle ear effusions in children. Laryngoscope 1973; 83:594-604.

20. Holm VA, Kunze LH. Effect of chronic otitis media on language and speech development. Pediatrics 1969; 43:833-839.

21. Andrus WS, Berry QC, Bluestone CD, et al. Middle ear effusions. Ann Otol Rhinol Laryngol 1974; 83[Suppl 11]:1-77.

22. Stool SE, Berg AO. Otitis Media with Effusion in Young Children: Clinical Practice Guideline , Number 12. Rockville: Agency for Health Care Policy and Research - USPHS, 1994.

23. Takata G, Chan LS. Diagnosis, Natural History, and Late Effects of Otitis Media With Effusion - Evidence Report/Technology Assessment. Rockville: Agency for Healthcare Research and Quality U.S. Department of Health and Human Services, 2003.

24. Rovers MM, Zielhuis GA, Bennett K, Haggard M. Generalisability of clinical trials in otitis media with effusion. Int J Pediatr Otorhinolaryngol 2001; 60:29-40.

25. Eagle WW. Secretory otitis media. Ann Otol Rhinol Laryngol 1946; 55:55-67.

26. Hoople GD. Otitis media with effusion; a challenge to otolaryngology. Laryngoscope 1950; 60:315-329.

27. Post JC, Stoodley P, Hall-Stoodley L, Ehrlich GD. The role of biofilms in otolaryngologic infections. Curr Opin Otolaryngol Head Neck Surg 2004; 12:185-190.

28. Hall-Stoodley L, Hu FZ, Gieseke A, et al. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. JAMA 2006; 296:202-211.

29. Black NA. Fashion, science and technical change: the history of the treatment of glue ear. Clin Otolaryngol 1985; 10:31-41.

30. Ruben RJ. Redefining the survival of the fittest: communication disorders in the 21st century. Laryngoscope 2000; 110(2 Pt 1):241-245.

Delayed prescribing: A sensible approach to the management of acute otitis media (for the first great debate)

Paul Little, M.D.

The Dutch have developed a policy of no prescription for acute otitis media (AOM) unless there was significant otaligia and/or fever 72 hours after seeing the doctor or if a prolonged discharge developed, and have shown that if such an approach is used there are likely to be very few cases of complications. The potential advantages of delayed prescribing is that it rationalizes antibiotic use (with reductions in antibiotic use from 50-75%); changes beliefs in antibiotics – since prescribing antibiotics probably fuels a vicious circle of belief in antibiotics, subsequent reattendance, further antibiotic use, etc.; achieves acceptable symptom control; and provides a back up and rapid access to antibiotics where there is uncertainty about which children will do poorly when no antibiotics are provided.

There is weak ecological evidence from the UK and European studies that either localities or countries that have lower prescribing of antibiotics have higher admission rates for mastoiditis. Even assuming such ecological data does provide secure evidence of a genuine problem, the data suggests that several thousand prescriptions would be required to prevent one case of mastoiditis in affluent, developed populations. The alternatives to delayed prescribing are all problematic: not to prescribe at all, which is probably less safe; to prescribe in most or all cases, which will lead to side effects, antibiotic resistance, and possibly more complications associated with antibiotic resistance; or to target antibiotics to those likely to suffer prolonged illness or adverse events. Unfortunately, there are very few good prospective clinical studies to confirm such at-risk groups, nor the benefit of antibiotics in such groups. Delayed prescribing can either be implemented by giving parents access to a prescription with clear guidance, or not to prescribe but advise patients to return for review if they are getting worse or not improving. The latter option provides the clinician with more control, but may result in higher reconsultation rates, and for no clear benefit. In conclusion, based on current evidence, delayed prescribing has its place. If parents are provided with clear information about the timing of antibiotic use, what should trigger review, it is acceptable to parents, is reasonably safe, and provides a significant help in the battle against antibiotic resistance.

Wait and see prescription for the treatment of acute otitis media - benefit or harm?

David Spiro, M.D.

Paul Little and I will discuss the benefit of the Wait and See Prescription Approach for acute otitis media, and we will debate Drs. Marchant and Pichichero who will discuss the harm of this approach.

Great debate 1: Current status of the observation option for acute otitis media

Colin Marchant, M.D.

The observation option has been practiced in some countries for many years. Recently, it has become more fashionable, particularly in the United States with the publication of practice guidelines by the American Academy of Pediatrics and the American Academy of Family Practice. Although acute otitis media (AOM) is usually a self-limited disease if not treated with antibiotics, the medical literature supports the efficacy of antibiotic therapy in AOM. Analgesics are also widely prescribed and undoubtedly have some effect on pain and suffering, but there are no rigorous randomized trials of analgesic therapy in AOM. The observation option has been studied and the evidence demonstrates that children with AOM have less pain when treated immediately with antibiotics. While the observation option is acceptable to many parents, it is not in the best interests of the child with symptomatic AOM. In Great Debate 1, the fallacies, distortions, misrepresentations, and misconceptions underlying the observation option will be revealed.

Diagnostic inaccuracy and subject exclusions render placebo/observational studies of acute otitis media inconclusive

Michael Pichichero, M.D.

The reliability of diagnostic accuracy of clinicians enrolling children with acute otitis media (AOM) in trials to evaluate placebo/observation is of paramount importance. Using video otomicroscopy recordings that included pneumatic otoscopy, our group has tested the diagnostic skills of otolaryngologists, pediatricians, and general practitioners from three countries. Their accuracy in distinguishing AOM, otitis media with effusion (OME), and variations of normal was remarkably variable and 60-80% accurate for ENTs, and 40-50% accurate for pediatricians and general practitioners (Table 1).[1] The lack of accuracy in recognizing AOM among clinicians entering children in placebo/observational trials creates a high likelihood of enrollment of subjects who do not have bacterial AOM, and therefore will respond satisfactorily to no treatment.

Criteria for establishing a clinical diagnosis of bacterial AOM have been lacking, although the evidence suggests that a bulging/full tympanic membrane (TM) with effusion has the best positive predictive value based on tympanocentesis findings.[2,3] A review of the diagnostic criteria used in observational (Table 2) and natural history (Table 3) studies shows that few required a bulging TM.

Examination of subject enrollment criteria for placebo/observation trials provides evidence that many of those included did not have AOM but rather the subjects had no middle-ear disease at all or they had OME. Concerns about diagnostic certainty as estimated by Rosenfeld and Kay for the natural history

and placebo/observational studies are shown in Tables 4 and 5.[4] These studies should not be included in a meta-analysis because there is significant statistical and clinical heterogeneity (Table 6).

The exclusion criteria of placebo/observational studies are also remarkable. Frequently children <2 years old were excluded; mean age also reflected an older age group, unlike the true epidemiology of AOM. Otitis-prone children, those with severe disease, with a bulging TM, with fever, with a definite need for antibiotics, with recent antibiotic treatment, with recent AOM, or with perforation of the TM were often excluded (Tables 7-8).[4] These exclusion criteria biased results and reduced the generalizability of findings.

Guidelines and some authorities have overlooked or discounted the importance of the issues of inaccurate diagnosis on study entry, broad inclusion criteria, and the creation of bias in exclusion criteria among placebo/natural history trials. Meta-analyses have been performed despite statistical heterogeneity of the studies. Antibiotics eradicate bacteria if the organisms are susceptible. Adults would not tolerate the proposal that they suffer one extra hour from AOM due to concern about over-treatment and antibiotic pressure on selection of resistant bacterial strains. So why should children be considered for such treatment? The current momentum of opinion favoring observation of children with AOM should be reconsidered until better studies are conducted.

Current Status of "Observation Option" for Acute Otitis Media

Table 1. Comparison of diagnostic accuracy in three different countries among otolaryngologists, pediatricians, and nurse practitioners

		US% Correct			South African % Correct			Greek % Correct		
Ear Exam #	Correct Diagnosis	Otolary. (n=273)	Ped. (n=2,190)	NP (n=360)	Otolary. (n=36)	Ped. (n=36)	NP (n=206)	Otolary. (n=58)	Ped. (n=115)	NP (n=126)
1	OME	89	46	78	89	88	58	70	52	43
2	OME	70	48	28	71	22	29	50	23	37
3	Retracted, Otherwise Normal	70	54	64	56	71	58	68	43	44
4	AOM	78	72	56	76	66	68	84	56	63
5	OME	81	54	20	85	35	34	50	39	39
6	OME	35	29	22	44	46	25	32	29	26
7	Retracted, Otherwise Normal	88	48	39	81	48	35	70	28	33
8	OME	83	49	42	89	35	25	65	28	38
9	Retracted, Otherwise Normal	74	58	66	56	68	46	61	26	33
OVERALL		74	51	46	72	53	42	61	36	39

Table 2. Observational studies required inclusion criteria

		Acute Onset	Otalgia	Middle Ear Effusion	TM Full or Bulging	TM Red	TM Opaque	TM Immobile
1958	Fry	✓	✓			✓		
1964	Townsend		✓			✓		
1979	Thomsen	✓	✓		✓	✓	✓	✓
1985	Van Buchem	✓	✓		✓ Or	✓		
1988	Ostfeld	✓		✓	✓ Or	✓		
1990	Froom		53%	15%	36%	87%	50%	
1991	Bollag	✓	✓		✓ Or	✓		
1997	Tilyard	✓	✓ Or		✓			
2001	Little	✓	✓		✓ Or	✓ Or	✓	
2003	Siegel				✓ Or	✓ Or		
2005	Marchetti		✓ Or		✓ Or	✓ Or	✓	
2005	McCormick		✓	✓				

Table 3. Natural history studies required inclusion

		Acute Onset	Otalgia	Middle Ear Effusion	TM Full or Bulging	TM Red	TM Opaque	TM Immobile
1968	Halsted				✓	✓		
1970	Laxdal					✓		
1972	Howie			✓				
1981	Mygind	✓	✓			✓		
1981	Van Buchem				✓	✓		
1986	Thalin			✓				
1991	Appleman	✓	✓					
1991	Burke		✓					
1991	Kaleida	✓	✓	✓	✓ Or	✓ Or	✓ Or	✓
2000	Damoiseaux	✓	✓		✓ Or	✓		
2001	Jacobs	✓	✓	✓				
2005	LeSaux	✓		✓	✓ Or	✓ Or	✓ Or	✓

Table 4. Observational studies of nonantibiotic treatment of AOM

Year	Author	Country	Age Range	% < 2 yr	Diagnostic Accuracy *	Specialty
1958	Fry	England	< 20 yr	28%	Low (o)	UK
1964	Townsend	USA	NS	NS	Low (o)	PEDs
1979	Thomsen	Denmark	6 mo – 10 yr	52%	High (e,o)	ENT
1985	Van Buchem	Netherlands	2-12 yrs	0%	Low (o)	GPs
1988	Ostfeld	Israel	NS	88%	High (n,p)	ENT
1990	Froom	Various	NS	< 34%	Low (o)	GPs
1991	Bollag	Switzerland	< 16 yr	x = 5 yrs	Low (o)	GPs
1997	Tilyard	New Zealand	< 16 yr	25%	Low (o)	GPs
2001	Little	England	6 mo -10 yr	< 40%	Low (o)	GPs
2003	Siegel	USA	1 – 12 yr	x = 5 yrs	High (p)	PEDs
2005	Marchetti	Italy	1 – 14 yr	x = 4.8 yrs	Low (o)	PEDs
2005	McCormick	USA	6 mo – 12 yr	57%	Low (o)	PEDs

1.* Studies with high certainty confirmed diagnosis of AOM with tympanometry (t), pneumatic otoscopy (p), otomicroscopy (m), needle aspiration (n), or referral to an ear, nose, and throat specialist (e); low certainty studies relied on nonpneumatic otoscopy (o) and clinical symptoms.

2. Adapted from Rosenfeld R and Kay D Evidence Based Otitis Media

Table 5. Natural history of AOM in children randomized to placebo or no drug in clinical trials

Year	Author	Country	Age Range	% < 2 yr	Diagnostic Accuracy *	Specialty
1968	Halsted	USA	2 mo – 6 yr	70%	Low (o)	PEDs
1970	Laxdal	Canada	< 14 yr	< 50%	Low (o)	PEDs
1972	Howie	USA	< 2.5 yr	~ 100%	High (o,n)	PEDs
1981	Mygind	Denmark	1 -10 yr	x = 49 mos	High (e,o)	ENT
1981	Van Buchem	Netherlands	2 -12 yr	0 %	High (e,o)	ENT
1986	Thalin	Sweden	2 -15 yr	0 %	High (e,m)	ENT
1991	Appleman	Netherlands	6 mo – 12 yr	46 %	High (e,o)	ENT
1991	Burke	England	3 – 10 yr	0 %	Low (o)	GPs
1991	Kaleida	USA	7 mo – 12 yr	100 %	High (p,t)	PEDs
2000	Damoiseaux	Netherlands	6 mo – 2 yr	100 %	Low (o)	GPs
2001	Jacobs	USA	18 mo – 6 yr	x = 40 mos	Low (o)	PEDs
2005	Le Saux	Canada	6 mo – 6 yr	28%	High (p,t)	PEDs/GPs

1.* Studies with high certainty confirmed diagnosis of AOM with tympanometry (t), pneumatic otoscopy (p), otomicroscopy (m), needle aspiration (n), or referral to an ear, nose, and throat specialist (e); low certainty studies relied on nonpneumatic otoscopy (o) and clinical symptoms.

2. Adapted from Rosenfeld R and Kay D Evidence Based Otitis Media

Table 6. Heterogeneity found among studies

Year	Author	Diagnostic Certainty	Managed w/out Antibiotics n/N9%)
1958	Fry	Low (o)	387/497 (78)
1964	Townsend	Low (o)	243/335 (73)
1979	Thomsen	High (e,o)	76/93 (82)
1985	Van Buchem	Low (o)	465/490 (95)
1988	Ostfeld	High (n,p)	397/693 (57)
1990	Froom	Low (o)	419/2,982 (14)
1991	Bollag	Low (o)	211/230 (92)
1997	Tilyard	Low (o)	74/2,441 (3)
2001	Little	Low (o)	114/285 (40)
RANDOM-EFFECTS METANALYSIS			
Combined sample size			8,101
Estimate of combined rate, (95% CI)		.59 (.31, .87)	
Test for heterogeneity , Q statistic			11,803.7, df =8
Test for heterogeneity, p value			<.001

1.* Studies with high certainty confirmed diagnosis of AOM with tympanometry (t), pneumatic otoscopy (p), otomicroscopy (m), needle aspiration (n), or referral to an ear, nose, and throat specialist (e); low certainty studies relied on nonpneumatic otoscopy (o) and clinical symptoms.

2. Adapted from Rosenfeld R and Kay D Evidence Based Otitis Media

Table 7. Observational Studies Required Exclusion Criteria

Year	Author	Recent Antibiotics	Perforated TM	Chronic OM	OME	Required Antibiotics	Unwell/Severe	Tympanostomy Tubes
1958	Fry					✓	✓	
1964	Townsend		✓				✓	
1979	Thomsen		✓				✓	
1985	Van Buchem	✓		✓	✓			
1988	Ostfeld	✓	✓	✓				✓
1990	Froom							
1991	Bollag						✓	
1997	Tilyard							
2001	Little	✓			✓		✓	
2003	Siegel		✓				✓	
2005	Marchetti	✓	✓	✓				
2005	McCormick		✓				✓	✓

Table 8. Natural history studies exclusion criteria

Year	Author	Recent Antibiotics	Perforated TM	Chronic OM	OME	Required Antibiotics	Unwell/Severe	Tympanostomy Tubes
1968	Halsted	✓	✓					
1970	Laxdal		✓					
1972	Howie					✓	✓	
1981	Mygind	✓	✓					
1981	Van Buchem	✓		✓	✓			
1986	Thalin	✓				✓		
1991	Appleman	✓						
1991	Burke	✓	✓			✓	✓	✓
1991	Kaleida	✓					✓	
2000	Damoiseaux	✓						
2001	Jacobs	✓	✓					✓
2005	Le Saux	✓	✓			✓	✓	

References

1. Pichichero ME, Poole MD. Comparison of performance by otolaryngologists, pediatricians, and general practitioners on an otoendoscopic diagnostic video examination. Int J Pediatr Otorhinolaryngol 2005; 69:361-366.
2. Arola M, Ruuskanen O, Ziegler T, et al. Clinical role of respiratory virus in acute otitis media. Pediatrics 1990; 86: 848-55.
3. Karma PH, Sipila MM, Kayaja MJ, Penttila MA. Premature otoscopy and otitis media: The value of different tympanic membrane findings and their combinations. In: Lim DJ, Bluestone CD, Klein JO, Nelson JD, Ogra PL, eds. *Recent advances in otitis media: proceedings of the fifth international symposium*. Burlington, Ontario: B.C. Decker; 1993: 41-5.
4. Rosenfeld RM, Kay D. Natural history of untreated otitis media. Laryngoscope 2003; 113:1645-1657.

Great debate: Which child benefits most from tympanostomy tubes?

Richard Rosenfeld, M.D., M.P.H., Mark Haggard, Ph. D., Jorgen Lous, M.D., Robert Ruben, M.D., Anne Schilder, M.D.

Objective

To offer a lively, entertaining "great debate" about the benefits (or lack thereof) of tympanostomy tubes for otitis media, based on current best evidence, expert opinion, and new research.

Design

Participants will have 8-10 minutes each to formally present their opinion, followed by case presentations with feedback from participants and the audience regarding management options.

Participants

Drs. Lous and Schilder will take the position that tube benefits are smaller than advertised, tubes are probably overused, and that greater caution should be exercised by clinicians regarding tube insertion. Conversely, Drs. Haggard and Ruben will argue that tube benefits can be larger than advertised in certain populations, and that tubes might even be underutilized. The moderator, Dr. Rosenfeld, will ensure that all fighting is fair and just, and that none of the presenters or attendees incurs bodily injury.

Main outcome measures

The side with the most participants alert, conscious, and oriented at the debate conclusion will be declared the winner.

Results

We are unlikely to eliminate all uncertainty about "which child benefits most from tympanostomy tubes," but the debate will facilitate an evidence-based approach to selecting children most likely to benefit from intervention.

Conclusions

To be determined by the participants and audience.

Which children benefit most from tympanostomy tubes (grommets)?

Jorgen Lous, M.D.

Background

Operation with tympanostomy tubes (TT) is the second most common operation in the world, only exceeded by circumcision. The use of TT vary greatly between countries, regions, and ENT specialists. A ten-fold variation has been described.[1] Denmark has a very high rate of insertion of TT, about 40,000 operations a year in a population of 5.5 million inhabitants. What is the right level? And which children benefit most from grommets?

Objective

The aim of this paper is to document why I believe we, at the moment, have an overuse of TT in some areas, and how we should be as fair as possible based on evidence.

Methods and materials

I refer to epidemiological research in otitis media with effusion (OME) in North Jutland, Denmark, and in other countries that has been done during the last 30 years, supplemented by prospective research of possible language and cognitive developmental disability after periods with OM. Finally, I present some the results from a systematic Cochrane Review, and point at some tracks forward.

Results

My history begins with two observations from 1974 at my residency at the ENT department in the very north of Jutland. All children seen with OME at the ENT department had an operation with TT and adenoidectomy. At the same time, we in the residents' department had 13 children, and 10 of them had or have had OME for shorter or longer periods. The two observations did not fit.

The idea to make a prospective survey to study the natural history of OME was born. All 523 3-year-old children in the municipality of Hjorring were invited to an examination at the ENT department in the city. A total of 98% showed up, and 12% had OME.[2] They were followed for 6 months without intervention. We found a great spontaneous normalization.[3] An epidemiological study in schoolchildren with 10 examinations during the first year in school showed hat nearly all OME disappeared during the summer season.[4] We found no association between duration of OME and reading (OS-400), Peabody vocabulary test, and verbal IQ.[5-7] Phonology at the beginning of school was lower in the very few children with bilateral OME just at the time of testing.

The Danish consensus conference on OME in 1987 concluded that the present use of TT of 23,000 per year in children--meaning about 20 TT per 1,000 children between ages 0-15 years per year--was too high.

A systematic Cochrane review[8] of randomized trials on the effect of TT in children with OME showed the effects of TT on hearing and time with OME. In a subgroup of randomized trials with bilateral OME and treatment with unilateral TT, the effect was an improvement in hearing of about 9 dB after 6 months and only a few dB after 12 months. Only four randomized studies on the developmental and cognitive consequences of OME fulfilled the inclusion criteria. They found no significant effect of early TT on development compared with 6- to 9-month delayed operation. At the moment we do not know if the excluded children are the ones who would benefit from TT. The children in the Maw study were included from the waiting list. They were apparently more affected than the children in the other three studies. Maw found a marginal significant effect of delayed TT on language development.

Discussion

The use of TT in very young children with recurrent AOM is purely documented from randomized studies. But, the use of this indication is increasing. At the moment, we in Denmark do nearly 40,000 operations for TT, more than 35,000 on children—or about 25-30 TT per 1,000 children per year.

In 2002 we found 25% of normal day care children at the age of 3 to 5 years, had TT or have had

them.[9] One Iceland a study found that up to 40% of children in some regions have had TT.[10]

The complications to TT have been described. A few dB hearing loss has been found in some prospective, well-controlled studies and not in others. Changes in the tympanic membrane are well known, but their long-term consequences on hearing are still discussed. Complications to the anaesthesia narcoses can be serious.

Some children really need TT. How to define them? How to identify them? As mentioned, were the children in the trial by Maw et al. more affected than children in the other three RCTs, where the children were found by screening? The effect of the TT was marginally significant. What we need is not more RCTs where the most affected children are excluded from the randomization because of ethical reasons. Instead, we need prospective cohort studies including all children with OME, follow them all, and do regular examination to characterize the children who benefit most from TT, including the children where the parents and the ENT physician did not find indication for operation during the observation period.

Another important question is the role of adenoidectomy as a supplement to TT or instead of TT. Some results show a more long-lasting effect of adenoidectomy compared to TT.

Also, we really need preoperative tests or indicators to identify the children at most risk for later complications that are anatomical, functional, and developmental.

Conclusions

Until further evidence is available, we should be very restrictive in the use of TT in otherwise normal children. Six months with bilateral OME and significant hearing loss should be present before treatment with TT.

At the moment, we have no evidence for the subgroups of children excluded from the RCTs, i.e., children with speech/language delays, behavior and learning problems, or other syndromes. Clinicians will need to make their own decisions regarding treatment of such children.

The situation just now, as I see it, is that some children are overtreated and some are undertreated. There is an urgent need for prospective cohort studies on children with OME to characterize the children who benefit most from TT.

References

1. Schilder AG, Lok W, Rovers MM. International perspectives on management of acute otitis media: a qualitative review. Int J Pediatr Otorhinolaryngol 2004; 68:29-36.

2. Fiellau-Nikolajsen M, Lous J, Pedersen SV, Schousboe HH. Tympanometry in three-year-old children. I. a regional prevalence study on the distribution of tympanometric results in a non-selected population of three-year-old children. Scand Audiol 1977; 6:199-204.

3. Fiellau-Nikolajsen M, Lous J. Prospective tympanometry in three-year-old children: a study of spontaneous course of tympanometry types in a non-selected population. Arch Otolaryngol 1979; 105:461-466.

4. Lous J, Fiellau-Nikolajsen M. Epidemiology of middle ear effusion and tubal dysfunction: a one-year prospective study comprising monthly tympanometry in 387 non-selected 7-year-old children. Int J Pediatr Otorhinolaryngol 1981; 3:303-317.

5. Lous J, Fiellau-Nikolajsen M. A 5-year prospective case-control study of the influence of early otitis media with effusion on reading achievement. Int J Pediatr Otorhinolaryngol 1984; 8:19-30.

6. Lous J. Silent reading and secretory otitis media in school children. Int J Pediatr Otorhinolaryngol 1993; 25:25-38.

7. Lous J, Fiellau-Nikolajsen M, Jeppesen AL. Secretory otitis media and language development: a six-year follow-up study with case-control. Int J Pediatr Otorhinolaryngol 1988; 15:185-203.

8. Lous J, Burton M, Felding J, Ovesen T, Rovers M, Williamson I. Grommets (ventilation tubes) for hearing loss associated with otitis media with effusion in children. Cochrane Database Syst Rev 2005; 1:CD001801.

9. Lauritsen MB. Identification of the preschool child at risk of developmental effects from otitis media with effusion. Evaluation and further development of a Danish speech-in-noise test. Copenhagen, Research unit for general practice: University of Copenhagen, 2006.

10. Arason VA, Sigurdsson JA, Kristiansson KG, Gudmundsson S. Tympanostomy tube placements, sociodemographic factors and parental expectations for management of acute otitis media in Iceland. Pediatr Infect Dis J 2002; 21:1110-1115.

Which child benefits from tympanostomy tubes?

Robert Ruben, M.D.

"All animals are equal but some animals are more equal than others"[1]

Physicians take care of individuals, yet much of the rationale for treatment decisions is based on population studies in which individual patient susceptibility is poorly represented. This occurs from two different problems in experimental design. One is the prohibitive costs of studies sufficiently large to include enough cases of various subgroups that are both susceptible to the acquisition of disease and to their sequelae/morbidity. Yet biological knowledge and clinical experience suggest many ways in which mechanisms of differential susceptibility to acquisition and sequelae/morbidity interact and need to be recognized. In treatment studies, the clinically reasonable presumption of benefit and obligation to treat leads to exclusions. Too frequently, this leads to a bland sample of milder cases, and this underestimates the benefits from treatments to susceptible patients. The second is that it is difficult to design ethical prospective and/or randomized studies for known or highly suspected susceptible populations.

The interactions of the patient's individual susceptibility characteristics with the variation of the disease agent are usually excluded from randomized controlled trials (RCTs)--the basis for evidence-based medicine (EBM)--for reasons of power, research costs, and more. Consequently, these studies usually do not examine the critical variables. To determine what the morbidity will be, a physician caring for the individual patient needs to consider the differences–the special intrinsic and extrinsic susceptibility of the patient for acquiring the disease and the other intrinsic and extrinsic patient characteristics.

First let us look at the long and diverse list of intrinsic factors for acquiring otitis media with effusion (OME). These include genetic traits, craniofacial malformations such as acute sphenopalatine angle, immune deficiency, previous history of acute otitis media (AOM) or OME, bilateral disease, low IgA or IgG2 levels with poor eustachian tube function and decreased levels of mannose-binding lectin, physiologic conditions such as muscular dystrophy, and others. Then, there are the extrinsic factors for acquiring OME--tobacco smoke exposure, day care attendance, lack of breast-feeding, winter season, prolonged endotracheal intubation, and others.

Another part of the decision-making process to determine which child will benefit from a tympanostomy tube must consider the characteristics that will exacerbate the morbidity from OME, i.e., given the same occurrences of OME, some children will be more adversely affected than others. In this differential morbidity, both the intrinsic factors (cognitive resources, linguistic development, psychiatric factors, sensory deficiencies, enzymatic disorders) and the extrinsic influences (inadequate parental/caretaker language input, poverty) play a role.

One of the most cited studies on the efficacy of tympanostomy tubes for OME correctly concluded from their data that:

"In otherwise healthy children younger than three years of age who have persistent middle-ear effusion within the duration of effusion that we studied, prompt insertion of tympanostomy tubes does not improve developmental outcomes at six years of age"[2]

However, close examination of this study reveals that the list of exclusions is so long that its relevance to clinical practice must be in doubt, or at least remains to be proven by exercises in generalization.

Firstly, this study excluded various intrinsic factors for acquiring OME such as birth weight < 5lbs, small for gestational age, other serious illness, major congenital malformation, chronic illness, multiple births, and having a sibling already enrolled. Secondly, this study excluded various extrinsic factors for acquiring OME such as foster care, adopted, mother dead, and mother a known drug or alcohol abuser. Thirdly, this study excluded the intrinsic factors for exacerbating the morbidity of OME of birth weight < 5lbs, small for gestational age, neonatal asphyxia, other serious illness, and a chronic illness. Fourthly, this study excluded the extrinsic factors for exacerbating the morbidity of OME including foster care, adopted, mother dead, mother a known drug or alcohol abuser, mother limited socially, mother limited intellectually, mother < 18 years old, and English not the only language spoken.

This study gives no information as to prevalence or incidence of the excluded cases, and thus we do not know how many of these are to be found in

the typical caseload that is referred for intervention. The families of very effected children will seek out intervention and would, to this observer, be much less likely to participate in a randomized study, for they see a greater need for care for their child. Most of the hearing losses in the Paradise study were minimal, as only 9% of subjects with early intervention had bilateral continuous OME with abnormal hearing loss of > 20 to 25 dB PTA for 60 days. The very impaired seemed to be absent from this cohort.

It is a reasonable expectation that those children with OME having one, two, or more complicating factors would receive particularly high benefit if treated. It may be true that they do, and clear illustrations of this would be useful. Need, and the relevant concern for care, will be proportional to the seeking of care; that is, the greater the need, the more care sought. Also the greater the need (morbidity), the greater effect the same benefit will have. One example may suffice. A severely retarded boy who was considered not trainable had a 45dB PTA conductive loss from OME. After an adenoidectomy and tube insertion, his hearing was restored to 5 dB PTA and, within a few months, he was reclassified as trainable. This was mother who saw a need, and the resultant hearing and communication improvement allowed for the quantal cognitive advance. This type of child was excluded from the Paradise study.

The decision for intervention for OME--a tympanostomy tube with or without an adenoidectomy--should be made from data that cover intrinsic and extrinsic host susceptibilities. There is yet another factor which must be accounted for, although there is sparse data in this area: information about the virulence of the disease agents and their interaction with the host. However, decisions must be made using what information is available.

The physician must determine whom and when to treat based upon on the knowledge of the patient's intrinsic and extrinsic risk factors for acquisition of the disease and the subsequent morbidities. The physician's assessment is based on knowledge of and experience with the synergy of these factors.

The next objective for medicine in the 21st century, built upon the RTC and EBM, will be knowledge-based medicine, which is the logical and worthy continuation of the movement begun by Cochrane's 1972 *Effectiveness and efficiency; random reflections on health services*.[3]

References

1. Orwell G. Animal Farm. London: Secker & Warburg; 1945.
2. Paradise JL, Campbell TF, Dollaghan CA, et al. Developmental outcomes after early or delayed insertion of tympanostomy tubes. N Engl J Med 2005; 353:576-586.
3. Cochrane AL. Effectiveness and efficiency; random reflections on health services. London: Nuffield Provincial Hospitals Trust; 1972.

Great debate: towards a balanced synthesis on surgical intervention in otitis media with effusion

Mark Haggard, Ph.D.

Data are often insufficient to provide a good evidence base for practice, but asserting this is a stock reflex, sometimes biased by the aspirations of research providers. Existing data are frequently misused (quoted selectively to confirm prior preconception) or are under-used (conceptually inappropriate or insufficiently detailed analysis, failure to apply theoretical constraint from known facts or principles, or failure to attempt rational interpretation). In otitis media with effusion (OME), it has long been appreciated that mere diagnosis is extremely common, so the attribution of severe impact and the basis for a surgical intervention policy must be restricted well beyond diagnosis e.g., comorbidities, severe and persistent cases, etc. The more remarkable fact is that many practitioners and some researchers have remained in denial of this principle. Absence of evidence is not evidence of absence. Hitherto, research formulated specifically to show with power and precision just what the "etc." above are and where

cut-offs might with clinical wisdom be drawn has been absent. This did not make the principle false.

Thankfully, some of the required evidence is now present from the UK TARGET trial, where the restricted-access healthcare system plus the entry criteria combined to locate enough severely and persistently affected children. It generally confirms the truth of the principle (see other papers at this meeting) and furthermore suggests a detailed clinical algorithm. Correct application of results cannot be guaranteed. Over-interventionists will probably continue to ignore the recommended restrictions and indulge or exploit biased beliefs. Anti-interventionists wishing to focus on those parts of the data showing null effectiveness will continue to throw out the baby with the bathwater and be left unable to reconcile effectiveness evidence with more explanatory and efficacy-oriented evidence for pathogenetic and therapeutic hypotheses. However, such misuses and under-uses of the data are not inevitable. Knowledge-based medicine is advanced as a framework for minimizing mis- and under-use.

Predictors of chronic suppurative otitis media in children

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Objective

To determine which factors predict development of chronic suppurative otitis media (CSOM) in children.

Design

Case control study.

Subjects

Prognostic factors for CSOM were identified in 1) 100 children with CSOM and 161 controls aged 1 to 12 years, and 2) 83 children who developed CSOM in the presence of a tympanostomy tube and 136 children with tympanostomy tubes who did not develop CSOM.

Methods of analyses

Univariate and multivariate logistic regression analyses were applied to evaluate which factors independently predict CSOM.

Results

Independent predictors for CSOM were: previous tympanostomy tube insertion (OR 121.4 [95% CI 38.9-379.3]); having had more than three upper respiratory tract infections in the past 6 months (OR 12.2 [95% CI 3.5-42.3]); having parents with a low education level (OR 14.1 [95% CI 2.9-68.6]); and having older siblings (OR 4.4 [95% CI 1.6-12.6]). Independent predictors for CSOM after tympanostomy tube insertion were: having experienced more than three episodes of otitis media in past year (OR 4.9 [95% CI 2.2-11.0]; attending day-care (OR 3.6 [95% CI 1.7-7.8]); and having older siblings (OR 2.6 [95% CI 1.2-5.5]).

Conclusions

In conclusion, treatment with tympanostomy tubes is the most important prognostic factor for CSOM in children. In children who are being treated with tympanostomy tubes for persistent middle-ear effusion this is a history of recurrent episodes of acute otitis media. This information should be taken into consideration and discussed with parents when considering insertion of tympanostomy tubes in children.

Molecular biological study of infectious mechanism of *S. pneumoniae* in conjunctivitis-otitis media syndrome in young children

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Introduction

Acute otitis media (AOM) is one of the popular infectious diseases among childhood. *Streptococcus pneumoniae* is a leading pathogen responsible for AOM, rhinosinusitis, and a variety of infections in the respiratory tract such as acute purulent exacerbation of bronchitis and pneumonia. Acute conjunctivitis also is a common ocular infection during infancy and childhood. *S. pneumoniae* are also responsible for bacterial acute conjunctivitis as well as acute rhinosinusitis and AOM.[1] However, the infectious mechanism of microorganisms in conjunctivitis-otitis media-rhinosinusitis syndrome has not been addressed clearly yet.[2] *S. pneumoniae* colonizes in the nasopharynx and will often infect the conjunctiva or middle-ear cavity.[2]

The purpose of this study is to elucidate the infectious mechanism in conjunctivitis-otitis media-rhinosinusitis syndrome by studying bacteria isolated from the conjunctiva, middle ear, and nasopharynx in each patient using bacteriological and molecular-biological techniques, with special emphasis on *S. pneumoniae*.

Materials and methods

Subjects. The subjects of this study were infants and young children at age ≤60 months who visited the Sugita ENT clinic. Their chief complaints were purulent nasal discharge, ear symptoms as otalgia, and/or conjunctivitis.

General designs. The nasal discharges from acute rhinosinusitis, acute conjunctival lavage from conjunctivitis, and middle-ear fluids from AOM were subjected to bacterial culture and further molecular analysis. *S. pneumoniae* as well as *Haemophilus influenzae* and *Moraxella catarrhalis* were identified according to the standard microbiological procedures. Antimicrobial susceptibilities of *S. pneumoniae* isolates were determined by the minimal inhibitory concentrations (MICs) recommended by Clinical Laboratory Standard Institute (CLSI). Polymerase chain reaction (PCR)-based genotypes of *S. pneumoniae* were also determined in each isolate. Pulsed-field gel electrophoresis (PFGE) was performed to determine the identical bacteria isolated from the three different sites of each patient.

PCR-based genotyping. Primers for *pbp1a*, *pbp2x*, and *pbp2b* were designed to amplify penicillin-binding protein 1A (PBP1A), PBP2x, PBP2B. To confirm the isolated pathogen as *S. pneumoniae*, identification of *letA* was applied.

A single colony of *S. pneumoniae* on chocolate agar plates were lysed in 30 μl of lysis solution (1M Tris pH 8.9, 4.5 v/v nonident P-40, 4.5 v/v Tween 20, 10 mg/ml Proteinase K) for 10 minutes at 60°C and for 5 minutes at 94°C in the programmable thermal cycler, Gene Amp PCR System 9700 (Parkin Elmer, Norwalk, CT). A total 50 μl of reaction mixtures consisted of 2 μl of bacterial lysate, 0.8 μl of 10 mM of dNTP mixture, 0.1 μl of *Taq* DNA polymerase, 2.5 μl of 10 x PCR buffer, 0.5 μl of 25 mM MgCl$_2$, 5.0 μl Q-solution (Qiagen, Valencia, CA), 0.125 μl (100 μM) of each primer and distilled water. The mixture was subjected to denaturation at 94°C for 10 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds and then further extension at 72 °C for 10 minutes. Amplified DNA fragments were analyzed using 3% agarose gel electrophoresis.

On the basis of the PCR-based genotyping, *S. pneumoniae* strains were classified into four genotypes. They were genetically penicillin-susceptible *S. pneumoniae* (gPSSP) without mutations in *pbp1a*, *pbp2x*, and *pbp2b*, genetically penicillin intermediately resistant *S. pneumoniae* (gPISP) with one or two *pbp* genes such as *pbp1a*, *pbp2x*, *pbp2b*, *pbp1a*+pbp2, *pbp1a*+pbp2b, *pbp2x*+*pbp2b*, and genetically penicillin-resistant *S. pneumoniae* (gPRSP) in the strains having mutations in all *pbp* genes.

Restriction fragment polymorphism of genomic DNA analyzed by PFGE. One colony of *S. pneumoniae* isolate was grown at 37°C for 6 hours in Todd Hewitt broth (Difco Laboratories, Detroit, MI). The cells were harvested by centrifugation at 4000 g at 4°C for 5 minutes, washed with phosphate buffered saline (PBS), and suspended in washing buffer (50 mM Tris-HCl, pH 7.5). An equal volume of low-

melting-point agarose (1.6% of agarose for *S. pneumoniae* and 2.0% of agarose for *H. influenzae*) (In Cert agarose, FMC BioProducts, Rockland, ME) was added to 50 μl of each cell suspension for plug preparation. The mixture was poured into disposable 100-μl scale plug molds (Bio-Rad, Laboratories, Hercules, CA) and chilled at 4°C for 20 minutes. After incubation with 2 ml of lysis buffer (0.25 M EDTA, 1% SDS, 10 mM Tris-HCl, pH 9.5, and 0.5 mg/ml of proteinase K) over night at 50°C, sample plugs were rinsed with 2 ml of washing buffer three times for 30 minutes. One-third of each plug was sliced off. The restriction of genomic DNA was carried out after equilibration of the sliced plugs with appropriate restriction buffer, then each slice was incubated with 80 U of *Sma*I (Takara Shuzo, Co. Ltd., Ohtsu, Japan) for 20 hours at 30°C. The reaction was stopped by an equal volume of 0.5 M EDTA (pH 8.0). Electrophoresis was performed with a GenePath PFGE apparatus (Bio-Rad Laboratories, Hercules, CA) in 0.5 × TBE buffer (1 × TBE buffer: 0.1 M Tris-HCl, 0.1 M boric acid, 2 mM EDTA, pH 8.0). A 48.5-kb bacteriophage lambda DNA ladder (FMC BioProducts, Rockland, ME) was used as a molecular size marker. The gels were stained with ethidium bromide for 20 minutes and were photographed under UV light at 302 nm.

Results

Populations. A total 170 patients with rhinosinusitis, 170 patients with conjunctivitis, and 116 patients with AOM were enrolled into this study. The ages ranged from 5 to 48 months (mean 20.2 months).

Identification of S. pneumonia. A total 172 *S. pneumoniae* was isolated from patients of conjunctivitis-otitis media-rhinosinusitis syndrome (Fig. 1). They were 97 isolates (30.3%) from 320 nasal discharges, 51 isolates (27.3%) from 187 conjunctival lavage, and 24 isolates (26.4%) from 91 MEFs. Among 130 isolates identified from nasal discharge, 27 isolates (27.8%) were gPSSP strains, 63 isolates (65.0%) were gPISP, and 7 isolates (7.2%) were gPRSP strains. Among 51 isolates identified from conjunctival lavage, 10 isolates (19.6%) were gPSSP strains, 36 isolates (70.6%) were gPISP strains, and 5 isolates (9.8%) were gPRSP strains. Among 24 isolates identified from middle-ear fluids, 8 isolates (33.3%) were gPSSP strains, and 16 isolates (66.7%) were gPISP strains.

Determination of S. pneumoniae clones in conjunctivitis-otitis media-rhinosinusitis syndrome. A total 161 patients with conjunctivitis-otitis media-rhinosinusitis syndrome were evaluated for determination of *H. influenzae* clones. They were 68 patients (71 pairs) with rhinosinusitis, conjunctivitis, and AOM; 70 patients (107 pairs) with rhinosinusitis and conjunctivitis; and 23 patients (23 pairs) with rhinosinusitis and AOM. All 12 pairs of *S. pneumoniae* from nasal discharges, conjunctival lavage, and middle-ear fluids showed identical clones. Among 31 pairs of *S. pneumoniae* from nasal discharge and conjunctival lavage, 29 pairs (93.5 %) showed identical clones of *S. pneumoniae*. All 13 pairs of *S. pneumoniae* from nasal discharge and middle-ear fluids showed identical clones (Table 1, Fig. 2).

Discussion

Conjunctivitis-otitis media syndrome was first reported by Bodor et al. in 1982.[1] About 73% of the patients with purulent conjunctivitis concurrently had AOM. An identical pathogen was isolated from approximately 87% of the patients with bacterial conjunctivitis. *H. influenzae* has been the most frequently isolated causative pathogen responsible for conjunctivitis-otitis media syndrome.[2] However, the positive predictive value of nasopharyngeal culture for positive middle-ear fluid is reported to be only 47%. On the other hand, the negative predictive value of nasopharyngeal cultures for negative middle-ear fluid was about 87%.

S. pneumonia, together with *H. influenza,* colonizes asymptomatically in the human nasopharynx and becomes a leading causative pathogen responsible for AOM.[3] Thus, the *S. pneumoniae* will also become an important pathogen for conjunctivitis-otitis media-rhinosinusitis syndrome. Bacterial isolates from conjunctival lavage fluids and middle-ear fluid in younger children were found to be identical with those from nasal discharge by the molecular biological analysis in this study. The current findings showed that about 93.5% to 100% of the patients had identical clones of *S. pneumoniae* in the nasopharnx, conjunctiva, and middle-ear fluid. These results strongly suggest that bacteria in the nasopharynx and nasal discharge will cause conjunctivitis-otitis media-rhinosinusitis syndrome by ascending infections via the eustachian tube to the middle-ear cavity resulting otitis media and via the naso-lacrimal duct to the conjunctiva resulting conjunctivitis.[4]

The increase of PRSP is causing serious clinical problems worldwide and also in Japan.[5] The countrywide surveillance of *S. pneumoniae* isolated from upper respiratory tracts in Japan between 1998 and 1999 showed that PSSP, PISP, and PRSP accounted for 49.6%, 28.5%, and 21.9% respectively.

Physicians should pay attention to recent increases in antimicrobial-resistant *S. pneumoniae* when they treat pediatric infectious diseases.[6] The present study demonstrated that nasal discharge serves as a source of bacterial transmission responsible for bacterial acute conjunctivitis and AOM.[7]

Figure 1. Bacterial pathogens identified from conjunctivitis-otitis media-rhinosinusitis syndrome.

ND: Nasal discharge
CL: Conjunctival lavage
MEF: Middle ear fluid
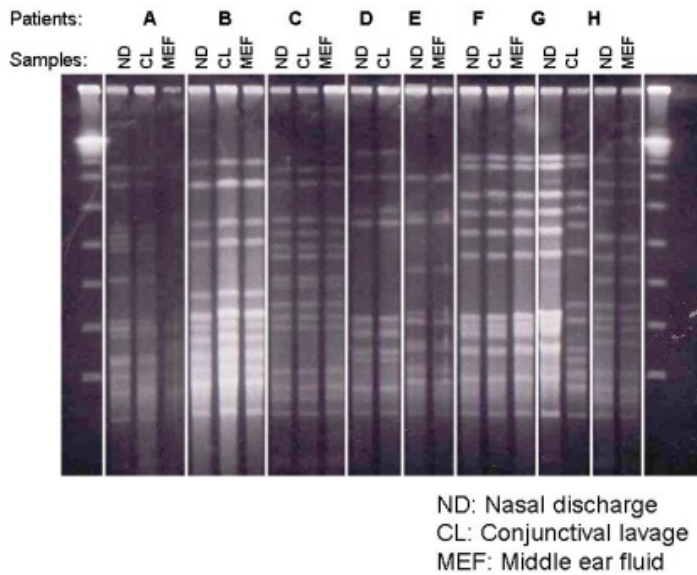
Figure 2. Clonal analysis of *S. pneumoniae* in conjunctivitis-otitis media-rhinosinusitis syndrome.

Rhinosinusitis
(n=170 patients, 320 isolates)

Conjunctivitis
(n=170 patients, 187 isolates)

Acute otitis media
(n=116, 91 isolates)

H. influenzae
S. pneumoniae
M. catarrhalis
S. pyogenes
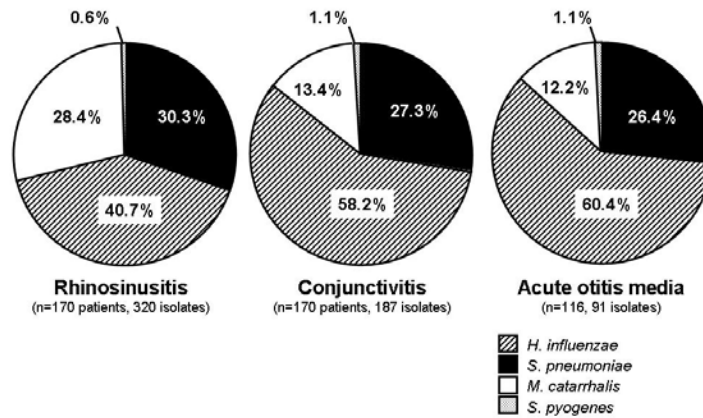
Table 1. Identification of *S. pneumoniae* in conjunctivitis-otitis media-rhinosinusitis syndrome.

Rhinosinusitis	Conjunctivitis	Acute otitis media	Identical PFGE pattern
●	●	●	12/12 pairs (100%)
●	●	×	29/31 pairs (93.5%)
●	×	●	13/13 pairs (100%)

References

1. Bodor FF. Conjunctivitis-otitis media syndrome. Pediatrics 1982; 69:695-698.
2. Bodor FF, Marchant CD, Shurin PA, Barenkamp SJ. Bacterial etiology of conjunctivitis-otitis media syndrome. Pediatrics 1985; 76:26-28.
3. Groothuis JR, Thompson J, Wright PF. Correlation of nasopharyngeal and conjunctival cultures with middle ear fluid cultures in otitis media. A prospective study. Clin Pediatr 1986; 25:85-88.
4. Harrison CJ, Hedrick JA, Block SL, Gilchrist MJ. Relation of the outcome of conjunctivitis and the conjunctivitis-otitis media syndrome to identifiable risk factors and oral antimicrobial therapy. Pediatr Infect Dis J 1987; 6:536-540.
5. Block SL, Hedrick J, Tyler R, Smith A, Findlay R, Keegan E, Stroman DW. Increasing bacterial resistance in pediatric acute conjunctivitis (1997-1998). Antimicrob Agents Chemother 2000; 44:1650-1654.
6. Bodor FF. Systemic antibiotics for treatment of the conjunctivitis-otitis media syndrome. Pediatr Infect Dis J 1989; 5:287-290.
7. Bingen E, Cohen R, Jourenkova N, Gehanno P. Epidemiology study of conjunctivitis-otitis syndrome. Pediatr Infect Dis J 2005; 24:731-732.

Molecular biological study of infectious mechanism of nontypeable *H. influenzae* in conjunctivitis-otitis media syndrome in young children

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Introduction

Acute conjunctivitis together with acute otitis media (AOM) and acute rhinosinusitis are common bacterial infectious diseases with a high incidence during infancy and childhood.[1] Reported symptoms accompanying bacterial acute conjunctivitis include acute upper airway infections such as nasal discharge and otalgia/otorrhea.[2] *Haemophilus influenzae* (*H. influenzae*) is a major pathogen responsible for AOM, acute rhinosinusitis, and variety of infections in the respiratory tract such as acute purulent exacerbation of bronchitis and pneumonia. However, less study of bacterial infections causing these conditions of combined acute conjunctivitis, AOM and acute rhinosinusitis complex named as conjunctivitis-otitis media-rhinosinusitis syndrome has been reported.[1] The infectious mechanisms of these microorganisms in the conjunctivitis-otitis media-rhinosinusitis syndrome have not been clear.

The present study was undertaken to demonstrate that bacteria isolated from nasal discharge were identical to those isolated from conjunctival lavage or middle ear fluids (MEFs) by use of bacteriological and molecular biological techniques based on the assumption that nasal discharge serves as a source of bacterial transmission responsible for bacterial acute conjunctivitis and AOM.

Materials and methods

Subjects

The subjects of this study were infants and young children at age ≤60 months who visited Sugita ENT clinic. Their chief complaints were nasal discharge and ear symptoms such as otalgia and/or conjunctivitis.

General designs

The nasal discharges from rhinosinusitis, conjunctival lavage from conjunctivitis and middle ear fluids from AOM were subjected to bacterial culture's. *influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*

were identified by the standard laboratory procedures. Antimicrobial susceptibilities of each bacterial isolates were determined by the minimal inhibitory concentrations (MICs). Polymerase chain reaction (PCR) based genotypes of *H. influenzae* were also determined in each *H. influenzae* isolates. When the pairs of *H. influenzae* isolated from nasal discharge and middle ear fluids or conjunctival lavage were identified, the genomic polymorphisms were further analyzed by pulsed-field gel electrophoresis (PFGE).

PCR-based genotyping

Primers for *ftsI* were designed to amplify both a variable mutated locus (Asn-526 or Arg-517) and a highly mutated locus (Ser-385).Primers to amplify *bla* were designed to confirm production of ß-lactamase. To confirm the isolated pathogen as *H. influenzae*, identification of *p6* was applied. The determination of type b *H. influenzae* strain, primers for *cpsb* was used in this study.

PCR was conducted as usual laboratory procedures. Briefly, a single colony of *H. influenzae* on chocolate agar plates were lysed in 30 µl of lysis solution (1M Tris pH 8.9, 4.5 v/v nonident P-40, 4.5 v/v Tween 20, 10 mg/ml Proteinase K) for 10 min at 60°C and for 5 min at 94°C in the programmable thermal cycler, Gene Amp PCR System 9700 (Parkin Elmer, Norwalk, Conn., USA). A total 50 µl of reaction mixtures consisted of 2 µl of bacterial lysate, 0.8 µl of 10 mM of dNTP mixture, 0.1 µl of *Taq* DNA polymerase, 2.5 µl of 10x PCR buffer, 0.5 µl of 25 mM MgCl$_2$, 5.0 µl Q-solution (Qiagen, Valencia, Calif., USA), 0.125 µl (100 µM) of each primer and distilled water. The mixture was subjected to denaturation at 94 °C for 10 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec and then further extension at 72°C for 10 mins. Amplified DNA fragments were analyzed using 3% agarose gel electrophoresis.

On the basis of the PCR-based genotyping, *H. influenzae* strains were classified into four genotypes. They were genetically ß-lactamase non-producing ampicillin susceptible (gBLNAS) strains without

mutations in *ftsI* gene, genetically ß-lactamase non-producing ampicillin resistant (gBLNAR) strains having amino acid substitution in *ftsI* gene, and genetically ß-lactamase producing (gBLP) strains having *bla* gene.

Restriction fragment polymorphism of genomic DNA analyzed by PFGE

One colony of *H. influenzae* isolate was grown at 37˚C for 6 h in Todd Hewit broth (Difco Laboratories, Detroit, Mich.). The cells were harvested by centrifugation at 4000 g at 4˚C for 5 min, washed with phosphate buffered saline (PBS), and suspended in washing buffer (50 mM Tris-HCl, pH 7.5). An equal volume of low-melting-point agarose (1.6 % of agarose for *S. pneumoniae* and 2.0% of agarose for *H. influenzae*) (In Cert agarose, FMC BioProducts, Rockland, Ma.) was added to 50 µl of each cell suspension for plug preparation. The mixture was poured into disposable 100-µl scale plug molds (Bio-Rad, Laboratories, Hercules, Calif.) and chilled at 4˚C for 20 min. After incubation with 2 ml of lysis buffer (0.25 M EDTA, 1 % SDS, 10 mM Tris-HCl, pH 9.5, and 0.5 mg/ml of proteinase K) over night at 50˚C, sample plugs were rinsed with 2 ml of washing buffer three times for 30 min. One-third of each plug was sliced off. The restriction of genomic DNA was carried out after equilibration of the sliced plugs with appropriate restriction buffer, then each slice was incubated with 80 U of *Sma*I (Takara Shuzo, Co. Ltd., Ohtsu, Japan) for 20 h at 30˚C.The reaction was stopped by an equal volume of 0.5 M EDTA (pH 8.0). Electrophoresis was performed with a GenePath PFGE apparatus (Bio-Rad Laboratories, Hercules, Calif.) in 0.5 × TBE buffer (1× TBE buffer: 0.1 M Tris-HCl, 0.1 M boric acid, 2 mM EDTA, pH 8.0). A 48.5 kb bacteriophage lambda DNA ladder (FMC BioProducts, Rockland, Ma.) was used as a molecular size marker. The gels were stained with ethidium bromide for 20 min and were photographed under UV light at 302 nm.

Results

Populations

A total 170 patients with acute rhinosinusitis, 170 patients with conjunctivitis, and 116 patients with acute otitis media were enrolled into this study. The ages of them were ranging from 5 to 48 months (mean 20.2 months).

Identification of *H. influenzae*

A total 294 *H. influenzae* was isolated from patients of conjunctivitis-otitis media-rhinosinusitis complex. They were 130 isolates (40.7%) from 320 nasal discharges, 109 isolates (58.2%) from 187 conjunctival lavage, and 55 isolates (60.4%) from 91 MEFs (Fig. 1). Among 130 isolates identified from nasal discharge, 76isolates (58.5%) were gBLNAS strains, 35isolates (26.9%) were gBLNAR, and 19 isoteas (14.6%) were gBLP strains. Among 109 isolates identified from conjunctival lavage, 67isolates (61.4%) were gBLNAS strains, 32isolates (27.8%) were gBLNAR strains, and 10 isolates (9.2%) were gBLP strains. Among 55 isolates identified from middle ear fluids, 38 isolates (69.1%) were gBLNAR strains, 10 isolates (18.2%) were gBLNAR strains, and 7 isolates (12.7%) were gBLP strains.

Determination of *H. influenzae* clones in Conjunctivitis-Otitis Media-Rhinosinusitis complex

A total 161 patients with conjunctivitis-otitis media-rhinosinusitis complex were evaluated for determination of *H. influenzae* clones. They were 68 patients (71 pairs) with rhinosinusitis, conjunctivitis, and AOM, 70 patients (107 pairs) with rhinosinusitis and conjunctivitis, and 23 patients (23 pairs) with rhinosinusitis and AOM.

Among 37 pairs of *H. influenzae* from nasal discharges, conjunctival lavage, and middle ear fluids, 34 pairs (91.9%) showed identical clones of *H. influenzae*. Among 48 pairs of *H. influenzae* from nasal discharge and conjunctival lavage, 46 pairs (95.0%) showed identical clones of *H. influenzae* (Table 1, Fig. 2).

Discussion

Conjunctivitis-otitis media syndrome was first reported by Bodor et al in 1982.[1] About 73% of the patients with purulent conjunctivitis concurrently had otitis media.[1] An identical pathogen was isolated from approximately 87% of the patients with bacterial conjunctivitis.[3] However, the positive predictive value of nasopharyngeal culture for positive middle ear fluid is reported to be only 47%.On the other hand, the negative predictive value of nasopharyngeal cultures for negative middle ear fluid was about 87%.[4] *H. influenzae* has been the most frequently isolated causative pathogens responsible for conjunctivitis-otitis media syndrome. The current study revealed that

about 98.9% of the patients had identical clones of *H. influenzae* in nasopharynx, conjunctiva, and MEFs by the molecular biological analysis. These finding strongly suggest that nasopharyngeal colonization with causative pathogens is the most important causes of acute conjunctivitis, AOM and acute rhinosinusitis.[5] Bacteria in nasopharynx will cause conjunctivitis-otitis media-rhinosinusitis syndrome by ascending infections via Eustachian tube to middle ear cavity resulting otitis media and via naso-lacrimal duct to conjunctiva resulting conjunctivitis.

In the present study, it is noteworthy that BLNAR strains, in which PBP3 mutation is responsible for development of resistance (MIC of ampicillin$\geq$4 μg/ml), accounted for one third (27.8%) of all *H. influenzae* strains isolated. Nasal discharge serves as a source of bacterial transmission responsible for bacterial acute conjunctivitis and AOM. Physician should take the infection mechanisms into account when treating conjunctivitis-otitis media-rhinosinusitis syndrome and pay attention to the nasopharyngeal conditions.[6,7]

Fig. 1 Bacterial pathogens identified from conjunctivitis-otitis media-rhinosinusitis syndrome

Fig. 1

Rhinosinusitis
(n=170 patients, 320 isolates)

Conjunctivitis
(n=170 patients, 187 isolates)

Acute otitis media
(n=116, 91 isolates)

- *H. influenzae*
- *S. pneumoniae*
- *M. catarrhalis*
- *S. pyogenes*
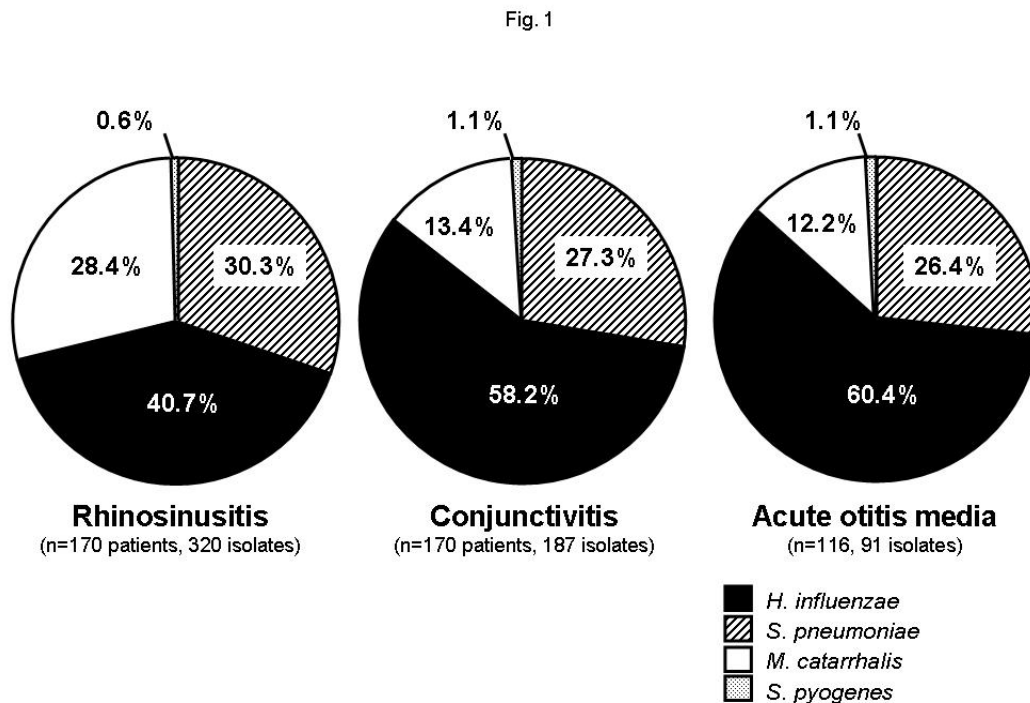
Fig. 2 Clonal analysis of *H. influenzae* in conjunctivitis-otitis media-rhinosinusitis syndrome

ND: Nasal discharge
CL: Conjunctival lavage
MEF: Middle ear fluid
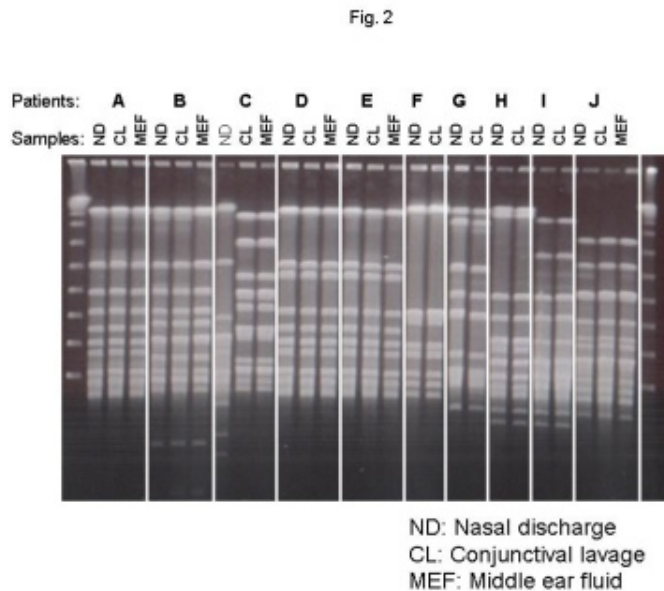
References

1. Bodor FF. Conjunctivitis-otitis media syndrome. Pediatrics 1982; 69:695-698.

2. Harrison CJ, Hedrick JA, Block SL, Gilchrist MJ. Relation of the outcome of conjunctivitis and the conjunctivitis-otitis media syndrome to identifiable risk factors and oral antimicrobial therapy. Pediatr Infect Dis J 1987; 6:536-540.

3. Bodor FF, Marchant CD, Shurin PA, Barenkamp SJ. Bacterial etiology of conjunctivitis-otitis media syndrome. Pediatrics 1985;76:26-28.

4. Groothuis JR, Thompson J, Wright PF. Correlation of nasopharyngeal and conjunctival cultures with middle ear fluid cultures in otitis media. A prospective study. Clin Pediatr1986;25:85-88.

5. Bingen E, Cohen R, Jourenkova N, Gehanno P. Epidemiology study of conjunctivitis-otitis syndrome. Pediatr Infect Dis J 2005; 24:731-732.

6. Bodor FF. Systemic antibiotics for treatment of the conjunctivitis-otitis media syndrome. Pediatr Infect Dis J 1989;5:287-290.

7. Block SL, Hedrick J, Tyler R, Smith A, Findlay R, Keegan E, Stroman DW. Increasing bacterial resistance in pediatric acute conjunctivitis (1997-1998). Antimicrob Agent and Chemothr 2000;44:1650-1654.

Expression of lysozyme in the rat eustachian tube and middle ear

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Introduction

Lysozyme (Lz) is a low molecular weighted (14.5 Kda) protein and has non-enzymatic antibacterial activity. It may play a role as an important component of innate mucosal immunity against pathogens. The eustachian tube (ET) and middle-ear mucosa are protected by complex biologic defense mechanisms such as mucociliary clearance, immune response, and secretory activity. And, they have important roles in otitis media pathogenesis. The purpose of the study was to assess the expression of Lz in the rat eustachian tube and middle ear and evaluate how much the Lz level increased in a lipopolysaccharide (LPS)-treated group compared to a normal group in the eustachian tube and middle-ear mucosa.

Materials and methods

Tissue preparation. Male Wistar-Kyoto rats weighing 250 to 350 g, with normal Preyer's reflex, were used for our studies. The animals were randomly divided into a normal group and a bacterial LPS-treated group. The LPS-treated group was injected intraperitoneally with a single dose of 0.1 mg of endotoxin (LPS) (Sigma Chemical, St. Louis, MO) dissolved in 1 ml of sterile phosphate-buffered saline (PBS). Animals were inoculated with a single dose of 0.3 mg LPS dissolved in 50 µl of sterile PBS through the tympanic membrane. For RNA isolation and quantitative, real-time polymerase chain reaction (PCR), the middle-ear mucosa, eustachian tube, and lung were dissected in RNAlaterTM (Ambion Inc., Austin, TX) at 1 hour and at 4 hours after inoculation.

RNA isolation and quantitative, real-time PCR. Total RNA was isolated by using the TRIzol® reagent (Gibco BRL, Rockville, MD). cDNA was generated by reverse transcription (RT) using the Superscript II-kit (Gibco BRL, Rockville, MD). All cDNAs were made from 1 µg RNA. The sequences of the PCR primers used for our studies were as follows: rat lysozyme forward: GGTGTAATGACGGCAAAACC, reverse: AGACTCCGCAGTTCCGAATA, rat 18S- rRNA forward: GTGGAGCGATTTGTCTGGTT, reverse:

CGCTGAGCCAGTCAGTGTAG. Quantitative, real-time PCR using ABI Sequence Detection Systems (ABI PRISM 7700; Applied Biosystems, Atlanta, GA) was performed with SYBR® Green PCR Master Mix (Applied Biosystems, Atlanta, GA). Rat lung cDNA was used as a positive control. Negative controls containing no cDNA water were included in all experiments. For the standard, the PCR products from lung cDNA after running gel were cut out. DNAs were extracted and purified using the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA). The numbers of molecules were calculated using the length of primer and molecule weight. After making 107 molecules/ml solution, serial diluted standards (100 to 106 molecules/ml) were prepared.

Immunohistochemistry. Following CO_2 inhalation, the animals were transcardially perfused with PBS, followed by ice-cold 4% paraformaldehyde in PBS. The temporal bones were removed and post-fixed, also in 4% paraformaldehyde in PBS. After decalcification in 125 mM EDTA in PBS for 2 months, the temporal bones were dehydrated and embedded in paraffin. Six µm-thick sections were obtained from paraffin block and mounted resin-coated slides. Antigen retrieval was performed by placing the samples into citrate buffer (0.01 M, pH 6.0) for 30 minutes. After blocking serum was put on the slides for 1 hour, rabbit polyclonal anti-human Lz antibody (1:100; DAKO, Carpinteria, CA) was used as a primary antibody. After washing three times in PBS for 5 minutes each, primary antibody was detected with AEC kit (Histostain-SP; Zymed Laboratories Inc., South San Francisco, CA). Rabbit serum was used as a primary antibody for negative controls. Samples were viewed and photographed using a Zeiss Axiovert 135 TV microscope and Axio Cam systems (Zeiss, Germany).

Western blotting. The middle-ear mucosa, eustachian tube, and lung were isolated and homogenized in lysis buffer (Cell Signaling Technology, Inc., Beverly, MA). Protein concentration was measured by Non-interfering Protein Assay kit (Bio-Rad, Hercules, CA). Total membrane proteins (20 µg/each lane) were solubilized in Tricine sample buffer (200 mM Tris-HCl, pH 6.8, 40% glycerol, 2% SDS, 0.04% Coomassie Blue G-250) and boiled for 5 minutes. SDS-polyacrylamide gel electrophoresis

(MiniProtean II apparatus; Bio-Rad, Hercules, CA) was performed using 10% tricine gel (2.6% Bis-Acrylamide, pH 8.3, Invitrogen, Carlsbad, CA). Electrotransfer was carried out for 2 hours on ice onto polyvinylidene difluoride membranes (Sequi-Blot; Bio-Rad, Hercules, CA). Membranes were blocked for 1 hour at room temperature in 2% bovine serum albumin with 1X Tris-buffered saline with 0.05% Tween 20, followed by overnight at room temperature with polyclonal antibody against Lz (DAKO, Carpinteria, CA). After washing in TBST (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.1% Tween 20) and 0.5% blocking solution, the membranes were incubated with 50 mU/ml anti-rabbit IgG horseradish peroxidase–linked secondary antibody, biotinlyated protein marker (Cell Signaling Technology, Inc., Beverly, MA) for 1 hour at room temperature, washed four times in TBST, and visualized via enhanced chemiluminescence (SuperSignal; Pierce Biotechnology, Inc., Rockford, IL) with variable film exposures (Hyperfilm; Amersham Pharmacia Biotech, England).

Results

RT-PCR and quantitative, real-time PCR. RT-PCR and quantitative, real-time PCR results from PBS-treated control tissues are shown in Figure 1. We could see the mRNA band of Lz at 194 bp in the lung, middle ear, and eustachian tube. The numbers of all mRNAs are normalized by one million 18S ribosome RNA molecules. Expression level of Lz in the middle ear, eustachian tube, and lung are 7380, 53435, and 322451 molecules per one million 18S ribosome RNA. Expression level of Lz in the lung is nearly six times higher than the eustachian tube level, and the expression level of the eustachian tube is about seven times higher than the middle-ear level. Four hours after LPS treatment, the Lz mRNAs amounts are increased in the middle ear (315000 molecules per one million 18S ribosome RNA) (Fig. 2). But in the eustachian tube, the Lz mRNA amount is not increased.

Immunohistochemistry. The Lz protein localization was determined by immunohistochemistry (Fig. 3). The Lz was detected in the eustachian tube epithelial cells. No signal in the eustachian tube epithelial cells was observed when the rabbit serum was used as a negative control.

Western blotting. The Lz protein was detected in the lung, middle ear, and eustachian tube (Fig. 4). The clear band was observed at the molecular weight of 14 Kda, which is known as authentic Lz.

Conclusions

Lz was expressed in the eustachian tube and middle ear. Lz was upregulated in the middle ear at the inflammatory state. The Lz level in the lung is 6 times higher than the eustachian tube, and the Lz level in the eustachian tube is 7 times higher than in the middle ear. In the inflammatory state, the Lz level in the middle ear is 6 times higher than in the eustachian tube. The middle ear showed more rapid and more sensitive responses compared to the eustachian tube. In the normal state, the eustachian tube has protective function against inflammation, but the middle ear shows more powerful action after inflammation.

The expression of Lz in the eustachian tube and middle ear means that they have important roles in otitis media pathogenesis. The middle ear showed more rapid and sensitive responses compared to the eustachian tube.

DNA microarray analysis of otitis media

Michelle Hernandez, M.D., Anke Leichtle, M.D., Kwang Pak, B.S., Nicholas Webster, Ph.D., Stephen Wasserman, M.D., Allen Ryan, Ph.D.

Introduction

The middle ear (ME) undergoes extensive modification during acute otitis media (OM). This includes the initial responses of ME cells to infection, followed by hyperplasia of the ME mucosa, infiltration of leukocytes into the ME tissue and lumen, and eventual recovery.[1,2] These phenomena potentially involve changes in the expression of many thousands of genes. Assessing these genes individually severely limits the number of expression changes that can be evaluated. However, advances in molecular biology and genomics have resulted in the development of techniques to address the assessment of very large numbers of genes using microarrays.

DNA microarrays, or gene chips, now allow the simultaneous assessment of virtually all genes in a single experiment. This technology allows not only the opportunity to discover novel genes involved in OM, but also to determine broad patterns of gene activation and to relate them to functional networks of genes. We evaluated the course of an acute episode of OM induced by nontypeable *Haemophilus influenzae* (NTHi) in the mouse, using gene chip technology.

Materials and methods

The MEs of 40 WBxC57Bl/B6, F1-hybrid mice per time point were inoculated with NTHi (strain 3566). F1 hybrids were used to avoid the recessive abnormalities that are present in many inbred mouse strains, while retaining genetic homogeneity. For each array, the ME contents from 20 mice were harvested at various time points after inoculation, and total RNA was extracted. After RNA quality was assured, the RNA was hybridized to Affymetrix 430-2.0 mouse whole-genome microarray sets. At each time point, RNA from two separate experiments was hybridized to two separate array sets. Fifty-five thousand expression transcript levels were evaluated using a variance-modeled posterior inference analysis (VAMPIRE) designed for very large arrays.[3] Expression changes were confirmed by quantitative polymerase chain reaction (PCR) or Western blot for selected genes.

Results

Expression profiles across time revealed distinct waves of gene activity. An initial gene cluster was activated at 3-6 hours after NTHi inoculation. These genes represented numerous ontology groups related to inflammation. A second cluster showed peak activity at 24 hours after inoculation. These genes represented ontology groups for chemotaxis; a variety of inflammatory pathways including innate immunity, neutrophil activation, and interleukin signaling; and phagocytosis. A third cluster peaked at 48-72 hours, and represented genes involved in interferon signaling, cell adhesion, angiogenesis, cell proliferation, and antigen presentation. A fourth cluster of genes were down-regulated, primarily from 3-48 hours. This included ontogeny groups related to tissue differentiation and apoptosis.

The gene ontogeny groups identified in this analysis represent broad categories that are related to biological processes, most of which would be expected to be involved in OM. However, within each ontogeny group, only a subpopulation of possible transcripts was differentially regulated. The identity of these genes will provide clues regarding the specific pathways that mediate ME defense, OM pathogenesis, and OM recovery.

Discussion

DNA microarray analysis provides simultaneous data on the expression of essentially all mouse genes during OM. Our results indicate that while many gene transcripts are differentially regulated during OM, gene activation occurs in discrete waves that are related to the progression of OM. Thus early inflammatory genes are followed by genes that mediate the recruitment of inflammatory cells and tissue proliferation. Finally, activation of genes that are involved in OM recovery occurs. In addition, early down-regulation of differentiation genes may represent the de-differentiation of the mucosa prior to hyperplasia. Detailed analysis should allow us to identify key genes and gene families that contribute to both generation of, and recovery from, this disease.

Acknowledgements

References

1. Lim DJ, Birck H. Ultrastructural pathology of the middle ear mucosa in serous otitis media. Ann Otol Rhinol Laryngol 1971; 80:838-853.
2. Palacios S, Pak K, Rivkin A, et al. Role of p38-Mitogen-Activated Protein Kinase in middle ear mucosa hyperplasia during bacterial otitis media. Infect Immun 2005; 72:4662-4667.
3. Hsiao A, Ideker T, Olefsky JM, Subramaniam S. VAMPIRE microarray suite: a web-based platform for the interpretation of gene expression data. Nucl Acids Res 2005; 33:W627-W632.

Distribution of collagens in healthy human tympanic membranes

Johan Knutsson, M.D., Magnus von Unge, M.D., Ph.D.

Introduction

The development of retractions of the tympanic membrane and cholesteatomas is not fully understood. Normally serous otitis media (SOM) and purulent otitis media (POM) heal without any sequelae, but previous longstanding SOM or recurrent episodes of POM may be a risk factor for the development of a cholesteatoma later in life. Several theories have been presented regarding the formation of cholesteatomas; the metaplasia theory, the retraction pocket theory, the migration and the papillary ingrowth theory, and the sniff theory. The retraction pocket theory proposes that a cholesteatoma is developed from a preformed retraction pocket where the squamous cell epithelium is inadequately exfoliating, thus leading to epithelial debris retention and subsequent inflammation.

The retraction pocket theory is supported by evidence from both patient observations and animal experiments.[1] A prerequisite for the genesis of retraction pockets is a weakening of the strength-bearing properties of the tympanic membrane.[2,3] Our previous studies have shown that tympanic membranes that have been subjected to experimentally induced OM lose stiffness. Morphological investigations have shown an intact number of collagen fibers and no structural deterioration of the fibers. A change in collagen type composition could be an explanation to the change in the tensile properties of the tympanic membranes that has been shown after induced inflammation.[4,5]

We have recently studied the histological distribution of collagens in the pars tensa and the fibrous annulus of the tympanic membrane in healthy Sprague-Dawley rats. Using immunohistochemistry, we found that the main collagens in the pars tensa of the tympanic membrane are collagen types II and IV. Judged by the intensity of the staining in the fibrous annulus, we found that there seems to be an outer and an inner part of the fibrous annulus. The outer part of the fibrous annulus has collagen type III and IV as its major collagen component, while the inner part is chiefly made up of collagen type II.[6]

The distribution of collagens in healthy human tympanic membranes has not been investigated before. The objective of this study was to investigate the histological distribution of collagens in the healthy human tympanic membrane and compare with results from the rats.

Materials and methods

The study is based on a unique material consisting of seven healthy human tympanic membranes from patients undergoing trans-labyrinth surgery for vestibular schwannomas. The patients had no history of retraction pathology or long-standing OM with effusion. The tympanic membranes were fixated in paraformaldehyde (4%) immediately after being taken out from patients. After embedding in paraffin and sectioning, immunohistochemical analyses were performed to identify collagen types I, II, III, and IV. The staining was analysed by light microscopy. The results were compared with our previous results from rat tympanic membranes that were analysed with the same method.

Preliminary results

The preliminary results (collagen types I, III, and IV) show a different pattern in the staining of the fibrous annulus as compared with rat. The most striking difference is that the staining of the human specimen show presence of collagen type I in the fibrous annulus.

The staining of the pars tensa shows clear evidence of collagen type III but not collagen type I in the lamina propria. In the rat, the collagen type I and III showed low staining intensity. Collagen type IV is present in the basal membrane of the pars tensa in both the human and rat tympanic membranes (Fig. 1-3). Staining for collagen type II has not yet been performed.

Discussion

A change in the stiffness of the tympanic membrane is a requirement for the formation of focal retraction pockets and is therefore of great interest in the search for a clue regarding the development of cholesteatomas. Prolonged SOM or repetitive episodes of POM have been shown to result in stiffness changes of the tympanic membrane in experimental settings.

The collagen fiber bundles have, in previous experimental studies, appeared intact histologically when analyzed using electronmicroscopy, but often spatially split from each other by edema. The stiffness alterations reported previously cannot though be completely explained by the spatial split of collagen fiber bundles.[7] Potentially, a change in the collagen fiber distribution resulting from OM may contribute to the stiffness alterations in tympanic membranes.

The collagen distribution in healthy human tympanic membranes has not been reported on before. Therefore, we wanted to investigate the collagen distribution of the healthy tympanic membrane to make possible a comparison with future analyses of human tympanic membranes that have been subjected to persistent SOM or repeated episodes of POM.

The method we used for the subtyping of collagens in the tympanic membranes is a well-described immunohistochemical method.[8] We focused on collagen types I-IV, which are the most widespread collagen types, and which all have different particular qualities. The role of collagen type I is chiefly to give a tissue resistance to force. Collagen type II provides shape and resistance to deformation. The function of collagen type III is to provide elasticity and support. Collagen type IV serves as a filtration barrier and also provides support.[9]

Primary antibodies are readily accessible for these four types of collagen. The primary antibodies demonstrate a high specificity for each of the collagen types tested for. The cross reactivity with other collagen types including the other types of collagen that were tested for is less than 10% according to the supplying company (Southern Biotechnology Associates Inc, Birmingham, AL). By using a normal blocking serum, the non-specific background staining was reduced, but it was still present to some degree in all specimens except the negative controls.

The results are still preliminary. The staining of more sections will follow to possibly confirm our preliminary results that are obtained from a very limited number of sections. If there are major differences in collagen composition between human and rat tympanic membranes, it could be questioned whether the rat model is the most relevant one for translational tympanic membrane studies on OM, and whether other species might provide better alternatives. It is important to remember, though, that staining for the most important collagen in the pars tensa, the type II, has not been successful yet. Analysis for collagen type II is under way.

This study is part of a series of investigations intended to clarify the pathophysiological mechanisms behind the of cholesteatoma formation in man – maybe the most important enigma of clinical middle-ear pathology.

Acknowledgements
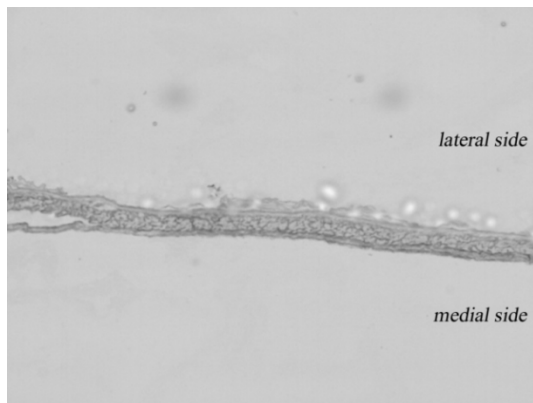
Figure 1. No distinct staining for collagen type I in the human pars tensa.
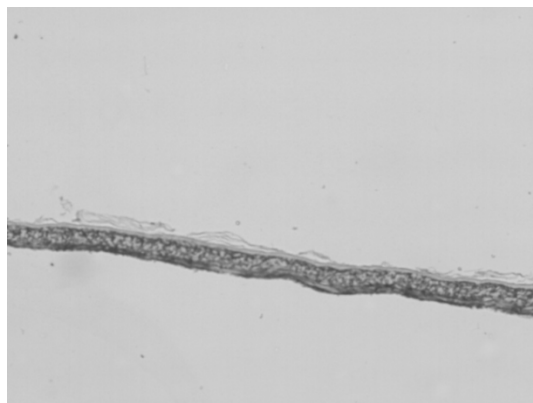
Figure 2. Presence of collagen type III in the lamina propria of the human pars tensa.
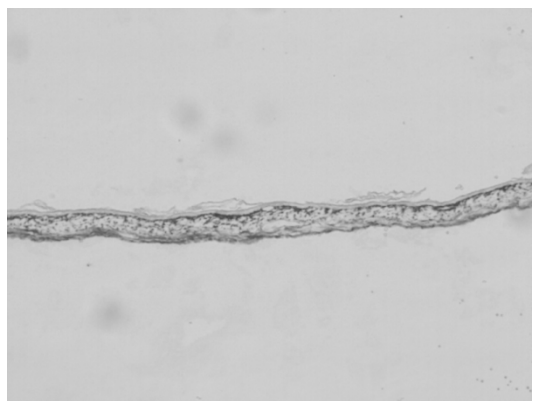
Figure 3. Presence of collagen type IV mainly in the basal membrane of the human pars tensa.

References

1. Wells MD, Michaels L. Role of retraction pockets in cholesteatoma formation. Clin Otolaryngol Allied Sci 1983; 8:39-45.
2. Ars B, De Craemer W, Ars-Piret N. The lamina propria and cholesteatoma. Clin Otolaryngol 1989; 14:471-475.
3. Ars BM. Tympanic membrane retraction pockets. Etiology, pathogeny, treatment. Acta otorhinolaryngol Belg. 1991; 45:265-277. Review.
4. von Unge M, Bagger-Sjoback D. Tympanic membrane changes in experimental otitis media with effusion. Am J Otol 1994; 15:663-669.
5. von Unge M, Decraemer WF, Dirckx JJ, Bagger-Sjoback D. Shape and displacement patterns of the gerbil tympanic membrane in experimental otitis media with effusion. Hear Res 1995; 82:184-196.
6. Knutsson J, Bagger-Sjoback D, von Unge M. Distribution of different collagen types in the rat's tympanic membrane and its suspending structures. Otol Neurotol. 2007; 28:486-491.
7. von Unge M, Decraemer WF, Bagger-Sjoback D, Van den Berghe D. Tympanic membrane changes in experimental purulent otitis media. Hear Res 1997; 106:123-136.
8. Wick G, Nowack H, Hahn E, Timpl R, Miller EJ. Visualization of type I and II collagens in tissue sections by immunohistologic techniques. J Immunol 1976; 117:298-303.
9. Kuhn K, Glanville RW. Molecular structure and higher organization of different collagen types. In: Forsknsingsrad SL, Viidik A, Vuust J, eds. Biology of Collagen. London: Academic Press; 1980:1-14.

Effect of inflammatory mediators on the inner ear

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Middle-ear infection is known to cause sensorineural hearing loss secondary to passage of inflammatory mediators and bacterial toxins into the inner ear via the round window membrane (RWM).[1] The existence of a blood labyrinth barrier (BLB) in the lateral wall of the cochlea and its involvement in the maintenance of cochlear homeostasis has been reported.[2] The specific mechanisms responsible for inner ear damage, however, are not clearly understood. The objective of our study was to examine the effect of inflammatory mediators on auditory function and on the permeability of the blood labyrinth barrier of the lateral wall. Both epithelial connective tissue and gap junction systems play crucial roles in maintaining fluid homeostasis within the cochlea. The gap junctions between the cells provide pathways for K^+ ions, which contribute to the recycling of K^+ through the cochlear lateral walls.[3] The recycling of K^+ ions from the hair cells after the auditory transduction process to the stria vascularis via the spiral ligament fibrocytes appears to be essential for the maintenance of high K^+ concentration in the scala media for auditory transduction.[4] Cytokines were selected to be placed on the RWM for the BLB permeability assessment.

Materials and methods

A total of 32 chinchillas were anesthetized (intramuscular injection of 40 mg/kg ketamine and 10 mg/kg xylazine) and their RWM surgically exposed with a posterior inferior approach through the tympanic bulla. Interleukin 1β (IL-1β, 10µl, 100µg/ml), tumor necrosis factor alpha (TNF-α, 10µl, 100µg/ml) or lipopolysaccharide (LPS, 10µl, 100µg/ml) were soaked into gelfoam and were applied to the RWM independently at periods of 5 hours or 1, 2, or 5 days. Functional testing via auditory brainstem response was performed by differential recording of electric signals in response to a broadband click stimulus and pure tone stimuli at 1, 2, 3, 4, 8, 16, and 24 kHz. The average of 512 responses was recorded with a Tucker-David Technologies System 3 recording station with TDT's BioSigRP as the software. Three ml of 2% Evans blue dye solution was injected intravenously in the femoral vein. One hour after injection, the animals were sacrificed and lateral walls were dissected out, homogenized and centrifuged. A spectrophotometer was used to measure the optical density of the Evans blue in the supernatant.

Results

An increase in the permeability of the BLB and in the ABR thresholds was observed following application of all inflammatory mediators compared to controls. Both the quantity of Evans blue (Fig. 1) and auditory brainstem response thresholds increased with time compared to saline and normal controls. Elevations in hearing thresholds across all tested frequencies appear and increases are proportional to exposure length of the inflammatory mediator. Low end frequencies (1-4 kHz) showed threshold increases of 5-10 dB after just 5 hours of inflammatory mediator application, which increased to a 20 dB shift after 5 days. High end frequencies (8-24 kHz) showed 10-20 dB increases after 5 hours of application and 25-30 dB shifts after 5 days of inflammatory mediator treatment.

Discussion

High molecular weight albumin does not pass through the RWM under normal conditions but can penetrate the RWM into perilymph in antigen-induced OM.[5] Endotoxins themselves, or the inflammatory reaction they induce, facilitate their penetration through the RWM. Histological changes in the cochlea with experimental otitis media have been reported.[6] Hindrance of passage of K^+ by the disruption of BLB can result in the disturbance of homeostasis of ions in the cochlear fluids leading to the disturbance of auditory transduction.[7]

Conclusion

Inflammatory mediators increase the permeability of the BLB and may be involved in the hindrance of K^+ recycling resulting in a disturbance of auditory transduction; however, it is not known at this time the exact mechanism of disturbance through which permeability is increased. Results of the present study may partially explain the mechanisms of sensorineural hearing loss in otitis media; however, further exploration with a broader variety of inflammatory mediators as well as the specific results with experimentally-induced otitis media should be pursued.

Fig. 1a. LPS on the RWM – Evans blue permeability.
*= Significant difference between LPS and PBS; p<0.0001, n=5. (Student's t-test)
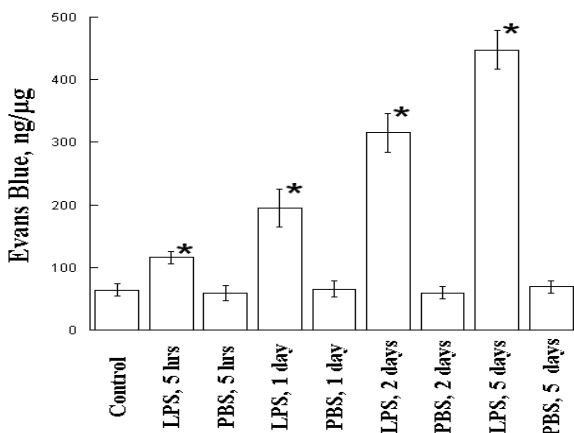
Fig. 1b. IL-1β/TNF-α on the RWM – Evans blue permeability.
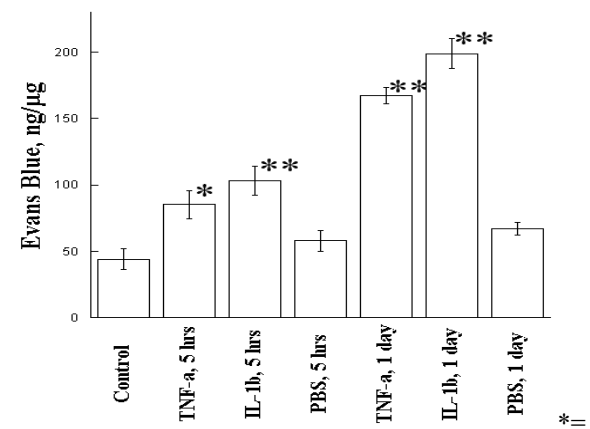
p<0.001; **= p<0.0001; n=5. (Student's t-test)

Figure 1. Statistically significant permeability increases are seen in all three inflammatory mediator applications versus saline and normal controls. Increases in permeability also correlate to duration of the application of the inflammatory mediator.

References

1. Cureoglu S, Schachern PA, Rinaldo A, Tsuprun V, Ferlito A, Paparella MM. Round window membrane and labyrinthine pathological changes: an overview. Acta Otolaryngol 2005; 125:9-15.
2. Juhn SK, Hunter BA, Odland RM. Blood-labyrinth barrier and fluid dynamics of the inner ear. Int Tinnitus J 2001; 7:72-83.
3. Couloigner V, Sterkers O, Ferrary E. What's new in ion transports in the cochlea? Pflugers Arch 2006; 453:11-22.
4. Zhao HB, Kikuchi T, Ngezahayo A, White TW. Gap junctions and cochlear homeostasis. J Membrane Biol 2006; 209:177-186.
5. Hamaguchi Y, Morizono T, Juhn SK. Round window membrane permeability to human serum albumin in antigen-induced otitis media. J Otolaryngol 1988; 9:34-40.
6. Grote JJ, van Blitterswijk CA. Acute otitis media: An animal experimental study. Acta Otolaryngol 1984; 98:239-249.
7. Hibino H, Kurachi Y. Molecular and physiological bases of the K^+ circulation in the mammalian inner ear. Physiology 2006; 21:336-345.

The effects of plasminogen deficiency on the healing of tympanic membrane perforations

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Background

The reason why some tympanic membrane (TM) perforations become chronic and others heal is yet unknown. Earlier studies on skin wound healing have shown that plasminogen (plg) plays an important role, and it has been suggested that the keratinocytes are dependent on plg to dissect their way through the provisional matrix that ultimately leads to a healed wound.

Objective

The aim of the present study is to evaluate the role of plg in healing of TM perforations, both *in vivo* and *in vitro*, mainly focusing on 1) the inflammatory response, 2) the keratinocyte migration, and 3) the fibrin(ogen) distribution in the TM.

Material and method

Plg deficient and urokinase-type plg activator (uPA) deficient mice were used for the studies. Specimens were prepared for regular light microscopy and immunohistochemistry (IHC). The latter was performed with antibodies targeting macrophages, neutrophils, T- and B-cells, cytokeratin and fibrin(ogen). All experiments were evoked by a perforation of the TM and thereafter observed for up to 143 days.

Results

When performing a myringotomy, occupying one quadrant of the TM, in plg deficient mice the perforation was still open after 143 days. The IHC revealed that there was an abundant and persistent recruitment of both macrophages and neutrophils to the perforation throughout the whole observation period. Furthermore the migration of keratinocytes was arrested and fibrin massively deposited along the TM. Reconstitution of plg to the plg deficient mice completely restored the healing capacity. Studies of the inflammatory response during the first 48 hours following the myringotomy showed no differences between the plg deficient and the wt mice. Neither did uPA deficiency alter the inflammatory cell recruitment or healing pattern of TM perforations. The *in vitro* experiment resulted in an initiated healing in all groups, regardless of plg concentration.

Conclusions

Our studies showed that plg plays an essential role in the healing of a TM perforation. Both the inflammatory response and the keratinocyte migration as well as the removal of deposited fibrin are affected. The *in vitro* study indicates that an intact circulation is necessary for the plg to enhance the healing process. Though speculative it is possible that administration of plg could be a possible treatment modality also in chronic perforations in humans.

Analysis of the cellular immune responses to influenza A virus infection in the middle ear

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Introduction

Several respiratory pathogens, including influenza virus, are known to infect respiratory epithelium, causing rhinorrhea, middle ear effusions, and alveolar edema. A significant number of individuals, especially infants and children, can develop acute otitis media (AOM). The role of influenza A virus in the pathogenesis of OM has been the focus of intensive research for many years. However, little is known about the effect of Influenza A virus infection on the immune responses in the middle ear. It is important to understand how influenza A virus modulates host cellular responses in efforts to explore the new strategy for AOM. In this study, we investigated the cellular immune responses in the middle ear after influenza A viral infection.

Materials and methods

Animals:

Specific pathogen-free male BALB/c mice, 5 weeks of age, were purchased from Charles River Laboratories (Atsugi, Japan) and used for all experiments.

Animal model:

Influenza A virus adapted to mice (PR8, H1N1) was provided by the Department of Microbiology, Oita University. The virus was suspended in 0.01 M phosphate-buffered saline (PBS, pH 7.2) at a concentration of 4×10^9 pfu/ml for intranasal inoculation. Mice were intranasally inoculated with influenza A virus suspension (PR8, H1N1), and killed on days 1, 5, 9, and 14. No treatment mice at day 0 were served as a control. The samples were obtained to examine the histological study and Flow cytometric analysis.

Histological evaluation:

Under anesthesia with intraperitoneal injection of pentobarbital, the mice were perfused transcardially with physiological saline containing 0.1% heparin, followed by perfusion with PLP fixative. The heads were immersed in same fixative for 6 hours and decalcified with 0.12 M ethylenediaminetetraacetic acid (EDTA, pH 7.0) for 2 weeks. The tissues were embedded in OCT compound (Sakura Finetechnical Co., Tokyo, Japan) and horizontal serial sections were prepared at 10 mm for immune staining with anti-mouse Thy 1, 2 and Pan B antibody to detect T/B cell distribution in these tissues. For histological investigation, frozen sections of the lymphoid and mucosal tissues were made and stained with anti-mouse Thy 1,2 and Pan B antibody to detect T/B cell distribution in these tissues.

Flow cytometric analysis:

To analyze the cellular phenotypes in lymphocytes responding to viral infection, mononuclear cells from the nose and middle ear mucosa were collected by teasing or digesting with collagenase, and were stained with anti CD3-, CD4-, CD8 and CD69-specific antibodies conjugated with a fluorescent label. Flow cytometric analysis of the cellular immunofluorescence was performed using a FACScan flow cytometer.

Results

Sections of the middle ear mucosa around the tympanic orifice and nasal turbinate were observed microscopically. In the control group, the nasal and middle ear mucosa was covered with a single layer of ciliated cells, and the subepithelial spaces were thin without inflammatory cell infiltration. After the inoculation, a part of the cilia on the nasal and middle ear mucosal surface gradually disappeared, and the subepithelial space appeared thickened with inflammatory cell infiltration from day 5 to day 14 after the inoculation (Fig.1). The severity of mucosal inflammation reached the peak at day 9 after the inoculation. In the control group, the Thy 1.2 and Pan B positive cells was seen a little in the subepithelial space of the nasal and middle ear mucosa. However, Thy 1.2 and Pan B positive cells increased in the subepithelial space of the both mucosal sites during the infection.

Using flow cytometry, we examined the phenotype of the infiltrating T cells into the mucosal tissues. In the nasal and middle ear mucosa, both of CD4+ and CD8+ T cells increased from day 5, and peaked at day 5 and 9 after the inoculation. On the contrary, CD4CD8 double negative T cell gradually decreased (Fig.2). However, CD4/CD8 ratio was not altered by the infection.CD4+CD69+ and CD8+ CD69+ T cells increased at day 9 after the inoculation in these mucosa.

Discussion

Influenza A virus, which infects primarily upper respiratory tract epithelial cells, is closely associated with acute otitis media (AOM) in children [1, 2]. The role of influenza A virus in the pathogenesis of OM has been the focus of intensive research for many years. Earlier studies suggest several possible mechanisms to explain this phenomenon including viral compromise of eustachian tube (ET) mucosal integrity, virus promoting a significant increase in bacterial nasopharyngeal colonization, and viral suppression of polymorphonuclear leukocyte function [3–7]. However, the details of the immune responses after influenza A viral infection involved in the middle ear have rarely been studied, very little is known as to how influenza A virus affects cellular immunity in the middle ear. The research work on the pathogenesis of influenza middle ear infection and analysis of host immune response against the virus are needed. In this study, we investigated how the immune responses are affected in the middle ear during influenza A virus infection in terms of CD4+ and CD8+ T lymphocytes. In our results, influenza A viral infection affected the mucosal immune responses, including the middle ear. These immune responses reached the peak at day 9 after viral inoculation in the nose and middle ear. This mice model may be useful for further study about acute otitis media with influenza A virus infection.

References

1. Heikkinen T. The role of respiratory viruses in otitis media. Vaccine 2001;19:S51–S55.
2. Heikkinen T, Thint M, Chonmaitree T. Prevalence of various respiratory viruses in the middle ear during acute otitis media. N Engl J Med 1999;340:260–264.
3. Abramson JS, Giebink GS, Quie PG. Influenza A virus-induced polymorphonuclear leukocyte dysfunction in the pathogenesis of experimental pneumococcal otitis media. Infect Immun 1982;36: 289–296.
4. Chung MH, Griffith SR, Park KH, Lim DJ, DeMaria TF. Cytological and histological changes in the middle ear after inoculation of influenza A virus. Acta Otolaryngol (Stockh) 1993; 113:81–87.
5. Giebink GS, Ripley ML, Wright PF. Eustachian tube histopathology during experimental influenza A virus infection in the chinchilla. Ann Otol Rhinol Laryngol 1987;96:199–206.
6. Ohashi Y, Nakai Y, Esaki Y, Ohno Y, Sugiura Y, Okamoto H. Influenza A virus-induced otitis media and mucociliary dysfunction in the guinea pig. Acta Otolaryngol (Stockh) 1991;486:135–148.
7. Hirano T, KuronoY, Ichimiya I, Suzuki M, Goro M. Effects of influenza A virus on lectin-binding patterns in murine nasopharyngeal mucosa and on bacterial colonization. Otolaryngol Head Neck Surg. 1999;121:616-621.

Figure 1

In the control group, the nasal and middle ear mucosa was covered with a single layer of ciliated cells, and the subepithelial spaces were thin without inflammatory cell infiltration. After the inoculation, a part of the cilia on the nasal and middle ear mucosal surface gradually disappeared, and the subepithelial space appeared thickened with inflammatory cell infiltration from day 5 to day 14 after the inoculation. The severity of mucosal inflammation reached the peak at day 9 after the inoculation.
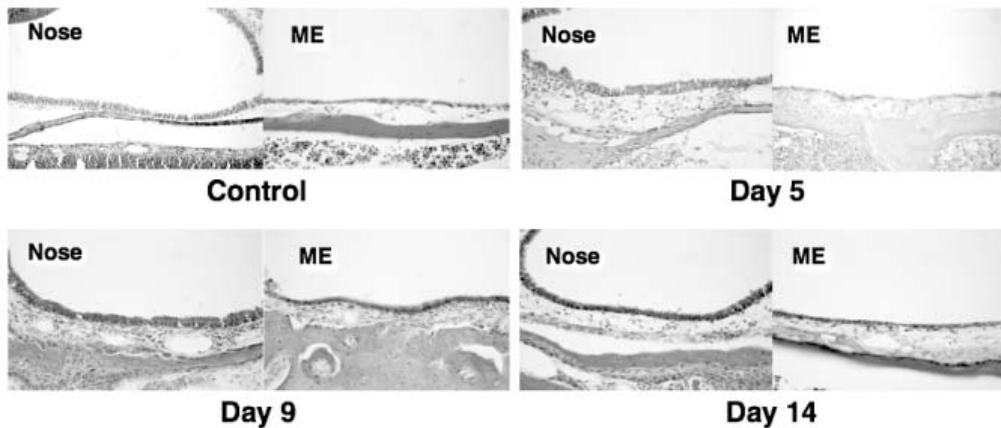
Figure 2

In the nasal and middle ear mucosa, both of CD4 and CD8 T cells increased at day 9 after the inoculation. On the contrary, CD4CD8 double negative T cell decreased. However, CD4/CD8 ratio was not altered by the infection.
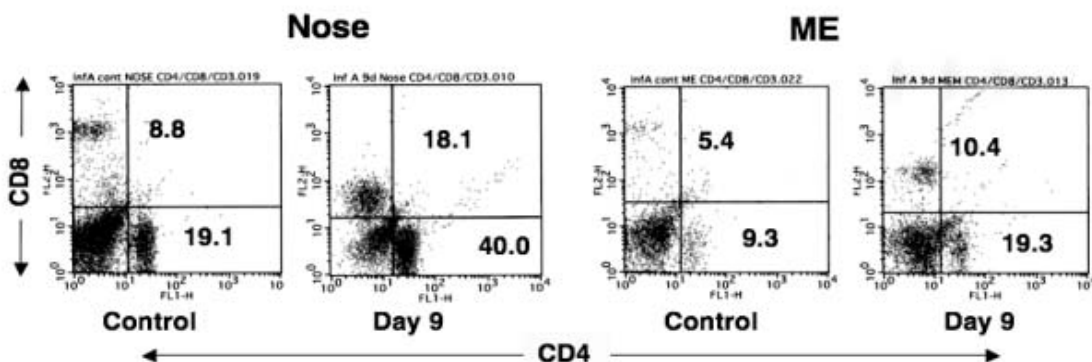
Cytokine profile of nasopharyngeal T cells in Igs-specific mucosal immune response

Takaya Yamada, Ph.D., Miki Tongu, M.D., Kazuhiro Hashigucci, M.D., Hideyuki Kawauchi, M.D., DMSc.

Introduction

Thanks to the development of molecular biological techniques, the nasal mucosa has become a focus of studies on mucosal immune responses in order to confirm theoretical bases and to pursue its clinical application for the vaccination therapy against microbial infections and hyposensitization therapy for nasal allergy. Despite the recent emphasis on elucidation of the molecular and cellular aspects of the mucosal immune system, very little information is yet available in regard to the nasopharyngeal mucosal immune system in comparison with in the gastrointestinal tract. Thus, for the last decade, we have been studying local immune responses in the nasopharynx and tubotympanum by animal experiments and employing human specimens such as nasal mucosae, paranasal sinus mucosae, and tonsillar tissues. In our series of animal experiments, we have already demonstrated that antigen-specific mucosal immunity (IgA response) is provoked by nasal protein antigen challenges together with cholera toxin (CT), and that T cell subsets as well as Ab-producing B cells are essential to mount a local antibody (Ab) production.[1, 2] Therefore, to better understand the exact mechanism of nasopharyngeal mucosal immunology, from T-cell aspects, the antigen-specific Ab response was investigated in T cell receptor transgenic mice (Tg-mice), OVA23-3,[3] and wild type BALB/c mice, in comparison, which were stimulated with repeated nasal antigen challenges of ovalbumin (OVA) together with CT.

Materials and methods

Antigen-specific Ab response to intranasal challenge. Tg-mice and BALB/c mice were intranasally immunized every two days with 2μL of phosphate-buffered saline (PBS) containing a mixture of 100μg OVA and 1μg of CT as a mucosal adjuvant, or OVA alone. OVA-specific IgA and IgG Ab titers in nasal washings were determined by ELISA. For an enumeration of OVA-specific immunoglobulin-producing cells, the number of OVA-specific IgA-producing and IgG-producing cells in the in nasopharyngeal-associated lymphoreticular tissue (NALT), nasal passage (NP), cervical lymph node (CLN), and spleen (SP) were respectively determined by ELISPOT assay. Lymphocytes obtained from NALT, NP, CLN, and SP were cultured with 200μg of OVA in 200μL of RPMI-1640 medium for 48 hours in flat-bottomed, 96-well culture plate. Commercial ELISA kits were respectively used to measure levels of IFN-γ, IL-4, IL-5, IL-6, and IL-13 in the culture supernatants and nasal washings.

Maintenance of memory of antigen-specific response. BALB/c mice were intranasally immunized seven times every 2days with 2μL of PBS that contained a mixture of 100μg OVA and 1μg of CT. After 6 months, mice were secondly challenged with 100μg OVA or PBS 3 times every 2 days. Each group of mice was sacrificed 1 week after the second immunization, and we irrigated the nostrils of the slaughtered mice and collected nasal washings. OVA-specific IgA and IgG Ab titers in the samples were determined by ELISA.

Results

Antigen-specific Ab response to intranasal challenge. OVA-specific IgA and IgG antibodies significantly increased in nasal washings of BALB/c and Tg-mice stimulated with OVA together with CT (OVA+CT-immunized group) and even in those of Tg-mice stimulated with OVA without CT (OVA-immunized group) (Fig. 1). The specific IgG- and IgA-producing B cells in NALT and NP cells significantly increased in number in BALB/c mice stimulated with OVA and CT and in Tg-mice stimulated with OVA with or without CT SP (Fig. 2). IFN-γ is noted as a representative of Th1 type cytokines and IL-4, 5, 6, and 13 production as Th2. Those cytokine levels were measured in the culture supernatants harvested from wells containing NALT, NP, CLN, and SP cells derived from each group of mice (Table 2). No significant Th1 or Th2 type cytokine production was detected in the culture supernatants of lymphocytes obtained from various tissues of BALB/c mice stimulated with OVA alone. The lymphocytes obtained from NALT, NP, CLN, and SP of Tg-mice stimulated with OVA together with or without CT, released the

highest levels of Th1 type (IFN-γ) and Th2 type (IL-4, 5, 6, 13) cytokines into the culture supernatants at each interval after intranasal immunization. These cytokine productions peaked at the second week. The lymphocytes obtained from NALT, NP, CLN, and SP of BALB/c mice stimulated with OVA and CT, showed significant IFN-γ production at each interval (1-3 week). In regard to Th2 cytokines, on BALB/c, no production was observed except IL-4, 6, and 13 productions were transiently detected in culture supernatants of NP and CLN stimulated with OVA and CT. On Tg-mice, IL-4, 5, 6, and 13 were detected in culture supernatants of NP, CLN, and CP in all groups. Lymphocytes from NALT of all Tg-mice groups did not show any Th2 type cytokine production.

Maintenance of memory of antigen-specific response. Generally, to provoke an antigen-specific IgA response in the nasal mucosa of wild type of mice, such as the BALB/c strain, a potent mucosal adjuvant like CT must be intranasally introduced together with the antigen. To examine the maintenance of memory of antigen-specific Ab response, OVA-specific IgG and IgA Ab titers at 6 months after the first immunization were measured. Ab titers with nasal washings were significantly higher in the OVA-immunized group than in the control group at 6 months after the first immunization ($p<0.05$). IgG values, indicated as reciprocal log2 titers, are 5.2±1.8 for OVA-stimulated group and 0.4±0.6 for the control group; in regard to IgA, 3.0±1.6 for the OVA-stimulated group and 0.0 for the control group.

Discussion

The important and interesting findings in this study are that antigen-specific IgA Ab-producing B cells in the NALT and NP were induced and that antigen-specific IgA Ab activity was detected in nasal washings, even though Tg-mice were intranasally immunized with OVA without CT. These findings strongly suggest that CD4$^+$ CD8$^-$ helper T cells play an important role in assisting antigen-specific IgA and IgG production of B cells in nasopharygeal mucosa, by way of Th1 and Th2 cytokine production.[4] From this point of view, the frequency of antigen-specific helper T cells is the key factor to mount the significant local IgA and IgG response in the nasopharyx. In Tg-mice, less OVA-specific IgA antibody-producing B cells were seen in the NALT rather than in the NP, and the production of Th2 type cytokines in the NALT cells was not detected. Antigen-specific IgG and IgA antibody response was obviously caused by the second antigen challenge even 6 months after the first immunization. This suggests that antigen-specific memory T cell was

maintained in nasal lymphoid for at least 6 months after initial immunization. Further experiments will be addressed to examine the persistence of longer term T cell memory. Taking all data into consideration, it can be concluded that helper T cells recruited into the nasal mucosa and/or locally activated in an antigen-specific fashion are essential for mounting antigen-specific IgA and IgG responses. In view of the anatomical difference in the existence of NALT and tonsils between rodents and humans, the results obtained from our animal experiments cannot be directly extrapolated to the human situations.
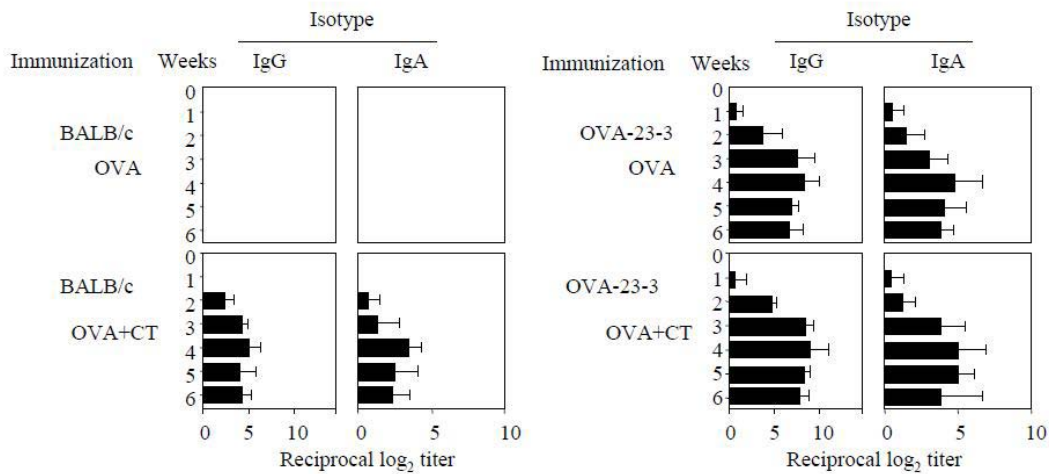
Figure 1. Induction of ovalbumin-specific IgG and IgA antibody in nasal washings from BALB/c or OVA-23-3 mice. The BALB/c mice were immunized intranasally with OVA (100 µg) with CT (1 µg) or OVA alone on days 0, 2, 4, 6, 8, 10, and 12. Values represent mean antibody title ± SEM. Each group consisted of 10 mice.
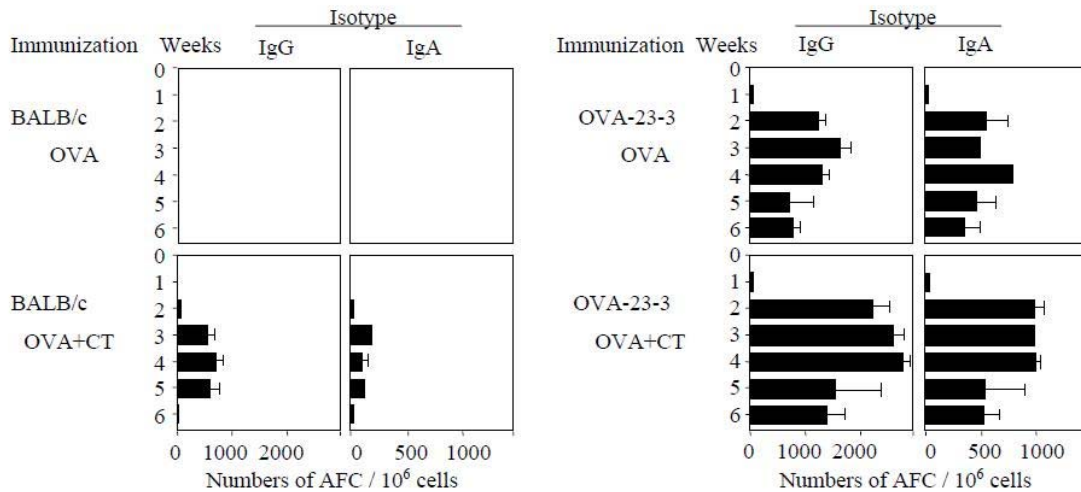
Figure 2. Nasal immunization induces ovalbumin-specific antibody-formed (AFC) in the NP from BALB/c or OVA-23-2 mice. Number of ovalbumin-specific IgG and IgA AFC were determined by ELISPOT assay. Each group consisted of five mice.

| | | BALB/c | | | | | | | | TCR-Tg | | | | | | | |
| | | OVA | | | | OVA+CT | | | | OVA | | | | OVA+CT | | | |
		NALT	NP	CLN	SP	NALT	NP	CLN	SP	NALT	NP	CLN	SP	NALT	NP	CLN	SP
INF-γ	0 weeks	0	0	0	0	0	0	0	0	7	5	61	250	7	5	61	250
	1 weeks	0	0	0	0	97	84	19	25	33	33	92	97	72	83	127	236
	2 weeks	0	0	0	0	391	1933	1457	105	498	1870	3052	2596	447	2356	11885	2342
	3 weeks	0	0	0	0	39	397	12	0	17	8	18	1391	46	2061	775	2106
	4 weeks	0	0	0	0	0	0	0	0	26	17	2	296	10	120	64	357
IL-4	0 weeks	0	0	0	0	0	0	0	0	0	5	7	36	0	5	7	36
	1 weeks	0	0	0	0	0	0	0	0	0	17	31	86	0	38	31	57
	2 weeks	0	0	0	0	0	12	0	0	0	213	321	119	0	126	69	74
	3 weeks	0	0	0	0	0	0	1	0	0	20	10	36	0	42	22	105
	4 weeks	0	0	0	0	0	0	0	0	0	5	1	27	3	27	21	42
IL-5	0 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2 weeks	0	0	0	0	0	0	0	0	0	88	122	68	0	32	2	3
	3 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IL-6	0 weeks	0	0	0	0	0	0	0	0	0	17	1	13	0	17	1	13
	1 weeks	0	0	0	0	0	0	0	0	0	10	0	37	0	143	0	41
	2 weeks	0	0	0	0	0	48	12	38	0	271	365	270	0	338	716	357
	3 weeks	0	0	0	0	0	0	0	0	0	10	18	222	0	276	35	351
	4 weeks	0	0	0	0	0	0	0	0	0	11	0	23	0	56	1	26
IL-13	0 weeks	0	0	0	0	0	0	0	0	0	11	4	28	0	11	4	28
	1 weeks	0	0	0	0	0	0	0	0	0	11	13	43	0	52	26	81
	2 weeks	0	0	0	0	0	4	26	121	0	239	402	340	0	241	506	206
	3 weeks	0	0	0	0	0	0	3	0	0	10	5	102	0	34	71	140
	4 weeks	0	0	0	0	0	0	0	0	0	11	5	58	0	11	11	71

(Pg/mL)

Table 1. Cytokines production of NALT, NP, CLN, and SP lymphocytes.

References

1. Kawauchi H, Sano K, Ishimitsu R. Mucosal Immunity of nasopharynx: an experimental study in mice. Bologna, Italy: Monduzzi Editore; 1997: 1717-1720.
2. Kawauchi H, Sano K, Shiba H, Sano C, Yamada T, Suzuki T. T-cell mucosal immunity in the nasopharynx. In: Veldman JE, Passali D, Lim DJ, eds. *New Frontiers in Immunobiology.* The Hague: Kugler; 2000:35-41.
3. Sato T, Sasahara T, Nakamura Y, et al. Naive T cells can mediate delayed-type hypersensitivity response in T cell receptor transgenic mice. Eur J Immunol 1994; 24:1512-1516.
4. Fujihashi K, Yamamoto M, McGhee JR, Kiyono H. Function of alpha beta TCR+ and gamma delta TCR+ IELs for the gastrointestinal immune response. Int Rev Immunol 1994;11:1-14.

Early inflammatory cell response to myringotomy in otitis media with effusion

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Background/aims

Otitis media with effusion (OME) is highly prevalent in children[1,2] and myringotomy (MYR), with or without ventilation tubes, is a commonly recommended treatment.[3] However, various sequelae have been reported in association with this surgical procedure.[4,5]

The rat tympanic membrane (TM), which is structurally similar to the human TM[6], has proved to react consistently to provoked conditions in the middle ear.[7, 8] In that respect, our research group has been devoted to the investigation of the early healing events that occur in the TM after MYR.[9-11]

The present immunohistochemical study aimed to compare the pattern of the early inflammatory cell permeation in the *pars tensa* (PT) of the tympanic membrane (TM) after MYR during OME in respect to normal TMs.

Material and methods

Twenty-eight Sprague-Dawley rats were used in the study after approval by the Regional Ethical Committee of the University of Umeå. Rats were anesthetized with an injection of methohexital sodium (Brietal®, Eli Lilly and Company, USA) via a tail vein. Through a ventral skin incision, the left tympanic bulla was reached, opened with a scalpel and the Eustachian tube blocked with a gutta-percha plug. The right bulla was left untouched.

Two days later, denominated as day zero, animals were re-anesthetized with Brietal®, had their ears checked under the otomicroscope, and were bilaterally myringotomized. The TM incisions, created with a sterile microlancet, were standardized in size and occupied almost the entire posterior superior quadrant of the PT. At 3, 6, 9, 12, 24 and 48 hours, four randomized animals from each group, respectively, were injected with an overdose of pentobarbital sodium (Pentobarbitalnatrium®, Apoteksbolaget AB, Sweden), had their TM status checked under the otomicroscope, and were promptly sacrificed.

After sacrifice, the TMs were dissected out, fixed and embedded in paraffin or Polybed® (Polysciences Inc., USA) as described previously.[10]

Horizontal sections (4 micron) were taken perpendicular to the malleus handle and immunohistochemistry was performed to analyze the ongoing cellular inflammatory cells permeation. Different antibodies were used to identify PMNs, monocytes/macrophages (ED1, ED2), lymphocytes B (OX33-CD45) and lymphocytes T (OX34-CD2) (Table 1). Incubation was performed with the avidin-biotin-peroxidase complex and the peroxidase activity was visualized by the addition of diaminobenzidine (DAB) (Vector Laboratories, UK). Rat spleen was used as positive and negative controls, respectively. Positive cells stained in a dark brown fashion. ED2 slides were not considered due to excessive background artifacts.

Morphometry

With a scale grid adapted to the binocular light microscope, under an oil immersion objective at a magnification of 100x, positively-stained cells were counted by two observers in three standardized spots at two different levels of the perforated quadrant of the pars tensa. Photographs were taken in a Zeiss Axiophot® (Karl Zeiss, Germany) light microscope equipped with an MTI3CCD® camera connected to a personal computer with Image Pro-Plus® software (Parameter AB, Sweden).

Statistical analysis

Cell counts in the left and right tympanic membranes were compared using the Wilcoxon signed-rank test (non-parametric test, paired-samples). The variations in counts over time were compared through linear regression separately for the left and right tympanic membranes. The regression coefficients represent the variation in the number of cells per hour. All analyses were performed using STATA®, version 9.2.

Results

Otomicroscopy

At day 0, manifest otomicroscopic signs of OME developed in all plugged ears, represented by opalescent TMs with retracted pars flaccida. Upon MYR a yellowish turbid fluid drained from the middle ear cavity in the OME specimens. The middle ear space was normal in the controls. From 12h on, no more fluid could be seen in the myringotomized OME TMs. At 24h, TMs from both groups showed faint otomicroscopic deposits of myringosclerosis in the umbo region of the PT and at 48h these sclerotic lesions were more noticeable in the control group.

Immunohistochemistry

On light-microscopy, at 3 and 6 hours after MYR, the TMs appeared almost quiescent in both groups, apart from a slight hemorrhage in the incised area on the posterior superior quadrant of the pars tensa. From 9 hours on, a progressive increase of cells staining positive, PMNs and macrophages, were noticed both at the malleus area and in the annulus region. This progressive invasion of inflammatory cells continued on subsequent hours, but lymphocytes were absent. There was a relatively more intense rise in the mean counts of PMNs at 24h in comparison to controls (PMN´s linear regression coefficient: OME=2, 47/h ($p<0$, 05), Control=0.96/h ($p<0$, 05)). Macrophages were also prevalent at 24h, and the annulus area was particularly populated with this type of cells, in both groups. At 48h the inflammation via PMNs and macrophages was still progressing with significant trends (Macrophages´ linear regression coefficient: OME=1, 13($p<0$, 05), Control=1.07($p<0.05$)), along with a strong stimulation on the keratinizing layer. Differences in the pattern of permeation of the pars tensa's structure between these two cell lines were also observed, with PMNs dispersed throughout the whole TM thickness, whereas macrophages were mostly confined to the basal lamina beneath the squamous epithelium (Figures 1, 2). Only occasional lymphocytes could be identified during these early stages of the TM healing process (Figure 3).

Comments

To our knowledge, this is the first study that evaluates the early inflammatory cell permeation in the TM following MYR in OME. In fact, MYR caused a fast and progressive infiltration of the rat pars tensa by PMNs and monocytic/macrophagic cells, both in the OME specimens and the normal control TMs. These cells appeared almost exclusively in the injured quadrant. Previous studies have shown that the presence of inflammation promotes the TM healing[10, 11], but probably scarring too.[12]

Interestingly, as reported by Eriksson et al.[8], the pars tensa also appeared devoid of inflammatory cells at the early stages of this study, indicating that the participation of this structure in the OME events, following tubal occlusion, is apparently very low. Also, lymphocytes were very seldom observed throughout this study, suggesting that their role during the early stages of TM healing is of little relevance. However, other researchers have also showed that even in more aggressive settings, such as provoked acute otitis media in the rat, the infiltration by the lymphocyte population, both in the middle ear mucosa and within the TM structure, is also later and less pronounced than that consubstantiated by PMNs and macrophages.[8,13]

Further similar studies are needed in the long-term to try to integrate these early findings with the capacity of the TM to heal and associated sequelae following myringotomy.

Acknowledgements

Figure 1 – Light micrographs of sections from the annulus border of the TM perforation, at 48hours post-myringotomy. Immunostaining for polymorphoneutrophis (PMN). (Fig.1. A-Control; Fig.1. B-OME). Stained cells are dispersed in the whole thickness of the specimens. (mec-middle ear cavity; eac- external auditory canal; bar=100 micron)

Figure 2 – Light micrographs of sections from the annulus border of the TM perforation, at 48hours post-myringotomy. Immunostaining for macrophages ED1. (Fig.2. A-Control; Fig.2. B-OME). Stained cells are more sparsely located in the basal lamina beneath the keratinizing epithelium. (mec-middle ear cavity; eac-external auditory canal; bar=100 micron)

Figure 3 – Light micrographs of sections from the annulus border of the TM perforation, at 48hours post-myringotomy. Immunostaining for lymphocytes CD45. (Fig.3. A-Control; Fig.3. B-OME). Note absence of staining lymphocytes at this time-period. (mec-middle ear cavity; eac- external auditory canal; bar=100 micron)

Table 1 – Immunohistochemistry sera database

FIGURES AND TABLES

Fig1A
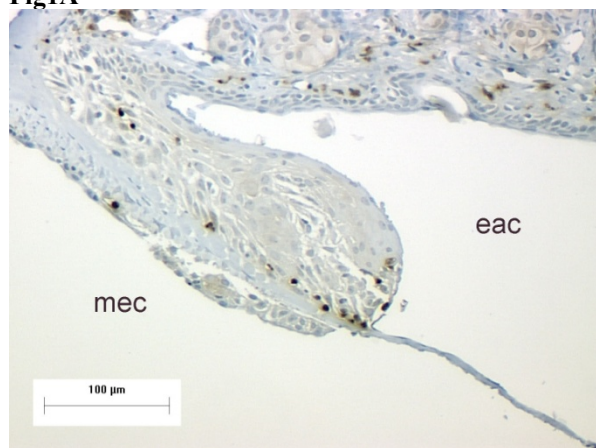
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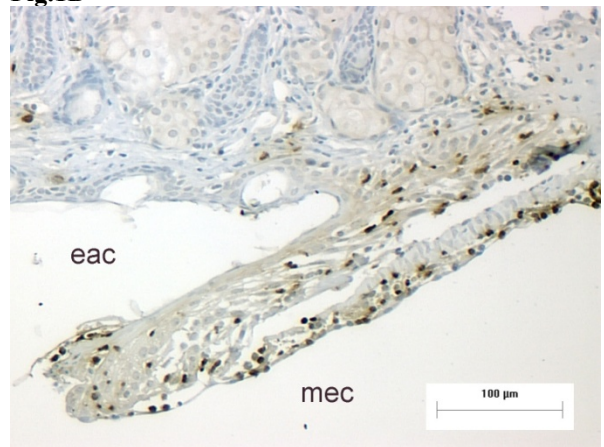
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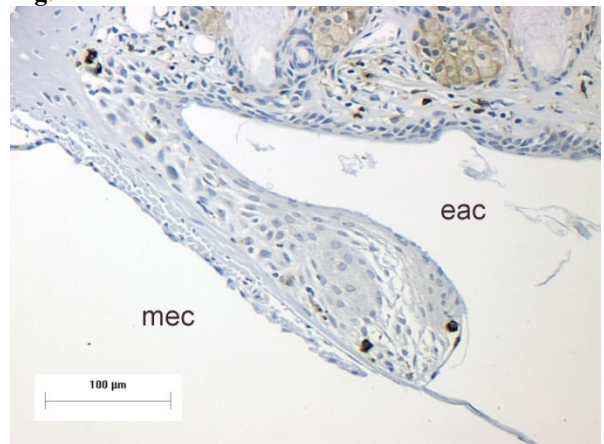
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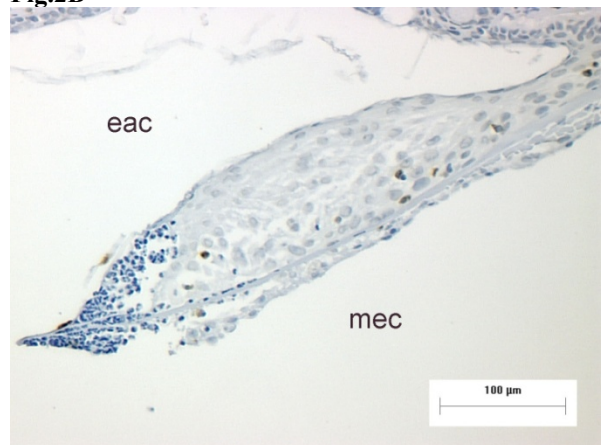
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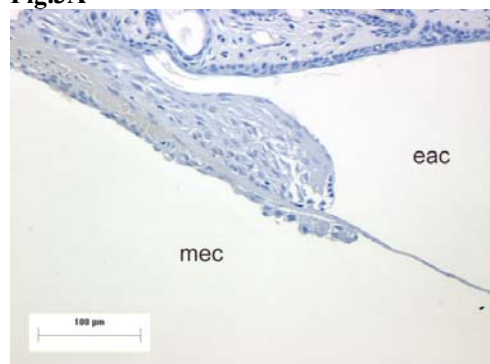
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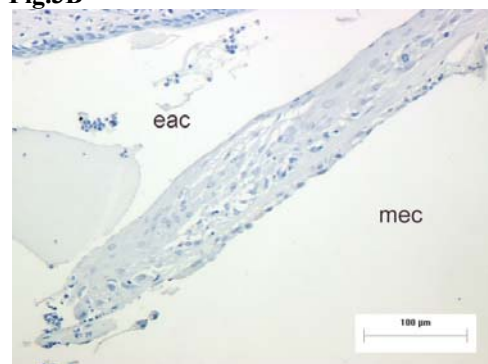
Table 1

Clone	Specificity	Form	Dilution	Source	Antigen Retrieval	Normal serum	Biotinylated secondary ab
PMN	Polymorphonuclear leucocytes	Polyclonal	1:2000	Cedarlane Laboratories Ltd. Homby, Ontario, Canada (CLAD51140)	0,01M citrate buffer pH 6.0, microwave oven, 630W, 101ºC, 15min	Swine	Swine anti-rabbit IgG ab
ED1	Tissue macrophages	Purified	1:100	Serotec, Novakemi, Enskede, Sweden (MCA341R)	0.1% trypsin, 8min, 37ºC	Rabbit	Rabbit anti-mouse IgG ab
ED2	Resident macrophages	Purified	1:100	Serotec, Novakemi, Enskede, Sweden (MCA341R)	0.1% trypsin, 8min, 37ºC	Rabbit	Rabbit anti-mouse IgG ab
OX33	Lymphocytes B CD45RA	Supernatant	1:100	Harlan Sera – Lab Ltd. Loughborough, England (MAS-258b)	None	Rabbit	Rabbit anti-mouse IgG ab
OX34	Lymphocytes T CD2	Purified	1:80	Cedarlane Laboratories Ltd. Homby, Ontario, Canada (CLO34AP)	0,01M citrate buffer pH 6.0, microwave oven, 630W, 101ºC, 15min	Rabbit	Rabbit anti-mouse IgG ab

References

1. Teele DW, Klein JO, Rosner BA. Epidemiology of otitis media in children. Ann Otol Rhinol Laryngol 1980; 89:5-6.
2. Daly K Definitions and epidemiology of otitis media with effusion. In: Roberts JE, Wallace I, Henderson (Eds). Otitis Media, Language, Skills, and Learning in Young Children. Baltimore, Paul H. Brooks, 1997.p3-41.
3. Bluestone CD. Myringotomy and tympanostomy tubes for otitis media with effusion. In: Alper CM, Bluestone CD, Casselbrant ML, et al. (eds.) Advanced Therapy of Otitis Media, BC Decker Inc, Hamilton 2004;p.194-202.
4. Mattsson C, Magnuson K, Hellström S. Myringotomy: a prerequisite for the development of myringosclerosis? Laryngoscope 1998; 108:102-106.
5. Kay DJ, Nelson M, Rosenfeld RM. Meta-analysis of tympanostomy tube sequelae. Otolaryngol Head Neck Surg 2001; 124:374-380.
6. Schmidt SH, Hellström S. Tympanic membrane structure – new views. A comparative study. ORL 1991; 53:32-36.
7. Hellström S, Mattsson C, Eriksson P-O. Early events in the development of middle ear inflammatory conditions as reflected by changes in the pars flaccida – A sequential experimental study. In: Veldman J, Passàli D & Lim D (eds.) New Frontiers in Immunobiology. Kugler Publications, The Hague, The Netherlands, 2000; p.3-7.
8. Eriksson PO, Johansson C, Hellström S. Inflammatory cells in developing otitis media. An experimental sequential study. In: Eriksson PO, Developing Otitis Media. Thesis, University of Umeå, paper IV; 2004.
9. Spratley J, Hellström S, Eriksson PO, Pais-Clemente M. Early structural tympanic membrane reactions to myringotomy: a study in an acute otitis media model. Acta Otolaryngol. 2002; 122:479-487.
10. Spratley J, Hellström S, Eriksson PO, Pais-Clemente M. Myringotomy delays the tympanic membrane recovery in acute otitis media: a study in the rat model.Laryngoscope. 2002; 112:1474-1481.
11. Spratley J., Hellström S., Eriksson P.-O., Pais-Clemente M.: "Tympanic Membrane Healing After Myringotomy in Various Middle Ear Conditions", Abstract, In: IXth International Congress of the European Society of Pediatric Otorhinolaryngology, Paris, France, 2006;p.125-126.
12. Forséni-Flodin M, Hultcrantz M. Possible inflammatory mediators in tympanosclerosis development. Int J Pediatr Otorhinolaryngol 2002; 63:149-154.
13. Forséni M, Hanson GK, Bagger-Sjöback D, Hultcrantz M. Infiltration of immunocompetent cells in the middle ear during acute otitis media. Am J Otol 1999; 20:152-157.

Lack of matrix metalloproteinase 9 does not affect the healing of tympanic membrane perforations

Cathrine Johansson, B.S., Per-Olof Eriksson, M.D. Ph.D., Jinan Li, Ph.D., Tor Ny, Ph.D., Sten Hellström, M.D. Ph.D.

Background

Cutaneous wound healing is associated with migratory and remodeling events that require the action of matrix metalloproteinases (MMPs) and their tissue inhibitors. MMPs catalyze the normal turnover of extracellular matrix and there are more than 20 different MMPs.

Objective

The objectives of the study were to investigate whether deficiency in MMP9, or gelatinase B, affected the healing of tympanic membrane (TM) perforations, and to describe the light- and electronmicroscopical appearance of the healed TM membrane.

Material and method

MMP9-/- and wild-type (MMP9+/+) mice in C57BL/6 background were myringotomized bilaterally at day 0. At days 4, 8, and 16 mice were otomicroscopically examined and sacrificed. After sacrifice the TMs and adjacent external ear canal skins were dissected out and immersed in glutaraldehyde fixative and later embedded in an Epoxy resin. Sections were prepared for light- and electron microscopy. The experiment was approved by the Regional Ethical Committee of Umeå University.

Results

At day 8, 18 out of 23 MMP9-/- and 19 out of 23 MMP9+/+ were otomicroscopically healed. At day 16, no perforations remained patent. Light microscopically, the healing initially showed a typical bridging by keratinocytes, guided by a keratin spur, followed by closure of the lamina propria in both genotypes. No difference in scar formation or epithelialisation was noticed.

Conclusion

The deficiency of MMP9, or gelatinase B, known to be of importance in wound-healing and scar tissue formation, did not seem to affect the healing time or the structural organization after TM perforations. An electron microscopical evaluation is in progress.

Effect of pneumococcal proteins on the inner ear

Patricia Schachern, B.S., Vladimir Tsuprun, Ph.D., Sebahattin Cureoglu, M.D., Patricia Ferrieri, M.D., Michael Paparella, M.D., Steven Juhn, M.D.

Introduction

Sensorineural hearing loss is a serious sequela of otitis media (OM). The round window membrane (RWM) is the only soft tissue barrier between the middle ear and the inner ear and has been shown to be permeable to a variety of substances. Passage of toxins through the RWM is considered the likely cause of inner ear damage. Streptococcus is one of the major pathogens in acute OM. Not only have pneumococcal proteins been shown to gain access to the inner ear by pore formation of the RWM, but intact bacteria have been shown to pass through the RWM and into the inner ear.[1] There are several pneumococcal proteins that contribute to its virulence and pathogenicity. Among these are pneumococcal surface protein A (PspA) and pneumolysin (Ply). PspA is involved in interactions with the host complement system[2] and Ply in degradation of the extracellular matrix and lysis of cholesterol-containing membranes.[3] The function of these proteins appears to facilitate significant aspects of pneumococcal colonization and invasion. Compromise of these functions may reduce pathogenicity of *Streptococcus pneumoniae*. We tested the hypothesis that *S. pneumoniae* mutants, deficient in PspA and Ply, are less virulent and less likely to cross the RWM and cause pathology of the RWM and inner ear.

Materials and methods

A total of 11 chinchillas were given bilateral intrabullar inoculations of 0.5 ml of 105 CFU of *S. pneumoniae* serotype 2 strain D39 (NCTC 7466) or its mutants, deficient in *PspA* or *Ply*. Three animals were inoculated for each strain. Two animals inoculated with the wild-type strain died prior to sacrifice and were replaced. Two days after inoculation, animals were euthanized, bullas removed, and the cochlea perfused via the apex and oval window with 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). Fixation was continued by emersion for 2 hours. Samples were decalcified in 10% EDTA on a rotator in a cold room for 3 days. EDTA was changed daily. Samples were washed in phosphate buffer and post-fixed in 1% OsO4 in phosphate buffer (pH 7.4) for 1 hour. They were washed again in buffer, dehydrated in a graded series of ethanol, followed by propylene oxide, and embedded in epoxy resin. Samples were cut at a thickness of 1 μm and stained with Toluidine blue for light microscopic assessment. For electron microscopy, samples were cut at a thickness of 20 nm, stained with uranyl acetate and lead citrate, and examined with a JEOL 1010 electron microscope.

Results

Bacterial infiltration of the RWM and scala tympani (ST) were observed in the wild-type strain and the Ply-deficient mutant but not in the PspA-deficient mutant. Inner ear changes in the wild-type and Ply-deficient mutant included bacterial infiltration of the scala tympani and scala vestibuli, endolymphatic hydrops and damage to the stria vascularis, particularly the intermediate cells, and outer and inner hair cell damage and loss. Pathologic changes were not observed in the inner ears of the PspA-deficient mutants.

Discussion

S. pneumoniae is a common pathogen in acute OM. We have previously shown that inner-ear damage and high-frequency hearing loss in humans and chinchillas can arise from passage of bacterial products and inflammatory mediators from the middle ear through the RWM into the inner ear.[4,5,6,7] Widespread use of oral antibiotics has resulted in an alarming increase of antibiotic-resistant bacterial strains increasing the potential for labyrinthitis and tympanogenic meningitis. Pneumococcal proteins facilitate significant aspects of pneumococcal colonization and/or invasion and can therefore serve as targets for the development of novel therapies to treat pneumococcal diseases.

Although Ply-deficient mutants have been shown to be less virulent in various organs, including the lung and blood[8], our findings showed no difference between the Ply-deficient and the wild-type strain in their ability to penetrate the RWM into the ST of the inner ear. However, the mutant, deficient in PspA, was

less virulent when compared to the wild-type or the Ply-deficient strains. Our findings support those of other investigators who showed the cell wall to be the most important contributor to pneumococcal pathogenicity in OM, with at most a modest additional effect of pneumolysin.[9]

PspA is attached to the cell surface by non-covalent binding to the choline residue of the lipoteinichoic or techoic acids via its C-terminal repeat region.[10] The N-terminal end extends from the cell wall and protrudes outside the capsule. PspA is involved in interactions with the host complement system, reducing complement-mediated clearance and phagocytosis.[11] Recent evidence has shown that a function of PspA in pneumococcal virulence is the prevention of the bactericidal effect of the host molecule, lactoferrin (L).[12] Lactoferrin is an iron storage glycoprotein that is predominantly found in mucosal secretions including middle-ear effusions.[13] The apo form of lactoferrin (apoL) is the form that is bactericidal against pneumococci.[12] PspA binding to apoL presumably protects pneumococci against its bactericidal effects. Due to high concentrations of apoL in secretions, the process presumably takes place at the mucosal surface and facilitates colonization and carriage of *S. pneumonia*.[14]

Although the 23-valent polysaccharide vaccine is immunogenic and protective in most adults and children over 5 years of age, it may fail to protect children under the age of 2 years.[15] Currently, several pneumococcal surface proteins are considered as alternative vaccine candidates because of their serotype-independence. PspAs of different *S. pneumoniae* strains are immunologically cross-reactive and immunization with a single PspA stimulates broadly cross-reactive antibodies[16], making it an extremely good vaccine candidate. Further studies regarding these bacterial proteins and their possible use in treatment of otitis media is warranted.

Conclusion

The mutant deficient in PspA did not penetrate the RWM or cause damage to the inner ear as compared to the wild-type strain or the mutant deficient in Ply. PspA may be a good vaccine candidate for the prevention of OM and related inner ear damage and sensorineural hearing loss.

Acknowledgements
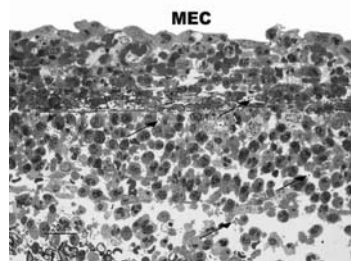
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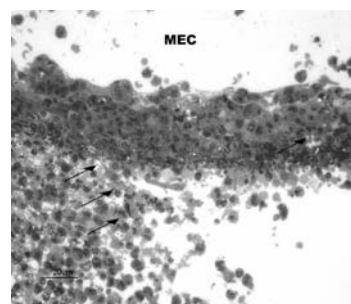
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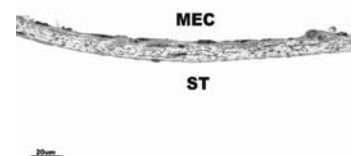
Fig. 1c.

Figure 1. Bacterial infiltration (arrows) of the round window membrane and scala tympani were observed in a) the wild-type strain and b) the Ply-deficient mutant but not c) in the PspA-deficient mutant. MEC = middle ear cavity. Stained with Toluidine blue.
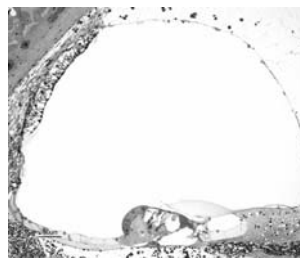
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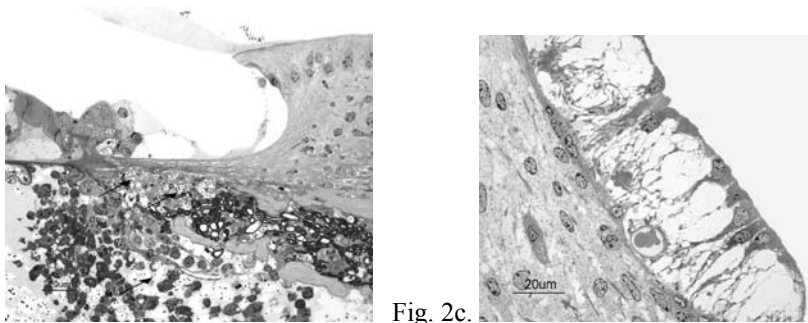
Fig. 2b.   Fig. 2c.

Figure 2. Changes in the inner ear in the wild-type and Ply-deficient mutant were similar and included: a) endolymphatic hydrops and damage to the stria vascularis and outer and inner hair cells, b) higher magnification shows bacteria (arrows) among the neurons near the habenula perforatae, and c) damage to the stria vascularis, primarily the intermediate cells. Stained with Toluidine blue.

References

1. Schachern PA, Paparella M, Hyberston R, Sano S, Duvall III AJ. Bacterial tympanogenic labyrinthitis, meningitis and sensorineural damage. Arch Otolaryngol Head Neck Surg 1992; 118:53-57.

2. Ren B, Szalai AJ, Hollingshead SK, Briles DE. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. Infect Immun 2004; **72:**114–122.

3. Rubins JB, Janoff EN. Pneumolysin: a multifunctional pneumococcal virulence factor. J Lab Clin Med 1998; 131:21-27**.**

4. Paparella M, Oda M, Hiraide F, Brady D. Pathology of sensorineural hearing loss in otitis media. Ann Otol Rhinol Laryngol 1972; 81:632-648.

5. Paparella MM, Morizono T, Le CT, et al. Sensorineural hearing loss in otitis media. Ann Otol Rhinol Laryngol 1984; 93:623-629.

6. Morizono T, Giebink GS, Paparella MM, Sikora MA, Shea D. Sensorineural hearing loss in experimental purulent otitis media due to *Streptococcus pneumoniae*. Arch Otolaryngol 1985; 111:794-798.

7. Juhn SK, Jung TT, Lin J, Rhee CK. Effects of inflammatory mediators on middle ear pathology and on inner ear function. Ann N Y Acad Sci 1997; 830:130-142. Review.

8. Kadioglu A, Gingles NA, Grattan K, Kerr A, Mitchell TJ, Andrew PW. Host cellular immune response to pneumococcal lung infection in mice. Infect Immun 2000; 68:492-501.

9. Sato K, Quartey MK, Liebler CL, Le CT, Giebink GS. Roles of autolysin and pneumolysin in middle ear inflammation caused by a type 3 *Streptococcus pneumoniae* strain in the chinchilla otitis media model. Infect Immun 1996; 64:1140-1145.

10. Yother J, White JM. Novel surface attachment Mechanism of the *Streptococcus pneumoniae* protein PspA. J Bacteriol 1994; 176:2976-2985.

11. Neeleman C, Sibyl PM, Geelen SP, et al. Resistance to both complement activation and phagocytosis in type 3 pneumococci is mediated by binding of complement regulatory protein factor H. Infect Immun 1999; 67:4517-4524.

12. Shaper MS, Hollingshead SK, Benjamin WH Jr, Briles DE. PspA protects *Streptococcus pneumoniae* from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin. Infect Immun 2004; 75:5031-5040.

13. Giebink GS, Carlson BA, Hetherington SV, Hostetter MK, Le CT, Juhn SK. Bacterial and polymorphonuclear leukocyte contribution to middle ear inflammation in chronic otitis media with effusion. Ann Otol Rhinol Laryngol 1985; 94:398-402.

14. Jedrzejas MJ. Unveiling molecular mechanisms of pneumococcal surface protein A interactions with antibodies and lactoferrin. Clinica Chimica Acta 2006; 367:1-10.

15. Paton JC. Novel pneumococcal surface proteins: role in virulence and vaccine potential. Trends Microbiool 1998; 6:85-88.

16. Nabors GS, Braun PA, Herrmann DJ, et al. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies. Vaccine 2000; 18:1743-1754.

Immunohistochemical study of the effect of 5-fluorouracil (5-FU) on cholesteatoma

Tomomi Yamamoto-Fukuda, M.D., Mariko Terakado, Yoshitaka Hishikawa, M.D., Takehiko Koji, Ph.D., Haruo Takahashi, M.D.

Objective

To investigate the cell-biological effect of 5-fluorouracil (5-FU) ointment (Kyowa, Roche) on the middle-ear cholesteatoma of humans.

Study design and setting

Two to three cubic millimeters of 5-FU topical cream was applied to 12 cases of attic cholesteatoma (12 patients) two to five times with an interval of 2 weeks, and its cell-biological efficacy was evaluated by using immunohistochemical analysis (5-FU group). Sixty-one cholesteatoma cases without treatment using 5-FU served as controls. Immunohistochemical analysis for Ki-67, Keratinocyte growth factor (KGF) and its receptor (KGFR) were performed to analyze pathological mechanism. Polyclonal antibodies against KGF and KGFR were prepared by immunization of rabbits against synthetic peptides in cooperation with Nichirei Co. (Tokyo, Japan).

Results

In the 5-FU group, the expression of KGF was significantly decreased ($p < 0.05$). The Ki-67 labeling index of cholesteatoma tissues was $37.5 \pm 0.2\%$ in the 5-FU group, which was significantly lower than that of the control group ($48.5 \pm 0.2\%$) ($p < 0.05$).

Conclusion

One of the mechanisms of the good effect of 5-FU on cholesteatoma may include a decrease in the expression of KGF in stromal cells and in the downregulation of the proliferative activity of the epithelial cells.

Eustachian tube form/function in cleft lip and palate

Anil Gungor, M.D., William J Doyle, Ph.D., J. Douglas Swarts, Ph.D.

Objective

The objective of this pilot project was to demonstrate the feasibility of studying the form-function identity for the eustachian tube (ET)-middle ear (ME) complex using magnetic resonance imaging (MRI) and various tests of ET function in repaired cleft palate (CP) children aged 7 to 10 years.

Study design

Survey.

Setting

Urban tertiary care hospital.

Subjects and methods

Twenty-two volunteer subjects (17 male), 7 to 10 years of age, with repaired complete clefting of the hard palate were enrolled. One-half of the subjects had bilateral clefts of the hard palate; the others had unilateral clefts equally distributed by side. Six subjects had tympanostomy tubes (TT) in place and patent. Thirteen completed the MRI. Eustachian tube function was evaluated using four tests: sonotubometry, the forced response test (FRT), the inflation-deflation test (IDT), and the continuous pressure test (CPT). The MRI was performed on a GE Signa system using a double oblique scanning protocol. ET measurements were taken from cross-sectional images aligned perpendicular to the long axis of the tube.

Results

One quarter of the 15 subjects with intact tympanic membranes were tympanometrically normal bilaterally. Fewer than one-third of the subjects had positive sonotubometric findings (at least one of eight opportunities produced increases in external auditory canal sound pressure levels ≥ 5 dB). The results of the IDT and CPT document an inability to transmit either applied ME or nasopharyngeally induced over- and underpressures. The low values of the passive function variables of the FRT contrast with higher than normal values of subjects with chronic OM without a CP. Despite these quantitative passive function differences, both samples showed a decrease in airflow during swallowing in the steady state phase of the FRT. Eight of the MRIs were of sufficient quality to perform a morphometric analysis. Intra- and inter-observer error was approximately 10%, with features of the cartilage showing the least variation. The cartilage height and tensor veli palatini muscle-cartilage angle were consistent with values obtained from histologic analyses of normal children.

Conclusions

These CP subjects display the same spectrum of ET dysfunction we identified in our previous studies, lower values for the passive function variables, and very poor active function. Although difficult, more than half of the subjects undergoing MRI produced images useful for morphometric analysis.

Acknowledgements

Interspecies comparisons of eustachian tube flow patterns during swallowing in the forced response test

J. Douglas Swarts, Ph.D., Samir Ghadiali, Ph.D.

Background

Eustachian tube (ET) dilation is caused by tensor veli palatini muscle (mTVP) contraction. Human and animal studies have demonstrated the causal relationship between mTVP function and middle-ear (ME) status; lack of an effective mTVP function results in otitis media (OM). Humans with persistent OM, with or without craniofacial dysmorphogenesis, show increased active resistance (a swallowing induced change) on the forced response test (FRT). Often a complete obstruction of flow through the ET occurs during swallowing in these individuals. However, in only a minority of instances (cancer invading the mTVP) is there evidence of mTVP dysfunction.

Objective

Compare the FRT flow patterns during swallowing across species for evidence of anomalous muscular activity (flow patterns during swallows).

Materials and methods

Over the past 15 years, the ENT Basic Sciences Laboratory at Children's Hospital of Pittsburgh has performed the FRT on a range of species including rats, chinchillas, cats, ferrets, monkeys, and humans. Here we report a preliminary analysis of the flow patterns that develop during swallowing in the steady state phase of the FRT. The flow rate was 6 ml/min (rats, ferrets), 11 ml/min (monkeys), and 11 or 23 ml/min for humans. Data from the FRT datasets was extracted, imported into a spreadsheet, and the ET dilation with the largest change in flow rate analyzed. Each swallow was categorized as to whether there was a restriction of ET steady state flow and, if so, whether it occurred prior to or after evidence of an increased flow rate. Continuous variables analyzed included maximum flow, ET dilation duration, and active resistance.

Results

Data were available for 10 rats, 20 ferrets, 6 monkeys, 6 human adults with no history of OM, and 38 older children with persistent OM. None of the rats, ferrets, or monkeys had flow completely obstructed during FRT swallows. One human adult had flow obstruction and another showed no evidence of a change in flow during swallowing. In contrast, 23 of the 32 subjects with COM evidenced complete obstruction of the ET. Dilation of the ET in ferrets and monkeys produced the largest median maximum flow during a swallow, and they had the lowest active resistances. In contrast, median flows of rats and humans with COM were about one-half that of ferrets and monkeys. The three non-human species had the shortest duration of ET dilation. Humans had longer median dilation times and they also displayed greater variability as measured by the 95% confidence interval.

Conclusion

The monkey and ferret possess the best active function of the species examined. They also showed little evidence of flow obstruction prior to the maximum flow rate achieved during a swallow. In contrast, humans have poorer ET function and a greater range of variability.

Otitis media and overweight in toddlers

Kathleen Daly, Ph.D., Cynthia Davey, M.S., Linda Bartoshuk, Ph.D., Heather Nelson, M.P.H.

The prevalence of overweight/obesity has doubled over the past 20 years among preschool children, predisposing them to numerous chronic diseases.[1,2] A history of otitis media (OM) has been shown to be significantly related to overweight/obesity in adults.[3] Repeated ear infections may damage the chorda tympani nerve, which passes through the middle-ear space. Food preference data show that sweet, high-fat foods are more palatable among those with OM histories, leading to the hypothesis that OM results in overweight/obesity by altering sensory perceptions to increase palatability of sweet and fat foods.[4]

The purpose of this study was to explore the relationship between recurrent OM/tympanostomy tube treatment and overweight using existing databases. Data were from two prospective cohorts followed from birth to age 2: the Early OM and Little Ears Cohorts. Those with outcome data (height and weight at ≥1 year of age) were included in these analyses; 108 from the Little Ears cohort and 416 from the early OM cohort.

OM and tympanostomy tube history, height, and weight from the last well-child visit were abstracted from the medical record. Height and weight measures were used to calculate weight-for-stature in children at 1.0 to 1.99 years in the Little Ears cohort, while BMI-for-age was calculated using height and weight data at 2.0-2.99 years. In both cohorts, the proportion of children with BMI-for-age or weight-for-stature >95th percentile was calculated.

In the Early OM cohort, 6% had BMI-for-age measures at age 2 years that exceeded the 95th percentile. Those with a history of tympanostomy tube treatment had a significantly increased risk of overweight after controlling for birth weight, maternal prenatal smoking, maternal education, and family income (OR 2.42, 95% CI 1.24-4.71). In the Little Ears cohort, 35% had weight-for-stature measures at age 1-2 years exceeding the 95th percentile. Similar to the Early OM cohort, multivariate analysis demonstrated that recurrent OM increased the risk of overweight in the Little Ears cohort at 1-2 years of age (OR 3.32, 95% CI 1.19 -9.28).

These findings are consistent with the hypothesis that recurrent OM and/or chronic otitis media with effusion treated with tympanostomy tubes can lead to overweight, possibly mediated by damage to the nerves involved in taste.

References

1. Ogden CL, Flegal KM, Carroll MD, Johnson CL. Prevalence and trends in overweight among US children and adolescents, 1999-2000. JAMA 2002; 288:1772-1773.
2. Strauss RS. Childhood obesity and self esteem. Pediatrics 2000; 105:e15.
3. Snyder DJ, Duffy VB, Bartoshuk LM. Localized oral pathology produces disinhibitory sensory changes promoting orofacial pain and obesity risk. Presented at the Society for Neuroscience meeting, San Diego California, 2004.
4. Landis BN, Beutner BN, Frasnelli J, Huttenbrink KB, Humell T. Gustatory function in chronic inflammatory middle ear diseases. Laryngoscope 2005; 115:1124-1127.

A community-based questionnaire study on the association between symptoms suggestive of otitis media with effusion, rhinitis, and asthma in primary school children

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Objectives

To evaluate the association between symptoms suggestive of otitis media with effusion (OME), rhinitis, and asthma in an unselected population of primary school children and investigate whether our previous observation of such an association in a secondary care setting was valid in the community.

Methods

A specifically designed questionnaire was administered to 332 new entrant primary school children across 11 state and independent primary schools in the East Berkshire district between March and June 1996. It had six sections, to ascertain symptoms suggestive of OME, rhinitis, asthma, other atopic features, treatment for any of these, and a possible family history of atopy. Within the first three sections, each question was scored and weighted depending on the importance of each in establishing the possible diagnoses, with three scoring bands: '0' indicating the absence; '1-5' the possibility and '≥6' a strong likelihood that the above conditions were present. The outcome measures were the number of children with or without symptoms suggestive of OME, rhinitis, asthma, and the correlation of these symptom scores with each other, OME with eczema, other atopic manifestations, family history of atopy and educational system.

Results

Data revealed that 32.8%, 36.6%, and 24% had symptoms suggestive of OME, rhinitis, and asthma respectively with scores of either 1-5 or ≥6. There was a highly significant correlation between otological (OME) and nasal scores ($p = 0.00000$), particularly obstructive nasal symptoms ($p = 0.00000$) and ≥ 6 upper respiratory tract infections/year ($p = 0.00000$); otological and chest scores suggestive of asthma ($p = 0.00001$), and a family history of asthma ($p = 0.0178$). No association was found between scores for OME and eczema, urticaria, food, or drug allergies. No differences were noted between the sexes or educational systems.

Conclusions

The highly significant association between the symptom scores suggestive of OME and rhinitis in this unselected population indicates the importance of inquiring about nasal symptoms in children with chronic OME, as appropriate treatment of rhinitis may improve the child's quality of life, reduce health care utilization, and possibly reduce the need for OME surgery.

Risk factors, treatments, and other conditions associated with frequent ear infections in U.S. children through 2 years of age: The Early Childhood Longitudinal Study-Birth Cohort (ECLS-B)

Howard J. Hoffman, M.A., Jennifer Park, Ph.D., Katalin G. Losonczy, M.A., May S. Chiu, B.S.

Background

ECLS–B is a nationally-representative, longitudinal study of U.S. births in 2001 with timely reporting of ear infections (EIs); most prior studies involved long recall intervals.

Methods

Data are currently available from the first two waves, 9 months and 2 years of age. Racial/ethnic groups, low birth weight and twin births were oversampled. Risk factors and treatments for EIs are analyzed in singleton births (N=8,076), using parents' report of medically-diagnosed otitis media. Treatment information is available for a maximum of 3 visits at each wave. We defined "frequent" EIs to be 3 or more at either age.

Results

At 9 months, 39.1% had medically-diagnosed EIs, which increased to 62.3% by 2 years. Treatments (multiple ones possible) for the most recent EIat age 2 were: antibiotics (96.4%), ear drops (7.8%), analgesics (7.6%), ear tubes (3.2%), decongestants or antihistamines (2.3%), other (1.7%), and "watch and wait"(0.6%). Multivariable logistic regression identified factors increasing the likelihood of frequent EIs: male sex (odds ratio [OR]=1.3; 95% confidence interval: 1.1-1.5), non-Hispanic white race (OR=2.0; 1.7-2.3), child care center 20+ hours/week (OR=2.9, 2.3-3.7), and birth weight <1500 g (OR=1.3, 1.03-1.6). Breastfeeding 9+ months (OR=0.7; 0.5-0.9) decreased the likelihood. Children with frequent EIs had increased other illnesses/conditions and were much more likely to have had ear tubes inserted (OR=39; 25-59). No association was found with Bayley mental or motorsummary T-scores at age 2.

Conclusion

This study documents the prevalence of recent treatment practices and identifies major EI risk factors among U.S. infants and toddlers.

Otitis-prone children: does the infectious liability persist?

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Background

It has been questioned whether otitis-proneness is associated with a partial or generalized immunological hyporesponsiveness. Although it has been reported that otitis media and respiratory morbidity is still common in schoolchildren, the relationship between otitis-proneness and infectious morbidity in school-age is not known.

Objective

The aim of the study was to study whether early initiation of otitis media and otitis- proneness are predictors for infectious respiratory morbidity in 10-year-old children.

Methods

A population-based study of 3754 children born in Oslo in 1992/93, of which 2549 children were followed from birth to 10 years. Main outcome measures were questionnaire-based information on acute otitis media (AOM) and respiratory infections at ages 6 months, 1, 1 ½ , 2 and 10 years. Recurrent acute otitis media (rAOM) was defined as >=4 episodes during a 12-month period. Follow-up rates were 95%, 92%, 86%, 81% and 68% respectively.

Results

Both children with otitis media the first year of life and recurrent episodes before age 2 had increased risk of AOM at age 10 with odds ratios (ORs) 1.3 (95%CI 1.0-1.6) and 1.6 (95%CI 1.0-2.7), respectively. The otitis-prone children had significantly increased probability for both later otitis media surgery and bronchial obstruction before age 2 (cORs 5.7 (95%CI 3.7-8.9) and 2.4 (95%CI 1.6-3.7). rAOM before age 2 was also associated with allergic rhinoconjunctivitis at age 10 with OR 1.7 (95%CI 1.0-2.7), but not significantly associated with any other respiratory infections in school-age children.

Conclusion

Otitis-prone children remain susceptible to otitis media, but do not confer increased risk for other respiratory infections in school-age children.

Otitis media in children: A third world experience

Olubunmi Akinpelu

Background

Otitis media (OM) constitutes the greatest clinical burden to an otolaryngologist practicing in Nigeria and is a common disease in children. The aim of this study is to describe the presentations of OM in Nigerian children.

Methods

A prospective, hospital-based study carried out in Obafemi Awolowo University Teaching Hospitals complex (OAUTHC), a tertiary hospital located in Southwestern Nigeria. All children from 6 months to 15 years with the diagnosis of chronic suppurative OM (CSOM) between January 2004 and December 2006 are included in the study. Information on the symptoms and signs, duration of otorrhea before presentation, mode of referral, and associated complications were obtained from an interviewer-administered questionnaire.

Results

A total number of 178 cases of CSOM were seen during the period, 95 (53.2%) of whom were children. Forty-one (43.2%) were below the age of 5 years. The average duration of otorrhea before presentation was 15 months. Sixty-one (64.2%) of the patients had one form of treatment or the other before presentation. The practice of plugging the ear discharge with a cotton bud was common among the patients seen (33.2%). Various other local remedies included topical application of goat nasal discharge, honey, traditional herbal preparations, and ear drops procured without prescription. A significant number (66/69.5%) were from the rural area. Poverty-related problems were strong predisposing factors noticed among the patients seen (31/32.6%). Bilateral disease was seen in 33 (34.7%) of the patients. The perforations were central in 87 (91.6%) cases and marginal in the rest. Cholesteatoma was associated in 4 (4.2%) patients. The complications seen included disabling hearing loss (7/7.4%), subperiosteal mastoid abscess (5/5.3%), intracranial suppurations (6/6.3%), meningitis (4/4.2%), facial nerve palsy (2/2.1%), otitis externa (3/3.2%), and external auditory meatal stenosis (1/1.1%).

Conclusion

CSOM is a challenge to an otologist practicing in Nigeria. Bringing specialist otolaryngologic care to the communities might be an approach to adopt to lessen the burden of CSOM among Nigerian children.

Burden of otitis in Dutch children ≤ 4 years old: an analysis of the 2005 LINH database

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Background

Otitis is a frequent childhood disease, but its full burden is difficult to assess as patients can be seen by different healthcare providers. In the Netherlands (NL), almost all individuals are registered with a general practitioner (GP).

Objectives

To estimate the rate and burden of otitis in children ≤ 4 years old at the GP level in NL.

Methods

The 2005 LINH database (medical records of a nationally representative population of 350,000 individuals from 85 general practices) was searched for otitis in ≤ 4 year-olds. The rate of otitis episodes and descriptive statistics on medical resources used were generated.

Results

One thousand eight hundred seventy-one otitis episodes were identified in 1,570 of 9,676 ≤ 4 year-olds. 85.3% episodes were acute, 1.7% were chronic, 13% were otitis media with effusion. The rate of otitis episodes was 193.4 (95% CI: 185.6-201.4) per 1,000 person-years. Children with otitis had up to 5 (average 1.2 ± 0.6) episodes of acute otitis/year with 1.3% having ≤ 4 episodes/year. Each episode required up to 9 (average 1.3 ± 0.7) visits. 41.6% of children were prescribed antibiotics (80% amoxicillin), and 3.9% were referred to an ear-nose-and-throat specialist.

Conclusions

In 2005, 16.2% of ≤ 4 year-olds were diagnosed with otitis by GPs in NL. As Dutch GPs are thought to see only 30% of otitis cases (Bos JM et al. Clin Ther 2003:25(10);2614-30), the rate of otitis is likely underestimated when using a GP database. With up to 5 episodes/year and up to 9 visits/episode, otitis and its complications represent a significant burden that needs to be properly addressed.

Candidate gene screening in a large family with otitis media

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Otitis media (OM) is one of the most prevalent inflammatory diseases in the pediatric population. The personal and societal costs for OM are significant. Evidence from studies in the human population and in mouse models reveals that there is a very significant genetic component predisposing to chronic/recurrent OM (COME/ROM). Here, we ascertained a four-generation family segregating for an autosomal dominant form of OM. Eleven of the 19 family members suffered from COME/ROM during childhood. Ten of them received myringotomy and tube placement at least once in their life time. None of them were reported to have any craniofacial abnormality. We have collected DNA samples from two affected individuals and one unaffected person for genetic study. The FBXO11 gene is the human homologue of the gene mutated in the novel deaf mouse mutant jeff (Jf), a single gene model of otitis media. To investigate the role of FBXO11 in human OM, we will sequence coding regions and exon/intron boundaries of FBXO11 for any possible OM-causing mutation in the present family.

Otitis media as a cause of significant hearing loss among Nigerians

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Introduction

Otitis media, defined as inflammation involving the mucosal lining of the middle-ear cleft is a recognized cause of significant hearing loss.[1] Studies examining hearing sensitivity in children with otitis media with effusion (OME) report that average pure tone hearing loss at 4 frequencies (500, 1000, 2000, and 4000 Hz) ranges from normal hearing to moderate hearing loss (0–55 dB). The 50th percentile is about 25 dB hearing level (HL), and approximately 20% of ears exceed 35 dB HL.[2] Non-complicated CSOM on the other hand causes conductive hearing loss in the mild to moderate category in more than 50% of cases.[3,4] Even though hearing loss due to chronic suppurative otitis media (CSOM) was believed to constitute a burden mainly in developing countries, there has been recent report from a developed country of bilateral deafness from CSOM necessitating cochlear implants.[5] Prolonged duration, severity, as well as type of otitis media are associated with cranial complications (ossicular destruction and ankylosis), and together with persistent tympanic membrane perforation contribute to the severity of hearing loss.[6] There are few reports examining the distribution of otitis media presenting with significant hearing loss among ears.

Methods and materials

This study is a retrospective analysis of prospectively collected data of individuals attending the hearing loss clinic of National Hospital, Abuja, between May 2005 and December 2006. Data matching the diagnosis of acute otitis media (AOM), otitis media with effusion (OME), and chronic suppurative otitis media (CSOM) were extracted from the database and analyzed. All individuals had clinical otoscopy, otomicroscopy, pure tone audiometry (Diagnostic Audiometer: Interacoustics AD229e), and tympanometry. Results were analyzed using SPSS software Version 13.0

Results

A total of 257 cases of self-reported hearing losses were seen. Of these, 54 cases fulfilling the criteria of self-reported significant hearing loss due to otitis media were analyzed. Twenty-nine were males and 25 females (male: female ratio 1:1.16). The age range of the cases studied was 1- 68 years (mean = 30.1, standard deviation = 5.29). Table 1 is a distribution of age according to diagnosis.

Twenty-six patients (10.1%) had OME, 24 (9.3%) had CSOM, while 4 (1.5%) had AOM. Seventy-five percent of cases with significant self-reported hearing loss had unilateral hearing loss. Of these, 53% (n=29) had the left ear primarily affected, while 22% (n=12) had the right ear primarily affected. The remaining 25% (n=13) of cases studied had bilateral involvement giving a total of 67 ears.

The observed distribution of the OME was left ear OME (5.4%, n=14), right ear OME (2.7%, n=7), and bilateral OME (1.9%, n=5), while the CSOM was distributed into left ear CSOM (5.4%, n=14), bilateral CSOM (2.3%, n=6), and right ear CSOM (1.5%, n=4), and AOM was distributed into right ear AOM (0.38%, n=1), left ear AOM (0.38%, n=1), and bilateral AOM (0.77%, n=2).

The average duration of symptoms prior to presentation was 43 weeks (OME), 280.5 weeks (CSOM), and 1 week (AOM). Table 2 is a distribution of symptom duration according to diagnosis.

The mean pure tone averages for the three groups were AOM (26.5dB), OME (39.5dB), and CSOM (53dB).

Summary of findings

20% of all significant hearing loss seen was due to otitis media.

75% of these were unilateral.

53% (71% of unilateral cases) of significant hearing loss due to otitis media affected the left ear.

Individuals with left ear hearing loss due to otitis media were relatively older and tended to present earlier.

Late presentation was common in OME and CSOM.

Conclusion

Otitis media is an important cause of preventable hearing loss in developing countries.

Delayed presentation is possibly responsible for the severity of hearing loss.

Predominance in the left ear was most likely due to trauma.

More studies are needed to clarify role of trauma in otitis media with significant hearing loss.

Table 1. Age Distribution versus Diagnosis in 54 Individuals with Significant Hearing Loss Due to Otitis Media

Diagnosis	Total No	Min. Age (yrs)	Max. Age (yrs)	Mean Age (yrs)
OME	26	1	63	28.6
Bilat	5	1	56	20.8
Right	7	3	61	33
Left	14	7	63	41.5
CSOM	24	9	68	36
Bilat	6	9	47	35.8
Right	4	20	49	29.3
Left	14	22	76	44.7
AOM	4	4	49	25.75

Table 2. Symptom Duration Versus Diagnosis in 54 individuals with significant hearing loss due to otitis media

Diagnosis	Min. Duration (wks)	Max. Duration (wks)	Mean Duration (Weeks)
OME	1	208	43
Bilateral.	2	156	45.1
Left	1	104	28.5
Right	1	208	50.25
CSOM	16	1040	280.5
Bilateral.	16	1040	420.8
Left	16	780	222.1
Right	52	780	224.4
AOM	1	2	1.25

References

1. Grundfast KM. Hearing loss. In: Bluestone CD, Stool SE, eds. Pediatric Otolaryngology. 2nd ed. Philadelphia: W.B. Saunders; 1990:203-239.
2. Thrasher RD III. Middle ear, otitis media with effusion. [Medscape Web site] Available at: http://www.emedicine.com/ent/topic209.htm
3. Ezeanolue BC, Okafor BC, Obiakor MN. Hearing loss in uncomplicated chronic suppurative otitis media among Nigerians. (Proceedings of the XVII World Congress of the International Federation of Oto-Rhino-Laryngological Societies (IFOS)) Otorhinolaryngol Int Congress Series 2003; 1240:81-87.
4. Chronic suppurative otitis media: Burden of illness and management options. World Health Organization, Geneva, Switzerland, 2004. Available at: http://www.who.int/entity/pbd/deafness/activities/hearing_care/otitis_media.pdf
5. Basavaraj S, Shanks M, Sivaji N, Allen AA. Cochlear implantation and management of chronic suppurative otitis media: single stage procedure? Eur Arch Otorhinolaryngol 2005; 262:1434-4726.
6. Paparella MM, Schumrick DA. Otolaryngology. 3rd ed. Philadelphia: W.B. Saunders Co., 1991.

The global burden of childhood otitis media and hearing impairment: a systematic review

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Introduction

The World Health Organization (WHO) resolved that chronic otitis media and resultant hearing impairment are significant global health problems. WHO called for more detailed epidemiological information, particularly the association between prevalence and socioeconomic variables.

Aim

To determine the worldwide prevalence of otitis media (OM), hearing impairment (HI) and their risk factors.

Methods

We searched Medline, Embase and Cinahl for population-based studies with incidence or prevalence data on OM and HI (>25dB) in children (<18 years), without language restrictions. Studies identified through reference lists were also included. We examined the effect of socioeconomic and health variables on OM and HI prevalence.

Results

The search strategy identified 1,504 studies with substantial methodological variation. The included studies (n=108) provided a combined sample size of 250,978 children. Acute OM incidence ranged from 0.6 to 1.7 episodes per child per year. The highest OM prevalence rates were in Inuits (81%) and Australian Aborigines (84%). HI prevalence ranged from <1% (Greece) to 23% (Australian Aborigines). HI was significantly more common in children with OM (OR 8.11, 95%CI 6.91-9.52). In meta-analysis increased OM prevalence was associated with not breastfeeding (OR 1.28, 1.03-1.59) and parental smoking (OR 1.73, 1.42-2.10), but male gender (OR 1.04, 95%CI 0.90-1.20) and urbanization (OR 0.72, 95%CI 0.28-1.83) were not significant. Some studies reported increased OM prevalence with overcrowding, lower maternal education and poorer household sanitation.

Conclusion

Indigenous children have the highest prevalence of OM and its complications. OM remains a significant cause of preventable childhood HI and many of the risk factors are modifiable.

Laterality of acute otitis media: different clinical and microbiological characteristics

David McCormick, M.D., Stephanie Chandler, M.D., Tasnee Chonmaitree, M.D.

Background

A large individual patient data meta-analysis recently showed that children aged less than two years with bilateral, as compared with unilateral, acute otitis media (AOM) were at higher risk for persistent symptoms without antibiotic treatment. Prior studies have shown a propensity for children with bilateral AOM to be infected with bacterial pathogens, specifically *Haemophilus influenzae*.

Objectives

To further characterize risk factors for bilateral AOM and to assess the propensity for specific viral and bacterial pathogens to predispose to bilateral versus unilateral AOM.

Methods

We performed a secondary data analysis on 1216 cases of AOM diagnosed and treated at our institution: 566 subjects underwent tympanocentesis and middle ear fluid (MEF) culture. We compared subjects with bilateral and unilateral AOM for demographic characteristics, clinical findings, parent/clinician perception of AOM severity, and MEF study results for bacteria and viruses.

Results

When compared with children who have unilateral AOM, children with bilateral AOM were more likely to be younger (P<0.001), have *Haemophilus influenzae* isolated from one or both middle ear fluids (P<0.0001), and have more severe inflammation of the tympanic membrane (TM) on otoscopic examination (P<0.0001).

Conclusion

Compared with children who have unilateral AOM, children with bilateral AOM are more likely to have bacteria in the MEF and have more severe inflammation of the TM. This may help explain why children with bilateral AOM are more likely to experience persistent symptoms without antibiotic treatment. Laterality of AOM should be considered when discussing treatment options with parents.

Acute otitis media: the incidence of hospital admissions, surgery and complications 1999-2005

Kari Kvaerner, M.D., Ph.D.

Background

It has been suggested that a decline in the use of antibiotics for young children affects the admission rates to hospital for complications. Uncomplicated acute otitis media is treated without antibiotics, in all Norwegian children aged one year and above.

Objective

To assess the incidence of hospital admissions for childhood acute otitis media by age and gender, surgery and disease complications.

Methods

Descriptive study of hospital admissions for acute otitis media in children aged 0 through 7, using national treatment data 1999 through 2005.

Results

The incidence of acute otitis media decreased from 85 to 74 per 100,000 children during the study period, and was more common in boys. Mean age for hospitalization decreased from 2.4 to 1.7 years in the same period of time. The incidence of acute mastoiditis media remained stable, approximately 6 per 100,000. The distribution of age, gender, surgical treatment and disease complications will be presented.

Conclusion

While hospitalization for acute otitis media occurs at a lower age and less frequently, the incidence of acute mastoiditis and cortical mastoidectomy remains stable.

Fine mapping of two loci influencing chronic otitis media with effusion and/or recurrent otitis media (COME/ROM)

Michele Sale, Ph.D., Peter Perlegas, B.S., Miranda Marion, M.A., Pamela Hicks, B.S., Stephen Rich, Ph.D., Kathleen Daly, Ph.D.

Background

We previously conducted a genome-wide linkage scan using 591 subjects that included 238 affected and informative relative pairs with chronic otitis media with effusion and/or recurrent otitis media (COME/ROM). Multipoint nonparametric linkage analyses exhibited greatest evidence for linkage on chromosome 19q (LOD=2.56, P=0.0006) at 98.8 cM near marker D19S254. The second highest linkage peak was located on chromosome 10q (LOD=1.80, P=0.004) at 168.3 cM near marker D10S212.

Objective

Our aim was to fine map the two linkage peaks on chromosomes 19 and 10.

Methods

The original 10 microsatellite markers on chromosome 19 were supplemented with 3 microsatellite markers and 12 single nucleotide polymorphisms (SNPs) in the region of interest. On chromosome 10, one additional microsatellite marker and 16 SNPs were added to the 19 original microsatellite markers.

Results

Multipoint linkage analysis of the fine-mapping data revealed two linkage peaks on chromosome 19: one at 109.8 cM (LOD=3.85; P=3.0×10^{-5}, near rs260462) and one at 107.3 cM (LOD=3.70; P=4.0×10^{-5}, near rs1542039). The support for linkage on chromosome 10 increased to LOD=2.20 at 169.8 cM (P=0.0015), and remained closest to D10S212.Some potential positional candidates under the chromosome 19q region of linkage include: Killer Cell Ig-like receptor (KIR) genes, and Ig-like transcripts (ILT), while the 10q region contains the A Disintegrin and Metalloproteinase Domain 8 (ADAM8) gene.

Conclusion

This study suggests these regions of 19q and 10q contain genes contributing to COME/ROM susceptibility.

Influence of viruses, host genetics, and environment on nasopharyngeal cytokines during upper respiratory infection

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Introduction

About 10-50% of young children with viral upper respiratory infection (URI) develop acute otitis media (AOM). Despite the well-known role of viruses in causation of AOM, the mechanisms of differential risks of AOM with different viruses and specific hosts remain poorly defined.

During viral URI, several cytokines and other inflammatory molecules are found in the nasal secretions of children, suggesting that these cytokines participate in regulation of virus-induced inflammation and/or recovery from it. IL-1β, IL-6, and TNF-α are found in significant quantities during viral URI[1]; however, the relationship between the presence of these cytokines in the nasopharynx with respect to pathogenesis of AOM has previously not been reported. Recently, we reported that specific high-cytokine–inducing single nucleotide gene polymorphisms of IL-6 and TNF-α are found more often in children who are prone to recurrent otitis media.[2] High levels of cytokines in nasal secretions during viral URI may be associated with risk for complications such as AOM, and host genetic background as well as environmental factors such as breastfeeding and passive exposure to cigarette smoke may have a relationship with cytokine levels in the nasopharynx.

In the present study, we performed quantitative measurements of IL-1β, IL-6, and TNF-α in the nasopharyngeal secretions (NPS) during URI episodes, and correlated them with the virus etiology, respective cytokine gene polymorphisms, other host factors, and the rate of AOM development.

Materials and methods

Clinical evaluation. This was a sub-cohort study of virus-positive URI episodes in children who were enrolled in a prospective, longitudinal study designed to capture all URI episodes that occurred during a one-year follow-up period to study the incidence and characteristics of AOM complicating URI in children. The study was performed at the University of Texas Medical Branch (UTMB) at Galveston and was approved by the UTMB Institutional Review Board.

Children were enrolled at 6 months to 3 years of age; demographic and AOM risk factors such as breastfeeding during infancy and ongoing exposure to cigarette smoke exposure were recorded at enrollment. Each child was followed for 1year for occurrences of URI and AOM. Children were seen by a study physician as soon as possible after the onset of symptoms and a few days later (days 3-7 in the course) for complications. Parents were advised to bring the child for examination whenever they suspected the child to have any symptom of AOM.

AOM complicating URI was considered when the episode occurred within 28 days of the onset of URI unless a new URI occurred within this period; in that case, AOM was considered as a complication of the most recent URI episode. Children diagnosed with AOM were observed or given antibiotic therapy consistent with standards of care.

Sample collection. A buccal swab was obtained at enrollment in the study for DNA analysis for cytokine gene polymorphisms. Nasal swabs and NPS from both nostrils were collected from children at the initial visit for each URI episode, and at subsequent visit only when AOM was diagnosed or if URI was persistent at follow-up visits. After NPS suction, additional 1 ml of sterile saline was aspirated through the suction tubing to collect the residual amount coated on the inner parts of the tubing. Nasal swabs and NPS samples were used for virologic studies. Aliquots of NPS were stored at -70^0 C until used for quantitative cytokine analysis.

Virologic studies. The nasal swab was processed for viral culture (performed in the Clinical Virology Laboratory at UTMB) while NPS was studied for respiratory syncytial virus (RSV) antigen detection by enzyme immunoassay (EIA) (performed at UTMB only during RSV season).

Quantitative cytokine analysis and gene polymorphism study. NPS samples from the initial visit for all virus-positive URI episodes were further analyzed to measure concentrations of IL-1β, IL-6, and

TNF-α by using multiplex EIA (BioSource kit,® Invitrogen Corp, Carlsbad, CA) on Luminex™ 100 platform (Luminex Corp, Austin, TX). An internal range of standards was used to establish the standard curve and to define the upper and lower detection limits of cytokine concentrations at each run. The lowest detection limit of the assay was <10 pg/ml for IL-6 and TNF-α, and <5 pg/ml for IL-1. All samples that were above the upper range of calculation were further diluted until within the range of assay. All NPS samples were processed in duplicate, and the average value of cytokine was used for analysis.

DNA from the buccal smear was studied for single nucleotide polymorphisms (SNP) for the high cytokine-producing genotype (TNF-α $^{-308}$, IL-1ββ^{+3953}, and IL- 6^{-174}) by restriction fragment length polymorphism assay as described previously from our laboratory.[2]

Results

Study population. Between January 2003 and March 2006, 158 episodes of URI in 119 children were documented to be due to a single virus. Episodes with multiple viruses were excluded in order to study of association of a specific virus on cytokine production and AOM development.

Cytokine concentrations vs. demographic and clinical factors. The levels of IL-1β, IL-6, and TNF-α correlated positively with each other (P<0.01). IL-6 levels were significantly higher in younger children (P= 0.01) and among those who had longer duration of fever (P<0.01). Ordinary least square regression analysis showed that after controlling for virus type, gender, age, and breastfeeding, cigarette smoke exposure was significantly associated with higher levels of IL-1ββ and TNF-α (P = 0.03 and 0.04, respectively). Similarly, while controlling for gender, age, breastfeeding, and cigarette smoke exposure, virus type was significantly associated with higher levels of IL-6 (P = <0.01). History of breastfeeding did not influence the levels of any of the cytokines; however, it provided significant protection against development of AOM.

Cytokine concentrations vs. virus type. Table 1 shows the concentrations of all three cytokines by virus type. Rhinovirus was the most common virus identified as a cause of URI (23%), while enterovirus was the least common (11%). Adenovirus and influenza virus were associated with the highest concentrations of IL-6 in NPS, although only adenovirus was significantly different as compared to parainfluenza, rhinovirus, RSV, and enterovirus.

Cytokine concentrations vs. gene polymorphism and development of AOM. 52 (33%) of URI episodes were associated with the development of AOM. Overall, the cytokine levels were not associated with the rate of AOM development. Figure 1 shows the levels of cytokines per genotype information. As the high-cytokine–producing homozygous polymorphisms were rare, both heterozygous and homozygous polymorphic gentoypes were combined and then compared with the homozygous normal genotypes. Only IL- 6^{-174} genotype was associated with higher levels of IL-6 in NPS (Fig. 1).

Discussion

Cytokines function in a complex network in which production of one cytokine will influence the production of several others. Several cytokines may have a similar action, and cytokines are rarely produced or act alone. Cytokine levels in NPS may directly or indirectly influence the pathophysiologic changes in the nasopharynx that are necessary for the development of AOM.

While the acute phase cytokines IL-1ββ, IL-6, and TNF-αα have previously been detected in the NPS during URI due to a variety of viruses, the relationship of these cytokines in NPS with the risk of developing AOM has not been reported previously. In this regard, we show that of all viruses studied, only adenovirus virus induces significantly higher quantities of IL-6. In this relatively modest study, we do not show an overall association between cytokine levels and AOM risk. However, we believe that to show such an association, a much larger study size will be needed.

Overall, all of the viruses induced significant quantities of IL-1ββ, IL-6, and TNF-αα which correlated with each other, suggesting their important role in the pathogenesis of acute URI symptoms. We also show that IL-6 levels are correlated with the duration of fever prior to NSP collection. Previous studies have also shown a relationship between IL-6 and URI symptoms. For example, in influenza virus infection, duration of virus shedding is associated with levels of IL-6 in nasal secretions and the levels of IL-6 are associated with increased local and systemic symptoms, including fever.[3]

Our study also shows that in addition to specific viruses, cytokine genotype and exposure to cigarette smoke influenced the levels of cytokines in NPS. Specifically, IL- 6^{-174} genotype was associated with higher levels of IL-6 in NPS during viral URI; this is a unique observation, as no prior study has explored the influence of cytokine polymorphic

genotypes on cytokine production during any type of viral infection. The influence of cigarette smoke exposure on cytokine production has been explored previously--we have shown that exposure to cigarette smoke increases the susceptibility for OM in children who have TNF-$\alpha\alpha^{-308}$ polymorphism.[2] Cigarette smoke regulates the production of TNF-$\alpha\alpha$ through activation of NF-$\kappa\kappa$B transcriptional factors in both airway epithelial cells and macrophages.[4,5]

In summary, our study highlights the role of respiratory viruses in the induction of proinflammatory cytokines in relation to specific viruses. The cytokines may be influenced by host factors such as cytokine gene polymorphisms, as well as by environmental factors such as exposure to cigarette smoke. Additional studies are needed to investigate the mechanism by which the cytokine network influences the development of AOM following viral URI.

Financial support

Table 1. Cytokine levels in NPS by virus type in 158 virus-positive episodes of URI

Virus type	N (%)[a]	Mean pg/ml ($\pm$ SD)		
		IL-1β	IL-6	TNF-α
Rhinovirus	37 (23%)	1412 $\pm$ 2069	2214 $\pm$ 3362	1619 $\pm$ 2885
RSV	29 (18%)	2017$\pm$ 2686	2950 $\pm$ 3346	2193 $\pm$ 3832
Adenovirus	28 (18%)	1978 $\pm$ 2255	14404 $\pm$ 18502[b]	1111 $\pm$ 958
Parainfluenza virus	25 (16%)	1155 $\pm$ 2130	4309 $\pm$ 4348	1036 $\pm$ 1700
Influenza virus	22 (14%)	1520 $\pm$ 1684	10215 $\pm$ 14552	1773 $\pm$ 2978
Enterovirus	17 (11%)	1621 $\pm$ 2406	4087 $\pm$ 12210	955 $\pm$ 1099

[a] Figures in parenthesis represent percent of column total

[b] Adenovirus > parainfluenza, rhinovirus, RSV or enterovirus, P = 0.05 (calculated by Tukey's post-hoc test for pairwise comparisons)

Figure 1. Cytokine concentrations as per respective cytokine polymorphic genotype.
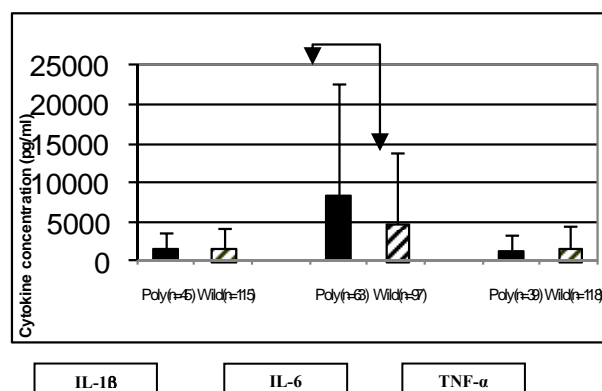
Poly = hetero or homozygous polymorphic genotype
Wild = normal genotype
The bars show mean +/- SD values of cytokines

References

1. Noah TL, Henderson FW, Wortman IA, et al. Nasal cytokine production in viral acute upper respiratory infection of childhood. J Infect Dis 1995; 171:584-592.

2. Patel J, Nair S, Revai K, et al. Association of proinflammatory cytokine gene polymorphisms with susceptibility to otitis media. Pediatrics 2006; 118:1-7.

3. Hayden F, Fritz RS, Lobo MC, et al. Local and systemic cytokine responses during experimental human influenza A virus infection. J Clin Invest 1998; 101:643-649.

4. Yang SR, Chida AS, Bauter MR, et al. Cigarette smoke induces proinflammatory cytokine release by activation of NF-kappaB and posttranslational modifications of histone deacetylase in macrophages. Am J Physiol Lung Cell Mol Physiol 2006; 291:L46-57.

5. Rahman I, Gilmour PS, Jimenez LA, MacNee W. Oxidative stress and TNF-alpha induce histone acetylation and NF-kappaB/AP-1 activation in alveolar epithelial cells: potential mechanism in gene transcription in lung inflammation. Mol Cell Biochem 2002; 234-235:239-248.

Mucin gene polymorphisms in otitis media patients

Matthew Ubell, M.D., Joseph Kerschner, M.D., Amy Burrows, B.S.

Objective

Mucin gene 2 (MUC2), MUC5B, and MUC5AC have been identified as major gel-forming mucins in the middle ear (ME). This study compared polymorphisms in MUC2, MUC5B, and MUC5AC genes in otitis media (OM) patients and controls.

Design

Case-controlled study.

Subjects

Patients age 6 months to 14 years undergoing routine ventilation tube insertion (VTI) for recurrent otitis media (RecOM) or otitis media with effusion (OME) were compared to control patients with no history of OM undergoing an unrelated procedure.

Methods

While under anesthesia, 5cc of blood was obtained from participants. DNA was extracted for analysis. Southern blots with gene-specific probes were used to determine sizes of MUC2 and MUC5AC genes.PCR was used to determine the size of MUC5B genes.

Study group

Children undergoing tympanostomy tube placement for chronic otitis media with effusion (COME) or recurrent otitis media (RecOM).

Control Group

Children matched by age, race and gender to the Study Group but without a history of otitis media.

Definitions

1) COME: Any patient with persistent middle ear effusion for greater than three months duration.
2) RecOM: Any patient with 3 or more episodes of acute OM over a 6 month period with resolution between episodes and middle ear fluid that does not persist greater than 3 months duration.
3) Absence of history of otitis media (Control Group): One or fewer episodes of otitis media requiring a visit to a health professional per 12 month period of life.

Results

Sixteen RecOM patients, 21 OME patients, and 40 control patients were analyzed. There were no statistically significant differences in polymorphism expression identified between groups for MUC2 and MUC5B mucin genes. OME patients were more likely than Controls or RecOM to carry the longer MUC5AC-b allele (all $p < 0.05$).

Conclusions

Differences in mucin polymorphisms have been demonstrated in other diseases. In otitis media, OME patients are more likely than Controls or RecOM to carry the longer MUC5AC-b allele. MUC5AC is a primary innate defense mechanism for ME epithelium and alterations in protein structure have the potential to affect that defense and predispose patients to disease. This study demonstrates that the properties of post-transcriptionally modified MUC5AC deserve further study and specific pathogen-host interactions studies should explore the impact of this polymorphism.

Pathogenesis

Impact of evolution on eustachian tube function

Charles Bluestone, M.D.

Abstract

I posit that humans appear to be the only species that develops otitis media. If animals in the wild had developed middle-ear disease to any significant degree, they would have been selected out during evolution, since they would not have survived their predators due to the associated hearing loss. Why do humans have otitis media? Evolution has had a significant impact. It is well known that humans are born 12 months too early, which is due to adaptations to bipedalism and our big brain that, over time, resulted in a relatively small female pelvic outlet compared to nonhuman primates. As a consequence of too early a birth, not only is our immune system immature, but the eustachian tube is too short and floppy in the first year of life.

But why is otitis media still common in older individuals? What other adaptation is uniquely human? We developed speech that was associated with descent of the larynx and hyoid bone, which, along with decrease in prognathism (i.e., facial flattening) resulted in a change in palatal morphology, as compared with other primates. Comparative anatomic and physiologic studies have demonstrated significant differences between humans and monkeys, especially in the muscles of the eustachian tube. Paradoxical constriction, as apposed to dilation, upon swallowing is a common tubal dysfunction in humans and certain monkey models with chronic middle-ear effusion. My hypothesis is that chronic otitis media with effusion in patients with tubal constriction is a consequence of adaptation for speech, and that most likely the levator veli palatini muscle is the cause.

Tympanometric findings in young children during upper respiratory tract infection with and without acute otitis media

Krystal Revai, M.D., M.P.H., Janak Patel, M.D., James Grady, Dr.P.H., Tasnee Chonmaitree, M.D.

Introduction

Acute Otitis Media (AOM) is one of the most common pediatric infectious diseases. It mostly affects young children and generally occurs as a complication of upper respiratory tract infection (URI).[1,2] The incidence of AOM peeks between 6 and 24 months of age. Children 6 to 12 months of age are at highest risk for AOM.[3,4] It is believed that URI leads to the disease by causing congestion of the nasopharynx and Eustachian tube leading to Eustachian tube dysfunction (ETD) or obstruction.[5]

Tympanometry is a useful tool for evaluation of the middle ear effusion (MEE) status[6] and has been recommended by the AAP as an adjunct to determine the presence of MEE in the child with suspected AOM and OME.[7,8] Tympanometry during URI has predominantly been studied in school children and those older than 2 to 3 years age.[9-12] We sought to compare tympanometric findings in young children (6 to 47 months of age) during URI with and without complicating AOM. Our hypothesis was that younger children are more likely to experience changes in the middle ear status during a URI compared to older children and this mechanism leads to higher AOM incidence.

Methods

This study is a secondary analysis of data collected at the University of Texas Medical Branch, Galveston (UTMB) during a prospective, longitudinal study of virus-induced AOM (Chonmaitree-unpublished data). Healthy children 6 to 35 months-old were enrolled in a study designed to capture all AOM following URI during a one year follow-up period. At enrollment, demographic and AOM risk factor information was collected and baseline tympanograms were taken. Parents were asked to notify the study office as soon as the child began to have a cold or URI symptoms (nasal congestion, rhinorrhea, cough, sore throat, or fever). Children were seen by a study physician as soon as possible after the onset of URI symptoms, and again a few days later (days 3 through 7 of the URI). Each

URI episode was monitored closely for at least 3 weeks for AOM development. This study includes data collected from the beginning of the study in January 2003 through November 2006.

During the first week of each URI episode, children had two visits for otoscopic examination. Tympanometry was performed using a Microaudiometrics Ear Scan II portable tympanometer (Micro Audiometrics Corp., Port Orange, FL). Home visits were performed weekly until the tympanogram became normal or the next URI episode. Right and left ear tympanograms were analyzed separately. Tympanograms were classified in accordance to the guidelines described by Finitzo et al.[12] AOM complicating URI was considered when AOM occurred within 21 days of the URI. AOM was defined by acute onset of symptoms (fever, irritability, or earache), signs of inflammation of the tympanic membrane and presence of fluid in the middle ear documented by pneumatic otoscopy and/or tympanometry. Children diagnosed with AOM were observed or given antibiotic therapy consistent with standard of care.[7]

Chi-square analysis was performed using STATA 9.0 © (Stata Corporation, College Station, TX) statistical software.

Results

This study included 204 patients who were enrolled in the study and had been seen at least once by the study group. Of these, 52% were male, 58% were Caucasian, 31% were African-American, 2% were Asian and 9% were mixed race. Forty-four percent were of Hispanic ethnicity. Twenty-nine percent of the children attended day care, 29% were exposed to cigarette smoke and 44% were breastfed for at least 2 weeks. The mean and median age at URI onset was 18 months.

During the study period, there were a total of 741 URI episodes and 2359 study visits. The total number of tympanometry performed on right and left ears was 4718. There were 279 cases of AOM; in other words, 38% of the URIs were complicated by AOM. Tympanogram types found in the first week of URI by age are shown in Table 1. Overall children from 6 to 11 months of age were more likely to have type B

tympanogram or MEE.

To avoid the possibility that the results shown in Table 1 could be skewed by the ears with persistent MEE prior to the URI episode, we sought to analyze a subgroup of children who had a documented normal tympanogram prior to the URI (Table 2). In this sub-group, 64% had type A tympanogram during the first week of URI. Among children with an abnormal tympanogram, the younger children tended to have a type B tympanogram while the older children had a type C tympanogram (p<0.001). The proportion of normal (type A) tympanograms by day of URI is shown in Figure 1. The change in normal tympanograms to abnormal (type B or C) was most pronounced on day 2 (p=0.09). Overall, although abnormal tympanograms occurred more often in the young age group the difference did not reach statistical significance.

Discussion

Young children (6 to 11 months) have the highest burden of disease in regards to AOM complicating URI. Our finding that abnormal middle ear status, including MEP and MEE occurred more frequently in young children may help explain the pathogenic mechanism. The proportion of children with a normal tympanogram decreased by 32 % from day 1 to 2, suggesting that abnormal MEP likely occurs from day 2 of URI. Interestingly, we found that approximately one-third of children 24 to 47 months of age have type C tympanograms during URI.

ETD is likely a key step in the pathogenesis of AOM following URI. The Eustachian tube functions include: middle ear pressure regulation, protection of the middle ear from sounds and secretions, and clearance of secretions which either enter from the nasopharynx or the middle ear. Inflammation in the nasopharynx can cause anatomic obstruction of the Eustachian tube, potentially disrupting any of its protective functions. Young children are at particular risk of ETD because their Eustachian tube is relatively shorter, straighter and more compliant that that of an older child or adult. It is believed that these anatomical differences in the Eustachian tube predispose young children to progress from ETD and abnormal MEP to AOM.[5]

A type B tympanogram (MEE) represents more advanced ETD and middle ear pathology than a type C tympanogram (negative middle ear pressure). Effusion formation occurs as a result of the following sequence of events: ETD, negative middle ear pressure, influx of infectious microorganisms into the middle ear, secretion of inflammatory mediators, increased vascular permeability and stimulation of epithelial secretory activity.[13] We found that during URI, young children (6 to 11 months of age) were likely to have more Type B tympanograms, while older children (24- to 47 months) tended to have more Type C tympanograms. These findings are consistent with those shown by Smith et al[14] who reported in children with URI a relationship between increasing age and increasing negative MEF, especially in children with MEE documented by pneumatic otoscopy. We speculate that older children have a higher prevalence of type C tympanograms because compared to younger children, their Eustachian tube is less compliant and less likely to become completely obstructed for a prolonged period of time. This may allow for intermittent drainage of the middle ear contents reducing the likelihood of AOM development.

In summary, we have shown that more severe ETD is associated with URI in younger children, who are at higher risk of AOM. The peak day of abnormal tympanogram coincides with the peak day of AOM diagnosis. These findings may add to the further understanding of role of the Eustachian tube function in AOM development in children. Because the Eustachian tube function in young children is the most vulnerable making them more susceptible to AOM following URI, efforts should be made to prevent URI in young children. Decreasing the frequency of exposure to respiratory viruses (e.g. avoidance of group child care), and the use of timely viral vaccines may be important steps in the prevention of AOM in children.

Acknowledgement

Table 1: Tympanogram Types in the 1st Week of URI by Age, Whole Cohort [a]

Age Group	Type A [b]	Type B [c]	Type C [c]	Total (n=2094)
6-11 mos.	158 (39)	178 (44)	67 (17)	403
12-23 mos.	467 (46)	322 (31)	241 (23)	1030
24-27 mos.	325 (49)	94 (14)	242 (37)	661

[a] Total number of patients = 204, Total URI episodes = 741 including 275 AOM (unilateral and bilateral) following URI; tympanograms were obtained during the first week of URI including episodes with AOM
[b] Number (row %)
[c] p<0.001 by Cochran-Armitage Trend Test

Table 2: Tympanogram types in the 1st Week of URI by Age, cases with documented normal TYM prior to the URI episode [a]

Age Group	Type A	Type B [b]	Type C [b]	Total (n=1031)
6-11 mos	87 (62)	26 (19)	27 (19)	140
12-23 mos	327 (65)	56 (11)	119 (24)	502
≥ 24 mos	241 (62)	21 (5)	127 (33)	389

[a] Total Patients= 123, Total URI Episodes= 322, AOM= 74
[b] p<0.001 by Cochran-Armitage Trend Test

References

1. Henderson, FW, Collier, AM, Sanyal, MA, Watkins, JM, Fairclough, DL, Clyde, WA, Jr., Denny, FW: A longitudinal study of respiratory viruses and bacteria in the etiology of acute otitis media with effusion. N Engl J Med 1982;306:1377-1383.
2. Teele, DW, Klein, JO, Rosner, B: Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study. J Infect Dis 1989;160:83-94.
3. Revai, K, Dobbs, LA, Nair, S, Patel, JA, Grady, JJ, Chonmaitree, T: Incidence of acute otitis media and sinusitis complicating upper respiratory tract infection: the effect of age. Pediatrics 2007;119:e1408-e1412.
4. Harrison, LM, Morris, JA, Telford, DR, Brown, SM, Jones, K: The nasopharyngeal bacterial flora in infancy: effects of age, gender, season, viral upper respiratory tract infection and sleeping position. FEMS Immunol Med Microbiol1999;25:19-28.
5. Bluestone CD, Klein JO: Physiology, pathophysiology and pathogenesis. In Otitis Media in Infants and Children. Bluestone CD, Klein JO, Eds. Hamilton, Ontario, BC Decker, Inc, 2007:41-72.
6. Ruuskanen O, Rouhola A: Diagnostic Methods. In Advanced Therapy of Otitis Media. Alper CM, Bluestone CD, Casselbrant ML, Dohar JE, Mandel EM, Eds. Hamilton, Ontario, BC Decker Inc, 2004:9-13.
7. Subcommittee on Management of Acute Otitis Media: Diagnosis and management of acute otitis media. Pediatrics 2004;113:1451-1465.
8. American Academy of Family Physicians, American Academy of Otolaryngology-Head and Neck Surgery, American Academy of Pediatrics Subcommittee on Otitis Media With Effusion: Otitis Media With Effusion. Pediatrics 2004;113:1412-1429.
9. Moody, SA, Alper, CM, Doyle, WJ: Daily tympanometry in children during the cold season: association of otitis media with upper respiratory tract infections. Int J Pediatr Otorhinolaryngol 1998;45:143-150.
10. Bylander, A: Upper respiratory tract infection and eustachian tube function in children. Acta Otolaryngol 1984;97:343-349.
11. Winther, B, Hayden, FG, Arruda, E, Dutkowski, R, Ward, P, Hendley, JO: Viral respiratory infection in schoolchildren: effects on middle ear pressure. Pediatrics 2002;109:826-832.
12. Finitzo, T, Friel-Patti, S, Chinn, K, Brown, O: Tympanometry and otoscopy prior to myringotomy: issues in diagnosis of otitis media. Int J Pediatr Otorhinolaryngol 1992;24:101-110.
13. Jung, TT: Prostaglandins, leukotrienes, and other arachidonic acid metabolites in the pathogenesis of otitis media. Laryngoscope 1988;98:980-993.
14. Smith, CG, Paradise, JL, Sabo, DL, Rockette, HE, Kurs-Lasky, M, Bernard, BS, Colborn, DK: Tympanometric findings and the probability of middle-ear effusion in 3686 infants and young children. Pediatrics 2006;118:1-13.

Experimental evidence that the composition of the nasopharyngeal bacterial flora affects the incidence of otitis media

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Objective

This study aimed to investigate whether the composition of the bacteria present in the nasopharynx affected both the incidence rate of otitis media and which bacteria appeared to be the causative agent.

Method

Mice were nasally inoculated with different combinations of *Streptococcus pneumoniae*, *Moraxella catarrhalis,* and/or nontypeable *Haemophilus influenzae*. These groups were also divided according to whether they had been infected 3 days pre-bacterial inoculation with a respiratory virus (rodent Sendai virus). Mice from each group were euthanized at intervals over a 14-day period for middle ear (MEL), nasal (NL), and bronchoalveolar (BAL) lavages.

Results

As predicted and previously demonstrated, pre-viral infection increased the incidence of middle-ear infection. Combining bacteria (with or without viral infection) also changed the incidence rate of infection. The concurrent presence of *M. catarrhalis* significantly increased both the incidence rate and severity of infection caused by *S. pneumoniae* and, to a lesser extent, NTHi. NTHi did not have the same effect as *M. catarrhalis* on *S. pneumoniae* infection. Nitric oxide assay indicated that even without detectable bacterial recovery, nasal inoculation resulted in inflammatory responses in the middle ear.

Conclusion

The presence of a second bacterium increases the incidence and severity of otitis media due to *S. pneumoniae* with *M. catarrhalis* > NTHi in affecting *S. pneumoniae* infection. In a nasal polymicrobial environment, the resulting causative agent of otitis media was *S. pneumoniae* > NTHi > *M. catarrhalis.*

Streptococcus pneumoniae desialylates and degrades the antimicrobial peptide sPLUNC-1 as a potential mechanism for immune evasion

Glen McGillivary, Ph.D., Samantha J. King, Ph.D., Robert S. Munson Jr., Ph.D., Lauren O. Bakaletz, Ph.D.

Antimicrobial peptides (APs) are expressed at mucosal surfaces and are key components of the host innate immune system. Herein, we provide the first description of a chinchilla homologue of the human short palate, lung, and nasal epithelium clone (csPLUNC-1). We detected csPLUNC-1 mRNA and protein in each URT tissue evaluated in the chinchilla, but not in non-airway samples, underscoring the relevance of this AP to the tubotympanum. Importantly, we detected the human homolog of csPLUNC-1 in pediatric middle-ear effusions, thus demonstrating that this AP was present in the human middle ear. Purified chinchilla and human sPLUNC-1 killed clinical isolates of nontypeable *Haemophilus influenzae* (NTHi), *Moraxella catarrhalis*, and *Streptococcus pneumoniae,* which suggested that sPLUNC-1 was an important component in defense of the airway.

We identified several isoforms of sPLUNC-1, which contained sialic acid as a post-translational modification. Purified bacterial neuraminidases removed this sugar from csPLUNC-1. When using conditions which abrogated the ability of csPLUNC-1 to kill microorganisms, *S. pneumoniae,* but not NTHi or *M. catarrhalis,* desialylated csPLUNC-1. Additionally, *S. pneumoniae* further degraded csPLUNC-1 using a bacterial cell-associated protease, a result not observed with NTHi or *M. catarrhalis*. Collectively, our data showed that the gram-positive pneumococcus deglycosylated and degraded csPLUNC-1 and suggested that this causative agent of otitis media (OM) could modulate the structure of other sialylated APs as well. We hypothesize that impairment of innate immune system function(s) via the mechanisms presented here, contributes to the disease progression of bacterial OM.

Critical role of LPS sialylation in pathogenesis of experimental otitis media

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We previously showed that complement is key in combating nontypeable *Haemophilus influenza* (NTHi) middle-ear infection in the chinchilla experimental otitis media (EOM) model; depleting C3 with cobra venom factor rendered otherwise avirulent and serum-sensitive NTHi siaB mutants (unable to sialylate lipopolysacharide (LPS)) rendered fully pathogenic. We examined the role of seven LPS biosynthesis genes (lic1, lic2A, lgtC, lpt6, lpsA, hmg, and opsX) on pathogenicity and the ability to resist killing by complement.

Material and methods

Using the chinchilla EOM model, we inoculated 50 cfu of WT 375 or respective isogenic mutants into the superior bullae. Killing by normal human serum (NHS), guinea pig serum (GPS), and complement C3 and C4 binding were performed.

Results

Despite being susceptible to NHS, lic1, lpt6, and lgtC mutants (all with sialylated LPS) retained comparable virulence to WT 375. However, lpt6 and lgtC resisted GPS, consistent with their virulence in chinchillas and demonstrated differences in killing by NHS and GPS. Lic1 (lacking phosphocholine, but sialylated) was killed by NHS and GPS, yet caused EOM. LpsA, lic2A, and opsX (all with unsialylated LPS) were sensitive to NHS and GPS and were attenuated in pathogenicity. The LPS of lpsA and lic1 mutants bound high amounts of C4 and C3, respectively.

Conclusions

Collectively, these data suggest that resistance to complement may not be an absolute requirement for virulence in the chinchilla EOM model. These findings underscore the critical importance of LPS sialylation in NTHi pathogenesis and suggest a role beyond resistance to complement mediated bacteriolysis.

The effects of gastroesophageal reflux on eustachian tube function, the development of otitis media, and the resolution of an induced episode of acute otitis media

David Chi, M.D., Cuneyt M. Alper, M.D., Sancak Yuksel, M.D., J. Douglas Swarts, Ph.D.

Background

The pathogenesis of otitis media (OM) is multifactorial. The etiology of persistent OM has been attributed to dysfunction of the eustachian tube (ET) via the disruption of its middle-ear (ME) pressure regulation, clearance, and protection mechanisms. Conditions that lead to edema and nasopharyngeal (NP) congestion, such as gastroesophageal reflux disease (GERD), are thought to cause functional ET obstruction leading to otitis media with effusion (OME). The chronic inflammation induced by GERD may also prevent the resolution of an acute otitis media (AOM) episode by disabling the clearance mechanisms of the ET.

Objective

To investigate the effects of GERD on ET function, the development of OM, and the resolution of AOM in the rat animal model.

Study design

Randomized controlled trial.

Subjects and methods

Twenty-four Sprague-Dawley rats with transcutaneous catheters were randomized to two groups treated with water or pepsin/hydrochloric acid solution, a surrogate for GER. Each group was infused three times a day for 17 days. After the first 7 days of treatment, the animals were inoculated bilaterally with *Streptococcus pneumoniae*. Middle-ear status was evaluated every other day for 10 days. Eustachian tube function was assessed with the forced response (FRT) and inflation-deflation (IDT) tests prior to: catheter implantation, the initiation of treatment, ME infection, and sacrifice.

Results

Treatment with the pepsin/HCl solution increased passive resistance (FRT) and residual negative pressure (IDT). No ME effusions developed in either group due to NP treatment. Otoscopic resolution of the AOM episode did not differ with respect to the experimental treatment. With respect to ET function, AOM reduced opening and closing pressures and passive resistance, but did not change active resistance or dilatory efficiency on the FRT. Residual pressures on the IDT were larger following AOM.

Conclusions

While GERD does induce ET dysfunction, this response is insufficient to cause OME or prolong an episode of AOM.

Acknowledgements

Negative pressure-induced secretion of inflammatory mediators by cultured middle-ear epithelial cells: Relevance to eustachian tube dysfunction and otitis media with effusion

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Background

Hydrops ex vacuo is a valid explanation for the development and persistence of otitis media with effusion (OME) under certain conditions.[1-4] Disrupting eustachian tube (ET) function in animals induces middle-ear (ME) pressure dysregulation reflected as underpressures, causing increased vasculature permeability and ME effusion. Our research group and others have reported ET dysfunction in children at risk for the development of OME, with concurrent OME or with a history of OME. Clinical studies of children with OME report improvement in mucosal inflammation and resolution of effusion after insertion of tympanostomy tubes (TTs), which bypass the ET to maintain stable ME pressures, and other methods of introducing air into the ME have been reported to aid disease resolution.[5] Animal studies also show that disrupting the normal function of the ET causes ME pressure dysregulation and ME underpressures, which in turn provoke changes in permeability of the mucosal vasculature and, at a critical level of approximately -200 mm H2O, effusion within the ME containing pro-inflammatory mediators.[6-8] However, the mechanism responsible for transducing this biological signal (ME underpressure) and thereby provoking ME increased vascular permeability and mucosal inflammation is not known and has not been studied.

Objective

The objective of this in vitro study is to determine whether ME epithelial cells respond to changes in ambient pressure, specifically to negative pressure, and release inflammatory mediators thereby contributing to the pathogenesis of OME.

Methods

Human ME epithelial cell cultures, hMEEC-1, a generous gift from the House Institute, Los Angeles, CA, were cultured in a 1:1 mixture of DMEM (Invitrogen) and bronchial epithelial basal medium supplemented with 52μg/ml bovine pituitary extract, 0.5μg/ml hydrocortisone, 0.5ng/ml hEGF, 0.5μg/ml epinephrine, 10μg/ml transferring, 5μg/ml insulin, 6.5ng/ml triiodothyronine, 0.1ng/ml retinoic acid, 50μg/ml gentamycin, and 50ng/ml amphotericin-B (Lonza), in 5% CO2 at 37-degC and atmospheric pressure.[9] They were grown to confluence in upper chambers of transwell plates, then brought to the air/liquid interface for 24 hours. Wells were exposed to individual treatments for an additional 24 hours. The negative control was atmospheric pressure, positive control was interleukin(IL)-1β (1ng/ml), a pro-inflammatory cytokine, at atmospheric pressure. The experimental treatment was negative (sub-atmospheric) pressure of -250daPa (decaPascal = mm H2O, measured with a manometer), achieved using a tissue culture incubator with computer-controlled gas and pressure regulation.

Secretions were collected individually from basal and apical sides of epithelial cultures (as shown in Figure 1) and analyzed using a Bio-Rad/Bioplex Human 27-Plex Multi-Cytokine Detection System to detect the following cytokines, chemokines, angiogenic factors, growth factors, and colony stimulating factors: IL-1β, IL-1 receptor antagonist (RA), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, basic FGF, Eotaxin, G-CSF, GM-CSF, IFNγ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNFα, and VEGF. Comparison was made of the effect of negative pressure alone with that of the positive control pro-inflammatory cytokine, IL-1β.

Results

A subset of the mediators in the multiplex assay were found to be significantly increased by ME epithelial cells exposed to cytokine or low pressure stimulation, as shown in Figure 2. Negative pressure alone induced secretion of pro-inflammatory macrophage chemokines, IL-8, IL-12 and RANTES, IL-10, an anti-inflammatory cytokine, and VEGF, an angiogenic factor. The mediators were predominantly secreted

from the apical surface; except for RANTES, which showed mostly basal side secretion.

In general, low pressure or IL-1β treatment showed somewhat similar secretion patterns for key inflammatory mediators. And both exhibited polarity of secretions, basal vs apical, with a trend for secretions to be higher from the basal (mucosal) side.

Conclusions

Exposure of ME epithelial cells to physiologically relevant negative pressure triggered the release of key inflammatory mediators, notably IL-8 and VEGF. The polarity of the secretions (predominantly apical) may also indicate potential mechanisms of action of individual cytokines. Translating to analogous in vivo conditions of ME underpressure, this response may contribute to persistent inflammation in ME mucosa during OME.

Acknowledgement
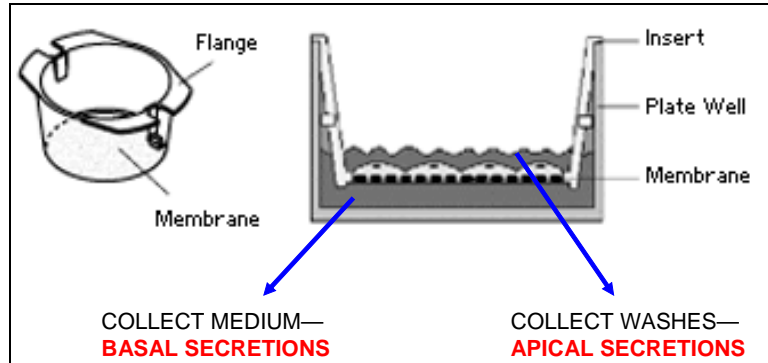
Figure 1. Cell culture on transwell inserts. H-MEEC-1 cells were seeded onto transwell inserts and grown under standard culture conditions to confluence. Then the medium was removed by gentle suction from both upper and lower chambers, and fresh medium added only to the lower chamber so that the cells were at the air/liquid interface. After 24 hours the plates were divided into groups for exposure to experimental conditions of either IL-1β (1ng/ml) or low pressure (-250daPa) for 24 hours.
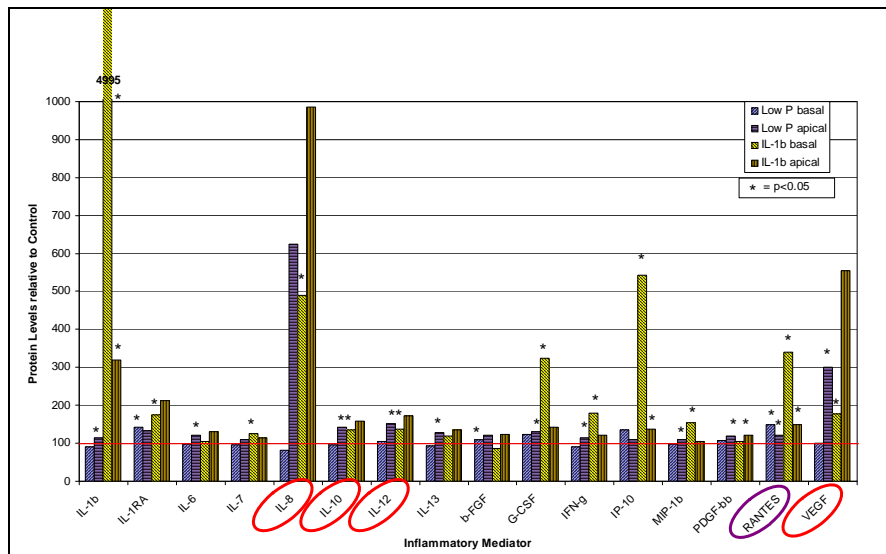
Figure 2. Effects of negative pressure on inflammatory signals from human middle-ear epithelial cells. The graph show the subset of mediators that were secreted by ME epithelial cells after exposure to IL-1β or to negative pressure (Low P). The red line marks the normalized baseline levels measured in control cultures. The circles indicate cytokines that were significantly induced by negative pressure, predominantly from the apical side except for RANTES, which was secreted from the basal side of the ME epithelial cell layer.

References

1. Ranade A, Lambertsen CJ, Noordergraff A. Inert gas exchange in the middle ear. Acta Otolaryngol 1989; (suppl 371):1-23.
2. Flisberg K. The effects of vacuum on the tympanic cavity. Otolaryngol Clin North Am 1970; 3:3.
3. Hiraide F, Eriksson H. The effects of the vacuum on vascular permeability of the ME. Acta Otolaryngol (Stockh) 1978; 85:10-16.
4. Lamkin R, Axelsson A, McPherson D, Miller J. Experimental aural barotrauma. Electrophysiological and morphological findings. Acta Otolaryngol (Stockh) 1975 (suppl 335):1-24.
5. Bluestone CD, Doyle WJ. ET Function: Physiology and role in otitis media. Ann Otol Rhinol Laryngol 1985; 94(suppl. 20):1-60.
6. Johnson MD, Contrino A, Contrino J, Maxwell K, Leonard G, Kreutzer D. Murine model of otitis media with effusion: Immunohistochemical demonstration of IL-1alpha antigen expression. Laryngoscope 1994; 10:1143-1149.
7. Piltcher OB, Swarts JD, Magnuson K, Alper CM, Doyle WJ, Hebda PA. A rat model of otitis media with effusion caused by eustachian tube obstruction with and without S. pneumoniae infection: Methods and disease course. Otolaryngol Head Neck Surg 2002; 126:490-498.
8. Hebda PA, Piltcher OB, Swarts JD, Alper CM, Zeevi A, Doyle WJ. Cytokine profiles in a rat model of otitis media with effusion caused by eustachian tube obstruction with and without S. pneumoniae infection. Laryngoscope 2002; 112:1657-1662.
9. Chun YM. Moon SK, Lee HY, Webster P, Brackmann DE, Rhim JS, Lim DJ. Immortalization of normal adult human middle ear epithelial cells using a retrovirus containing the E6/E7 genes of human papillomavirus type 16. Ann Otol Rhinol Laryngol 2002; 111:507-517.

Middle ear pressure-regulation: a computational model

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Introduction

For adequate transduction of environmental sound pressures to the inner ear (IE) and maintenance of normal hearing levels, the potential middle ear (ME) airspace needs to be free of fluid and pressurized with gas to an approximate local ambient. Changes in environmental pressure resulting from changing weather fronts and elevation and changes in ME pressure (MEP) secondary to physiological exchange of ME gas with adjacent compartments continuously disturb the ME-ambient total pressure balance. The resulting pressure imbalances can cause mild to moderate hearing loss while ME underpressures on the order of 200 to 300 mmH2O cause the transudation of capillary fluids from the ME mucosa (MEM) into the ME airspace with the complete loss of ME transductive function.[1] MEPregulation is a homeostatic process that, when well-functioning, periodically adjusts total MEP to local ambient before the development of significant pressure imbalances and ME pathology.[2] This function can be expressed as $|P^{AMB}\text{-}P^{ME}| < P^{CRIT}$ for all times, t where $|P^{AMB}\text{-}P^{ME}|$ is the absolute value of the difference between ambient (P^{AMB}) and ME (P^{ME}) pressure and P^{CRIT} is the critical pressure for hearing loss and pathology. Here we present a 3-tiered computational model that describes MEP regulation in health and disease (See Figure 1).

Modeling

The calculation of total MEP is given by the general gas law, $P^{ME}=N^{ME}RT^{ME}/V^{ME}$, where R is a gas constant and, P^{ME} is pressure, VME is volume, N^{ME} is the number of moles of all contained gases and T^{ME} is temperature of the ME airspace.[2] Each represented gas (g) species independently contributes to MEP such that $N^{ME}=\Sigma Ng^{ME}$ and $P^{ME}=\Sigma Pg^{ME}$ where Pg^{ME} is gas partial-pressure and Σ indicates summation over all gases. Because T^{ME} is relatively constant and, under normal conditions, changes in VME (δV^{ME}; e.g. tympanic membrane[TM] displacements) are small *vis a vis* VME, change in MEP (δP^{ME}) is primarily driven by the change in N^{ME} ($\delta N^{ME}=\Sigma\delta Ng^{ME}$) and the rate of change in MEP ($\delta P^{ME}/\delta t$) is a direct, approximately linear function of $\delta N^{ME}/\delta t$. In our model, the gas exchange pathways that contribute to δN^{ME} are

represented as submodels of tier-1. The operation of these submodels requires, at a minimum, information on extant (time=t) P^{ME} and Pg^{ME} and calculatesδNg^{ME} for a specified time-interval (δt).A tier-2 Integrator sums the δNg^{ME} contributed from each submodel with the existing Ng^{ME}, references these values to extant VME and calculates P^{ME} and Pg^{ME} for time t+δt. The Integrator outputs the revised P^{ME} and Pg^{ME} values to the tier-1 submodels and the P^{ME} value to a tier-3 Evaluator. The Evaluator compares extant P^{ME} to local ambient, calculates a revised VME based on expected TM displacements and adjusts ME volume at tier-2 for TM displacements, and determines if the ME-ambient pressure gradient is sufficient to cause pathology, i.e. $|P^{AMB}\text{-}P^{ME}| > P^{CRIT}$. If pathology is detected, the predicted VME resulting from MEM swelling and accumulation of effusion in the ME airspace are fed back to the Integrator and other changes are fed back to the tier-1 submodels. The complete set of model equations is iterated at successive time-intervals, δt, to generate a representation of MEP behavior over time. Ideally, the model operates over infinitely small time intervals ($\delta t\rightarrow 0$), but in practice, the equations in the model are iterated at a time interval sufficiently short to capture MEP trajectory. Adequate MEP regulation is defined for all time periods where the MEP-ambient pressure gradient is maintained within specified limits that exclude hearing loss and ME pathology, i.e. $|P^{AMB}\text{-}P^{ME}| < P^{CRIT}$ at all times, t.

Tier-1 Submodels

Figure 2a shows a schematic of gas exchange within the healthy ME (tympanum with Mastoid Air Cell System [MACS]), and between the healthy ME and adjacent compartments (local blood via the ME mucosa [MEM], ambient air via the TM, and nasopharynx [NP] via the Eustachian tube [ET]).Theory predicts and the results of experiments agree that gas admixture in the airphase (ME-MACS) is extremely rapid and this is enhanced by the pumping action associated with TM displacements.[3,4] Consequently, the gas composition of the tympanum and MACS can be considered to be identical over time and the dynamics of this exchange is ignored in our model. Figure 2b shows the approximate gas partial-pressures for the various compartments in a healthy ME at a total pressure of 760mmHg.[5] In developing our model, we omitted the ME-IE gas exchange

pathway via the round window membrane because the IE gas-partial pressures are nearly identical to those of blood and the surface area of round window is vanishingly small compared to that of the MEM. Consequently trans-round window exchange is subsumed under transMEM exchange in the model. Thus, tier-1 consists of 3 independent submodels that describe ME gas exchange with the atmosphere, the local blood and the Naphtha first 2 submodels operate on each represented gas species independently and describe passive, bidirectional, gradient (partial-pressure [ΔPg]) dependent exchange across a biological barrier. In contrast, the transET exchange model is characterized by active or passive, total pressure gradient (ΔP) dependent exchange of mixed gases with source gas (NP or ME) composition.

transTM Gas Exchange

TransTM exchange rates of the physiological gases were measured to be extremely slow *vis a vis* the corresponding transMEM exchange rates, but the results of animal experiments suggest that these rates are significantly increased by TM pathologies such as scaring and atrophy.[6,7] The fine structure of the transTM exchange model is not known, but most simply (e.g. ignoring a possible contribution of intra-TM blood perfusion), gas flux can be described by the Fick equation for diffusion across a biological barrier. The fundamental parameters of the submodel are the geometric (area [A], thickness [H], regional distribution of H over A) and physical properties (e.g. lipid/water content) of the TM. The latter properties uniquely specify the solubility (Sg) and diffusivity (Dg) of each gas in the TM. Gas flux across the TM relative to the ME is then modeled as: $\delta Ng^{ME}/\delta t = -\Delta Pg^{TM}Sg^{TM}Dg^{TM}A^{TM}/H^{TM}$ and the total ME gas gain or loss is $\Sigma\delta Ng^{ME}/\delta t$. The only free parameter for this submodel is ΔPg^{TM} since $Sg^{TM}Dg^{TM}A^{TM}/H^{TM}$ is a constant, Kg^{TM}, for a given TM. The inputs for submodel are the extant environmental and ME gas partial-pressures and the outputs are the change in ME gas moles for each species over a specified interval. Because Pg^{ME} is affected by ongoing gas exchanges via the other pathways, the free parameter needs to be continuously updated which is done by iterating the model equation with revised inputs from the Integrator (Pg^{ME}) and Evaluator (Pg^{AMB})and new outputs calculated at very short intervals ($\delta t \rightarrow 0$).In calibrating the submodel, the constant Kg^{TM} is measured empirically for each gas species[6]

transMEM gas Exchange

TransMEM gas exchange has been a focus of past modeling efforts. Early attempts to apply the Fick diffusion equation to all physiological gases produced results that were not in agreement with experiment.[8-10] Specifically, experimental measurements made in monkeys and confirmed in humans showed that a diffusion equation similar to that developed above for the TM predicted the transMEM exchange rate of reactive gases (O2, CO2) but not inert gases (N2, N2O) whose exchange rate depended on MEM blood perfusion (Q^{MEM}).[11-13] This difference was explained by the fact that reactive gases are quickly converted in blood to other chemical compounds (reaction products [RP])such that their partial-pressure gradient between ME and blood is a function of the ME partial-pressure alone (i.e. partial-pressure in blood is constant).Thus, as a first approximation, transMEM gas exchange rate can be described by the diffusion-limited exchange equation,$\delta Ng^{ME}/\delta t = -\Delta Pg^{MEM}Sg^{MEM}Dg^{MEM}A^{MEM}/H^{MEM}$, where ΔPg^{MEM} is the partial-pressure gradient of a reactive gas, g, between ME airspace and venous blood, or the perfusion-limited exchange equation, $\delta Ng^{ME}/\delta t = -\Delta Pg^{MEM}Sg^{B}Q^{MEM}$, where ΔPg^{MEM} is the partial-pressure gradient of an inert gas between ME airspace and arterial blood and Sg^{B} is the solubility of the gas in blood. Because Sg^{MEM}, Dg^{MEM}, $Sg^{B}$and Pg^{B} are known constants, the free parameters of this transMEM submodel are Pg^{ME}, Q^{MEM},A^{MEM}, and H^{MEM}. In calibrating this submodel to the basal state, the values of the products of $Sg^{MEM}Dg^{MEM}A^{MEM}/H^{MEM}$ and of $Sg^{B}Q^{MEM}$ are measured empirically for each gas species.[11]Operation of the submodel outputs $\delta Ng^{ME}/\delta t$ to the Integrator and inputs $Pg^{ME}$from the Integrator and the other free parameters (Q^{MEM}, A^{MEM}, H^{MEM}) from the Evaluator.

It should be noted that this formulation of the submodel provides reasonable estimates of transMEM gas exchange rates for the effusion-free MEs but not for MEs with effusion. In the latter case, the effusion represents a new ME compartment which introduces capacitance (i.e. gas storage) effects and unpredictable changes in average barrier (MEM+effusion) thickness and surface area. A more complicated system of equations based on an electrical analog can be used to solve gas exchange rates between the ME airspace and local blood for the diseased ME, and a schematic of one such circuit is shown in Figure 2c.[2] Primary assumptions in constructing the circuit are that: 1) the ME exchanges gas with adjacent compartments that in turn exchange gas with more peripheral compartments; 2) each compartment is characterized by a specific volume represented as an effective capacitance for

each represented gas species; 3) all compartments have essentially 0 compliance, i.e. capacitance is fixed, and 4) inter-compartmental exchange is limited by an effective, route specific resistance for each gas. More specifically, compartment voltage represents gas partial-pressure (Pg^c), current represents inter-compartmental gas flow ($\delta Ng^{c1c2}/\delta t$), resistance ($Rg^{c1c2}$) represents the barrier impediment to gas flow and capacitance represents the effective compartment volume (Vg^c) which in the fluid and tissue phase is gas specific (Sg^cVg^c). The ground state represents the gas partial-pressures in blood. Note that the subcircuits represented by R1-R2-R3 and R1-R2-R4-R5 yield the same transMEM gas flows for inert and reactive gases as the simpler model presented above, but that the parallel circuit allows for the effects of effusion on gas transfer rates. For each gas species, the resistors are calibrated using the Fick equations as is relevant to extant conditions. The model outputs $\delta Ng^{ME}/\delta t$ to the Integrator and inputs Pg^{ME}, Q^{MEM}, A^{MEM}, H^{MEM}, A^{EFF}, H^{EFF} and the compartment volumes at t+dt from the higher level models. In calibrating this submodel to the basal state, the values of the resistance for each subcircuit are measured empirically for each gas species.[11]

transET Gas Exchange

Gas exchange across the ET has been studied for many years, yet remains poorly conceptualized by many with an interest in ME physiology.[14-16] It is clear to most that the ET is a requisite element for MEP regulation as shown in animal experiments where disabling the active, muscle assisted opening of the ET causes the progressive development of ME underpressures (ref. ambient), hearing loss and ME effusion.[1,17] This is reproduced by operation of our calibrated model without the ET component or with a poorly functional ET component. Our submodel of transET gas exchange is parameterized by the anatomical relationships among components of the ET system. The basic anatomy of ET system has been described in previous publications[18,19], and, more recently, the functional anatomy of the ET was more fully described by us.[20,21] Briefly, the posterior portion of the ET is a mucosa lined, bony tube continuous with the anterior tympanum while the anterior portion is cartilaginous medially and membranous laterally. The cartilaginous portion is usually closed by a tissue pressure (P^{ET}) equal to the sum of the ambient pressure and a vascular pressure which imposes a distributed force on the ET lumen (F^{ETc}). A muscle, the mTVP takes origin from the membranous wall of the ET, rounds the hamular process and terminates within the palatine

aponeurosis. Activation of the muscle during swallowing exerts an anterior-lateral-inferior force (F^{TVP}) on the membranous wall of the ET. Figure 3 depicts these functional relationships. F^{ETc} is a function of P^{ET} such that $F^{ETc}=A^{Lc}P^{ETc} = A^{Lc}(C_2P^{AMB}+f^M(P^{VASc}))$, $0<C_2<1$, where $f^M(P^{VASc})$ is a functional operation on P^{VASc}, A^{Lc} is the surface area of the collapsed lumen and C_2 is a constant relating ET basal tissue pressure to ambient pressure.

ET opening can be affected by passive, pressure-driven processes and by active, pressure-driven or muscle-assisted mechanisms. The former occurs when either F^{ME} or F^{NP} exceeds that of F^{ETc} as described by $A^{NP}P^{NP}$ or $A^{ME}P^{ME}>A^{Lc}P^{ETc}$ where the superscript placed on A indicates the surface area of the ET lumen exposed to the pressure of the respective compartment. Active, pressure-driven ET opening occurs when the force of P^{NP} is increased to greatly exceed that of P^{ETc} during maneuvers such as Valsalva or Toynbee, or for some individuals, when P^{ETc} is reduced by yawning or mandibular repositioning. Active, muscle-assisted ET opening occurs when the mTVP contracts with sufficient inferiolateral force (F^{TVPv}) to overcome the closing force, $F^{TVPv}>F^{ETc}$ where F^{TVPv} indicates the effective vector force of the mTVP. For all mTVP contractions satisfying this condition, the magnitude of the effective force determines the ET lateral wall displacement as approximated by Hooke's law: $X^{ET}=F^{TVPv}C^{ET}$ where C^{ET} is the compliance of the ET mucosa-cartilage and X^{ET} is the lateral lumen wall displacement. Assuming that transET gas exchange follows Hagen-Poiseuille flow between two parallel plates (22), the volume gas transferred, Q^{ET}, is described by $Q_{ET}=(2/3)\Delta P^{ET}(X^{ET})^3W(\mu L)^{-1}$ where W is the superior-inferior height of the tube lumen, X^{ET} is the lumen width at opening, μ is the viscosity of air and ΔP^{ET} is the driving gradient for transfer. Thus, the volume flow during mTVP muscle contractions is given by $\delta N^{ET}/\delta t=K^OT_s(2/3)\Delta P^{ET}(F^{TVPv}C^{ET})^3W(\mu L)^{-1}$ where T_s is the duration of effective mTVP contraction and K^O is the probability of mTVP contraction during the interval. Similarly, for passive openings, the volume flow is given by $\delta N^{ET}/\delta t=T_p(2/3)\Delta P^{ET}(C^{ET})^3W(\mu L)^{-1}$ where T_p is time that $A^{NP}P^{NP}$ or $A^{ME}P^{ME}>A^{Lc}P^{ETc}$. For both types of exchange, $\delta Ng^{ET}/\delta t$ is distributed among component gases in proportion to the fractional gas partial-pressures of the source compartment. The basal function of this submodel is specified by system geometry, C^{ET}, F^{TVPv}, K^O and T_s which are measured empirically.[22, 23] The model outputs δNg^{ET} for a specified interval to the Integrator and inputs P^{ME}, P^{NP} and F^{ETc} from the Evaluator.

Tier-2 Integrator

The Integrator serves the simple function of storing Ng^{ME} and VME at time t and calculating Ng^{ME}, Pg^{ME} and P^{ME} for time, $t+\delta t$. The operation requires summing the specific gas flows across all paths $\Sigma^{Path}\delta Ng^{ME}$ for the time interval, summing these flows across all gases $\Sigma^g\Sigma^{Path}\delta Ng^{ME}$ and then adding these flows to the extant Ng^{ME} and N^{ME} to obtain revised values for Ng^{ME} and N^{ME} at time, t. A first estimate of P^{ME} is then calculated using the general gas law, $P^{ME}=N^{ME}RT^{ME}/V^{ME}$ where VME is the ME volume at time, t. This estimate is outputted to the Evaluator and VME is returned at time, t (i.e. a nested calculation). ME gas pressure and partial-pressures at the revised volume are calculated and outputted to the tier-1 submodels for operation at $t+\delta t$.

Tier-3 Evaluator

The Evaluator continuously samples ambient, vascular and nasopharyngeal pressure and monitors the nasopharyngeal environment for pathological changes (virus infection, allergy) that affect MEM blood flow and the ET closing pressure.[24-27] The first process assesses the change in ME volume associated with TM displacements by comparing the pressure of the ME and ambient environment at time t, determining the TM displacement volume and adding this to the ME volume at t=0. This is done using a variant of Hooke's Law, $K(P^{ME}-P^{AMB})=\delta V^{ME}$ and then summing $V_{t=0}^{ME}+\delta V^{ME}=V^{ME}$.[28] The revised volume is returned to the Integrator at time t (i.e. a nested calculation). The second process assesses the nasopharyngeal environment for inflammatory changes and, if present, revises Q^{MEM} (increased) and P^{VASc} (increased) to reflect those changes. Q^{MEM} is returned to the Integrator and, for all conditions, the ET closing force, $F^{ETc}=(C_2P^{AMB}+f^M(P^{VASc}))$ is calculated using extant P^{AMB} and P^{VASc} and outputted to the transET exchange submodel for operation at $t+\delta t$. The final process compares the extant ME and environmental pressures to determine if the pressure gradient is sufficient to initiate pathology, $|P^{AMB}-P^{ME}| > P^{CRIT}$. If the inequality is satisfied, the Evaluator estimates the effect on Q^{MEM} (as adjusted for nasopharyngeal pathology) and on the volumes, thicknesses and surface areas of the ME compartments (airspace, effusion, MEM). These values are returned to the Integrator (submodel 2) and used to recalibrate the resistors and capacitors of the circuit. In practice, most of the functions of the Evaluator are qualitatively evaluated as the required mathematical relationships describing the effects of pathology on the model parameters have not been sufficiently developed for quantitative application.

Discussion

While conceptually simple, MEP regulation is poorly understood by many with an interest in ME physiology. Our model is based on two fundamental principles; passive gas exchange between ME and adjacent compartments is gradient dependent and, with the exception of ET openings, no active gas exchange processes are operative. An analysis of the operation of our model for the healthy ME shows that the established partial-pressure gradients between ME and adjacent compartments cause a net loss of ME gas while effective ET openings will resupply those gases. Thus, operation of these processes can be assigned to the domains of ME gas demand (passive exchanges) and ME gas supply (ET openings) with effective pressure-regulation representing a balance between the two. This homeostasis can be disrupted by changes in ET function as caused by viral infections and allergy[25,26] or by changes in the rate of the passive gas exchange as caused by changes in blood flow within the MEM or introduction of an effusion compartment to the ME.[27,29-31] The model accurately predicts the MEP and ME gas partial-pressures measured by experiment, the trajectory of MEP change for the normal ME, the complex interplay between extant ME gas supply and demand in the development of ME pathology and the efficacy of certain interventions designed to prevent or cure ME pathology.[32-34] Importantly, the model can reproduce all experimental evidences that others have used to support gas generation by the MEM[35-40] without the need to include the proposed exotic and unphysiological mechanisms.[2,33,34,41,42] For these reasons, this model and its future revisions is a useful tool for understanding MEP regulation and for dissecting those factors that disrupt that homeostasis.

Acknowledgement

Figure 1: A schematic diagram of the operation of the three-tiered model of MEP regulation. Arrows indicate the direction of information flow. See text for details.

Figure 2: The pathways for ME gas exchange (a), the partial-pressures of gas for the ME and adjacent compartments at a total ambient pressure of

760mmH2O (b) and an electrical circuit representing gas flow between ME and blood for ears with and without effusion (c). The capacitors represent compartment volume, the resistors represent barriers to gas flow and current represents gas flow. See text for details. Subscripts: atm=atmospheric, np=Nasopharynx, tm-TM, ie=IE, as-airspace, eff=effusion, m=mucosa, lb=local blood rp=reaction product in local blood.

Figure 3: A schematic of the functional relationships for the ET. Note that the ET is treated as a pressure valve between the ME and NP and that the TVP muscle attaches to the membranous wall of the ET. The ET pressure exerts a distributed force along the ET lumen and the TVP muscle is positioned to exert an inferio-lateral force on the ET lumen during contraction. The ET can be opened when either the ME or NP pressure exceeds the ET pressure or the TVP muscle force exceeds the distributed force acting on the ET lumen.

FIGURE 1
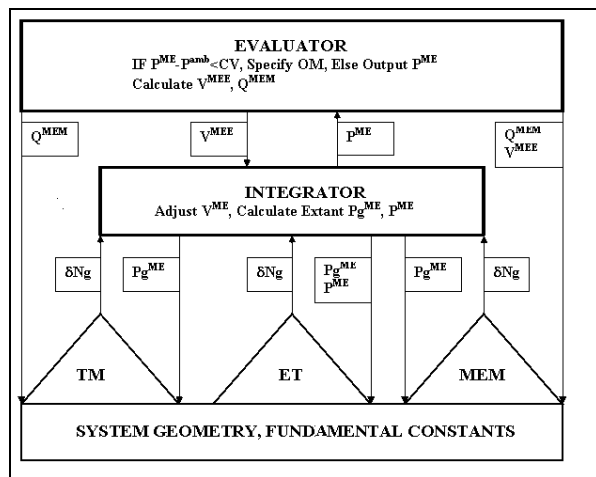
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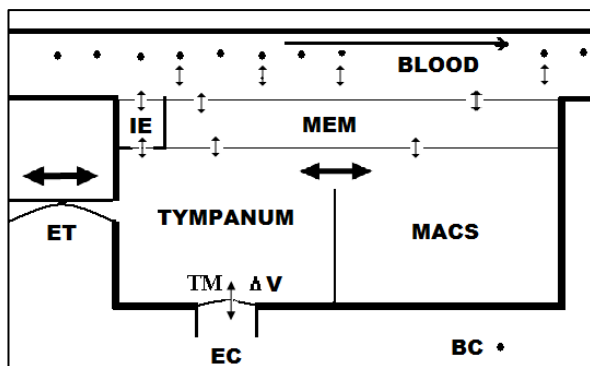
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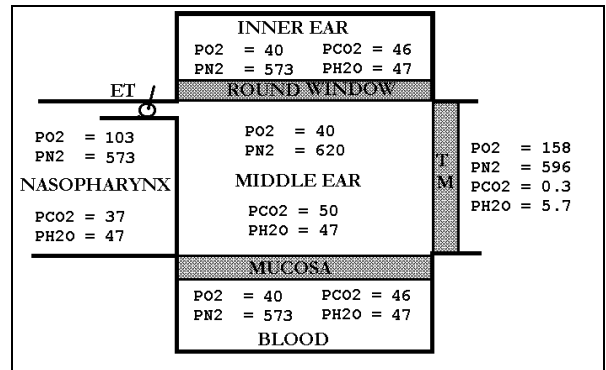
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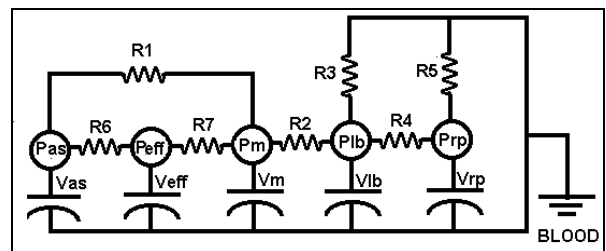
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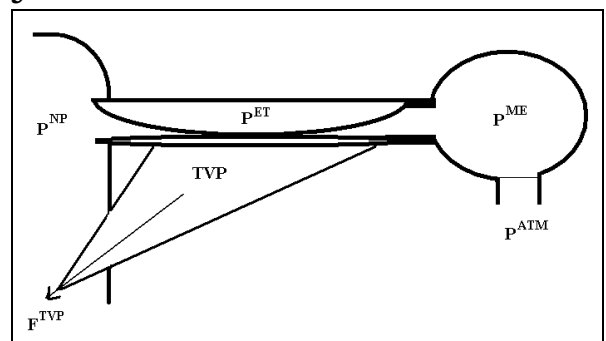
References

1. Swarts JD, Alper CM, Seroky JT, Chan KH, Doyle WJ. In vivo observation with magnetic resonance imaging of middle ear effusion in response to experimental underpressures. Ann Otol Rhinol Laryngol 1995;104:522-528.

2. Doyle WJ. Mathematical model explaining the sources of error in certain estimates of the gas exchange constants for the middle ear. Ann Otol Rhinol Laryngol 2000;109:533-541.

3. Raveh E, Sade J, Mover-Lev H, Guney S. Mastoid buffering properties: I. Gas partial pressures. Ann Otol Rhinol Laryngol 1999;108:750-755.

4. Felding UN, Banks JM, Doyle WJ. Middle ear gas exchange in the air phase. Acta Otolaryngol 2003;123:808-811.

5. Felding JU, Rasmussen JB, Lildholdt T. Gas composition of the normal and the ventilated middle ear cavity. Scand J Clin Lab Invest Suppl 1987;186:31-41.

6. Doyle WJ, Alper CM, Seroky JT, Karnavas WJ. Exchange rates of gases across the tympanic membrane in rhesus monkeys. Acta Otolaryngol 1998;118:567-573.

7. Felding UN, Banks JM, Doyle WJ. Gas diffusion across the tympanic membrane in chinchillas: effect of repeated perforations. Auris Nasus Larynx 2004;31:353-359.

8. Ostfeld EJ, Silberberg A. Gas composition and pressure in the middle ear: a model for the physiological steady state. Laryngoscope 1991;101:297-304.

9. Ostfeld EJ, Silberberg A. Transient pressure changes in the middle ear. Arch Otolaryngol Head Neck Surg 1991;117:1390-1394.

10. Ostfeld EJ, Silberberg A. Theoretic analysis of middle ear gas composition under conditions of nonphysiologic ventilation. Ann Otol Rhinol Laryngol 1992;101:445-449.

11. Doyle WJ, Seroky JT, Alper CM. Gas exchange across the middle ear mucosa in monkeys. Estimation of exchange rate. Arch Otolaryngol Head Neck Surg 1995;121:887-892.

12. Doyle WJ, Alper CM, Seroky JT. Trans-mucosal inert gas exchange constants for the monkey middle ear. Auris Nasus Larynx 1999;26:5-12.

13. Doyle WJ, Banks JM. Middle ear pressure change during controlled breathing with gas mixtures containing nitrous oxide. J Appl Physiol 2003;94:199-204.

14. Ingelstedt S, Ivarsson, Jonson B. Mechanics of the human middle ear. Pressure regulation in aviation and diving. A non-traumatic method. Acta Otolaryngol 1967; Suppl 228:1-58.

15. Bylander A. Function and dysfunction of the eustachian tube in children. Acta Otorhinolaryngol Belg 1984;38:238-245.

16. Cantekin EI, Bluestone CD, Saez CA, Doyle WJ, Phillips DC. Normal and abnormal middle ear ventilation. Ann Otol Rhinol Laryngol Suppl 1977;86(4 Pt 3 Suppl 41):1-15.

17. Alper CM, Tabari R, Seroky JT, Doyle WJ. Magnetic resonance imaging of the development of otitis media with effusion caused by functional obstruction of the eustachian tube. Ann Otol Rhinol Laryngol 1997;106:422-431.

18. Prades JM, Dumollard JM, Calloc'h F, Merzougui N, Veyret C, Martin C. Descriptive anatomy of the human auditory tube. Surg Radiol Anat 1998;20:335-340.

19. Rood SR, Doyle WJ. Interspecific study of the eustachian tube/middle ear system. Trans Pa Acad Ophthalmol Otolaryngol 1979;32:55-61.

20. Ghadiali SN, Swarts JD, Federspiel WJ. Model-based evaluation of eustachian tube mechanical properties using continuous pressure-flow rate data. Ann Biomed Eng 2002;30:1064-1076.

21. Kanick SC, Doyle WJ. Barotrauma during air travel: predictions of a mathematical model. J Appl Physiol 2005; 98:.1592-1602.

22. Cantekin EI, Saez CA, Bluestone CD, Bern SA. Airflow through the eustachian tube. Ann Otol Rhinol Laryngol 1979;88:603-612.

23. Ghadiali SN, Federspiel WJ, Swarts JD, Doyle WJ. Measurement of the viscoelastic compliance of the eustachian tube using a modified forced-response test. Auris Nasus Larynx 2002;29:1-5.

24. Seroky JT, Alper CM, Tabari R, Doyle WJ. Effects of intranasal challenge with histamine, bradykinin and prostaglandin on middle ear pressure and blood flow in cynomolgus monkeys. Acta Otolaryngol 1995;115:83-87.

25. Doyle WJ. The link between allergic rhinitis and otitis media. Curr Opin Allergy Clin Immunol 2002;2:21-25.

26. Doyle WJ, Alper CM, Buchman CA, Moody SA, Skoner DP, Cohen S. Illness and otological changes during upper respiratory virus infection. Laryngoscope 1999;109:324-328.

27. Yuksel S, Doyle WJ, Banks J, Seroky JT, Alper CM. Nasal prostaglandin challenge increases N(2)O exchange from blood to middle ear. Auris Nasus Larynx 2005;32:29-32.

28. Kanick SC, Doyle WJ. Barotrauma during air travel: predictions of a mathematical model. J Appl Physiol 2005;98:1592-1602.

29. Alper CM, Doyle WJ, Seroky JT. Higher rates of pressure decrease in inflamed compared with noninflamed middle ears. Otolaryngol Head Neck Surg 1999;121:98-102.

30. Alper CM, Doyle WJ. Repeated inflation does not prevent otitis media with effusion in a monkey model. Laryngoscope 1999;109:1074-1080.

31. Alper CM, Swarts JD, Doyle WJ. Middle ear inflation for diagnosis and treatment of otitis media with effusion. Auris Nasus Larynx 1999;26:479-486.

32. Doyle WJ, Seroky JT. Middle ear gas exchange in rhesus monkeys. Ann Otol Rhinol Laryngol 1994;103:636-645.

33. Doyle WJ. The mastoid as a functional rate-limiter of middle ear pressure change. Int J Pediatr Otorhinolaryngol 2007;71:393-402.

34. Doyle WJ, Alper CM. A model to explain the rapid pressure decrease after air-inflation of diseased middle ears. Laryngoscope 1999;109:70-78.

35. Magnuson B. Functions of the mastoid cell system: auto-regulation of temperature and gas pressure. J Laryngol Otol 2003;117:99-103.

36. Sade J, Luntz M. Middle ear as a gas pocket. Ann Otol Rhinol Laryngol 1990;99:529-534.

37. Sade J, Luntz M. Gas diffusion in the middle ear. Acta Otolaryngol 1991;111:354-357.

38. Buckingham RA, Stuart DR, Geick MR, Girgis SJ, McGee TJ. Experimental evidence against middle ear oxygen absorption. Laryngoscope 1985;95:437-442.

39. Hergils L, Magnuson B. Regulation of negative middle ear pressure without tubal opening. Arch Otolaryngol Head Neck Surg 1988;114:1442-1444.

40. Hergils L, Magnuson B. Morning pressure in the middle ear. Arch Otolaryngol 1985;111:86-89.

41. Doyle WJ. Increases in middle ear pressure resulting from counter-diffusion of oxygen and carbon dioxide into the middle ear of monkeys. Acta Otolaryngol 1997;117:708-713.

42. Doyle WJ. Mucosal surface area determines the middle ear pressure response following establishment of sniff-induced underpressures. Acta Otolaryngol 1999;119:695-702.

Intranasal challenge with prostaglandin D2 and bradykinin increases middle ear to blood inert gas exchange rates

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Introduction

Nasal allergic responses cause physiological changes in adjacent anatomical compartments including the lungs and middle ear (ME). For example, epidemiological studies frequently identify nasal allergy as a risk factor for otitis media with effusion (OME) and other studies reported that OME is more common in children with allergy when compared to non-allergic children.[1,2] Experimental studies show that, while the ME mucosa can sustain a Type I allergic reaction, the duration of the provoked inflammation is short-lived[3] and a feasible mechanism for allergen delivery to the ME has not been identified.[4] For these and other reasons, most, but not all, investigators agree that OME is not a direct result of an *in situ* allergic response, but is mediated by allergen provoked nasal inflammation and the generation of inflammatory chemicals that have non-local, peripheral effects. This hypothesis is consistent with the results of studies in humans and animals that documented impaired Eustachian tube (ET) pressure-regulating and clearance functions during natural[5,6] and experimental[7,8] exposure to allergens, and after intranasal challenges with histamine[9] and other inflammatory chemicals produced during the allergic response.[10,11]

Less easily measurable, but equally important otological effects were reported for preliminary studies in sensitized animals that showed nasal allergen challenge to cause increased ET and ME mucosal blood flow.[12,13] This has the predicted pathophysiological correlate of increasing the rate of inert gas exchange between the ME ear and blood, which in conjunction with ET dysfunction destabilizes ME pressure and promotes conditions that favor the development of OME.[4] Here, we use previously developed methods[14] to determine the effect of nasal exposure to a single dose of prostaglandin D2 (PgD2), capsaicin and bradykinin on the ME-blood exchange of the inert gas, N_2O. The pathophysiological implications of the results for OME are discussed within the context of the *hydrops ex vacuo* mechanism.[15]

Methods

Eight juvenile cynomolgus monkeys weighing between 2.8 and 3.9 kg completed a total of 5 bilateral ME inflation experiments at a minimal interval of 2 weeks. For each experiment, the monkey was sedated with 30 mg Ketamine®, anesthetized with "monkey mix" (ketamine-10mg/kg, xylazine-2mg/kg, acepromazine-0.3mg/kg), intubated and placed on a circulating-water heating pad. Over a 60 minute acclimation period, the animal was monitored for ME pressure using a GSI-33 Middle Ear Analyzer (Model 1733, Littleton, MA USA), body temperature using a Electrotherm (Model TM99A) rectal thermometer and vital signs using a Dynamap neonatal vital signs monitor coupled to a thigh cuff.

Then, the endotracheal tube was placed online to the output of a Harvard respirator (model 661, South Natick, MA. USA) that delivered room air at 50 cc gas/stroke at 20 strokes/ minute and the nasal cavities were bilaterally challenged with the designated chemical [saline (Dulbecco's Phosphate Buffered Saline .25ml/nostril; Sigma), bradykinin (5mg/nostril; Bachem), PgD2 (.5 mg/nostril; Biomol), histamine (10 mg/nostril; Sigma), or capsaicin (5 ug/nostril; Sigma)]. For challenge, a 250 ul dosing volume was sprayed into each nostril using a pre-primed atomizer (Wolf Tory Medical, Salt Lake City, UT).

Ten minutes later, the ME was bilaterally inflated with the test gas mixture (20% N_2O, 6% O_2, 6% CO_2, balance N_2 as previously described.[14] Briefly, a probe on-line to a pressurized mixed gas source was introduced into the monkey's nose and a low flow of the designated gas mixture was begun to washout gases of other composition. Then, the velopharyngeal port was closed by manually elevating the palate, the gas flow was increased for approximately 5 seconds, and the palate was released. This procedure causes an increased nasopharyngeal pressure that passively opens the ET and introduces a bolus of test gas into the ME airspace. The success of the inflation procedure was confirmed by repeat tympanometry (i.e. increased ME pressure over the pre-maneuver value). ME pressure, body temperature and vital signs were recorded at 5-minute intervals for 90 minutes, and then the animal was returned to the vivarium.

This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C. et seq). The protocol was approved by the Animal Care and Research Committee at the Children's Hospital of Pittsburgh.

The primary data used in the analysis were the ME pressure measurements made after bilateral N_2O inflation. From these data, the time-constant for transmucosal N_2O exchange was estimated using published methods.[14] In brief, the total pressure in the ME is equal to the sum of the partial-pressures of all contained gases, or:

EQ1a: $P^m = \Sigma P^m_g$

and the change in ME pressure is equal to:

EQ1b: $\delta P^m/\delta t = \Sigma \delta P^m_g/\delta t$

where P^m is the total ME pressure, P^m_g is the partial-pressure of gas g in the ME and Σ is an operator indicating summation over all represented gas species (i.e for these experiments, O_2, CO_2, H_2O, N_2 and N_2O). Under physiological conditions and at constant O_2 and CO_2 pressures in blood (as is true for the acclimated state), the ME is saturated with water vapor and the extremely rapid ME-blood exchange of O_2 and CO_2 cause their gradients to approach 0 mmHg. Because there is no gradient to drive the transmucosal exchange of these gases, $\delta P^m_{O2}/\delta t = \delta P^m_{CO2}/\delta t = \delta P^m_{H2O}/\delta t = 0$ pressure/time. Direct measurements show that the change in ME N_2 pressure at extant ME-blood gradients as high as 50mmHg is not measurable in experiments lasting for 4 hours,[14] and therefore in short duration experiments, EQ1b reduces to:

EQ2: $\delta P^m/\delta t \approx \delta P^m_{N2O}/\delta t$

Transmucosal exchange of inert gases (N_2, N_2O etc) is perfusion-limited and can be described by:

EQ3: $\delta P^m/\delta t \approx F_{N2O} R Q T^m S^b_{N2O}(P^a_{N2O} - P^m_{N2O})/V^m$

where P^m is total ME pressure; R is the general gas constant; T^m is ME temperature; V^m is ME volume, Q^m is mucosal blood flow rate; S^b_{N2O} is the N_2O solubility in blood; P^m_{N2O} and P^a_{N2O} are the N_2O pressures in the ME and arterial blood and F_{N2O} is the ratio of the arterial-venous to arterial-ME N_2O gradient. By rearranging terms, EQ3 can be written as an expression for the transmucosal N_2O time-constant:

EQ4: $K^m_{N2O} = (\delta P^m/\delta t)/(P^a_{N2O} - P^m_{N2O}) = F_{N2O} R Q^m T^m S^b_{N2O}/V^m$

or for purposes of calculation:

EQ5: $(\delta P^m/\delta t) = -K^m_{N2O} P^m_{N2O} + K^m_{N2O} P^a_{N2O}$.

This form of the equation shows that, for constant P^a_{N2O}, the N_2O time-constant is equal to $F_{N2O} R Q^m T^m S^b_{N2O}/V^m$ and can be calculated as the negative slope of the line relating the rate of ME pressure change to extant ME pressure. Note that violation of any assumptions (i.e. within session changes in gradient of other gases, P^a_{N2O}, Q^m or F_{N2O}) will be reflected as a non-linear relationship between the rate of ME pressure change ($\delta P^m/\delta t$) and gradient ($P^a_{N2O} - P^m_{N2O}$), and consequently, linearity of the function is a testable hypothesis of assumption validity.

The data for all experiments were analyzed by least-square, linear regression of the change in ME pressure (per sequential, overlapping 10 minute intervals) on the average ME pressure for the respective 10-minute interval. To allow time for re-establishing the near 0 mmH_2O ME-blood gradients for O_2 and CO_2 that could have been disturbed by inflation with the N_2O gas mixture, the data were analyzed for the post-inflation period between 10 and 70 minutes (linear region of the rate-pressure function; see above). Between-session differences in measured time-constants were tested for statistical significance using a paired Student's t test. Where reported, the Pearson Product Moment Correlation Coefficient is used to represent the degree of relationship, and the format mean±standard deviation is used in data summaries.

Results

During the 60-minute, pre-inflation period the majority of experiments documented a decrease in ME pressure to relatively stable values. Also, while temperature, blood pressure and heart rate were affected by the anesthesia, these measures were relatively stable during the post-inflation time-period. The Figure shows the pressure-time functions for the right and left ears of animal #46 at the baseline and saline challenge test sessions (a) and the four corresponding rate-pressure functions derived from the transformed data (b). For the pressure-time functions, note the pre-inflation decrease in ME pressure (acclimation to anesthesia), the rapid pressure increase immediately after inflation (gas transfer through ET into the ME), and the curvilinear decrease over the post-inflation period (ME to blood exchange of N_2O).As expected from the mathematical derivations described in the previous section, the corresponding rate-pressure functions were relatively linear over the post-inflation time-period, a requisite observation if the model assumptions are valid.

The N_2O time-constant for each ear and experiment was estimated as the value of the slope for the corresponding rate-pressure function. The Table reports the transmucosal N_2O time-constants calculated for both MEs of all animals at the five test sessions, the average and standard deviations of these

measures and the significance levels for the paired saline-mediator challenge results. While nasal saline and capsaicin challenges had no effect on the N_2O time-constant, PgD2 and bradykinin challenges caused a significant increase in that measure; i.e. an increased rate of transmucosal inert gas exchange per unit pressure gradient.

Discussion

As reviewed in recent publications, there is continuing interest in nasal allergy as a "risk factor" or precipitant cause of ME pathologies including OME.[4] However, direct linkage has not been established and evidence based on efficacious responses to anti-allergy treatments is not convincing.[4,15] The ET represents a potential physical communication between the nose and ME, but allergens delivered to the nose even under substantial driving pressures cannot enter the ET orifice because: the nasal anatomy favors particle deposition on the nasal mucosa, the ciliary tracts of the ET and nasal mucosa are opposed with both directing mucus laden secretions to the oropharynx, the large pressures required to open the nasopharyngeal orifice of the ET and the significant pressure drop across the resistor represented by the nose. Because there is no direct pathway by which nasal allergens can gain access to the ME airspace, most mechanisms relating nasal allergy to otological pathologies require intermediate responses that culminate in OME by *hydrops ex vacuo*. However, while provoking ET dysfunction, neither natural exposure to nasal allergens, or experimental nasal exposures to allergens and/or associated inflammatory chemicals caused OME.[5-11]

Hydrops ex vacuo is a mucosal response to abnormal ME pressure-regulation that is well described for animals with persistent ET dysfunction and humans with ET dysfunction caused by experimental respiratory virus infection.[4] Because the ME is a mucosa lined, non-collapsible, gas cavity that exchanges gases continuously with the mucosal blood and intermittently with the nasopharynx during transient ET openings, its total pressure is determined by the rate of gas exchange across these pathways. At normal (ambient) pressure, the ME gas partial-pressures of O_2, CO_2 and H_2O are in approximate equilibrium with local blood, but the N_2 partial-pressure exceeds that of blood by approximately 50 mmHg.[4] This gradient has the net effect of driving total ME pressure to equilibrium with blood, an underpressure not realized because either the periodic openings of the ET allow for gas flow from nasopharynx to ME and reestablishes near ambient pressure, or hydrostatic pressures within the mucosa

cause capillary dilatation, mucosal swelling and the leakage of transudative fluids into the ME. The provoked ME pathology is not recognizably different from the clinical presentation of OM, leading many to suggest identity of the two disease expressions.

While long recognized as a consequence of ET dysfunction, *hydrops ex vacuo* has only recently been redefined as a disturbance in ME pressure-regulation caused by either decreased gas supply (ET dysfunction) or increased gas demand (increased ME-blood N_2 exchange). As discussed above, nasal inflammation decreases ME gas supply (ET dysfunction) and the results of the present study suggest that nasal inflammation also increases the ME demand for gas (increased transmucosal inert gas exchange).

The higher rate of transmucosal inert gas exchange reported in this study was anticipated from the results of two earlier studies that showed nasal antigen challenge in sensitized animals to provoke increased ME and ET mucosal blood flow[12, 13]; (see Equation 4). Ideally, transmucosal N_2 exchange would have been measured, but this is not feasible given the extremely slow rate of N_2 exchange measured in earlier experiments.[14] The inert gas, N_2O was used as a surrogate for N_2 because, like N_2, transmucosal N_2O exchange is perfusion-limited, its exchange rate is much faster than N_2 (much greater solubility in blood, see EQ4) and the N_2O time-constant can be converted to that for N_2 by a simple, linear transformation.[14]

At present, it is not known if the documented effects of nasal inflammation on mucosal blood flow and transmucosal inert gas exchange are simply interesting physiological observations or play a contributing role in the pathogenesis of OME. However, one envisioned scenario has the inflammatory chemicals released during nasal allergic inflammation maintaining a high ME mucosal blood flow, a correspondingly high N_2 exchange rate and a consequent increased demand for gas replacement during ET openings. While constitutive ET function may be sufficient to satisfy this level of demand, impaired function caused by allergy, viral respiratory infection or other causes may not. This and other possibilities derived from the results of this experiment provide new leads for the design of future studies that focus on mechanistic links between allergy and OME.

Acknowledgement

TABLE 1
Transmucosal N2O Time-Constants (/min) Measured for the Left (L) and Right (R) Ears of all Animals (#) for the Baseline and Nasal Challenge Sessions

#	EAR	BASELINE	SALINE	CAPSACIAN	PGD2	BRADYKININ
37	L	0.016	0.023	0.017	0.033	0.019
38	L	0.020	0.032	0.027	0.023	0.040
39	L	0.015	0.015	0.009	0.025	0.029
40	L	0.008	0.007	0.012	0.034	0.033
41	L	0.022	0.024	0.030	0.031	0.041
44	L	0.016	0.021	0.024	0.049	0.027
46	L	0.020	0.025	0.024	0.026	0.019
47	L	0.025	0.029	0.027	0.033	0.047
37	R	0.018	0.020	0.017	0.078	0.026
38	R	0.021	0.026	0.024	0.037	0.023
39	R	0.014	0.013	0.021	0.038	0.025
40	R	0.016	0.010	0.012	0.052	0.031
41	R	0.023	0.025	0.029	0.034	0.024
44	R	0.020	0.027	0.031	0.071	0.036
46	R	0.031	0.027	0.033	0.035	0.034
47	R	0.022	0.028	0.023	0.028	0.062
AVG	T	0.019	0.022	0.023	0.039	0.032
STD		0.005	0.007	0.007	0.015	0.011
P value		-------	------	0.681	0.002	0.002

ME pressure (referenced to ambient) as a function of time (a) and the rate of change in ME pressure as a function of ME pressure (b) for the right (circles) and left (squares) ears of animal 46 at the baseline (open) and saline challenge sessions (filled). Solid lines represent solutions to linear regression.
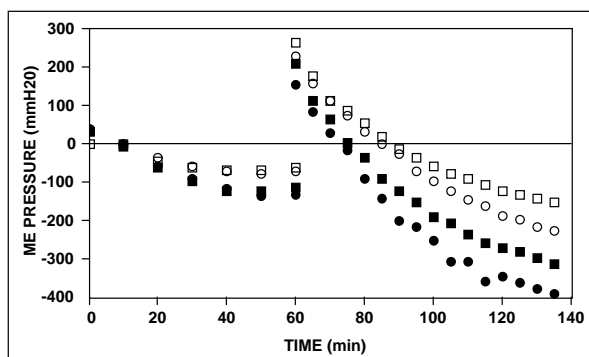
FIGURE 1a
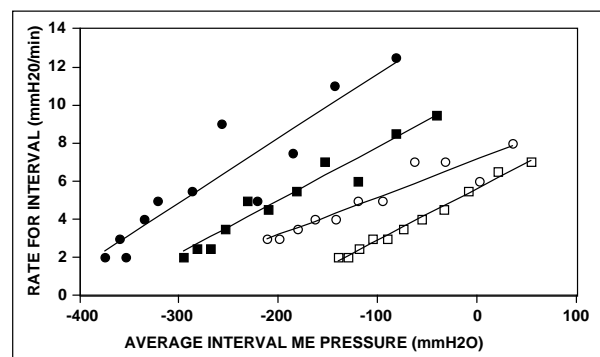
FIGURE 1b

References

1. Kvaerner KJ, Tambs K, Harris JR, Mair IW, Magnus P. Otitis media: relationship to Tonsillitis, sinusitis and atopic diseases. Int J Pediatr Otorhinolaryngol 1996;35:127-141.
2. Bernstein JM, Lee J, Conboy K, Ellis E, Li P. Further observations on the role of IgE-mediated hypersensitivity in recurrent otitis media with effusion. Otolaryngol Head Neck Surg 1985;93:611-615.
3. Doyle WJ, Takahara T, Fireman P. The role of allergy in the pathogenesis of otitis media with effusion. Arch Otolaryngol 1985;111:502-506.
4. Doyle WJ. The link between allergic rhinitis and otitis media. Curr Opin Allergy Clin Immunol 2002;2:21-25.
5. Osur SL, Volovitz B, Dickson S, Enck DC, Bernstein JM. Eustachian tube dysfunction in children with ragweed hay fever during natural pollen exposure. Allergy Proc 1989;10:133-139.
6. Knight LC, Eccles R, Morris S. Seasonal allergic rhinitis and its effects on eustachian tube function and middle ear pressure. Clin Otolaryngol 1992;17:308-312.
7. Friedman RA, Doyle WJ, Casselbrant ML, Bluestone C, Fireman P. Immunologic-mediated eustachian tube obstruction: a double-blind crossover study. J Allergy Clin Immunol.1983;71:442-447.
8. Doyle WJ, Skoner DP, Seroky JT, Fireman P. Reproducibility of the effects of intranasal ragweed challenges in allergic subjects. Ann Allergy 1995; 74:171-176.
9. Walker SB, Shapiro GG, Bierman CW, Morgan MS, Marshall SG, Furukawa CT etal. Induction of eustachian tube dysfunction with histamine nasal provocation. J Allergy Clin Immunol 1985;76:158-162.
10. Doyle WJ, Boehm S, Skoner DP: Physiologic responses to intranasal dose-response challenges with histamine, methacholine, bradykinin and prostaglandin in adult volunteers with and without nasal allergy. J Allergy Clin Immunol 1990; 86:924-935.
11. Vayonis AG, Seroky JT, Doyle WJ, Skoner DP, Fireman P. Intranasal Histamine, Methacholine and Bradykinin Challenge in Children with and without Allergy. Am J Rhinology 1995; 9:1-7.
12. Tanner EP, Skoner DP, Doyle WJ, Fireman P:Intranasal challenge of cynomolgus monkeys with ascaris, ragweed, bradykinin and histamine. Am J Rhinol 1990; 4:33-37.
13. Seroky JT, Alper CM, Tabari R, Doyle WJ. Effects of intranasal challenge with histamine, bradykinin and prostaglandin on middle ear pressure and blood flow in cynomolgus monkeys. Acta Otolaryngol 1995; 115:83-87.
14. Doyle WJ, Seroky JT, Alper CM. Gas exchange across the middle ear mucosa in monkeys: Estimation of exchange rates. Arch Otol Head Neck Surg 1995; 121:887-892.
15. Bernstein JM, Doyle WJ. Role of IgE-mediated hypersensitivity in otitis media with effusion: pathophysiologic considerations. Ann Otol Rhinol Laryngol 1994;163:15-19.

Interaction of pneumococcal phase variation and middle ear pressure/gas composition: An *in vitro* model of otitis media pathogenesis

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Background

Streptococcus pneumonia, a leading cause of otitis media (OM), undergoes spontaneous intrastrain variations in colony morphology. Transparent (T) variants are more efficient in colonizing the nasopharynx (NP), while the opaque (O) variants exhibit greater virulence during systemic infections. Because the middle ear (ME) is a fixed- volume, air-filled cavity, its pressure is determined by contained gas volume, and processes that affect gas volume such as ME-blood gas diffusion and NP-ME gas transfers during eustachian tube (ET) opening change both ME gas volume (pressure) and composition. It is possible that changes in ME gas volume/composition during ET dysfunction and treatment for that dysfunction (tympanostomy tube (TT) insertion) play a role in selecting the *S. pneumonia* variant that can efficiently colonize/infect the ME mucosa.

Aim

This study tested that hypothesis.

Methods

Human middle-ear epithelial cells were: preconditioned for 24 hours under one of three different ME gas/pressure conditions designed to reflect those for the 1) normal ME, 2) ME with ET dysfunction, and 3) ME with patent tympanostomy tubes (TT); subsequently exposed to a fixed dose of ($\sim 10^7$ CFU/ml) of either transparent (T) or opaque (O) *S. pneumoniae*, and then incubated for 1 and 3 hours. Host cell viability, bacterial growth, phenotypic variation, adhesion, invasion, and associated virulence gene expression were assessed.

Results

Viability of the T-variant–infected epithelial cells under conditions 2 and 3 decreased with time, whereas O-variant–infected epithelial cells exposed to those conditions continued to proliferate. For condition 2, the T variants exhibited a higher growth rate and greater percent adherence to ME epithelial cells than did O variants. Different pathogen-host interactions for these variants and conditions were documented by scanning and transmission electron microscopy. Genes coding for selected virulence factors (lytA, psaA, pspA, spxB, and ply) were differentially induced in T-variant–infected epithelial cells that were exposed to the three gas/pressure environments.

Conclusions

The link between the ME gas/pressure environment and the selection of phenotype variants for *S. pneumoniae* was demonstrated, and the differential expression of virulence factors during pathogen-host interactions was established *in vitro*. The ME mucosal response to pneumococcal infection is a complex process involving interaction of the pathogen with the extant local pressure/gas environment. These data support the T-variant as being a key player in pneumococcal OM pathogenesis.

Acknowledgements

Trans-mucosal gas exchange rate of the middle ear is nitrogen dependent

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The role of nitrogen (N2) in the transmucosal gas exchange of the middle ear (ME) was studied using an experimental rat model to measure gas volume variations in the ME cavity at constant pressure. The steady state gas composition was disturbed with either air or N2 to measure resulting changes in volume at ambient pressure. Changes in gas volume over time could be characterized by three phases: a primary transient increase with time (phase I), followed by a linear decrease (phase II), then a gradual decrease (phase III). The mean slope of phase II was –0.128 $\mu L \cdot min{-1} \pm 0.023SD$ in the air group (n=10) and –0.105 $\mu L \cdot min{-1} \pm 0.032SD$ in the N2 group (n=10), but the difference was not significant (p=0.13), which suggests that the rate of gas loss can be attributed mainly to the same steady state partial pressure gradient of N2 reached in this phase. Further, a mathematical model was developed analyzing the transmucosal N2 exchange in phase II. The model takes gas diffusion into account, predicting that, in the absence of change in mucosal blood flow rate, gas volume in the ME should show a linear decrease with time after steady state conditions and gas composition are established. In accordance with the experimental results, the mathematical model also suggested that transmucosal gas absorption of the rat ME during steady state conditions is governed mainly by diffusive N2 exchange between the ME gas and its mucosal blood circulation.

Potential for preventing otitis media as a complication of viral upper respiratory tract infections

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Introduction

Rhinovirus infection is the major cause of acute upper respiratory tract illness and young children suffer from 5 to 7 rhinovirus infections per year. No antirhinoviral treatment or vaccine development is on the horizon. In contrast to bacteria, respiratory viruses are not commensal flora in the nasopharynx and cause infections in non-immune individuals.[1,2] Illness expression during viral infections in adults varies remarkably with a spectrum ranging from no symptoms to acute viral sinusitis, whereas children seem more prone to viral otitis media.[3,4] There is a large body of data supporting a causal relationship between viral upper respiratory tract infections and the onset of new otitis media episodes.[5-8]

Prevention of the incidence of acute otitis media by bacterial vaccination against common otopathogens has demonstrated an overall reduction of less than 10% of AOM episodes.[9] Prevention of acute otitis media with influenza vaccine has, on the other hand, demonstrated an impressive reduction of 33-36% in the number of AOM during the influenza season when compared to placebo.[10,11] In addition, early treatment with an oral neuraminidase inhibitor in children with influenza infections has shown a 40% reduction in the development of AOM during influenza illness.[12] This suggests that respiratory viruses may have a higher impact on the pathogenesis of AOM than previously anticipated. Otitis media may be avoidable by preventing viral infections, modifying expression or infection, or interfering with the mechanism for OM pathogenesis during a viral respiratory tract infection.

The overall lack of knowledge regarding the symptom profile of children with colds is simply astonishing.[13] During the past four years, we have performed observational cohort studies with 60 children in 30 families per year. One of the objectives was to assess the potential of prevention strategies for development of AOM during colds. The prevention strategies we considered were: 1) prevention of infectious exposure; 2) prevention of illness expression by upregulation of biologic filters to decrease symptom development during infection; 3) antiviral treatment to decrease viral load; and 4) prophylaxis for complication of OM.

Study design

Each year, an observational cohort of 30 families with 2 young children (age 1 to 5 years) were identified by advertisement and enrolled by October 1st and followed for 7 months during the fall, winter and spring seasons. Parents recorded their children's respiratory signs and symptoms on diaries and measured middle ear pressure by tympanometry each day. Otoscopy was performed weekly and nasal secretions for virology testing were obtained from both siblings when one child had a new cold.[14] Rhinovirus, RSV, Coronavirus 229E and OC43, Parainfluenza 1-3, Influenza A and B and adenovirus genome were examined by PCR[1] Cold-like illness was defined as more than two consecutive days with a positive report by the parents of cold symptoms separated from other episodes of at least seven "cold-free" days. A new OM was assigned when two previous weekly otoscopies had been normal. The length of a new OM episode was estimated as the number of days from the new OM diagnosis to the first non-OM observation by otoscopy. The criteria for diagnosing AOM was based on otoscopic changes of the tympanic membrane suggestive for acute inflammation combined with a history of new onset of upper respiratory symptoms.[15]

Results

The incidence of cold-like illnesses per month in 100 children were highest in November and December (60%), gradually declining to 35% in April [Figure 1A], whereas the incidence of OME in the same cohort was relatively constant between 25-32% from November through May. The incidence of AOM peaked in February [Figure 1B].

Cold viruses were commonly transmitted within the family. Siblings frequently expressed cold-like illness simultaneously (18-21.7%). Detection of a clear index case and a secondary case occurred in 25-30% of 69 siblings [Figure 2]. The transmission of colds between children did not occur immediately, but over a 10 day period [Figure 3]. The median cold

transmission time was 3 days.

The overall incidence of OM during cold-like illness was 73.2% in the youngest sibling and 67.4% in the older sibling [Figure 4]. The daily monitoring of middle ear pressure by tympanometry showed changes towards more negative middle ear pressure prior to the onset of a new cold-like illness [Figure 5].

Detection of respiratory viruses in nasopharyngeal secretions from children with OM showed that a fourth to a third had a viral infection without simultaneous expression of cold-like illness. The distribution of different respiratory viruses detected with new OM episodes was not different from that for colds.

Summary

Our data of normal children ages 1-5 years show that about 40% of colds involve OM episodes, and that about 70% of new OM episodes occur during colds. Rhinovirus infections are manifested by OM episodes without cold-like illness in about 1/4 to 1/3 of OM episodes.

Conclusion

The efficiency and efficacy of intervention strategies to prevent OM as a complication of a viral URI are limited by the frequency absence of signaling event and short time between signal and OM. Therefore, high efficacy strategies must focus on prevention or treatment of rhinovirus colds in order to decrease OM episodes, and high efficiency strategies require a target population with a high risk of OM episodes.

Acknowledgements
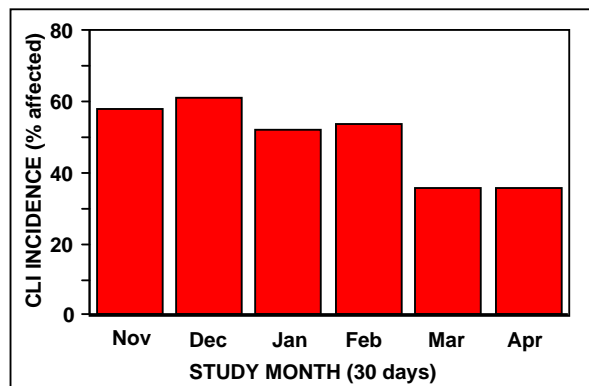
Figure 1A. Incidence of cold-like illness in 100 children during one season
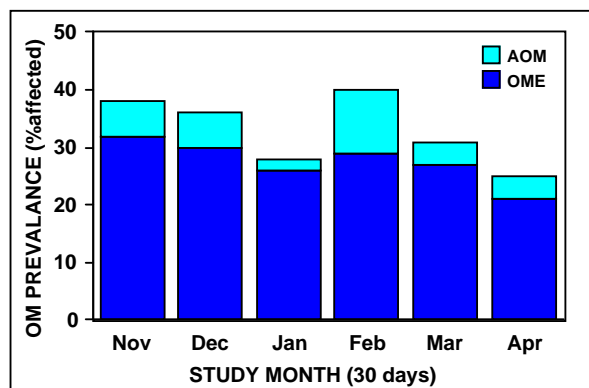
Figure 1B. Incidence of OME and AOM in 100 children during one season
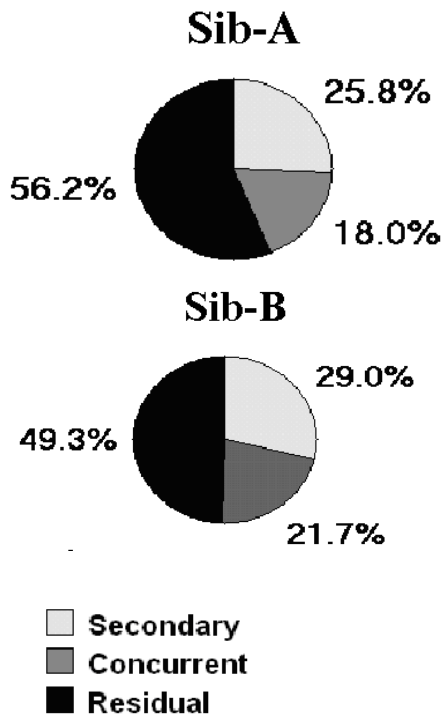
Figure 2. Relative relationship between index case and secondary cases among siblings.

Figure 3. The time frame for transmission of cold-like illness from index case to secondary case.

Figure 4. Relative proportion of OM in relation to the present of cold like illness in 69 sibs.
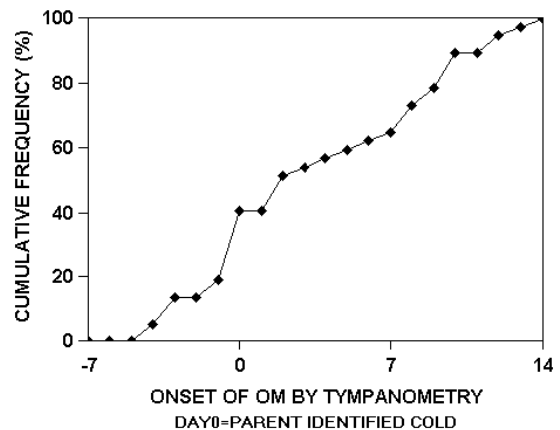
Figure 5. Occurrence of "flat tympanograms" in relation to onset of cold like illness. N= 37 ears.

OM DURING CLI (N=55)   **OM WITHOUT CLI (N=24)**
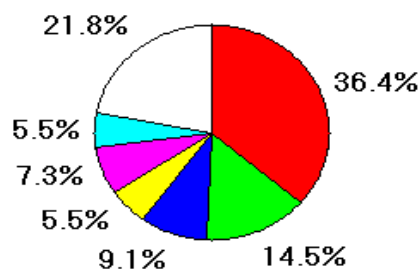
OM DURING CLI (N=55)   **OM WITHOUT CLI (N=24)**
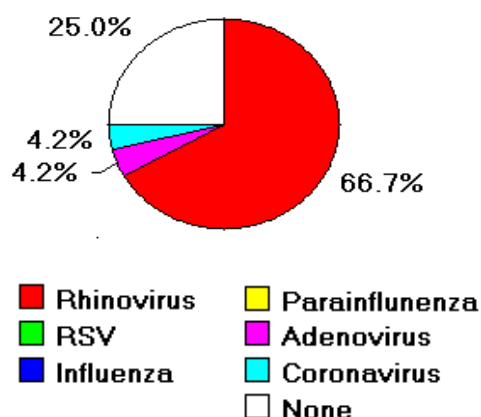
Figure 6. Relative proportion of different respiratory viruses from nasopharyngeal secretion in 60 children with OM and with or without simultaneous presence of cold-like illness

References

1. Winther B, Hayden FG, Hendley JO. Picornavirus infection in children diagnosed by RT-PCR during longitudinal surveillance with weekly sampling: association with symptomatic illness and effect of season. J Med Virol 2006;78:644-650.
2. Chi DH, Hendley JO, French P, Arnago P, Hayden FG, Winther B. Nasopharyngeal reservoir of bacterial otitis and sinus pathogens in adults during wellness and viral respiratory illness (VRI). Am J Rhinology 2003;17:209-214.
3. Pitkaranta A, Arruda E, Malmberg H, Hayden FG. Detection of rhinovirus in sinus brushings of patients with acute community acquired sinusitis by reverse transcription-PCR. J Clin Microbiol 1997;35:1791-1793.
4. Pitkaranta A, Virolainen A, Jero J, Arruda E, Hayden FG. Detection of rhinovirus, respiratory syncytial virus and coronavirus infection in acute otitis media by reverse transcriptase polymerase chain reaction. Pediatrics 1998; 102:291-295.
5. Wald ER, Guerra N, Byers C. Upper respiratory tract infections in young children: duration of and frequency of complications. Pediatrics 1991;87:129-133.
6. Hendley JO. Otitis media. New Engl J Med 2002; 347:1169-1174.
7. Vesa S, Kleemola M, Blomqvist S, Takala A, Kilpi T, Hovi T. Epidemiology of documented viral respiratory infections and acute otitis media in a cohort of children followed from two to twenty-four months of age. Ped Infect Dis J 2001;20:574-581.
8. Winther B, Alper CM, Mandel EM, Doyle WJ, Hendley JO. Temporal relationship between colds, upper respiratory viruses detected by polymerase chain reaction and otitis media in young children followed through a typical cold season. Pediatrics 2007; 119:1069-1075.
9. Eskola J, Kilpi T, Palmu A, Jokinen J, Haapakoski J, Herva E, et al. Efficacy of a pneumococcal conjugated vaccine against acute otitis media. New Engl J Med 2001; 344:403-409.
10. Heikkinen T, Ruuskanen O, Waris M, Ziegler T, Arola M, Halonen P. Influenza vaccination in the prevention of acute otitis media in children. Am J Dis Child 1991; 145:445-448.
11. Clements DA, Langdon L, Bland C, Walter E. Influenza A vaccine decreases the incidence of otitis media in 6-30 month old children in daycare. Arch Pediatr Adolesc Med 1995; 149:1113-1117.
12. Winther B, Hayden FG, Whitley R, Reisinger K, Dutkowski R, Ward P, et al. Oseltamivir reduces the risk of developing acute otitis media following influenza infection in children. Fortieth annual ICAAC, Toronto, Ontario, Canada, September 2000, abstract # 977.
13. Pappas D, Hendley JO, Hayden FG, Winther B. Symptom profile of common colds in schoolage children. Pediatr Infect Dis J 2008; 27:8-11.
14. Winther B, Doyle WJ, Alper CM. A high prevalence of new onset otitis media during parent diagnosed common colds. Int J Ped Otorhinolaryngol 2006: 70:1725-1730.
15. Alper CM, Winther B, Mandel EM, Doyle WJ. Temporal relationship for cold-like illnesses and otitis media in sibling pairs. Pediatr Infect Dis J 2007; 26:778-781.

Synergy of Id1 and NF-κB in the proliferation and migration of cholesteatoma keratinocytes

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A hallmark of cholesteatoma is proliferation and migration of keratinocytes in the middle ear. This study demonstrates that Id1 (a transcription factor that counters cell differentiation) and nuclear factor kappa B (NF-kB, a key pro-inflammatory and immune transcription factor) were both highly expressed in middle-ear cholesteatoma specimens, and both synergistically promoted the proliferation and migration of keratinocytes *in vitro*. Their actions include the upregulation of the cell division cycle (Cdc) and cyclin D1 that drives cell cycle progression, coupled to the downregulation of p16[Ink4a] that normally inhibits cell cycle progression, and the upregulation of extracellular matrix proteins important in cellular migration. Specifically, Id1 increased the expression of vascular endothelial growth factor (VEGF), inteleukin-8 (IL-8), epithelial membrane protein 2, collagens (type VI), and intercellular adhesive molecules (CD54), laminin (LAMA1), and fibronectin (FN1), which are involved in angiogenesis, cell adhesion, and migration. NF-kB upregulated the expression of cyclin D1 and downregulated the expression of p16[Ink4a], leading to cell cycle progression. Id1 and NF-kB may serve as new therapeutic targets of cholesteatoma.

Materials and methods

Cell line. The Rhek-1A cell line, representative epidermal keratinocytes[1], was maintained in Eagle's minimal essential medium (MEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (50 μg/mL), and 2 mM L-glutamine (full growth medium, FGM). During transient transfection of cells, Opti-MEM supplemented with 6 μg/mL of Polybrene® (transfection medium, Invitrogen) was used. Rhek-1A was chosen because it is derived from human skin keratinocytes as is cholesteatoma.

Stable transfection of Rhek-1A cells with Id1, p65, and Id1+p65. Id1 cDNA was constructed as previously described.[2] The p65 cDNA construct was a gift from the Dr. Frank Ondrey lab at the Cancer Center, University of Minnesota. Cells cultured in T25 flasks with 60% confluency were transfected with

pEGFP (plasmids with enhanced green fluorescent protein, empty vectors), Id1, p65, and Id1+p65 cDNA at 1.4 μg/mL for induction of corresponding protein expression. After transfection, cells were incubated with FGM for one week and submitted for selecting the transfected cells (GFP^{+} cells) via a cell sorter (FACsMaria[TM], BD Biosciences). After two~three rounds of selecting and continuously culturing for >6 months, cells remaining GFP[+] under a flow cytometry were regarded as stably transfected cells and submitted for microarray analyses.

Affymetrix microarrays. Experiments of Affymetrix microarrays were performed as previously described.[3] Briefly, cDNA, prepared from 10 μg total RNA using the double-strand DNA synthesis kit (Life Technologies, Rockville, MD), was reverse transcribed into cRNA and labeled with biotin-streptavidins using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY). Biotin-streptavidin labeled cRNA was then hybridized to the human U133A arrays. Intensity of global gene expression on the U133A arrays was normalized to a battery of housekeeping genes using Affymetrix software 4.0. U133A microarray data were uploaded onto GeneSifter in a SMA format (vizXlabs, a normalization of gene expression within chips). A comparison between pEGFP, Id1, p65, Id1+p65 was made to extract genes that had been changed more than 1.5-fold. A pathway analysis tool (PEGG) was used for evaluation of biological changes over all the genes (>1.5 fold) to determine which pathways are affected in specimens in comparison with controls (pEGFP). Genes changed <1.5 folds were generally considered as technical reasons and not included for analysis. Once the entire pathway was analyzed with PEGG, important genes in that particular pathway were individually analyzed using *t*-test (embedded in GeneSifter), with p value less than 0.05 being considered significant.

Results

It was found that the Id1 gene was involved in the upregulation of vascular endothelial growth factor

(VEGF) and inteleukin-8 (IL-8) compared with controls (pEGFP). Id1 also upregulated the expression of cell adhesion molecules such as epithelial membrane protein 2, collagens (type VI), and intercellular adhesive molecules (CD54) compared with controls. In addition, extracellular matrix proteins such as laminin (LAMA1) and fibronectin (FN1) genes were highly upregulated by Id1. P65, a heterodimer of the NF-κB transcription factor, is mainly involved in the regulation of cell cycle progression-related genes, upregulating cyclin D1 and proliferating cell nuclear antigen (PCNA) but downregulating p16Ink4a.

Discussion

Abundant angiogenesis, aggressive proliferation, and aggressive migration are characteristic features of middle-ear cholesteatoma keratinocytes. What causes these changes is not clearly understood at the present time. Id1, induced by middle-ear infection[4], is involved in middle-ear epithelial metaplasia[5], which may participate in the aggressive behaviors of middle-ear cholesteatoma. These prompted us to examine whether Id1 plays a role in the aggressive proliferation of keratinocytes, as our microarray data indicated that Id1 is related to the expression of angiogenic factors (VEGF and IL-8), adhesive molecules (epithelial membrane protein 2, collagens (type VI), intercellular adhesive molecules (CD54), and extracellular matrix proteins (LAMA1 and FN1). Our latest studies indicated that Id1 regulates the promoter activity of both VEGF and IL-8, suggesting that Id1 is a major transcription factor that controls the proliferation of new blood vessels in the middle-ear mucosa. While p65 regulated the proliferation of keratinocytes, it is frequently observed in the clinic that middle-ear cholesteatoma tissues frequently "expand" into the neighborhood and cause complications if not treated timely. It is suggested in this study that the Id genes play a role in the middle-ear submucosal pathologies: production of abundant extracellular matrix proteins plus proliferation of new capillary brood vessels, which lays a ground for the granulation tissue formation and subsequent fibrosis. It is well accepted that chronic otitis media is frequently accompanied by granulation tissues or fibrotic disorders. In our most recent studies, Id1 regulated the promoter activity of NF-κB by luciferase assays[2] and activation of NF-κB by immunohistochemistry.[6] NF-κB then increases the expression of cyclin D1 via regulating the cyclin D1 promoter activity. Cyclin D1, together with cyclin-dependent kinases (4 and 6), promotes the cell cycle progression in keratinocytes.[2] The process is associated with the expression of proliferating cell

nuclear antigen (PCNA).[6] Microarray data analyses also indicated that Id1 activates the signaling pathway of transforming growth factor-beta (TGF-β). TGF-β is known as a trigger for fibrotic disorders by increasing extracellular matrix protein production. TGF-β is perhaps linked to the production of collagen VI, LAMA1, and FN-1 in this study. This may explain why middle-ear cholesteatoma is almost always accompanied by granulation tissues. On the other hand, p65 increased the proliferation of keratinocytes. With the assistance of angiogenic factors, new blood vessels were continuously formed, which further supports the aggressive behaviors of keratinocytes in cholesteatoma. Further studies regarding the effects of Id genes on granulation tissue formation is warranted.

Acknowledgements

References

1. Gamou S, Shimizu N. Change in metabolic turnover is an alternate mechanism increasing cell surface epidermal growth factor receptor levels in tumor cells. J Biol Chem 1987; 262:6708-13.
2. Hamajima Y, Komori M, Preciado DA, et al. The role of inhibitor of DNA-binding (Id1) in hyperproliferation of keratinocytes: the pathological basis for middle ear cholesteatoma from chronic otitis media. Cell Prolif 2010; 43:457-463.
3. Lee Y-W, Ozeki M, Juhn SK, Lin J. Expression of platelet-derived growth factor in the developing cochlea of rats. Acta Otolaryngol 2004; 124:558-562.
4. Lin J, Tsuboi Y, Pan W, Giebink SG, Adams GL, Kim Y. Analysis by cDNA microarrays of altered gene expression in middle ears of rats following penumococcal infection. Int J Pediatr Otorhinolaryngol 2002; 65:203-211.
5. Hamajima Y, Toyama K, Kim Y, Lin J. Id1 induces epithelial cell hyperplasia in the middle ear of rats. In: Lim DJ, Bluestone CD, Klein JO, Nelson JD, eds. *Recent Advances in Otitis Media*. Hamilton, Ontario: B. C. Decker, 2003.
6. Zhang QA, Hamajima Y, Zhang Q, Lin J. Identification of Id1 in acquired middle ear cholesteatoma. Arch Otolaryngol Head Neck Surg 2008; 134:306-310.

The Sap transporter is critical for the commensal and pathogenic behavior of nontypeable *Haemophilus influenzae* (NTHi)

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Nontypeable *Haemophilus influenzae* (NTHi) persists in the host airway as a commensal microorganism and yet is able to cause diseases of both the upper and lower respiratory tracts. We have previously shown that the Sap (sensitivity to antimicrobial peptides) operon gene products mediate NTHi resistance to antimicrobial peptides (APs), in part by SapA binding APs and subsequent up-regulation of sap gene transcription. We further show that the putative Sap transporter ATPase protein, SapD, is required for AP resistance and potassium uptake in NTHi. Importantly, NTHi mutants impaired in Sap transporter function are rapidly cleared in a chinchilla model of otitis media (OM), indicating a role for the Sap transporter as a critical virulence factor in the pathogenesis of experimental OM. We hypothesize that NTHi couples a mechanism of AP resistance with rapid potassium uptake to counter potassium loss and thus oppose AP lethality. Here, we extended our functional studies and showed that components of the Sap transporter played a direct role in potassium acquisition in NTHi, thus providing a critical and necessary function to mediate NTHi survival in response to attack by key components of the host's innate immune response. We further showed that SapA bound the iron-containing compound, heme. An NTHi mutant lacking SapA displayed an iron-starved phenotype, characterized by up-regulation of multiple iron acquisition systems. Our observations suggested that the Sap proteins may play an additional role in iron acquisition pathways of NTHi, likewise essential to its survival in the iron-limited environments of the host. Thus, we've shown that components of the Sap transporter equip this commensal for AP resistance, for potassium uptake, for iron homeostasis, and for survival in vivo. These data describe a critical role for the Sap transporter in NTHi survival strategies, which may prove to be similarly essential in other diverse mucosal pathogens of clinical importance.

Expression of platelet-activating factor receptor and nitric oxide synthases in adenoid tissues of children with middle ear effusion

Kyung Wook Heo, M.D., Seong Kook Park, M.D., Ph.D., Sae Gwang Park, M.D., Ph.D., Tae Hee Han, M.D.

Introduction

Otitis media with effusion (OME) is one of the most common diseases, and one of the most frequent causes of hearing loss, in children. The pathogenesis of OME, however, is still not fully understood.[1]

The role of adenoids in middle ear disease is complex. The removal of large adenoids results in better effusion resolution than removing smaller adenoids, suggesting the importance of mechanical obstruction of the Eustachian tube.[2] Recurrent or chronic infection in the adenoids without obstructive hypertrophy, however, may also manifest as recurrent acute otitis media, persistent OME, or chronic rhinosinusitis, indicating that the adenoids are reservoirs of pathogenic organisms leading to tubal edema and malfunction.[3]

Nitric oxide (NO) is a simple inorganic radical that is produced in many cells by different isoforms of the NADPH-dependent enzyme, NO synthase (NOS). Three isoforms of NOS have been characterized to date. The endothelial (eNOS) and neuronal (nNOS) types are constitutively active isoforms that release trace quantities of NO and are involved in signal transduction mechanisms, such as those responsible for regulating vascular tone and neurotransmission. Inducible NOS (iNOS) is usually not expressed constitutively, but can be induced in almost every type of cell by stimulation with bacterial lipopolysaccharide (LPS) and cytokines.[1] Since NO can react with superoxide, sustained NO release may be injurious to healthy tissue and may be responsible for certain pathologic conditions.[4] NO is thought to mediate OME, including mucin secretion in endotoxin-induced OME.[5]

Platelet-activating factor (PAF) is a biologically active phospholipid that is elevated in many hypersensitivity and inflammatory diseases.[6] PAF transmits signals between cells, thus functioning similarly to hormones, cytokines, interleukins, and other signaling molecules.[6] PAF is not stored in most cells, but is made only upon activation. The biological effects of PAF are mediated by binding to a specific transmembrane PAF receptor, which belongs to the family of G-protein-coupled receptors.[7] Moreover, PAF appears to be important in acute inflammatory processes occurring in otitis media.[8,9]

To determine the role of adenoid tissues in the pathogenesis of OME, we have assayed the expression of PAF receptor and NOS isoforms in hypertrophied adenoids of children with OME.

Materials

Fourteen children who underwent consecutive adenoidectomy and/or tonsillectomy with or without tympanostomy tube insertion were enrolled in this study. Informed consent was obtained from each child and his/her parents, and the study was approved by the Institutional Review Board. Of these 14 children, nine (OME group) had received simultaneous tympanostomy tube insertion for microscopic findings compatible with OME, B or C type tympanogram, and/or conductive hearing loss greater than 20 dB lasting at least three months despite proper medical treatment. The remaining five children, who underwent only adenoidectomy and/or tonsillectomy, were considered as the control group.

Adenoid tissue was surgically removed from each patient presenting with adenoid hypertrophy (AH) secondary to nasal obstruction, snoring, mouth breathing, and pausing of breathing during sleep. AH was defined radiologically as an adenoidal-nasopharyngeal ratio of >0.6.[10] Before surgery, each child underwent routine ear, nose, and throat examination, as well as paranasal sinus and neck lateral X-rays. Subjects with sinusitis or nasal polyps were excluded, as were those who had received any steroids (systemic or topical), non-steroidal anti-inflammatory drugs, antihistamines, leukotriene receptor antagonists, or macrolide antibiotics in the month prior to this study.

Methods

Western blotting

Adenoid tissues were ground using a microtissue grinder in homogenization buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% SDS, 1 mM DTT and 1 mM PMSF). A 0.3 mg protein aliquot of each sample was denatured at 100oC for 5 minutes. The samples were electrophoresed on 10% SDS-PAGE gels and electrophoretically transferred to nitrocellulose membranes for one hour. The membranes were washed with 2% PBST for five minutes, blocked overnight with blocking buffer at room temperature, and incubated with 0.1 ng/ml polyclonal antibody to PAF receptor, iNOS, or eNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed with 2% PBST for five minutes and incubated with secondary antibody prior to chemiluminescence detection using ECL reagent according to the manufacturer's recommendations (Amersham, Buckinghamshire, UK). All data were normalized to the actin content of the same sample.

RNA isolation and cDNA preparation

RNA was isolated using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD, USA) according to the manufacturer's protocol. One μgof total RNA was reverse transcribed using MMLV-Reverse Transcriptase (Promega, Madison, WI, USA) using oligo-dT$_{16-18}$ primers.

Primer sequences

The cDNA sequences of the genes of interest were obtained from GeneBank. The primer sequences used for real-time PCR analysis were those recommended by Primer Bank.

Real-Time PCR

Quantitative RT-PCR was performed on an iCycler machine (Bio-Rad, Hercules, CA, USA) using SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The amplification protocol consisted of an initial 10-second denaturing step, followed by 45 cycles of denaturation for 10 seconds at 95oC, annealing for 15 seconds at the annealing temperature specified for each gene, and extension for 30 seconds at 72oC; melting point analysis was conducted in 0.1oC steps, followed by a final cooling step. Each reaction mixture consisted of 10 μl of Supermix, 2 μl of cDNA, 7 μl of H2O, and 1 μl of primer mix (5 μM of each primer). The final amount of cDNA per reaction corresponded to 10 ng of total RNA used for reverse transcription. The mRNA levels of PAF receptor, eNOS, and iNOS were calculated as logarithmic values or log10 transformation of raw data and further normalized to the level of GAPDH mRNA. Data were expressed as the ratio between the samples and GAPDH.

Statistical analysis

All results are reported as mean ± SD. Between group differences were analyzed using the Mann-Whitney U-test. A p value of < 0.05 was considered statistically significant.

Results

Expression of PAF receptor, eNOS, and iNOS protein

The PAF receptor/actin protein ratio was significantly higher in the OME group than in the control group (0.326 ± 0.135 vs. 0.132 ± 0.038 P = 0.014), whereas the eNOS/actin protein ratio was significantly lower (0.043 ± 0.049 vs. 0.238 ± 0.066 P = 0.003). Adenoid tissues from both the OME and control groups showed no expression of iNOS protein.

Quantification of GAPDH mRNA

Expression of GAPDH mRNA, a housekeeping gene, was used as an internal control for expression of PAF receptor, eNOS, and iNOSmRNA. The molecular mass of each RNA standard was calculated based on its sequence, and solutions containing 102 to 108 copies of standard GAPDH mRNA were made. Sample cycle threshold (CT) values were determined from plots of relative fluorescence units (RFU) versus PCR cycle number during exponential amplification, in order to make comparisons between sample measurements. The standard curve represents one of the experiments showing a linear correlation between the log of the matrix gene copy number and the CT, with a regression line showing a slope of 3.297. The correlation coefficient of determination was calculated as 95.7%, and the copy numbers for sample quantification ranged from 102 to 106. These values were considered as reliable for quantitative analyses.

Expression of PAF receptor, eNOS, and iNOSmRNA

Expression of specific mRNA in these samples was quantified using a standard control curve constructed from cloned PCR products. The resultant slopes of the standard curves for PAF receptor, eNOS, and iNOS were 3.410, 3.660, and 3.459, respectively, and their PCR correlation coefficients were 99.3%, 99.3%, and 99.9%, respectively. The PAF receptor/GAPDH mRNA ratio was similar in the OME and control groups (0.912 ± 0.206 vs. 1.028 ± 0.275 $P = 0.162$), as was the eNOS/GAPDH mRNA ratio (1.029 ± 0.037 vs. 0.830 ± 0.240 $P = 0.72$) and iNOS/GAPDH mRNA ratio (0.897 ± 0.152 vs. 0.821 ± 0.287 $P = 0.386$).

Discussion

Because of the anatomic continuity of the nasopharynx with the middle ear through the Eustachian tube, the role of the latter in the pathogenesis of OME is unclear, with effusion in OME most often attributed to an ascending infection from the nasopharynx into the middle ear.[11] One factor related to upper airway infections in children may be hypertrophy of the adenoid tissue, which may provoke the production of an inflammatory mucoexudate, often accompanied by mucus metaplasia of the local epithelium.[11]

NO has a dual function in cells. It is important in many biochemical processes, such as the regulation of blood vessel dilation and immune response, in which it functions as a neurotransmitter.[12,13]Although NO also has bactericidal or tumoricidal functions, under pathologic conditions excess NO production may be involved in the pathogenesis of several diseases, including septic shock, autoimmune diseases, cerebral infarction, and diabetes mellitus.[13] NO is also generated in adenotonsillar tissue. For example, serum arginase and iNOS activities were found to be significantly higher in children with adenotonsillar hypertrophy than in the same children post-operatively and in healthy controls.[14] Moreover, tonsillar tissues had a significantly higher arginase activity than did adenoidal tissues of the same patients.[15]

NO is an important immune modulator (IM) in the pathogenesis of OME. For example, iNOS has been found to be critical for the development of LPS-induced middle ear effusions[5] and NO in middle ear effusions is produced by iNOS.[1] Other studies have yielded inconsistent results about the role of eNOS in airway diseases and OME.[5] Antibodies to eNOS have been localized in the epithelium of human nasal mucosae[16] and ultrastructural studies have localized eNOS to the basal membranes of ciliary microtubules[17]where it is thought to contribute to the regulation of ciliary beat frequency.[18] NO production is also decreased in the upper airways of patients with chronic sinusitis[19]and low nasal NO levels have been found to indicate dysfunction in the mucociliary system.[20] In this study, eNOS was significantly decreased in the OME group, suggesting that decreased eNOS activity in adenoid tissues may reduce ciliostimulatory clearance in hypertrophied adenoids leading to OME.

PAF is an inflammatory mediator generated by the enzyme phospholipase A_2. Among the functional actions of PAF are airway constriction, microvascular leakage, chemotaxis (especially for eosinophils), and bronchial hyperresponsiveness.[21] PAF appears to be an important mediator of mucus hypersecretion in the middle ear cavity and Eustachian tube.[22,23] Likewise, experimental OME could be prevented by PAF inhibitor.[24] PAF has been found to stimulate NOS to release NO, and this endogenous NO mediates reactions including endotoxic shock, cardiopulmonary actions, microvascular permeability, and bowel injury in animal experiments.[25-28] Recent findings have suggested that PAF may act through NO-dependent and NO-independent mechanisms.[29] In our study, PAF receptor protein was significantly increased in the OME group, suggesting that hypertrophied adenoids act as a source of PAF in OME.

In conclusion, this study shows that the expression of PAF receptor and eNOS is altered in hypertrophied adenoids of children with OME. These findings suggest that PAF receptor present in hypertrophied adenoids of OME patients may contribute to the pathogenesis of OME. Moreover, a decrease in eNOS in adenoid tissues may lead to OME due to altered mucociliary clearance.

Acknowledgement

References

1. John EO, Russell PT, Nam BH, Jinn TH, Jung TT. Concentration of nitric oxide metabolites in middle ear effusion. Int J Pediatr Otorhinolaryngol 2001;60:55-58.
2. Maw AR, Parker A. Surgery of the tonsils and adenoids in relation to secretory otitis media in children. Acta Otolaryngol Suppl 1988;454:202-207.
3. Gates GA. Adenoidectomy for otitis media with effusion. Ann Otol Rhinol Laryngol Suppl 1994;163:54-58.
4. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol 1996;271:C1424-1437.
5. Ball SS, Prazma J, Dais D, Rosbe KW, Pillsbury HC. Nitric oxide: a mediator of endotoxin-induced middle ear effusions. Laryngoscope 1996;106:1021-1027.
6. Imaizumi T, Stafforini DM, Yamada Y, McIntyre TM, Prescott SM, Zimmerman GA. Platelet-activating factor: a mediator for clinicians. J Intern Med 1995;238:5-20.
7. Honda Z, Ishii S,Shimizu T. Platelet-activating factor receptor. J Biochem (Tokyo) 2002;131:773-779.
8. Rhee CK, Jung TT, Miller S, Weeks D. Experimental otitis media with effusion induced by platelet activating factor. Ann Otol Rhinol Laryngol 1993;102:600-6055.
9. Jung TT, Hwang AL, Miller SK, Rhee CK, Park YS. Effect of leukotriene inhibitor on cochlear blood flow in salicylate ototoxicity. Acta Otolaryngol 1995;115:251-254.
10. Fujioka M, Young LW, Girdany BR. Radiographic evaluation of adenoidal size in children: adenoidal-nasopharyngeal ratio. AJR Am J Roentgenol 1979;133:401-404.
11. Elsheikh MN, Mahfouz ME. Up-regulation of MUC5AC and MUC5B mucin genes in nasopharyngeal respiratory mucosa and selective up-regulation of MUC5B in middle ear in pediatric otitis media with effusion. Laryngosocpe 2006;116:365-369.
12. Moncada S, Higgs EA. Molecular mechanisms and therapeutic strategies related to nitric oxide. FASEB J 1995;9:1319-1330.
13. Gotoh T, Mori M. Arginase II downregulates nitric oxide (NO) production and prevents NO-mediated apoptosis in murine macrophage-derived RAW 264.7 cells. J Cell Biol 1999;144:427-434.
14. Unal M, Cimen MY, Dogruer ZN, Cevik T. The potential inflammatory role of arginase and iNOS in children with chronic adenotonsillar hypertrophy. Int J Pediatr Otorhinolaryngol 2005;69:381-385.
15. Elgun S, Guney K, Tuncer M, Gokhun IH. A potential role for arginase in recurrent infections and hypertrophy of tonsillar and adenoidal tissue. Eur Arch Otorhinolaryngol 2000;257:158-160.
16. Kawamoto H, Takumida M, Takeno S, Watanabe H, Fukushima N, Yajin K. Localization of nitric oxide synthase in human nasal mucosa with nasal allergy. Acta Otolaryngol Suppl 1998;539:65-70.
17. Xue C, Botkin SJ, Johns RA. Localization of endothelial NOS at the basal microtubule membrane in ciliated epithelium of rat lung. J Histochem Cytochem 1996;44:463-471.
18. Jain B, Rubinstein I, Robbins RA, Leise KL, SissonJH. Modulation of airway epithelial cell ciliary beat frequency by nitric oxide. Biochem Biophys Res Commun 1993;191:83-88.
19. Lindberg S, Cervin A, Runer T. Nitric oxide (NO) production in the upper airways is decreased in chronic sinusitis. Acta Otolaryngol 1997;117:113-117.
20. Lindberg S, Cervin A, Runer T. Low levels of nasal nitric oxide (NO) correlate to impaired mucociliary function in the upper airways. Acta Otolaryngol 1997;117:728-734.
21. Barnes PJ, Chung KF, Page CP. Platelet-activating factoras a mediator of allergic disease. J Allergy Clin Immunol 1988;81:919-934.
22. Lin J, Kim Y, Lees C, Juhn SK. Effect of platelet-activating factor on secretion of mucous glycoprotein from chinchilla middle ear epithelial cells in vitro. Eur Arch Otorhinolaryngol. 1995;252:92-96.
23. Rhee CK, Jeong PS, Kim YH, Chung PS, Jung TT. Effect of platelet activating factor and its antagonist on the mucociliary clearance of the eustachian tube in guinea pigs. Ann Otol Rhinol Laryngol 1999;108:453-458.
24. Jung TT, Park SK, Rhee CK. Effect of inhibitors of leukotriene and/or platelet activating factor on killed H. influenzae induced experimental otitis media with effusion. Int J Pediatr Otorhinolaryngol 2004;68:57-63.
25. Fabi F, Calabrese R, Stati T, del Basso P. Nitric oxide (NO) modulation of PAF-

induced cardiopulmonary action: interaction between NO synthase and cyclo-oxygenase-2 pathways. Br J Pharmacol 2001;134:777-788.

26. Jeon SY, Kim EA, Ma YW, Kim JP, Jung TG, Hwang EG. Nitric oxide mediates platelet activating factor-induced microvascular leakage in rat airways. Ann Otol Rhinol Laryngol 2001;110:83-86.

27. Klabunde RE, Anderson DE. Obligatory role of nitric oxide in platelet-activating factor-induced microvascular leakage. Eur J Pharmacol 2000;404:387-394.

28. Qu XW, Rozenfeld RA, Huang W, Sun X, Tan XD, Hsueh W. Roles of nitric oxide synthases in platelet-activating factor-induced intestinal necrosis in rats. Crit Care Med 1999;27:356-364.

29. Maniscalco M, Sofia M, Faraone S, Carratu L. The effect of platelet-activating factor (PAF) on nasal airway resistance in healthy subjects is not mediated by nitric oxide. Allergy 2000;55:757-761.

Seasonality of allergy in patients with chronic otitis media with effusion

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Introduction

Evidence that allergy contributes to the pathogenesis of OME is derived from epidemiologic, mechanistic, and therapeutic lines of investigation.[1] Recent studies support the hypothesis that there is a relation of atopy to chronic otitis media with effusion (OME), and that the middle ear, similar to the rest of the upper respiratory system is itself capable of an allergic immune response.[2-5] In-vitro and in-vivo evidence indicates that like asthma and allergic rhinitis, a Th-2 mediated allergic response is found in MEE in many OME patients[6], supporting the hypothesis that there is a relation of atopy to chronic otitis media with effusion (OME). Despite this science, current texts and AAO-HNS approved slide series continue to debunk this relationship and state that allergy cannot be related to OME because, among other reasons totally unsubstantiated by any reference, "OM is common in winter and major allergens (grass, trees) occur later."[7]

The purpose of this study was to determine if there was any truth to this statement by looking at 1) the seasonality of those allergens that seem to be responsible for OME in patients whose atopy was determined by intradermal testing and in whom, 2) the OME then resolved after being treated by immunotherapy (IT) directed at those specific allergens.

Patients

These patients were selected from a previous study[8] of a group of 74 patients referred with (A) chronic OME >6 months during a one-year period and no previous myringotomy and tubes (M&T) or OME >3 months warranting a second M&T, or (B) chronic suppurative otitis media (CSOM) with persistent drainage from a perforation or tympanostomy tube which had been otherwise unresponsive to oral and/or topical antibiotics. Allergy immunotherapy directed at those allergens specifically identified by intradermal skin testing had been effective in completely controlling OME for 63 patients. Since their middle ear disease had totally responded to immunotherapy in that their ears remained free of recurrence of fluid or drainage from myringotomy tubes during the follow up period ranging from 2 to 8 years, it was assumed that there must logically be some correlation of the allergens

found on skin testing to their middle ear disease. The treatment group had been compared to a control cohort of 24 patients receiving no immunotherapy, none of whom resolved spontaneously (P<0.005). [8] We then looked at the seasonality of the treatment group's allergens.

Methods

Testing was performed for a battery of 11 inhalants (dust mites der P and der F, cat, dog, cockroach, Timothy and Meadow Fescue grass, long and short ragweed, birch, oak) and 5 mold mixes. (Table 1) Injection consisted of an allergenic extract in volumes of 0.01 ml to produce a 4-mm wheal according to American Academy of Otolaryngic Allergy (AAOA) guidelines. The skin tests were performed with 1:500 wt/vol and successive serial 5-fold weaker dilutions of the allergenic extracts. For each challenge results were considered positive when a wheal of 7 mm or larger was observed that was at least 2 mm larger than the wheal of a glycerin control of the same dilution strength, all measured after 10 minutes. Patients were categorized as atopic if they reacted positively to at least two allergens at a class 2 or greater. This definition was biased so as to intentionally exclude borderline atopic patients. Patient outcomes were assessed after a minimum of one year on IT and patients were followed for 2 to 8 years.

Intervention consisted of immunotherapy according to AAOA criteria. IT dosage for those antigens eliciting a class 2(1:500 dilution) or higher reaction was administered at the maximum tolerated dosage. Injections were maintained weekly throughout the first year and advanced until the patient could maintain a symptom free state while on a 3-week dosage interval for a year, after which therapy was discontinued. Mean dosage time was 4.5 years. Patients were all seen initially at 4 months after initiation of therapy and then on a bi-annual schedule.

Results

Most (85.7%) patients (Table I) were panallergic to an average of 9 allergens including dust (94%), animals (47%), ragweed (67%), and molds (88%). Nine were allergic only to seasonal pollens. Associated allergic diseases included asthma (21%), allergic rhinitis/sinusitis (66%) but limited to otitis only in

37%. Surprisingly, allergic otitis presented as unilateral disease in 13%.

Discussion

This study is important in that patients with chronic OME are demonstrated to have allergens that can affect them throughout the year. Winter months are particularly problematical because of dust and mold allergy compounded by increased viral exposure. Prior AAO-HNS Academy teaching[7] that allergies bother people more in Spring (grass and trees) and fall (goldenrod, ragweed) and therefore do not correlate with the winter season of OME was made with no supporting references and fails to recognize the importance of dust and mold allergy in this population.

OME is a multifactorial disease, of which symptoms of respiratory allergy, otitis heritability and IgE sensitization are all independently associated.[9] The middle ear, which is identical in its mucosal lining to a sinus, would be expected to behave similarly as a part of a unified airway.[6] Th-2 inflammatory mediators including eosinophil cationic protein, tryptase, and/or IL-5 mRNA cells, CD3+ T cells[2], eosinophils[4], and mast cells[3], Rantes, prostaglandins[6], and IgE[10] have all been shown to be present in middle ear mucosa and/or effusion. It is the host's *immunologic status* (atopic or non-atopic) that holds the key to understanding the underlying mucosal response that is responsible for the immunologic processes that produce his/her sinusitis, asthma or middle ear disease. In chronic otitis media, as in chronic sinusitis, for each individual case of "infection" the particular organism is of utmost importance. Yet the bacteriology tells us little about the pathophysiology of the disease itself. Allergy adds unique co-morbidity in that a child who gets an episode of acute otitis media is up to 3 times more likely to develop OME if that same child is also allergic.[11] Asthma incidence increases with increased viral URI episodes in atopics in winter months. Similarly, this study demonstrated OME patients to have allergies to predominantly winter allergens. More research is needed to determine if the processes we know to occur in atopic asthmatics also occurs in otitis patients.

In order to understand the relative high percentage of atopics we report in this series it is necessary to recognize the relative advantages of intradermal testing. Chronic otitis has been shown to be a low level IgE mediated disease similar to allergic rhinitis, with 2/3 of OME patients having a serum IgE < 100µg/l. [4] Prick testing is designed to identify high level IgE patients such as asthmatics with a mean IgE exceeding 100µg/l. It is not surprising that prick testing, considered to be equivalent to a class 4 (1:12,500) or less dilution would at most have only detected 10% of these patients as being atopic. This demonstrated the imperative to use intradermal testing, in order to successfully diagnose atopy in many of these patients as prick and RAST testing would certainly have missed most all of these. RAST testing is generally considered to correlate with prick testing and has an even lower sensitivity in diagnosing allergy as compared to IDT, especially when trying to detect allergy to molds and foods.[12] The majority (88%) of these OME patients were mold allergic. Furthermore, 44% of these OME patients were positive only at a dilution of C2 or 1:500. Our success was also dependent on treating C2 level allergy or a significant number of these patients would not have resolved.

Conclusion

Current teaching that allergy cannot be related to chronic OME because of seasonal variation is FALSE. Most patients with chronic OME are panallergic. The majority of OME patients is allergic to dust and molds, the two most prevalent allergens in winter months, which does indeed coincide with the predominant season of cases of OME. This adds to the evidence that chronic OME is an allergic disease.

Table 1
63 Patients whose OME resolved on Immunotherapy. Sorted by Allergen and Concentration of Reaction End Point

CLASS + IDT	1;312,500 #6	1:62,500 #5	1:12,500 # 4	1:2,500 #3	1:500 #2	%
DUST P		2	7	10	22	62
STD CAT			1	3	27	47
AP DOG			11	2	5	27
DUST F	2	1	12	17	30	94
GRASS		2	4		35	62
RAGWEED	2	1	5	3	33	67
TREE			3	7	25	53
WEED			1	4	17	33
GOLDENROD		1		8	29	58
COCKROACH	1	1	1	9	30	64
MOLD A				3	34	56
MOLD B				3	40	65
MOLD C			1	5	30	55
MOLD D			1	18	39	88
TRICHO				3	23	39
TOTALS	5	8	47	95	419	

Class = reaction 2 mm greater than glycerin control of same dilution
MOLD A = Aspergillus, Alternaria, Hormodendrum, Penicillum
MOLD B = Helminthosporium, Mucor, Rhizopus, Spondylocladium
MOLD C = Curvularia, Epicoccum, Fusarium, Stemphyllium
MOLD D = Cephalosporium, Chartomium, Phoma, Pullularia, Rhodotorula
TRICHO = Trichoderma

References

1. Tewfik TL, Mazer B. The links between allergy and otitis media with effusion. Curr Opin Otolaryngol Head Neck Surg 2006; 14:187-90.
2. Wright ED, Hurst D, Miotto D, Giguere C, Hamid Q. Increased expression of major basic protein (MBP) and interleukin-5(IL-5) in middle ear biopsy specimens from atopic patients with persistent otitis media with effusion. Otolaryngol Head Neck Surg 2000; 123:533-538.
3. Hurst DS, Amin K, Sevéus L, Venge P. Evidence of mast cell activity in the middle ear of children with otitis media with effusion. Laryngoscope 1999; 109:471-477.
4. Hurst DS, Venge P. Evidence of eosinophil, neutrophil, and mast-cell mediators in the effusion of OME patients with and without atopy. Allergy 2000; 55:435-441.
5. Rosenfeld RM, Culpepper L, Doyle KJ, et al. Clinical practice guideline: Otitis media with effusion. Otolaryngol Head Neck Surg 2004; 130:S95-118.
6. Nguyen LH, Manoukian JJ, Sobol SE, et al. Similar allergic inflammation in the middle ear and the upper airway: evidence linking otitis media with effusion to the united airways concept. J Allergy Clin Immunol 2004; 114:1110-1115.
7. McKenna M. Otitis Media. In: Bailey B, Johnson J, Kohut R, Pillsbury H, Tardy E, eds. Head and Neck Surgery—Otolaryngology. Vol. 2: Lippincott Williams & Wilkins, 1998;1597.
8. Hurst DS. The efficacy of allergy immunotherapy for patients with chronic OME. Arch Otolaryngol Head Neck Surg *Submitted 2007*.
9. Chantzi FM, Kafetzis DA, Bairamis T, et al. IgE sensitization, respiratory allergy symptoms, and heritability independently increase the risk of otitis media with effusion. Allergy 2006; 61:332-336.
10. Hurst DS, Ramanarayanan MP, Weekley M. Evidence of possible localized specific IgE production in middle ear fluid as demonstrated by ELISA testing. Otolaryngol Head Neck Surg 1999; 121:224-230.
11. Stenstrom C, Ingvarsson L. General illness and need of medical care in otitis prone children. Int J Pediatr Otorhinolaryngol 1994; 29:23-32.
12. Chinoy B, Yee E, Bahna SL. Skin testing versus radioallergosorbent testing for indoor allergens. Clin Mol Allergy 2005; 3:4.

Antibodies directed against the type IV pilus protein of nontypeable *Haemophilus influenzae* can prevent or reverse biofilms formed *in vitro*

Molly Bruggeman, Joseph Jurcisek, B.S., Lauren Bakaletz, Ph.D.

Type IV pili (Tfp), which are expressed on the surface of some Gram-negative pathogens, are multifunctional, filamentous appendages comprised of helically arranged pilin subunits. Tfp mediate competence, adherence, colonization of mucosal surfaces, and also biofilm formation. Biofilms are aggregates of bacteria that are sheathed in a protective glycocalyx matrix and are highly resistant to immune-mediated clearance. It has recently been demonstrated by our laboratory that nontypeable *Haemophilus influenzae* (NTHI), an opportunistic pathogen that causes multiple respiratory tract diseases, including otitis media (OM), produce Tfp that provide twitching motility and contribute to biofilm formation both *in vitro* and *in vivo*.

In this study, we examine the ability of anti-recombinant soluble PilA (rsPilA) antibodies to prevent and/or reverse the formation of biofilms by NTHI. We incubated NTHI with rabbit anti-rsPilA, naïve rabbit serum or medium alone for one hour in a continuous flow chamber and grew these bacteria further *in vitro*. After 24 hours, biofilms formed under all three conditions, however there was a marked reduction in biofilm density and height, as well as an altered architecture when NTHI were preincubated with immune serum, compared to either control. Since anti-rsPilA was able to diminish the ability of NTHI to form a biofilm, we were interested in determining if the anti-rsPilA could also *reverse* an established biofilm. Mature NTHI biofilms incubated with rabbit anti-rsPilA for 16 hours showed a complete loss in structural integrity and areas of biofilm eradication, compared with either control. As it has been previously reported that TFP-mediated twitching motility is necessary for biofilm development, we also investigated whether anti-rsPilA could block twitching motility. In the absence of antiserum, NTHI were able to form satellite colonies on an agar surface, whereas in the presence of the antiserum, no satellite colonies formed suggesting that twitching motility was inhibited. In further support of our data, in an experimental chinchilla model of OM, animals immunized with rsPilA showed a statistically significant reduction in OM as compared to the cohort that received adjuvant alone. Collectively, our data suggested that immunization against NTHI type IV pilin protein may not only help *prevent* biofilm formation, but may also confer a therapeutic effect by facilitating eradication (or *reversal*) of an existing biofilm.

Format and compliance in a study to investigate the relationship between viral upper respiratory tract infections and otitis media

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Introduction

New episodes of otitis media (OM) are most commonly diagnosed in association with signs and symptoms typical of a viral upper respiratory tract infection (vURI). A causal relationship between viral upper respiratory tract infections (vURI) and otitis media (OM) is supported by studies in animals and humans. Because of the implied causal association between OM and vURI, many believe that most OM episodes are a complication of a vURI, and thus theoretically preventable by preventing vURIs and/or by interfering with OM pathogenesis during a vURI. A variety of strategies to achieve that goal has been proposed, but the relative efficacy and efficiency of each strategy depends on the currently unknown values of a number of parameters. Existing data do not provide the high resolution, time-series evaluations for the wide range of contributing variables necessary to demonstrate this temporal relationship. The feasibility of such a study depends in part on the family/subject willingness and compliance with this study format. As part of our study to establish various links between vURI and OM, enrolled families are expected to keep daily symptom diaries, perform daily tympanometry on their enrolled children, accept a weekly in-home (Pittsburgh) or in-clinic (Virginia) ear exam by a study MD or nurse practitioner on the enrolled children, and attend enrollment and exit visits at the local study office site.

Materials and methods

The study took place at 2 enrollment sites (University of Pittsburgh, University of Virginia), over a period of 5 years. The study protocol was approved by the Institutional Review Boards at the University of Pittsburgh and the University of Virginia. Thirty families with 2 or more children aged 1 to 5 years were recruited for participation in each year. Families were recruited in September of each year by newspaper, radio, flyer, and word-of-mouth advertisement. Families were provided monetary compensation for participation. Anonymous exit surveys were collected to document family satisfaction.

At study entry, informed consent, demographics, and family history were obtained. Buccal swabs were collected on each child for assessment of cytokine genotype, and a baseline audiogram was performed to document absence of sensorineural loss. A user-friendly tympanometer (RaceCar, American Electromedics Corporation, Amherst, NH) was placed in each home.

The children were followed from the time of enrollment (October) through April 30 (the "typical cold season") by:

- Weekly assessments of middle ear (ME) status by pneumatic otoscopy;
- Daily parental assignment of each child's status regarding the presence/absence of signs of a vURI;
- Daily parental recording of ME pressures by tympanometry and periodic recording of temperature, and
- Periodic collection of nasal secretions for bacteriology by culture, virus detection by PCR and inflammatory mediator assay. Secretions were sampled if either of the enrolled children developed a URI, or if OME or AOM was noted on otoscopy. In the first year of the study, samples were also collected at random times during wellness.

At the conclusion of each study period in May, families were again seen at the study site office. A follow-up audiogram was performed on each child, and parents participated in an informal exit interview and filled out an exit questionnaire which they could choose to sign or not.

Results

In years 1-3, 99 families consented to study participation. Nine withdrew prior to onset of data collection and 6 dropped out or were withdrawn during the study period. Of the remaining 84 families (85% of enrolled families, 190 children, average age 3.8 ± 1.6 years, 96 male, 151 white), the average completion rate (Nov 1-April 30) for the genetic assays was 100%, the daily recordings of signs was $94\pm5\%$, the daily tympanograms was $77\pm23\%$, and the weekly otoscopic exams was $86\pm18\%$ (Figures 1 and 2). In years 1 and

2, a total of 869 nasal wash samples were collected and assayed for bacteria and viruses. Exit interviews documented parental satisfaction with study participation and no significant concerns (Figure 3).

Conclusions

This study format provided a great deal of data, capturing temporal information on both asymptomatic and symptomatic presentations of vURIs and OM during the typical season for cold-like illnesses. Though the study required intensive effort on the parts of both families and study personnel, compliance was high and most families reported the experience to be enjoyable. Potential reasons for the positive responses include the financial compensation; 1:1 attention to their child regularly by health care providers, especially for those children who have had ear disease in the past; and the opportunity for parents to learn about normal and pathological ear processes.

Acknowledgment
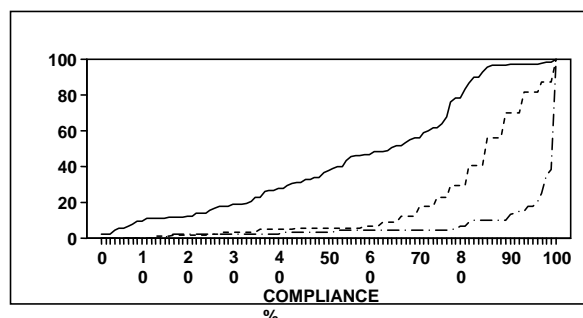
Figure 1. Cumulative percent compliance (y-axis) with daily symptom scoring (straight line), temperature ascertainment (short dashed lines) and weekly visits (long dashed lines)
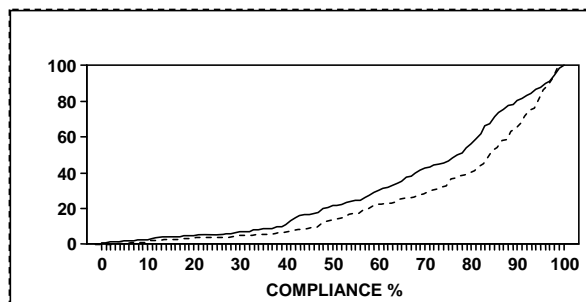
Figure 2. Cumulative percent compliance (y-axis) with tympanometry. "Attempted measures" were all tympanograms recorded (straight line); "accepted measures" were all those deemed to be evaluable (dashed line).
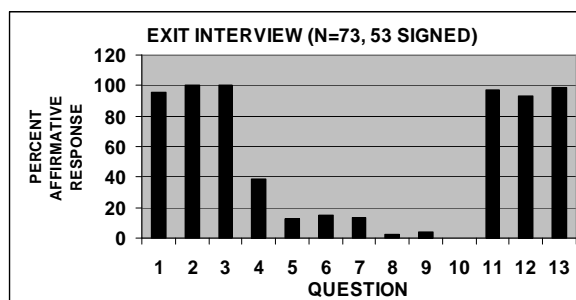
KEY

1	Adequate Information (advertisement)
2	Adequate Information (first visit)
3	Enrollment Visit Satisfactory
4	Technical Problems with Equipment
5	Problems with Measuring Tympanometry
6	Problems with Nasal Wash
7	Problems Keeping Symptom Diary
8	Problems with Visit Scheduling
9	Problems Contacting Study Personnel
10	Problems with Interactions with Study Personnel
11	Satisfactory Experience
12	Participate in a Future Study
13	Recommend the Study to Other Families

Modulation of cytokine activity in middle ear by formalin-killed Haemophilus influenzae or its lipooligosaccharide htrB and rfaD mutants during acute otitis media in the rat

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Introduction

NTHi LOS plays an important role in activating middle ear epithelium, resulting in early production of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) that are thought to be of central importance during the pathogenesis and regulation of proliferation, chemotaxis, and activation of inflammatory cells during the course of middle ear infections.[1,2,3] We have previously demonstrated that the role of nontypeable Haemophilus influenzae (NTHi) lipooligosaccharide (LOS) in the induction of proinflammatory cytokine gene expression and production in human middle ear epithelial (HMEE) cell in vitro.[4] Strain DK-1 is an rfaD gene mutant, expresses a truncated LOS consisting of only three deoxy-D-manno-octulosonic acid residues, a single heptose, and lipid A. Strain B29 is an isogenic htrB mutant, possesses an altered oligosaccharide core and an altered lipid A.[5] In order to define the relative contributions of the oligosaccharide and lipid A portions of LOS to this disease at the molecular level, we used these mutant strains differing in virulence to assess the role of NTHi LOS in the gene expression of the proinflammatory cytokines and to map the in vivo kinetics of induction of the inflammatory cytokine genes by NTHi LOS in the rat middle ear during the course of acute OM.

Methods

We compared the ability of formalin-killed NTHi strain 2019 and its LOS htrB and rfaD mutants in the modulation of complement and cytokine gene expression in the middle ear during the early stage of AOM. At 3, 6, 12, 24 and 48 h after transbulla inoculation with NTHi, expression of genes for the cytokines tumor necrosis factor alpha (TNF-α), interleukin lbeta (IL-1β), and IL-6 interleukin-1alpha (IL-1α), IL-10, and inducible nitric oxide synthase (iNOS) were quantitated by real-time PCR. NTHi 2019 and its LOS mutants induced a significant up-regulation in gene expression for the cytokines examined in this study compared to saline sham-inoculated controls at 3, 6 and 12 h post inoculation (P < 0.05 in all cases). Furthermore, NTHi 2019 cohort demonstrated a significant increase in gene expression for the cytokine examined relative to animals inoculated with NTHI B29 at 3 and 6 h post infection (P < 0.01 [2019 versus B29].). Enzyme-linked immunosorbent assay results confirmed the gene expression data as determined by real-time PCR. There were no significant differences in mRNA expression of the cytokines between the 2019- and DK-1-treated groups.

Results

These data demonstrate that the LOS of NTHi 2019 parent strain induced significant up-regulation of cytokine and iNOS gene expression within the middle ear in the rat OM model. Moreover, we report, for the first time, a differential induction of cytokines and iNOS mediated by two NTHi LOS mutant strains. These data are also consistent with our previous report, which demonstrated that expression of TNFα, IL-6 and IL-1β genes was significantly induced in HMEE cells stimulated with formalin-killed NTHi 2019 in vitro compared to that induced by NTHi B29 strain[4]. However, there is a difference in the potency of B29 in the induction of cytokine genes in HMEE cells in vitro and in rat middle ear epithelium in vivo, which may reflect the complex of environment in middle ear and the orchestral interactions between the host inflammatory response and pathogens. It is noteworthy that the ability of NTHi 2019 and each LOS mutant strain to induce cytokine gene expression seems closely related to the local inflammatory response in the middle ear. Histological examination revealed significant differences in influxes of inflammatory cells in the middle ear between the rats infected with NTHi 2019 and B29. Our data strongly suggest that NTHi LOS htrB gene products are responsible for the induction of proinflammatory cytokine gene expression in the rat middle ear epithelium and the middle ear inflammation after T.B. inoculation of nonviable NTHi cells.

Conclusions

In conclusion, the significant decline in gene transcripts observed with the NTHi B29 mutant

indicates that the product of the NTHi LOS *htrB* gene plays a major role in the induction of these particular inflammatory mediators. These data will contribute to our understanding of the role of various LOS gene disruptions on OM pathogenesis and may thus provide potential new targets for future protection and intervention strategies.

Acknowledgment

References

1. Melhus A, Ryan AF. Expression of cytokine genes during pneumococcal and nontypeable *H. influenzae* acute otitis media in the rat. Infect Immun 2000; 68:4024-4031.
2. Sato K, Kwana M, Nonomura N, NakanoY. Course of IL-1β, IL-6, IL-8, and TNF-α in the middle ear fluid of guinea pig otitis media model induced by nonviable H. influenzae. Ann Otol Rhinol Laryngol 1999; 108:599-563.
3. Long JP, Tong HH, Shannon PA, DeMaria TF. Differential expression of cytokine genes and inducible nitric oxide synthase induced by opacity phenotype variants of streptococcus pneumoniae during acute otitis media in the rat. Infect Immun 2003;71:5531-5540.
4. Tong HH, Chen Y, James MA, Van Deusen J, Welling DB, DeMaria TF. Expression of cytokine and chemokine genes by human middle ear epithelial cells induced by formalin-killed H. influenzae or its LOS htrB and rfaD mutants. Infect Immun 2001; 69:3678-3684.
5. DeMaria TF, Apicella MA. Nichols WA, Leake ER. Evaluation of the virulence of nontypeable H. influenzae lipooligosaccharide htrB and rfaD mutants in the chinchilla model of otitis media. Infect Immun 1997; 5:4431-4435.

Nasopharyngeal biofilm density in the pediatric nasopharynx: otitis media with effusion versus recurrent acute otitis media and obstructive sleep apnea

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Objective

To compare the extent of biofilm infection in terms of percent mucosal surface area of adenoids removed from children with otitis media with effusion (OME) versus those with recurrent acute otitis media (RAOM) and obstructive sleep apnea (OSA).

Design

Comparative micro-anatomic investigation of adenoid mucosa using scanning electron microscopy from patients with OME, RAOM and OSA.

Setting

University-affiliated hospitals and ambulatory surgery center.

Patients

17 boys and 13 girls ranging in age from 9 months to 10 years.

Main outcome measure

Measurements of biofilm coverage of the entire adenoidal surface.

Result

Adenoids removed from patients with OME had moderately dense mature biofilms covering the mucosal surface with a mean of 28.8% of their mucosal surface covered with mature biofilms. These results were distinct from results obtained from patients diagnosed with RAOM and OSA with means of 97.6% and 0.12% of their mucosal surfaces covered with mature biofilms, respectively. These differences were statistically significant at P<0.0001.

Conclusions

Adenoids removed from patients with OME were characterized by distinctly different percentage of biofilm mucosal surface area coverage, with significantly more biofilm presence than adenoids obtained from OSA patients but significantly less biofilm presence than adenoids obtained from RAOM patients. Our previous investigations suggest that nasopharyngeal biofilm mediated infections play a dominant role in the pathogenesis of RAOM. This data indicates that although nasopharyngeal biofilms were identified in patients with OME, biofilms were not found in abundance. These results suggest nasopharyngeal biofilms may play a different role in the pathogenesis of OME and that this clinical entity may be more multifactorial in nature.

Detection of *Helicobacter pylori* in middle ear fluids in patients with otitis media with effusion

Keehyun Park, M.D., Yun-Hoon Choung, M.D., Hun Yi Park, M.D., Sang Eun Lee, M.D., Choi Sung Jun, M.D.

Introduction

Otitis media with effusion (OME) is defined as the presence of fluid in the middle ear without signs or symptoms of acute ear infection.[1,2] And, it is the most common cause of hearing difficulty affecting up to 1/3 of preschool children in the developedworld.[3] However, its pathogenesis remains unsettled. Bacterial and viral infections, adenoid hypertrophy or adenoiditis, Eustachian tube dysfunction, allergy, gastroesophageal reflux are thought to play a role in its pathogenesis.

Gastroesophageal reflux (GER) is believed to be an important contributing factor in many disorders of the upper respiratory tract: pharyngolaryngitis, croup, oropharyngeal dysphagia, rhinosinusitis, otalgia, and different forms of otitis, among others.[4] Tasker et al[5] showed pepsinogen and pepsin in the middle ear fluid of patients with OME, indicating that gastric fluid could reach as far as middle ear and this might be involved in the pathogenesis of OME.

Helicobacter pylori (HP) is a microaerophilic, Gram negative spiral organism that has been shown to be the causative factor in a large portion of patients with stomach ulcers, gastritis and gastric cancer[6] Recently, HP was detected in the upper airway mucosa (tonsil, adenoid tissue and sinus mucosa) of patients with tonsillitis, adenoiditis and chronic sinusitis, which indicates a possible role in pathogenesis of tonsillitis, adenoiditis and chronic sinusitis.[6,7] Because middle ear is an extension of upper airway tract, HP could also be expected to be found in the middle ear of patients with OME and GER.

The aim of this study is to determine the presence of HP in the middle ear fluid of OME and evaluate possible role of HP in the pathogenesis of OME.

Methods

The study was performed in 56 patients with OME (25 children and 31 adults) who were referred to Ajou University Medical Center from Oct. 2005 to Jan. 2007. In all cases, myringotomies (with or without placement of ventilation tube) were performed. The effusion samples aspirated from the middle ear were analyzed with rapid urease test (RUT) and PCR assay using 16sRNA primer or multiplex PCR assay (VacAS, VacAM, CagA).

Results

A total 64 samples from 56 patients were included in RUT and36 (56.3%) of 64 samples were shown to be *H. pylori*-positive.

In PCR assay using 16sRNA primer, a total 31 samples from 25 patients were included and 12 (38.7%) of 31 samples were shown to be *H. pylori*-positive. Both PCR and RUT were carried out in 31 samples. Of 12 PCR-positive samples, 8(66.7%) were RUT-positive and 4 (33.3%) were RUT-negative. Of 19 PCR-negative samples, 8 (42.1%) were RUT-positive and 11 (57.9%) were RUT-negative. (Figure 1)

In multiplex PCR assay, a total 16 samples from 16 patients were included and 13 (81.3%) were shown to be *H. pylori*-positive. Of 13 PCR-positive samples, 9 (69.2%) were RUT-positive and 4 (30.8%) were RUT-negative. Of 3 PCR-negative samples, 2 (66.7%) were RUT-positive and 1 (33.3%) were RUT-negative. (Figure 2)

Conclusions

This study showed that H. pylori were frequently present in the middle ear effusion in OME and multiplex PCR was more sensitive than PCR using 16sRNA primer to detect *H. pylori*. These results indicate that *H. pylori* has a possible role in the pathogenesis of OME.

Figure 1. PCR with 16S rRNA

PC, positive control; H. pylori ATCC 43504   NC, negative control; DW

PS, positive sample   NS, negative sample
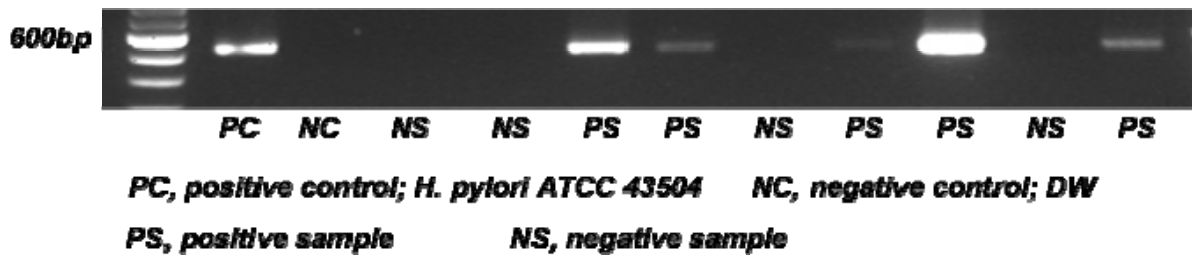
Figure 2. Multiplex PCR
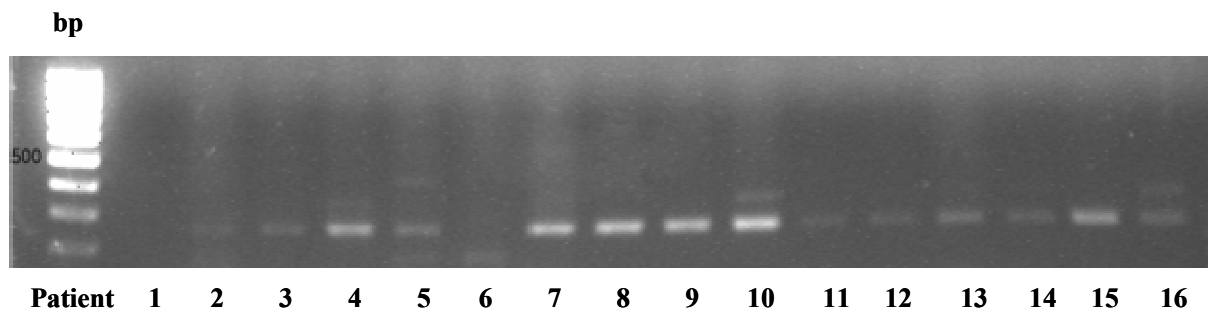
References

1. Stool SE, Berg AO, Berman S, et al. Otitis media with effusion in young children. Clinical Practice Guideline, Number 12. Rockville, MD: Agency for Health Care Policy and Research, Public Health Service, US Department of Health and Human Services; 1994; AHCPR Publication No. 94-0622.
2. Shekelle P, Takata G, Chan LS, et al. Diagnosis, natural history and late effects of otitis media with effusion. Evidence report/technology assessment no. 55. Rockville, MD: Agency for Healthcare Research and Quality; 2003. AHRQ Publication No. 03-E023.
3. Maw AR, Otitis media with effusion. In: AL Kerr AL, ed.*Scott-Brown's Otolaryngology, vol. 6-7,*Oxford: Butterworth-Heinemann; 1997:1-10.
4. Rozmanic V, Velepic M, Ahel V, Bonifacic D, Velepic M.Prolonged esophageal pH monitoring in the evaluation ofgastroesophageal reflux in children with chronic tubotympanaldisorders, J Pediatr Gastroenterol Nutr 2002; 34:278-280.
5. Tasker A, Dettmar PW, Panetti M, et al. Is gastric reflux a cause of otitis media with effusion in children? Laryngoscope 2002; 112:1930-1934.
6. Ozdek A, Cirak MY, Samim E, et al. A possible role of Helicobacter pylori in chronicrhinosinusitis: a preliminary report. Laryngoscope 2003; 113:679-682.
7. Cirak MY, Ozdek A, Yilmaz D, et al. Detection of Helicobacter pylori and its CagA gene in tonsiland adenoid tissues by PCR. Arch Otolaryngol Head NeckSurg 2003; 129:1225-1229.

Production of biofilm by *Haemophilus influenza* isolated from children with intractable otitis media

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Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is a human specific respiratory commensal bacteria and a major causative pathogen responsible for acute otitis media (AOM).[1] The genomic DNA of NTHi is highly identified in middle ear effusions while conventional bacterial culture failed to identify NTHi. Recently, an increase of intractable AOM caused by NTHi despite of proper antimicrobial treatments becomes a serious problem among young children in Japan.[2] A high prevalence of a drug resistant strain as ß-lactamase non-producing ampicillin resistant (BLNAR) *H. influenzae* in young children is a leading factor for an increasingly intractable AOM.[2] The immaturity of immune responses specific to NTHi is also a major cause for intractable AOM.[3]

Recently, biofilm is considered to be involved in the persistent bacterial infections caused by NTHi.[3] Recent reports suggest that biofilm produced by NTHi will be involved in the pathogenesis of AOM.[4]

In this study, we evaluated formations of biofilm by NTHi isolated from children with AOM in comparison with clinical outcome of the disease.

Materials and methods

Populations

Pediatric patients with AOM were enrolled in the study. They were divided into two groups. One was AOM cases that improved by treatment with amoxicillin (AMP) (16 patients, 56 NTHi strain) and another was AOM cases that did not improve by treatment with AMP (12 patients, 14 NTHi strain).

Biofilm formation assay

Formations of biofilm by NTHi were evaluated by a modification of the microtiter plate assay with crystal violet staining reported by O'Toole and Kolter in this study. The assay is based on the ability of bacteria forming biofilm on solid surfaces of polystyrene or polyvinyl chloride. NTHi isolates grown overnight in brain heart infusion (BHI) broth were washed with sterile PBS and diluted 1:200 in fresh BHI medium. Two hundred µl of NTHi suspension was inoculated into wells of a non-tissue culture-treated polystyrene flat-bottomed 96-well microtiter plates (Nunc, Kracker Scientific, Inc, Albany, NY). Plates were incubated at 37°C for 12 to 24 h aerobically in 5 % CO_2. Before quantifications of biofilm formations, growth of NTHi was assessed by measuring the absorbance of OD at 600 nm. Media including unattached bacteria were then decanted from the wells, and remaining planktonic NTHi cells were removed by rinsing with distilled water. Wells were air-dried and adherent bacteria were stained with 0.5 % (wt/vol) crystal violet (Fisher Scientific, Pittsburgh, PA.) solution for 15 min. After rinsing with distilled water, bound crystal violet was released from stained NTHi cells by 30% of glacial acetic acid. This allowed indirect measurement of biofilm formed on both bottom and sides of the well. The level of biofilm formations was quantified by measuring absorbance of OD at 562 nm.

In this study, we defined a ratio of OD_{570} of clinical isolates to OD_{570} of positive control as Biofilm formation index (BFI). The strain showing BFI ≥ 0.8 was defined as the strain forming biofilm (biofilm positive).

Results

Contribution of biofilm forming NTHi to clinical outcomes of AOM

The detection rate of NTHi forming biofilm among children with AOM not improved by treatment with AMPC (87.5%) was higher than those among children with AOM improved by treatment with AMPC (25.0%) (p<0.01).

In contrast, there was no significant difference in the detection ration between the cases with febrile episodes (fever ≥ 37.5°C) and without febrile episodes, 20.0% and 18.8%, respectively (Table 1).

Contribution of biofilm forming levels of NTHi to clinical outcomes of AOM

The level of biofilm formation was expressed as biofilm forming index. Biofilm forming index of NTHi isolated from AOM not improved by treatment with AMPC were higher than those of NTHi isolated from AOM improved by treatment with AMPC (p<0.05). The median, 95% value and 5% value of NTHi isolated from AOM that were not improved by treatment with AMPC and NTHi isolated from AOM improved by treatment with AMPC were 0.97, 3.05, 0.30, and 0.60, 0.97, 0.31, respectively(Fig. 1).

Discussion

Acute otitis media (AOM) is the most common reason for children to receive antibiotics. The clinical outcomes of AOM are closely related with eradication of pathogens from middle ear. The evidence that intractable AOM is associated with persistent bacterial infection in spite of antimicrobial treatments led to the development of the biofilm hypothesis.

Biofilm consists of aggregated bacteria, usually adherent to a surface, surrounded by an extracellular matrix, and have been implicated in several chronic bacterial infections.[5] Recent studies suggest persistent infections are associated with the formation of *in vivo* biofilm which renders the bacteria resistant to antibiotic treatment. Several studies focused on the production of biofilm by NTHi.[6] Previous laboratory studies with clinical isolates of *H influenzae* have demonstrated that biofilms form on the middle ear mucosa (MEM) of the chinchilla.[7] However, few studies have directly examined human AOM for biofilms. The impact of biofilm on clinical courses of AOM has not yet been well documented.

The current results suggest that formation of biofilm by NTHi would be associated with clinical outcome of AOM. NTHi will exist in both planktonic and biofilm states in the middle ear cavity. NTHi biofilm formation may enable it to survive and cause a chronic or intractable clinical course of AOM. Recent studies demonstrated that approximately 30% of cases of recurrent AOM result from relapses attributable to the original organism.[8,9] NTHi persists in middle ear fluids despite intensive antibiotic therapies. Therefore, clinically attainable antibiotic concentrations may not adequately clear infections. The current finding that biofilms are present in most of the intractable cases of AOM may help to explain the lack of antibiotic efficacy for this disorder, given that biofilm bacteria are more antibiotic resistant than single cells in suspension.

Fig. 1   Contribution of biofilm forming levels of NTHi to clinical outcomes of AOM

P value by Mann-Whitney U test
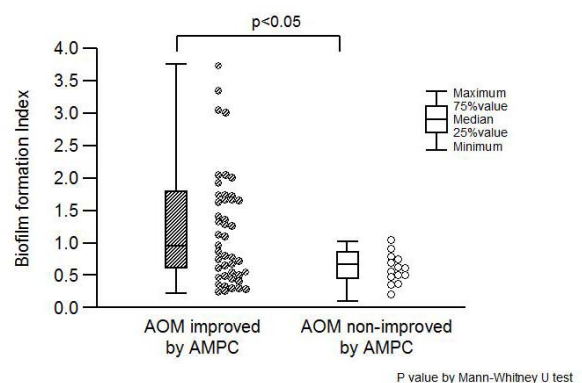
Table 1. Contribution of biofilm foriming NTHi to clinical outcomes of AOM

	Biofilm +	Biofilm -	%
AOM improved by AMPC	3	9	25.0%
AOM non-improved by AMPC	14	2	87.5%**

**p<0.01 by Chi-square test

	Febrile episode (+) (≥37.5°C)	Febrile episode (-) (<37.5°C)	%
Biofilm +	2	10	20.0%
Biofilm -	6	16	18.8%

Not significant

References

1. Erwin AL, Smith AL. Nontypeable Haemophilus influenzae: understanding virulence and commensal behavior. Trends Microbiol 2007; 15:355-362.
2. Hotomi M, Fujihara K, Billal DS, Suzuki K, Nishimura T, Baba S, YamanakaN. Genetic characteristics and clonal dissemination of ß-lactamase non-producing ampicillin resistant (BLNAR) *Haemophilus influenzae* isolated from the upper respiratory tract in Japan. Antimicrob Agents Chemother 2007; 51:3969-3976.
3. Gallaher TK, Wu S, Webster P, Aguilera R. Identification of biofilm proteins in non-typeable Haemophilus Influenzae. BMC Microbiol; 6:65.
4. Hall-Stoodley L, Hu FZ, Gieseke A, Nistico L, Nguyen D, Hayes J, Forbes M, Greenberg DP, Dice B, Burrows A, Wackym PA, Stoodley P, Post JC, Ehrlich GD, Kerschner JE. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. JAMA 2006; 296:202-211.
5. Webster P, Wu S, Gomez G, Apicella M, Plaut AG, St Geme JW 3rd. Distribution of bacterial proteins in biofilms formed by non-typeable Haemophilus influenzae. J Histochem Cytochem 2006; 54:829-842.
6. Ehrlich GD, Veeh R, Wang X, Costerton JW, Hayes JD, Hu FZ, Daigle BJ, Ehrlich MD, Post JC. Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. JAMA 2002; 287:1710-1715.
7. Erdos G, Sayeed S, Antalis P, Hu FZ, Hayes J, Goodwin J, Dopico R, Post JC, Ehrlich GD. Development and characterization of a pooled Haemophilus influenzae genomic library for the evaluation of gene expression changes associated with mucosal biofilm formation in otitis media. Int J Pediatr Otorhinolaryngol 2003; 67:749-755.
8. Post JC. Direct evidence of bacterial biofilms in otitis media. Laryngoscope 2001; 111:2083-2094.
9. Starner TD, Shrout JD, Parsek MR, Appelbaum PC, Kim G. Subinhibitory concentrations of azithromycin decrease nontypeable Haemophilus influenzae biofilm formation and diminish established biofilms. Antimicrob Agents Chemother 2008; 52:137-145.

Gas loss from the middle ear in normal and inflammatory conditions: the role of mucosa thickness and blood flow

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The aim of this study was to compare the trans-mucosal gas exchange in normal and inflamed ears and to evaluate the role of gas diffusion path in the middle-ear (ME) mucosa and mucosal blood flow rate on ME gas economy. A trans-mucosal gas exchange method was used to record the ME volume changes at constant pressure. Mucosa thickness was measured as an estimate of gas diffusion path between the ME space and the ME circulation. Inflammation was inflicted using lipopolysaccharide (LPS) in one ear. The contralateral ear served as control. ME gas volume decreased significantly faster with time in inflamed ears (-0.107mL·min-1) vs control ears (-0.067mL·min-1). Mucosa thickness was significantly thicker in inflamed ears (48.4µm) vs controls (20.5µm). A mathematical model was used to estimate the relative effective mucosal blood flow rate. It predicted that in spite of almost doubling mucosa thickness of LPS-treated ears, the increased gas loss may be explained by doubling mucosal blood flow. We suggest that the quantitative estimate of ME blood flow presented is relevant to medical managing of an inflamed ME.

Up-regulation of proinflammatory and profibrotic mediators in a mouse model of otitis media with effusion induced by eustachian tube obstruction

Patricia Hebda, Ph.D., Chia-Yee Lo, M.S., Joseph Dohar, M.D., M.S., Ha-Sheng Li-Korotky, M.D., Ph.D.

Background

Disrupting eustachian tube (ET) function causes middle-ear (ME) pressure dysregulation, which in turn causes increased permeability of the mucosal vasculature and ME effusion, otherwise called *hydrops ex vacuo*. However, the mechanism(s) responsible for transducing the biological signal(s) associated with underpressure and initiating ME mucosal inflammation is not known.

Objective

To use our mouse model of otitis media with effusion (OME) to assess the inflammatory response of ME mucosa to the effects of ME pressure dysregulation induced by ET obstruction, and to compare with other animal models of OME and with the clinical disease.

Materials and methods

Unilateral ET obstruction was established in female C57BL/6 mice by electrocautery of the ET. Sham animals underwent the surgical procedure but without ET obstruction. Samples were collected from sets of animals at time points up to 8 weeks. The ME mucosa and surrounding bulla were collected individually, flash frozen, and processed for mRNA analysis by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), to measure TNF-a, IFN-g, IL-1b, IL-6, IL-10, TGF-b1, MCP-1, IL-8 (mouse equivalent), MIP-2, and COX-2.

Results

At 8 weeks, the ears with ET obstruction vs sham-treated controls demonstrated higher levels of key pro-inflammatory cytokines, chemokines, and TGF-b1 (pro-fibrotic mediator), as shown in Figure 1. Results are expressed in fold increase (or decrease) compared to normal control tissues. In the ETO group, all of the mediators of interest showed moderate increases in fold expression of mRNA, whereas only half the mediators were upregulated in the sham group. Pro-inflammatory mediators TNF-a, IFN-g, IL6, IL8, and pro-fibrotic mediator TGF-b1 were selectively increased with ETO. The anti-inflammatory cytokine IL10 was also upregulated in the ETO group.

Conclusions

Increases in pro-inflammatory and pro-fibrotic mediators following ET obstruction of non-infected ears strongly suggest that *hydrops ex vacuo* contributes to the pathogenesis of OME. This mouse model of OME is both feasible and effective, showing a consistent pattern of upregulation of pro-inflammatory cytokines and chemokines, comparable to previous results in rat OME, thereby paving the way for future mechanistic studies of key inflammatory pathways and relevant interventions.

Acknowledgements:

Figure 1. Fold Change in mRNA of selected mediators in the middle-ear mucosa of mice with obstructed (ETO) vs non-obstructed (sham) eustachian tubes by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR).
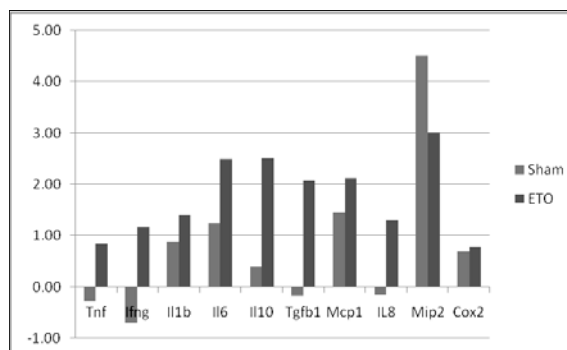
Entry into and survival of nontypeable *Haemophilus influenzae* in epithelial cells

Paul Webster, Ph.D.

Nontypeable *Haemophilus influenzae* (NTHi) is a major otitis media pathogen, particularly for recurrent infections and for chronic otitis media with effusion. While not generally considered to be an intracellular pathogen, NTHi has been reported to invade epithelial cells, which could be one explanation for its ability to persist within the upper respiratory tract.

In this study, the entry and intracellular fate of NTHi in Chang conjunctival epithelial cells has been examined. Small numbers of viable intracellular bacteria were detectable at 4 hours after exposure, and their numbers were increased by several-fold at 24 hours, indicating a capacity to adapt to intracellular survival. At 4 hours after exposure, while some intracellular NTHi were enclosed within vacuoles, the majority of NTHi were observed to be free in the cell cytoplasm. Even when enclosed within a vacuole, the NTHi did not always appear to be in typical phagosomal/endocytic compartments, and the membranes enclosing the bacteria showed limited labeling with mannose 6-phosphate receptor (MPR) and lysosome-associated membrane protein (LAMP1) endocytic pathway markers. Cells exposed to NTHi displayed clustering of LAMP1-positive structures and a reduced ability to accumulate horseradish peroxidase in the endocytic pathway. These data indicate that NTHi may have the ability to enter the cytoplasm and survive within epithelial cells for up to 24 hours.

CD14-dependent upregulation of ICAM-1 in human monocytes by *Moraxella catarrhalis* lipooligosaccharide

Hang Xie, Ph.D., Xin-Xing Gu, M.D.

Moraxella catarrhalis is a common human pathogen that causes otitis media (OM) in young children and pulmonary infections in the elderly. Lipooligosaccharide (LOS) from the outer membrane of *M. catarrhalis* is an endotoxin and has been suggested to play a role in the pathogenesis of *M. catarrhalis*. However, its exact underlying molecular mechanisms remain to be identified. In this study, we investigated the effects of *M. catarrhalis* LOS on the expression of adhesion antigens of human monocytes. It was found that *M. catarrhalis* LOS preferentially enhanced ICAM-1 expression on human monocytes. Time course studies suggested that the augmentation of ICAM-1 expression started as early as 3 hours and peaked at 16 hours following LOS stimulation. Using different monoclonal anti-human toll-like receptors (TLRs) and their accessory molecules, we found that such up-regulation of ICAM-1 on human monocytes was completely blocked by anti-CD14, though it could be partially down-regulated by anti-TLR4 at a different extent. Further analysis also indicated that the LOS-stimulated monocytes showed no difference in adherence to lung epithelium compared to non-stimulated human monocytes, suggesting cell surface ICAM-1 was not involved in monocyte adherence. We conclude that *M. catarrhalis* LOS upregulates ICAM-1 expression on human monocytes through CD14-dependent pathway.

Introduction

M. catarrhalis is one of the leading pathogens of OM in children and chronic obstructive pulmonary disorder in elderly people.[11] Several virulence factors of *M. catarrhalis* are believed to be involved in colonization, adherence, or complement resistance; however, the exact pathogenic mechanisms are not well understood.[1] Similar to other Gram-negative bacteria, *M. catarrhalis* possesses an endotoxin-lipooligosaccharide (LOS) in its outer membrane. *M. catarrhalis* LOS consists of an oligosaccharide core and lipid A without repeated O-antigen polysaccharide side chains, which is different from enteric bacterial lipopolysaccharide (LPS). While *M. catarrhalis* LOS is assumed to have the general biological properties of

endotoxins, its role in the pathogenesis of *M. catarrhalis*-associated diseases remains undefined.

Intercellular adhesion molecule 1 (ICAM-1, or CD54) is a member of the immunoglobulin superfamily.[1] It is constitutively expressed at low levels on the surface of epithelial and endothelial cells. By binding to CD11a/CD18 (LFA-1) and CD11b/CD18 (MAC-1), ICAM-1 regulates leukocyte infiltration and migration.[3,4,10,14] Recent evidence has suggested that some respiratory and mucosal pathogens can establish attachment and colonization by interacting with ICAM-1 or modulating its expression on epithelium and endothelium.[2,5,7,16]

In the present study, we showed that *M. catarrhalis* LOS selectively up-regulated surface ICAM-1 on human monocytes, which was mediated through a CD14-dependent pathway.

Materials and methods

A clinical isolate of *M. catarrhalis* strain O35E (serotype A) provided by Eric Hansen (University of Texas Southwestern Medical Center, Dallas, TX,) was grown on chocolate agar plates at 37°C, 5% CO_2. Ultrapure LOS was extracted from overnight culture by phenol-water extraction as described previously.[8]

Human monocytic cell line THP-1 and human lung epithelial cell line A549 were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium supplemented with 10% HI FBS, 2 mM L-glutamine,1 mM sodium pyruvate, 1% nonessential amino acids, 10 mM HEPES buffer (pH 7.3), 50 μM 2-mercaptoethanal, and antibiotics at 37 °C, 5% CO_2.

Cells (2×10^6 cells ml^{-1}) were stimulated with purified *M. catarrhalis* LOS at a final concentration of 1 μg ml^{-1} overnight. After 16-18 hours of incubation, cells were harvested and stained with the following fluorescence directly labeled anti-human mAbs: anti-ICAM-1 PE (eBioscience). The samples were resolved on an EPICS XL-MCL flow cytometer (Beckman Coulter), and data were analyzed by FlowJo 7.2 (Tree Star, Inc., Ashland, OR).

In the blocking experiments, cells (2×10^6 cells ml^{-1}) were pre-treated with (1) 20 μg ml^{-1} of the following functional grade purified mouse mAbs: 1)

anti-human TLR4 mAb, anti-human MD2 mAb, anti-human TLR2 mAb (eBioscience), and anti-human CD14 mAb (R&D Systems, Minneapolis, MN) or 2) matched isotype controls at $37^{o}C$ for at least 30 minutes before being stimulated with purified LOS.

The adherence of LOS-activated THP-1 cells to epithelial cell monolayer was determined as described previously with minor modifications.[1,7] A549 lung epithelial cells (5 x 10^4 cells per well) were seeded in 96-well black-framed ViewPlate microplates (PerkinElmer Life and Analytical Sciences, Shelton, CT) overnight until 100% confluence. Meanwhile, THP-1 cells labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) were stimulated with or without 1 μg ml^{-1} of LOS for 6 h. Epithelial monolayer was then overlaid with 5 x 10^5 CFSE labeled THP-1 cells in 100 μl per well of PBS containing 0.1% FCS and incubated at $37^{o}C$ for 30 min. Unbound THP-1 cells were removed by gently washing each well three times. Remaining fluorescence was determined by a VICTOR3™ multilabel microplate counter (PerkinElmer Life and Analytical Sciences). Autofluorescence from wells without CFSE labeled THP-1 was subtracted from all experimental wells. Adherence (%) was calculated based on the fluorescence signals of medium treated THP-1 cells adherent to epithelial surface.

All the data were expressed as the mean ± SEM, and analyzed by a *t* test with two-tailed *p* value. A *p* value < 0.05 was considered significant.

Results and discussion

M. catarrhalis LOS significantly up-regulates ICAM-1 expression on human THP-1 cells and primary monocytes

As shown in Figure 1, incubation of THP-1 cells with 1 μg ml^{-1} of *M. catarrhalis* LOS resulted in significantly enhanced expression of ICAM-1.

M. catarrhalis LOS induces a time-dependent up-regulation of ICAM-1 on THP-1 cells

The kinetics of *M. catarrhalis* LOS-induced surface ICAM-1 expression on THP-1 cells is shown in Figure 2. The mean fluorescence intensity (MFI) of ICAM-1 expression increased as early as 3 hours following LOS stimulation, which peaked at 16 hours (Fig. 2).

M. catarrhalis LOS induced up-regulation of ICAM-1 requires CD14 and TLR4

Toll-like receptor (TLR)-4, along with its co-receptor CD14 and accessory molecule MD2, mediate the signaling of *E. coli* LPS and *Neisseria meningitides* LOS.[6,12,13,18] However, it is unclear whether *M. catarrhalis* LOS induced up-regulation of ICAM-1 in THP-1 cells was also regulated in a similar way. Compared to the cells pre-treated with matched isotype control, neutralizing anti-human TLR4 mAb only partially down-regulated LOS-induced surface ICAM-1 expression in THP-1 cells (Fig. 3). Blocking MD2 or TLR2 alone had no effects on ICAM-1 up-regulation induced by LOS. In addition, blocking MD2 in addition to TLR4 did not show any further inhibition on ICAM-1 expression than anti-human TLR4 mAb alone pre-treated THP-1 cells. The most marked down-regulation on LOS-induced surface ICAM-1 expression was observed in THP-1 cells pre-neutralized by anti-human CD14 mAb (Fig. 3). Taken together, it suggested that *M. catarrhalis* LOS-enhanced surface ICAM-1 expression in human monocytes through a CD14-dependent pathway, a process which also involved TLR4.

Surface ICAM-1 of THP-1 cells is not required in the adherence of monocytes to pulmonary epithelial monolayer

Increased surface expression of ICAM-1 on epithelial and endothelial cells has been reported to contribute to an enhanced adherence of PMN and neutrophils to epithelial and endothelial monolayers.[1,7,9] However, in the current study, LOS stimulated THP-1 cells showed no difference in the adherence to the epithelial monolayer from medium-treated control cells (data not shown). Our results indicated that ICAM-1 on the surface of monocytes was not required in the adherence to the epithelial surface.

Conclusion

The results presented in this study suggested that *M. catarrhalis* LOS selectively enhanced surface ICAM-1 expression on human monocytes, which was CD14-dependent and partially regulated through TLR4. The information may bring new insight into developing vaccines and therapies against *M. catarrhalis* infections.

Acknowledgements

Figure 1. *M. catarrhalis* LOS up-regulated ICAM-1 expression on human THP-1.
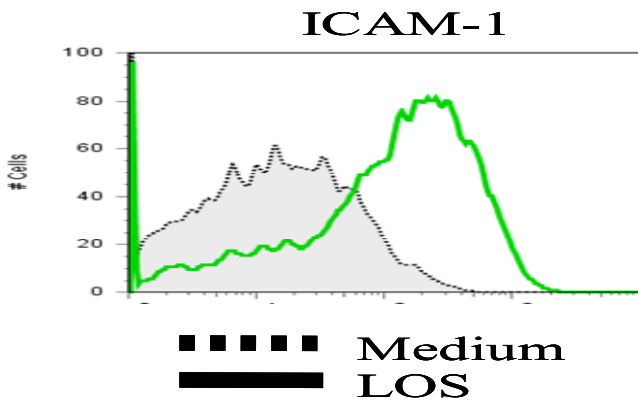
Figure 2. The kinetics of surface ICAM-1 expression on human THP-1 cells following *M. catarrhalis* LOS stimulation.
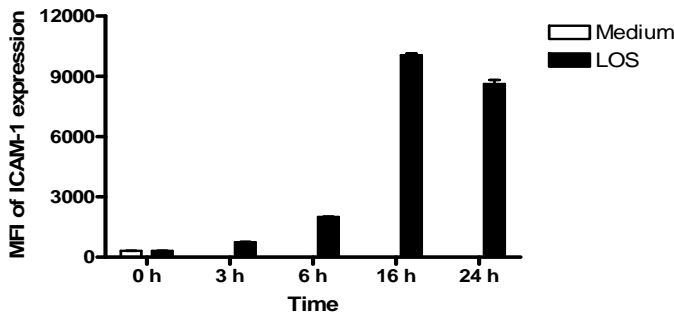
Figure 3. The role of CD14 and TLR4 in M. catarrhalis LOS-induced ICAM-1 expression in human monocytes.
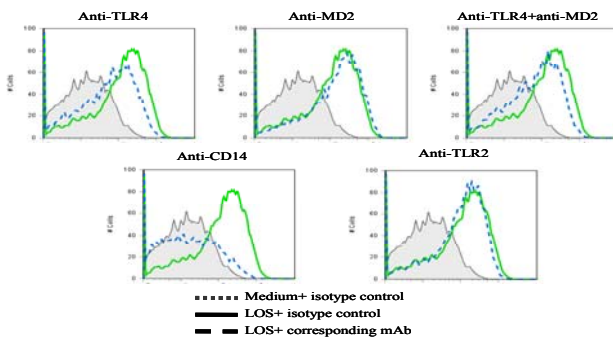
References

1. Arnold R, König W. Repiratory syncytial virus infection of human lung endothelial cells enhances selectively intercellular adhesion molecule-1 expression. J Immunol 2005; 174:7359-7367.

2. Avadhanula V, Rodriguez CA, Ulett GC, Bakaletz LO, Adderson EE. Nontypeable Haemophilus influenza adheres to intercellular adhesion molecule 1 (ICAM-1) on respiratory epithelial cells and upregulates ICAM-1 expression. Infect Immun 2006; 74:830-838.

3. Boyd AW, Wawryk SO, Burns GF, Fecondo JV. Intercellular adhesion molecule 1 (ICAM-1) has a central role in cell-cell contact-mediated immune mechanisms. Proc Natl Acad Sci USA 1988; 85:3095-3099.

4. Chang YJ, Holtzman MJ, Chen CC. Interferon-gamma-induced epithelial ICAM-1 expression and monocyte adhesion. Involvement of protein kinase C-dependent c-Src tyrosine kinase activation pathway. J Biol Chem 2002; 277:7118-7126.

5. Chavakis T, Hussain M, Kanse SM, et al. Staphylococcus aureus extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. Nat Med 2002; 8:687-693.

6. Dobrovolskaia MA, Vogel SN. Toll receptors, CD14, and macrophage activation and deactivation by LPS. Microbes Infect 2002; 4:903-914.

7. Frick AG, Joseph TD, Pang L, Rabe AM, St. Geme III JW, Look DC. Haemophilus influenza stimulates ICAM-1 expression on respiratory epithelial cells. J Immunol 2000; 164:4185-4196.

8. Gu XX, Chen J, Barenkamp SJ, Robbins JB, Tsai CM, Lim DJ, Battey J. Synthesis and characterization of lipooligosaccharide-based conjugates as vaccine candidates for Moraxella (Branhamella) catarrhalis. Infect Immun 1998; 66:1891-1897.

9. Kocabas C, Katsenelson N, Kanswal S, et al. Neisseria meningitides type C capsular polysaccharide inhibits lipooligosaccharide-induced cell activation by binding to CD14. Cell Microbiol 2007; 9:1297-1310.

10. Madjdpour C, Oertli B, Ziegler U, Bonvini JM, Pasch T, Beck-Schimmer B. Lipopolysaccharide induces functional ICAM-1 expression in rat alveolar epithelial cells in vitro. Am J Physiol Lung Cell Mol Physiol 2000; 278:L572-579.

11. Murphy TF, Bakaletz LO, Kyd JM, Watson B, Klein DL. Vaccines for otitis media: proposals for overcoming obstacles to progress. Vaccine 2005; 23:2696-2702.

12. Palsson-McDemott E.M, O'Neill LA. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. Immunology 2004; 113:153-162.

13. Pridmore AC, Jarvis GA, John CM, Jack DL, Dower SK, Read RC. Activation of toll-like receptor 2 (TLR2) and TLR4/MD2 by Neisseria is independent of capsule and lipooligosaccharide (LOS) sialylation but varies widely among LOS from different strains. Infect Immun 2003; 71:3901-3908.

14. Rosseau S, Wiechmann K, Moderer S, et al. Moraxella catarrhalis-infected alveolar epithelium induced monocyte recruitment and oxidative burst. Am J Respir Cell Mol Biol 2005; 32:157-166.

15. Rothlein R, Dustin ML, Marlin SD, Springer TA. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. J Immunol 1986; 137:1270-1274.

16. Thornton J, McDaniel LS. THP-1 monocytes up-regulate intercellular adhesion molecule 1 in response to pneumolysin from Streptococcus pneumoniae. Infect Immun 2005; 73:6493-6498.

17. Verduin CM, Hol C, Fleer A, van Dijk H, van Belkum A. *Moraxella catarrhalis*: from emerging to established pathogen. Clin Microbiol Rev 2002; 15:125-144.

18. Zughaier SM, Tzeng YL, Zimmer SM, Datta A, Carlson RW, Stephens DS. Neisseria meningitides lipooligosaccharide structure-dependent activation of the macrophage CD14/Toll-like receptor 4 pathway. Infect Immun 2004; 72:371-380.

In vitro formation of NTHi biofilms

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The ability of the Gram-negative nontypeable *Haemophilus influenzae* (NTHi) bacteria to form biofilms *in vitro* and *in vivo* is well documented. Evidence that otitis media is a biofilm infection is supported by the direct demonstration of biofilm structures taken from the middle ears of children with persistent, antibiotic-resistant infections. Although the link with biofilm formation and persistent infections is accepted, the process by which bacteria form biofilm structures is not well understood.

In this study, the early stages of biofilm formation using NTHi as a model organism has been examined. Biofilms that formed on a variety of abiotic and biotic surfaces were examined at the electron microscopic level to compare morphologies. The formation process of colony biofilms on membrane filters over a 4-day period was examined by electron microscopy and by estimating total biofilm mass and viable bacteria in the forming biofilms. Biofilm formation was characterized by a rapid production of extracellular material that covered the bacteria. At 6 hours, the number of viable bacteria multiplied rapidly until reaching a peak at 12 hours, after which time there was a rapid drop in viable bacteria. The lower number of viable bacteria remained constant over a 4-day period.

Although the total biomass of biofilm reached a peak before 12hours and remained constant over a 4-day period, the ultrastructure of the biofilms altered over time. More mature biofilms were characterized by a less dense packing of bacteria and by the presence of extracellular spaces. In some instances, regularly-spaced partition walls formed from the extracellular material. If it can be established that *in vitro*-formed biofilms, which are not contaminated with mammalian host proteins, are similar in composition to those associated with infection, these biofilms may be useful for proteomic and other studies.

Role of extra-esophageal reflux in chronic otitis media with effusion

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Background/objectives

Otitis media with effusion is the most common cause of childhood hearing loss. Despite its prevalence, the enormous health care expenditures resulting from its treatment, and the increasing therapeutic challenges imposed by antimicrobial resistance, very little is known regarding the cellular and molecular immunologic and inflammatory events in this disease process. Extra-esophageal reflux (EER) has been implicated in the pathogenesis of chronic otitis media with effusion (OME). The objective of this study was to confirm that children with chronic OME have EER into the middle ear as measured by the presence of pepsin in middle ear effusions (MEE) removed during tympanostomy tube (TT) placement.

Methods

MEE were obtained from children undergoing TT placement for OME. The fluid was lysed in a urea buffer and the presence of pepsin quantitatively determined by Western blot analysis using a specific anti-pepsin antibody. The pH of the samples was recorded before lysis.

Results

Pepsin protein was detected in 18/32 (56%) samples analyzed, with 12/20 (60%) patients having at least one positive sample for pepsin. Pepsin levels ranged from 80ng/mL to 1000ng/mL. The pH of the samples ranged from 6.0 to 7.6, mean pH = 6.8.

Conclusions

Pepsin was detected in 60% of patients with OME, confirming that EER into the middle ear occurs in these children. The pepsin present would have little or no activity at pH6.0 to 7.6; however, pepsin is stable below pH8.0 and thus could be reactivated following a decrease in pH.

Bactericidal efficacies of azithromycin on nontypeable *Haemophilus influenzae* adhered to and invaded cultured human epithelial cells

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Introduction

Nontypeable *Haemophilus influenzae* (NTHi) colonizes in the nasopharynx and accounts for the majority of localized infectious disease such as acute otitis media (AOM), sinusitis, bronchitis, and conjunctivitis.[1] Intimate association of NTHi with respiratory tract epithelia might be a necessary prerequisite factor for developing infection. Some of NTHi strains can internalize into epithelial cells *in vitro* following their adhesion onto epithelial cells.[2] Recent investigations showed that NTHi resided and replicated in human macrophage like cells found subepithelially in human adenoid tissue.[3] NTHi might be shielded from the local immune response and antibiotics by entering macrophages and epithelial cells. These possibilities may explain the persistence of NTHi in prolonged AOM, sinusitis and bronchitis.

In the current study, we evaluated the invasion of NTHi into human epithelial cells and further performed bacteriological efficacy of azithromycin (AZM) against NTHi.

Materials and methods

Bacterial strains and human epithelial cell line

Nine wild-type isolates of *H. influenzae* isolated from the nasopharynx of children with AOM were used in this study. *Escherichia coli* K-12 (ATCC 10798) and *Pseudomonas aeruginosa* PAO1 (ATCC BAA-47) as negative or positive control, respectively. The human nasopharyngeal epithelial cell line Detroit562 (ATCC CCL-138) was used in this study.

Trans-well chamber assay

Approximately 1×10^5 cells/cm^2 of Detroit 562 were cultured in each well of 24 trans-well chambers plates at 37˚C under a 5% CO_2 environment for 10 to 14 days until monolayer growth occurred. The NTHi suspensions were inoculated onto epithelial cell monolayer at 1×10^7 CFUs per 10 µl. The monolayer was then incubated at 37˚C under a 5% CO_2 environment for 3 h. After rinsing, the monolayer was used for three additional assays as follows: (i) The infected epithelial cells were lysed with Saponin for cell-associated NTHi. (ii) The infected monolayer was treated with 200µg/ml gentamicin for 2h for internalized NTHi.

Inhibition assay

The monolayer of Detroit562 cells prepared as for trans-well chamber assay was incubated in culture media containing various concentrations of AZM for 2 h. An epithelial cell monolayer containing various concentrations of AZM was used for a transwell chamber assay for evaluating inhibition of adherence and internalization of NTHi.

Results

Internalization of NTHi though cultured human epithelial cells

NTHi strains adhered to Detroit562 cell monolayer and subsequently internalized into the epithelial cells to various degrees. Out of nine strains, four strains (I-168, I-193, I-270, and I-275) adhered to Detroit562 cell monolayer and subsequently internalized into the epithelial monolayer (Fig. 1).The positive control of *P. aeruginosa* PAO1 strains showed the time-dependent increase of internalization and the negative control of *E. coli* K-12 strain did not show any internalization.

Bacteriological effects of azithromycin on NTHi internalized into cultured human epithelial cells

There were no significant differences in the adherence and subsequent internalization between Detroit562 cells treated with 1 µg/ml of AZM and the controls (Fig. 2).When the Detroit562 cells were treated with ≥10 µg/ml of AZM, the adherence and subsequent

internalization of strain I-270 were significantly reduced compared with the controls.

Discussion

It is of great interest to evaluate the bactericidal efficacies of antibiotics against intercellular NTHi. Recent observations suggest that NTHi can enter a variety of eukaryotic cells.[3] In this study, NTHi clinical isolates showed various degree of adherence on and subsequently internalization into cultured human epithelial monolayer. NTHi may possess the ability to invade and survive inside human epithelial cells. Intracellular localization of NTHi might serve as a reservoir and play a role in the pathogenesis of this pathogen for promoting recurrent infections. For the treatment of infections caused by facultative intracellular pathogens, both the accumulation of antibiotics and their magnitude become important.

Ahrén et al investigated the intracellular activity of antibiotics against NTHi and demonstrated excellent bactericidal activity of low-dose quinolones but only a limited effect of high-dose ampicillin.[4] Kratzer et al reported the importance of combined high extracellular and intracellular activities against intracellular NTHi.[5] However, these studies focused on only bactericidal activities of antibiotics against intracellular NTHi. AZM accumulates higher tissue and plasma concentration ratios, has a longer half-life than some other antibiotics but sustains lower levels of concentrations in extracellular locations and shows enhanced phagocytic killing.[6] The agent showed

marked bacteriological efficacy against not only adherence of NTHi on to cells but also NTHi internalized into epithelial cells. AZM has sufficiently relevant therapeutic efficacy to be used as an antibiotic against persistent infections.
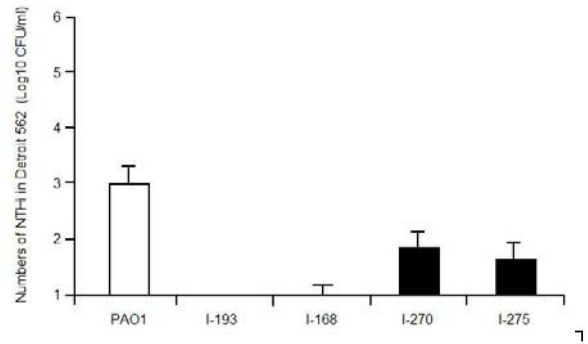
FIG. 1. (B)
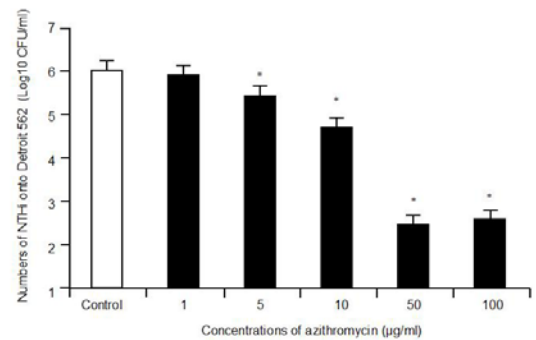
FIG. 2. (A)
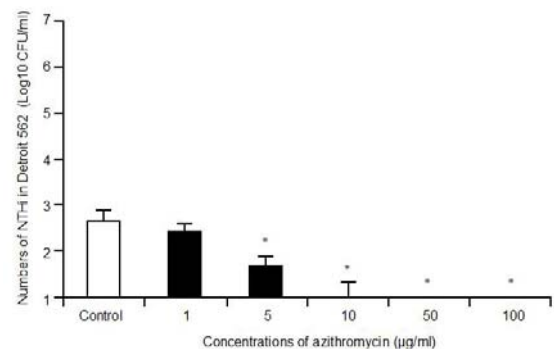
FIG. 2. (B)

References

1. Murphy TF. Respiratory infections caused by nontypeable *Haemophilus influenzae*. Curr Opin Infect Dis 2003; 16:129-314.
2. St. Geme III JW, Falkow S. *Haemophilus influenzae* adhered to and enters cultured human epithelial cells. Infect Immun 1990; 58:4036-4044.
3. Forsgren J, Samuelson A, Ahlin A, Jonasson J, Ryne-Dagoo B, Lindberg A. *Haemophilus influenzae* resides and multiplies intracellularly in human adenoid tissue as demonstrated by in situ hybridization and bacterial viability assay. Infect Immun1994; 62:673-679.
4. Ahrén IL, Karlsson E, Forsgren A, Riesbeck K. Comparison of the antibacterial activities of ampicillin, ciprofloxacin, clarithromycin, telithromycin and quinupristin/dalfopristin against intracellular non-typeable *Haemophilus influenzae*. J Antimicrob Chemother 2002; 50:903-906.
5. Kratzer C, Graninger W, Macfelda K et al. Comparative activities of antibiotics against intracellular non-typeable *Haemophilus influenzae*. Wien Klin Wochenschr 2007; 119:297-302.
6. Gladue RP, Sinder ME. Intracellular accumulation of azithromycin by cultured human fibroblast. Antimicrob Agents Chemother 1990; 34:1056-1060.

Prevalence of otitis media in young children during the respiratory season

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Introduction

Otitis media is a broad disease category that is often subclassified clinically on the basis of concurrent presentation with (acute otitis media) or without (otitis media with effusion) signs/symptoms thought to be associated with *in situ* infection of the middle ear (ME) cleft. However, because some of the signs/symptoms that are presumably diagnostic for acute otitis media (AOM) overlap with those of viral upper respiratory tract infections -- a known precipitant of otitis media (OM) -- clinical certainty with respect to subcategory assignment is undermined. Moreover, it is clear from natural history studies that uncomplicated AOM progresses to an expression diagnostically indistinguishable from otitis media with effusion (OME) both with and in the absence of effective antibiotic treatment, though the latter may favor a more rapid transition. Therefore, without extensive historical information and/or intrusive sampling for pathogens, it is reasonable to consider OM to be a single entity with different etiologies that converge onto the established presentation of middle ear mucosal inflammation with effusion.

There has been a long held interest in defining the incidence and prevalence of OM in the population and for abstracting from these data certain "risk factors" that circumscribe subpopulations most likely to include affected individuals. Because OME is largely asymptomatic, the most common method for establishing prevalence is population screening using various methods and either single or multiple assessment intervals. However, incidence estimates of OME are compromised by the typically long inter-assessment intervals employed in most studies where resolution with reacquisition of the disease would be assigned to a single episode.

In contrast, AOM presumably is associated with symptoms, making incidence and prevalence estimates easier. However, many of the AOM studies abstracted the reported presentations from large databases representing the combined data from multiple health care providers, and given the assumed relative and variable skills of the contributing physicians, the quality of the data may be compromised.

The purpose of this study was to define the incidence and prevalence of OM in an unselected cohort of children followed by pneumatic otoscopy at approximately 1 week intervals over the typical cold/flu months of November through April.

Materials and methods

The data for this report were abstracted from those available for the first 3 years of our ongoing, 5-year study entitled "Role of Virus and Genetic Susceptibility in Otitis Media". In this study, families with at least 2 children aged 1 to 5 years were recruited by newspaper advertisement and by word of mouth for participation in each year. Exclusion criteria included the presence in either child of a serious medical condition, a medical condition that predisposed them to persistent OM, a non-intact or structurally abnormal tympanic membrane, a pre-existing sensorineural hearing loss, or an inability to cooperate sufficiently with the examination and test procedures. After affirmation of willingness to participate and acquisition of written informed consent, families were entered into the study and were followed from October through April of the respective study year. The two index children who satisfied the enrollment criteria for the family and any older siblings less than 10 years who provided assent were followed. Families were reimbursed $100/month for their participation. The study protocol was approved by the Institutional Review Boards at the University of Pittsburgh and the University of Virginia.

Bilateral otoscopic examinations on all followed children were scheduled at approximately weekly intervals at an "in home visit" (Pittsburgh) or at a study clinic visit (Virginia). At each observation time, both ears were classified dichotomously by the otoscopist with respect to the presence/absence of OME or AOM based on ratings of the tympanic membrane with respect to visibility, other conditions, position, appearance, color, vascularity, light reflex and mobility. A positive otoscopic diagnosis for OM

was made when ME effusion was observed irrespective of the presence or absence of concurrent signs or symptoms of AOM. AOM was diagnosed by the presence of middle-ear effusion with at least one sign and one symptom of ME infection. Criteria considered in the diagnosis of AOM were parent-reported symptoms of ear pulling, irritability and fussiness, and fever, and otoscopic signs of bulging, erythema and/or white opacification - other than from scarring - of the tympanic membrane, and otorrhea from a perforation of a previously intact tympanic membrane.OME was assigned to OM episodes without concurrent signs and symptoms of infection. Episodes of AOM but not OME were treated empirically with antibiotics. Because otoscopy was not necessarily done at a time when the child presented with otologic symptoms (the children were seen and treated by their primary care physician for many of their acute illnesses), assignments of OM were biased to expressions of OME. Data were coded for the left and right ears as OME present/absent and AOM present/absent.

The purpose of the current analysis was to determine the prevalence and incidence of OM in the study population during the study period. Because of the variable entry times during the month of October, this report focuses on the period between November 1 and April 30 of each year. Daily OM status for each ear was determined as the status at one visit until the status at another visit. A new OM episode was defined as a diagnosis of OM at a visit which was preceded by a diagnosis of no OM. An episode of AOM was defined as a newly introduced diagnosis of AOM preceded by a visit at which AOM was not diagnosed.

Results

This report is based on 180 children enrolled from 84 families followed during the first 3 years of this study. The average age at entry was 3.8 years and age ranged from 1-9 years. Fifty-three percent were male; 84% were white and 13% were black.

There were8582 otoscopic observations on these 180 children. Twenty-five percent of children had 22 or fewer bilateral observations while 75% of children had more than 22 bilateral otoscopic examinations. OM was diagnosed at 13.3% of observations, 12.2 % as OME and 1.1% as AOM. The rest of the time, no OM was diagnosed. When OM was diagnosed, it was bilateral 54.6% of the time, and unilateral 45.4% of the time.

The daily prevalence of OM (all subjects and subjects with bilateral only) is shown as a function of time for the interval between November 1 and April 30 (Figure 1). For OM, the prevalence floor was

approximately 15% with peaks approaching 25% in mid-December and early March. A similar, but less well-characterized pattern was documented for bilateral OM where the prevalence varied between 5 and 10% and peaked at approximately 16% in early March. Sixty-three percent of effusions were noted to have cleared by 2 weeks; 83% were clear by 4 weeks, and 92 % resolved by 8 weeks. This distribution of OM duration is downwardly biased by the fact that OM sequences were begun before or terminated after the period of observation, and consequently, the durations reported for those episodes are less than or equal to the true value.

Age versus the number of OM episodes is shown in Figure 2. As expected, the younger children had more episodes of OM than did the older children.

Conclusions

This is just a preliminary look at the voluminous data obtained in this study. We found that most otoscopic examinations of the children enrolled in the study did not reveal OM. When OM was found, it was of short duration, and the younger children had more OM than the older children. The peak times of OM diagnosis were December and March.

A previous study using daily tympanometry for 1 month found that 8 of 43 (19%)OM episodes lasted for more than 26 days.[1]Another study using daily tympanometry and weekly otoscopy found only 24% of episodes lasted longer than 30 days, while 9% and 1.5% lasted more than 60 and 90 days, respectively.[2] Our finding of 17% of effusions lasting for more than 4 weeks is in agreement with these other studies.

That younger children have a higher incidence of OM has been shown in several studies. When children 2-4 years of age in the Netherlands were screened, MEE was found in 28-39% of children[3], while MEE was found in 10% of school-aged (7-8-year old) children in the Netherlands.[4]A longitudinal study of Danish children reported the point prevalence of a type B tympanogram at various ages: 13% at 1 year of age, 11-18% at 2-5 years of age, and 7% at 6-7 years of age.[5]

This study, using a limited number of observers and short observation intervals, provides reliable data on the prevalence of OM and duration of episodes in a carefully monitored population of children.

Acknowledgement

Figure 1. Prevalence of OM in subjects (%) by time of year during the observation period of November 1 – April 30. (―all OM; ---- bilateral OM)

Figure 2. Number of OM episodes (with 95% confidence interval) by age group (years).
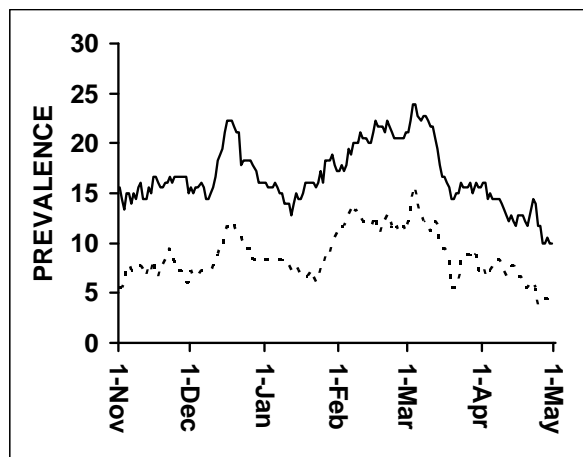
References

1. Elbrond O, Birch L.Daily impedance audiometric screening of children in a day-care institution. Auris Nasus Larynx 1985;12 Suppl 1:S216-218.
2. Antonio SM, Don D, Doyle WJ, Alper CM. Daily home tympanometry to study the pathogenesis of otitis media. Pediatr Infect Dis J 2002;21:882-885.
3. Zielhuis GA, Rach GH, vanden Broek P. The occurrence of otitis media with effusion in Dutch pre-school children. Clin Otolaryngol 1990;15:147-153.
4. Schilder AGM, Zielhuis GA, van dan Broek P. The otological profile of a cohort of Dutch 7.5-8-year olds. Clin Otolaryngol 1993;18:48-54.
5. Tos M.Epidemiology and spontaneous improvement of secretory otitis. Acta Otorhinolaryngol Belg 1983;37:31-43.

Diagnostic practice and formal medical education in acute otitis media: an Italian survey

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Background

The diagnosis of acute otitis media (AOM) has become increasingly important in the last few years for both pediatricians (PEDs) and otolaryngologists (ENTs). The 2004 AOM Clinical Practice Guideline reminds that clinical signs and symptoms alone are poorly predictive of the presence of AOM and that middle-ear effusion (MEE) is commonly confirmed with the use of pneumatic otoscope. Tympanometry and/or acoustic reflectometry can be considered diagnostic supplements. Few data are available regarding the diagnostic practice and formal medical education on AOM outside of the United States.

Aim

To determine the diagnostic practice, formal medical education, and attitude toward guidelines regarding AOM among Italian PEDs and ENTs.

Methods

In 2005 and 2006, a cross-sectional survey was administered to a national network of 1,000 PEDs (either primary or academic pediatricians) and 1,000 ENTs. We used a questionnaire to gather information on the diagnostic practice, formal medical education, and attitude toward guidelines regarding AOM. For preliminary analysis, we randomly selected 500 questionnaires from each specialty group.

Results

PEDs and ENTs were similar regarding age, years in practice, and experience with children with AOM. Children younger than 2 years were seen more frequently by PEDs (19.2%) than by ENTs (5.2%) (p< 0.0001). Most ENTs saw children only after a previous pediatric visit. Pneumatic otoscopy was indicated as a first diagnostic tool for AOM by 10.4% of PEDs and 3.2% of ENTs (p < 0.0001), static otoscopy by 87.4% of PEDs and 85.6% of ENTs (p=0.45). Potential diagnostic supplements were tympanometry (1.8% of PEDs and 26.4% of ENTs, p<0.0001) and otomicroscopy (27.2% of ENTs, no PEDs).

Overall, 57.2% of PEDs and 85.4% of ENTs (p<0.0001) had received a formal medical education on AOM, only minimally during medical school (18.4% PEDs and 21.0% ENTs, p=0.34), and more largely during residency (38.8% of PEDs and 64.4% of ENTs, p< 0.0001).

Diagnostic and therapeutic management of AOM was declared to be based on local guidelines by 35.2% of PEDs and 16.4% of ENTs (p < 0.0001), on international guidelines by 12.4% of PEDs and 12.4% of ENTs, on both local and international guidelines by 9.0% of PEDs and 2.2% of ENTs (p < 0.0001), and mainly on personal clinical experience by 40.2% of PEDs and by 68.8% of ENTs (p <0.0001).

Conclusion

These preliminary data indicate substantial variation in AOM diagnosis between Italian PEDs and ENTs, only partially explainable by the second-level approach of ENTs. The diagnostic recommendation by 2004 AAP guidelines are only rarely adopted by both PEDs and ENTs. Moreover, formal otitis media-related education is far from optimal in Italy, and this is reflected by the high number of PEDs and ENTs relying on their personal clinical experience. Educational strategies that could potentially lessen inter-specialist differences and compliance with published recommendations are needed.

Visualization of bacterial cells and glycocalyx in biofilms

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Introduction

Bacterial biofilms are structured communities of bacteria encased in a self-produced matrix that adhere on surfaces. Biofilm production is a major clinical problem because of the resistance of bacteria in biofilms to antibiotic treatments and host defenses. The presence of biofilms on human mucosal surfaces of the airways has been studied using a limited number of microscopic techniques, failing the visualization of the glycocalyx. Double staining for both bacterial cells and the biofilm matrix can demonstrate the presence of a biofilm, but so far has not been used for the study of mucosal surfaces in human tissue.

Therefore, the aim of this study is to demonstrate mucosal biofilms in human tissue by direct visualization of bacteria and the glycocalyx using confocal laser scanning microscopy with double fluorescence staining on tonsils and to compare it with the results of scanning electronic microscopy analysis.

Methods and materials

Specimens. Tonsils obtained during routine tonsillectomy from children with chronic or recurrent tonsillitis (n=24) were washed in phosphate-buffered saline (PBS) and cut in two parts to be respectively prepared for scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). The Committee of Medical Ethics of Leiden University Medical Center approved the protocol.

Scanning electron microscopy. The specimens for SEM were fixed with 1.5% glutaraldehyde in 0.2 M sodium cacodylate buffer pH 7.4 for 24 hours at 4°C on a rotary shaker. The samples were then subsequently dehydrated through a graded series of acetone solutions (70%, 80%, 90%, 96%, and 100% acetone) for 20 minutes at room temperature (RT), and critical point drying was performed. The specimens were then orientated, mounted on metal stubs, and sputter coated with gold using a Polaron 5000 Sputtering System (Watford, England) prior to imaging. The specimens were examined in a JSM6400 scanning electron microscope (JEOL, Tokyo, Japan) with digital imaging capabilities. The images were collected at an acceleration voltage of ~5.0 kV, a filament current of ~10^{-10}A, a working distance of ~39 mm.

Confocal laser scanning microscopy. Specimens for CLSM were immediately snap-frozen in cold isopenthane on dry ice and stored at –80°C. The frozen tissue specimens were then cut to a thickness of 10μm at –24°C using a cryostat CM 3050S (Leica, Bensheim, Germany) and fixated in 70% acetone. The obtained sections were then processed for double staining. They were washed three times with PBS, and first stained with propidium iodide 15 μM for 5 minutes at RT to detect bacterial cells in red. The sections were washed again with PBS and incubated with 50 μg mL^{-1} Concanavaline A fluorescein isocyanate-conjugated (ConA-FITC, C7642, Sigma, St. Louis, MO) for 5 minutes at RT to stain the glycocalyx in green. The sections were then successively washed in PBS and demineralized water and embedded in Gelvatol/DABCO. The sections were examined using an Axioplan upright microscope (Zeiss, Germany) equipped with a Biorad MRC1024ES scan head (Hercules, CA) with a krypton/argon laser for visualization of ConA-FITC (excitation 488 nm and emission 522 DF 32 nm) and propidium iodide (excitation 568 nm and emission 605 DF 32 nm). Digital images of the CLSM optical sections were collected using the Lasersharp 2000 software (Biorad, Hercules, CA).

Results

*Surface analysis of tonsils by **scanning electron microscopy**.* Attached bacteria were present on the surface of the specimen and could be clearly distinguished from smaller anorganic materials or irregularities of the epithelium nearby (Figs. 1A and 1B). The number of attached bacteria was highly variable between specimens. The cells were not evenly distributed over the entire surface of the specimen but rather clustered, forming microcolonies. These microcolonies were mostly located in small depressions between epithelial cells. Higher magnifications confirmed that the microcolony formations were preferably located at the junction of the epithelial cells, in little holes and/or crypts (Figs. 1C & 1D). Bacterial cells were connected by extracellular material, which could be representing a glycocalyx. Bacteria appeared to be organized in a scaffolding network located in small crypts.

Confocal laser scanning microscopy. To

visualize bacterial cells and a surrounding glycocalyx (which is indicative for bacterial biofilm formation) present on the surface of tonsillar tissue, a double staining was performed using propidium iodide and FITC-labeled ConA (Fig. 2). Bacterial cells and nuclei of tonsillar cells stained red, whereas binding of ConA resulting in green staining indicated the presence of a bacterial glycocalyx. Biofilms were observed both at the outside (Fig. 2A) and in crypts (Fig. 2B) of the tissue section. Most of the bacteria were cocci-shaped (Fig. 2). Interconnected bacteria were encased in a scaffolding network composed of extracellular matrix suggesting a three-dimensional (3D) architecture of biofilm formations (Figs. 2C and 2D).

All tonsils (24) were suitable for CLSM analysis; 17 (70.8%) showed evidence of mucosal biofilms according to the above-mentioned criteria.

Discussion

The main findings of the current study is the demonstration of mucosal biofilm *in vivo* using double staining in human tissue allowing in situ visualization of both the bacteria and the glycocalyx. At first glance, CLSM combined with double fluorescent staining provides the best anatomical evidence of what a biofilm is, according to its anatomical definition of bacteria encased in a self-produced exopolymeric matrix.

When compared to previous publications reporting the existence of mucosal biofilm, our results appeared to be conclusive. The comparison of the results achieved in our series, with the yet reported data documenting the use of CLSM in mucosal biofilm, noticeably shows the absence of double staining in the medical literature. Ehrlich et al.[1] used an animal model to monitor biofilm formation using a Live/Dead BacLight bacterial viability kit. Whereas he reported dead bacteria in red and living bacteria in green, no staining of the glycocalyx was provided. Post et al.[2] observed tympanostomy tubes removed from three children by CLSM using green fluorescent staining only for bacteria identification, without any staining at all for the glycocalyx. This lack of demonstration of the glycocalyx was the impetus for our study to provide clear demonstration of mucosal biofilm, i.e., bacteria encased in a glycocalyx at an air/specimen interface.

For bacterial identification, cells were easily and individually recognized by their size and morphology. One could argue that propidium iodide not only indicates bacterial cells because it is also taken up by the host cell nuclei.[1] However in our series, according to the point of view of all three investigators during the image analysis, no confusion existed between host cell nuclei and bacteria. However, the protocol we used only showed dead bacterial cells. Therefore, more living bacteria may exist, and the biofilm formations we showed here, may be underestimated in their bacterial cell content. For glycocalyx identification, because secreted exopolysaccharides are the most common reported component of the extracellular matrix,[3, 4] we used the Concanavalin A, a lectin that binds specifically to α-mannose.[5] One may argue that this staining is not specific of the mannose of the glycocalyx itself since those sugars are ubiquitous in tissue. In our series, bright areas within the bacteria or surrounding the bacterial colonies visualized the glycocalyx. Although no quantification was performed, the bright fluorescence between the bacteria emerged from the green background with a good signal to background ratio. However, the staining of the matrix with ConA-FITC was not homogeneously distributed. Some dark areas also were present within the biofilm. Unstained parts of the biofilm matrix may exist due to (i) existing water channels, (ii) heterogenous production of the matrix and the types of exopolysaccharides within the biofilm, and (iii) unbinded matrix to ConA as uneven distribution of lectin-stained structures has been reported.[6, 7] Therefore, CLSM not only permits the observation of hydrated and preserved structures,[8] but allows proper visualization of mucosal biofilms.

From an anatomical point of view, the presence of both the bacteria and glycocalyx were demonstrated, either attached to and integrated in the mucosal surface, or located in some crypts of the specimen. From a functional point of view, our data showed representative images of the dynamic process of biofilm formation and development. In an apparent process of division, bacterial cells exhibited metabolic activity. Our results also confirm the well-known 3D architecture of biofilm formations in a scaffolding network. All these images strengthen the notion that CLSM, combined with double fluorescent staining provides a clincher to make obvious the presence of biofilms and assert itself as the benchmark for in vivo demonstration of mucosal biofilms.

Surprisingly, in the medical literature, the presence of biofilm in human tissue was mainly reported using SEM and/or transmission electronic microscopy (TEM), both techniques that require a dehydration process. Because the matrix is highly hydrated (typically 95 to 99%), it is prone to dehydration artifacts. Therefore, when using either SEM or TEM, only the remnants of the original hydrated structure can be visualized.[5,8] Whereas bacterial microcolonies can be visualized with these techniques, water channels are obviously, if there are any, much more difficult to identify.

Despite these limitations, electron microscopy techniques are often the unique mean of investigation used to report first anatomical evidence of biofilm formations. Ramadan et al[9] as well as Cryer et al[10] have reported biofilm in chronic sinusitis in 5 and 16 patients respectively. Spherical bodies within the size range of bacterial organisms 0.05μm to 5μm were noted. But, in most of the images provided, a thick coating, which was uniformly distributed all over the surface of the specimen, severely hampered proper identification of bacterial colonies. In a series of 24 specimens of cholesteatomas, [11] some densely packed bacteria where shown using Gram staining and TEM, both techniques also requiring a dehydration process. Whereas they described a homogeneous amorphous background substance, there was no clear demonstration of the glycocalyx itself. Biofilm formations of indwelling silicone rubber tracheoesophageal voice protheses have also been reported in an electron microscopical study[12], but the images provided are limited to 100μμm magnification, which is not sufficient to identify bacteria. Similarly, an electron microscopy analysis was conducted on endotracheal tubes removed from intubated neonates.[13] Although image magnification is satisfactory, layers described as accretions, amorphous material, and debris may mimic coccal colonies that could actually correspond to anorganic materials. Also using SEM, Perloff et al. reported bacterial biofilms on frontal recess stents in 5 patients with chronic rhinosinusitis.[14] Although SEM images of silastic stent material in culture clearly showed colonies of bacteria, those from stents removed from patients after surgery were not convincing. Therefore, when compared with the SEM images reported in the medical literature, our results were much clearer to describe the presence of bacterial microcolonies.

In our series, according to the three investigators, the attached bacteria organized in microcolony formations that were identified on the surface of the specimen, could be clearly distinguished from smaller anorganic materials or irregularities of the epithelium nearby. Although higher magnifications clearly demonstrated interspersed and interconnected bacteria by extracellular matrix, SEM was limited to visualize the matrix which is logical considering the process of dehydration. Overviews of the surface of the specimen detected microcolony formations spread out in different areas, as if the superior part of the biofilm formation had been removed during the washing of the specimen. Concurrently, well-defined 3D biofilm formations were not seen, which could be explained by the fact that SEM gives only images of the surface without going through the thickness of the specimen.

Our data also suggest that biofilms play a role in tonsillitis. Chole and Faddis[15] raised the possibility that biofilms found in 73.3% of tonsillitis specimens could account for the recalcitrant nature of some cases of recurrent tonsillitis. Such data parallel our series, as the proportion of specimens, found to harbor mucosal biofilm, was comparable (70.7%). Also, as largely described in the literature, bacteria existing in the anatomical and functional state of mucosal biofilm, are commonly described as highly resistant to host defenses and antibiotics. These findings may have insightful implications for the development of rational treatment modalities toward the biofilm bacteria and glycocalyx.

Conclusion

From a technical point of view, CLSM combined with fluorescent staining of both the bacteria and the glycocalyx provides a clincher for the demonstration of mucosal biofilm. In our opinion, SEM is not the best technique for identification of bacteria, and CLSM with double staining should be retained as the benchmark for mucosal biofilm demonstration in human tissue.
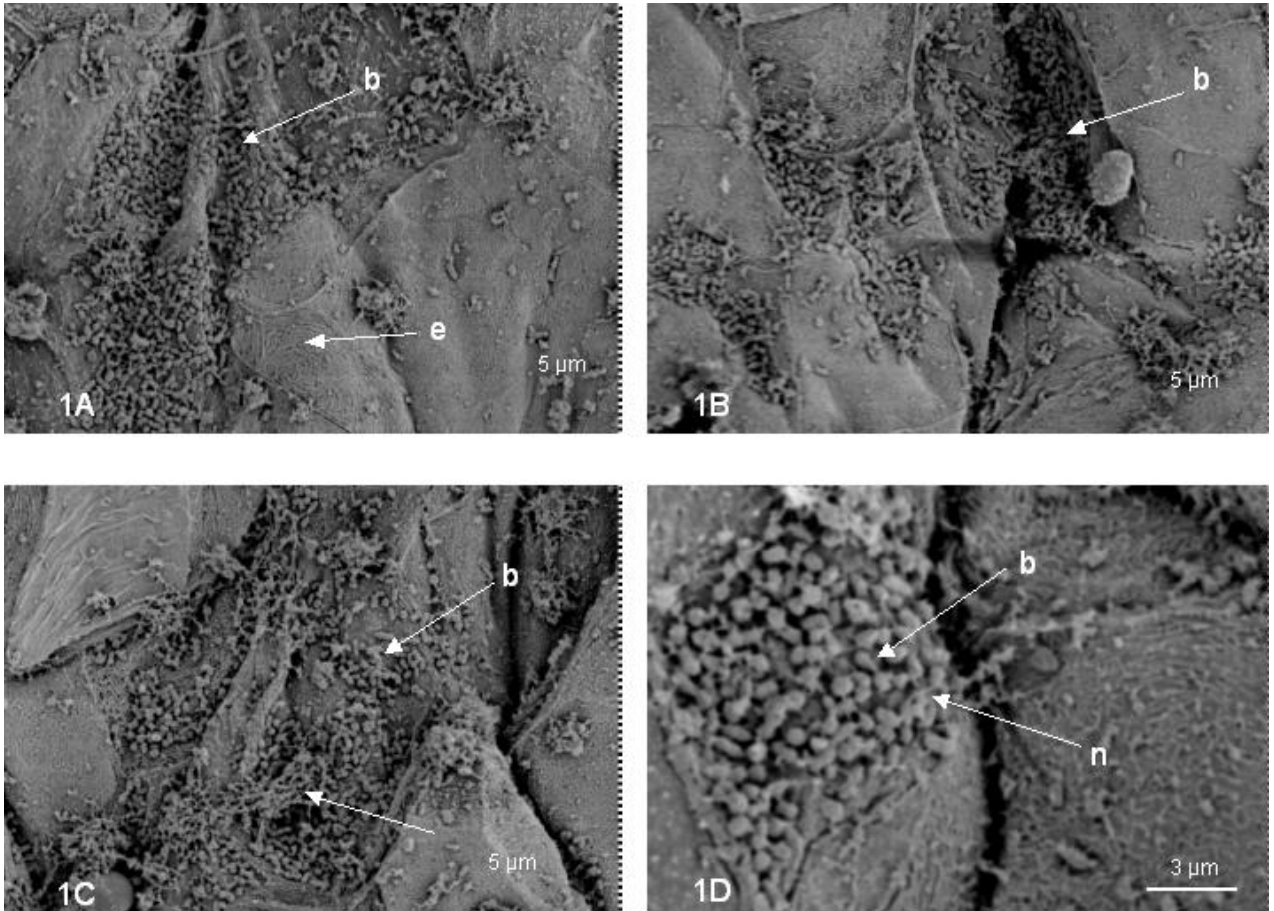
Figure 1. Scanning electron microscopy imaging of human tonsil surfaces. Attached bacteria were organized in microcolony formations mostly located in small depressions (pointed out by "b"-labeled arrow) between epithelial cells and in little depressions and/or crypts (Figs. 1A & B) with a widespread colonization of the tonsil surface. Images showed that cells were connected by extracellular materials resulting in a network-like organization (n-labeled arrow) (Figs. 1C and 1D).
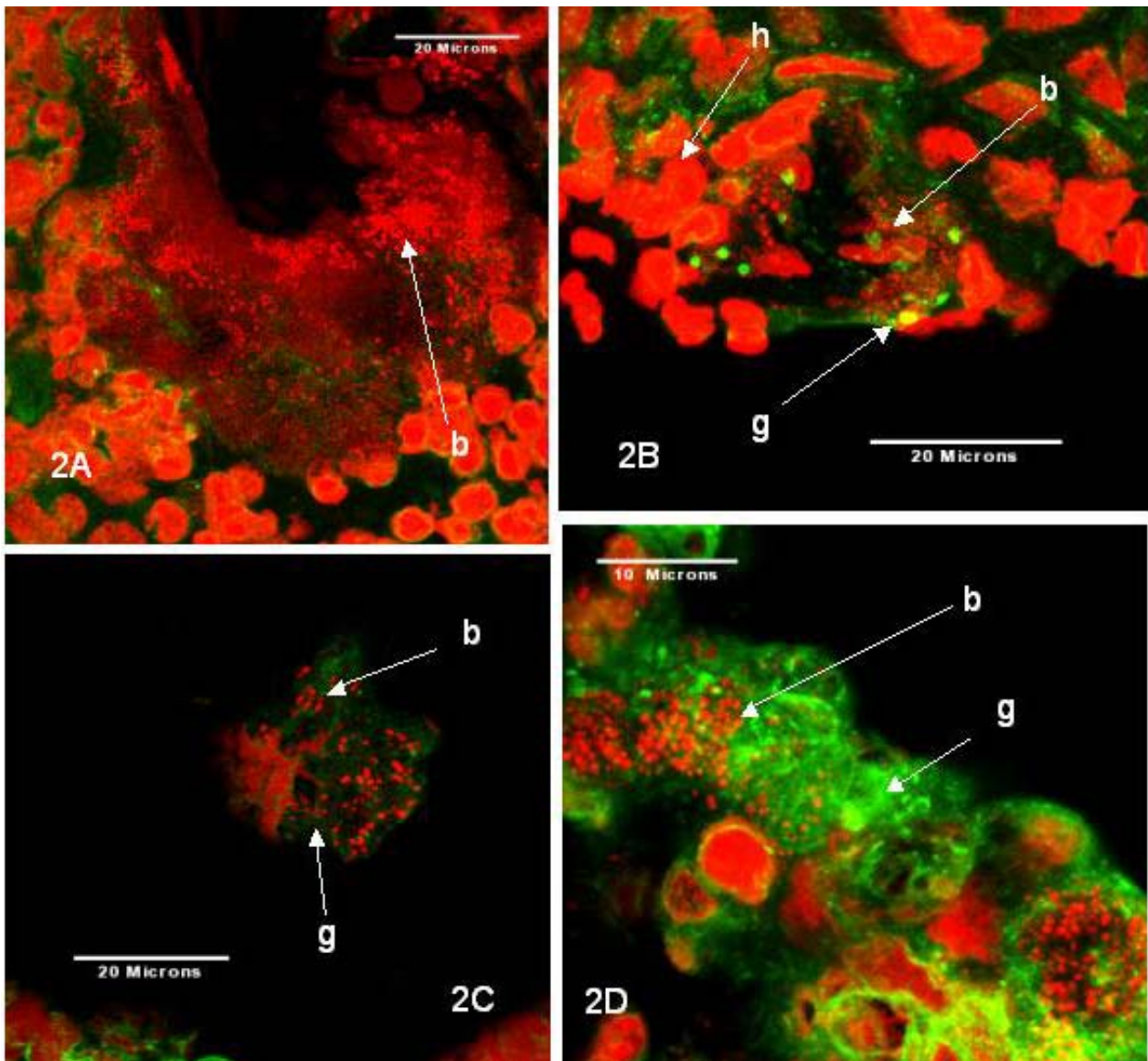
Figure 2. Confocal laser scanning microscopy images demonstrating the presence of bacterial biofilms on human tonsils. Tissue sections were stained with propidium iodide (red) for detection of DNA and FITC-conjugated Concanavaline A (green) for detection of bacterial glycocalyx. Both bacterial cells in biofilm (b-labeled arrow, as well as surrounding nuclei of tonsillar cells (h-labeled arrow) stained red. Green fluorescent staining around bacterial cells indicates the presence of a bacterial glycocalyx (g-labeled arrow). Biofilm formations are shown either in crypts (Fig. 2A) or on the outside of the tissue (Fig. 2B). Interconnected bacteria encased in a scaffolding network of glycocalyx forming a three-dimensional structure were observed (Figs. 2C and 2D).

References

1. Ehrlich GD, Veeh R, Wang X, et al. Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. JAMA 2002; 287:1710-1715.
2. Post JC. Direct evidence of bacterial biofilms in otitis media. Laryngoscope 2001; 111:2083-2094.
3. Christensen BE. The role of extracellular polysaccharides in biofilms. J Biotechnol 1989; 10:181-182.
4. Starkey M, Gray KA, Chang SI, Parsek MR. A sticky business: the extracellular polymeric substance matrix of bacterial biofilm. In: Ghannoum M, O'Toole GA, eds. *Microbial Biofilms*. Washington, DC: ASM Press; 2004: 174-191.
5. Akiyama H, Hamada T, Huh WK, et al. Confocal laser scanning microscopic observation of glycocalyx production by Staphylococcus aureus in skin lesions of bullous impetigo, atopic dermatitis and pemphigus foliaceus. Br J Dermatol 2003; 148:526-532.
6. Lawrence JR, Neu TR. Confocal laser scanning microscopy for analysis of microbial biofilms. Methods Enzymol 1999; 310:131-144.
7. Neu TR, Lawrence JR. Lectin-binding analysis in biofilm systems. Methods Enzymol 1999; 310:145-152.
8. McLean RJC, Bates CL, Barnes MB, McGowin CL, Aron GM. Methods of studying biofilms. In: Ghannoum M, O'Toole G.A., eds. *Microbial Biofilms*. Washington, DC: ASM Press; 2004: 379-413.
9. Ramadan HH, Sanclement JA, Thomas JG. Chronic rhinosinusitis and biofilms. Otolaryngol Head Neck Surg 2005; 132:414-417.
10. Cryer J, Schipor I, Perloff JR, Palmer JN. Evidence of bacterial biofilms in human chronic sinusitis. ORL J Otorhinolaryngol Relat Spec 2004; 66:155-158.
11. Chole RA, Faddis BT. Evidence for microbial biofilms in cholesteatomas. Arch Otolaryngol Head Neck Surg 2002; 128:1129-1133.
12. Leunisse C, van Weissenbruch R, Busscher HJ, van der Mei HC, Dijk F, Albers FW. Biofilm formation and design features of indwelling silicone rubber tracheoesophageal voice prostheses--an electron microscopical study. J Biomed Mater Res 2001; 58:556-563.
13. Zur KB, Mandell DL, Gordon RE, Holzman I, Rothschild MA. Electron microscopic analysis of biofilm on endotracheal tubes removed from intubated neonates. Otolaryngol Head Neck Surg 2004; 130:407-414.
14. Perloff JR, Palmer JN. Evidence of bacterial biofilms on frontal recess stents in patients with chronic rhinosinusitis. Am J Rhinol 2004; 18:377-380.
15. Chole RA, Faddis BT. Anatomical evidence of microbial biofilms in tonsillar tissues: a possible mechanism to explain chronicity. Arch Otolaryngol Head Neck Surg 2003; 129:634-636.

Prediction of upper respiratory tract bacteria in acute otitis media

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Conclusions

Thorough otomicroscopical examination of the tympanic membrane in AOM might distinguish AOM episodes caused by different bacteria. It thus might be a way to select proper treatment for each patient without raising the number of dangerous complications. Objectives: The main bacteria causing acute otitis media are Streptococcus pneumoniae (S. pneumoniae), Haemophilus influenzae (non-typeable Haemophilus influenzae), Moraxella catarrhalis (M. catarrhalis) and Streptococcus pyogenes, (GAS). The aim of this study was to see if it might be possible to predict the causative bacterium by judging the otomicroscopical appearance of the tympanic membrane in episodes of acute otitis media.

Methods

Patients suffering from non-perforated AOM were included. The tympanic membrane was photographed. A prediction of the causative bacterium was made and tympanocentesis was performed. Effusion from the middle ear and a nasopharyngeal swab were obtained for bacterial culturing. The causing bacteria were categorized into capsulated (S. pneumoniae and S. pyogenes) or nonencapsulated (NTHi and M. catarrhalis).

Results

A total of 82 patients were included in the study (44 male and 38 female, median age 2 years). A correct prediction was made in 47/63, a false prediction in 16/63 (kappa 0.48, p<0, 001). Sterile cultures were found in 9/82 and not conclusive cultures in 10/82.

Otological signs, changes, and treatment effectiveness in acute otitis media (AOM)

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Background

The medical literature describes many morphological criteria used to characterize the severity of acute otitis media (AOM) including redness, bulging, opacity and lack of movement of the tympanic membrane (TM), loss of normal TM structure, and the lack of the light reflex. Of these criteria, bulging and opacity of the TM are the most relevant for proper diagnosis of AOM.[1] Several studies were previously conducted to determine if it is possible to predict the etiology of AOM based on symptoms and clinical findings. Some studies suggest that the symptoms and findings in AOM due to *S. pneumoniae* were more severe[2,3], whereas other studies found that it is impossible to determine etiology based on clinical and otologic factors.[4,5]

A survey of the literature revealed only one study in which otologic changes were followed during the course of AOM.[6] In this study, the course of AOM was analyzed in 31 children at high risk for developing complications. They reported that all the children without perforation of the TM had moderate to severe bulging of the TM. In 77% of the cases, the pathological otologic signs were still present 14 days after the diagnosis, and very few returned to their normal state. No connection was found between the otologic findings and treatment failures. The study did not deal with the relationship between the otologic findings and the etiology of the disease.

Objective

To evaluate the otologic findings and their relationship with the bacterial etiology, treatment efficacy and prior infections in infants with AOM.

Patients and Methods

Study population included 111 children aged 3-36 mo. (mean 14±6m, 93% <2y) enrolled during 2001-2004 in 4 studies evaluating the efficacy of various antibiotics. Otological signs (redness, bulging and opacity of TM) were recorded at enrollment and during a 1-month follow-up. Middle ear fluid (MEF) was cultured by tympanocentesis pre-treatment (Day 1) and 3-5 days during treatment (Day 4-6) in initially culture-positive patients. Patients were also examined at day 11-14 and followed until Day 22–28. To guarantee maximal diagnostic precision and follow-up consistency and continuity, only the files of participants whose first and second examination were performed by the same two otorhinolaryngologists (YS, SR) were included in this study. The third and fourth examinations were performed by a senior pediatrician from the Pediatric Infectious Diseases Unit. Demographic data were recorded, along with information on tympanic membrane findings (redness, bulging and opacity). In addition, the microbiologic results of the MEF cultures were recorded.

Results

One hundred seventy three MEF cultures were obtained at visit 1: 150 Cx(+) and 23 Cx(-). The most common bacteria isolated was *H. influenzae* (61.2%) followed by *S. pneumoniae* (34.1%). *M. catarrhalis* (3.9%) and Group A streptococcus (0.83%) were found at low percentages. 129 tympanocenteses were performed on Day 4-6. Severe redness and severe bulging of TM were more common at diagnosis among patients with culture(+) *vs.* those with culture(-) MEF: 105/150,70%, *vs.* 10/23, 43.3%, P=0.01 and 150/107,71%*vs.*13/23, 56%, P=0.05, respectively)-Figure 1. No similar difference was found at the second visit (P=0.15 and 0.8, respectively). Severe TM bulging was less common among patients with ≥3 previous AOM episodes compared with those with <3 episodes, (62/113, 54.9% *vs.* 58/83. 69.9%, P=0.03).

Discussion

In this study, we attempted to follow children with AOM during antibiotic therapy, not just at the time of diagnosis (as described in the majority of the medical literature), in order to document the severity of findings, and to assess the relationship between the pathogens causing the disease and the otoscopic findings. This study included 111 patients who were followed during the antibiotic therapy period. Throughout the follow-up period the patients were examined and underwent tympanocentesis by the same 2 ENT physicians to allow for the greatest possible

continuity of care and precision of the procedure. At every visit a list of clinical signs and symptoms was generated and the severity of the otologic signs was assessed. All of the patients were treated by the double tympanocentesis method that allows a bacteriologic diagnosis at enrollment and determines whether sterilization of the middle ear fluid was achieved during treatment. Regarding the redness and opacity of the TM, the course of the disease was such that severe redness and opacity were found at diagnosis in the majority of cases, whereas by the second visit an equal number of severe and less severe observations was recorded. Regarding TM bulging, the course of the disease was characterized by severe bulging at the first visit and, in most cases, no bulging from the second visit and on. At diagnosis and at the second visit, the percentage of cases with severe redness and bulging was higher among the culture-positive ears compared with those with a sterile culture. Opacity, however, was not found to be affected by culture positivity at the first, second or third visit. These findings confirm the findings previously reported by Polachek and his group[3] who reported a stronger inflammatory systemic response (as expressed in numbers of peripheral blood WBC) in the cases of AOM where the MEF cultures were positive compared with cases in which the cultures were negative. Similarly, Leibovitz and his group[4] analyzed 372 children with AOM by means of a clinical scoring system based on symptoms (restlessness and ear tugging), systemic signs (body temperature) and otoscopic signs (redness and bulging). They reported that the clinical/otologic scores were higher among children with positive MEF cultures compared with children with negative cultures. Additionally, they found that redness and bulging of the TM were the determining factors in the scoring system, capable of distinguishing between a bacterial etiology and a non-bacterial etiology.

The variation among the otoscopic findings can be attributed to the fact that redness and opacity are due to an inflammatory process occurring in the middle ear and depend on the presence or absence of MEF whereas bulging also depends on the response of the TM to stretching, a characteristic that can change following repeated infections. This hypothesis clearly demands histopathological support in future studies.

Conclusions

1) Otological signs were more severe among patients with Cx (+) MEF. 2) On follow-up no differences were found in the TM severity of redness, opacity and bulging between the cases where MEF sterilization and those without MEF sterilization.

Figure 1. The influence of etiology on the severity of the otologic signs
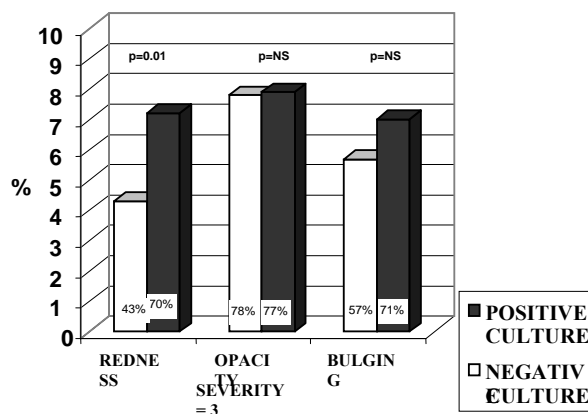
References

1. Rothman R, Owens T, Sinel D. Does this child have acute otitis media? JAMA 2003;290:1633-1642.
2. Rodriguez WJ, Schwartz RH. Streptococcus pneumoniae causes otitis media with higher fever and more redness of tympanic membranes than Haemophilus influenzae or Moraxella catarrhalis. Pediatr Infect Dis J 1999;18:942-944.
3. Polachek A, Greenberg D, Lavi-Givon N, Broides A, Leiberman A, Dagan R, Leibovitz E. Relationship among peripheral leukocyte counts, etiologic agents and clinical manifestations in acute otitis media. Pediatr Infect Dis J2004;23:406-413.
4. Leibovitz E, Satran R, Piglansky L, Raiz S, Press J, Leiberman A, Dagan R. Can acute otitis media caused by Haemophilus influenzae be distinguished from that caused by Streptococcus pneumoniae. Pediatr Infect Dis J2003;22:509-514.
5. Palmu AAI, Herva E, Savolainen H, Karma P, Makela H, Kilpi TM. Association of clinical signs and symptoms with bacterial findings in acute otitis media. CID2004;38:234-242.
6. Gibney KB, Morris PS, Carapetis JR, Skull SA, Smith-Vaughan HC, Stubbs E, Leach AJ. The clinical course of acute otitis media in high-risk Australian Aboriginal children: a longitudinal study. BMC Pediatr 2005;5:16.

Comparison of extended high frequency DPAOEs and behavioral thresholds in children with and without histories of significant otitis media

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Introduction

Compared to other research in otitis media, there is a relative paucity of data that exist regarding extended high frequency hearing of children with histories of otitis media and/or tympanostomy tube insertions. Several studies have shown that children with numerous bouts of acute otitis media, had more time with ear disease or who had multiple sets of tympanostomy tubes had more chance of having a hearing loss in the extended high frequencies i.e. above 8000 Hz. [1,2,3,4] Margolis et al.[5], reported similar findings of decreased auditory sensitivity in the extended high frequency region, and surmised that the cochlea was involved as the site of lesion. Ryding et al.[3], also reported that the extended high frequency hearing loss was mainly sensorineural and that the loss is consistent with the suggestion by Engel and colleagues[6] that middle ear toxins can diffuse through the round window membrane and affect the basal turn of the cochlea.

The functional consequences of hearing loss in the extended high frequencies are not well understood, but there is some speculation that the extended high frequency auditory nerve fibers are important for encoding speech in noise. More information is needed not only on the hearing in the extended high frequencies, but also etiology of the loss and the impact of such hearing loss on communication abilities. Consequently, this study was conducted in an attempt to gain more insight into high frequency hearing in children with histories of otitis media and the ability to detect the loss with the use of otoacoustic emissions (OAEs). OAEs are an objective means of detecting presence or absence of hearing loss. Given the young age when otitis media typically occurs, having an objective means of detecting hearing loss is critical. Furthermore, it is believed that otoacoustic emissions could potentially shed light on the issue of site of lesion.

The children in this study were part of a larger study to determine normative values for vestibular testing and the effect of otitis media on vestibular testing. Two different groups of children were enrolled in the study. One group included children with known middle-ear status since early infancy that were followed longitudinally with yearly assessment of hearing, and monthly assessment of middle ear status. The second group included children with patent tympanostomy tubes who were evaluated six months after tube insertion and compared to age-matched controls, who had two or less episodes of otitis media since birth.

Methods

All children were examined with pneumatic otoscopy and tympanometry prior to hearing evaluation to confirm that the middle ear status was normal, or that the tympanostomy tubes were patent at the time of testing. Behavioral hearing testing was performed at 8 different frequencies from 500 Hz to 20k Hz, encompassing both traditional and extended high frequencies, using conventional audiometry testing methods of 10 down and 5 up. Distortion Product Otoacoustic Emissions (DPOAEs) frequency sweeps were measured from roughly 1500 to 16,000 Hz (F2) using either a Biologic Scout System or a Mimosa Otoacoustic System. The f2/f1 was equal to 1.22 and the stimulus levels were (L1/L2) of 65/55 and 55/45. Two stimulus levels were used in an attempt to evaluate if the lower stimulus levels better separated the groups.

Results

Ninety-nine children were enrolled in the longitudinal study. The children were tested yearly within six months of their birthday from 5 years through 9 years of age. There were slightly more males than females with 57 males and 42 females. The majority of the children were white; 77 white, 17 African American and 5 biracial. Of the children followed longitudinally, complete audiologic data including both behavioral measures and otoacoustic emissions were obtained on 34 children for one year, 24 children for two years, 27 children for three years but only 10 children for four years and 4 children provided no complete data. The other groups were 17 children who served as a normal control group matched for age and 16 children who

had a history of otitis media that necessitated tympanostomy tubes insertion. The mean age was 6 years 4 months in the control group and 6 years in the tube group. In the group of children who received tympanostomy tubes, conventional and extended high frequency hearing was assessed 6 months after tympanostomy tube insertion.

The mean hearing thresholds for the different testing frequencies from 500 Hz through8000 Hz were within the normal range. As expected, the hearing levels increased as a function of frequency for frequencies above 8000 Hz. Hearing levels also varied as a function of group. The hearing levels for the longitudinal cohort group fell between the hearing levels for these two groups. Figure 1shows the average hearing levels plotted by group (longitudinal cohort, tube and control groups) and by ear. As can be seen, there is a clear separation in hearing threshold levels at 12 through 20 kHz for the groups, with the widest separation occurring between the groups with the most dissimilar histories of otitis media, namely, the control and the tube group. The longitudinal cohort group, who had a range of histories of otitis media, was plotted only as an illustrative comparison group to the control and tube who have the more extreme histories with respect to otitis media. Testing for significance between the otitis media group and the tube group and using a linear regression model that took into consideration multiple observations i.e. right and/or left ears per child and included adjustment of age, a significant difference in hearing levels for the extended high frequencies was found.

Distortion Product Otoacoustic Emissions.

We measured the amplitude of the distortion product otoacoustic emission, the relative amplitude minus the noise floor and the noise floor. Figure 2 shows a comparison of the tube and control group for the measure of absolute amplitude and noise with absolute amplitude differences clearly noted between the tube and control groups. Figure 3 shows the DPOAE amplitude minus the noise floor (DP-NF) for the tube and otitis groups at the lower stimuli intensity levels of 55/45 dB (left) compared to the 65/55 dB(right). Similar to the audiometric data, there are differences noted between the groups with the higher amplitude found for the control group and the lower amplitude noted for the tube group. The amplitude changes are expected with stimuli intensity changes and in fact follow what we might expect from normally hearing ears.

Table 1 shows the p values for the DPAOEs between the control and the tube groups. There are statistically significant differences between the tube and control group at most frequencies tested.

Discussion

The results of this descriptive study show that both hearing thresholds and distortion product otoacoustic emission differences are seen between children with varying histories of otitis media. Children with no substantial history of otitis media (control group) had significantly better hearing thresholds in the extended high frequency region compared to children who had tympanostomy tubes inserted. The differences were roughly 12-15 dB, with the greatest difference observed at 16,000 Hz. The comparison group of children in the longitudinal cohort group who had varying degrees of time with middle ear effusion falls between these two groups, indicating that some history of otitis media does influence the hearing levels in the extended high frequency region, but not to the extent found in the tube group. Others have reported on high frequency hearing for children with histories of otitis media and tympanostomy tube insertion and have shown that hearing is worse in the frequency region above 8000 Hz. The amount of difference between children with and without histories of otitis media varied from 9 dB to a high of 25 dB. The high value of 25 dB was reported in a group of child with multiple sets of tympanostomy tubes. The differences found in this study are in line with those previous reports.

The findings for the distortion product otoacoustic emissions in the conventional range of hearing (approximately 1500 to 8000 Hz) mirror the extended high frequency hearing threshold, with the control group having the highest amplitudes (DP-NF) and the tube group having the lowest amplitudes, and most frequencies were found to be significantly different. Of interest though, is that there was relatively little difference found between groups for the extended high frequency region, i.e. above 8000 Hz. This may be reflective of calibration issues rather than reflecting actual similarities between groups. Further review of the calibration needs to be undertaken.

The lower stimulus intensity level used for the DPOAEs, i.e. 55/45 did not seem to add to our findings of helping to differentiate possible site of lesion as all groups showed an increase in response amplitude with increase in stimulus level, with similar increase found in all groups.

For children who are unable to participate in behavioral audiologic testing, it appears from the findings of the present study that recording otoacoustic emissions is a viable option for monitoring hearing levels and that recording emissions in the conventional

frequency range may be reflective of hearing in the higher frequencies. Additional analysis will be conducted to further evaluate the otoacoustic emissions in the conventional range of hearing with respect to hearing in the extended high frequency range. In addition, since the children in the tube group were tested when their tubes were in place, repeated testing is necessary in this group when their tubes are extruded to see if the differences found in this study hold.
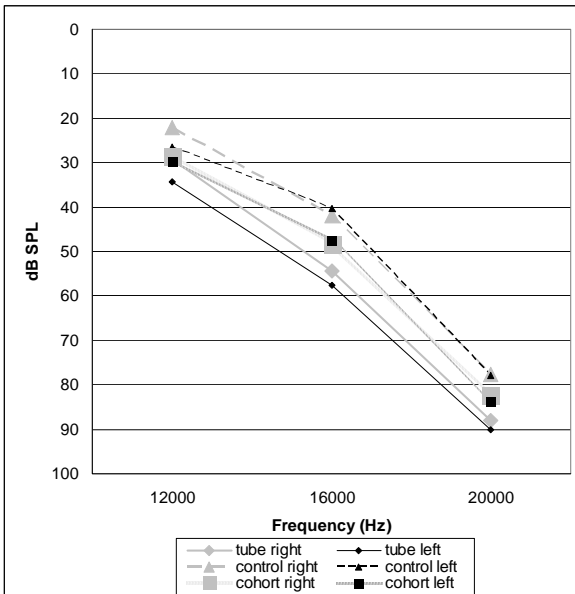
Figure 1. Average hearing levels in the extended high frequencies by group & by ear.
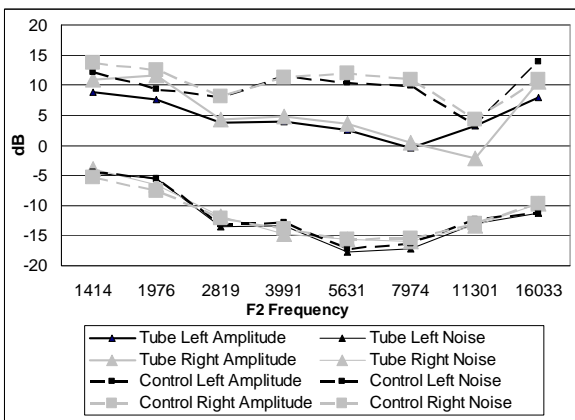
Figure 2. Comparison of tube and control group's DPOAE plotted showing amplitude and noise floor for the right and left ears.
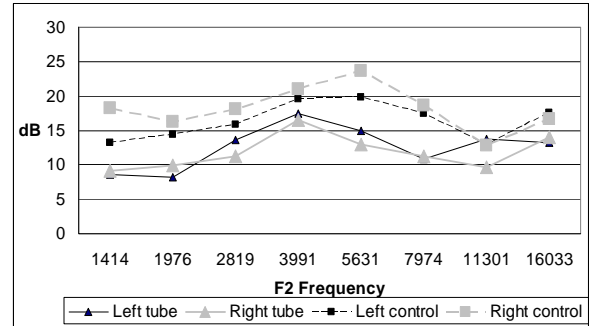
Figure 3. DPOAE (DP-NF) Comparison by stimulus level, and by ear for tube and control Group. The stimulus level was 55/45 on the left and 65/55 on the right.
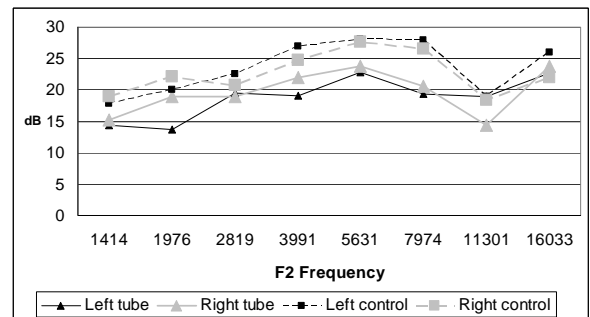
F2 Frequency	1414	1976	2819	3991	5631	7974	11301	16033
Control vs. tube groups 65/55	0.10	0.006*	0.16	0.02*	0.01*	0.002*	.030	0.52
Control vs. tube groups 55/45	0.04*	<0.01*	0.02*	0.19	0.004*	0.006*	0.54	0.04*

Table 1.P values for DPOAE (DP-NF) * denotes significance at 0.05.

References

1. Hunter LL, Margolis RH, Rykken JR, Le CT, Daly DA & Giebink GS. High frequency hearing loss associated with otitis media. Ear Hear 1996;17:1-11.
2. Laitila P, Karma P, Sipila M, Manninen M & Rahko T. Extended high frequency hearing and history of acute otitis media in 14-year-old children in Finland. Acta Otolaryngol (Stockh) 1997;Suppl529:27-29.
3. Ryding M, Konradsson K, White P & Kalm O. Hearing loss after "refractory" secretory otitis media. Acta Otolaryngol 2005;125:250-255.
4. Gravel JS, Roberts JE, Roush J, et al. Early otitis media with effusion, hearing loss, and auditory processes at school age. Ear Hear 2006;27:353-368.
5. Margolis RH, Saly GL, Hunter LL. High-frequency hearing loss and wideband middle ear impedance in children with otitis media histories. Ear Hear 2000;21:206-211.
6. Engel F, Blatz R, Schliebs R, Palmer M, Bhadki S. Bacterial cytolysin perturbs round window membrane permeability barrier in vivo: possible cause of sensorineural hearing loss in acute otitis media. Infect Immun 1998;66:343-346.

Hearing in unilateral cleft lip and palate children

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Introduction

Conductive hearing loss accompanied by otitis media with effusion (OME) is the usual finding in cleft palate population[1] and its severity correlates with the severity of the cleft type.[2] The natural history of OME is still unclear and often described as multi-factorial. Unilateral cleft lip and palate (UCLP) is characterized with asymmetries of the soft tissue of nasomaxillary and lower facial structures. The structural and functional changes of hearing and middle ear condition in cleft vs. non- cleft side are still unclear. The aim of the study was to find out does hearing condition in the presence of the structural changes on the cleft side shows higher frequency and severity of hearing loss than non-cleft side.

Method

We measured medians (MHL) and average thresholds (AHL) at 250 up to 4 kHz, for the left and right ears respectively in 101 unilateral cleft lip and palate children (74 with left and 27 with right cleft side) according to age subgroups: 1-3 years, 4-7 years, 8-12 years, >12years, (68 males, 33 females, both with median age of 6 years). The hearing level was classified as normal (0-10 dB), mild (11-20dB), moderate (21-40 dB) and severe >40 dB[3]. Non-cleft group of ears served as control, right cleft side ears served as double control for the groups of the left cleft side ears. Tympanometry confirmed otitis media with effusion by tympanogram of B type. Descriptive statistics determined the main features of audiologic data before ventilation tubes were placed.

Results

In all tested children age group of 1-3 years have highest number of ears with moderate and severe AHL. Right ears had median hearing level higher for lower register at 500Hz while left ears had higher at middle register of 1 kHz, 2 kHz, 4 kHz (Table 1). Right ears of UCLP (L) show significant improvement of AHL until 4-7 years. Left (cleft) ears of UCLP (L) showed no age related improvement of median hearing loss until 12 years. MHL at 250 Hz showed less improvement with aging if compared to other frequencies. Left ears of UCLP (L) showed age related MHL improvement for middle register (10 dB at 500

Hz and 1 kHz, 7.5dB at 4 kHz) which is lower than MHL improvement of right ears for low register (15dB for 500 Hz and 1000 Hz). Improvement of AHL is twice bigger for right vs. left ears. All tested ears showed age related improvement of AHL and MHL. (Table 2.) Left ears in UCLP (L) and UCLP shoved significant age related decrease of ears with moderate and severe AHL (p>0.001) but with no increase of mild and normal hearing ears (Figure 1, Figure 3). Right ears in UCLP (L) have age related decrease of moderate and severe hearing ears with significant age related increase of mild and normal hearing ears (p<0.005) (Figure 2, Figure 4.)

Discussion

Previous studies described changes of the length and angulations of the cranial base, more backward and upward position of the maxilla and smaller sphenopalatine angle as additional etiological factors to OME in UCLP patients. Kemaloglu[4] found difference of the scull base in cleft vs. non-cleft side patients. Our results suggested that cleft lip and palate could not be considered only as a local defect. Left (cleft) ears in UCLP (L) are more pronounced for hearing impairment than right ears because of developmental phenomena during which a primary defect in the orofacial region is associated by secondary defects of contiguous structures of the 1st branchial arch of the cleft side. According to our findings, left cleft ears (UCLP) (L) have a different mechanism of hearing loss then non-cleft ears. Literature showed that middle frequencies are affected mostly because of accumulation of the fluid in the middle ear, which covers and increases tympanic membrane mass.[5] Non-cleft right ears have less middle ear effusion and have more prominent edema of mucosa, which affects the hearing at the lower frequencies register. The presence of the cleft is an additional etiological factor, which contributes to the higher frequency and universality of the OME in cleft palate individuals.

Conclusion

Left ears on cleft side are prone to higher frequency of moderate and severe hearing disturbance than right ears groups and have not significant increase in number of ears with normal average hearing level with aging. Characteristics of hearing loss and its improvement in UCLP children depend of cleft type, ear side and age group.

Table 1. Median hearing level and ranges by age groups, absolute values in dB.

Cleft type age	N	Right Ears						Left ears					
		AHL R	250 Hz	500 Hz	1 kHz	2 kHz	4 kHz	AHL L	250 Hz	500 Hz	1 kHz	2 kHz	4 kHz
UCLP	101	20	20	20	20	20	20	21	20	25	20	20	20
1-3	11	35	30	40	30	30	25	33	25	30	30	30	30
4-7	51	21	25	20	20	20	20	25	25	25	25	20	25
8-12	24	18	20	20	20	15	20	18	20	20	20	15	20
>12	15	16	20	15	15	10	10	16	15	15	10	10	15
UCLP (R)	101	20	20	20	20	20	20	21	20	25	20	20	20
1-3	11	35	30	40	30	30	25	33	25	30	30	30	30
4-7	51	21	25	20	20	20	20	25	25	25	25	20	25
8-12	24	18	20	20	20	15	20	18	20	20	20	15	20
>12	15	16	20	15	15	10	10	16	15	15	10	10	15
UCLP (L)	74	21	25	23	20	20	20	24	25	25	25	20	23
1-3	10	35	30	40	33	30	30	33	28	30	33	33	33
4-7	37	21	25	25	20	20	20	25	25	30	30	25	25
8-12	16	20	20	23	20	18	23	16	20	20	20	13	18
>12	11	16	20	15	15	10	10	17	15	15	15	15	15

Legend: AHL R- = average hearing level - right ears; AHL L = average hearing level - left ears; UCLP = unilateral cleft lip and palate; UCLP (R) = UCLP right side); UCLP (L) = UCLP left side; N = number of ears; M = median.

Table 2. Absolute difference in AHL and MHL (dB) between age groups.

Cleft type Age-UCLP	AHL R	AHL L	Right ear					Left ear				
			250 Hz	500 Hz	1 kHz	2 kHz	4 kHz	250 Hz	500 Hz	1 kHz	2 kHz	4 kHz
§ 1-3 vs. 4-7	-14*	-8	-5	-20*	-10*	-10*	-5*	0	-5	-5	-10	-5
4-7 vs. 8-12	-3	-7**	-5	0	0	-5	0	-5*	-5**	-5*	-5**	-5*
8-12 vs. >12	-2	-2	0	-5	-5	-5	-10*	-5	-5	-10	-5	-5
§§ 1-3 vs. 4-7	-	-	-	-	-	-	-	-	-	-	-	-
4-7 vs. 8-12	-6	-3	-5	0	-5	-8	-8	-8*	-5	-2	-5	0
8-12 vs. >12	-	-	-	-	-	-	-	-	-	-	-	-
§§§1-3 vs. 4-7	-14*	-8	-5	-15**	-13*	-10**	-10*	-3	0	-3	-8	-8
4-7 vs. 8-12	-1	-9*	-5	-3	0	-3	3	-5	-10*	-10*	-12	-7*
8-12 vs. >12	-4	1	0	-8	-5	-8	-13	-5	-5	-5	2	-3

* Statistically significant at p≤0.05; ** p≤0.01; § - UCLP; §§ - UCLP (R); §§§ - UCLP (L).
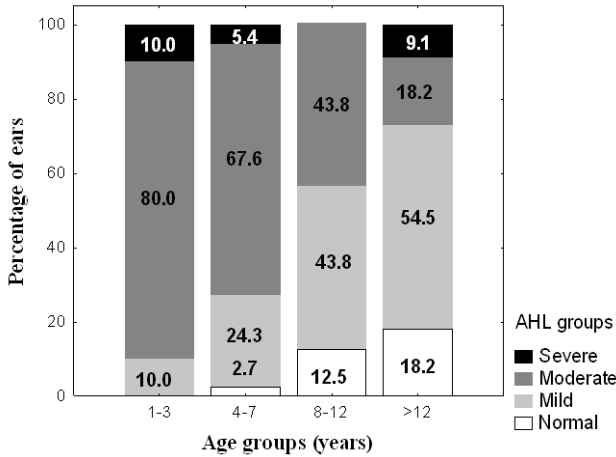
Figure 1. UCLP (L) - LEFT ear - proportions of ears with mild, moderate and severe AHL and normal hearing by age groups.
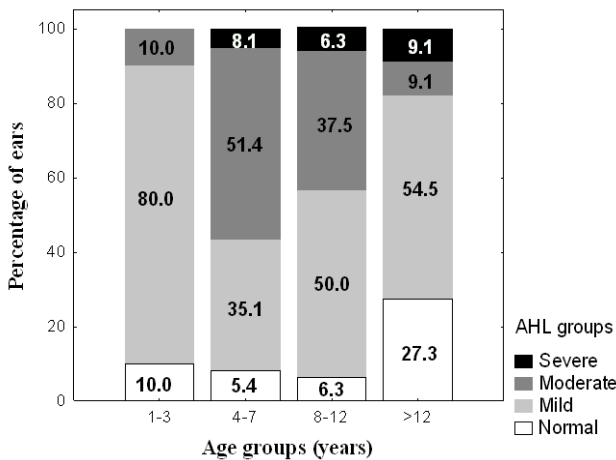
Figure 2. UCLP (R) - RIGHT ear - proportions of ears with mild, moderate and severe AHL and normal hearing according to age groups.
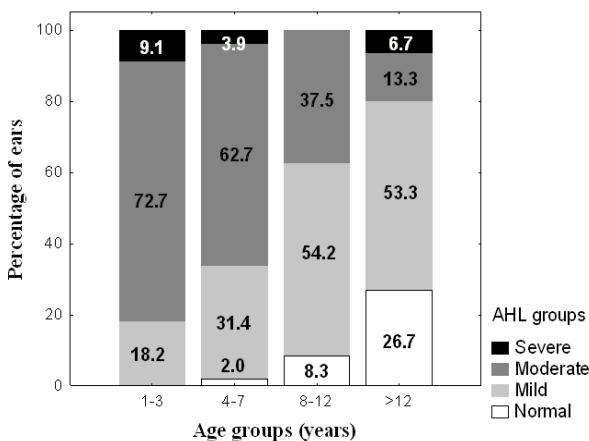
Figure 3. UCLP - LEFT ear - proportions of ears with mild, moderate and severe AHL and normal hearing according to age groups.
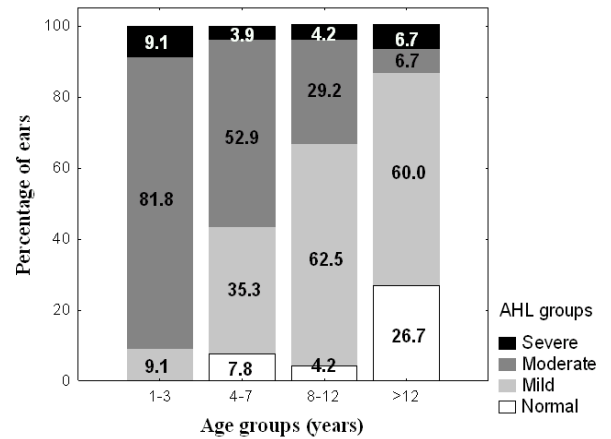
Figure 4. UCLP - RIGHT ear - proportions of ears with mild, moderate and severe AHL and normal hearing according to age groups.

References

1. Paradise J, Bluestone C, Felder H. The universality of otitis media in 50 infants with cleft palate. Pediatrics 1969; 44:35-42.
2. Handzic-Cuk J, Cuk V, Risavi R, Katusic D, Stajner-Katusic S. Hearing levels and age in cleft palate patients. Int J Pediatr Otorhinolaryngol 1996; 37:227-242.
3. Ravicz ME, Rosowski JJ, Merchant SN. Mechanisms of hearing loss resulting from middle-ear fluid. Hear Res 2004; 195:103-130.
4. Kemaloglu YK, Kobayashi T, Nakajima T. Analysis of the craniofacial skeleton in cleft children with otitis media with effusion. Int J Pediatr Otorhinolaryngol 1999; 47:57-69.
5. Heerbeek N, Akkerman AE, Ingels JAO, Engel JAM, Yielhuis GA. Left-right differences in Eustachian tube function in children with ventilation tubes. Int J Pediatr Otorhinolaryngol 2003; 67:861-866.

Investigation of Eustachian tube function in chronic otitis media with cholesteatoma

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Eustachian tube (ET) dysfunction has been linked to being a cause of middle ear pathology, and it is considered that ET dysfunction causes otitis media with effusion (OME). Research has shown that 70% of children who have chronic OME suffer mild-to-moderate hearing loss. Moreover, the sequelae of ET dysfunction is tympanic membrane retraction, and this physiological state provoked chronic inflammation in some cases, leading to adhesive otitis media followed by debris collection and fulminate cholesteatoma. It is important to understand the ET functions of the patients who suffer from the middle ear diseases. In this report, we investigate retrospectively the ET function of the patients who underwent tympanoplasty for otitis media with cholesteatoma, which is one of the sequelae of chronic OME, from 1996 to 2005. Ninety-three patients (59 were male, 34 were female) underwent tympanoplasty, and we reviewed the medical records and the ET function of each patient by sonotubometry before the surgery, such as the duration of opening period and the sound pressure level on swallowing. The patients were divided into 2 groups according to the level of these factors, graded as "good" and "poor". Twenty-eight cases were identified as "good" group, and 65 as "poor" group. There is no difference in the expanding of cholesteatoma between the two groups. However, there is some relation between ET function and improvement of the hearing levels after the surgery. The recurrences of cholesteatoma were seen in 7 cases, and six in the 7 cases showed "poor" ET function. These results indicated that sonotubometry did not reflect the precise condition of the cholesteatoma, but may be related to the prognostic factor for the hearing levels and the recurrence of cholesteatoma.

Validity, reliability, and acceptance of the Galker test of speech reception in noise for Danish preschool children

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Objective

To evaluate the validity, reliability and acceptance of the "Galker test of speech reception in noise" developed for primary health care to preschool children with otitis media with effusion (OME).

Methods and materials

The Galker test is an audio-visual, computerized, word discrimination test in background noise comprising 50 word pairs. Some 388 children attending a daycare center and aged 3 to 5 years were included after parental consent. Children were examined with the Galker test, tympanometry, audiometry, and the Reynell language scale of verbal comprehension.

With the Rasch item response model, it was examined whether the total score of the Galker test (Galker50) validly reflected item responses across subgroups defined by sex, age, bilingualism, middle-ear effusion, hearing level, and Reynell score. In item response theory, the Rasch model is considered a gold standard during scale development and validation against which invalid items can be identified. The idea is that a test person can be assigned a single value on a single continuum assumed to reflect the unobservable construct. In item response theory, this is referred to as unidimensionality. It then follows that the response pattern of test persons can be ordered in a hierarchy that allows distinction between persons of high ability and persons of low ability and between easy and difficult items on the scale. All persons must be more likely to answer easy items correctly than difficult items and all items must be more likely to be passed by persons of high ability than those of low ability.

Reliability was examined with Cronbach's Alpha and the effect of noise on test results was examined by re-tests of 40 children without noise and 67 children with noise. The total score of the Galker items fitting the Rasch model was examined for associations with the other variables.

Results

A total of 370 children (95%) were tested with the Galker50, and 339 (87%) completed all 50 items.

Mean age was 4.1 years, 11% were bilingual, and 10% received speech therapy. The parents stated that 90% had good or very good health. A total of 94% had tympanometric examination; 46% were bilaterally normal (type A or C1), 18% were unilaterally and 20% bilaterally abnormal (type C2 or B), and 9% had open ventilation tubes in one or both ears. A total of 24% had been treated with ventilations tubes.

Analysis showed that 35 items (Galker35) fitted the Rasch model well and 15 items were problematic. Reliability was 0.75 before and after exclusion of the 15 non-fitting items. Some 31 children did not complete the test; 28 of them were younger than 4 years of age.

Marginal (unadjusted) relationships between Galker35 to other variables are seen in Table 1. The test-retest analysis with or without noise at retesting showed a retest effect of around 1 point and a noise effect of about 6 points in the Galker35 score. In the adjusted graphical model of relationships, Galker35 still had a strong relationship to age (Partial y-coefficient = 0.35) and Reynell score (Partial y-coefficient = 0.33).

Conclusion

The results indicate that the Galker35 provides valid and reliable measurement of speech reception in noise. The method may potentially be useful to detect children who are disabled in daily communication due to OME or other reasons.

Further research will focus on the associations between the Galker35 and measures of language development and hearing, and the effect of using the test in daily practice.

Table 1. Marginal (unadjusted) relationships between variables of the graphical analysis and the Galker35 test.

Variables	Gamma Coefficient	P value
Age	0.49	<0.0001
Gender, girl	0.19	0.005
Education, mother	0.08	0.116 (ns)
Education, father	0.11	0.034
Tympanometry	0.20	0.011
Audiometry	0.30	<0.0001
Reynell scale	0.59	<0.0001
Teacher-rated hearing	0.11	0.13 (ns)
Teacher-rated pronunciation	0.34	<0.0001
Teacher-rated sentence construction	0.34	<0.0001
Teacher-rated vocabulary	0.37	<0.0001
Teacher-rated ability to follow instructions during group activities	0.35	<0.0001
Teacher-rated ability to understand messages given to a group	0.26	<0.0001
Parent-rated hearing	0.09	0.26 (ns)
Parent-rated pronunciation	0.13	0.02
Parent-rated sentence construction	0.15	0.01
Parent-rated vocabulary	0.15	0.01
Parent-rated bother from ear problems through childhood	0.03	0.32 (ns)

Telemedicine and digital imaging as tools to study inflammatory conditions in the middle ear--a method study

Thorbjorn Lundberg, M.D., Goran Westman, M.D., Sten Hellstrom, M.D., Ph.D., Herbert Sandstrom, M.D.

Objective

To assess the quality of digital imaging of the tympanic membrane (TM) by telemedicine technology.

Design

The study was open, consecutive, and prospective.

Setting

Three rural health care centers in Northern Sweden.

Subjects

Sixty-four children, 2-16 years who presented with otalgia.

Interventions

As part of a larger study of the course of inflammatory ear disease, patients were examined with endoscopic video photography of TMs in a telemedical environment. One hundred twenty-four images were stored in a database and later assessed regarding image quality by three different viewers, an ENT specialist, a general practitioner, and a GP registrar. An overall grading (0–2) of image quality was assessed and eight different components, four image-related components and four anatomically related components, were separately evaluated. Image quality over time in the study and in different age groups was assessed. Interpersonal agreement was calculated.

Results

Overall image quality was good, with 82.3% of acceptable or excellent quality. The position and thickness of the TM were found to be the most important factors of the images to be able to assess inflammatory disease. Image quality tended to be higher later in the study and in older patients. Interpersonal agreement between examiners was acceptable.

Conclusions

The image quality of video endoscopy of the TMs was good overall. Interpersonal agreement in evaluating image quality was acceptable but not excellent. The use of digital imaging of good quality in clinical studies can offer an objective grading of the TM in retrospect by independent reviewers using strict criteria.

Convenient method of measuring ventilatory function of the eustachian tube: preliminary study of updated sonotubometry (SNT) and Valsalva maneuver (VAL) in tubo-tympanum-aero-dynamography (TTAG)

Tomoko Tsutsumi, M.D., Masahiro Morita, M.D., Naoyuki Kohno, M.D.

Introduction

Sonotubometry (SNT) is one of the most convenient methods of measuring ventilatory function of the eustachian tube (ET). Tubo-tympanum-aero-dynamography (TTAG) is one of the most useful methods of adapting pathophysiological function of the ET. A recently updated SNT and Valsalva maneuver (VAL) in TTAG were tested in patients with ear problems described below.

Methods

The test population comprised 76 patients aged 11 to 79 years. Patients who complained of ear fullness, autophony, or hearing one's own respiratory sounds were included in this study. SNT testing took place in two sessions of three acts of swallowing each. In the same series of sessions, we also examined TTAG to measure tubal opening pressure and equilibrating ability of the inflated pressure in the middle ear after VAL. We diagnosed tubal stenosis (TS), patulous eustachian tube (PET), otitis media with effusion (OME), and adhesive otitis media from the combined results of SNT and TTAG.

Results

Thirty-one cases revealed PET, while 50 cases revealed TS. Opening of the ET was recorded in at least two of the three measurements in 27 out of 31 cases with PET, and in 17 out of 50 cases with TS. The type of tubal function resulting from both SNT and VAL of TTAG were matched in one-half of the cases of PET and two-thirds of the cases of TS. Mean values of base line sounds (BSL), meaning sound pressure level of nasal sound source at the nasal orifice, were lower in PET than in TS, especially in cases that showed lower or no sound change during swallowing.

Conclusions

Measurement results in TS had relatively high correlation between the results of both SNT and VAL in TTAG. On the other hand, BSL in SNT could be a good indicator of finding PET. Therefore, the combined tests of both SNT and VAL in TTAG form a useful method to assess ET ventilatory function in various ear diseases. The performance of this updated version needs to be established in patients with otological diseases.

Prevalence of acute otitis media among children with pyrexia in a Nigerian hospital

Alabi Sulyman, F.W.A.C.S.

Aim

This study was to determine the prevalence of acute otitis media (AOM) in children with pyrexia (most of whom are treated as cases of malaria) and the relevance of socioeconomic factors on AOM.

Patients and methods

This prospective study was conducted over a 10-month period (January to October, 2004) at the emergency pediatric unit of the hospital among children ages 0-15 years presenting with pyrexia. All the children had full ENT examinations in addition to an evaluation by the emergency pediatricians. Diagnosis of AOM was based on clinical history, physical examinations, and otoscopic findings.

Results

One hundred children with pyrexia were seen over the period, age range was 3 months to 15 years; 16 had features of AOM. Male/female ratio was 1.7:1.0. Two-thirds of the children were from a low socioeconomic class.

Conclusions

Prevalence of isolated AOM was 16% among children with pyrexia, most of whom were from a low socioeconomic background. There is a need for detailed otoscopic examinations in children with pyrexia as AOM could be one of the differentials; most of them are diagnosed as having malaria due to the endemicity in the tropics.

Current trends in research methods (design and analysis) for clinicians and biologists

Mark Haggard, Ph.D.

Much published science contains errors, about which the professional and academic systems have become more concerned than in the past. The responsibility for these has to be seen as shared among authors – but not equally. With the exception of simple group-comparisons or cross-tabs, data analysis in clinical and biological research tends to require involvement of a professional bio-statistician or data-analyst. However, the need for genuine interaction with these third parties, especially in the vital planning stage, means that the researcher is not absolved from some understanding of statistical ideas. Those ideas evolve. In addition to the basic principles taught in the past (still poorly observed in much medical research), there have been major developments in the last 20 years. These changes have several drivers: critiques (e.g., from EBM) of science as practiced, new developments in statistical techniques, more powerful software

implementations, and general intellectual movements (e.g., away from excessive hypothesis-testing and bias-prone seeking of $P < 0.05$ findings).

This briefing session does not attempt detailed practical teaching, but general awareness-raising in four areas where standards have been rising so rejection by editors is increasingly risked. It emphasizes intuitive understanding of the basis for error; hence the need for appropriate analyses and changing some "unknown unknowns" into "known unknowns"! The four areas are: (1) *a priori* analysis protocols and penalties in multiple testing, (2) what to do about missing data, (3) meta-analysis and publication bias, and (4) the reduction of measurement error and projection of findings to new circumstances by statistical modeling.

Adenoidectomy for otitis media with effusion in 2-3 year-old children

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Background

There are very little data regarding the efficacy of adenoidectomy for middle ear disease in young children. Recent guidelines for the treatment of otitis media in children have not recommended adenoidectomy for first-line surgical treatment in young children due to inadequate information on which to base a decision.

Objective

To compare the efficacy of three surgical treatment combinations – myringotomy and tympanostomy tube insertion (M&T), adenoidectomy with M&T (A-M&T), and adenoidectomy with myringotomy (A-M) – in reducing middle ear disease in young children with chronic OME.

Methods

Children 24-47 months of age, with a history of bilateral middle-ear effusion (MEE) for at least 3 months, unilateral for 6 months or longer or unilateral for 3 months after extrusion of a tympanostomy tube, unresponsive to recent antibiotic, were randomly assigned to either M&T, A-M&T, or A-M. Treatment assignment was stratified by age (24-35 months, 36-47 months), nasal obstruction (no, yes) and previous history of M&T (no, yes).Subjects were followed monthly and with intercurrent ear disease.

Results

Ninety-eight subjects were randomly assigned to the 3 treatment groups. Fifty-six subjects (57%) were 24-35 months of age; 63% had nasal obstruction, and 36% had previously undergone M&T. During the 18 months after entry, subjects were noted to have MEE for the following percentages of time:11.9% in the M&T group, 18% in the A-M&T group, and 35.7% in the A-M group.

Conclusions

Adenoidectomy with or without tube insertion provided no advantage to young children with chronic MEE in regard to time with effusion compared to tube insertion alone.

Relationship between persistence of pathogens despite clinical improvement or cure (CI/C) in antibiotic-treated acute otitis media (AOM) and clinical and bacteriologic recurrence of the disease

Eugene Leibovitz, M.D., Elad Asher, M.D., Noga Givon-Lavi, Ph.D., Alberto Leiberman, M.D., Ron Dagan, M.D.

Background

In studies with evaluation of bacteriologic and clinical outcome, early eradication of pathogens (after 3-5 days of treatment) from middle-ear fluid (MEF) was associated with a reduced risk of clinical failures.[1-3] On the other hand, bacteriologic failures were not associated with clinical failure in ~60-70% cases, and therefore a considerable amount of children in whom MEF cultures remain positive after 3-5 days of treatment show clinical improvement or cure (CI/C) at the end-of-treatment visit.[1-3]

Objectives

To investigate the relationship between MEF culture-positivity during treatment in patients with CI/C and the occurrence of subsequent acute otitis media (AOM).

Methods

The initial baseline population included all the patients aged 3-36 months with AOM presenting at the Pediatric Emergency Room of the Soroka University Medical Center from January 1, 1995, through December 31, 2003, who were enrolled in prospective double-tympanocentesis antibiotic efficacy studies and had an initial positive MEF culture. After tympanocentesis, one of the following antibiotic drugs was administered: oral cefaclor (40 mg/kg/day tid for 10 days), oral amoxicillin (80 mg/kg/day tid for 10 days), oral trimethoprim-sulfamethoxazole (8 mg/kg/day of trimethoprim bid for 10 days), oral amoxicillin/clavulanate (45/6.4 mg/kg/day or 90/6.4 mg/kg/day bid for 10 days), oral azithromycin (10 mg/kg for 3 days or 10 mg/kg/day for the first day followed by 5 mg/kg/day for the next 4 days), oral gatifloxacin (10 mg/kg/day once daily for 10 days), oral levofloxacin (20 mg/kg/day bid for 10 days), oral cefdinir (25 mg/kg once daily for 10 days), and intramuscular ceftriaxone (50 mg/kg/day once daily for 1 or 3 days).Of these, all children with clinical recurrence of AOM occurring within 3 weeks from completion of therapy for the initial AOM episode and who could be studied bacteriologically at recurrence were included in a retrospective analysis and constituted the study population. Successful completion of therapy for the initial AOM episode was defined as CI/C at end of treatment (as established at an additional visit on Day 11-14) with no need for additional systemic anti-infective drugs. Clinical failure was defined as failure to improve or clear signs and symptoms after ≥2 days of therapy requiring the use of an alternative antibiotic. Clinical recurrence was defined as the occurrence of an AOM episode during a 3-week follow-up period after the end of therapy in those patients who were considered CI/C at the completion of antibiotic therapy and were prescribed additional antibiotic therapy for the treatment of AOM. Phenotypic (serotyping and serogrouping for *Streptococcus pneumoniae* and b-lactamase production for *Haemophilus influenzae*) and genotypic characterization methods (pulsed field gel electrophoresis, PFGE) were used to establish the similarity (or disimilarity) between the MEF pathogens (*S. pneumoniae* and *H. influenzae* only) isolated at recurrence of AOM and the MEF isolates obtained during the initial AOM episode.

Results

Overall, 673 patients with culture(+) MEF were enrolled in double-tympanocentesis studies and followed for 3 weeks after completion of treatment. On Day 4-6, 189/673 (28%) patients had culture(+) MEFs. Patients with CI/C on Day 11-14 (end of treatment) and culture(+) MEF had more recurrent AOM episodes (53/151, 35%) than those with culture(-) MEF (114/476, 24%), P=0.007.

This represented a 72% higher risk of clinical recurrence of AOM (OR 1.72, CI 95% 1.16 to 2.5) compared with patients without bacteriologic eradication during treatment. In the logistic models used (controlling for antibiotic treatments administered at enrollment, ethnicity, gender, age, and AOM history), the higher risk to recurrence among patients without bacteriologic eradication was preserved. 41/53 (77%) culture(+) patients with CI/C on Day 11-14 underwent tympanocentesis when AOM recurred and

29/41 (71%) were culture(+). PFGE identity between pathogens at recurrence and those persisting on Day 4-6 was found in 19/29 (66%) vs. 31/86 (36%) of the evaluable patients with recurrence and culture(-) MEF on Day 4-6 (P=0.005).

Discussion

In this study we found that AOM patients with clinical improvement or cure despite failure to eradicate the causing organisms after 3-5 days of antibiotic therapy had a significantly higher rate of subsequent early recurrent AOM episodes than those who achieved culture-negative MEF on Day 4-6 of treatment. Our results therefore emphasize the importance of early bacteriologic eradication in the outcome of AOM, even in patients who appear to do well clinically at the end of treatment.

Recent studies showed that when clinical recurrences of AOM occurred early following eradication of the initial AOM pathogens, the majority represented new infections. Leibovitz et al.[4] reported on the bacteriology of 108 episodes of recurrent AOM during the 1-3 week time period following successful (bacteriologic eradication and clinical improvement or cure) completion of antibiotic therapy for the initial AOM event and found that 71% of these recurrences occurred early (the first 2 weeks after the end of therapy) during the follow-up period. True bacteriologic relapses were found in 28% of patients, mostly during the first 2 weeks after completion of therapy. However, this scenario was demonstrated only in patients successfully treated with antibiotics (achieving bacteriologic eradication within 3-5 days).

Since our analysis included patients that participated in various clinical trials with penicillins, cephalosporins, azalides, TMP/SMX, and quinolones, we described the distribution of the different antimicrobials and their specific bacteriologic and clinical efficacy among the two main patient groups of the study (the group with during treatment MEF eradication and the group with during treatment MEF pathogen persistence). However, after correction for the administered drugs, our findings remained significant. Furthermore, even after correction by multiple logistic regression after additional factors such as age, ethnicity, gender, and previous AOM history, the relative risk of AOM recurrence in patients who were defined as improvement or cure despite failure to eradicative the MEF organisms during therapy was considerably increased.

We conclude that: 1) Lack of eradication of MEF pathogens is associated with clinical recurrences, even in patients showing CI/C at end of treatment; 2) These recurrences are mostly caused by pathogens initially present in MEF and not eradicated during treatment.

References

1. Carlin SA, Marchant CD, Shurin PA, Johnson CE, Super DM, Rehmus DIM. Host factors and early therapeutic responses in acute otitis media: does symptomatic response correlate with bacterial outcome? J Pediatr 1991; 118:178-183.

2. Dagan R, Leibovitz E, Greenberg D, Yagupsky P, Fliss DM, Leiberman A. Early eradication of pathogens from middle ear fluid during antibiotic treatment of acute otitis media is associated with improved clinical outcome. Pediatr Infect Dis J 1998;17:776-782.

3. Dagan R, Leibovitz E. Bacterial eradication in the treatment of acute otitis media. Lancet Infect Dis 2002;2:593-604.

4. Leibovitz E, Greenberg D, Piglansky L, et al. Recurrent acute otitis media occurring within one month from completion of antibiotic therapy: relationship to the original pathogen. Pediatr Infect Dis J 2003;22:209-215.

Efficacy of trimethoprim-sulfamethoxazole in children with active chronic otitis media: a randomized placebo controlled trial

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Objective

To determine the clinical effectiveness of prolonged treatment with trimethoprim-sulfamethoxazole (TMP-SMX) in children with active mucosal chronic otitis media (COM).

Material and methods

We performed a randomised placebo controlled trial in 101 children with COM aged 1 to 12 years. They were randomized to either 6 to 12 weeks TMP-SMX orally (18mg/kg, 2dd) or placebo. The primary outcome was otomicroscopic signs of otorrhea at 6, 12 weeks and 1 year follow-up. Secondary outcomes were nasopharyngeal carriage of potential pathogens and resistance to TMP-SMX.

Results

At 12 weeks follow-up, 15(32%) children in the TMP-SMX group and 23 (47%) children in the placebo group had otomicroscopical signs of otorrhea (NNT7).

At 1 year follow-up, the percentage of children with otorrhea was similar in both groups. In the TMP-SMX group, nasopharyngeal carriage of H. influenzae, M. catarrhalis and S. pneumoniae decreased significantly during the first 12 weeks of follow-up as compared to the placebo group, whereas resistance to TMP-SMX of H. influenza increased from4 at baseline to 44% at 12 weeks follow-up. At 1 year follow-up, carriage of potential pathogens as well as the prevalence of TMP-SMX resistant H. influenzae returned to baseline levels.

Conclusions

A 6-to-12-week course of oral trimethoprim-sulfamethoxazole reduced otorrhea by 68% at 12 weeks follow-up. At the same time a reduction of nasopharyngeal carriage of potential pathogens and an increase in TMP-SMX resistance was found. The effects of TMP-SMX were most pronounced with the shorter course, and disappeared if the medication was discontinued.

Development of the antimicrobial peptide OP-145 for chronic otitis media

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Background

OP-145 is a novel antimicrobial peptide based on the structure of human LL-37 that was developed for the treatment of mucosal infections. The peptide neutralizes the proinflammatory activity of bacterial products such as lipopolysaccharide and lipoteichoic acid, and displays antimicrobial activity. Therefore, it targets microbial products and bacteria that are associated with chronic otitis media (COM).

Objectives

To expand on the mechanism of action of OP-145 with respect to antibacterial activity and its effect on bacterial biofilms and to evaluate its potential application for the treatment of COM in a phase I study.

Methods

Antimicrobial activity was determined against a panel of relevant micro-organisms. Biofilm inhibition and degradation assays were performed with Pseudomonas aeruginosa PAO1. COM patients were exposed to various doses of OP-145 administered locally using eardrops in a phase I study.

Results

Antimicrobial activity was detected against P. aeruginosa, Moraxella catarrhalis, and Streptococcus pneumoniae (MIC <3 µM). OP-145 inhibited formation of biofilms by P. aeruginosa, and caused degradation of these biofilms. No toxicity was observed in a phase I study in 16 COM patients after a 2-week treatment period with OP-145 eardrops, and treatment was well-tolerated.

Conclusions

OP-145 showed antimicrobial activity against relevant upper respiratory tract pathogens and affected formation and degradation of biofilms formed by P. aeruginosa. Eardrops with OP-145 were found to be safe and well-tolerated in COM patients. Based on our in vitro analysis, the potential mechanism of action of this peptide in COM is mediated by bacterial toxin neutralization and antimicrobial and anti-biofilm activities.

Efficacy of air inflation with/without corticosteroids for treating otitis media with effusion

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Introduction

Otitis Media with Effusion (OME) is a common disease in the pediatric age group and remains resistant to current medical and surgical treatments. Past studies show that the onset and/or persistence of OME is related to an insufficiency in middle ear (ME) pressure regulation that results in the development of ME underpressures (ref. ambient) that in turn cause capillary dilatation with transudation of capillary fluids into the ME airspace as an effusion (MEE) by *hydrops ex vacuo*. These considerations have generated a renewed interest in alternative, noninvasive methods of maintaining stable middle ear pressures (MEP) to prevent or treat Olefin that regard, a variety of methods to introduce gas via the Eustachian tube (ET) into the diseased ME has been described. However, the reported efficacy of such procedures for treating OME in past studies was contradictory, with reports of both no benefit as well as high clinical cure rates. For example, Gottschalk[1] reported achieving 81.5% success in clearing the MEE in serous OM (OME). A more acceptable and appealing (to children) method of inflation using a carnival blower was described by Hunt-Williams.[2] He monitored the achieved nasopharyngeal pressure with a manometer and ET openings with an otophone, and showed that nasopharyngeal pressure can be increased above the passive opening pressure of the ET by blowing through the nose into a balloon. He reported a good response for intermittent catarrhal cases of OM but a poor response for OME.

A number of clinical trials were conducted to assess the efficacy of autoinflation with respect to resolving OME. Chan[4] reported no efficacy of a two-week treatment in a randomized clinical trial of children with OME between the ages of 3 and 18. Stangerup[5] reported a 64% improvement for OME in the group of children that used an autoinflation device (Otovent®) composed of a balloon mounted to a nose tube, as compared to a control group. However, two weeks after the cessation of autoinflation, the difference between the groups declined to non-significant levels. Brooker and McNeice[6] reported a worse outcome with respect to disease resolution for autoinflation compared to control in a randomized trial of 40 children between the ages of 3 and 10 years. The

Otovent® device was used by Blanshard[7], who reported in a randomized trial of children between 3 and 10 years, a tympanometric (only) improvement at 1 month and an otoscopic improvement at 1 and 2 months. In a non-randomized, non-controlled trial, Kaneko et al.[8] compared the results of two months of inflation treatment for OME in groups of children who could and could not inflate their MEs as confirmed by tympanometry. That study demonstrated that OME resolved in the successfully-inflated ears at a higher proportion when compared with non-inflated ears.

The reasons for these discrepant results may be related to the differences in the age of the study populations and in study design. The latter include differences in disease definition, entry criteria, the type of maneuver used to inflate the ME, documentation of the efficiency of the maneuver in establishing gas transfer to the ME, the frequency, duration and compliance in performing the inflationary maneuver, and the methodologies used to diagnose disease at entry and resolution at termination.

Recent evidences document a self-amplifying feedback between ME inflammation and ME pressure dysregulation.[9-12] Specifically, the presence of effusion and ME mucosal inflammation causes a more rapid loss of gas from the ME (i.e. a more rapid decrease in MEP) when compared to the healthy ME. This can overtax the ability of the ET to resupply the ME with gas and limit the effectiveness of other procedures designed to introduce gas into the ME and stabilize MEP. There, it is expected that successful autoinflation will increase MEP but, in the absence of mucosal healing, the effect will be short-lived as MEP rapidly decreases to the pre-maneuver values. On the other hand, corticosteroid treatment has the potential to reduce ME inflammation, but is not expected to exhibit this action under conditions of marginal ET function that favor continued inflammation by *hydrops ex vacuo*. We hypothesized that combining these modalities would exhibit a synergistic effect with respect to their potential efficacy in resolving OME.

The objective of the current study was therefore to evaluate the efficacy of a two-pronged strategy including repeated ME air inflations with the Otovent® device to increase ME pressures to approximate ambient levels and anti-inflammatory treatment with oral steroids to resolve the underlying

Methods

Children 3 to 12 years of age suspected of having persistent OME were evaluated using pneumatic otoscopy, tympanometry and audiometry. If the child qualified on the basis of preset criteria (≥2 months of unilateral or bilateral effusion), the study design was explained to the parents and child, and the advantages and disadvantages of study participation were outlined. If expressing a willingness to participate, the child was instructed in the use of the Otovent® device and he/she attempted to inflate the MEs. If successful inflation was documented by tympanometry, informed consent was obtained from the parent and, where applicable, assent was obtained from the child. The enrolled children were stratified for age (3-6 years, 7-12 years), laterality of MEE (unilateral or bilateral) and duration of MEE (2-3 months, ≥4 months, unknown), and then randomly assigned to one of three (original study design) or to one of two treatments (alternative study design): 1) amoxicillin (28 days) + placebo steroid (28 days) + ineffective ME inflation (28 days); 2) amoxicillin (28 days) + placebo steroid (28 days) + effective ME inflation (28 days), and 3) amoxicillin (28 days) + prednisone (28 days) + effective ME inflation (28 days), or 1) amoxicillin (28 days) + ineffective ME inflation (28 days); 2) amoxicillin (28 days) + effective ME inflation (28 days).The children were scheduled for repeat evaluations at the Ear Nose Throat Research Center (ENTRC) at 4 weeks and 8 weeks after enrollment.

In designing this study, we chose a commonly prescribed steroid, prednisolone, and a relatively safe dosing regimen (2 mg/kg/day x 7 days, 1 mg/kg/day x 7 days, 0.5 mg/kg/every other day x 7 days and 0.25 mg/kg/every other day x 7 days). The prednisolone dose was chosen to approximate the physiologic replacement doses[13] and the gradual weaning to lower doses was included to eliminate the risk for ACTH-adrenal axis suppression.[14-15] We included an antimicrobial (amoxicillin) treatment because of the synergistic effects of antibiotics and steroids noted in animal studies[16] and to decrease the risk of acute ME infection during and immediately following the time of steroid administration. For autoinflation treatments, parents were given an Otovent® device with a spare balloon. The active treatment group received an intact balloon and the placebo group received a balloon with a hole. At the first treatment (day 0), parents were asked to have their child perform repeated bilateral inflations (via the left and right nostrils) a total of 10 times at 15 minute intervals for purposes of "priming"

(i.e. saturating) the ME effusion with N_2. After the first session, the treatments were continued under supervision of the parent for 28 days on a TID regimen.

At entry, pneumatic otoscopy was done to assess ME status, an audiological exam was done to document hearing and tympanometry was performed with the Grasen Stadler GSI 33. Children were scheduled to return to the ENTRC on or about 4 weeks for a compliance review and re-examination (pneumatic otoscopy, tympanometry, and audiometry), and again at 8 weeks for the same procedures. Those with effusion at 8 weeks were referred to their otolaryngologist for consideration of tympanostomy tube placement. All children who did not have MEE at the 8 week visit were asked to return at 12 weeks to ascertain recurrence rates. If symptoms of AOM occurred at any time, the child was seen at the clinic and, if confirmed, he/she was treated with appropriate antimicrobial therapy.

Results

A total of 31 children aged 3.6 to 11.5 years (avg±std = 7.4+2.2 years) with documented OME (bilateral = 16 subjects) for greater than 2 months duration were entered into this pilot study. There were 19 (61%) males, 22 were white (7 black, 2 Asian) and all were pre-screened for the ability to perform autoinflation using the Otovent® device. These children were randomly assigned to placebo-placebo treatment (GR1; n=12), placebo-autoinflation (GR2; n=12) and steroid-autoinflation (GR3; n=7) groups. The number of subjects with bilateral OME was 5 (45.5%), 5 (50%) and 6 (85.7%) for GR1, GR2 and GR3 respectively.

Three subjects withdrew from study participation prior to the 30 day endpoint evaluation. Of the remaining 28, 3 of 11 in GR1 (27%), 4 of 10 in GR2 (40%) and 3 of 7 in GR3 (43%) had resolved their OME by the 30 day endpoint (see Table 1).

Despite randomization and stratification at enrollment, subject characteristics in each group demonstrated some variability. Because of randomization and uneven drop-outs across study groups, the percentage of ears with OME (reflecting differences in bilateral OME) was different at entry; 73%, 75% and 93% in GR1, GR2 and GR3, respectively (see Table 2). At the 30 day endpoint, the proportion of ears with OME for the 3 groups was 59.1%, 30% and 50%, and there was one new OME in a patient with unilateral OME at entry. One subject in Group 1 did not report for the 60 day visit. At that time, the percentage of ears with OME was 45% (decreased from 30 day endpoint), 50% (increased from the 30 day endpoint) and 79% (increased from

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the 30 day endpoint) for GR1, GR2 and GR3, respectively. For these groups, the percentage of ears with persistent OME throughout the follow-up period was 35%, 25% and 43%, and the recurrence rate between the two follow-up visits was 5%, 20%, and 14%, respectively.

Discussion

Recruitment for the present study was much lower than expected and this was related to a number of factors, some of which required changes in recruitment and in the protocol. First, the target population for recruitment was those children presenting to the ENT clinic with persistent OME. During the time when the study was ongoing, a number of studies that reported no significant long term effects of OME on child hearing and speech were published and given broad media coverage.[17] This reduced the enthusiasm for parents to seek treatment for their children with persistent OME and for primary care physicians to refer their patients to pediatric ENT clinics. To counter this, attempts were made to educate families and primary care physicians about the nature of the study and to solicit direct referrals to the research center. Second, parents of children referred to the ENT clinic were expecting to discuss the insertion of ventilation tubes, having been frustrated with the failure of medical management and other options over an extended time period. Presentation of an experimental, non-surgical option which could delay OME "cure" in their child was not well accepted by most eligible families. Third, the theoretical underpinnings of the treatments were difficult to communicate to families and this was especially true of the potential value of autoinflation "treatments". This was further complicated by including the information available from a recently published study that showed only a marginal efficacy of steroid treatment for OME.[18] Many parents were extremely cautious about accepting the steroid treatment arms in light of the information provided to them on possible side effects of that treatment. A protocol modification was introduced in year 3 allowing parents to accept randomization to either the original 3 arm study or to a 2 arm study that excluded the steroid+inflation arm. Acceptance of the 2 arm study protocol effectively unblinded the family to their child's treatment but study physicians were kept blinded for unbiased assessment of the ME status at follow-up. Even with modifications, the families that accepted study participation was but a small fraction of those with a qualified child.

From this small group of interested families, an additional requirement for study entry was that the child could perform a successful inflation of the

Otovent® balloon. This proved to be a major impediment to study enrollment in that younger children often refused to try this maneuver, or when pressured by their parents to try, made only a limited effort. Other younger children and some older children could not perform the maneuver consistently. Some families were not eager to go through with the required efforts and inducements to enforce this procedure three times per day for 28 days in their children. This is reflected in the bias to older children (average age 7.4±2.2 years) despite the facts that the eligible study population was defined as 3-12 years and the greatest benefit of successful OME treatment is expected to be realized in younger children.

Despite these problems with recruitment, the compliance of those enrolled was quite high, only 3 (10%) subjects withdrew prior to the 30 day endpoint and 1 subject (3%) withdrew prior to the 60 day endpoint. While the total population sample size was limited, the results for "cure" at 28 days was consistent with past reports for the placebo (antibiotic only) treatment used in this study, 27%. The "cure" rates for the two experimental treatments, steroid+inflation+antibiotics and inflation+antibiotics were 43% and 40%, respectively and the relative efficacy for these treatments (when compared to placebo) was not different from other medical treatments evaluated in clinical trials.[19-20] A similar trend was observed when the analyses were repeated using ears as the experimental unit. Also similar to other clinical trials, the higher cure rates for the experimental treatments did not persist with approximately equal or lesser cure rates for the experimental treatments when compared to the placebo treatment at the 60 day assessment (approximately 30 days post-treatment).

In conclusion, the intensive protocol for effective autoinflation of the ME in children is not readily accepted by parents and the addition of a steroid to that treatment further dampens existing enthusiasm. Few parents whose child had been referred to the ENT clinics for ventilation tube insertion secondary to persistent OME were willing to forgo immediate relief of their child's disease in anticipation of the success of a prolonged, intensive non-surgical treatment option. Autoinflation in young children is technically difficult and for this reason holds very limited potential as a treatment for those patients most likely to maintain a persistent disease condition. While the results support a short-term, limited efficacy of autoinflation for OME, the benefit does not extend beyond the period of active treatment. The addition of anti-inflammatory treatment with steroid is not acceptable to most parents and did not act synergistically with the inflation procedure in

promoting either short or long-term OME resolution. A caveat to these conclusions is that the treatment protocol was a based on existing theory and was therefore extremely complex. Other autoinflation methods, durations and adjunctive treatments may yet prove to be effective in treating persistent OME.

Acknowledgement

Table 1. Outcome of bilateral and unilateral OME in subjects at entry, at 30 and 60 day time-points based on the treatment group.

STUDY GROUPS	Entry			30 Day			60 Day		
				Bilateral OME (+)	Unilateral OME (+)	OME (−)	Bilateral OME (+)	Unilateral OME (+)	OME (−)
GR1 Placebo and Placebo	Bilateral OME (+)	5		4	0	1	2	2	1
	Unilateral OME (+)	6		1	3	2	0	3	2
	Sum	11*		5	3	3	2	5*	3
GR2 Inflation and Placebo	Bilateral OME (+)	5		0	3	2	3	0	2
	Unilateral OME (+)	5		0	3	2	0	4	1
	Sum	10		0	6	4	3	4	3
GR3 Inflation and Steroid	Bilateral OME (+)	6		3	0	3	2	3	1
	Unilateral OME (+)	1		0	1	0	0	1	0
	Sum	7		3	1	3	2	4	1
TOTAL	Bilateral OME (+)	16		7	3	6	7	5	4
	Unilateral OME (+)	12		1	6	4	0	8	4
	Sum	28*		8	10	10	7	13*	7

* One subject failed to follow up at 60 day visit

Table 2. Number and percentage of ears with OME at entry, at 30 and 60 day time-points based on the treatment group.

	Status of Ear	GR1 Placebo and Placebo n (%)		GR2 Inflation and Placebo n (%)		GR3 Inflation and Steroid n (%)	
Entry	Number of Ears	22	100	20	100	14	100
	OME (+) Ear	16	72.7	15	75	13	92.9
Day30	OME (−) Ear	4	18.2	9	45	6	42.9
	New OME	1	4.55	0	0	0	0
	OME (+) Ear	13	59.1	6	30	7	50
Day60	OME (−) Ear	11*	55	10	50	3	21.4
	Persistent OME	7	35	5	25	6	42.9
	Remained OME (−)	3	15	4	20	4	28.6
	Resolved Late	4	20	1	5	1	7.1
	New OME	0	0	0	0	0	0
	Recurred OME	1	5	4	20	2	14.3
	OME (+) Ear	9*	45	10	50	11	78.6

* One subject failed to follow up at 60 day visit

References

1. Gottshalk GH. Serous otitis: treatment by controlled middle ear inflation. Laryngoscope 1962; 72:1379-1390.
2. Hunt-Williams R.A method for maintaining middle-ear ventilation in children. J Laryngol Otol 1968;82:921-926.
3. Bylander A, Tjernstom O, Ivarsson A.Pressure opening and closing functions of the Eustachian tube by inflation and deflation in children and adults with normal ears. Acta Otolaryngol 1983;96:255-268.
4. Chan KH and CD Bluestone. Lack of efficacy of middle-ear inflation: treatment of otitis media with effusion in children. Otolaryngol Head Neck Surg 1989;100:317-323.
5. Stangerup SE, Sederberg-Olsen J, Balle V. Autoinflation as a treatment of secretory otitis media. a randomized controlled study. Arch Otolaryngol Head Neck Surg 1992;118:149-152.
6. Brooker DS, McNeice A. Autoinflation in the treatment of glue ear in children. Clin Otolaryngol 1992;17:289-290.
7. Blanshard JD, Maw AR, Bawden R. Conservative treatment of otitis media with effusion by autoinflation of the middle ear. Clin Otolaryngol 1993;18:188-192.
8. Kaneko Y, Takasaka T, Sakuma M, Kambayashi J, Okitsu T. Middle ear inflation as a treatment for secretory otitis media in children. Acta Otolaryngol (Stockh) 1997;117:564-568.
9. Doyle WJ, Alper CM. A model to explain the rapid pressure decrease after air-inflation of diseased middle ears. Laryngoscope 1999;109:70-78.
10. Alper CM, Doyle WJ, Seroky JT. Higher rates of pressure decrease in inflamed compared to non-inflamed middle ears. Otolaryngol Head Neck Surg 1999;121:98-102.
11. Alper CM, Doyle WJ.Repeated middle ear inflation does not prevent otitis media with effusion in a monkey model. Laryngoscope 1999;109:1074-1080.
12. Alper CM, Swarts JD, Doyle WJ. Prevention of otitis media with effusion by repeated air inflation in a monkey model. Arch Otolaryngol Head Neck Surg 2000; 126: 609-614.
13. Linder BL, Esteban NV, Yergey AL, et al. Cortisol production rate in childhood and adolescence. J Pediatr 1990; 117:892-896.
14. Harter JG, Reddy WJ, Thorn GW. Studies on an intermittent corticosteroid dosage regimen. N Engl J Med 1963269:591-596.
15. Schurmeyer TH, Tsokos GC, Augerinos PC, et al. Pituitary-adrenal responsiveness to corticotropin-releasing hormone in patients receiving chronic, alternate day glucocorticosteroid therapy. J Clin Endocrinol Metab 1985; 61:22-27.
16. Jung TTK, Hwang SJ, Olson D, et al. Effects of penicillin, ibuprofen corticosteroid, and tympanostomy tube insertion on experimental otitis media. In: Lim DJ, Bluestone CD, Klein JO, Nelson JD (eds.) Proceedings of the Fourth International Symposium on Recent Advances in Otitis Media. Toronto: B.C. Decker, 1988:231-235.
17. Paradise JL, Feldman HM, Campbell TF, et al. Effect of early or delayed insertion of tympanostomy tubes for persistent otitis media on developmental outcomes at the age of three **years.** *New Engl JMed2001;344:1179-1187.*
18. Mandel EM. Casselbrant ML. Rockette HE. Fireman P. Kurs-Lasky M. Bluestone CD. Systemic steroid for chronic otitis media with effusion in children. Pediatrics2002; 110:1071-1080.
19. Mandel EM. Rockette HE. Paradise JL. Bluestone CD. Nozza RJ. Comparative efficacy of erythromycin-sulfisoxazole, cefaclor, amoxicillin or placebo for otitis media with effusion in children. Pediatr Infect Dis J1991;10:899-906.
20. Mandel EM. Casselbrant ML. Kurs-Lasky M. Bluestone CD. Efficacy of ceftibuten compared with amoxicillin for otitis media with effusion in infants and children. Pediatr Infect Dis J1996; 15:409-414.

Lifetime of subannular ventilation tubes in treatment of chronic otitis media with effusion and tubal dysfunction

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Background

In chronic cases of otitis media and tubal dysfunction it is often preferable to insert long-term ventilation tubes in an attempt to achieve lasting normalization of the middle-ear pressure. However, these tubes are usually associated with higher complication rates than short-term tubes. Hence, there is an ongoing search for efficient, durable, and low-risk methods that are convenient for both the physician and the patient.

Objective

To describe the insertion of the subannular ventilation tubes (SVT) and their functional lifetime, and secondarily to describe the complication rates and the effect on hearing.

Methods

We used a retrospective case series of 121 SVT insertions in 84 patients. The procedure was performed by trained otosurgeons in a tertiary referral center. Under general anesthesia, a Per-Lee ventilation tube was placed subannularly in a groove in the posteroinferior part of the bony annulus. The minimum follow-up time was 1 year.

Results

The median functional lifetime was 33 months (95% CI 24-53 months). On average, 4.3 outpatient controls per year were needed to maintain the patency of the tubes. The rate of persisting tympanic membrane perforation was 13%. The median hearing gain was 15 dB.

Conclusion

This method is capable of lasting aeration of the middle ear with a lifespan that is equal to or better than other long-term tubes, and serves as a decent alternative to other long-term tubes. Compared to common T-tubes, the rate of persisting tympanic membrane perforation was significantly lower for the SVT.

Treatment outcome of acute otitis media in children treated with a newly released guideline in Japan

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Background

A recent increase in multidrug-resistant pathogens in Japan is considered due to inappropriate use of antibiotics, especially overuse of cephalosporins. Otologic Society of Japan, Japan Society for Pediatric Otorhinolaryngology, and Japan Society for Infectious Diseases in Otolaryngology released "Clinical Practice Guideline for Acute Otitis Media in Children" in 2006 to support daily clinical practice in the era of antimicrobial resistance.[1] This new guideline recommends that disease severity of acute otitis media (AOM) is classified into three categories - mild, moderate, or severe - determined by the scores for age, their clinical symptoms and their tympanic membrane (TM) findings. Each category offers its own treatment course, including not only antibiotic choice but also application of myringotomy (Table 1, Fig. 1-3). In Japan, where most pediatric patients with AOM come to see otolaryngologists and not pediatricians, this guideline was designed mainly for otolaryngologists to fit the Japanese environment of prevalent drug-resistant pathogens with evidence-based recommendations.

Objectives

The goal of this study is to clarify how appropriate this guideline is and whether it has any problems for use in daily clinical practice.

Methods

Treatment outcome of AOM in children was assessed when treated according to the guideline. Twelve hospitals in Hokkaido, the northern-most prefecture of Japan, joined in this study between May and September 2006. Patients were diagnosed and treated according to the guideline. Swab samples were obtained from the nasopharynx at their first visits to detect pathogens. Patients were asked to visit the clinic at 3 days, 1 week, 2 weeks, and 4 weeks after their first visits.

Results

One hundred forty-six patients with AOM were analyzed on the initial disease severity, initial scores of TMs, isolated bacteria, and treatment information including antibiotics and myringotomy they underwent. Twenty-five patients dropped out of the study, leaving 121 patients who were followed until their TM scores reached zero. One hundred ninety-eight bacteria were isolated from 133 patients. Fifty-seven percent of *S. pneumoniae* were penicillin-resistant and 59% of *H. influenzae* were β–lactamase non-producing ampicillin resistant strains. Three (2%), 77 (53%) and 66 cases (45%) out of 146 were classified as mild, moderate and severe respectively. One hundred twenty-two of 146 patients (84%) underwent antibiotic therapy recommended in the guideline. Sixty-three of 76 recommended cases (83%) underwent myringotomy. Eleven cases (9%) still had inflammatory changes in their TMs even a week after their first visits. However, 8 of these 11 cases achieved TM score 0 in 2 weeks, and so did the others within 4 weeks after their first visits. Moreover, their symptom scores reached zero in 1 week for all patients (Figure 4).

Conclusions

Improvement of symptoms and TM findings was satisfactory when patients with AOM in children were treated according to the guideline. This guideline could play an important role in decreasing the prevalence of non-susceptible strains of AOM pathogens as the result of the spread of appropriate use of antimicrobial agents in this country.

1. Scores for Symptoms

Otalgia	0: none
	1: mild pain
	2: long-lasting severe pain
Fever	0: < 37°C
	1: 37-38°C
	2: ≥ 38°C
Crying or Irritability?	0: yes
	1: no

2. Scores for Findings

Redness of TM	0: none
	2: partial
	4: entire ear drum
Bulging	0: none
	4: mild
	8: severe or just before burst
Otorrhea	0: none
	4: yes but TM is visible
	8: TM is invisible

3. Age

< 3 years old?	0: no
	3: yes

Disease Severity (1+2+3)

Mild	< 5 points
Moderate	6-11 points
Severe	≥ 12 points

Table 1. Scoring system for the guideline.
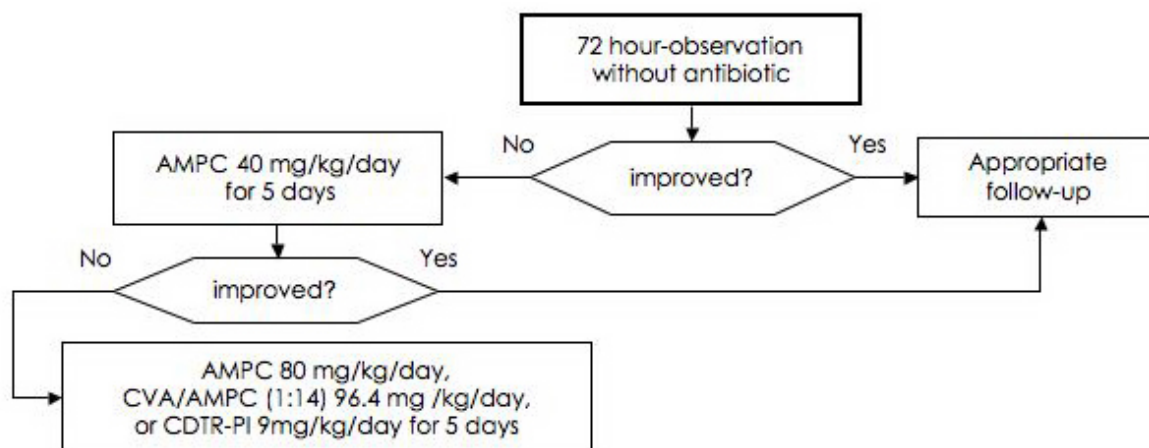
Figure 1. Treatment course for mild AOM
   -For otalgia and/or high fever (>38.5°C); acetaminophen 10mg/kg/dose.
   -For sinonasal infection; treatment for the disease.
   - Bacterial testing from nasopharynx; at the first visit at least.
  AMPC: amoxicillin, CVA/AMPC: amoxicillin clavulanate, CDTR-PI: cefditoren pivoxil

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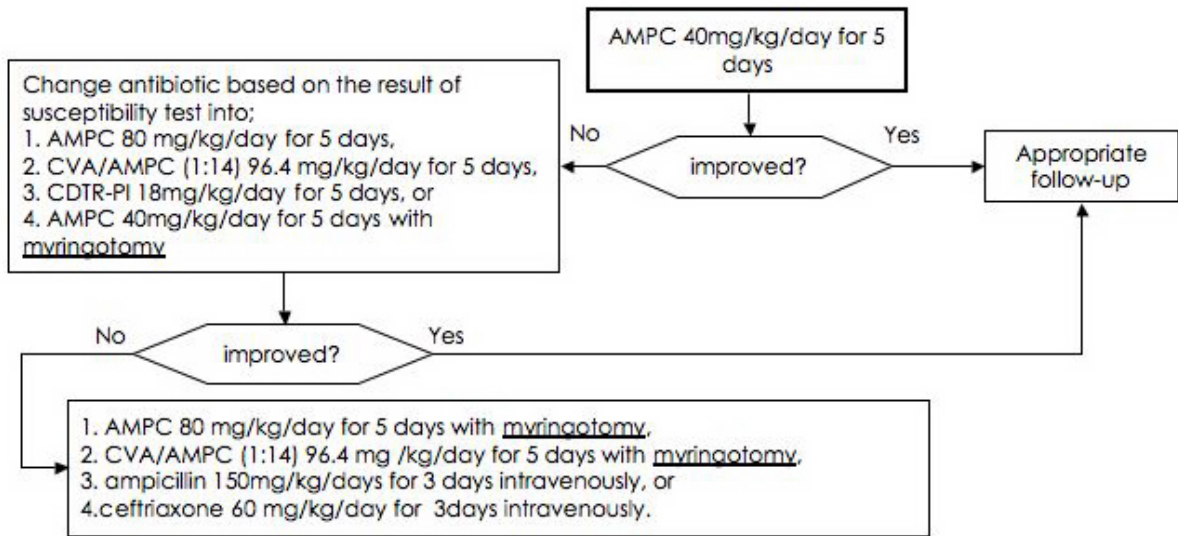
Figure 2. For moderate AOM Myringotomy is recommended to the moderate cases with severe TM findings, such as a TM score of 8 or more,
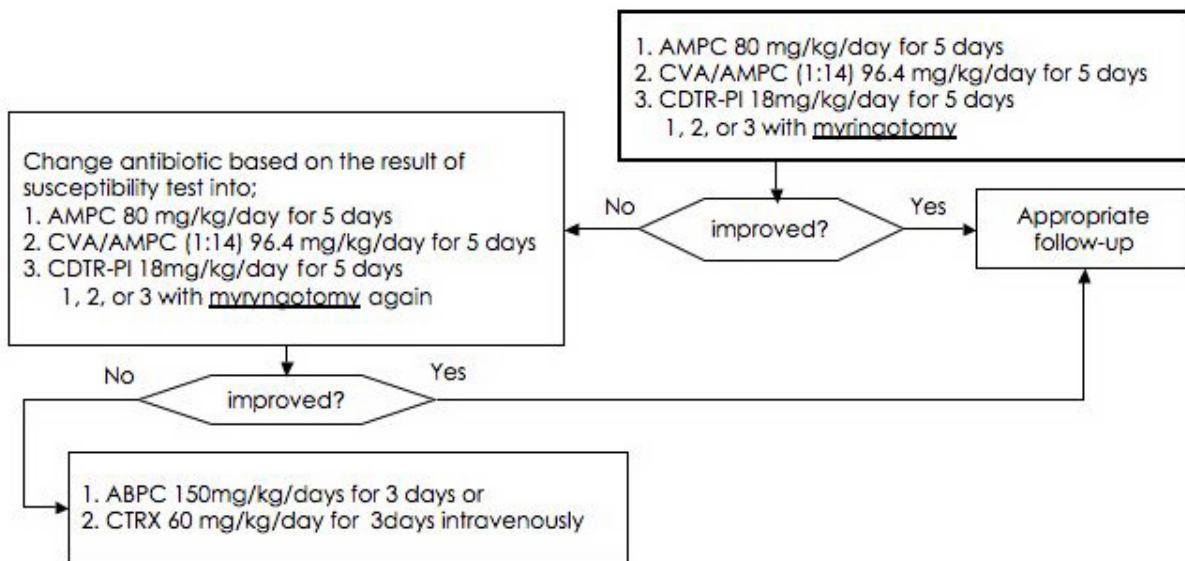
Figure 3. For severe AOM.
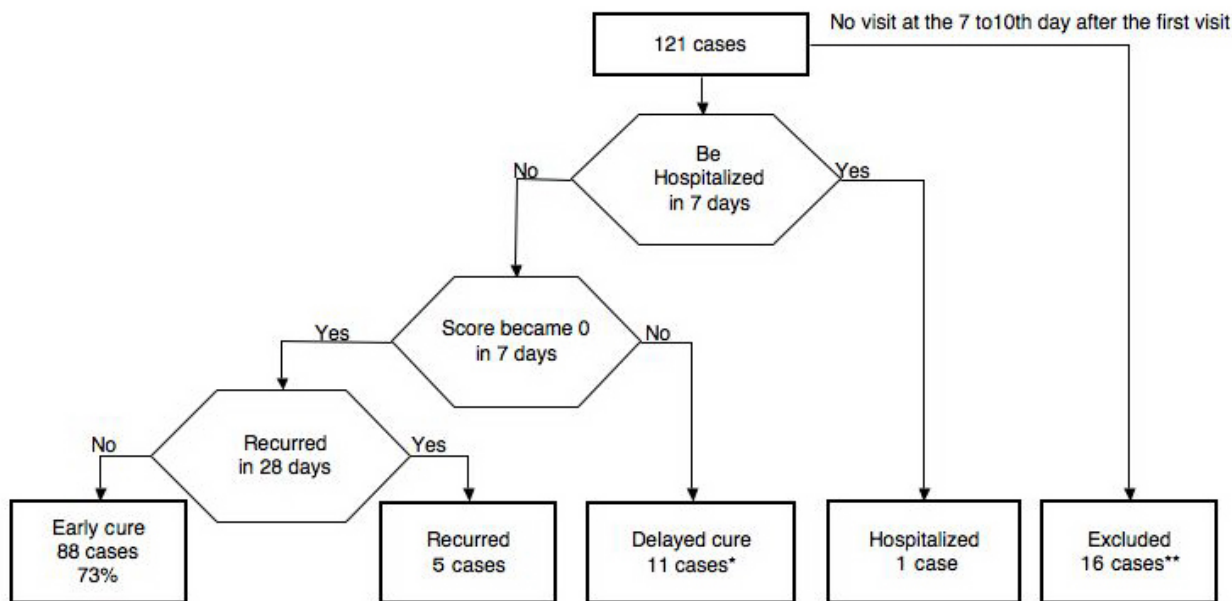
Figure 4. Treatment outcome
　*: 8 cases reached score 0 in 14 days, so did 3 cases in 28 days.
　**: All 16 cases reached score 0 in 15 days.

References

1. Otologic Society of Japan, Japan Society for Pediatric Otorhinolaryngology, and Japan Society for Infectious Diseases in Otolaryngology. "Clinical practice guideline for acute otitis media in children." (in Japanese). Otol Jpn 16 (suppl 1): 1-34, 2006.

Effect of antibiotic choice: changes in antimicrobial susceptibility and genetic characteristics of *Streptococcus pneumoniae* isolated from nasopharynx of pediatric patients

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Background

A recent increase in multidrug-resistant pathogens in Japan is considered due to inappropriate use of antibiotics, especially overuse of cephalosporins. In order to prevent a further increase in prevalence of drug-resistant pathogens, amoxicillin (AMPC) has been recommended as the first-line agent for children with acute otitis media (AOM) and other respiratory tract infection. At the last meeting in Amsterdam we reported obvious decrease in penicillin non-susceptible strains of *S. pneumoniae* after a switch of the first-line antibiotics from cephalosporins to AMPC. However, genetic characteristics of *S. pneumoniae* when the decreasing in non-susceptible strains was observed are uncertain.

Methods

Ninety strains of *S. pneumoniae* isolated from nasopharynx of pediatric patients were evaluated in this study. Forty-two swab samples were collected between March 24 and May 31, 2005, then 48 specimens were obtained between November 7, 2005 and January 25, 2006. Minimal inhibitory concentrations (MICs) of these samples to penicillin G (PCG) for S. pneumonia were determined. The susceptibility of *S. pneumoniae* to PCG was defined according to the criteria of the National Committee for Clinical Laboratory Standards (NCCLS). Gene mutations of penicillin binding protein (PBP) genes (pbps; pbp1a, pbp2x, and pbp2b) were analyzed by polymerase chain reaction (PCR) technique simultaneously.

Results

Nine of 42 strains (21%) in the first period and 13 of 48 strains (27%) in the second period were penicillin non-susceptible *S. pneumoniae*, while mutations of pbp genes were identified in 83% and 85% of isolates, respectively.

Conclusions

These data suggest that an appropriate use of antibiotics have to be the important first step for the decrease in drug resistant pneumococci and also this is not a goal. Pneumococcal strains classified as penicillin susceptible with pbp gene mutations could change their biologic behavior easily by application of antibiotics. Different modality of treatment such as vaccination might be necessary to gain this battle against drug resistant pathogens.

Effect of various corticosteroids in LPS-induced otitis media

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Introduction

Otitis media (OM) is one of the most common diseases during early childhood. Incidence of otitis media with effusion (OME) decreases with age.[1] OM is caused by multiple factors such as infection, eustachian tube dysfunction, allergy, and barotraumas. These factors stimulate the middle-ear mucosa and inflammatory cells to secrete inflammatory mediators (IMs), which, in turn, increase vascular permeability and secretory activity, resulting in middle-ear effusion (MEE).[2,3] The medical treatment of OM includes antibiotics, corticosteroids, and antihistamines with or without decongestants.[4-6] Previous studies have shown that combination antibiotic and steroid preparations have been more effective in the treatment of OM than antibiotic preparations alone.[7-9]

Oral corticosteroids have been tried for the treatment of OM. Many side effects are known with systemic use of corticosteroids. Corticosteroid systemic use in OM calls for high doses, whereas for topical use does not. Topical application of corticosteroids keeps the side effects, if any, to a minimum. Corticosteroids reduce inflammation and have been found to be effective in the treatment of OME in both animal models and in humans.[9,10]

The purpose of this study was to determine the effectiveness of topically applied corticosteroids on the outcome of lipopolysaccharide (LPS)-induced OM in chinchillas. The long-term goal of this study is to find better method of treatment of OM especially using topical treatment to avoid systemic side effects.

Materials and methods

OME was induced in all experimental groups by injecting 300 µl (1 mg/ml) of *Salmonella typhimurium* LPS (Sigma, St. Louis, MO) into the superior bullae of chinchillas with a turberculin syringe and a venting needle. All test substance (TS) formulae were supplied by Alcon® Research, Ltd., Fort Worth, TX. Before the inoculation of LPS and TS into the bullae, the chinchilla's hair was removed from the superior bullae, and the area was thoroughly prepared and disinfected with Povidone iodine 10% solution. To summarize, 200 µL of each TS was inoculated at -2, 24, and 48 hours relative to the 300 µl LPS injection at zero hour. All animals were examined under an operating microscope before each inoculation for the presence or absence of MEE. At 96 hours, the chinchillas were euthanized, and MEE fluid was individually collected after removing the superior bullae bone through needle aspiration by direct visualization. Temporal bones were harvested and processed for histopathology.

Five experimental groups were studied in healthy adult male and female chinchillas weighing 400-600g that were randomly assigned to each group. Each group consisted of an equal number of male and female animals. Throughout the study, the sample size (*n*) refers to the number of ears. Group 1 consisted of controls in two subgroups: saline *n*=38 (Control-LPS alone); vehicle *n*=48 (Vehicle Substance-LPS). Group 2 was treated with dexamethasone and included subgroups of separate concentrations of dexamethasone: 0.1% *n*=44 and 1% *n*=46 solutions. Group 3 was treated with dexamethasone sodium phosphate and included subgroups of separate concentrations of dexamethasone sodium phosphate: 0.1% *n*=28 and 1% *n*=24 solutions. Group 4 was treated with rimexolone and included subgroups of separate concentrations of rimexolone: 0.1% *n*=20 and 1% *n*=20 solutions. Group 5 was treated with hydrocortisone and included subgroups of separate concentrations of hydrocortisone: 0.1% *n*=22 and 1% *n*=26 solutions. A total of 158 animals were used, 43 for controls and 115 for experimental groups.

Four temporal bones from each of Group 2 (dexamethasone) and Group 4 (rimexolone) were sent for processing to the Otopathology Laboratory at the University of Minnesota School of Medicine, and the remaining temporal bones were processed in our facility.[10] At the Otopathology Laboratory, whole temporal bones were processed by the usual method using celloidin embedding. In our facility, part of the

temporal bone with middle-ear mucosa (MEM) was dissected out, fixed in buffered 10% formalin, decalcified in 5% ethylene diamine tetraacetic acid for 5 days, rinsed four times with the same buffer, dehydrated in graded ethanol, and embedded in paraffin. Each paraffin block was sectioned at 20 μm, and every 10th section was stained with hematoxylin and eosin (H&E) for microscopic examination. The slides were examined in a blinded manner to the examiners for the mucosal thickness under a personal computer-attached inverting microscope (Zeiss, Goettingen, Germany) and a digital image was captured by a SIT-66 camera (Dage MTI; Indianapolis, IN) and a MicroPublisher 5.0 RTV camera (Q Imaging, Surrey, BC, Canada) of a representative portion of MEM.

Examiners measured the thickness of MEM from the digital picture with Image-Pro Plus 3.0 (Media Cybernetics, Silver Spring, MD) and Q Capture Pro (QImaging, Surrey, BC, Canada). Averages were taken from all the measurements obtained for mucosal thickness and MEE collected. The outcome of each treatment was determined by the amount of MEE present and MEM thickness. Statistical Package for Social Sciences (SPSS) and Analyse-it for Microsoft Excel (Analyse-it Software Ltd) were used to run Student's t tests.

Results

Initial otomicroscopic examination revealed healthy, intact tympanic membranes in all animals. At the end of 4 days, only in 14 ears (0.1% dexamethasone $n=2$, 1% dexamethasone $n=3$, 0.1% dexamethasone sodium phosphate $n=1$, 1% dexamethasone sodium phosphate $n=4$, 0.1% rimexolone $n=1$, 1% rimexolone $n=1$, 0.1% hydrocortisone $n=1$, 1% hydrocortisone $n=1$) the MEE was unobservable. The volume of MEE fluid collected differed for each TS. The maximum mean amount of MEE was found in the saline control group 488 μL. The overall volume of MEE in TS was significantly ($p<0.03$) less (lowest in 1% rimexolone group, 133 μL, and second lowest in 0.1% dexamethasone group, 278 μL) compared to the saline and vehicle control groups except for 0.1% hydrocortisone group (428 μL). The MEM thickness TS was significantly ($p<0.01$) less (least in 1% dexamethasone sodium phosphate group), compared to the saline and vehicle control groups (51.6 μM) except for 0.1% and 1% rimexolone (56.1 and 45.8 μM respectively).

Conclusion

Topical corticosteroid treatment was effective in resolving inflammation of the middle-ear mucosa in the LPS-induced experimental otitis media compared to the control. There was a difference between the various corticosteroids tested. In terms of reducing mucosal thickness, 1% dexamethasone sodium phosphate was most effective and 1% rimexolone was the most effective in terms of reducing the volume of MEE. Overall, the higher corticosteroid concentration, the better the outcome was.

Acknowledgement

References

1. Daly KA, Rovers MM, Hoffman HJ, Uhari M, Casselbrant ML, Zielhuis G, Kvaerner KJ. Recent advances in otitis media. 1. Epidemiology, natural history, and risk factors. Ann Otol Rhinol Laryngol Suppl. 2005; 194:8-15.
2. Jung TT. Prostaglandins, leukotrienes, and other arachidonic acid metabolites in the pathogensis of otitis media. Laryngoscope 1988; 98:980-93.
3. Jung TT, Park YM, Schlund D, Weeks D, Miller S, Wong O, Juhn SK. Effect of prostaglandin, leukotriene, and arachidonic acid on experimental otitis media with effusion in chinchillas. Ann Otol Rhinol Laryngol Suppl. 1990; 148:28-32.
4. Fliss DM, Dagan R, Houri Z, Leiberman A. Medical management of chronic suppurative otitis media without cholesteatoma in children. J Pediatr 1990; 116:991-6.
5. Kenna MA, Rosane BA, Bluestone CD. Medical management of chronic suppurative otitis media without cholesteatoma in children--update 1992. Am J Otol 1993; 14:469-473.
6. Berman S. Otitis media in children. N Engl J Med 1995; 332:1560-1565.
7. Jung TTK, Giebink GS, Juhn SK. Effects on ibuprofen, corticosteroid, and penicillin on the pathogenesis of experimental pneumococcal otitis media. In: Lim, DJ, ed. *Recent Advances in Otitis Media with Effusion.* Philadelphia: BC Decker, Inc., 1984:269-272.

8. Jung TTK, Hwang SJ, Poole D, et al. Chinchilla Models of Otitis Media. In: Lim DJ, Bluestone CD, Klein JO, Nelson J, eds. *Recent Advances in Otitis Media with Effusion*, Philadelphia: BC Decker, Inc., 1988:450-453.
9. Dohar J, Giles W, Roland P, et al. Topical ciprofloxacin/dexamethasone superior to oral amoxicillin/clavulanic acid in acute otitis media with otorrhea through tympanostomy tubes. Pediatrics 2006; 118:e561-569.
10. Florea A, Zwart JE, Lee CW, et al. Effect of topical dexamethasone versus rimexolone on middle ear inflammation in experimental otitis media with effusion. Acta Otolaryngol 2006; 126:910-915.

What is the best method of repairing tympanic membrane perforation?

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Introduction

One of the common sequelae of chronic otitis media is tympanic membrane (TM) perforation, which can cause hearing loss and otorrhea. The two traditional methods for reconstruction of TM perforation have been medial (underlay) or lateral (overlay) graft techniques. In the underlay technique, the graft is placed entirely medial to the remaining TM and annulus, which is the most common and easiest technique. It is typically used for posterior perforations. In the overlay technique, the graft is placed laterally to the annulus, and any remaining fibrous middle layer after the squamous layer has been carefully removed. The anterior canal wall is widened with a drill to minimize blunting, and the graft is placed just medially to the long process of malleus to prevent lateralization. Each of these techniques has its advantages and disadvantages.[2,3]

The anterior or subtotal TM perforation is difficult to repair because of less vascularity than posterior tympanic membrane[4] and anterior bony overhang that blocks visualization. Because of reduced vascularity in the anterior tympanic membrane, there is a greater risk of necrosis and reabsorption of the fascia graft.[4] When the medial graft technique is used to repair anterior or subtotal TM perforation, the anterior portion of the fascia graft may fall away, resulting in reperforation and obliteration of anterior portion of the middle-ear cavity.[5] Although the lateral graft technique has a higher success rate for the reconstruction of anterior or subtotal TM perforation, serious lateralization of graft may occur. During the past 11 years, we have been using medio-lateral graft tympanoplasty for repair of anterior or subtotal tympanic membrane perforation. In the medio-lateral graft technique, the fascia graft is placed medially to the posterior half of the TM perforation and laterally to the anterior half of the perforation. This method is a hybrid of the medial and lateral graft techniques that takes advantage of both methods. The purpose of this study is to describe and evaluate the medio-lateral graft tympanoplasty for anterior or subtotal TM perforation and medial graft tympanoplasty for reconstruction of posterior TM perforation.

Materials and methods

Patients. The charts of 200 patients who underwent the medial tympanoplasty (100 cases) and medio-lateral tympanoplasty (100 cases) during the past 11 years (1995 to 2006) were retrospectively reviewed. The main outcome measure was intact TM without lateralization or anterior blunting. All patients underwent preoperative and postoperative audiograms. Concomitant mastoidectomy was performed whenever necessary. In addition, ossiculoplasty was also done as needed. The choice of prosthesis was made according to the condition and anatomy of the middle ear cleft at the time of the surgery. Prostheses used were various types made of either hydroxylapatite (Applebaum, Wehr, or Goldenberg design) or titanium. Patients with less than 6 months follow up were excluded.

Surgical technique. The procedure is usually performed under general anesthesia. Transcanal, endaural, or postauricular approaches are used, depending on the clinical situation. A rim of tissue is removed from the perforation edge to de-epithelialize and encourage migration of the mucosal layer and epithelium. Vertical canal incisions are made at the 12- and 6-o'clock positions. A posterior tympanomeatal flap is elevated, and ossicles are evaluated. Mastoidectomy or ossiculoplasty are performed at the appropriate time if needed. In posterior TM perforation, temporalis fascia is grafted as a medial graft under the tympanic membrane perforation. In medio-lateral cases, first a horizontal incision is made in the anterior canal skin with a curved round knife. The distance of the anterior-horizontal canal incision from the anterior annulus should be about the same as the diameter of the perforation. After the incision, the anterior canal skin is elevated medially and laterally, then canalplasty is performed by drilling the anterior bony overhang with diamond burrs and suction irrigator until a full view of the anterior annulus is possible. The anteromedial canal skin flap is elevated up to the annulus or margin of the TM. At the annulus, only the squamous epithelial layer of the TM is carefully elevated to the anterior half of the perforation edge, leaving the anterior annulus intact. The middle ear cavity is packed with Gelfoam soaked in

nonototoxic fluoroquinolone antibiotic otic drops. Unlike in the case of usual medial graft technique, the middle-ear packing does not have to be tight. The temporalis fascia is grafted medially from the long process of malleus and under the annulus in cases of medial graft tympanoplasty. In medio-lateral tympanoplasty the temporalis fascia is grafted medially for the posterior half of the perforation and is grafted laterally over the annulus anterior half of the perforation. To avoid anterior blunting, the fascia graft is brought only to the anterior sulcus on the annulus and not above the annulus. As a second layer of closure, anteromedial canal skin is rotated to cover perforation and fascia as a superiorly based flap. Anterolateral canal skin is replaced, and packings are placed. Traditional rosebud packing is inserted by using otosilk strips with small to medium-sized cotton ball inside, soaked in the antibiotic otic drops. The rest of the ear canal is packed with a gauze strip soaked in antibiotic ointment or a Xerofoam gauze. The incision site is closed in the usual manner.

Results

Medial graft tympanoplasty. There were four failures (96% success rate) in medial graft method for posterior TM perforation due to infection and reperforation.

Medio-lateral graft tympanoplasty. There were 3 failures (97% success rate), caused by a postoperative infection, recurrent cholesteatoma, and anterior blunting, respectively. There was no lateralization and anterior fall-away of fascia. Two cases of the epithelial pearls on the tympanic membrane were easily removed in the office setting. It was noted that healing of the reconstructed TM took place much faster with the medio-lateral graft method compared with the case of the traditional medial or lateral graft technique.

Discussion

Over the years, various techniques have been attempted to improve tympanoplasty results. These include overlay tympanoplasty, underlay tympanoplasty, Gelfilm sandwich tympanoplasty, Crowncork tympanoplasty, swinging door tympanoplasty, sandwich graft tympanoplasty , window shade tympanoplasty.[5-8] Among them, underlay and overlay techniques are most commonly used. The advantages of medial (underlay) graft include ease of learning the technique, avoidance of the risk of lateralization and blunting of the anterior sulcus, and high success rate, especially for the

posterior perforation. The disadvantages of medial graft are inadequate visualization of the anterior meddle ear, possible anterior graft fall-away, reduction of middle-ear space with consequent increased risk of adhesions, and less suitability for reconstruction of anterior perforation. The lateral (overlay) graft provides superior exposure, suitable for all perforations, and minimizes reduction of the middle-ear space. This technique has a high success rate and has been particularly effective for large, anterior perforations. The disadvantages of lateral graft include anterior blunting, possible lateralization of graft, tendency to create more epithelial pearls, need for malleus manipulation, longer healing time, increased operation time, and complexity for repair of small posterior perforations.

One of the most serious complications of the graft techniques is lateralization of the TM. Lateralization of the TM is a condition in which the visible surface of the TM is located to the bony annular ring and loses contact with the conducting mechanism of the middle ear. Lateralization of TM may be associated with considerable morbidity, including hearing loss and cholesteatoma. Surgical repair is often necessary for significant underlying disease, but reestablishment of a normal TM can be challenging. Medio-lateral graft tympanoplasty avoids lateralization of the graft tympanoplasty avoids lateralization of the graft and TM by placing fascia medially to the posterior half of the TM and perforation, as well as the long process of malleus, and laterally to the anterior half of the perforation to prevent lateralization. In our study, there was no lateralization of the graft.

The medio-lateral graft tympanoplasty has many advantages over traditional medial or lateral graft: (1) prevention of anterior fall-away of fascia, (2) stability of the graft, like a "button in a button hole," (3) no need for tight Gelfoam packing to support the graft, (4) prevention of lateralization of graft, (5) better blood supply because anterior canal skin is rotated as a rotational flap rather than free graft, (6) easier because the epithelial layer of only the anterior half of the TM remnant is elevated rather than the entire TM, and (7) less malleus manipulation.

Conclusion

The medial graft tympanoplasty is the method of choice for the posterior TM perforation. For the large anterior or subtotal TM perforations, the medio-lateral graft method seems like a superior method than the traditional medial or lateral graft technique.

References

1. Jung TT, Park SK. Mediolateral graft tympanoplasty for anterior or subtotal tympanic membrane perforation. Otolaryngol Head Neck Surg 2005; 132:532-536.
2. Rizer FM. Overlay versus underlay tympanoplasty. Part I: historical review of the literature; Part II: the study. Laryngoscope 1997; 107:1-36.
3. Wehrs RE. Grafting techniques. Otolaryngol Clin North Am 1999; 32:443-455. Review.
4. Applebaum EL, Deutsch EC. An endoscopic method of tympanic membrane fluorescein angiography. Ann Otol Rhinol Laryngol 1986; 95:439-443.
5. Farrior JB. Sandwich graft tympanoplasty: experience, results, and complications. Laryngoscope 1989; 99:213-217.
6. Weider DJ. Use of the Williams microclip in various aspects of tymanoplastic surgery. Laryngoscope 1981; 91:2106-2125.
7. Kartush JM, Michaelides EM, Becvarovski Z, LaRouere MJ. Over-under tympanoplasty. Laryngoscope 2002; 112:802-807.
8. Schraff S, Dash N, Strasnick B. "Window shade" tympanoplasty for anterior marginal perforations. Laryngoscope 2005; 115:1655-1659.

Simple tympanotomy or grommet insertion in chronic secretory otitis media - a 25-year follow-up on eardrum pathology, hearing and Eustachian tube function

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Introduction

The proper indication for treatment of chronic otitis media with effusion/secretory otitis media (SOM) by myringotomy or ventilation tube insertion remains controversial. Benefits must be weighed against side effects of treatment, as well as sequelae induced by the treatment itself. While it seems clear that ventilation tubes improve hearing in the short term[1], beneficial long-term effects have been elusive.

Information on short-term hearing, tympanometric profiles and eardrum pathology related to the treatment of SOM is readily available[2-12], whereas long-term findings and the dynamics of outcome over time are scarcely documented in the literature. Only a few centers have presented contemporary data spanning more than 10 years.[13-20] On diverse materials, Maw and Bawden reported on a follow-up of 12 years[13], while Valtonen et al had a follow-up of 14 years[16,17], Skinner and Lesser of 15 years[20], de Beer et al of up to 16 years[18,19], Ryding et al of 18 years in a small group with extremely long-standing SOM[15], while Daly et al have published the hitherto longest follow-up of up to 23 years.[14] In terms of change of sequelae over time, the previous reports have described both improvement[19] and deterioration.[10,14]

This report documents the dynamics of ear drum pathology, hearing acuity and Eustachian tube function during 25 years after treatment of SOM. The included patients were treated by myringotomy in one ear and ventilation tube insertion in the other ear at a mean of 3.9 years of age. Follow-up examinations were performed 3, 7 and 25 years after treatment, when all patients were well into their adulthood.

Materials and methods

The materials and methods of the study have been thoroughly reported previously.[21] During 1977 and 1978, 224 consecutive children (91 girls and 134 boys) with chronic bilateral SOM (bilateral type B tympanogram for at least 3 months) were treated by adenoidectomy and bilateral myringotomy with evacuation of the middle ear effusion, and insertion of a Donaldson type ventilation tube on the right side only. The myringotomy was performed similarly on both sides, with a radial orientation in the posterior-inferior quadrant, length approximately 2-3 mm. Due to waiting time for surgery, the pre-operative tympanometric profile had normalized in some ears (93% had a type B or type C2 tympanogram, 72% had symmetric tympanograms and there were no significant differences between left and right ears). Tonsillectomy was performed in 41 children, because of recurrent acute tonsillitis. Mean age at surgery was 3.9 years (range 11 months -13 years).

The children were seen at follow-up examinations every three months for at least one year (longer in case of persisting disease). The ventilation tubes were functioning for an average of 10 months. Children with persisting or recurrent disease (type B tympanogram and hearing impairment for at least 3 months during the follow-up period) received either re-insertion of a ventilation tube on the right side or a secondary ventilation tube insertion on the left side, or both. A total of 10% had a tube re-inserted on the right ear, whereas 21% had a tube inserted on the left, previously myringotomized ear and 2% had a tube re-inserted after extrusion of the first tube on the left ear. Of the total patient material, 168 children (75%) had only the initial treatment (Table 1). Fifteen percent had discharge during the ventilation tube period, but in most cases short and uncomplicated. After extrusion of the ventilation tube, AOM occurred in 20% (bilateral in 9%, right side in 5% and left side in 6%).

The series were evaluated on 3 occasions, in 1980 (193 patients – 86%), in 1984 (146 patients – 65%) and in 2002 (104 patients – 46%). On all occasions, the group of patients not attending the examination was compared to those who did, with respect to initial severity of disease and recurrence

rate, as well as hearing impairment and tympanometric profile during the post-operative 1-year follow-up period. No statistically significant differences (see below for statistical methods) occurred between the attendees and the non-attendees.

To avoid confounding differences in disease severity and potential influence of repeated treatments, the major part of the following results focuses on the 168 patients receiving only the initial treatment (adenoidectomy, myringotomy left ear, myringotomy and ventilation tube insertion right ear). Of these 168 patients, 146 (87%) attended the 1980 examination, 115 (68%) the 1984 examination and 80 (48%) the 2002 examination. Again, comparisons between attendees and non-attendees revealed no significant differences.

Otomicroscopy, tympanometry, as well as pure tone audiometry at 250, 1000 and 4000 Hz were performed at each evaluation. Forty-six of the 168 children were too young to corporate for a pure tone audiometry before treatment, leaving 122 for the initial hearing test. All eardrum changes, such as perforation, atrophy, tensa retraction, atelectasis, myringosclerosis and flaccida retraction were meticulously recorded during otomicroscopy, including the extension of pathology throughout the four quadrants of the drum. The shape and extension of pathologic changes was recorded by sketching on a schematic print of an eardrum. Perforation was defined as presence of a visible hole, atrophy as a localized area of thinning, retraction as an area of inward displacement, atelectasis as fusion of a drum retraction with a medial part of the middle ear, and myringosclerosis as a whitish, calcific plaque in the drum. Flaccida retraction was graded according to the classification suggested by Tos.[22]

At the last evaluation in 2002, the Eustachian tube function was evaluated using the nine-step inflation/deflation tympanometric test and the Toynbee test.[23] If a patient could alter the middle ear pressure by at least 10 mmH$_2$O by swallowing during any of the steps of the nine-step test or the Toynbee test, the Eustachian tube function was considered "good". The function was considered "poor", if pressure changes were absent or below 10 mmH$_2$O.

Part of the results of the 3- and 7-year follow-up examinations have been published before.[2,4,6,7] The Chi-square and Fisher's exact test were used for comparisons of prevalence/frequency between ears and examinations. The Student's t-test was used for comparisons of hearing levels and pathology extension. A p-value<0.05 was considered significant.

Results

The results of the study have been thoroughly reported previously.[21]

Pathology of the eardrum

Overall pathology

The overall prevalence of eardrum pathology was higher in tube ears, primarily due to more ears with myringosclerosis. The prevalence of atelectasis, perforation and cholesteatoma was not significantly related to treatment.

Focusing the 168 patients who in 1977-78 received the initial treatment only (myringotomy left ear and tube right ear), the difference in overall pathology between sides was significant at all points of follow-up (right more than left ear, p<0.0003; Chi-square and Fisher's exact test), whereas a decrease of overall pathology over time for left ears was non-significant.

All the following data are on the 168 patients who in 1977-78 received the initial treatment only (myringotomy left ear and tube right ear) (see Materials and Methods for explanation).

Myringosclerosis

The prevalence of myringosclerosis was around 50% in the previously tubed right ears, which was significantly higher than the 10-20% in the myringotomized left ears, at all points of follow-up (p<0.0001; Chi-square and Fisher's exact test). The prevalence of sclerosis did not change over time in tubed ears, whereas an increase was noted between 1980 and 2002 in the myringotomized ears (p=0.0029; Chi-square and p=0.0024; Fisher's exact test).

The mean overall drum extension of sclerosis was around 1.1-1.5 quadrants in the tubed right ears, which was significantly higher than the 0.3-0.6 quadrants in the myringotomized left ears, at all points of follow-up. A significant increase of sclerosis extension was seen over time on both sides. Comparing the left and right ears with sclerosis, the extension increase over time was confirmed and no significant difference existed between sides, although an apparent tendency was more extensive sclerosis on the tube side.

Atrophy

The prevalence of atrophy was around 13-27% in the previously tubed right ears and 8-12% in the

myringotomized left ears. An increase occurred over time in tubed ears, as the prevalence of atrophy was significantly higher in 2002, compared to the previous points of follow-up (p<0.028;Chi-square and p<0.044;Fisher´s exact test). This was not seen in the myringotomized ears, leading to a significantly higher prevalence of atrophy in the tubed ears at the 25-year follow-up in 2002 only (27 vs.12%; p=0.0093; Chi-square and p=0.0154; Fisher´s exact test).

The mean overall drum extension of atrophy was around 0.2-0.4 quadrants on both sides, which remained unchanged over time. However, looking specifically at the ears with atrophy, the mean extension decreased significantly over time, from around 2.2 quadrants in 1980 to around 1.7 quadrants in 2002. Still, no difference existed between sides.

Tensa retraction

The prevalence of tensa retraction decreased significantly over time on both sides, from around 12% in 1980 to a few percent in 2002 (p<0.02; Chi-square and Fisher´s exact test).

The mean overall drum extension of tensa retraction also decreased significantly over time on both sides, from around 0.4 quadrants in 1980 to below 0.1 quadrant in 2002. Looking specifically at the ears with tensa retraction, the decrease of extension was confirmed, from around 3.3 quadrants in 1980 to around 1.8 quadrants in 2002. No difference existed between sides.

Flaccida retraction

The prevalence of flaccida retraction also decreased significantly over time on both sides, from around 30% in 1980 to around 15-20% in 2002 (p<0.046;Chi-square and Fisher´s exact test).

The mean overall grade of flaccida retraction was equal between sides and did not change over time. However, in ears with retraction, a tendency towards increasing grade was noted over time on both sides, from a mean of around 1.7 in 1980 to around 2.1 in 2002. No difference existed between sides.

Hearing acuity

Before treatment, the hearing was significantly poorer on the right side (PTA 28.8 vs. 26; p<0.01; Student´s t-test). Treatment improved the hearing on both sides (p<0.001; Student´s t-test). The improvement was greater on the right, tubed side (PTA 16.9 vs. 10.2; p<0.001; Student´s t-test), leading to a better right-sided hearing after treatment (PTA 11.9 vs. 15.8; p<0.001; Student´s t-test). However, this difference

had levelled out at the first follow-up in 1980. A continuous, bilateral hearing improvement occurred over time between the subsequent follow-up examinations, which revealed no significant differences between sides.

Eustachian tube function

A marked improvement in tympanometric findings occurred over time. No differences occurred between sides and almost all ears had a normal type A tympanogram at the 25-year follow-up in 2002. The nine-step Eustachian tube function test was performed in 2002. Again, no significant difference existed between sides, whether or not the drum was pathologic. A majority of ears (around 2/3) had a poor tubal function according to the nine-step test, even though the initial tympanometry was normal.

Discussion

The results of the presented 3-, 7- and 25-year follow-up on eardrum pathology, hearing and Eustachian tube function after myringotomy or ventilation tube treatment for chronic secretory otitis media can be summarized in the following 7 statements:

1. Myringosclerosis and late atrophy are more prevalent in ears treated with a ventilation tube.
2. Prevalence of eardrum retraction decreases over time, while that of sclerosis remains unchanged in tube ears and increases in myringotomy ears.
3. Prevalence of atrophy increases over time, but only in ventilation tube ears.
4. Extension of myringosclerosis increases, whereas that of atrophy and tensa retraction decreases over time, regardless of treatment modality.
5. Hearing is better in the ventilation tube ear 3 months after treatment (with the tube in place), but not at 3 years or beyond (after tube extrusion).
6. Tympanometric findings are not related to treatment.
7. Eustachian tube function at 25 yearsis not related to treatment.

It can thus be concluded, that the insertion of a ventilation tube leads to better hearing until extrusion, but also to an increased prevalence of long-term pathologic changes of the ear drum. The dominating pathologic change is myringosclerosis, which seem to be of negligent clinical importance.

The prevalence and extension of some subtypes of pathology appear to change over time. However, the pathologic changes do not seem to be detrimental to the overall hearing acuity. In addition, the treatment modality has no impact on short- or long-

term tympanometric profile or Eustachian tube function.

Previous research

Naturally, several pre-defined or attendant conditions may differ between studies of the present type, e.g. morbidity, co-morbidity, social class, race and use of kindergarten. This is important to keep in mind, even when comparing studies with equivalent objectives. As an example, we found that ears treated repeatedly demonstrated more pathology, which has also been reported by other authors.[10,13] This may, however, also be a sequelae of more severe disease[13], which is the major reason for focusing our attention on the patients only receiving the initial treatment.

Thus, the variability of patient inclusion criteria, specific type and timing of the treatment, additional treatment, characteristics of the control group, calculation of PTA and definitions of pathologic lesions between studies are all potential confounders to be considered in the comparisons below. Equivalent caution should be paid when interpreting the overall conclusions of meta-analyses on the subject.[24]

Eardrum pathology

It is noteworthy, that no cholesteatoma and only small numbers with atelectasis or eardrum perforation were found in the present series, and that these were without statistical relation to the type of treatment.

We found an overall prevalence of eardrum pathology of around 40% in myringotomized ears and around 70% in tubed ears, which is comparable to the 70-75% found in tubed ears after 3-4 years by Johnston et al.[11] In a study of very severe, longstanding SOM with repeated treatments, Ryding et al found pathology in 76%, 18 years after the first tube insertion,[15] whereas Stenstrom et al recorded pathology in 81% of cases 6-9 years after treatment with T-tubes.[12] Although the prevalence of individual subtypes of pathology did change over time, as detailed above and below, our prevalence of pathology overall did not change significantly, from 3 over 7 to 25 years after treatment. This is not in agreement with Daly et al, who found increasing prevalence of pathology up to 8 years after treatment, although the incidence decreased over time, from 3 to 8 years.[10] In de Beer et al´s long-term follow-up at 8 and 18 years of age, a decrease in the prevalence of pathology was found, as 92% of tubed ears were pathologic after 8 years, decreasing to 72% at 18 years.[19] The numbers for non-treated ears with SOM were 46 and 17%, respectively.

Along with atrophy, myringosclerosis is one of the two most frequent pathologic long-term finding following SOM, whether treated or not.[12,19,25-27] Ventilation tube treatment leads to a marked increase of sclerosis prevalence, which has been documented by the present and a number of previous studies.[3,11-13,19] We found sclerosis in around 50% in tube ears versus around 15% in myringotomy ears. In comparison with the other long-term studies, de Beer et al found 56-59%,[19] Maw and Bawden 52%[13] and Daly et al 44-54%.[10,14] with sclerosis following tube treatment, all numbers higher than the calculated 32% in the meta-analysis by Kay et al[23], which however also includes short-term follow-ups.

In opposition to the latter implication, this paper documents that the prevalence remains unchanged for tube ears in the very long term, but also that the extent of the sclerotic plaque increases over time, regardless of treatment, which is a novel finding. The stable prevalence beyond the first few years after treatment is in agreement with the majority of other reports on the subject, although with shorter observation periods.[10,14,19,20,26,27]

For the myringotomized ears, surprisingly, the sclerosis prevalence will increase over time when observing the patients for 25 years, which is an unprecedented finding.

The number of ears with atrophy of the eardrum remains stable over time in myringotomy ears (around 8-12%), whereas an increase occurs in tube ears, leading to more tube ears with atrophy 25 years after treatment (12 vs. 27%). This increase is in agreement with the shorter-term studies[8,10,20], but in opposition to de Beer et al, who found a decrease of atrophy frequency over two points of observation, up to 16 years after treatment (from 63 to 20% in tube ears).[19] Maw and Bawden found 24% with atrophy at 4 years and 22% at 10 years.[13] We have no explanation for this discrepancy, although differences in the patient material and pathology definitions may be in play, as considered above. In their meta-analysis, Kay et al calculated that 25% of tube ears display atrophy[24], which is well in accordance with present numbers, as opposed to the high 75% found a few years after treatment by Johnston et al.[11] and Schilder et al.[28] The mean extension of the atrophic drum area decreases with time, regardless of treatment modality, which seems to indicate the existence of an inherent tympanic membrane repair mechanism.

While the extension of atrophy decreases over time, the incidence increases. Concurrently, the extension of myringosclerosis increases. Thus, the increasing extension of sclerosis over time seems to be associated with more frequent atrophy, but also a reduction in size of the atretic patches. This may again

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implicate an intrinsic mechanism to maintain unchanged dynamic properties of the drum, facing changes of pathology over time. This is supported by the fact that the overall hearing is equal between sides, although pathology is more prevalent and more extensive on the tube side.

Eardrum retraction is an established feature of SOM and as expected, we found a continuously decreasing prevalence of pars tensa and flaccida retraction over the years, concurring with resolving disease and time following treatment. Shorter-term studies have shown an initial increase of retraction prevalence[10], followed by stabilization[13] and decrease.[19] The type of treatment had no impact on the occurrence of retraction, which is also the conclusion of other[13,29], but not all papers on the subject, as de Beer et al found a higher prevalence in tubed ears.[19]

Hearing acuity

The hearing level improve over the years following SOM, regardless of treatment modality, and it is noteworthy, that the mean group hearing level at 25 years is equivalent to normal subjects. The hearing is better in tubed ears 3 months after insertion (when the tube is still in place), otherwise not. The temporary hearing improvement may, however, reduce the risk of detrimental effects on speech and language development. The more frequent and extensive pathology found following tube treatment does not affect the overall hearing level in a clinically significant manner, which is the main clinical implication of this report. This conclusion is in keeping with previous reports on shorter observation periods[17,30], although questioned by some authors.[18,31]

Eustachian tube function

Tube treatment does not alter the tympanometric profile over the years or Eustachian tube function after 25 years, which is in agreement with short-term functional studies[32]. In line with the findings of Ryding et al[15], the Eustachian tube function is not related to eardrum pathology when adulthood is reached. During childhood, however, there is a clear association between poor tubal function and the development of eardrum pathology.[33] The lack of association in adulthood can be partly explained, as the Eustachian tube matures and function improves in some ears over the years, while the pathology is less likely to disappear. Still, it is striking that around 25 years after disease, the majority of patients have a poor tubal function according to the nine-step test, regardless of the type of treatment given. Even the group of ears with a normal drum has a high proportion with poor

tubal function, as almost half of the tube ears and 2/3´s of the myringotomy ears with a normal drum belong to this category.

Potential study bias

The main advantage of this unique 25-year follow-up is the fact that the children serve as their own control. The left myringotomized ear serves as an appropriate control for the ventilation tube treatment on the right ear, as good pre-treatment symmetry of tympanometric findings existed between the two ears. A number of potential patient selection biases are thereby avoided.

The main potential of bias in the present study derives from a 25-year follow-up attendance on the low side, i.e. 46-48%. However, the group of patients not attending each follow-up examination was compared to those who did, with respect to initial severity of disease and recurrence rate, as well as hearing impairment and tympanometric profile during the post-operative 1-year follow-up period. No statistically significant differences occurred between the attendees and the non-attendees.

Only a few percent of the patients had emigrated or could not be found (all citizens have a social security number, which is linked to the national registry of addresses). Three letters were posted and even telephone calls were made to those not showing up. However, the mean age at the 25-year follow-up was around 29 years, which is an age which in our country typically implies various combinations of heavy academic studies and exams, week-end parties or clubbing (the examinations were performed during week-ends), busy careers and small children. Thus, the inclination to attend may be small, especially if ear problems are non-existent. The attendance at the 3- and 7-year follow-up (when the children were 7 and 11 years, respectively) was comparably better at 86-87% and 65-68%, respectively. These examinations were performed while the children were still living with their parents and had a higher probability of ear-related symptoms.

References

1. Lous J, Burton MJ, Felding JU, Ovesen T, Rovers MM, Williamson I. Grommets (ventilation tubes) for hearing loss associated with otitis media with effusion in children. Cochrane Database Syst Rev 2005;(1):CD001801.
2. Bonding P, Tos M, Poulsen G. Unilateral insertion of grommets in bilateral otitis media. Tympanometric findings after 1 to 3 years. Acta Otolaryngol Suppl 1982;386:161-2.

3. Lildholdt T. Ventilation tubes in secretory otitis media. A randomized, controlled study of the course, the complications, and the sequelae of ventilation tubes. Acta Otolaryngol Suppl 1983;398:1-28.

4. Tos M, Bonding P, Poulsen G. Tympanosclerosis of the drum in secretory otitis media after insertion of grommets. A prospective, comparative study. J Laryngol Otol 1983;97:489-96.

5. To SS, Pahor AL, Robin PE. A prospective trial of unilateral grommets for bilateral secretory otitis media in children. Clin Otolaryngol Allied Sci 1984;9:115-117.

6. Bonding P, Tos M. Grommets versus paracentesis in secretory otitis media. A prospective, controlled study. Am J Otol 1985; 6:455-460.

7. Tos M, Stangerup SE. Hearing loss in tympanosclerosis caused by grommets. Arch Otolaryngol Head Neck Surg 1989;115:931-935.

8. Sederberg-Olsen JF, Sederberg-Olsen AE, Jensen AM. Late results of treatment with ventilation tubes for secretory otitis media in ENT practice. Acta Otolaryngol 1989;108:448-455.

9. Riley DN, Herberger S, McBride G, Law K. Myringotomy and ventilation tube insertion: a ten-year follow-up.J Laryngol Otol1997;111:257-261.

10. Daly KA, Hunter LL, Lindgren BR, Margolis R, Giebink GS. Chronic otitis media with effusion sequelae in children treated with tubes. Arch Otolaryngol Head Neck Surg 2003;129:517-22.

11. Johnston LC, Feldman HM, Paradise JL, Bernard BS, Colborn DK, Casselbrant ML, Janosky JE. Tympanic membrane abnormalities and hearing levels at the ages of 5 and 6 years in relation to persistent otitis media and tympanostomy tube insertion in the first 3 years of life: a prospective study incorporating a randomized clinical trial. Pediatrics 2004;114:58-67.

12. Stenstrom R, Pless IB, Bernard P. Hearing thresholds and tympanic membrane sequelae in children managed medically or surgically for otitis media with effusion. Arch Pediatr Adolesc Med 2005;159:1151-1156.

13. Maw AR, Bawden R. Tympanic membrane atrophy, scarring atelectasis and attic retraction in persistent, untreated otitis media with effusion and following ventilation tube insertion. Int J Ped Otorhinolaryngol 1994;30:189-204.

14. Daly KA, Hunter LL, Lindgren BR, Giebink GS. Relationships between otitis media sequelae and age. Laryngoscope 1998;108:1306-1310.

15. Ryding M, White P, Kalm O. Eustachian tube function and tympanic membrane findings after chronic secretory otitis media. Int J Ped Otorhinolaryngol 2004;68:197-204.

16. Valtonen H, Tuomilehto H, Qvarnberg Y, Nuutinen J. A 14-year prospective follow-up study of children treated early in life with tympanostomy tubes: Part 1: Clinical outcomes. Arch Otolaryngol Head Neck Surg 2005;131:293-298.

17. Valtonen H, Tuomilehto H, Qvarnberg Y, Nuutinen J. A 14-year prospective follow-up study of children treated early in life with tympanostomy tubes: Part 2: Hearing outcomes. Arch Otolaryngol Head Neck Surg 2005;131:299-303.

18. de Beer BA, Schilder AG, Ingels K, Snik AF, Zielhuis GA, Graamans K. Hearing loss in young adults who had ventilation tube insertion in childhood. Ann Otol Rhinol Laryngol 2004;113:438-444.

19. de Beer BA, Schilder AGM, Zielhuis GA, Graamans K. Natural course of tympanic membrane pathology related to otitis media and ventilation tubes between ages 8 to 18 years. Otol Neurotol 2005;26:1016-1021.

20. Skinner DW, Lesser TH, Richards SH. A 15 year follow-up of a controlled trial of the use of grommets in glue ear.Clin Otolaryngol Allied Sci1988;13:341-346.

21. Cayé-Thomasen P, Stangerup SE, Jørgensen G, Drozdziewic D, Bonding P, Tos M. Myringotomy versus ventilation tubes in secretory otitis media – eardrum pathology, hearing and Eustachian tube function 25 years after treatment. Otol Neurotol 2008;29:649-657.

22. Tos M, Poulsen G. Attic retractions after secretory otitis media. Acta Otolaryngol 1980;89:479-486.

23. Bluestone CD. Assessment of Eustachian tube function. In: Jerger J, ed. Handbook of Clinical Impedance Audiometry. New York: American Electromedics Corporation, 1975:127-148.

24. Tos M, Stangerup SE, Holm-Jensen S, Sørensen CH. Spontaneous course of secretory otitis and changes of the eardrum. Arch Otolaryngol 1984;110:281-9.

25. Kay DJ, Nelson M, Rosenfeld RM. Meta-analysis of tympanostomy tube sequelae. Otolaryngol Head Neck Surg 2001;124:374-380.

26. Maw AR. Development of tympanosclerosis in children with otitis media with effusion and ventilation tubes. J Laryngol Otol 1991;105:614-617.

27. Tos M, Stangerup SE, Larsen P. Dynamics of eardrum changes following secretory otitis. A

prospective study.Arch Otolaryngol Head Neck Surg 1987;113:380-385.

28. Schilder AG, Zielhuis GA, Haggard MP, van den Broek P. Long-term effects of otitis media with effusion: otomicroscopic findings.Am J Otol 1995;16:365-372.

29. Brown MJ, Richards SH, Ambegaokar AG. Grommets and glue ear: a five-year follow up of a controlled trial.J R Soc Med 1978;71:353-356.

30. Hunter LL, Margolis RH. Effects of tympanic membrane abnormalities on auditory function.J Am Acad Audiol 1997; 8:431-446.

31. Stenstrom R, Bernard PAM, Feldman W, Durieux-Smith A, Pless IB, Beauregard Y. Long-term sequelae of ventilation tube insertion for the treatment of otitis media with effusion and recurrent acute otitis media. In: Lim D, Bluestone CD, Casselbrant M, Klein J, Ogra P, eds. Recent Advances in Otitis Media. Proceeding of the 6th International Symposium. Hamilton, Ontario: BC Decker, Inc., 1996:373-375.

32. van Heerbeek N, Ingels KJ, Snik AF, Zielhuis GA. Eustachian tube function in children after insertion of ventilation tubes.Ann Otol Rhinol Laryngol 2001; 110:1141-1146.

33. Stangerup SE, Tos M, Arnesen R, Larsen P. A cohort study of point prevalence of eardrum pathology in children and teenagers from age 5 to age 16. Eur Arch Otorhinolaryngol 1994; 251:399-403.

Ventilation tubes in OME benefit development in older but not younger children

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Background and aim

In sensory deprivation and its effects on development, early experience has for 60 years been thought paramount, with the assumed "partial irreversibility" justifying early intervention for secondary prevention. With statistically reliable and material treatment effects with ventilation tubes (VTs) in otitis media with effusion (OME) on hearing and developmental impact in a large randomized controlled trial, we could test dependence of treatment outcomes on age, and thus evaluate whether age is relevant to surgical intervention in OME.

Methods

Three hundred seventy-six children aged 42 to 84 months were randomized in the UK TARGET randomized controlled trial to further watchful waiting controls, VTs (Shepard), or VTs+adenoidectomy and followed for five occasions over 2 years. Outcomes for hearing loss and developmental impact (a well-developed and bias-adjusted 49-item parental questionnaire score) were imputed for attrition on a 2-period basis: 0-6 months and 12-24 months. Here VTs and VTs+adenoidectomy were combined for power to test the interaction of treatment with age at baseline, this both as continuum and dichotomy.

Results

For period 0-6 months post-randomization, the age-by-treatment interaction for developmental impact was significant, and was strongest for age dichotomized at 5 years ($p < 0.001$). As standard deviation affects sizes, the older children received a substantial benefit of + 0.63 SD but the younger a dis-benefit (-0.10 SD; NS). Results for the full 2-year period were consistent. Age-dependency for hearing loss was totally absent.

Discussion

The dis-benefit in younger children is too small to be considered material harm. The nullity of age-dependence for hearing loss as outcome shows that the therapeutic efficacy cannot be the basis of the obtained age-dependence. Rather, it must lie in the developmental domain, with the younger cases that present mostly not having sufficient impact (due to insufficient persistence or severity) to make a difference.

Conclusions

The study does not address the biological issue of irreversibility as justification of early prevention, but does question its relevance. The false assumption of overall benefit to development in younger children must be offset by rigorous selection of a minority of those for severity, persistence, and physical health that treatments can benefit. TARGET results overall justify a highly selective restrictive treatment policy with VTs and adjuvant adenoidectomy for a high proportion, on the basis of other outcomes. TARGET is totally consistent with two other high-quality trials (Paradise et al., US; Rovers et al., NL) showing null benefit to development in younger children.

Tympanostomy tube outcomes in children at-risk and not-at-risk for developmental delays

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Introduction

Tympanostomy tube insertion is one of the most common surgical procedures in children, with more than 10,000 cases performed weekly in the United States. The efficacy of tubes for reducing ear infections, hearing loss, and middle-ear effusion is well-documented, but the impact on child development and quality of life is less clear. Published studies are limited by short-term follow-up, loss to follow-up, and limited generalizability because of restricted cohorts that include mainly healthy, asymptomatic children with middle-ear effusion identified by screening or intense surveillance.

Although current guidelines for otitis media with effusion (OME) emphasize prompt intervention in children at-risk for developmental delays, there are very limited data on outcomes of tympanostomy tube insertion in this sub- population. The paucity of data stems from ethical concerns about including children with delays in speech, language, learning, or development in randomized trials. Nonetheless about 15% of children in the United States have developmental delay. Accordingly, evaluating the benefit from tube insertion in this large group of candidates is an important goal of current otitis media research. Our study is the first to compare tympanostomy tube outcomes in children at-risk and not at-risk for developmental delays using the national criteria proposed by the American Academies of Pediatrics, Family Physicians, and Otolaryngology – Head and Neck Surgery.

Methods and materials

The study was conducted at a hospital-based pediatric otolaryngology practice in Brooklyn, New York. A historical cohort was identified by chart review of all children aged 6 months or older who had bilateral tympanostomy tubes inserted as a sole surgical procedure by the principal investigator (RMR) as part of routine clinical care for otitis media between March 1, 2004, and September 1, 2005. The cutoff date was selected to allow a minimum of 6 months follow-up. Children were excluded if they had concurrent surgery (e.g., adenoidectomy or tonsillectomy), if tubes were inserted for an indication other than otitis media, or if the caregiver could not speak English. Institutional review board approval was obtained before starting the study.

The office charts were reviewed to obtain demographic data and information about the child's clinical history, which was recorded on a de-identified data form. All children had baseline audiometric testing performed by a licensed pediatric audiologist. Air conduction testing was done using age appropriate techniques, including visual reinforcement audiometry (6 to 36 months), play audiometry (30 months to 5 years), and standard audiometry (5 years and older). Immittance tests were performed using a Grason Stadler 33 bridge with a 226 Hz probe tone.

Baseline hearing levels were calculated as the pure tone average (PTA) of 500 Hz, 1,000 Hz, and 2,000 Hz when tested in sound field, or for the better-hearing ear when tested with headphones. The presence or absence of middle-ear effusion was determined using pneumatic otoscopy by a validated otoscopist (RMR). All children underwent bilateral insertion of tympanostomy tubes under general anesthesia as part of routine clinical care. Armstrong beveled fluoroplastic tubes were used, which have a median intubation period of about 13 months. Caregivers received written instructions for an office visit 1 month after surgery, and for routine visits to check the tubes every 6 months thereafter until they extruded.

The primary outcome was caregiver responses about changes observed after ear tubes using a 5-point Likert scale (much worse, a little or somewhat worse, no change, a little or somewhat better, much better, don't know), with questions modeled after a validated instrument for assessing patient benefit from ear, nose, or throat surgery. The survey questions were:

- Have the results of tubes made your child's life overall better or worse?
- Has the result of tubes affected the things your child can do?
- Has the result of tubes affected your child's hearing?
- Has the result of tubes affected your child's speech and language?

- Has the result of tubes affect your child's learning or school performance?

Secondary outcomes concerned the child's progress after tubes regarding speech therapy, physical therapy, and occupational therapy (if applicable). Expectations about ear tubes were categorized as not met, met, or exceeded. Last, the child's at-risk status was determined using recommended criteria. An at-risk child was defined as having one or more of the following factors: (a) developmental delay, (b) speech and language delay or disorder, (c) permanent hearing loss not related to middle-ear fluid, (d) autism-spectrum disorder or pervasive developmental disorder, (e) Down syndrome or other syndrome affecting the ears, nose, or throat, (f) blindness or uncorrectable visual impairment, or (g) cleft palate.

Survey responses were obtained using telephone interview by an investigator (KT or DJ) other than the surgeon. The child's at-risk status was not assessed until the end of the survey to avoid ascertainment bias in obtaining responses. If a telephone response could not be obtained, a mail survey was forwarded with a cover letter and self-addressed stamped return envelope.

All statistical analyses were performed using SPSS software. Normally distributed data were summarized with the mean and standard deviation (SD), and skewed data with the median and interquartile range (IQR). Overall comparisons between at-risk vs. not at-risk children were performed using the Mann-Whitney U test. Responses on the primary outcome survey were also dichotomized into a most favorable category (much better) vs. all others, and odds ratios were calculated based on at-risk status. Last, multiple logistic regression was used to adjust the odds ratios (ORs) and 95% confidence intervals (CIs) for the effects of gender, age, and baseline hearing level. All tests were performed using a two-sided alpha level of 0.05 for statistical significance.

Results

Baseline characteristics of the 229 children meeting inclusion criteria are summarized in Table 1. Follow-up questionnaires were completed by 168 parents, with 93 (55%) reporting the presence of one or more factors that would place their child at-risk for speech, language, or learning problems because of middle-ear effusion (Table 2). The respondent was the child's mother for 87% and the father for 13%. Outcome questionnaires were completed a median of 2.0 years after tube insertion, with upper and lower quartiles of 1.6 and 2.4 years, respectively.

Overall changes perceived by caregivers after tube insertion were favorable (Table 3). The median response for all items was "much better." Nearly all caregivers reported their expectations regarding the impact of ear tubes were met (59%) or exceeded (38%). For children receiving *speech therapy* (n=82), caregivers reported that the progress after tubes was unchanged for 15 (18%), a little or somewhat better for 15 (18%), and much better for 52 (64%). For children receiving *physical therapy* (n=44), progress was unchanged for 13 (29%), a little or somewhat better for 17 (39%), and much better for 32%). Last, for children receiving *occupational therapy* (n=51), progress was unchanged for 18 (35%), a little or somewhat better for 11 (22%), and much better for 22 (43%).

Caregivers of at-risk children reported better outcomes after tubes than did caregivers of not at-risk children for speech and language (median response "much better" vs. "no change," P<.001) and for learning or school performance (median response "much better" vs. "a little or somewhat better," P=.004). Similarly, an outcome of "much better" vs. all other responses (Table 4) was reported more often for at-risk children regarding changes in speech and language (OR 4.59, 95% CI 2.39-8.85) and for learning or school performance (OR 3.13, 95% CI 1.65-5.94). No differences were reported between groups for hearing, the child's life overall, or for things the child can do.

Multiple logistic regression was performed to adjust the relationship between at-risk status and outcomes for confounding effects of gender, age, otitis media duration, hearing levels, and survey respondent (Tables 5 and 6) . An a priori decision was made to include all variables in the model, regardless of statistical significance. After adjustment, the ORs related to at-risk status increased for both speech and language outcomes (OR 5.11, 95% CI 2.43-10.75) and for learning and school performance outcomes (OR 3.53, 95% CI 1.75-7.13). The odds of reporting a "much better" response for hearing in at-risk vs. not at-risk children also improved after adjustment (OR 2.29, 95% CI 1.12-4.70), but the lower limit of the CI approached unity and the overall regression model was not significant (P=.066).

Discussion

Our results show that caregivers of children with otitis media and factors placing them at increased risk for developmental difficulties (Table 2) perceive greater changes after tympanostomy tube insertion than do caregivers of children who are not at-risk. A "much better" response was 5.1 times more likely for speech

and language in an at-risk child, and 3.5 times more likely for learning and school performance (Tables 5 and 6). Caregivers of at-risk children also reported improved progress after tube insertion for 82% of children receiving speech therapy, 71% receiving physical therapy, and 65% receiving occupational therapy. Conversely, there was no association with at-risk status for changes reported by caregivers in their child's hearing, life overall, or things they could do (Table 4).

The favorable changes reported after tube insertion in all children *cannot* be interpreted as overall tube effectiveness without an untreated comparison group to control for natural history, spontaneous resolution, or a regression-to-mean statistical artifact. However the enhanced improvement seen for at-risk vs. not at-risk children suggests that the former are more susceptible to the detrimental effects of middle-ear effusion on auditory function, and that removing this impediment facilitates progress. These outcomes remained highly significant for speech and language and for learning or school performance, core aspects of development thought to be influenced by OME, after multivariate adjustment for age, gender, hearing level, mother/father as respondent, and otitis media duration.

Current treatment guidelines from the American Academy of Pediatrics (AAP), American Academy of Family Physicians (AAFP), and American Academy of Otolaryngology – Head and Neck Surgery (AAO-HNS) advise clinicians to "distinguish the child with OME who is at risk for speech, language, or learning problems from other children with OME" and to more promptly "evaluate hearing, speech, language, and need for intervention." This statement was based on low-level evidence that included case series, observational studies, and expert opinion. The guideline panel, however, noted significant potential benefits that included "optimizing conditions for hearing, speech, and language; enabling children with special needs to reach their potential; and avoiding limitations on the benefits of educational interventions because of hearing problems from OME." They concluded an "exceptional preponderance of benefits over harm based on subcommittee consensus because of circumstances to date precluding randomized trials." The present results provide stronger evidence than previously available that some restriction of intervention to children with manifest problems in the domain of development is indeed justified.

Management guidelines need to be based on the best available evidence, yet the quality and quantity of published research for otitis media is highest for children least likely to benefit from intervention: "innocent bystanders" who are otherwise healthy and not at increased risk for developmental difficulties. Randomized trials, the highest level of treatment evidence, typically exclude at-risk children with OME because of ethical concerns. When trials are performed, they include primarily innocent bystanders, for whom tympanostomy tubes have a null or trivial impact on developmental outcomes. Such trials offer convincing evidence to restrict *routine* intervention in otherwise healthy children unless middle-ear effusion is highly persistent, but may be inappropriately extrapolated to at-risk populations. Current guidelines and expert panels highlight the need for new otitis media research applicable to at-risk children, which may have to be observational or use other types of design than the between-participant randomized controlled trial.

Our results can be generalized to children with characteristics similar to those in Tables 1 and 2 who are referred to an otolaryngologist for assessment of otitis media. The applicability to children evaluated in a primary care setting is unknown, as is the relevance to children with a lesser burden of otitis media. Most children studied had bilateral middle-ear effusion, mild hearing loss, and a prolonged duration of recurrent AOM, OME, or both. The "at-risk" designation for children with otitis media includes a diverse spectrum of disorders and conditions (Table 2), many of which occurred infrequently in our study sample. The most common at-risk condition was suspected or diagnosed speech and language delay or disorder, which may explain the favorable outcomes observed in this domain. Additional research is needed to assess outcomes in children with other at-risk conditions, particularly those with cognitive impairment.

The finding of high caregiver satisfaction and favorable developmental outcomes after tympanostomy tubes in at-risk children *cannot be translated into* a recommendation for liberal tube insertion, even in this population. Rather, it is a first step in validating current guidelines for prompt treatment of otitis media in at-risk children. Although tube insertion is generally safe and well-tolerated, the potential benefits of surgery for a given child must be balanced against the small, but nontrivial, risks of anesthesia, tube sequelae, and structural changes in the tympanic membrane. Until additional research is available, the preliminary evidence in our study should help caregivers and clinicians make more informed management decisions for at-risk children with otitis media.

Table 1. Characteristics of 229 children with otitis media as a chief complaint

Characteristic	At-risk	Not at-risk	Lost to follow-up	Total	Statistical test, P value
Total subjects, n(%)	93(40)	75(33)	61(27)	229(100)	—
Child age in years, median (IQR)	2.4 (1.5-3.8)	2.2 (1.3-5.0)	1.9 (1.3-4.0)	2.3 (1.3-4.1)	KW ANOVA, P=.812
Male gender, n(%)	62(67)	40(53)	35(57)	137(60)	Chi-square, P=.194
Otitis media duration in months, median(IQR)	12(6-24)	12(6-18)	12(6-18)	12(6-f9)	KW ANOVA, P=.806
Indication for tubes, n(%):					
Recurrent AOM	22(24)	30(40)	22(36)	74(32)	Chi-square, P=.042
OME	47(50)	21(28)	24(39)	92(40)	
Both	24(26)	24(32)	15(25)	63(28)	
Middle-ear effusion, n(%):					
None	6(6)	2(3)	4(6)	12(5)	Chi-square, P=.381
Unilateral	7(8)	12(16)	7(12)	26(12)	
Bilateral	80(86)	61(81)	49(82)	190(83)	
Flat (B) tympanogram, n(%):					
None	17(20)	7(10)	7(13)	31(15)	Chi-square, P=.430
Unilateral	19(22)	14(20)	14(26)	47(22)	
Bilateral	50(58)	48(70)	33(61)	131(63)	
Valid audiogram, n(%)	89(96)	73(97)	58(95)	220(96)	Chi-square, P=.509
Hearing level in dB,[†] mean(SD)	34(10)	31(10)	31(10)	32(11)	ANOVA, P=.312
Prior tube insertions, n(%):					
None	71(76)	66(88)	52(85)	189(82)	Chi-square, P=.384
1 prior	15(16)	8(11)	6(10)	29(13)	
2 prior	5(6)	0	2(3)	7(3)	
3 prior	2(2)	1(1)	1(2)	4(2)	

ANOVA, one-way analysis of variance; AOM, acute otitis media; dB, decibels; IQR, interquartile range; KW, Kruskal-Wallis; OME, otitis media with effusion; SD, standard deviation

[†]Pure-tone average at 500, 1000, and 2000 Hz in sound field, or for the better-hearing ear

Table 2. Risk factors for developmental difficulties in 168 children with otitis media

Risk factor	Prevalence, n(%)[†]
Suspected or diagnosed speech and language delay or disorder	86(51)
Developmental delay	39(23)
Autism-spectrum or other pervasive developmental disorder	8(5)
Permanent hearing loss	6(4)
Down or other syndrome affecting the ear, nose, or throat	4(2)
Blindness or uncorrectable visual impairment	3(2)
Cleft palate, with or without associated syndrome	2(1)

*Sensory, physical, cognitive, or behavioral factors that place children with otitis media at increased risk for developmental difficulties (delay or disorder).

[†]More than one risk factor could be present in a given child

Table 3. Distribution of responses for the caregiver-reported changes after ear tubes

Survey item	Item response, n (%)					
	Much worse	A little or somewhat worse	No change	A little or somewhat better	Much better	Don't know
Child's life overall	—	—	2(1)	16(10)	150(89)	—
Things child can do	—	—	44(26)	28(17)	88(52)	8(5)
Child's hearing	—	—	26(15)	25(15)	109(65)	8(5)
Child's speech & language	—	—	52(31)	22(13)	83(49)	11(7)
Child's learning or school performance	—	—	29(17)	34(20)	77(46)	28(17)

NA, not applicable

Table 4. Changes reported by caregivers a median of 2.0 years after tympanostomy tubes

| | "Much better" response, n (%) | | | |
| | At-risk | Not at-risk | Odds ratio | P |
Survey item	n=93	n=75	(95% CI)	value
Child's life overall	82(88)	68(91)	.77(.28, 2.09)	.603
Things child can do	54(58)	34(45)	1.67(.90, 3.08)	.100
Child's hearing	66(71)	43(57)	1.82(.96, 3.45)	.066
Child's speech & language	61(66)	22(29)	4.59(2.38, 8.85)	<.001
Child's learning or school performance	54(58)	23(31)	3.13(1.65, 5.94)	<.001

CI, confidence interval

Table 5. Multiple logistic regression for "much better" response for speech and language*

Variable	Coefficient (SE)	Wald X^2	P value	Odds ratio (95% CI)
Gender, female vs. male	-.59(.38)	2.45	.117	.56(.27, 1.16)
Age, years	-.17(.11)	2.57	.109	.84(.68, 1.04)
OM duration, months	-.01(.02)	.68	.409	.99(.96, 1.02)
Hearing in better ear[†]	.01(.02)	.29	.591	1.01(.97, 1.05)
Respondent, father vs. mother	-.98(.56)	3.01	.083	.38(.13, 1.14)
At-risk status, yes vs. no	1.63(.38)	18.48	<.001	5.11(2.43, 10.75)
Constant term	-.11(1.21)	.01		

CI, confidence interval; OM, otitis media; SE, standard error

*Overall model chi-squared 36.6, df=6, P<.001; R^2=.284; goodness of fit, P=.439

[†]Pure-tone average at 500, 1000, and 2000 Hz in sound field, or for the better-hearing ear

Table 6. Multiple logistic regression for "much better" response for learning and school performance

Variable	Coefficient (SE)	Wald X^2	P value	Odds ratio (95% CI)
Gender, female vs. male	-.04(.35)	.01	.908	.96(.48, 1.92)
Age, years	-.01(.09)	.01	.976	1.00(.83, 1.19)
OM duration, months	-.01(.01)	.48	.488	.99(.96, 1.02)
Hearing in better ear[†]	-.01(.02)	.02	.892	1.00(.97, 1.03)
Respondent, father vs. mother	-.60(.53)	1.32	.251	.55(.20, 1.53)
At-risk status, yes vs. no	1.26(.36)	12.32	<.001	3.53(1.75, 7.13)
Constant term	-1.11(1.17)	.92		

CI, confidence interval; OM, otitis media; SE, standard error

*Overall model chi-squared 15.3, df=6, P=.018; R^2=.127; goodness of fit, P=.868

[†]Pure-tone average at 500, 1000, and 2000 Hz in sound field, or for the better-hearing ear

References

1. Hall MJ, Lawrence L. Ambulatory surgery in the United States, 1996. Advance Data National Center for Health Statistics; 1998:300:7.
2. Gebhart DE. Tympanostomy tubes in the otitis media prone child. Laryngoscope 1981; 91:849-66.
3. Gonzalez C, Arnold JE, Woody EA, et al. Prevention of recurrent acute otitis media: chemoprophylaxis versus tympanostomy tubes. Laryngoscope 1986; 96:1330-1334.
4. Mandel EM, Rockette HE, Bluestone CD, Paradise JL, Nozza RJ. Myringotomy with and without tympanostomy tubes for chronic otitis media with effusion. Arch Otolaryngol Head Neck Surg 1989; 115:1217-1224.
5. Casselbrant ML, Kaleida PH, Rockette HE, et al. Efficacy of antimicrobial prophylaxis and of tympanostomy tube insertion for prevention of recurrent acute otitis media: results of a randomized clinical trial. Pediatr Infect Dis J 1992; 11:278-286.
6. Mandel EM, Rockette HE, Bluestone CD, Paradise JL, Nozza RJ. Efficacy of myringotomy with and without tympanostomy tubes for chronic otitis media with effusion. Pediatr Infect Dis J 1992; 11:270-277.
7. Gates GA, Avery CS, Prihoda TJ, Cooper JC Jr. Effectiveness of adenoidectomy and tympanostomy tubes in the treatment of chronic otitis media with effusion. N Engl J Med 1987; 317:1444-1451.
8. Lous J, Burton MJ, Felding JU, Ovesen T, Rovers MM, Williamson I. Grommets (ventilation tubes) for hearing loss associated with otitis media with effusion in children. Cochrane Database of Systematic Reviews 2005, Issue 1. Art. No.: CD001801. DOI: 10.1002/14651858.CD001801. pub2.
9. Rosenfeld RM, Bhaya MH, Bower CM, et al. Impact of tympanostomy tubes on child quality of life. Arch Otolaryngol Head Neck Surg 2000; 126:585-592.
10. Witsell DL, Stewart MG, Monsell EM, et al. The Cooperative Outcomes Group for ENT: A multi-center prospective cohort study on the outcomes of tympanostomy tubes for children with otitis media. Otolaryngol Head Neck Surg 2005; 132:180-188.
11. Rovers MM, Krabbe PF, Straatman H, Ingels K, van der Wilt GJ , Zielhuis GA. Randomised controlled trial of the effect of ventilation tubes (grommets) on quality of life at age 1-2 years. Arch Dis Child 2001; 84:45-49.
12. Paradise JL, Feldman HM, Campbell TF, et al. Effect of early or delayed insertion of tympanostomy tubes for persistent otitis media on developmental outcomes at the age of three years. N Engl J Med 2001; 344:1179-1187.
13. Rosenfeld RM, Culpepper L, Doyle KJ, et al. Clinical practice guideline: otitis media with effusion. Otolaryngol Head Neck Surg 2004; 130:S95-S118.
14. Rosenfeld RM. A practical classification of otitis media subgroups. Int J Pediatr Otorhinolaryngol 2005; 69:1027-1029.
15. Sices L, Feudtner C, McLaughlin J, Drotar D, Williams M. How do primary care physicians manage children with possible developmental delays? A national survey with an experimental design. Pediatrics 2004; 113:274-282.
16. Rosenfeld RM, Bluestone CD, Casselbrant ML, et al. Panel 8. Treatment, 8th Intl Symposium on Recent Advances in Otitis Media. Ann Otol Rhinol Laryngol 2005; 114(Suppl 194):114-139.
17. Robinson K, Gatehouse S, Browning GG. Measuring patient benefit from otorhinolaryngological surgery and therapy. Ann Otol Rhinol Laryngol 1996; 105:415-422.
18. SPSS Inc. SPSS 11.5 Brief Guide. Upper Saddle River, NJ: Prentice Hall Inc; 2002.
19. Ruben R. Host susceptibility to otitis media sequelae. In: Rosenfeld RM, Bluestone CD, eds. Evidence-Based Otitis Media. 2nd ed. Hamilton, Ontario: BC Decker Inc; 2003:505–514.
20. Roberts JE, Rosenfeld RM, Zeisel SA. Otitis media and language sequelae: a meta-analysis of prospective studies. Pediatrics 2004; 113:e238-e248.

"Long-term" benefits of adjuvant adenoidectomy (with ventilation tubes) in OME: the appropriate time scale(s) for evaluation

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Introduction

Otitis media with effusion (OME) is the most common cause of hearing loss and of surgical operations in children. The prevailing current treatment strategy, recommended in the U.S. but also predominantly followed in the UK, is ventilation tubes only (VTO) as a first procedure, reserving ventilation tubes plus adenoidectomy (VTA) to accompany any second set of ventilation tubes (VT) if fluid recurs after tubes fall out. This is because the role of adjuvant adenoidectomy (+ad) is set in part by issues of risk rather than purely by cost-effectiveness. There is not an evidence-based consensus about exactly who should receive adenoidectomy or when.

The complex evolution of OME histories raises difficulties for the evaluation of treatments. The natural efficacy of short- to medium-stay VTs is in the region of 6-9 months, but this is only a notional central band of stay-times, within a very wide range set by individual characteristics and tube-design factors. Even if there seems to be a clinical rationale for differing stay-times for different case-types, the issue of tube type is much less clear-cut than is widely thought. The entire intervention strategy is based on the premise that even a short period of good hearing soon makes up much of what may have been lost in a long period of bad hearing. This makes it slightly inconsistent to then argue, without strong evidence, in favour of relatively longer (i.e. middling) stay-time as a general policy. The best quality evidence suggests that the knock-on from hearing to development is in general modest, and there is reason to expect diminishing returns for increments in stay-time. The fact that many children do not require a second set of tubes is used to argue against adenoidectomy, but is equally applicable against medium-stay tubes. Hence the argument for medium-stay tubes is not highly convincing. The necessary evidence does not exist, so a trial is required. If it is ever carried out, it should include longer-term follow-up to assess whether risk of eardrum pathology increases into the zone of serious concern. Amid these uncertainties, the possible slight

further benefit from longer average tube stay times does not reduce the priority for evaluating of benefits from +ad, and its incorporation into appropriate policies. Such policies should include criteria for using +ad in two domains: (a) predictable magnitude or likelihood of benefit; (b) specific risk. Age might contribute to both.

Any randomised clinical trial (RCT) faces the issue of appropriate time scale through which to evaluate a treatment. Generally, after preliminary evidence of efficacy, possibly short-term, effectiveness trials attempt to span the period within which benefits may have reduced to the clinically trivial. In OME, the timing issues are particularly challenging because it is a variably manifesting, fluctuating and eventually resolving condition. For short-stay tubes, up to 12 months is a sensible trial duration. However there is no such clearly defined evaluation period for +ad, within which to examine the magnitude and duration of hearing and wider health benefits, both being required. The complexity is raised by the fact that +ad reduces the numbers of VT re-insertions[1], so the time over which this benefit can or should be quantified may be constrained by guidelines and current practices on clinical review and re-insertions. Early-phase RCTs may take a more limited efficacy-based short-term perspective. Planning and piloting should address the definitions of "short", "medium", and "long" timescales for the treatment(s) under evaluation. The appropriate follow-up period for an RCT must be long enough to capture a fairly complete clinical picture and to determine all benefits or risks of material public health value or concern, if these exist. On the other hand, having too long a time-frame could be counterproductive, creating practical and resource problems in realising the trial, and setting up an unrealistic scale of lasting and large benefit; against which an actual modest and short-term (though possibly worthwhile) result will appear to have a diluted treatment effect size. In the long term, all therapeutic effects can be expected to wash out biologically, in relation to variance in growth and development. In particular, as time passes, the drop-out rate increases and averages for subsamples who actually attend become so prone to attendance/attrition

bias as to be meaningless. Those attending are usually the worst cases still having a problem; and this may compress apparent magnitude of treatment effects. But attendance may also offer a methodological solution, as we later show.

The literature on +ad to date has taken some account of these principles. Gates et al[2] followed up 254 children aged 4-8 yrs for 2 years. VTA reduced time with effusion (26% of visits vs 35%) and halved the need for further surgery (14% vs 28%). Maw[1] followed up 222 children aged 3-9 yrs for up to 10 years. At 5 yrs, VTA had half the re-treatment rate of VTO: 34 vs 68%. At 10 years mean numbers of VTs needed were 1.5 and 2.5, respectively; +ad also appeared to benefit hearing, the probable chief basis for the reduced further treatment, for up to 4 years.

There are two justifications for further examination of the time-scales over which benefits of +ad should be evaluated, and hence justifications for this methodological work: (a) the need to delineate the extent to which short-term trials are representative of long-term outcomes; and (b) the increasing importance of the public health perspective, which requires an increased emphasis on the duration of benefits even if the ideal approach of a cost-per-QALY (quality-adjusted-life-year) is not followed. These sit in the context of two justifications for further clinical research on the adjuvant adenoidectomy question: (a) the need for controlled statistical analysis with adjustment for control variables, and sufficient numbers to have statistical power to address interactions of treatment with baseline characteristics, ie evidence-based indicators; and (b)the wish to increase the total quantity of evidence and establish generality, by documenting multiple outcomes from +ad through a medium-term perspective as well as any feasible and unbiased later outcomes.

TARGET, the Trial of Alternative Regimens in Glue Ear Treatment[3,4], was designed to evaluate OME treatment over a 2-year period on children aged 3.5 to 7 years. TARGET's main entry criterion (after applying preliminary less restrictive ones for efficiency) was 20 dBHL or worse in the better-hearing ear on initial visit, and the same again after 3 months. Those qualifying and accepting randomisation (376 children) were then randomised to VTO, VTA, or further watchful waiting (FWW). A further adjunct cohort of 56 children with hearing worse than 40dBHL, where there was thought to be overriding need for treatment, were also treated and given discretionary adenoidectomy. Subject to reservations about non-randomised analysis, this group, known as "obliged to treat" (OTT), boosts the numbers for comparing VTA with VTO or VTO+FWW. Subject to slight attrition, all were followed up for 2 years. There

was spontaneous remission in about a third of FWW children; however, overall 62% of children allocated to FWW switched to a surgical treatment arm within 1 year. Where trial entry is so selective, this high switching figure questions the allegation that treatment is not needed in OME. VTO led to better hearing outcomes initially, but after 1 year there was no advantage of VTO over FWW, permitting the pooling of these arms for certain analyses, particularly when a long-term perspective for +ad is adopted.

In addition to short (6 months) and medium-term (6-24 months) scheduled follow-up, TARGET obtained long-term outcomes in an adjunct study that examined need for further treatment Post Visit 7 ("PV7"). This last scheduled visit in TARGET at randomisation plus 2 years gives the adjunct study its acronym. However, as the PV7 length of follow-up was variable (1.2-5.5 years after trial exit) and in some cases short, validation of theses data against longer follow-up is required, to ensure that PV7 fairly represents the long-term. The present further adjunct study (Leicester in focus, "LIF") analysed consultations even beyond PV7, on a subset, to assess the extent to which PV7 is representative of the longer term, to potentially corroborate results seen in PV7.Study of the distribution of further visits assists the selection of cut-off points in the number-of-visits for the logistic regression analysis (in a related future paper) on the +ad treatment outcomes.

Methods

LIF examined notes of Leicester TARGET children only, collecting data on clinic visits and operations up to 30th April 2006; the records search was performed by a single clinician (MD). The PV7 study called upon the notes of TARGET children in all participating centres, reviewing these up to the end of January 2002 through the clinicians guided by a system of data queries from the central trial data manager. LIF thus gives longer-term data, but necessarily only on a subset ofPV7. The representativeness of LIF children as a sample of all PV7 children was also addressed in terms of descriptors: age, hearing level, socio-economic group, referral source (family doctor, community audiology).

Results

The group of 432 randomised plus OTT children in TARGET was chosen as lying between a slightly larger total having baseline data plus clinical follow-up (eg some who refused randomisation), and the smaller randomised only group. Of these, 102 were enrolled in Leicester (almost one quarter of the total): 93

randomised and 9 OTT (Leicester had higher-than-average willingness to randomise children over 40dB). The above-listed descriptors and the treatment effects were similar for randomised and OTT. LIF follow-up was successful on 98/102 children, for from 5.7 to 9.5 years after trial exit, with minimum period from randomisation of 7.5 years. Characteristics of the LIF vs other TARGET children are shown in Table 1.

Examination of the number of visits captured by PV7 and the eventual total on the same children captured by LIF (Table 2), shows good correspondence between the two data-sources. On-diagonal grey shading in Table 2 locates those children where the number of visits captured by PV7 is identical to the eventual final number of visits assessed by LIF. All omissions involve visits missed by PV7 but eventually found in LIF, but most of these remain close to the total in LIF. The distribution of number of visits over time after the scheduled trial follow-up is exponential, i.e. the number of children having return visits per unit time falls roughly exponentially over time (Fig 1). There is also a slight seasonal ripple.

Overall, the mean duration of care, measured by LIF from randomisation to last clinical contact (including the TARGET 2 year period itself), was 3.81 years (range 0.27-11.03). If, unlike the tables, we also include scheduled trial visits within the prior 2-year trial follow-up, then the mean number of visits becomes 8.77 and of operations become 1.68, respectively (ranges 2-31 and 0-7). The OME caseload contains a spike in the tail of this exponential: over 15% of cases meeting entry criterion (20dBHL twice over 3 months) had 9 or more further consultations (Table 2); this lifts the average number of consultations and duration of care considerably beyond the median. However, the numbers of children receiving further visits or operation after TARGET 2-year follow-up ended is the measure subject to least ambiguity. This is because with scheduled visits, the requirement for other healthcare could be suppressed due to seamless incidental healthcare being delivered. Table 3 compares PV7 (all 432 children) versus LIF (subset of 98 children) after TARGET ended. Only one of the 37 children (1% of total, 3% of those receiving a further operation) had clearly non-ear-reasons only, four having combined reasons (Table 4).

Discussion

The overall research strategy of TARGET has adopted three periods for the outcome analyses: short (up to 6 months), medium (6-24 months), and long terms, with the 2-pronged approach set out here addressing the difficulties over long-term data. PV7 analysed all children but over a variable slightly longer term,

whereas LIF followed longitudinally the largest single-clinic subset of children over an ultra-long term that, according to known cross-sectional epidemiology, should exceed the natural history of the disease, as seen in all but exceptional cases. The overlap set forms an efficient basis to evaluate the adequacy of the PV7 data from all participating centres as describing "the long term". To give a valid picture of associations, it is not necessary for PV7 to be totally representative of case histories, eg via a very high association between methods (ie here data-sources). But it should not be seriously un-representative. The data presented here show that PV7 gives an adequate representation of histories to establish as relevant the more powerful treatment analyses available with the larger numbers in PV7. The baseline hearing levels of Leicester children are highly similar to those of PV7 children from other centres, probably as a result of the same audiometric criterion being applied for inclusion, and the overall percentage re-consulting is similar. However the samples are not identical. Given the slightly younger age and lower socio-economic group in LIF, the higher percentage of further visits or treatments is understandable, and illustrates that HL, although necessary, may not offer a comprehensive severity metric. This is probably due to the greater involvement of community paediatricians, reaching out to populations with higher needs but also exercising more of their own pre-filter on severity (including prior watchful waiting and audiometry) than GPs can.

The length of records-based follow up in PV7 was between 1.2 and 5.5 years after trial exit, because data capture ended at one point although children had been recruited over 4.3 years. PV7 analyses therefore require a statistical adjustment for the actual length of window, to adjust for the fact that a child followed up for longer has more chance of re-consulting; whatever other factors such as treatment may also determine this. In LIF, the corresponding period was from 5.7 to 9.5 years after trial exit, leaving a minimum follow-up period from randomisation of at least 7.5 years. Because of the resolving natural history of OME, differences beyond 7.5 years (i.e. minimum age 3.5 + 7.5 yrs follow-up = 11 years of age) are trivial, so adjustment is not needed within LIF (and the duration term was not statistically significant when tested). In fact, LIF shows that by 5.5 years after trial exit, more than 90% of visits that were going to happen have already happened (Fig 1).Due to the differing durations, LIF inevitably captures more visits per case than PV7. Despite this, PV7 captured 74.49% of cases adequately, adequacy being here defined as PV7 having either the same number, or no more than 1 fewer than LIF. On this definition, 9/25 cases with some visits not captured in PV7 involve a shortfall of

only 1. As defined by numbers of children needing any further visits, PV7 captured 96.94% of case histories accurately (3 patients had no further visits recorded in PV7 but were found to have had at least one in LIF). It still correctly captures 93.88% of cases as measured by children needing $0/1$ vs ≥ 2 further visits (6 cases coded in PV7 as needing $0/1$ visits, but eventually requiring ≥ 2). However, where focusing on long histories, PV7 will indeed under-count.

The few cases missed by PV7 are due both to the expected undercounting from the shorter average time frame (adjustable in the analysis), and to the exceptional rigour of the LIF search (a clinician repeatedly probing all available record sources within the hospital — an effect not adjustable without "correcting" the data). In general we would expect "corrected" data to better show trends of a biologically predicted type, and also properly not to show any trends that are not real. However, the difference between LIF data and the adjusted PV7 data is small except for a minority of long-term cases. We did not have the opportunity to conduct rigorous supplementary searches at the centres providing the other three quarters of the trial cases. Therefore we decided not to incur the complexity of "correcting" data in this quarter of the sample, nor of adjusting values in the remaining three quarters from a predictive model with re-sampling, but just to note the slightness of shortfall and accept the present checks as general validation of the PV7 data-source.

A roughly exponential distribution is seen of number of cases, whether the counting basis is the total number of visits or the particular number of visits per 6-month period (Fig. 1). This distribution shape is the general basis of satisfactory correspondence between LIF and PV7 data. As such a distribution is not transformable to one with a central peak, multiple linear regression cannot be used, so multiple logistic regression is necessary to examine the effects of +ad, in turn requiring appropriate cut-off points for the dichotomy needed in logistic regression. The median is usually chosen as the valid measure of central tendency. Table 2 shows that the median region will be well bracketed by two analyses, one with 0 vs ≥ 1 visits, and another with 0 or 1 vs ≥ 2. These two cut-off locations span the median ($39/98$ below, $[39+10+1]/98$ above).

The vast majority of operations recorded were ear-related, with only one further procedure being entirely for non-ear reasons. Hence, and very reasonably given the entry criteria used here, it cannot be argued that VTO children are likely to have more later procedures than VTA merely on the basis that a proportion will have adenoid tissue that may be thought to require removal for a different, non-ear reason. A main contributor to removal of this ambiguity may have been the explicit pathway definition by protocol, not as seen in routine practice. In addition to the OTT category explained above, but included an additional "overriding concern" category was defined for patients severely affected by other problems, many of which could be adenoid-related, such as sore throats, nasal obstruction or sleep apnoea. These were discretionarily treated but excluded. Detailed analysis of external validity[4] has established that this poses no threat to applicability of the trial. Here we seem to see an actual advantage of exclusion to narrow the trial cases with respect to the clinical caseload. The clinical and reasonable allocation of +ad to such (here excluded) children would have undermined equipoise, hence the numbers randomised, introducing an unnecessary threat to a trial primarily about OME.

For long term outcomes, clinical records allow the efficient capture of affordable and relevant data. They offer information that may not otherwise be available if parents were recalled for a further visit. Parents may not respond to a recall, and those that attend are likely to be the ones that have ongoing problems. Both records and recall strategies will miss children that have moved away, although this is not a serious (biasing) form of attrition: it is unlikely that children from one randomised treatment arm are more likely to move than those from another. Record-based follow-up reflects problems for which children attended hospitals with, but not any problems managed in the community; for health-economic studies those have to be captured separately. It is thus an effectiveness measure having (incomplete) economic implications, one that complements the trial outcomes measured within formal scheduled assessment.

Conclusions

(1) Records-based follow up allows affordable and efficient capture of broadly based long-term outcomes in children with OME.

(2) Intensive records-based follow-up on a sub-sample showed that the shorter and necessarily variable-length variable period of records used in the PV7 data-source for all TARGET children is generally valid (assuming further precision via adjustment for length in analyses).

(3) The roughly exponential distribution that guarantees (2) entails that the two appropriate dichotomisation points for treatment analyses fall just below any or 2+ further consultations. Extreme caution should be exercised when reporting averages.

	Leicester (102)	Other centres(330)
Route of referral: Numbers (%)		
Community	68 (66.67)	150 (45.46)
General practitioner (GP)	2 (1.96)	126 (38.18)
Missing	32 (31.37%)	54 (16.36)
Socio-economic group: N & %		
Non-manual	28 (27.45)	117 (35.46)
Manual/unemployed	73 (71.57)	208 (63.03)
Missing	1 (0.98)	5 (1.52)
Characteristics at randomisation		
Mean age months (SD)	60.28 (10.88)	63.67 (10.22)
Average HL (SD)	34.50 (5.84)	34.74 (6.10)
Randomised allocation: N & %		
FWW	31 (30.39%)	91 (27.58%)
VTO	33 (32.35%)	121 (36.67%)
VTA	38 (37.26%)	118 (35.76%)
Outcomes assessed by PV7		
Mean N clinic visits+operatons (SD)	1.97 (2.48)	1.59 (2.23)
Mean N operations (SD)	0.43 (0.78)	0.20 (0.48)

Table 1: Characteristics of Leicester TARGET children compared with non-Leicester children.

	LIF											
PV7	0	1	2	3	4	5	6	7	8	9	≥10	TOTAL
0	39	1	1	0	0	1	0	0	0	0	0	42
1	0	10	2	1	0	0	1	0	0	0	0	14
2	0	0	5	1	0	0	1	0	0	0	2	9
3	0	0	0	6	2	2	0	0	0	2	0	12
4	0	0	0	0	2	0	1	1	0	1	0	5
5	0	0	0	0	0	2	1	0	2	1	1	7
6	0	0	0	0	0	0	0	1	0	0	1	2
7	0	0	0	0	0	0	0	0	0	1	2	3
8	0	0	0	0	0	0	0	0	0	0	2	2
9	0	0	0	0	0	0	0	0	0	0	1	1
≥10	0	0	0	0	0	0	0	0	0	0	1	1
TOTAL	39	11	8	8	4	5	4	2	2	5	10	98

Table 2: Correspondence between the number of visits per child captured in PV7, and the number for the same child captured in LIF. Above-diagonal entries show greater capture by LIF.
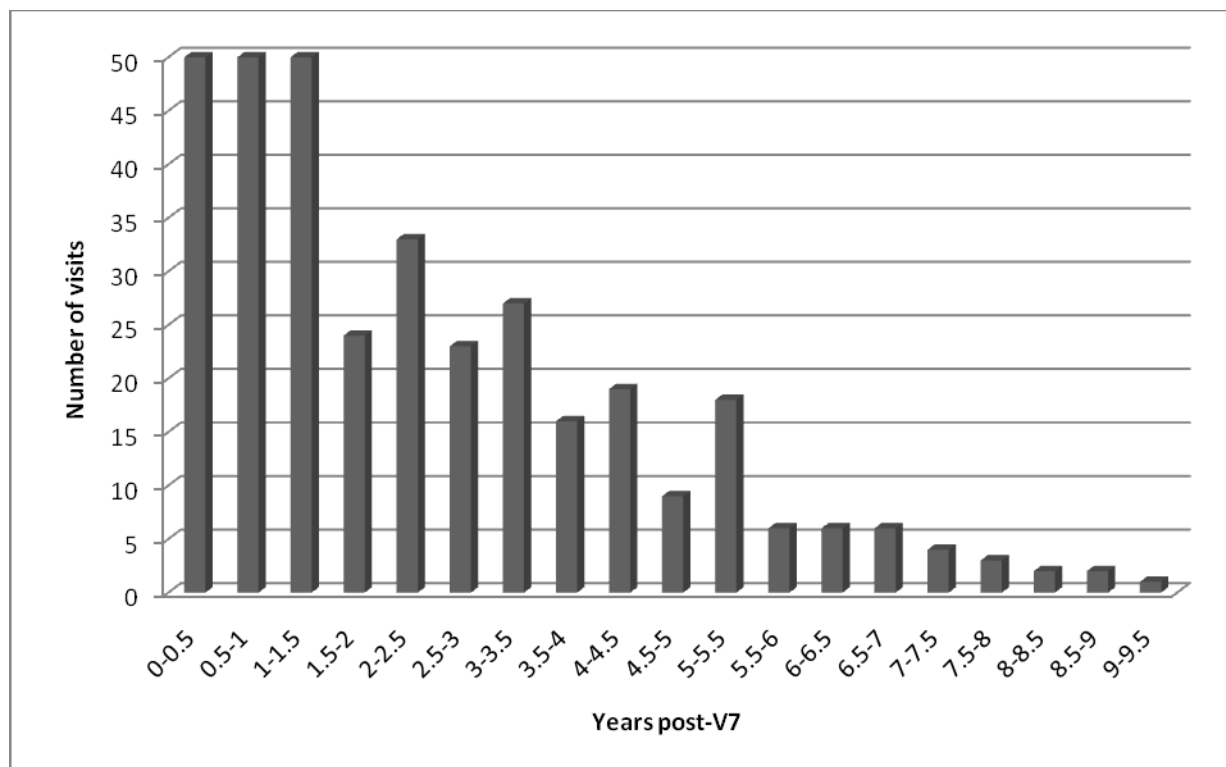
Figure 1: Total number of visits taking place during each 6 month period after V7.

Numbers (%) receiving further ENT care	PV7 (N=432)	LIF (N=98)
Clinic or surgery after TARGET visit 7	231 (53.47)	60 (61.22)
Clinic after TARGET visit 7	229 (53.01)	59 (60.20)
Surgery after TARGET	88 (20.37)	37 (37.76)
Clinic or surgery after 6 yrs	-	19 (19.39)
Clinic after 6 yrs	-	18 (18.37)
Surgery after 6 yrs	-	9 (9.18)

Table 3: Numbers (percentages) of children needing further treatment after TARGET visit 7, comparing the activity recorded in PV7 and LIF studies. Any ENT operation was counted.

NUMBER of PATIENTS	PROCEDURES	INDICATIONS
1	Adenotonsillectomy	Recurrent tonsillitis, snoring, nasal obstruction
1	VTA plus submucous diathermy to inferior turbinates	OME, nasal obstruction
3	VTA plus tonsillectomy	OME, recurrent tonsillitis

Table 4: Patients in LIF having non-ear operations, or no
n-ear indications for bivalent surgery.

References

1. Maw AR, Bawden R. The long term outcome of secretory otitis media in children and the effects of surgical treatment: a ten year study. Acta ORL Belg 1994;48:317-324.
2. Gates GA, Avery CA, Prihoda TJ, et al. Effectiveness of adenoidectomy and tympanostomy tubes in the treatment of chronic otitis media with effusion. New Engl J Med 1987;317:1444-1451.
3. MRC Multi-Centre Otitis Media Study Group. Risk factors for persistence of bilateral otitis media with effusion. Clin Otolaryngol 2001;26:147-156.
4. MRC Multicentre Otitis Media Study Group. Surgery for persistent otitis media with effusion: generalisability of results from the UK trial (TARGET). Clin Otolaryngol 2001;26:417-424.

Tube-associated otorrhea in children with recurrent otitis media--results of a prospective randomized treatment study

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Fifty infants, not older than 3 years, with tympanostomy tubes as prophylaxis for recurrent acute otitis media (RAOM) were included in a prospective randomized study of two different treatments of otorrhea. One group had topical treatment in cases of otorrhea and the other group had topical treatment combined with systemic antibiotics. The study period was 6 months per child. The aim of the study was to establish if there is a clinically significant difference between a topical treatment alone and systemic antibiotics in combination with topical treatment for otorrhea. The aim was also to determine the bacteriology of otorrhea in this group in order to assess whether otorrhea episodes were a symptom of acute otitis media (AOM).

In total, 51 episodes occurred and were analyzed. A vast majority of the cases were cured within 7 days. We found no statistically significant difference between the two study groups. Bacterial cultures from the draining ears showed bacteria originating from the upper airways in all positive cultures. Although topical treatment alone often seems to suffice as treatment, we also noted that systemic antibiotics had to be added in about 30% of the episodes in the topical treatment group, due to fever and general malaise. The study results indicate that otorrhea episodes in this group of RAOM children is similar to AOM. Cultures from ear discharge is recommended, and a watchful approach if you choose to use topical treatment alone.

Effect of round window membrane application of dornase alfa on cochlear function

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Introduction

Otitis media (OM) is one of the most common diseases, especially in children. Two of the most important factors in the pathogenesis of OM with effusion (OME) are infection and eustachian tube dysfunction. Other factors include allergy and barotraumas. These factors stimulate inflammatory cells and the middle-ear mucosa to secrete inflammatory mediators (IMs) and enzymes in the middle-ear (ME) cavity resulting in the development of OME. The composition of the middle-ear effusion (MEE) reflects the degree of inflammatory process taking place in the ME cavity. Treatment options of OME include trial of antibiotics and tympanostomy tube (TT) insertion. Myringotomy and tympanostomy tube insertion is the treatment of choice for chronic or recurrent OME. For most patients, TTs will take care of OME until the tube extrudes. However, a complication of TT placement is otorrhea and MEE that can cause them to plug. The frequency of otorrhea has been noted to involve 74.7% of the population with a mean of 2.17 episodes 12 months after TT insertion.[1] The key in treating clogged TT is to understand the make-up of the blockage material itself. Reid et al.[2] reported that the lumen of plugged tubes consists of eosinophilic material with a polymorphonuclear leukocyte infiltrate in 56% of the cases. The occurrence of luminal blockage was noted to be further associated with the presence of MEE. Therefore, altering the rheological properties of the MEE may restore the function of the TT.

The failure to deal with plugged tubes successfully may in large part be due to viscous MEE that is frequently encountered behind the plug--the so-called "glue ear" that likely was encountered during the original surgical procedure. The components within MEE responsible for its viscoelasticity have been studied. Analysis of the constituents of the MEE showed that glycoprotein and DNA, but not protein nor lipid, were significantly higher in mucoid effusions compared to the serous effusions.[3] Therefore, the success in treating blocked tubes goes beyond the dissolving the material occluding the lumen of the tube. It also involves managing the mucoid MEE.

Unfortunately, a host of methods have been described to unblock clogged tubes without any scientific credence and without much success. Empirically, clinicians rely on ototopical antibiotic drops. An in vitro laboratory study showed vinegar and hyaluronidase solutions are more likely to clear plugged tympanostomy tubes than water and ototopical antibiotic drops.[4]

Dornase alfa (Pulmozyme® by Genentech), approved by the FDA since 1994 for treating cystic fibrosis (CF), offers a unique potential for treating OME. It is a sterile, clear, colorless, highly purified solution of recombinant human deoxyribonuclease 1 (rhDNase), an enzyme which selectively cleaves DNA. The rationale behind its efficacy lies in the presence of high concentrations of extracellular DNA released by degenerating leukocytes that accumulate in response to infection present in airway secretions of patients with CF. The effect of dornase alfa provides CF patients relief by making it easier to expel airway secretions and sputum by reducing its viscoelasticity aiding in the prevention of mucus plug formation. Potential effects of dornase alfa in hydrolyzing dessicated MEE rich in DNA from leukocytes exist. Off-label use of dornase alfa for the treatment of non-CF lung diseases have been reported. They include respiratory syncytial virus bronchiolitis[5], empyema thoracis[6], and plastic bronchitis.[7] In addition, the treatment of CF patients with chronic sinusitis has also been reported.[8]

Ototoxicity of dornase alfa has to be assessed prior to human application. This study aimed at evaluating the potential ototoxicity of dornase alfa by monitoring the cochlear function utilizing auditory brainstem response (ABR) and distortion-product otoacoustic emissions (DPOAEs) before and after its application on the round window membrane (RWM) using chinchillas.

Materials and methods

Healthy adult chinchillas weighing 400–600g were randomly assigned to various treatment groups, each group consisting of an equal number of male and female animals. A total of 21 adult chinchillas were used for this study. The research design consisted of three groups: saline control (five animals), full-strength dornase (eight animals), and dornase diluted, 1:10 (eight animals). Under ketamine/xylazine combination anesthesia, baseline ABR, and DPOAE recordings were taken before application of test substances on RWM. RWMs were exposed by posteroinferior approach to the bulla. Saline or test substances were soaked into the Gelfoam and applied on the RWM. Effects on hearing were monitored and recorded for both operated and non-operated (contralateral) ears hourly by ABR and DPOAE testing for up to 8 hours. Contralateral (non-operated) ears of animals were used as an additional control. After 8 hours the animals in each group were properly euthanized.

ABR measurements were taken using the Nicolet Spirit™ Evoked Potential System, Version 1.4. ABR potentials were recorded using dermal needle electrodes at the vertex and the ipsilateral mastoid portion of the bulla. Responses were amplified, bandwidth filtered (10 kHz), and averaged (1125 stimulus presentations per trial). Stimuli consisted of broad band (150 to 1500 Hz) clicks generated by alternating polarity of 100 μsec pulses to a closed-field ear probe at a rate of 33.3/sec. Measurements were made in 5 dB decreasing increments. The hearing threshold was determined by the lowest auditory stimulus that produced detectable and reproducible ABR waveforms. Otoacoustic emissions were recorded by Grason-Stadler's GSI 60™ DPOAE System in a sound-proof audiobooth.

Results

ABR measurements showed that hearing deterioration was at its maximum 6 hours after dornase alpha application on the RWM for all groups. The mean hearing losses for full-strength dornase and dornase 1:10 were 32±2.9 dB and 13±0.36 dB, respectively. Hearing loss was not significantly different for either dornase [full-strength, $p=0.14$] or dornase [1:10, $p=0.20$] when compared to the saline control (24 ± 2.1 dB), $p>.05$. DPOAE data for hour 2 were compared to saline control at low (875 Hz), middle (2531 Hz), and high (4000 Hz) frequencies yielding statistically non-significant results ($p>.05$).

Discussion

As done in previous animal studies, RWM application of the test drug for ototoxicity screening provides one of the best direct tests of ototoxicity. In this case, dornase alfa was tested at full strength and at 1:10. Both of these were compared to the saline control group delineating the lack of ototoxic effects using the chinchilla model.

Conclusion

Dornase alfa is deemed non-ototoxic when tested by RWM application method in chinchillas. Therefore, it is most likely safe to test its effects on clogged tympanostomy tubes in humans.

Acknowledgement

References

1. Ah-Tye C, Paradise JL, Colborn DK. Otorrhea in young children after tympanostomy-tube placement for persistent middle-ear effusion: prevalence, incidence, and duration. Pediatrics 2001; 107:1251-1258.
2. Reid AP, Proops DW, Smallman LA. Why do tympanostomy tubes block? Clin Otolaryngol Allied Sci 1988; 13:279-283.
3. Carrie S, Hutton DA, Birchall JP, Green GG, Pearson JP. Otitis media with effusion: components which contribute to the viscous properties. Acta Otolaryngol 1992; 112:504-511.
4. Westine JG, Gainnoni CM, Gajewski B, Antonelli PJ. Opening plugged tympanostomy tubes. Laryngoscope 2002; 112(8 Pt 1):1342-1345.
5. Nasr SZ, Strouse PJ, Soskolne E, Maxvold NJ, Garver KA, Rubin BK, Moler FW. Efficacy of recombinant human deoxyribonuclease I in the hospital management of respiratory syncytial virus bronchiolitis. Chest 2001; 120:203-208.
6. Simpson G, Roomes D, Reeves B. Successful treatment of empyema thoracis with human recombinant deoxyribonuclease. Thorax 2003; 58:365-366.
7. Manna SS, Shaw J, Tibby SM, Durward A. Treatment of plastic bronchitis in acute chest syndrome of sickle cell disease with intratracheal rhDNase. Arch Dis Child 2003; 88:626-627.
8. Raynor EM, Butler A, Guill M, Bent JP III. Nasally inhaled dornase alfa in the postoperative management of chronic sinusitis due to cystic fibrosis. Arch Otolaryngol Head Neck Surg 2000; 126:581-583.

Effects of TNF-alpha antagonist, PAF antagonist and NOS inhibitor on experimental otitis media with effusion

Dong-Hyun Kim, Yong-Soo Park, M.D., Eun-ju Jeon, M.D., Sang-Won Yeo, M.D., Ki-Hong Chang, M.D.

Objectives

Tumor necrosis factor(TNF)-¥á antagonist , platelet activating factor(PAF) antagonist and nitric oxide synthase(NOS) inhibitor have been reported as effective for suppressing otitis media with effusion(OME). However there has been no systemic comparative study on the treatment and prevention of OME. We studied the inflammatory responses in OME induced by lipopolysaccharide (LPS) in rats, and compared the preventive effects of the TNF-¥á antagonist(TNF soluble receptor type I; sTNFRI), PAF antagonists(A-85783), and NOS inhibitor(N(G)-nitro-L-argine methyl ester; L-NAME).

Methods

We used 2 control groups of Sprague Dawley rats (untreated and saline- treated) and 4 experimental groups, which all received an intratympanic injection of LPS, followed in 3 groups by experimental treatment in the same ear. The LPS group had no additional treatment; the L-NAME group received intraperitoneal injection of L-NAME and reinjected after 12 hours. The A-85783 group was first given an intraperitoneal injection of A-85783, and the sTNFRI group was first given an intratympanic injection of sTNFRI. Twenty-four hours after the initial intratympanic injection of LPS, temporal bones from each group were examined histopathologically and vascular permeability of the middle ear mucosa was measured by Evans blue vital dye staining.

Results

The L-NAME, A-85783 and sTNFRI groups showed significantly reduced capillary permeability, subepithelial edema, and infiltration of inflammatory cells in comparison with the LPS group. There were no differences in capillary permeability, subepithelial edema, or infiltration of inflammatory cells between the A-85783 and sTNFRI groups. The L-NAME group showed no difference in vascular permeability or subepithelial edema comparison with the A-85783 and sTNFRI groups, but showed more infiltration of inflammatory cells.

Conclusions

We concluded that sTNFRI, A-85783 and L-NAME can be proposed as alternative future treatments for otitis media with effusion. However, L-NAME may be least effective of these agents.

Myringotomy by radiofrequency microfiber technique

Klaus Vogt, M.D., D.D.S., Ph.D.

Background

Ventilation of the tympanic cavity is the most important principle in treating secretory otitis media. In cases where cleansing of the nasopharynx and drum ventilation are performed simultaneously, healing time of the incision can be prolonged, thus avoiding paracentesis and/or insertion of a ventilation tube. Similar problems may exist in chronic serous otitis media in adults. Radiofrequency-myringotomy (RF-MT) accomplishes ventilation by acreating a self-healing perforation. The patency of the perforation is dependent on the reestablishment of tubal function as well as on the size of the artificial perforation.

Objectives

RF-myringotomy and CO_2-lasermyringotomy create a longer lasting drum perforation than a "cold" myringotomy. Because of the size of the instruments, RF-MT surgery is difficult to perform in ears with a small-diameter external auditory meatus. The development of a new "microfiber-technique" (Ellman, STORZ) leads to a greater precision in performance. RF-MT was refined by developing a microfiber of 0.5mm (Ellman Intl.) with a combination of suction and a guidance instrument (Karl Storz GmbH.(Fig.1). The instrument consists of two parallel tubes. The upper one guides the microfiber, which is fixed at the distal end to prevent movement; the lower one provides the removal of smoke. The instrument can be used not only for myringotomies but also for the removal of middle ear polyps and for the coagulation of small vessel during middle ear surgery. Another way to utilize RF-MT is to combine the microfiber with a small laserendoscope.

Figure 1
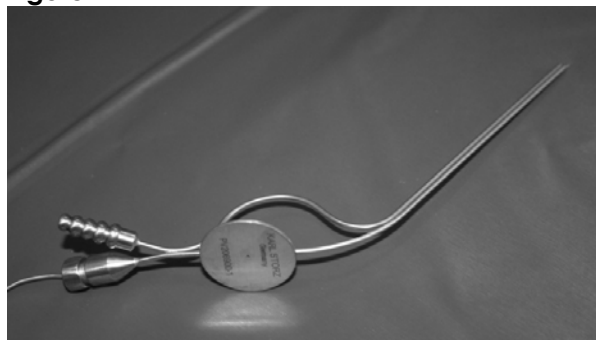
Figure 1
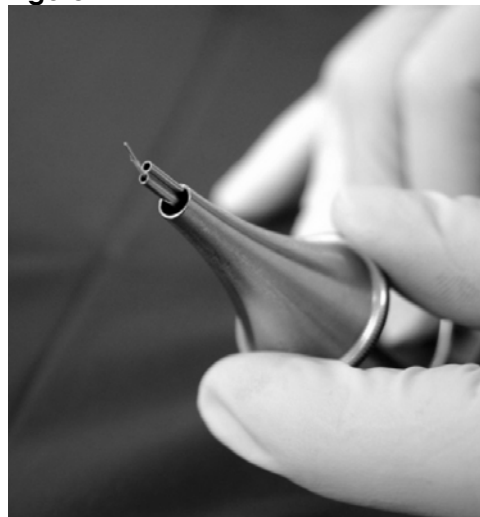
Methods and Patients

Using this technique, 122 myringotomies. In 78 children were performed. A setting of 8 (Surgitron IEC II, cutting mode) was found to be the optimum to deliver the energy. Fifty-nine children have been treated by additional adenoidectomy and/or RF-tonsillectomy.

Results

The closure time after RF-MT was between 5 and 28 days, in some cases with effusion of the middle ear. Antibiotics were given in only 12 cases. Impairment of inner ear function was not observed. At this time, a statistically significant relation between the size of the perforation and the duration of the perforation cannot be determined.

Discussion

The results are comparable with COHEN (2-3weeks), SILVERSTEIN (3-14 weeks) or SEDLMAYER (1-7weeks), performing a "hot" myringotomy by using lasers. (see references 1-3). A still longer-lasting perforation can be achieved by the topical application of Mitomycine C (RAGAB et al. (3), 5-7-weeks) before RF-MT or 5-Fluorouracile (KANEMARA, NAKANMURA (4). The authors reported an opening time of 7 days after "cold" myringotomy and 20 days after combination with 5-fluorouracile treatment.

Conclusion

RF-Myringotomy is a safe and cost-effective procedure to generate a drum perforation with longer persistence. Particularly in cases where a causative obstruction in the pharynx was removed, use of RF-MT avoided the need for drum tubes.

Clinical practice guideline for diagnosis and management of acute otitis media in children in Japan

Subcommittee on Management of Acute Otitis Media, Otological Society of Japan, Japan Society for Pediatric Otorhinolaryngology, Japan Society for Infectious Diseases in Otolaryngology

Introduction

Bacteriological conditions and clinical practice differs between Japan and other countries, but a clinical practice guideline for acute otitis media (AOM) had never been developed in Japan. Recently, an evidence-based clinical practice guideline for uncomplicated AOM in children under the age of 15 years, intended for use by otorhinolaryngologists, has been developed in Japan.

Methods

A committee was convened in cooperation with Otological Society of Japan, Japan Society for Pediatric Otorhinolaryngology, and Japan Society for Infectious Diseases in Otolaryngology. A systematic literature search was performed and pathogens from patients with AOM in Japan were reviewed. The recommendation was proposed according to GRADE (Grades of Recommendation, Assessment, Development, and Evaluation) and the guideline was described based on Conference on Guideline Standardization (COGS).

Level of evidence

Ia: Meta-analysis (with homogeneity) of randomized controlled trials
Ib: At least one randomized controlled trial
IIa: At least one well designed, controlled study but without randomization
IIb: At least one well designed, quasi-experimental study
III: At least one well designed, non-experimental descriptive study (e.g. comparative studies, correlation studies, case studies)
IV: Expert committee reports, opinions and/or experience of respected authorities

Level of recommendation

A: Strongly recommended, B: Recommended, C: Not recommended, D: Recommended against, I: Undetermined

Results

Definition, diagnosis and grading of the severity of AOM judged by eardrum findings and clinical signs and symptoms were addressed, and recommended treatments to each grade were established.

Definition

AOM is defined as an acute inflammation in the middle ear sometimes accompanying otalgia, fever and/or otorrhea.

Diagnosis and Examinations

(1) For the diagnosis of AOM, detailed inspection of the eardrum is indispensable, and AOM is diagnosed when the following findings were observed on the eardrum (recommendation B).
    Hyperemia, protrusion, diminution of light reflex, thickening, formation of bulla, cloudiness, perforation, collection of effusion, otorrhea, edema of tympanic mucosa
(2) Scoring of severity of AOM
    * 3 points are given when < 3 years old
    Otalgia – 0, 1, 2, Fever – 0, 1, 2, Crying, bad-tempered – 0, 1,
    Hyperemia of eardrum – 0, 2, 4, Protrusion of eardrum – 0, 4, 8,
    Otorrhea – 0, 4, 8
    Mild: < =5, Moderate: – 6 to 11, Severe : >=12
(3) Tympanometry
    Tympanometry should be done after the diagnosis of AOM, and is recommended to be used to determine the presence or absence of middle ear effusion (Recommendation B, Level IIa).
(4) Sufficient history taking is recommended because it is useful to know the background and past history of the patient for inferring the drug resistance of the bacteria and disease intractability (Recommendation B, Level IIa).

Treatment

(1) Exclusively to mild AOM, it is recommended to observe the natural course of AOM without administrating antibiotics (AB) (Recommendation A, Level Ia & Ib).

(2) Efficacy of AB specifically to otalgia is unknown (Recommendation I)

(3) Recommended AB depends on bacterial resistance and severity of AOM, but the followings are often recommended. P.O.: amoxicillin (AMPC), amoxicillin/clavulanate (AMPC/CVA), cefditoren pivoxil (CDTR-PI), DIV: ampicillin (ABPC), ceftriaxone (CTRX) (Recommendation A, Level Ib).

(4) It is recommended to administer AB for 5 days and to observe its effect on the third or forth day (Recommendation A, Level Ia).

(5) Myringotomy (myr) could be a choice of treatment in the severe stage of AOM (Recommendation I).

(6) Others
  * Watchful care must be taken with young children and also with those in day care (Recommendation A, Level Ia).
  * Treatment of the nose could be a choice of treatment when the patient has a nasal disease (Recommendation I).

(7) Algorithm of treatment of AOM
    Algorithm of treatment of AOM for mild, moderate and severe cases of AOM were shown in Figures 1 to 3.
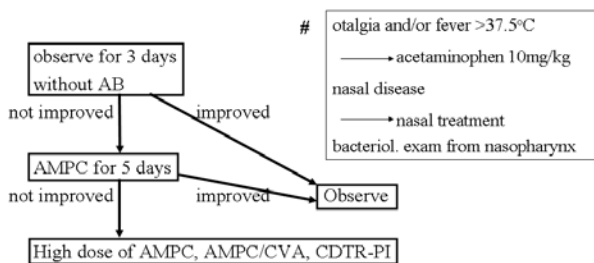
Fig. 1. Algorithm of treatment of AOM (mild case with score <=5)
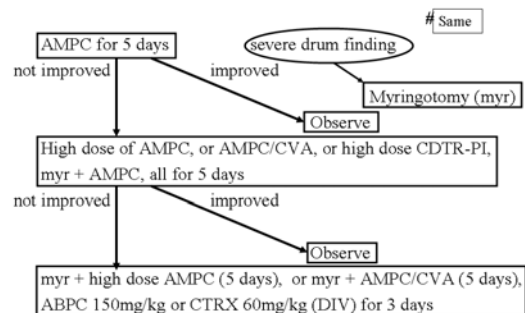
Fig. 2. Algorithm of treatment of AOM (moderate case with score 6-11)
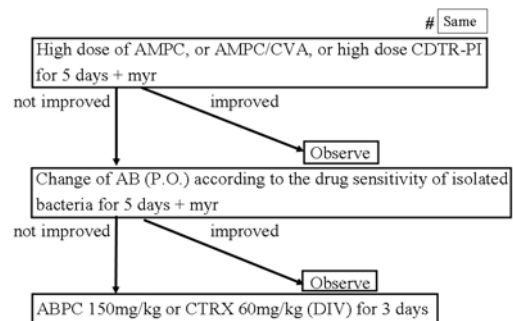
Fig. 3. Algorithm of treatment of AOM (severe case with score >=12)

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A novel peptide inhibitor of middle ear inflammation in experimental otitis media

Steven Hefeneider, Ph.D., Carol MacArthur, M.D., Dennis Trune, Ph.D., Sharon McCoy

Acute otitis media (AOM) is an inflammatory disease of the middle ear characterized by pain and hearing loss. Antibiotics treat infection and often result in symptomatic relief, but do not target inflammatory parameters such as middle ear effusion (MEE), which can cause hearing loss and other complications. Inflammation is initiated by engagement of Toll-like receptors (TLRs) on immune cells by bacterial ligands, such as LPS and PGs. We have recently identified a peptide, termed P13, which was previously shown to inhibit *in vitro* TLR-signaling and significantly reduced *in vivo* inflammation in a mouse model of AOM induced by *S. pneumoniae*. In this study, we demonstrate that this novel peptide also significantly reduces the *in vivo* middle ear inflammation and fluid accumulation in a mouse model of AOM induced by *H. influenza*. Assessment of route of administration and delayed treatment studies demonstrate the potential efficacy of peptide P13 as an anti-inflammatory therapy. Simultaneous injection of bacteria and peptide P13, administered through the TM, results in a significant reduction in fluid accumulation, infiltrating cells, and TM thickness. Fluid accumulation within the Eustachian tubes was also significantly reduced following P13 treatment. Subcutaneous and oral administrations of P13, but not intravenous administration, were also efficacious in reducing inflammation. Administration of P13 after initiation of an inflammatory response was effective at reducing inflammation and MEE. These results demonstrate the therapeutic potential of peptide P13 as a potential novel therapy for reducing inflammation and fluid accumulation in AOM.

Ribosomal therapy in the treatment of otitis media with effusion in children affected by chronic adenoiditis

Renzo Mora, M.D., Francesco Mora, M.D., Massimo Dellepiane, M.D., Barbara Crippa, M.D., Angelo Salami, M.D.

Introduction

Although the aetiology of otitis media with effusion is known to be multifactorial, adenoids infections and Eustachian tube dysfunction have been frequently associated with the incidence of middle-ear effusion 1). The most common bacterial pathogens involved in adenoiditis are: *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and group *A Streptococci*.[2,3]

In children with moderate or discrete adenoid hypertrophy, adenoidectomy should not be performed. In these conditions, the causes of possible nasal obstructions are usually due to either dysmorphic, allergic or phlogistic pathologies. For severe adenoid obstructions, surgery is always recommended. Moreover, the therapeutic strategy can be conditioned not only by nasal respiratory difficulties but also by frequent concomitant complications such as otitis, sinusitis, sleep apnea, etc. These disorders may be caused by both nasal obstruction and/or phlogistic problems.[3]

Therapy for otitis media with effusion is appropriate only if persistent and clinically significant benefits can be achieved beyond spontaneous resolution: meta-analysis of several randomized trials show no significant long term benefit for antihistamines, decongestants, oral steroids and antimicrobial therapy.[4,5] Adenoidectomy is not recommended for initial otitis media surgery unless a distinct indication exists, such as adenoiditis, postnasal obstruction, or chronic sinusitis.[3]

The aim of this study was to evaluate the efficacy of oral ribosomal immunotherapy in the prophylaxis of recurrent otitis media with effusion affecting children with chronic adenoiditis.

Methods

We have enrolled 60 children aged between 6 and 14 years (9 years old on average) with recurrent otitis media with effusion and chronic adenoiditis. The patients were equally divided at random into two groups (A and B).

After signing an informed consent at the beginning, at the end and six months after start, all patients underwent medical history, ENT examination, plasma levels of immunoglobulins class G, A, M (IgG, IgA, IgM), and subjective assessment of symptoms by patients' parents on the basis of a 0-4 scale (much better-much worse).

Frequency, duration, severity and social impact of adenoiditis episodes were also adopted asvalidcriteria. At each control the following parameters were checked: number of episodes (1, 2 or> 2), duration (<3 /3-6/ > 6 days), fever (yes/no), medical consultation (yes/no), disease frequency (once / twice / > twice per month), ancillary therapy(none /symptomatic only / antibacterials)and absence from school (none /1-3 / > 4 days).

On the basis of previous testing, group A children underwent ribosomal prophylaxis with Immucytal (1 tablet daily, 8 days a month for 3 months), while group B received a placebo (same dosage for the same period) (6-8). In order to maintain double blind conditions, Immucytal and placebo tablets were identical in shape and size.

Results

Serum concentration of immunoglobulins was significantly higher in the Immucytal recipients compared to group B when measured after 3 months (IgG and IgA; $P>0.05$) and at the end of the study (IgG and IgA; $P>0.01$). There was a moderate increase of IgM titers in group A compared to group B, which nonetheless did not reach statistical significance.

At the end of the study, each patient treated with Immucytal (group A) presented a subjective decrease of symptoms. The mean value on the subjective evaluation scale fell from 3.8 before the treatment to 1.8 after it, while in group B the same value fell from 3.9 at the beginning of the treatment to 3.1 at the end of it.

Compared with the placebo patients, the Immucytal recipients experienced a significant improvement of some clinical parameters. From the second visit (i.e. at the end of the therapy) the reduction of the infection episodes in group A proved

to be more significant compared to group B (0.68 versus 1.67; P<0.02). At the end, a significant improvement was also observed as far as the incidence of fever (P<0.02), duration of episodes (P<0.05), ancillary therapy (P< 0.01) and school absence (P<0.05) were concerned. No significant difference was found for medical consultation, which decreased in both groups. No patient experienced side effects from the treatment.

Comparisons of groups were made by the unpaired t-test and correlations were analyzed by regression analysis. Probability values < 0.05 were regarded as significant.

Discussion

Immucytal contains both proteoglycans from Klebsiella pneumoniae and ribosomes from four different bacterial strains (K. pneumoniae, Streptococcus pneumoniae, Haemophilus influenzae, and Streptococcus pyogenes A). Because of the presence of proteoglycans and ribosomes from four frequently encountered bacterial strains, the drug has specific immunostimulant properties. It can stimulate both the nonspecific immune response and the specific antibody production.[6-8]

Our data confirm that oral immunization generates a rapid and lasting immune response, building increased numbers of memory cells that are readily available to respond to further challenges by either more ribosomal preparations or potential pathogens.

With the emergence of resistant strains and the change in the distribution of rhinopharyngeal bacteria over the time, preventive strategies such as ribosomal immunotherapy may represent a valid alternative approach: several studies demonstrated that immunoglobulins influence the colonization of pharynx bacteria and highlighted their inhibitory effects against pharyngeal colonization.[9,10]

The results obtained in the present study are in agreement with the findings of the other principal investigations of ribosomal immunotherapy and support Immucytal use in the prophylaxis for recurrent otitis media with effusion in children affected by chronic adenoiditis.[6-8]

References

1. da Costa JL, Navarro A, BrancoNeves J, Martin M. Otitis medias with effusion: association with the Eustachian tube dysfunction and adenoiditis. The case of the Central Hospital of Maputo. ctaOtorrinolaringolEsp 2005;56:290-294.
2. Prellner K, Kahlmeter G, Marchisio P, Giebink SC. Update of microbial problems in pediatric otorhinolaryngology. Int J Pediatr Otorhinolaryngol1999;49S:31S-35S.
3. Ilki A, Ulger N, Inanli S, et al. Microbiology of sinusitis and the predictive value of throat culture for the aetiology of sinusitis. Clin Microbiol Infect 2005;11:407-410.
4. Levy SB. The Antibiotic Paradox. How the Misuse of Antibiotic Destroys Their Curative Powers. Cambridge, MA: Perseus Publishing; 2002.
5. Plasschaert AIO, Rovers MM, Schilder AGM, Verheij TJM, Hak E. Trends in doctor consultations, antibiotic prescription, and specialist referrals for otitis media in children: 1995-2003. Pediatrics 2006; 117:1879 -1886.
6. Mora R, Barbieri M, Passali GC, et al. A preventive measure for otitis media in children with upper respiratory tract infections. Int J Pediatr Otorhinolaryngol 2002;63:111-118.
7. Mora R, Ralli G, Passali FM, et al. Short ribosomal prophylaxis in the prevention of clinical recurrence of chronic otitis media in children. Int J Pediatr Otorhinolaryngol 2004;68:83-89.
8. Mora R,Dellepiane M, Crippa B, Salami A. Ribosomal therapy in the prophylaxis of recurrent pharyngotonsillitis in children. Int J Pediatr Otorhinolaryngol 2007;71:257-261.
9. Swidsinski A, Gogdas O, Bessler C, et al. Spatial organization of microbiota in quiescent adenoiditis and tonsillitis. J Clin Pathol 2007;60:253-260.
10. Kurono Y, Shimamura K, Shigemi H, Mogi G. Inhibition of bacterial adherence by nasopharyngeal secretions. Ann Otol Rhinol Laryngol 1991;100:455-458.

Otological flogosis and intradermal injection

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Acute otitis media (AOM) is one of the most common otological illnesses which affects the middle ear, presenting with a rapid onset of local, loco-regional and/or systemic symptoms and requiring a prescription for antimicrobial and anti-inflammatory agents.[1,2,3] The data reported recently by the Centers for Disease Control and Prevention show that there are an estimated 24.5 million visits made per-year to office-based physicians in the United States at which the principal diagnosis was otitis media–nearly one visit for every 10 people. These visits accounted for 3.5 percent of all office visits and represented the second most frequent illness diagnosis. For children under age 15, AOM represented the most frequent diagnosis in physician office practices. Since 1975, the first year these data were collected, the number of AOM visits has increased by almost 150 percent and the annual visit rate has more than doubled.[4]Children experience an average of two to three episodes a year, almost always accompanied by a viral upper respiratory infection. This inflammation often begins when infections that cause sore throats, colds, or other respiratory or breathing problems spread to the middle ear. The infection of the eustachian tube causes swelling and compromises the pressure equalization, which is the normal function of the tube. In general, the more severe and prolonged the compromise of eustachian tube function, the more severe the consequences are to the middle ear. In people with a poor eustachian tube function, there is a greater increase of the likelihood of more frequent and severe episodes of otitis media. Progression to chronic otitis media is much more common in this group of people, who often have a family history of middle ear disease. The main bacteria causing acute otitis media are Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis and Streptococcus pyogenes.[5] The AOM may be recurrent, when the episode of flogosis occurs every two months, with at least 6 months of evolution history, or persistent, when the microbial agent is the same, both in the first and in the second episode.

An oral high-dose of antibiotics (80 to 90 mg/kg/d of amoxicillin, divided twice daily, for five to seven days) remains first-line therapy for uncomplicated acute otitis media, despite increasing antimicrobial resistance. For persistent or recurrent acute otitis media, guidelines recommend high-dose amoxicillin/clavulanate (90/6.4 mg/kg/d), cefdinir, cefprozil, cefpodoxime, cefuroxime, or ceftriaxone). Increasing the dose of amoxicillin does not cover an infection with beta-lactamase-producing pathogens; it is necessary to add the beta-lactamase inhibitor clavulanate to amoxicillin, or to choose a cephalosporin with good activity against S. pneumoniae and good beta-lactamase stability. For clinical treatment failures after 3 days of amoxicillin, recommended antimicrobial agents include oral amoxicillin/clavulanate, cefuroxime axetil, cefprozil, cefpodoxime proxetil, and intramuscular (i.m.) ceftriaxone.[6,7,8] The administration of a corticosteroid in association with the antibiotic improves the resolution of AOM. The contribution of the corticosteroid in achieving a 20% a reduction in time to cessation of otological flogosis is clinically meaningful and represents an important advance over single-agent antibiotic therapy.[2,9,10]

These guidelines of systemic therapy are often inadequate or insufficient and may lead to the persistence of otalgia and of signs of flogosis. The choice of an alternative medical approach is emphasized by the need to obtain real clinical efficacy, avoiding side effects, failure of the therapy and the recurrence and the persistence of the flogosis. Moreover it is of great importance to reduce the dose of the drug, the frequency and the duration of the treatment.

Intradermic drug injection, performed on the skin projection of the organ or part of the damaged organ, allows longer drug permanence in the treatment area and greater bioavailability of the active substance which is transferred directly to the tissue and does not go through the hepatic filter and, therefore, is not partly inactivated. The high drug concentration obtained in this way in the tissue where the pathology is located increases the pharmacological effect, thus leading to greater rapidity and efficacy of action and avoiding side effects. The intradermic therapeutic approach produces two different types of effects. The first is a mechanical "physical" stimulation given by the needle, which is able to activate different cutaneous and subcutaneous receptors with induction of segmentary reflex effects; the second one is the local action of the drugs, given in small doses, which is achieved through both a chemical mechanism and mechanical extension of the tissues, on the peripheral receptors, on the preterminal tracts of the sensorial fibres and on the modulatory efferent fibres.[11-18] This

method was officially proposed by Pistor: he verified that "the introduction of a drug injected on the surface, near the pathology, gives quite superior and much more favourable results than if the same drug is given in a systemic way".[19] Since 1974 he has utilized this technique for the therapy of affliction affecting auditory apparatus.

Intradermal therapy allows a strengthening of the pharmacological effect and a reduction in the quantity of drug used. Moreover the rhythmic repeated therapeutic sessions required by this technique allow one to establish very good doctor-patient relation, providing encouraging support.

Method

Applying the intradermal approach in case of AOM, we perform the injections on two pretragal points, two retroauricular points and one in the posterior surface of the pinna. The drugs injected are antibiotics and corticosteroids (the same used systemically); the injections are repeated at least three times at intervals of 2 days (Fig. 1, 2, 3, 4, 5). The controls required by this therapy protocol allows us to establish the moment of both complete recovery from the infection and the symptoms of the patient and so to decide whether or not to continue the therapy.

Clinical examples

Fig. 6 shows the otoscopy of an ear affected by AOM observed after 10 days of oral administration of antibiotics and corticosteroids at the usual dosage. Despite the therapy there is the persistence of the relevant signs of flogosis and the severe pain referred by the patient.

Fig. 7 shows the otoscopy of a case of AOM performed at the time of the first observation. The patient was complaining of great pain and fever.

Four days later, after 2 sessions of infiltrations, a complete recovery of the infection was established. Already after the first session of the local treatment the patient noted a great reduction in the pain and the fever broke.(Fig.8) The follow-up performed at 10, 15 and 20 days confirmed the complete recovery from the flogosis.

Conclusion

Auricular flogosis can be difficult to heal with classic systemic therapy: long-term and high dose therapies are usually necessary for clinical and symptomatic resolution of the infectious process. Moreover in some physiological (i.e pregnancy) and pathological (i.e. hepatic and renal failure) conditions, the systemic administration of high doses of antibiotics and anti-inflammatory drugs are not recommended. Long-time persistence of the infection can lead to some local as well as systemic complications.

The administration of intradermal therapy, as in the example reported above, gives excellent results. The control of pain, the most significant symptom complained of by the patient is obtained usually within the second injection. A rapid, daily improvement in the loco-regional signs of infection is typically observed. The intradermic injection of small quantities of the same drugs, which, when used systematically are in greater doses, allows a strengthening of the pharmacological effect and a reduction in the quantity of drug used; in this way a great rapidity and efficacy of action are described, avoiding side-effects. The administration of an intradermal-therapy protocol requires periodic check-ups of the patient, at intervals of two days; in this way the duration of the protocol can be decided case by case according to its effectiveness.

Intradermal therapy may be a safe alternative approach for a rapid symptomatic and clinical resolution of auricular flogosis, even when complicated.

Fig. 1   Pretragal local infiltrations

Fig. 2 Retroauricular local infiltrations

Fig. 3 Local infiltration in the posterior part of the pinna
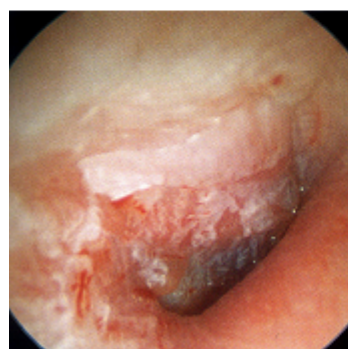
Fig. 4 Otoscopy showing the persistence

Fig. 5 Otoscopy of a case of AOM

Fig. 6 Otoscopy of the case of flogosis after 10 days of oral treatment at the moment of the first observation showed in Figure 7, four later, after two sessions of infiltration

References

1. Powers JH. Diagnosis and treatment of acute otitis media: evaluating the evidence. Infect Dis Clin North Am 2007;21:409-426, vi.
2. Aronovitz GH.Antimicrobial therapy of acute otitis media: review of treatment recommendations. Clin Ther 2000; 22:29-39.
3. Yaman H, Ozturk K, Uyar Y, Gurbilek M.Effectiveness of corticosteroids in otitis media with effusion: an experimental study. J Laryngol Otol 2007, 20:1-6.
4. Anderson JR, Mandans JH, Feldman JJ, Fisher GF, Hurley PL, Hartford RB, Nieberding SE, Rothwell CJ, Sirken MG. Vital and health statistics:advance data from vital and health statistics numbers 211-220, *Centers for Disease Control and Prevention* (1995) Available at :http://www.cdc.gov/nchs/data/series/sr_16/sr16_0 22.pdf Accessed May 16, 2007.
5. Cervera J, Villafruela MA, del Castillo F, Delgado Rubio A, Rodrigo C, de Liria G, Picazo JJ.Consenso Nacional sobre otitis media aguda. Acta Otorrinolaringol Esp2007; 58:225-231.
6. Gupta BD, Singh A.Otitis media. Indian J Pediatr2001; 68 Suppl 3:524-531.
7. Pichichero ME, Casey JR.Acute otitis media: making sense of recent guidelines on antimicrobial treatment. J Fam Pract 2005; 54:313-322.
8. Hoberman A, Marchant CD, Kaplan SL, Feldman S. Treatment of acute otitis media consensus recommendations. Clin Pediatr 2002; 41:373-390.
9. Roland PS, Anon JB, Moe RD, Conroy PJ, Wall GM, Dupre SJ, Krueger KA, Potts S, Hogg G, Stroman DW. Topical ciprofloxacin/dexamethasone is superior to ciprofloxacin alone in pediatric patients with acute otitis media and otorrhea through tympanostomy tubes. Laryngoscope 2003; 113:2116-2122.
10. van Cauwenberge P, van Hoecke H, Vandenbulcke L, Van Zele T, Bachert C. Glucocorticosteroids in allergic inflammation: clinical benefits in allergic rhinitis, rhinosinusitis, and otitis media. Immunol Allergy Clin North Am 2005; 25:489-509.
11. Bartoletti C.A. Editoriale. *Medicina Estetica* 1977; 1: 2-3.
12. Crenna P, Mancia M. Aspettiriflessologicidellamesoterapia. *Giornale di Mesoterapia*. 1981; 1:29-40.
13. Biondi G. Consider azioniedipotesisulmeccanismod'azionedellamesot erapia. *In*: Bartoletti CA, Maggiori S, Tomaselli F, eds. *Mesoterapia*. Roma: Ed. Salus, 1982: 66-68.
14. Maggiori S. Ruolo del farmaco in mesoterapia e suorazionaleimpiego. *In:* Bartoletti CA, Mggiori S, Tomaselli F, eds. *Mesoterapia* Roma: Ed. Salus, 1982: 30-31.
15. Daaloz-Bourguignon. *La Mesotherapie.* Ed. Maloine: Paris, 1983.
16. Maggiori S, Tomaselli F, Bartoletti CA. L'infiltrazioneintradermica. *Medicina Estetica* 1988; 1: 377-381.
17. Tomaselli F, Caputo M, Cogliano M *et al*. Il microcircolo: correlazionifisiopatologiche. Approcciofarmacologico. *Medicina Estetica* 1997; 4: 267-273.
18. Fasani U. *Mesoterapia- Metodologia e pratica.* Milano: Ed. OEMF Intern, 1997.
19. Pistor M. Exposè sommaire des proprietès nouvelles de la procaine locale en pathologie humaine. *La PresseMedicale* 1958; 66(44):4-6.

Clinical effectiveness of ototopical application of mupirocin ointment in the MRSA-infected ear

Katsuhisa Ikeda, M.D., Masayuki Furukawa, M.D., Akira Minekawa, M.D., Takuo Haruyama, M.D., Ph.D., Yuya Narui, M.D.

Otorrhea of methicillin-resistant *Staphylococcus aureus* (MRSA)-infected ear has become increasing problems regarding infection after tympanostomy tube placement or through tympanic membrane perforation, and postsurgical infection. In particular, dry ear at the preoperative stage is considered to be a crucial factor for surgery. We evaluated how to control MRSA-infected ear before and after ear surgery. Seventeen patients (21 ears) having MRSA otorrhea were enrolled in the present study, and randomly divided into two groups, namely mupirocin ointment therapy for 10 patients (14 ears) and ofloxacin ear drops for 7 patients (7 ears). Approximately, 3-5 mg of mupirocin ointment was locally applied on to the tympanic membrane around the perforation with its adjacent external ear canal 3 to 4 times for a few weeks at the clinic. On the other hand, ofloxacin ear drops were daily self-medicated for a few weeks at home. Complete elimination of MRSA from the ear was obtained in all patients of the mupirocin group, which was significantly improved as compared with the ofloxacin group (elimination rate:14%). Local application of mupirocin did not aggravate hearing acuity of any patients, who were evaluated by pure-tone audiometry before and after treatment. The present findings first indicated that minimally essential application with mupirocin ointment is an extremely useful ototopical agent against MRSA-infected ear without ototoxicity.

Results of simple myringoplasty with fibrin glue

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Simple underlay myringoplasty with fibrin glue was introduced in 1989 as a less invasive procedure of myringoplasty than conventional methods in Japan. In Juntendo University Hospital, simple myringoplasty with fibrin glue using temporalis muscle fascia was performed in 70 ears from June 2005 to February 2007, and 36 of these ears were observed for more than six months. The most commonly used anesthesia was general anesthesia. In 9 ears, endoscope-aided myringoplasty was performed since the margin of the perforation was not visible with an operating microscope. In 3 ears, the small tympanomeatal flap was created since the perforation edge was minimal, and the underlay technique was combined with the swing-door technique. The success rate was 91.7% regarding permanent closure of the tympanic membrane.

Tissue-engineered mastoid air cell plasty for intractable otitis media

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Aim

To effect a complete recovery from intractable otitis media, regeneration of the mastoid air cells (MACs) gas exchange functions is thought to be needed. Our previous study showed that MACs could be regenerated by *in situ* tissue engineering. In this study, we assessed whether or not MAC plasty affected regeneration of MACs gas exchange functions.

Materials and methods

Collagen-coated hydroxyapatite (Co-HA) of a honeycomb-like structure was used as artificial pneumatic bones. At the first stage of tympanoplasty, Co-HA was implanted into the newly opened mastoid cavity. Twelve patients (5 males and 7 females) with cholesteatoma (n=6), cholesteatoma with adhesive otitis media (n=4), and severe chronic otitis media (n=2) underwent MAC plasty. At the second-stage operation, a nitrous oxide gas study was performed for measurement of the changes of the middle-ear pressure. By CT scan images, we estimated whether or not MACs were regenerated. The observation period was from 9 to 12 months after the second-stage operation.

Results

At the second-stage operation, in 5 cases in which MACs were regenerated, gas exchange functions were regenerated. In 7 cases in which MACs were not regenerated, no gas exchange functions were observed. Regeneration of gas exchange functions of MACs accorded with regeneration of MACs. In the final assessment by CT scan images, MACs were regenerated in 8 of 12 patients.

Conclusions

MAC plasty is useful for regeneration of MACs with gas exchange functions.

Effect of endoscopic microdebrider-assisted adenoidectomy on tympanometry of secretory otitis media

Takuo Haruyama, M.D., Toru Yao, M.D., Masayuki Furukawa, M.D., Katsuhisa Ikeda, M.D.

There is accumulating evidence that the surgical technique for microdebrider-assisted adenoidectomy is a precise, rapid, and safe procedure as compared with conventional curette techniques using Beckman and La Force adenotomes. Furthermore, transoral endoscopic application is expected to provide a better visualization of the nasopharynx, leading to the precise removal of enlarged adenoid tissues around the eustachian tube. The present study aimed to evaluate the healing effect of endoscopic microdebrider-assisted adenoidectomy on tympanometry in patients with secretory otitis media. Twenty-four patients underwent the microdebrider-assisted adenoidectomy under transoral endoscopic control. We also performed conventional adenoidectomy in 22 patients. No statistically significant differences in operative time or intraoperative blood loss were observed. The healing rate of tympanometry in the microdebrider group was 80%, which was significantly higher than that of the conventional procedure (50%). In conclusion, the precise and meticulous removal of the adenoid enlargement using both microdebrider and endoscope resulted in satisfactory outcome of the treatment of secretory otitis media.

Effectiveness of the new medical treatment of patulous eustachian tube (PET): preliminary studies of treating PET with adenosine 5'-triphosphate (ATP) and/or Kamikihito, a Japanese Kampo-medicine

Takehiro Matsuda, M.D., Masahiro Morita, M.D., Naoyuki Kohno, M.D.

Introduction

Most of the forms of medical therapy for PET were poorly tolerated, produced inconsistent results, or required surgical intervention. To assess the effectiveness of the new formula for medical treatment of patulous eustachian tube (PET), 24 patients with the diagnosis of hyperpatent eustachian tube syndrome were evaluated.

Methods

Twenty-four patients were documented to have a continuous history of a plugged or stopped-up ear and autophony for at least 2 months. Eleven had bilateral patulous tube symptoms. Tympanic membrane movement, like fluttering on Toynbee maneuver or movement in synchrony with respirations, were confirmed under microscopic otologic examination. Moreover, in the tubal function testing (TFT), prolonged amplitude of sound pressure level on swallowing in sonotubometry and/or lower tubal opening pressure during the Valsalva maneuver were based on the diagnosis of patulous eustachian tube. Treatment consisted of oral medicine, adenosine 5'-triphosphate (ATP), and/or Kamikihito, a Japanese Kampo-medicine, as a medicine acting as a promoter of the peripheral circulation at least for 4 weeks.

Results

Three of the 24 patients reported excellent results (complete elimination of symptoms, more than partially normalizing in TFT) with no side effects. Eighteen patients reported satisfactory results (control of symptoms and/or relief in TFT). The remaining three patients had no effects on their symptoms.

Discussion and conclusion

This study evaluated a new medical treatment developed to improve peripheral circulation to produce closure of the tubal orifice resulting in eliminate the symptom. The results of the study demonstrated that the medication is effective for treating PET disorders. The treatment with medicine in this study is safe, effective, predictable, and produces few or no side effects.

Effects of an artificial eustachian tube on diseases in the middle ear due to tubal dysfunction

Masahiro Morita, M.D., Naoyuki Kono, M.D., Tomoko Tsutsumi, M.D., Takehiro Matsuda, M.D., Yasuhiko Takei, M.D.

An artificial eustachian tube (AET) was developed with a goal of restoring the ventilatory and drainage functions of the eustachian tube (ET) and becoming an alternative treatment to inserting tympanostomy tubes. We used the AET in nine cases of middle-ear disorders caused by ET dysfunction in patients with stenosis or occlusion of the ET. The nine cases consisted of three cases of tubal stenosis with no other complicated diseases in the middle ear, three cases of otitis media with effusion (OME), two cases of adhesive otitis media (AOM), and one case of otitis media with cholesteatoma (CHO) in patients whose symptoms like ear fullness, hearing loss, and/or ear discharge were resistant to other conservative therapies for more than 2 months. AETs made from polyurethane were inserted in the ET through the tympanic membrane, or directly into the ET in cases that received tympanoplastic surgery. AETs consisted of a tubular body with a proximal end placed protruding out of the tympanic membrane or just beneath the tympanic membrane, and with a distal end inserted into the ET and placed through the isthmus to face the inside of the cartilaginous ET. The distal and the proximal end of the AET communicated with each other through an internal cavity.

Objective evaluation of the efficacy was performed by measuring ET functioning with sonotubometry, tubotympanum aerodynamograms (TTAG), and the Valsalva maneuver. The use of AETs to improve ET function resulted in seven effective cases with no ear discharge and/or recovery of hearing loss out of total of nine cases. In the seven cases that were effective, two cases had tubal stenosis, two cases had OME, two cases had AOM, one case had CHO; one each of the cases of AOM and CHO received tympanoplastic surgery.

When we detect ET dysfunction, we can increase the cure rate by using AETs to treat middle-ear disease.

Sanitation of mesopharyngeal and retropharyngeal space by radiofrequency tonsillotomy and adenectomy

Klaus Vogt, M.D., D.D.S., Ph.D.

Objectives

In young children with chronic functional disorders of the eustachian tube, adenoids and tonsil hyperplasia are the most frequent causes implicating the sanitation of the tubal ostia region. The recent papers of a German workgroup using laser technology and the publications of E. Hultcrantz emphasize the high-level effectiveness of tonsillotomy in children ages 3-10 years. Although which technique is used to perform an effective tonsillotomy seems of secondary importance, the appliance of radiofrequency (RF) in general has some remarkable advantages. The delivered energy can be exactly adapted to the operation field and to the surgical process.

Material

Between August 2002 and August 2006, 147 RF-tonsillotomies in young children have been carried out. In 112 cases, additional adenectomy was done. In 58, cases additional RF-myringotomy or insertion of drum tubes (nine cases) was performed.

Technique

The Ellman Surgitron IEC II was used for all cases. The partial rectified (Cut/Coag) modality was found to be the most effective for this procedure. Additional adenectomy was performed by using RF-adenotomes by Ellman. All operations have been outpatient procedures with general anesthesia.

Results

There was no postoperative bleeding. In two cases, a recurrence of the hyperplasia was observed; unilateral tonsillectomy had to be carried out in one case. Two children suffered later from an acute tonsillitis. Essential results of a questionnaire that was answered by 71 parents are as follows:

- Painkillers have been necessary 2.1 (0-6) days.
- The child returned to normal daily life after 4.1 (1-7) days.
- Sixty-eight parents would recommend the procedure to others or want to have done it again if necessary.

Discussion

The results reported here, using the Ellman 4 MHz radiofrequency technique, are in accordance with previous results reported about tonsillotomy in young children with laser techniques. They are supporting the renewed discussion about conservative surgical methods successfully providing more safety as the classic tonsillectomy. The application of RF-generators is easier to manage and more cost-effective because of the simple maintenance. The danger of an unwanted violation of the posterior pillars or the pharynx backwall is reduced because the transferred RF energy ends immediately at the tip of the tool.

Conclusions

1. Radiotonsillotomy is comparable to lasertonsillotomy and is a safe surgical procedure for the treatment of tonsillar hyperplasia in 4-6 year old children, which preserves the immunologic function of the tonsils.
2. Radiotonsillotomy is easy to perform with low risk for the unwanted violation of the pillars.
3. Radiotonsillotomy is a cost-effective outpatient procedure and can be combined with adenectomy and drainage of the middle ear.

Rate of acute otitis media following upper respiratory tract infection by type of associated viruses

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Introduction

Healthy infants and children are prone to developing common cold or upper respiratory tract infection (URI). Information generated in the past few decades suggests the close link between URI and acute otitis media (AOM). Approximately 29-50% of all URIs develop into AOM[1-3] and a variety of viruses have been detected in the nasopharynx and middle-ear effusion (MEE) of children with AOM.[4,5] One way to prevent OM is to prevent URI in children. Because specific viruses may differ in their ability to induce OM, understanding the relative importance of common URI causative viruses in this regard will be useful in designing appropriate viral vaccines for OM prevention.

Our objective was to use comprehensive viral diagnostic methods to detect the viruses associated with URI and to determine the rate of AOM complicating URI by specific viruses.

Materials and methods

Healthy children 6-35 months of age were enrolled into a prospective, longitudinal, cohort study; each child was followed for 1 year for occurrences of URI and AOM. Children were seen as soon as possible after each URI onset and were monitored closely for 3 weeks for the development of AOM. Active and passive surveillance was used to capture all events. AOM complicating URI was considered when the episode occurred within 28 days of the URI unless a new onset of URI occurred within this period; in that case, AOM was considered the complication of the most recent URI episode. AOM was defined by acute onset of symptoms (fever, irritability, or earache), signs of tympanic membrane inflammation, and presence of fluid in the middle ear documented by pneumatic otoscopy and/or tympanometry.

Nasopharyngeal secretion (NPS) samples were collected at the initial URI visits and AOM visits for virus studies. Conventional viral diagnostic methods included viral culture and respiratory syncytial virus (RSV) antigen detection by enzyme immunoassay (EIA). Molecular diagnostic assays included real-time polymerase chain reaction (PCR) for adenovirus, rhinovirus, enterovirus, and coronavirus (OC43, 229E, NL63), and microarray assay for RSV, parainfluenza virus types 1, 2, 3 and influenza A and B.

The relationship of AOM and virus was analyzed using the general estimating equations (GEE) approach, which treats the child as the unit of analysis, and a subject's OM episodes as a correlated cluster of data. Analyses were conducted in the GENMOD procedure in SAS.[6]

Results

Two hundred and ninety-four children (6-36 months of age) were enrolled in the study between January 2003 and March 2006. Mean age at enrollment was 13.7 months (median age = 12 months). There were 150 (51%) males, 31% were White; 8%, Black; 2%, Asian; and 8%, biracial. Hispanic or Latino accounted for 56%. Sixty-eight percent of subjects were followed for the entire 12-month period, while 15% dropped from the study before 6 months. Overall, the follow-up period for 294 children was 256 child-years (mean duration=9.8 months, median=12 months).

A total of 1,295 URI episodes were documented (5.06/ child-year); median age at URI onset was 17.7 months; 67% of URI episodes were seen by the study group and were studied for the URI causative virus. There was a total of 440 AOM episodes documented overall (1.72 episodes/child-year), and 94% occurred with URI symptoms. Median age at the time of AOM diagnosis was 15.9 months.

Virologic studies were performed on 988 respiratory specimens collected during 864 URI episodes from 214 patients. Viral culture and/or RSV-EIA were positive in 24.8%. Six hundred specimens were tested by molecular diagnostics; 587 of these were culture- and RSV-Ag– negative specimens; 359 (59.8%) of these were positive for one or more viruses. Virus yield was 70% for 587 specimens tested by conventional methods and by molecular diagnostics (when conventional assays were negative). Overall, viruses were detected from NPS specimens collected

during 558 of 864 URI episodes (64.6%); in 11 episodes, only cytomegalovirus (CMV) was detected. CMV was also detected along with other respiratory viruses in another 16 episodes. Considering CMV shedding may be from congenital or acquired CMV infection and the virus may not be the cause of URI, CMV data were excluded from the subsequent analyses. After exclusion of CMV, 547 URI episodes were associated with detection of respiratory viruses. There were 708 respiratory viruses detected (in single or in combination) in 547 of 864 (63%) URI episodes. One virus was detected in 422 (77%); two viruses were detected in 92 URI episodes, three viruses in 30 episodes, and four viruses in three episodes. Adenovirus, rhinovirus, and enterovirus led other viruses as the cause of URI.

Rate of AOM complicating URI by specific viruses, median age, and median day of AOM in the course of URI are shown in Table 1. For each virus (except HSV, n=2), children who developed AOM were younger children. Coronavirus, RSV, and adenovirus were among respiratory viruses associated with higher rates of AOM complication. The GEE analysis indicated that age (P=0.0002) was the strongest predictor of AOM development after a URI, followed by virus type (P=0.05), controlling for gender (P=0.19), race (P=0.92), and ethnicity (P=0.75). For each additional month of age at URI episode, the chances of developing AOM decreases by 4% (OR=0.96; 95% CI: 0.94-0.98). In other words, as a child gets older, for each additional month of age, the chances of developing AOM decrease by 4%. This applies for the same child growing older, as well as comparing two children at different ages, for example, a 12-month-old child is 24% less likely to have AOM following URI than when he or she was 6 months old, and an 18-month-old child has a 24% lower chance of having AOM following URI than a 12-month-old.

We also computed pairwise differences among viruses. While rhinovirus was the second most common cause of URI, the risk of AOM after rhinovirus URI was lower than that caused by RSV, coronavirus, and adenovirus. The odds ratios were 2.7 for coronavirus vs rhinovirus (95% CI, 1.16-6.21, P=0.02); 2.2 for RSV vs rhinovirus (95% CI, 1.16-4.33, P=0.02); and 1.8 for adenovirus vs rhinovirus (95% CI, 1.07-3.19, P=0.03).

Discussion

In this longitudinal study of children at the peak age incidence of OM over a 4-year period, we documented nearly 1,300 URI episodes, two-thirds of which were studied virologically and closely monitored for AOM.

We documented a 37% rate of AOM complicating URI. Our statistical model identified age of the child as the most important factor of AOM complicating URI and predicted an approximate decreased rate of AOM by 4% per month the child grew older. We identified the two most common viruses causing URI in our population: adenovirus and rhinovirus. While adenovirus was also associated with high rate of AOM complicating URI, rhinovirus was found to have lower rate of AOM complication than coronavirus and RSV.

Results of previous studies on the relative role of specific virus types in AOM have been inconclusive as different methods were used for virus detection. Henderson et al.[7] used tissue culture for virus isolation and reported RSV, adenovirus, and influenza to be more closely associated with AOM; the incidence of AOM in children with rhinovirus URI was the lowest. Ruuskanen et al.[8], using conventional viral assays, reported similar results on incidence of AOM-associated viruses. Vesa et al.[9] found that URI associated with RSV and rhinovirus had higher rates of AOM than with adenovirus, but they used PCR for rhinovirus detection only and used antigen detection for other respiratory viruses. Pitkaranta et al.[10] used RT-PCR for selected viruses and reported rhinovirus to be the most common virus found in NPS and/or MEE of children with AOM, compared to RSV and coronavirus. Age of children in that study was older than in other studies (3 months to 7 years, with median age of 30 months). In our study, we used more comprehensive diagnostic methods, including conventional assays and more sensitive molecular assays for common respiratory viruses. Unlike any previous study, we identified adenovirus as the most common URI cause and also one of the viruses associated with the highest rate of AOM complicating URI. Our data suggest that prevention of adenovirus URI has a potential to make a significant impact on AOM incidence.

Although RSV-URI was not diagnosed as frequent in our study, compared to adenovirus, enterovirus, and parainfluenza viruses, the rate of AOM complicating URI is among one of the highest. These data are consistent with previous reports[7,11,12] and suggest that effective prevention of RSV-URI may also impact AOM incidence. Previous studies on coronavirus and AOM included data on types OC43 and 229E only[10]; our data also included coronavirus NL-63. Interestingly, while we found a high rate of AOM complicating coronavirus URI (12/24=50%), the relative high rate was driven by the OC43/229E coronavirus. Of 14 cases of OC43/ 229E URI, the AOM rate was 64%; the AOM rate associated with NL-63 was only 22%.

Research studies of viral etiology of URI in adults and children using a variety of viral diagnostics, including molecular techniques, have given virus yield between 42 to 73%.[9,13,14] Virus yield depends on a variety of factors from sensitivity of the technique, specificity of the primers, and the number of viruses targeted. New viruses have recently been discovered as causes of URI and/ or OM: human metapneumovirus, bocavirus, and NL-63.[15-17] The limited amount of samples and high costs were prohibitive to us to test for all viruses. In this study, when molecular techniques were applied to culture- and RSV-EIA–negative samples, the yield was 70%. We did not test for human metapneumovirus or bocavirus, which could have lowered the overall virus yield by 5-10%.[15,17]

Conclusion

We reported a high prevalence of viral URI in young children, more than one-third of whom developed AOM complication. Young age at URI onset and specific types of URI causative virus were found to be associated with the risk for AOM development. Preventive strategies for OM should be through preventing viral URI in very young children. The strategy may be more effective when significance of specific virus types is considered.

References

1. Heikkinen T, Ruuskanen O. Temporal development of acute otitis media during upper respiratory tract infection. Pediatr Infect Dis J 1994; 13:659-661.
2. Koivunen P, Kontiokari T, Neimelä M, et al. Time to development of acute otitis media during an upper respiratory tract infection in children. Pediatr Infect Dis J 1999; 18:303-305.
3. Winther B, Doyle WJ, Alper CM. A high prevalence of new onset otitis media during parent diagnosed common colds. Int J Pediatr Otorhinolaryngol 2006; 70:1725-1730.
4. Chonmaitree T. Viral and bacterial interaction in acute otitis media. Pediatr Infect Dis J 2000; 19 S24-30.
5. Ruohola A, Meurman O, Nikkari S, et al. Microbiology of acute otitis media in children with tympanostomy tubes: prevalences of bacteria and viruses. Clin Infect Dis 2006; 43:1417-1422.
6. SAS Institute INC. 2004. SAS/STAT® 9.1 User's Guide. Cary, NC: SAS Institute Inc.
7. Henderson FW, Collier AM, Sanyal MA, et al. A longitudinal study of respiratory viruses and bacteria in the etiology of acute otitis media with effusion. N Eng J Med 1982; 306:1377-1383.
8. Ruuskanen O, Arola M, Putto-Laurila A, et al. Acute otitis media and respiratory virus infections. Pediatr Infect Dis J 1989; 8:94-99.
9. Vesa S, Kleemola M, Blomqvist S, Takala A, Kilpi T, Hovi T. Epidemiology of documented viral respiratory infections and acute otitis media in a cohort of children followed from two to twenty-four months of age. Pediatr Infect Dis J 2001; 20:574-581.
10. Pitkaranta A, Virolainen A, Jero J, Arruda E, Hayden FG. Detection of rhinovirus, respiratory syncytial virus, and coronavirus infections in acute otitis media by reverse transcriptase polymerase chain reaction. Pediatrics 1998; 102:291-295.
11. Heikkinen T, Thint M, Chonmaitree T. Prevalence of various respiratory viruses in the middle ear during acute otitis media. N Eng J Med 1999; 340:260-264.
12. Patel JA, Nguyen D, Revai K, Chonmaitree T. Role of respiratory syncytial virus in acute otitis media: Implications for vaccine development. Vaccine 2007; 25:1683-1689.
13. Makela MJ, Puhakka T, Ruuskanen O, et al. Viruses and bacteria in the etiology of the common cold. J Clin Microbiol 1998; 36:539-542.
14. Winther B, Alper CM, Mandel EM, Doyle WJ, Hendley JO. Temporal relationships between colds, upper respiratory viruses detected by polymerase chain reaction, and otitis media in young children followed through a typical cold season. Pediatrics 2007; 119:1069-1075.
15. Williams JV, Wang CK, Yang CF, et al. The role of human metapneumovirus in upper respiratory tract infections in children: a 20-year experience. J Infect Dis 2006; 193:387-395.
16. Williams JV, Tollefson SJ, Nair S, Chonmaitree T. Association of human metapneumovirus with acute otitis media. Int J Pediatr Otorhinolaryngol 2006, 70:1189-1193.
17. Kesebir D, Vazquez M, Weibel C, et al. Human bocavirus infection in young children in the United States: molecular epidemiological profile and clinical characteristics of a newly emerging respiratory virus. J Infect Dis 2006; 194:1276-1282.

Genome-wide search for otitis media-associated pneumococcal genes

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Background and objective

S. pneumoniae is the most common bacterial cause of acute otitis media (OM) in children. Pneumococcal factors specific for ear infection are poorly described. Pneumococcal isolates representing the 19F serotype have been frequently associated with OM. The main goal of this study is to identify pneumococcal genes that are associated with middle ear infection in a type-9F isolated ST556 from an OM patient.[1]

Experimental Design and Methods

We screened for OM-associated pneumococcal genes by signature-tagged mutagenesis (STM) in a chinchilla infection model (outlined in Fig. 1). STM has been successfully used to identify virulence determinants in many other bacterial pathogens. Approximately 6,000 tagged mutants were constructed by insertional mutagenesis in a serotype 19-F isolate from an OM patient.[1] Sixty uniquely tagged mutants were separately grown *in vitro* and mixed as an input pool to infect the middle ears of chinchillas through transcanal tympanotomy. The pneumococci were recovered from the middle ear fluids of infected animals as outputs three days postinfection. The input and output pools were compared to determine the retention of each STM strain in the output pools by PCR amplification (see Fig. 4).

    Construction of tagged mutants in strain ST556. STM strains were generated in a 19F *S. pneumoniae* strain ST556 using derivatives of the suicide pID701t plasmid \by insertion duplication essentially as described.[2] The technical approach is outlined in Fig. 2.

    Negative screening of the S. pneumoniae STM strains in an ear infection model. The STM strains were screened in a chinchilla middle ear infection model as illustrated in Fig. 3.

    Detection of the S. pneumoniae STM mutants. The input and output pools of the ST556 STM strains were compared by a PCR based method as described in our previous study.[3] The technical approach is outlined in Fig. 4.

Results

Out of approximately 5,500 STM strains tested thus far, 687 STM mutants were consistently missing in the output pools from multiple chinchillas, thus confirming the reliability of the STM screening approach. We are currently evaluating this mutant list by confirming the attenuation phenotype and mapping out the mutation sites. Deletion mutants will be further constructed to determine the mechanisms by which the pneumococcal genes identified in this study contribute to bacterial survival and replication in the middle ear.

Summary

We have been pursuing an STM-based high throughput screening study to identify pneumococcal genes that are necessary for ear infection. Our preliminary experiments have demonstrated the feasibility of the STM approach in identifying OM-associated genes of *S. pneumoniae*. Considering the unique anatomic and physiological features of the middle ear, we expect to discover pneumococcal genes that are specifically required for bacterial infection in the middle ear.

Figure 1. Overall strategy of the study.
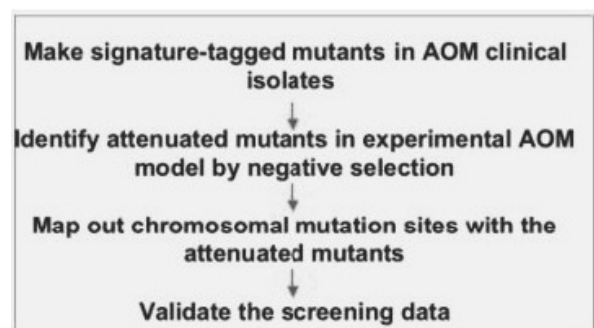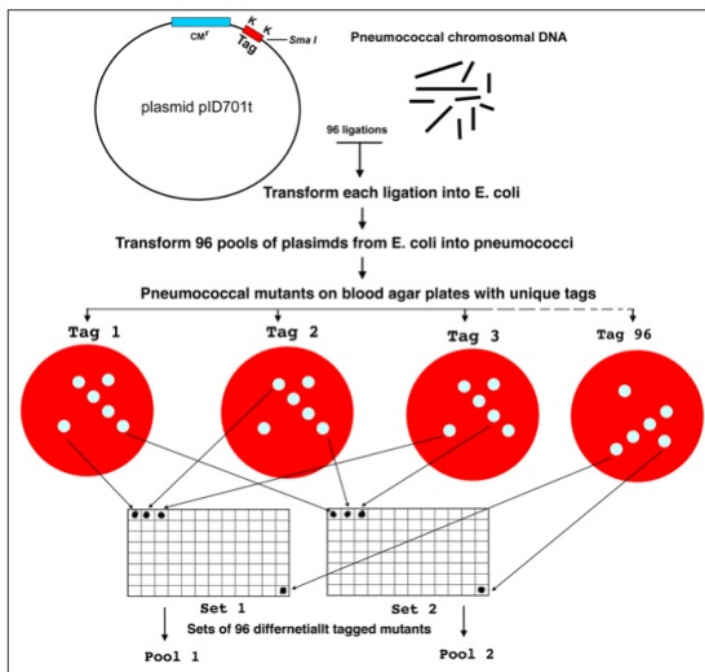
Figure 2. Mutagenesis of *Streptococcus pneumoniae.*

References

1. Joloba, M. L., A. Windau, S. Bajaksouzian, P. C. Appelbaum, W. P. Hausdorff, and M. R. Jacobs. Pneumococcal conjugate vaccine serotypes of Streptococcus pneumoniae isolates and the antimicrobial susceptibility of such isolates in children with otitis media. Clin Infect Dis 2001; 33:1489-1494.
2. Lau, G. W., S. Haataja, M. Lonetto, S. E. Kensit, A. Marra, A. P. Bryant, D. McDevitt, D. A. Morrison, and D. W. Holden. A functional genomic analysis of type 3 Streptococcus pneumoniae virulence. Mol Microbiol 2001; 40:555-571.
3. Su, J., J. Yang, D. Zhao, T. H. Kawula, J. A. Banas, and J. R. Zhang. Genome-wide identification of Francisella tularensis virulence determinants. Infect Immun 2007; 75:3089-3101.
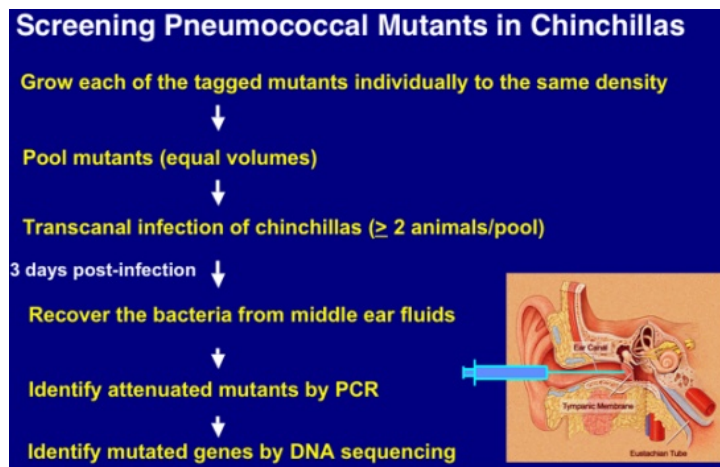
Figure 3. Middle ear infection of chinchillas with S. pneumonia STM strains.

Classification of PspA families of Streptococcus pneumoniae isolated from children with acute otitis media

Masamitsu Kono, M.D., Muneki Hotomi, M.D., Ph.D., Susan Hollingshead, Ph.D., David Briles, Ph.D., Noboru Yamanaka, M.D., Ph.D.

Introduction

Streptococcus pneumoniae is one of the major causative pathogens responsible for acute otitis media (AOM). The pathogen frequently colonizes in the healthy human nasopharynx. Under some predisposing conditions following viral infections, pneumococci cause symptomatic AOM. Recent alarming increases in antimicrobial resistance in *S. pneumoniae* bring an urgent demand to develop effective vaccines against this pathogen. The tremendous success of capsular polysaccharide vaccine against type b *Haemophilus influenzae* (Hib) has suggested the efficacy of a pneumococcal vaccine. Vaccines are required to protect AOM by not only protecting infections in the middle ear but also preventing or reducing nasopharyngeal colonization. Although the antibody to pneumococcal capsular polysaccharides (PCP) is highly protective against pneumococcal infections, PCPs are not immunogenic in children. Furthermore, there are at least 90 different types of PCPs in pneumococci, while only a single polysaccharide is required for Hib.

Among several pneumococcal proteins, pneumococcal surface protein A (PspA) is unique in eliciting protective immunity. This protein antigen is also considered as a virulent factor for pneumococci. PspA is divided into 3 families and 6 different clades.[1] While PspA is divided into serological distinguishable groups, each PspA families show better serological cross-reactivity with each other. Immunization with a single PspA family could elicit some antibodies cross-reactive with other PspA families. Thus, PspA are the attractive antigens to develop the effective vaccine against *S. pneumoniae*.[2] However, the prevalence of the PspA family among the clinical isolates from AOM children has not been well evaluated yet. In this study, we investigated the distribution of the PspA family among *S. pneumoniae* strains isolated from AOM children.

Methods and materials

S. pneumoniae strains. One hundred and fifty-seven pneumococcal isolates from AOM patients were used in this study. All isolates were serotyped or serogrouped by the capsular quelling method with pneumococcal capsule-specific antisera (Statens Seruminstitut, Copenhagen, Denmark) as recommended by the manufacturer.

Classification of PspA family by polymerase chain reaction (PCR). PspA were classified into three types named as "family" by PCR. Briefly, genomic DNA of pneumococci were extracted with TE buffer (10mM Tris HCL, 1mM EDTA, pH 8.0) containing 10% SDS and precipitated with 5 M potassium acetate in a cold 95% ethanol. The PCR is carried out in a standard PCR mixture of 25 µl containing 2.5 mM $MgCl_2$, 200 µM dNTPs (each), 50 pmol of primers, and 2.5 U of *Taq* DNA polymerase. The PCR conditions were 95°C for 3 minutes, then 30 cycles of 95°C for 1 minute, 62°C or 58°C for 1 minute and 72°C for 3 minutes, and finally 72°C for 10 minutes. The amplified PCR products for family 1 were usually around 1000 b.p. and for family 2 were approximately 1200 b.p.[2]

Results

Serotype 6A, 6B, 9V, 14, 19F, and 23F were the major serotypes isolated from the upper respiratory tract infections. On the other hand, 45.3% and 49.6% of pneumococcal isolates possessed PspA family 2 or PspA family 1, respectively. PspA family 3 was identified only in 3.1% of strains. About 0.4% of pneumococcal strains had PspA but were unclassified, non-family, PspA. About 1.6% of pneumococcal strains did not possess PspA and were determined as PspA-null strains (Fig. 2).

Discussion

Urgent demand to develop an effective vaccine has also been further emphasized by recent studies demonstrating a rapid increase in prevalence and level of resistance of multiple antibiotic-resistant pneumococci.[3,4] Currently available pneumococcal vaccines are based on pneumococcal capsular polysaccharides. The 23-valent pneumococcal

polysaccharide vaccine is immunogenic and protective in most adults but not in children under 2 years of age. Conjugation of capsular polysaccharides to certain protein carriers improves their immunogenicity in infants. A recently developed 7-valent pneumococcal conjugate vaccine (7-PCV) is highly efficacious in preventing invasive disease in children under 5 years of age.[5] Promising results regarding the prevention of pneumonia and AOM have also been published.[5] However, the protection is restricted to only a limited number of serotypes included in the vaccine. The vaccine has not achieved the desired success in reducing nasopharyngeal colonization. Furthermore, serotype replacement has been observed in vaccinated populations and an increase of infections caused by pneumococcal serotypes not included in the 7-PCV has been described.[6,7]

PspA is unique in eliciting protective immunity. PspA can be classified into three families including 6 clades; family 1 (clade 1 and 2), family 2 (clade 3, 4, and 5), and family 3 (clade 6) according to the genetic and serological diversity.[2] While PspA exhibit variations in sequences and epitopes, PspA are immunologically very cross-reactive.[2] Immunization with a single PspA family could elicit some antibodies cross-reactive with other PspA families.[8] In rat models, this protein also has been reported to elicit the protection against otitis media.[9] In the present study, both PspA family 1 and family 2 were the predominant families of PspA among pneumococci isolated from AOM children. About 94.9% of pneumococci isolated from AOM children possessed either types of PspA family. The human phase I trial with recombinant PspA (rPspA, family 1, clade 2) to adults in 2000 showed that immunization with 5 - 125 μg rPspA induced a significant anti-PspA antibodies in sera, as well as antibodies reactive to heterologous rPspA molecules.[10] The current findings suggested that PspA, especially PspA family 2, would be an attractive candidate for developing a pneumococcal vaccine against AOM.

Figure 1. Distribution of PspA family.
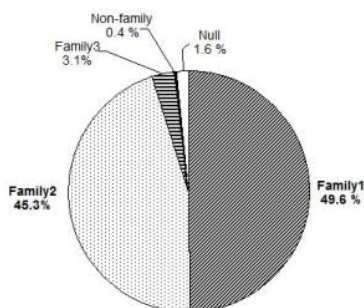
References

1. Briles DE, Tart RC, Swiatlo E, et al. Pneumococcal diversity: considerations for new vaccine strategies with emphasis on PspA. Clin Micro Review 1998; 11:645-657.
2. Hollingshead SK, Becker R, Briles DE. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. Infect Immun 2000; 68:5889-5900.
3. Hotomi M, Billal DS, Shimada J, Suzumoto M, Yamauchi K, Fujihara K, Yamanaka N. High prevalence of *Streptococcus pneumoniae* with mutations in pbp1a, pbp2x, and pbp2b genes of penicillin-binding proteins in the nasopharynx in children in Japan. ORL J Otorhinolaryngol Relat Spec 2006; 68:139-145.
4. Hotomi M, Yamanaka N, Faden H, et al. Nasopharyngeal carriage of drug-resistant *Streptococcus pneumoniae* in children with acute otitis media evaluated by polymerase chain reaction-based genotyping of penicillin-binding proteins. Acta Otolaryngol 2002; 122:72-77.
5. Eskola J, Kilpi T, Palmu A, et al. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N Engl J Med 2001; 344:403-409.
6. Pelton SI, Huot H, Finkelsstein JA, et al. Emergence of 19A as virulent and multidrug resistant pneumococcus in Massachusetts following universal immunization of infant with pneumococcal conjugate vaccine. Pediatr Infect Dis J 2007; 26:468-472.
7. Farrell DJ, Kulgman KP, Pichichero M. Increased antimicrobial resistance among nonvaccine serotypes of streptococcus pneumoniae in the pediatric population after the introduction of 7-valent penumococcal vaccine in the United States. Pediatr Infect Dis J 2007; 26:123-128.
8. Briles DE, Hollingshead SK, Brooks-Walter A, et al. The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection. Vaccine 2000; 18:1707-1711.
9. Briles DE, Hollingshead SK, Nabors GS, Paton JC, Brooks-Walter A. The potential for using protein vaccine to protect against otitis media caused by *Streptococcus pneumoniae*. Vaccine 2001; 19:S87-S95.
10. Nabors GS, Braun PA, Herrmann DJ, et al. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. Vaccine 2000; 18:1743-1754.

Continuing shifts in the pathogens causing acute otitis media in 2003-2006

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Introduction

Two studies published in 2004 in described a shift in the pathogens causing AOM in children undergoing tympanocentesis during 2001-2003 compared to the 1990s.[1,2] This shift in the frequency distribution of AOM pathogens coincided with the widespread use of the conjugated heptavalent pneumococcal conjugate vaccine (PCV7) in the United States. Among children, mostly with acute otitis media (AOM) antibiotic treatment failure (AOMTF) and/or recurrent AOM who had a tympanocentesis performed, the predominant pathogen became β-lactamase–producing *Haemophilus influenzae,* accounting for about 57% of isolates. The proportion of children with AOM due to penicillin intermediately resistant *Streptococcus pneumoniae* (PISP) and penicillin-resistant *S. pneumoniae* (PRSP) to decrease to 13% and 6%, respectively.[1,2] AOMTF and recurrent AOM decreased after the introduction of PCV7 by 24%[1] and the proportion of AOM caused by *S. pneumoniae* serotypes contained in the PCV7 vaccine declined by about 50%.[2]

We report continuing shifts in the frequency distribution of isolated pathogens from middle-ear specimens obtained from U.S. children with AOM between 2003 and 2006.

Methods and materials

We included all available AOM pathogens isolated from middle-ear fluid (MEF) specimens collected by tympanocentesis between September, 2003, and June, 2006. Participating centers included Rochester, NY; Pittsburgh, PA; and Vienna, VA.

We included only isolates of *S. pneumoniae, H. influenzae,* and *Moraxella catarrhalis* as AOM pathogens in the analysis. We identified isolates and determined antibiotic minimum inhibitory concentrations using standard laboratory methods.

Results

Demographics and history of AOM episodes for 262 children included in the study are presented in Table 1. Combining the three respiratory seasons, *H. influenzae* was the most frequently isolated pathogen, followed by *S. pneumoniae* (Fig. 1). The proportion of *H. influenzae* strains that produced β-lactamase remained unchanged overtime. In contrast, the proportion of children with PNSP increased, the highest proportion occurring in 2005-06 (p = 0.0001; Table 2).

Discussion

The relative proportion of pathogens causing AOM in the United States has continued to shift during a time of increasing and widespread use of PCV7 vaccine. *H. influenzae* continues to be the predominant pathogen, a change previously reported after the introduction of PCV7.[1,2] However, in 2005-2006 an upswing in *S. pneumoniae* isolates occurred, with a simultaneous increase in the proportion of strains that were PRSP.

Limitations of this study include the fact that tympanocentesis was not routinely performed in the United States for uncomplicated, previously untreated children with AOM or children experiencing infrequent infections. Tympanocentesis was undertaken generally because the child was in severe pain or toxic, was failing antibiotic treatment, or had experienced recurrent episodes of AOM. Accordingly, our results may not be generalized to other types of children with AOM. In general, children with AOM for whom tympanocentesis is considered are more likely to have AOM caused by resistant pathogens and/or multiple pathogens.

Acknowledgment

Figure 1. Changes in frequency and pathogens causing AOM (Rochester, NY)
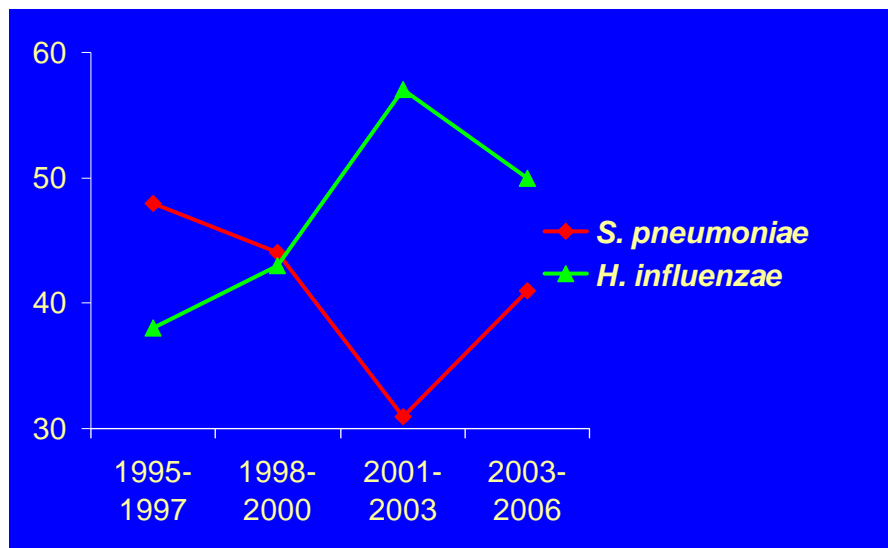
Table 1. Selected demographics and clinical characteristics

2003-2006				
	New York (n = 162)	Pennsylvania (n = 65)	Virginia (n = 37)	Total (n = 264)
Age Distribution				
2-24 months	133 (82%)	48 (74%)	24 (65%)	205 (77%)
2-5 years	23 (14%)	17 (26%)	10 (27%)	51 (19%)
> 5 years	6 (4%)	0	3 (5%)	10 (4%)
Mean Age ± SD (mo)	19 ± 18	18 ± 11	22 ± 12	19 ± 9
Gender				
Male	94 (58%)	33 (51%)	21 (57%)	148 (56%)
Female	68 (42%)	32 (49%)	16 (43%)	116 (44%)
PCV7 History				
<3 doses	16 (10%)	19 (29%)	13 (35%)	48 (18%)
3 doses	96 (59%)	27 (42%)	14 (38%)	137 (52%)
4 doses	50 (31%)	19 (29%)	10 (27%)	79 (29%)
AOM History				
≥3 AOM in 6 mo	62 (138%)	N/A	15 (41%)	77 (39%)
≥5 AOM in 6 mo	35 (22%)	N/A	3 (8%)	38 (19%)
Antibiotic History				
Antibiotic >2 d, failing	38 (23%)	18 (28%)	10 (27%)	66 (25%)
Antibiotic within 30 d	158 (98%)	26 (40%)	21 (57%)	205 (78%)

Table 2. Distribution of AOM pathogens isolated from middle-ear fluid of US children with AOM according to time period and site

Isolate	2003-2004				2004-2005				2005-2006				2003-2006			
	NY	PA	VA	Total	NY	PA	VA	Total	NY	PA	VA	Total	NY	PA	VA	Total
S. pneumoniae																
PSSP	14	9	5	28 (64%)	6	0	5	11 (64%)	5	4	6	15 (50%)	25	13	11	49 (56%)
PISP	4	2	0	6 (14%)	2	0	0	2 (11%)	5	2	0	7 (23%)	11	4	0	15 (17%)
PRSP	8	2	0	10 (23%)	4	1	0	5 (28%)	8	0	0	8 (27%)	20	3	0	23 (26%)
Total	26	13	5	44 (30%)	12	1	5	18 (34%)	18	6	6	30 (45%)	56	20	11	87 (33%)
H. influenzae																
β-Lactamase +	26	7	0	33 (48%)	19	1	0	20 (67%)	12	0	1	13 (50%)	57	8	1	66 (53%)
β-Lactamase -	18	11	7	36 (52%)	9	0	1	10 (33%)	10	3	0	14 (54%)	37	14	8	59 (47%)
Total	44	18	7	69 (48%)	28	1	1	30 (57%)	22	3	1	26 (40%)	94	22	9	125 (47%)
M. catarrhalis	3	0	5	8 (6%)	1	1	2	4 (8%)	3	0	5	8 (12%)	7	1	12	20 (8%)
2 or more pathogens				5 (3%)								2 (3%)				7 (3%)
Total	78	50	17	145	42	3	8	53	42	12	12	66	162	65	37	264

References

1. Casey JR, Pichichero ME. Changes in frequency and pathogens causing acute otitis media in 1995-2003. Pediatr Infect Dis J 2004; 23:824-828.
2. Block SL, Hedrick J, Harrison CJ, Tyler R, Smith A, Findlay R, Keegan E. Community-wide vaccination with the heptavalent pneumococcal conjugate significantly alters the microbiology of acute otitis media. Pediatr Infect Dis J 2004; 23:829-833.

Genetic characteristics and clonal dissemination of ß-lactamase non-producing ampicillin-resistant (BLNAR) *Haemophilus influenzae* isolated from acute otitis media

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Introduction

Haemophilus influenzae is a frequently isolated bacterium responsible for various infections of the respiratory tract, including acute otitis media (AOM)[1]. The ß-lactamase non-producing ampicillin-resistant (BLNAR) strain of *H. influenzae* is a rapidly increasing pathogen responsible for intractable and/or persistent upper respiratory tract infections. Systematic surveillance studies are essential tools in the effort to define the trends in the antimicrobial resistance of bacteria. Recent studies characterizing mutations of *ftsI* gene encoding PBP3 classified the BLNAR strains into the three major genotypes.[2] Of the various missense mutations of the *ftsI* gene, resistance to ß-lactam antibiotics largely depends on the substitution of Arg-517-His (Group I), Asp-526-Lys (Group II), and Ser-385-Thr (Group III). Intermediate ampicillin (ABPC) resistance is commonly found in strains with group I/II substitutions, while the isolates with group III substitutions are associated with a higher level of resistance to ABPC.[2]

In 2003, the Japanese Society of Infectious Diseases in Otolaryngology conducted the fourth nationwide surveillance to define the causative pathogens of infectious diseases of the upper respiratory tract (URT) and their contemporary resistance status in Japan. In this study, we evaluated the recent prevalence of antimicrobial-resistant *H. influenzae* isolated from the upper respiratory tract infections.

Materials and methods

H. influenzae strains. A total of 264 *H. influenzae* isolates were collected from 264 patients. The patients ranged in age from 0 to 83 years old, with 143 males and 121 females. Among the isolates, 66 (25.0 %) were from the middle-ear fluid (MEF), 77 (29.1 %) were from the nasopharynx of patients with AOM, 58 (22.0 %) were from the crypt of the palatine tonsil of patients with pharyngotonsillitis, and 63 (23.9 %) were

from the nasal discharges or sinus aspirates from patients with acute rhinosinusitis.

Antimicrobial susceptibility. Antimicrobial susceptibilities were determined by measuring the minimal inhibitory concentration (MIC) using the microbroth dilution method according to the procedure recommended by the Clinical and Laboratory Standards Institute (CLSI) (M 100-S17; 2007).

Polymerase chain reaction (PCR) based genotyping. Genotypes depending on the mutations in *ftsI* gene were evaluated by PCR. Primers used in this study were to amplify both the variable mutated locus (Asn-526 or Arg-517) and a highly mutated locus (Ser-385).[2] To determine the strain having ß-lactamase gene (*bla*), the loci were amplified by specific primers targeting ß-lactamase. To confirm that the isolated pathogen was *H. influenzae*, outer membrane protein P6 gene was identified.

Depending on the PCR amplification, *H. influenzae* were categorized into six genotypes. They were genetically ß-lactamase–nonproducing ampicillin-susceptible (gBLNAR), BLNAR with group I/II substitution (gBLNAR Group I/II), BLANR with group III substitution (gBLNAR Group III), ß-lactamase–producing ampicillin clavulanate-resistant (gBLPACR Group I/II and Group III), and ß-lactamase–producing (gBLP) (Fig. 1).

Restriction DNA fragment polymorphism analyzed by pulsed-field gel electrophoresis (PFGE). The restriction fragment polymorphisms of *SmaI*-digested chromosomal DNA from *H. influenzae* isolates were evaluated by PFGE.

Results

Susceptibility to ampicillin (ABPC). Out of the 264 *H. influenzae* isolates, 259 (98.1 %) were *bla* gene negative. Only five (1.9 %) strains had *bla* genes encoding TEM-type ß-lactamase. According to the criteria for the susceptibility of *H. influenzae* to ABPC recommended by the CLSI, *H. influenzae* isolates were divided into 161 (61.0 %) susceptible strains (MIC ≤1 µg/ml), 37 (14.0 %) intermediately resistant strains (MIC =2 µg/ml), and 66 (25.0 %) resistant strains

(MIC ≥4 µg/ml). Only five strains produced the TEM type of ß-lactamase.

PCR-based genotypes and susceptibility to ampicillin. The prevalence of each PCR-based genotype among the 264 *H. influenzae* isolates were as follows: 87 (33.0 %) were gBLNAS strains, 98 (37.0 %) were Group I/II gBLNAR strains, 74 (28.0 %) were Group III gBLNAR strains, one (0.4 %) was a Group I/II gBLPACR strain, two (0.8 %) were Group III gBLPACR strains, and two (0.8 %) were a gBLPAR strain (Fig. 2).

Clonal dissemination of BLNAR. Among the 61 BLNAR strains, six clones including closely related strains were identified. As the MIC to AMP was increasing, the frequencies of the clonal dissemination were also getting high. Among the 24 strains with MIC to ABPC were four µg/ml, 26 strains with MIC to ABPC were 8 µg/ml, and strains with MIC to ABPC were ≥16 µg/ml, six (25 %), 6 (23.0 %) and seven (63.6 %) were the strains with either type of similar PFGE patterns, respectively (Fig. 3).

Discussion

The increase in the percentage of BLNAR strains leads to serious problems in the treatment of infectious disease in Japan[3]. In the current surveillance, we focused on BLNAR strains isolated from the URT in Japan. The BLNAR strains were identified in 25.0% of *H. influenzae* samples isolated from the URT while *H. influenzae* strains having the *bla* gene was identified in only 5 (1.9%) isolates. Unfortunately, there is still limited information about the precise prevalence and dissemination of antimicrobial-resistant pathogens.

The detection of BLNAR strains with decreased susceptibilities to ß-lactams is controversial. An effort to acquire a genetic understanding of the intricacies of the resistance mechanism led to the development of reliable tests for detecting BLNAS strains. In this study, we applied a PCR-based method to determine *H. influenzae* genotypes. This method allowed us to evaluate mutations of the *ftsI* gene encoding PBP3. According to the PCR-based genotyping of *H. influenzae*, gBLNAR strains were highly prevalent in Japan (65.1%), and about 86.9% of gBLNAR strains were classified into Group III gBLNAR. About 62.2% of strains intermediately resistant to ABPC were also classified as Group I/II gBLNAR strains, and about 41.6% of strains susceptible to ABPC were classified as the Group I/II gBLNAR strains. The PFGE profiles showed a clonal dissemination among strains with increased resistance to AMP (MIC =16 µg/ml). The inappropriate use of oral antibiotics for the treatment of URT infections appears to be responsible for the selection for BLNAR strains. The usage of antibiotics might be related to the dissemination of gBLNAR strains in Japan.

Conclusions

In conclusion, there is an alarming increase in Japan of the occurrence of BLNAR strains with mutations of their *ftsI* gene. Consequently, we need to continue the careful surveillance for BLNAR strains of *H. influenzae*. PCR-based genotyping and the study of the clonal dissemination bring us beneficial information to continue our surveillance of this resistant pathogen.

Figure 1. PCR-based genotypes of *H. influenzae*. Lane 1: *p6* encoding outer membrane protein P6. Lane 2: *bla*, Lane 3: *pbp3-S*, Lane 4: *pbp3-BLN*. MM: molecular weight marker.
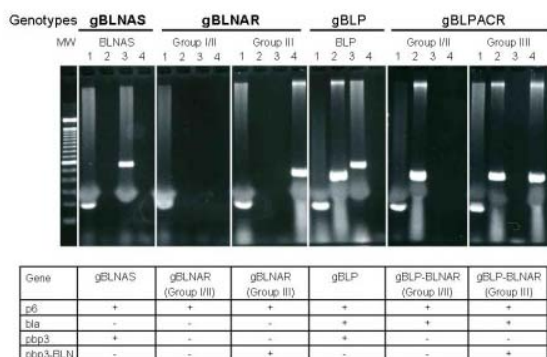
Fig.1

Figure 2. Susceptibilities of *H. influenzae* to AMP and PBP gene mutations.
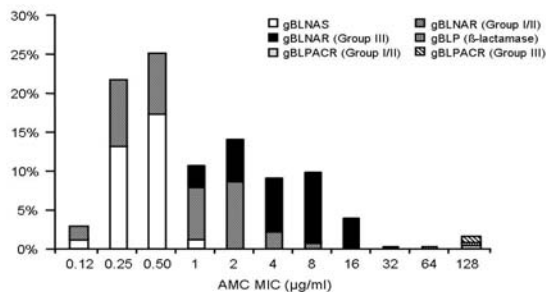
Fig.2

Figure 3. Clonal dissemination of BLNAR isolates.
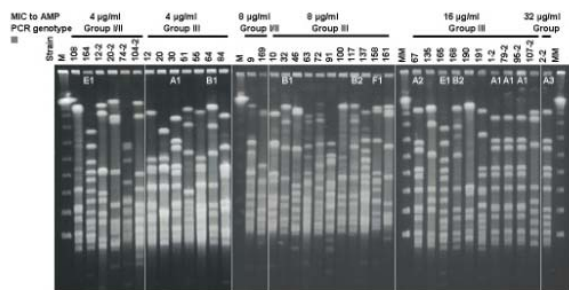
Fig.3

References

1. Hotomi M, Sakai A, Fujihara K, Billal DS, Shimada J, Suzumoto M, Yamanaka N. Antimicrobial resistance in *Haemophilus influenzae* isolated from the nasopharynx among Japanese children with acute otitis media. Acta Otolaryngol 2006;126: 130-137.
2. Ubukata K, Shibasaki Y, Yamamota K, et al. Association of amino acid substitutions in penicillin binding protein 3 with ß-lactam resistance in ß-lactamase negative ampicillin-resistant *Haemophilus influenzae*. Antimicrob Agents Chemother 2001; 45:1693-1699.
3. Hotomi M, Fujihara K, Billal DS, Suzuki K, Nishimura T, Baba S, Yamanaka N. Genetic characteristics and clonal dissemination of ß-lactamase non-producing ampicillin resistant (BLNAR) *Haemophilus influenzae* isolated from the upper respiratory tract in Japan. Antimicrob Agents Chemother 2007; 51:3969-3976.

Alloiococcus otitidis and biofilm in otitis media

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Background

The most common organisms involved in recurrent acute otitis media (rAOM) and otitis media with effusion (OME) are *S. pneumoniae*, *M. catarrhalis* and *H. influenzae*. *Alloiococcus otitidis*, more recently identified in middle ear effusions (MEE) of children may also play a role in OM pathogenesis.

Objectives

Evaluate bacterial prevalence in MEE and determine biofilm presence on mucosal biopsies.

Methods

MEE and mucosal middle ear biopsies were taken from children undergoing ear surgery. Bacterial presence in MEE was assessed using standardized culture techniques and 16S rRNA sequencing. Fluorescent in situ hybridization and confocal laser scanning microscopy (CLSM) were used to determine biofilm presence in biopsies.

Results

MEE samples from 39 ears (25 subjects) and biopsies from 31 ears (23 subjects) were collected. Indication for surgery was OME (12), rAOM (3), both (7) and CSOM (3). Microbiological culture revealed no bacteria (17 ears), mixed bacterial flora (11), *S. pneumoniae* (2), *M. catarrhalis* (2), *H .influenzae* (1), *S. aureus* (3) and *P. aeruginosa* (2).16S rRNA sequencing detected *A. otitidis* (18 ears), *S. pneumoniae* (5), *M. catarrhalis* (3), *H. influenzae* (2), Riemerella sp.(4) other bacteria (19), and no organism (7). To date we have successfully identified *H. influenzae* and *M. catarrhalis* plus other unidentified bacterial species in biofilm formation using CLSM, this is the subject of ongoing study.

Conclusions

Using 16S rRNA sequencing, *A. otitidis* is the most commonly detected bacterium, indicating this to be a sensitive and specific detection method whilst also showing multi-specie involvement in OM. Multi-specie biofilm is likely to be important in OM pathogenesis.

Vaccine/Microbiology

Protection of pneumococcal infection by maternal intranasal immunization with pneumococcal surface protein A (PspA)

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Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is responsible for a significant proportion of the bacterial infectious diseases. The acquisition of *S. pneumoniae* is as asymptomatic nasal colonization and transmission to others is thought to be primarily from carriers. Pneumococcal infections invariably begin with nasopharyngeal colonization on mucosal surfaces. Children colonized *S. pneumoniae* in their nasopharynx in the first year of life were at increased risk of developing acute otitis media (AOM) compared with children who remained free from *S. pneumoniae*.

Current polysaccharide based pneumococcal vaccines can evoke less immune responses to infants younger than 2 years old because of the weak immunogenicity as T cell dependent antigens. Moreover, otitis prone children usually showed subnormal levels of serum IgG antibody against the causative pathogens.[1]

Children younger than 2 years old also usually have lower levels of pathogen specific IgG antibody in sera depending on the age-related immaturity of immune responses.[2] If these younger children are suffering from pneumococci, especially antimicrobial resistant *S. pneumoniae*, it is difficult to eradicate the pathogen by immunological host defense as well as antibiotics use. It is important to induce effective protective immune responses against pneumococci during early childhood.

Pneumococcal surface protein A (PspA) is a surface-exposed antigen and is a virulence factor for invasive disease. The antigen is capable of eliciting protection against invasive disease and at least partial protection against carriage.[3,4] Thus, PspA is an attractive candidate for future protein based pneumococcal vaccines.

In this study, we evaluated the protection of pneumococcal infections during infant period by maternal intranasal immunization with PspA.

Materials and method

Construction of recombinant PspA

Recombinant PspA was constructed according to the sequence of TIGR4 strain. The pspA gene was amplified by PCR and the pspA gene fragment was inserted in vector pET20b (Novagen, USA).To purify the protein over-expressed by construct plasmid, we are currently using the Novagen system of binding buffers for the affinity nickel chromatography step. Screening by comassie blue staining shows a single band and we estimated rPspA to be above 90% purity.

Experimental design and immunization

BALB/c bjy mice, 4 wks old female, were used in this experiment. Mice were intranasally immunized with rPspA (1 μg) mixed with cholera toxin B subunit (CTB) (4 μg) on the Mondays and Fridays of 3 consecutive weeks. These immunization included CTB for the first 2 weeks of immunization. During the last week (i.e., the last two immunizations), mice received antigen alone. Control mice received only CTB for the first 2 weeks and only saline for the last weeks.

After the final immunization, they were mated with male mice for two weeks. Approximately 3 weeks after mating, we obtained offspring.

Systemic infection model

Offspring at 10 days old were inoculated intra-peritoneally with variable pneumococcal cells in 200 μl of sterile phosphate buffered saline (PBS) under the anesthesia. After the inoculation, these offspring were monitored for their health every 6 to 8 hours. The survival periods were expressed as times from inoculations of pneumococci to death.

Statistics

The comparisons between two groups were calculated by Mann-Whitney U test. Statistical values were calculated with Prism (SAS Inc.).

Results

Survival of offspring after infection with pneumococci at 1×10^4 CFUs per mouse was evaluated. The median, 75% value, and 25% value of survival times of offspring delivered from PspA immunized mother were 32h, 43h, and 28h. On the other hand, those of offspring delivered from control mother were 16h, 18h, and 16h (Fig. 1). Times to death of PspA immunized offspring were significantly prolonged rather than controls ($p<0.05$).

Discussion

In spite of the tremendous success of capsular polysaccharide vaccine against type b encapsulated *Haemophilus influenzae* (*H. influenzae*), the polysaccharide-based vaccines against pneumococci have some limitation for their efficacies due to wide variety of capsular serotypes of this pathogen. The polysaccharide-based vaccines can evoke less immune response to infants younger than 2 years old because of the weak immunogenicity as T cell dependent antigens. Thus, it is important to induce effective protective immune responses against pneumococci during early childhood. The maternal immunization is thought to be the most suitable approach to induce effective immune protections against pneumococcal infections.

The present study was the first report the effect of maternal intranasal immunization with PspA and CTB on systemic pneumococcal infections. Our previous study using outer membrane protein P6 of *H. influenzae* showed that maternal intranasal immunization with P6 evoked anti-P6 specific sIgA and IgG antibodies in breast milk and anti-P6 specific IgG antibody in sera. Anti-P6 specific IgG antibody in sera further transferred to their offspring via placenta.[5] In mice, IgG in mother's sera is transferred from mother to fetus through placenta by neonatal Fc receptor, FcRn, which is expressed in the yolk sacs of mice and rats.[6,7,8] Moreover, IgG in mother's breast milk is transferred from intestine lumen to systemic circulation and maintain the immune responses in neonate mice.[9] In this study, we have not addressed the levels of anti-PspA specific sIgA and IgG in mother's

breast milk and IgG in sera of offspring yet. However, maternal intranasal immunization with PspA enhanced survival times in mice with systemic lethal pneumococcal infections.

The current findings suggest that maternal intranasal immunization would be an attractive procedure against pneumococcal infections during early childhood because transplacental immunoglobulin is transferred during pregnancy and after birth.

Fig. 1
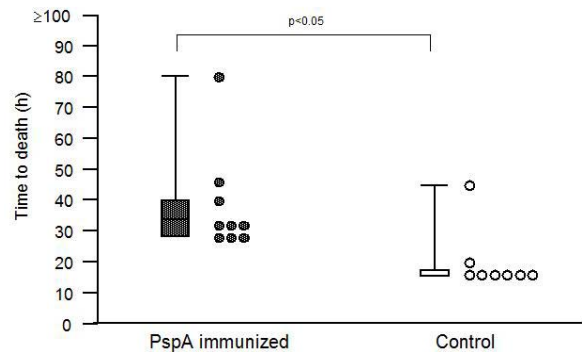
References

1. Hotomi M, Saito T, Yamanaka N. Specific mucosal immunity and enhanced nasopharyngeal clearance of nontypeable *Haemophilus influenzae* after intranasal immunization with outer membrane protein P6 and cholera toxin. Vaccine 1998; 16:1950-1956.
2. Yamanaka N, Faden H. Antibody response to outer membrane protein of nontypeable *Haemophilus influenzae* in otitis-prone children. J Pediatr 1993; 122:212-218.
3. Briles DE, Tart RC, Swiatlo E, Dillard JP, Smith P, Benton KA, Ralph BA, Brooks-Walter A, Crain MJ, Hollingshead SK, McDaniel LS. Pneumococcal diversity: considerations for new vaccine strategies with emphasis on PspA. Clin Micro Review 1998; 11:645-657.
4. Briles DE, Hollingshead SK, Brooks-Walter A, Nabors GS, Ferguson L, Schilling M, Gravenstein S, Braun P, King J, Swift A. The potential to use PspA and other pneumococcal protiens to elicit protection against pneumococcal infection. Vaccine 2000; 18:1707-1711.
5. Yamauchi K, Hotomi M, Billal DS, Suzumoto M, Yamanaka N. Maternal intranasal immunization with outer membrane protein P6 maintains specific antibody level of derived offspring. Vaccine. 2006; 24:5294-5299.

6. Roberts DM, Guenthert M, Rodewald R. Isolation and characterization of the Fc receptor from fetal york of the rat. J Cell Biol 1990; 111:1867-1876.
7. Israel EJ, Patel VK, Taylor S, Marshak-Rothstein A, Semister NE. Requirement for a beta 2-microglobulin-associated Fc receptor for acquisition of maternal IgG by fetal and meonatal mice. J Immunol 1995; 154:6246-6251.
8. Israel EJ, Taylor S, Wu Z, Mizoguchi E, Blumberg RS, Bhan A, Simister NE. Expression of the neonatal Fc receptor, FcRn, on human intestinal epithelial cells. Immunology 1997; 92:69-74.
9. Simister NE, Rees AR. Isolation and characterization of an Fc receptor from neonatal rat small intestine. Eur J Immunol 1985; 15:733-738.

Maternal intranasal immunization with outer membrane protein P6 maintain specific antibody level of derived offspring

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Introduction

Acute otitis media (AOM) is one of the most common infectious diseases in children below 2 years of age. Nontypeable *Haemophilus influenzae* (NTHi) is a leading causative pathogen responsible for AOM.[1]Recent studies of vaccines against NTHi have focused on outer membrane proteins (OMPs) which are highly conserved among NTHi and surface exposed. P6 is a peptidogly can-associated lipoprotein of 16600 dalton and shows a high degree of sequence conservation among strains.[2] Anti-P6 specific IgG antibodies had bactericidal activities, however, subnormal levels of anti-P6 specific IgG in sera were observed among otitis-prone children.[3] Furthermore, children younger than 2 years old usually have lower levels of anti-P6 specific IgG in sera depending on their immunological immaturity and result in being vulnerable to infections.[3] Thus, it is very important to induce effective protective immunity among children younger than 2 years old.

In this study, we investigated the enhancement of pathogen specific immune responses to infant mice by maternal immunization. Mother mice were intranasally immunized with P6.The induction of specific antibody in sera and breast milk in mother mice and transfer of their specific immune responses to offspring were investigated.

Materials and methods

Experimental design and immunization

BALB/c female mice were immunized with P6 and cholera toxin (CT) intranasally every two days for two weeks. After the final immunization, they were mated with male mice. After the birth, the mice were divided into 4 groups (Group A to D). Mother mice immunized with P6 breast-fed their own offspring (Group A). Mother mice immunized with P6 breast-fed offspring delivered from non-immunized mother (Group B). Mother mice with sham immunization breast-fed offspring delivered from immunized mother (Group C). Mother mice with sham immunization breast-fed their own offspring as control (Group D).

Sera and breast milk were collected from mother mice at birth (day 0), 3,7,14 days after birth.

Determination of specific antibody

Anti-P6 specific IgG, IgA and IgM were evaluated by the solid phase enzyme-linked immunosorbent assay (ELISA).

Results

Anti-P6 specific antibodies in sera of mother mice

Anti-P6 specific IgG in sera were highly induced at the birth.Anti-P6 specific IgA and IgM were also identified among P6-immunized mother mice. The levels of anti-P6 specific antibodies among pre-immunized mother mice were below the detection limits (Fig. 1).

Anti-P6 specific antibodies in breast milk of mother mice

Anti-P6 specific IgG were predominantly induced in breast milk (colostrums) rather than anti-P6 specific IgA and IgM. Anti-P6 specific IgG, IgA and IgM in sera and breast milk of control mothers were below the detection limits (Fig. 2).

The changes of anti-P6 specific IgG in sera of offspring according to feeding status.

Offspring delivered from P6-immunized mothers (Group A and Group C) already had anti-P6 specific IgG in sera at the birth. The levels of anti-P6 specific IgG in sera from offspring breast-fed by P6-immunized mothers (Group A) were then increased until day 14 and then decreased on day 21. The anti-P6 specific IgG in sera from offspring breast-fed by non-

immunized mothers (Group C) were rapidly decreased after the birth. Offspring delivered from non-immunized mother (Group B and Group D) did not have anti-P6 specific IgG in sera at the birth. The P6 specific IgG in sera from offspring breast-fed by P6-immunized mothers (Group B) gradually increased and reached the similar levels of offspring in Group A on day 3 to day 14 and then decreased on day 21. The control offspring (Group D) did not have anti-P6 specific IgG in sera (Fig. 3).

Discussion

Nasopharyngeal colonization with *H. influenzae* is the first step to develop AOM caused by this pathogen. Children colonized NTHi in the nasopharynx during the first year of life were at increased risk of developing AOM compared with children who remained free of NTHi. Effective protection against NTHi adhesion by immunization with common antigens of NTHi could results in selective reduction of NTHi load in nasopharynx.[4] Pathogen specific antibody appeared to be protective against nasopharyngeal colonization with NTHi.[5] However, young children below 2 years old show the lowest serum IgG level throughout their lives. Maternal immunization is thought to be the most appropriate approach to enhance specific immunity in early infancy.

The current findings indicate that P6-specific IgG transferred not only via placenta during pregnancy but also via breast milk after birth was very important to maintain high levels of serum anti-P6 specific IgG in offspring mice. However, predominant anti P6 specific antibody isotype was IgG. Differing from human, mice colostrums or breast milk contain high amount of IgG compared to IgA and IgM. Moreover, milk IgG is transferred from intestinal lumen to systemic circulation in neonate mice.[6] This was confirmed in our experiment that serum anti-P6 specific IgG levels of offspring were elevated by taking colostrums containing anti-P6 specific IgG. In contrast, offspring in group C nursed by control mother could not keep their serum anti-P6 specific IgG levels in spite of having the transplacental anti-P6 specific IgG at birth. These results indicate that the IgG of breast milk in mice plays a very important role to maintain the serum IgG levels of offspring.

The current findings strongly suggest that maternal intranasal immunization with P6 would be an attractive strategy against NTHi infections during early childhood. It can supply protective antibodies via transplacental during pregnancy and via breast milk after birth.

Fig. 1
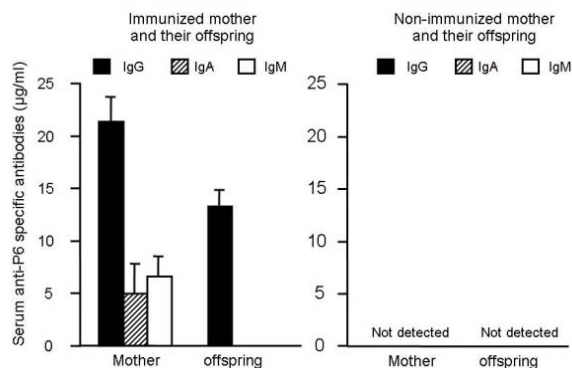
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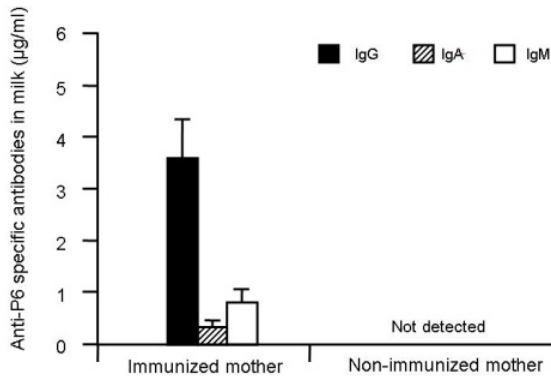
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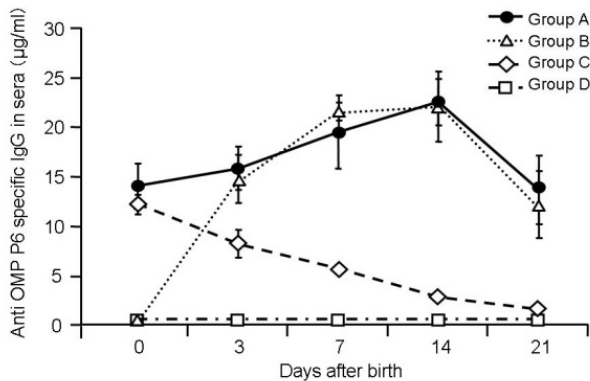
References

1. Murphy TF, Apicella MA. Nontypable *Haemophilus influenzae*: a review of clinical aspects, surface antigens, and the immune response to infection. Rev Infect Dis ; 9:1–15.
2. Hotomi M, Saito T, Yamanaka N. Specific mucosal immunity and enhanced nasopharyngeal clearance of nontypeable *Haemophilus influenzae* after intranasal immunization with outer membrane protein P6 and cholera toxin. Vaccine 1998; 16:1950-1956.
3. Yamanaka N, Faden H. Antibody response to outer membrane protein of nontypeable *Haemophilus influenzae* in otitis-prone children. J Pediatr 1993; 122:212-218.
4. Bernstein JM, Faden HS, Ogra PL. Nasopharyngeal colonization by nontypeable *Haemophilus influenzae* in children: the effect of serum bactericidal antibody. Otolaryngol Head Neck Surg 1991; 105:406-410.
5. Harabuchi Y, Faden H, Yamanaka N, Duffy L, Wolf J, Krystofik D. Nasopharyngeal colonization with nontypeable *Haemophilus influenzae* and recurrent otitis media. J Infect Dis 1994; 170:862-866.
6. Simpson-Morgan MW, Smeaton TC. The transfer of antibodies by neonates and adults. Adv Vet Sci Comp Med 1972; 16:355-386.

Evidence of biofilm in chronic suppurative otitis media in Greenland

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Objectives

Chronic suppurative otitis media (CSOM) is very frequent in the Inuit populations all over the Arctic. Risk factors are identified and the microbiology is known. Topical antibiotics and irrigation are the treatments of choice and surgery is also possible. The nature of CSOM is long lasting episodes of otorrhea with silent periods. Due to this chronic appearance we wanted to investigate CSOM patients in Greenland for presence of biofilm.

Methods

We examined six children with CSOM who met for examination and treatment at the yearly ENT-specialist visit in Ammassalik district in East Greenland. Four children with chronic OM with effusion (COME) who came for tubulation and adenoidectomy served as controls. Middle ear pus and effusions were aspirated together with a culture swab. Smears were immediately prepared on slides and heat fixated.

Results

The CSOM children were 9 - 15 years old (5 girls and 1 boy). The COME children were 2 - 5 years old (2 girls and 2 boys). We found microscopic evidence of biofilm formation in all six CSOM specimens but in none of the seven COME specimens (bilateral from three patients). In four CSOM children we cultured *Staph. aureus* with corresponding Gram-pos. cocci in the biofilms and in two we cultured *E. coli* with Gram-negative rods in the biofilms. All COME patients were culture negative.

Conclusions

The findings support the clinical appearance and the difficulties in the treatment of CSOM. Future anti-biofilm antibiotics may be of value in the treatment of CSOM.

Association between nasopharyngeal bacterial colonization during upper respiratory infection and acute otitis media

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Background

Acute Otitis Media (AOM) is one of the most common pediatric infectious diseases. Although the disease is primarily considered a bacterial infection, it is well known that viral upper respiratory tract infections (URI) predispose children to AOM and viruses alone can cause AOM 1). Three most common AOM pathogenic bacteria are *Streptococcus pneumoniae*, Non-typeable NT-*Haemophilus influenzae*, and *Moraxella catarrhalis*. These bacterial pathogens readily colonize in the nasopharynx and gain access into the middle ear during or after the URI episode. Is has been shown that nasopharyngeal bacterial colonization (NPC) during healthy state is different than NPC at the time of URI[2] and AOM.[3] Although previous studies have compared NPC during health state and URI or AOM, they have not followed children from URI onset to the development of AOM complication. We studied the relationship between NPC during URI and subsequent AOM occurrence to determine if NPC data at URI onset can be used to predict the occurrence of AOM complicating URI.

Methods

This study is a secondary analysis of data collected from January 2003 to March 2007at the University of Texas Medical Branch, Galveston (UTMB) during a prospective, longitudinal study of the pathogenesis of virus-induced AOM (Chonmaitree-unpublished data). The primary study was designed to capture all URI episodes occurring during a one-year period in healthy children aged 6 to 35 months to study the rate and characteristics of AOM following URI. Children were seen by a study physician as soon as possible after the onset of URI symptoms, followed a few days later (days 3-7 of the URI) and monitored closely for 3 weeks for AOM development.

AOM complicating URI was considered when the episode occurred within 21 days of the URI. AOM was defined by acute onset of symptoms (fever, irritability, or earache), signs of inflammation of the tympanic membrane and presence of fluid in the middle ear documented by pneumatic otoscopy and/or tympanometry. Children diagnosed with AOM were observed or given antibiotic therapy consistent with standard of care.[4]

Included in this study were URI episodes for which the child was seen by the study physician and had nasopharyngeal (NP) swabs collected within 7 days of URI onset. Excluded from the analysis were: URI episodes with NP cultures taken within 7 days of antibiotic therapy and AOM episodes without preceding URI.

Chi-square analysis was performed using STATA 9.0 © (Stata Corporation, College Station, TX) statistical software. Logistic Regression modeling was performed using SAS 9.1 (SAS Institute Inc., Cary, NC) statistical software.

Results

During the study period, there were a total of 1295 URI episodes documented in 294 patients enrolled and followed in our study. Of the total URI episodes, 709 URI episodes from 198 patients met the inclusion criteria for this analysis. Of 198 patients, 49% were male, 58% Caucasian, 29% Black, 10% Bi-racial, 3% Asian. Forty-three percent were of Hispanic or Latino ethnicity. Median age at enrollment was 12 mos.

NP culture was positive for pathogenic bacteria in 607 (85.6%) of cases. *S. pneumoniae* was isolated alone and in combination during 209 episodes (49%), NT- *H. influenzae*, 205 (34%) and *M. catarrhalis,* 417 (69%).

Two hundred forty-seven URI episodes(35%) were complicated by AOM (93 of them are bilateral). AOM diagnosis peaked on day 3 and 85% were within 7 days of URI onset. The odds ratios showed increased risk of AOM when NP were colonized with pathogenic bacteria (*S. pneumoniae,* NT- *H. influenzae* or *M. catarrhalis*), compared to no pathogen (Table 1). AOM rates were adjusted for breastfeeding, day care attendance and cigarette smoke exposure. Of the AOM diagnosed, 168 (68%) were diagnosed on the day of NP swab collection. To determine the predictive value of NP bacterial culture on AOM occurrence prior to AOM diagnosis, we excluded the URI episodes for which AOM was diagnosed at the time of NP swab

collection. In this subset of data (Table 2), odd ratios and adjusted odds ratios were similar; reduced numbers changed the significant differences in a few categories.

Discussion

Of 709 URI episodes included in this study, approximately one-third resulted in AOM. We obtained NP cultures early in the course of URI and clearly showed what had long been speculated; presence of pathogenic bacteria in the NP during URI increases the risk for AOM complicating URI. Compared to children with no pathogenic bacteria, children colonized with *S. pneumoniae,* NT- *H. influenzae*, and *M. catarrhalis* concurrently were at the highest risk for AOM, in their NP. Our data suggest that NPC results at the early URI onset may be helpful in predicting the risk of AOM complicating URI.

NPC is dynamic and the rate of NP flora turnover may differ among children and among bacterial strains.[5] NPC changes as children progress from healthy state through URI or AOM. Harrison et al[2] found that during URI children have more bacterial types and higher bacterial colony counts in the NP. Syrjanen et al[3] found that 87% of healthy *S. pneumoniae* carriers has *S. pneumoniae* at the time of AOM and only 26% of the non-carriers has *S. pneumoniae* at the time of AOM. Interestingly, the majority of *S. pneumoniae* associated AOM were due to newly acquired *S. pneumoniae* strains, not the one found during health state. These data demonstrate that in order to better understand the causal relationship between NPC and AOM; NP cultures should be obtained closer to the onset of AOM, ideally during URI.

Children in our study were seen and NP cultures obtained as early as possible after the onset of URI symptoms, usually within 2-5 days. AOM was already diagnosed at the initial visit in 68% of cases. Nevertheless, we were still able to compare NP cultures in 32% of children with URI prior to AOM development (cases diagnosed later in the first week through the third week of URI onset) to children with URI who never developed AOM. There was significant correlation between positive NPC with multiple pathogenic bacteria and AOM occurrence even after exclusion of cases diagnosed AOM at the time of NP culture collection.

It has now been accepted that viruses play a major role in the pathogenesis of AOM[1,6] and, AOM occurs mostly as a bacterial complication of viral URI. Viruses alone may also cause AOM[6]; this could be the cases of AOM for which there was no bacteria colonized in NP in our study. In general, both the URI causative virus and the colonized bacteria together play major roles in AOM pathogenesis.[1] Therefore, effective prevention of AOM will need to include both prevention of viral URI and prevention and/ or elimination of NPC with pathogenic bacteria. Further studies are required to better understand how these interventions effect bacterial and viral interactions in the pathogenesis of viral-induced AOM. Such understanding will lead to better ways for effective prevention of this high prevalent pediatric disease.

Acknowledgements

Table 1. Risk of AOM Complicating URI by Pathogenic Bacteria Colonized in the Nasopharynx at the Time of URI.

NP Bacterial colonization (n=646[a])	AOM Incidence (%)	OR, (95% CI)	p-value	Adjusted (95% CI)	OR,[b]	p-value
No Bacteria (102)	10	REF	REF	REF		REF
Sp only (55)	29	3.7 (1.6-9.0)	<0.01	3.8 (1.6 -9.1)		<0.01
Hi only (54)	43	6.8 (2.9-15.9)	<0.01	6.7 (2.8-15.9)		<0.01
Mc only (148)	32	4.4 (2.1-9.2)	<0.01	4.2 (2.0-8.8)		<0.01
Sp, Hi (18)	50	9.2 (3.0-28.5)	<0.01	8.0 (2.1-30.0)		<0.01
Sp, Mc (136)	41	6.4 (3.1-13.4)	<0.01	5.7 (2.7-12.2)		<0.01
Hi, Mc (45)	51	9.6 (4.0-23.0)	<0.01	8.4 (3.2-21.5)		<0.01
Sp, Hi , Mc (88)	51	9.6 (4.4-20.9)	<0.01	15.3 (6.0-39.6)		<0.01

[a] 63 cultures that grew other bacteria including *Staphylococcus aureus*, *Group A beta hemolytic Streptococcus*, *Group B Streptococcus*, *Diptheriods*, and *Bacillus species* are not included. Multiple bacteria listed are from the same sample. In 168 (68%) cases AOM was diagnosed at the time of NP swab collection.

[b] OR adjusted for breastfeeding, smoking and day care exposure

Abbreviations: *Sp: Streptococcus pneumoniae, Hi: Haemophilus influenzae, Mc*: *Moraxella catarrhalis.*

Table 2. Risk of AOM Complicating URI by Pathogenic Bacteria Colonized in the Nasopharynx at the Time of URI (excluding NP swabs collected at the time of AOM diagnosis).

NP Bacterial colonization (541[a])	AOM incidence%	OR, (95% CI)	p-value	Adjusted OR, [b] (95% CI)		p-value
No Bacteria (97)	5	REF	REF	REF		REF
Sp only (46)	15	3.3 (0.99 – 11.0)	0.04	3.5 (1.0 – 12.3)		0.05
Hi only (39)	21	4.7 (1.4- 15.6)	<0.01	5.2 (1.5- 17.9)		<0.01
Mc only (119)	16	3.5 (1.2 -9.7)	<0.01	3.7 (1.3 -10.3)		0.01
Sp, Hi (11)	18	4.1 (0.7 – 24.2)	0.09	10.5 (1.1 – 101.8)		0.04
Sp, Mc (96)	17	3.7 (1.3-10.5)	0.01	3.7 (1.2- 10.9)		0.02
Hi, Mc (26)	15	3.3 (0.8-13.4)	0.07	3.6 (0.8-16.3)		0.1
Sp, Hi , Mc (53)	19	4.3 (1.4-13.3)	<0.01	7.5 (1.9-30.4)		<0.01

[a] 57 cultures that grew other bacteria including *Staphylococcus aureus*, *Group A beta hemolytic Streptococcus*, *Diptheriods*, and *Bacillus species* are not included. Multiple bacteria listed are from the same sample.

[b] OR adjusted for breastfeeding, smoking and day care exposure

Abbreviations: *Sp: Streptococcus pneumoniae, Hi: Haemophilus influenzae, Mc*: *Moraxella catarrhalis.*

References

1. Chonmaitree T, Heikkinen T: Viruses and acute otitis media. Pediatr Infect Dis J2000; 19:1005-1007.
2. Harrison LM, Morris JA, Telford DR, Brown SM, Jones K: The nasopharyngeal bacterial flora in infancy: effects of age, gender, season, viral upper respiratory tract infection and sleeping position. FEMS Immunol Med Microbiol 1999; 25:19-28.
3. Syrjanen RK, Auranen KJ, Leino TM, Kilpi TM, Makela PH: Pneumococcal acute otitis media in relation to pneumococcal nasopharyngeal carriage. Pediatr Infect Dis J 2005; 24:801-806.
4. Subcommittee on Management of Acute Otitis Media: Diagnosis and management of acute otitis media. Pediatrics2004; 113:1451-1465.
5. Trottier S, Stenberg K, Svanborg-Eden C: Turnover of nontypable Haemophilus influenzae in the nasopharynges of healthy children. J Clin Microbiol1989; 27:2175-2179.
6. Chonmaitree T: Viral and bacterial interaction in acute otitis media. Pediatr Infect Dis J 2000; 19:S24-S30.

Bilateral acute otitis media (BAOM): epidemiologic, microbiologic and clinical characteristics and comparison with unilateral acute otitis media (UAOM)

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Background

Information regarding the specific characteristics of bilateral involvement in acute otitis media (AOM) is missing. Howie et al.[1] described 858 instances of AOM during 1965-1968 and reported that unilateral and bilateral ear disease were equally frequent and the mean age in bilateral AOM (BAOM) was significantly lower than in unilateral disease. Nontypeable *Haemophilus influenzae*(Hi) represented 26% of all pathogens in BAOM and only 15% in unilateral AOM (UAOM), whereas there was no significant difference for *Streptococcus pneumoniae* (Sp). We hypothesized that bilateral ear involvement in children is related to a different entity compared with UAOM in terms of its microbiology and clinical presentation.

Objectives

To describe the epidemiologic, microbiologic, and clinical characteristics of BAOM in children and compare it with unilateral AOM (UAOM).

Patients and methods

The study group consisted of 1,026 children aged 3-36 months (61% <1 year of age) with AOM seen at the pediatric emergency room during 1995-2003. Diagnosis of AOM was made if the patients had: 1) symptoms and physical findings consistent with AOM (symptoms: fever, irritability, and tugging of the ear; signs: redness and bulging of the tympanic membrane with blurring of the anatomic landmarks) and 2) an acute illness for ≤7 days. Only AOM patients with intact bulging tympanic membranes at enrollment (or purulent otorrhea of < 24 hours) were enrolled. Patients with tympanostomy tubes were excluded from the study.

All patients had tympanocentesis and middle-ear fluid (MEF) culture (Cx) performed at enrollment. Clinical status was determined by a clinical/otologic score (COS) evaluating severity (0=absent to 3=severe, maximal score 12) of patient's fever and irritability and tympanic membrane redness and bulging (Table 1). COS in BAOM evaluated the ear with more severe findings. Multivariate logistic regression models were used to estimate the risk for the BAOM patients (compared with UAOM patients) to present with a high severity COS (≥8).

Results

Forty-nine percent of patients had ≥3 previous AOM episodes and 660 (64%) did not receive antibiotic Rx for at least 48 hours before enrollment. Six hundred twenty-three (61%) patients had BAOM (Table 2). Positive MEF Cx were recorded in 786 (77%) patients. More patients with BAOM had positive MEF Cx than patients with UAOM (517/623, 83% vs. 269/403, 67%, P<0.01). Nontypeable Hi, Sp, mixed Hi and Sp, *Moraxella catarrhalis,* and *Streptococcus pyogenes* were isolated in 43%, 18%, 20%, 1%, and 1% of all BAOM patients vs. 30%, 21%, 12%, 4%, and 1% of all UAOM patients. Overall, Hi (alone and in mixed infections) was more commonly isolated in BAOM than in UAOM patients (390/623, 63% vs. 170/430, 42%, P<0.01). COS data was available in 991 (390 UAOM and 571 BAOM) patients. Overall, COS was higher in Cx (+) than in Cx (-)(8.2 vs. 7.7, P<0.001) and in BAOM than in UAOM (8.3 vs. 7.8, P=0.001) patients. More BAOM patients had COS ≥8 than UAOM patients (371, 61.7% vs. 200, 51.3%, P=0.001). COS ≥8 was found in 115 (49.1%) Cx (-) vs. 456 (60.2%) Cx (+) patients (P= 0.003). In multi-regression analysis, the estimated risk for BAOM patients (compared with UAOM patients) to present with a COS ≥8 was 1.5. The correlation between BAOM and COS ≥8 was maintained after adjustment for age, previous AOM history, and MEF Cx results at enrollment.

Discussion

The clinical differentiation between a bacterial and a non-bacterial AOM and, more specifically, between Sp and Hi AOM, may help in choosing an appropriate management for this disease. This includes a possible decision to delay treatment or to choose an appropriate antibiotic if such a treatment is considered necessary.[2] In the present study, we showed that BAOM is more frequently diagnosed in young children than UAOM, and more patients with BAOM had positive MEF cultures than did patients with UAOM. In addition, BAOM was caused more frequently by Hi and also associated with higher clinical and otologic severity than was UAOM. We showed in our study, by multivariate analysis, that the presence of bilateral ear involvement in a patient diagnosed with AOM is by itself an additional independent factor increasing the severity of the disease regardless of its pathogens. Furthermore, in a patient with fewer than three previous AOM episodes, BAOM is strongly associated with disease severity (OR 2.9 compared with UAOM), particularly in culture-positive patients.

Conclusions

1) Bilateral ear involvement in children with AOM is frequent; 2) Nontypeable Hi is more frequently involved in the etiology of BAOM than in UAOM; and 3) The clinical picture of BAOM is often more severe than that of UAOM. We suggest that the primary care physicians, at the time of diagnosis, consider bilateral ear involvement as an additional severity factor when deciding on the appropriate management of AOM.

Table 1. AOM clinical/otologic score (COS)

Score	Temperature (^{0}C)	Irritability	Redness	Bulging
0	<38.0	absent	absent	absent
1	38.0-38.5	mild	mild	mild
2	38.6-39.0	moderate	moderate	moderate
3	>39.0	severe	severe	severe*

Table 2. Characteristics of 623 patients with BAOM vs. 403 patients with UAOM

	Total (n = 1,026)	BAOM (n = 623)	UAOM (n = 403)	P value (BAOM vs. UAOM)
Age (mean ± SD)		10.6 ± 5.4	11.8 ± 6.1	< 0.001
<12 months	623/1,026 (61)*	398/623 (64)	230/403 (57)	0.03
Gender				
males	618/1,026 (60)	369/623 (59)	249/403 (62)	0.4
Ethnicity				
Muslim Bedouins	587/1,026 (57)	364/623 (58)	223/403 (56)	0.4
Jews	436/1,026 (43)	259/623 (42)	178/403 (44)	0.4
Previous AOM history				
≥ 3 episodes	493/1,008** (49)	302/615** (49)	191/393** (49)	0.9
No antibiotic treatment during last 48 hours before enrollment	660/1,025** (64)	394/623 (63)	266/402** (66)	0.3

*   in parentheses: % of all cases
**   data not available from some patients

References

1. Howie VM, Ploussard JH, Lester RL Jr. Otitis media: a clinical and bacteriological correlation. Pediatrics 1970; 45:29-35.
2. American Academy of Pediatrics and American Academy of Family Physicians. Clinical Practice Guideline. Subcommittee on Management of Acute Otitis Media. Diagnosis and Management of Acute Otitis Media. Pediatrics 2004; 113:1451-1465.

Detection of respiratory virus in acute otitis media

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Introduction

Acute otitis media (AOM) is a leading infectious disease among children and the most frequent reason for outpatient antibiotic therapy. Despite proper antibiotic treatment, middle-ear effusion may persist for weeks or months, often result in repeated courses of antibiotics, and eventually require surgical intervention. Although AOM is generally considered a bacterial infection, there is evidence that respiratory viruses have a crucial role in the etiology and pathogenesis of AOM.[1] Virus may profoundly affect the outcome of AOM. Recent studies have documented close interactions between the pathogenesis of AOM and viral infections in the upper respiratory tract.[2] Several studies have documented the presence of viruses in the middle-ear fluid (MEFs) of children with AOM. However, it is not known whether there are differences in the ability of various respiratory viruses to invade the middle-ear cavity. Knowledge of the relative frequencies of viral involvement in the middle ear would be important for the development of effective strategies to prevent AOM. In this study, we investigated the prevalence of respiratory virus in AOM children.

Materials and methods

Patients. We conducted a prospective study organized by a nationwide study group, the Advanced Treatment of Otitis Media Study Group (ATOMS) involving 20 ENT clinics from January 2006 to obtain clinical evidence of AOM in Japan; we are currently carrying on the study.

Pediatric patients with AOM were prospectively enrolled into the study. They were examined for nasopharyngeal pathogens and middle-ear pathogens at the first visit to the outpatient clinic. Nasopharyngeal swabs (NPSs) and MEFs in indicated cases were obtained in 5 ml of virus transportation medium and immediately transported to the Infection and Immunity Research Center of Wakayama Medical University.

We completed microbiological and viral analysis of 287 children who were under age 6 years and treated for AOM between January 2006 and October 2006. In total, 228 MEFs and 280 NPSs belonging to 287 AOM patients were evaluated for virus detection.

Detection of respiratory virus by polymerase chain reaction (PCR). Conventional PCR or reverse transcriptase (RT)-PCR was applied to identify respiratory syncytial virus (RSV), influenza virus type A and B (FluA and FluB), adenovirus, and human metapneumovirus (hMPV) in the NPSs and MEFs of AOM children.[3] All samples were amplified by three steps of PCR procedures as follows. All samples for determining respiratory virus were kept in -80 °C until examination. Viral DNA or RNA was extracted from 200 µl of samples by using the High pure Viral Nucleic Acid kit (Roche, Japan). The complementary DNA was synthesized from 5 µl of nucleic acid extract by using Superscript first-strand synthesis system for RT-PCR (Invitrogen, Japan). PCR was conducted as usual laboratory procedures. The PCR products were fractionated by size by means of electrophoresis on a 2 % agarose gel, and then visualized by ethidium bromide under ultraviolet light (Fig. 1.).

Results

Detection of virus among AOM children. A total 287 children with AOM were enrolled into this study. Among the 287 children, the respiratory viruses were detected in 67 children (23.3%).

Detection of virus in nasopharyngeal swabs. In 280 NPS samples, the respiratory viruses were found in 57 samples (20.4%) (Fig. 2). We detected 4 RSV, 11 hMPV, and one adenovirus in NPSs. Adenovirus was detected only in NPS. The rate of samples with virus alone, combined virus and bacteria, and bacteria alone were 0.4%, 20.0%, and 76.8%, respectively (Fig. 2). There were very few numbers of

samples that contained virus only. Most of the samples contained both virus and bacteria.

Detection of virus in MEFs. In 228 MEF samples, respiratory viruses were identified in 22 MEF samples (9.6 %) (Fig. 3). We detected 4 RSV and 5 hMPV in MEFs. The rate of samples with virus alone, combined virus and bacteria, and bacteria alone were 1.8%, 7.9%, and 55.7%, respectively (Fig. 3).

Discussion

While AOM is generally considered a bacterial disease, AOM is more likely a bacterial complication of viral upper respiratory infections.[1] However, a virus agent alone will cause signs and symptoms of AOM. A better understanding of the mechanism of viral and bacterial interaction in AOM will lead to new strategies for more effective treatment. Previous studies have shown various evidence regarding the role of viruses in AOM.[1,2] Several mechanisms and hypothesis have been suggested for roles of virus in the pathogenesis of AOM. They delay the clearance of bacteria from middle-ear cavity, decrease of antibiotic penetration due to local inflammation caused by viral infection, and increase nasopharyngeal colonization of causative bacteria. Case control studies that investigate the spectrum and prevalence of viruses between upper respiratory tract infections with and without complicated AOM would reveal much more important evidence concerning the role of viruses in AOM.[1]

In the literature, overall viruses have been detected in 8-25% of MEFs in children with AOM. Uhari et al found significantly higher RSV in AOM patients compared with controls.[3] Furthermore, differences in the methods for detecting viral infections used in earlier studies limit the comparability of the frequencies. In this study, we applied PCR to identify particular respiratory viruses (RSV, hMPV, FluA, FluB, and adenovirus) and found that at least 23.3% of the children with AOM were positive for these viruses. In our samples, hMPV appeared as the most frequent virus both in the middle ear (16%) and nasopharynx (33%). hMPV is a novel paramyxovirus described in 2001 that has been associated with acute upper and lower respiratory infections. Previous studies reported that 0.7-2.3% of hMPV in MEF samples belong to AOM patients, which is relatively lower than our frequency of 7%.[3] One reason would be the existence of four subtypes of hMPV. If the design of PCR primers will not cover all these subtypes, the PCR analysis may fail to detect all hMPV cases. Another reason, as a common characteristic of RNA virus, hMPV is a very fragile virus, and the detection in old sample materials stocked for a long time may cause loss of virus particles.

Most of the previous AOM studies reported that viral infections frequently co-existed with different types of bacteria rather than isolated virus-alone infections.[4] Our findings also showed similar frequency with 0.4 and 1.8% of virus-alone isolation in MEFs and NPSs, respectively. These results show that virus may function as triggering co-factor for the super-infection of bacteria rather than primary pathogens in the pathogenesis of AOM. The detection spectrum of our study could not cover all types of viruses related to upper respiratory tract infections including coronavirus, rhinovirus, parainfluenza virus, and enterovirus.[3] Development of procedures for detecting novel virus types may increase the prevalence of viruses.
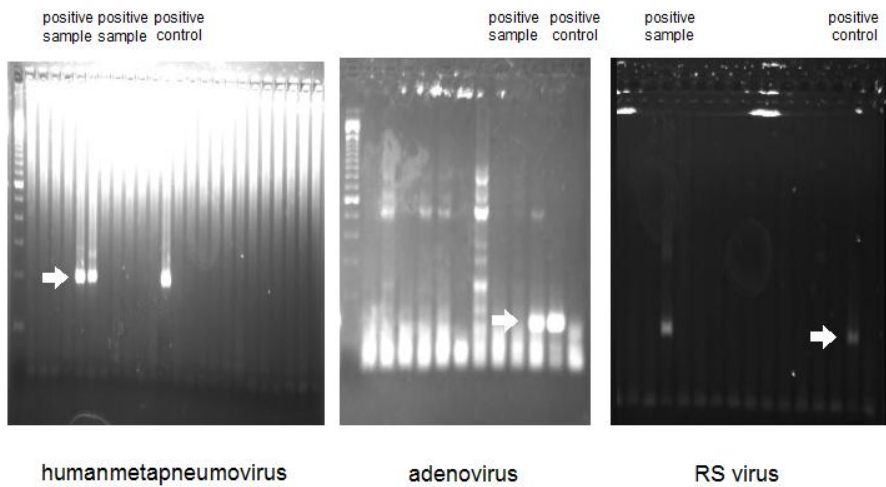
Figure 1. The PCR products were fractionated by size by means of electrophoresis on a 2 % agarose gel, and then visualized by ethidium bromide under ultraviolet light.
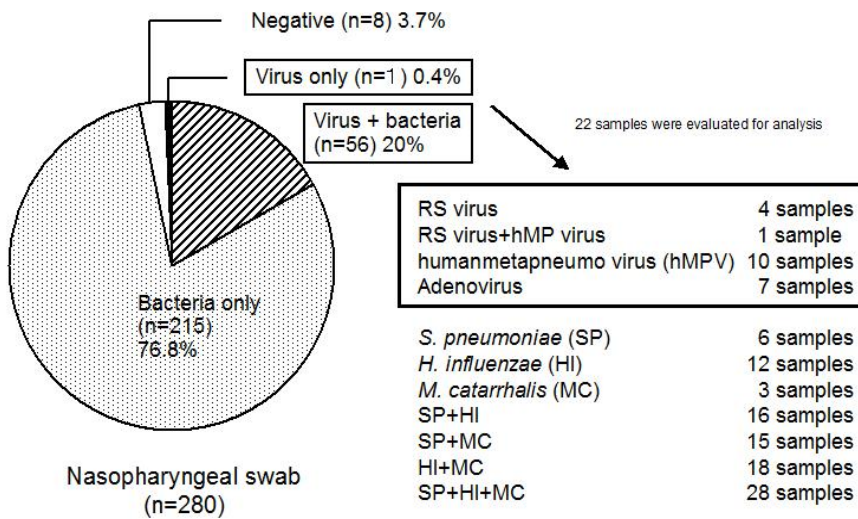
Figure 2. In 280 NPS samples, the respiratory viruses were found in 57 samples (20.4%).
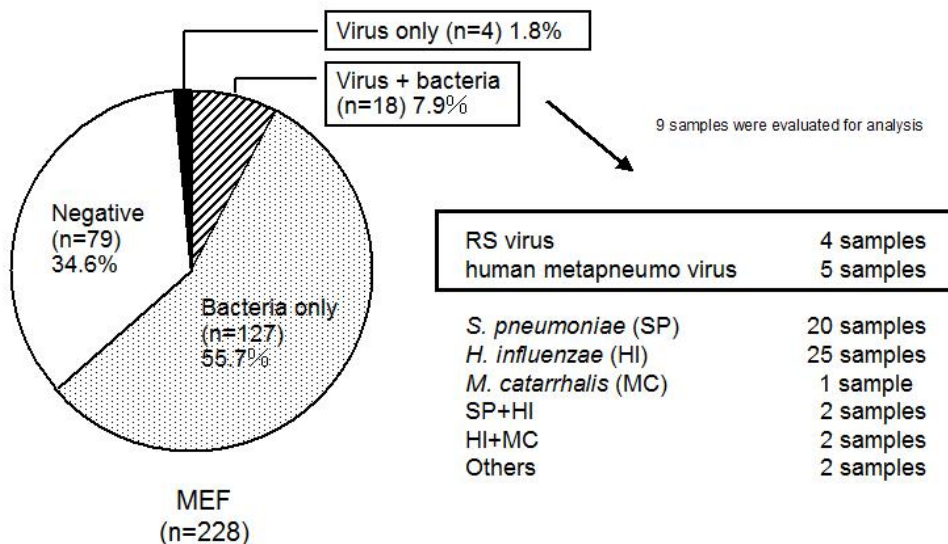
Figure 3. In 228 MEF samples, respiratory viruses were identified in 22 MEF samples (9.6 %).

References

1. Heikkinen T, Thint M, Chonmaitree T. Prevalence of various respiratory viruses in the middle ear during acute otitis media. N Engl J Med 1999; 340:260-264.
2. Uhari M, Hietala J, Tuokko H. Risk of acute otitis media in relation to the viral etiology of infections in children. Clin Infect Dis 1995; 20:521-524.
3. Ishibashi T, Monobe H, Nomura Y, Shinogami M, Yano J. Multiplex nested reverse transcription polymerase chain reaction for respiratory viruses in acute otitis media. Ann Otol Rhinol Laryngol 2003; 112:252-527.
4. Sung BS, Chonmaitree T, Broemmelling LD, Owen MJ, Patel J, Hedgpeth DC, Howie VM. Association of rhinovirus infection with poor bacteriologic outcome of bacterial-viral otitis media. Clin Infect Dis 1993; 17:38-42.

Methicillin-resistant *Staphylococcus aureus* otorrhea in chronic suppurative otitis media – a multicenter study

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Objectives

With the development and widespread use of antibiotics, the types of pathogenic microorganisms and their resistance to antibiotics have changed. Knowledge of methicillin-resistant *Staphylococcus aureus* (MRSA) and resistance rates of MRSA are important for determining the appropriate treatment for patients with chronic suppurative otitis media (CSOM). We investigated the MRSA of CSOM and evaluated the trends of annual isolation rates of MRSA during a 5-year period.

Subjects and methods

A retrospective study of 1,360 patients with CSOM seen at six hospitals in Korea from January 2001 to December 2005.

Results

A total of 1,161 bacteria were identified from 1,360 patients with CSOM. Staphylococcus accounted for 628 (54.1%) from all identified bacteria. MRSA were in 288 (45.9%) along with Staphylococcus. Detection rate of MRSA was not decreased. MRSA isolated both from patients with noncholesteatomatous otitis media and those with cholesteatomatous otitis media showed 100% sensitivity to vancomycin, and high sensitivity to teicoplanin (85.3%) and to sulfamethoxazole/trimethoprim (85.1%). In contrast, these strains showed no or low sensitivity to oxacillin, clindamycin, penicillin, and erythromycin. MSSA showed 100% sensitivity to vancomycin, teicoplanin, and oxacillin; high sensitivity (>80%) to sulfamethoxazole/trimethoprim and cephalothin; and low sensitivity only to penicillin. Coagulase-negative Staphylococcus showed 100% sensitivity to vancomycin and teicoplanin, but lower sensitivity to the other antibiotics in comparison with MRSA.

Conclusions

MRSA were most the most frequent identified bacteria from all staphylococcus in CSOM. The detection rate of MRSA is unchanged during the most recent 5 years. Continuous and periodic surveillance of MRSA is necessary.

Nasopharyngeal flora in otitis-prone children

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In the debate of choosing the right and optimal treatment for the odd episode of acute otitis media (AOM) as well as for recurrent AOM episodes, cultures from the nasopharynx are sometimes retrieved. To evaluate the use of these cultures, samples from young, otitis-prone children during healthy periods and episodes of AOM were studied. All bacteria were typed according to standard methods and the treatment possibilities were recorded. The children were followed during a period of 3 years with scheduled "healthy" visits and visits at suspected AOM.

All children were seen by the investigators at the ENT department. At all visits, samples from the nasopharynx were retrieved. The samples were divided into healthy/AOM, winter/summer, and the age of the children. In some instances of AOM, samples from the middle-ear effusions were also studied and compared to the findings in the nasopharynx. In young children (under age 12 months) suffering from AOM there was predominance of pneumococci, while *Haemophilus influenzae* was increasingly common in the older children. *Moraxella catarrhalis* was present in more than 70% of all healthy visits and in slightly fewer samples from AOM visits, demonstrating the very high carriage rate of these bacteria in small children.

Vaccines for otitis media: mining the *Moraxella catarrhalis* genome

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Moraxella catarrhalis is the third most common cause of otitis media, causing ~4.5 million episodes annually in the US. *M. catarrhalis* is also an important cause of lower respiratory tract infection in adults with chronic obstructive pulmonary disease (COPD), causing 2 to 4 million exacerbations of COPD annually in the US, second in frequency only to *Haemophilus influenzae*. Therefore, in order for vaccines to have a maximum impact on otitis media and COPD, preventing *M. catarrhalis* infections is critical. Such a vaccine, combined with effective vaccines for *H. influenzae* and the pneumococcus would have an enormous human and economic benefit.

Analysis of the genome of *M. catarrhalis* identified 348 open reading frames that encode potentially surface expressed proteins. These genes have been studied using the following approaches to identify novel vaccine antigens: 1) A microarray has been constructed and competitive hybridization has identified 147 genes that encode conserved putative surface proteins. 2) Analysis of bacterial RNA from bacteria grown in human sputum has identified genes that encode surface proteins whose transcription is up-regulated in the human respiratory tract. 3) Immunoassays have identified surface proteins that are expressed during human infection. 4) Novel recombinant proteins have been purified and antiserum has been raised. 5) Antisera to selected surface proteins are bactericidal. 6) Immunization with selected proteins induced protection in the mouse pulmonary clearance model.

This work has identified conserved surface proteins of *M. catarrhalis* that are expressed during human infection and that induce protective responses in animal model and *in vitro* systems. These proteins represent excellent potential vaccine antigens.

Promiscuous epitope peptides of p6 outer membrane protein of non typeable *Haemophilus influenzae*

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Background

Nontypeable *Haemophilus influenzae* (NTHi) is the cause of recurrent episodes of acute otitis media during childhood. In recent years, the development of a vaccine against NTHi infection has become an urgent necessity owing to the prevalence of antibiotic-resistant strains. The P6 outer membrane protein has been proposed as a possible candidate for vaccine formulation.[1-5] However, otitis-prone children show an impaired immune response to P6.[6] Therefore, the enhancement of antigen immunogenicity may be necessary for developing an effective P6-vaccine formulation. Recently, a peptide vaccine containing several T cell and/or B cell epitopes has garnered attention for a more effective immune response and fewer side effects. We previously identified human T-cell epitope peptide and highly immunogenic analog peptides on P6 which were restricted by HLA-DR9.[7]

Purpose

The aim of this study was to determine epitope peptides on P6 recognized by multiple HLA class II molecules.

Methods

P6 or peptide specific T cell lines (TCLs)were established with CD4-positive T cell and the dendritic cells (DCs) as reported previously.[7] To determine HLA class II restriction, we used monoclonal antibodies specific to HLA molecules and EBV-transformed B lymphoblastoid cell lines as antigen presenting cells.

Results

We established P6 specific TCLs from 2 healthy donors. These TCLs responded to same peptide in the context of HLA-DR4 and HLA-DR15 which are major HLA haplotype in the world, respectively. These data indicate that this epitope peptide was promiscuous. In addition to stimulating with each of the 2 peptides which are potentially promiscuous in the selection by SYFPEITHI, using a software program for a database of MHC ligands and peptide motifs, we obtained

peptide specific TCLs. Interestingly, each TCL also responded to P6.

Conclusions

On the basis of these findings, it suggested that the use of a polypeptide vaccine conjugated to these promiscuous peptides could be effective for a broad population.

References

1. Murphy TF, Nelson MB, Dudas KC, Mylotte JM, Apicella MA. Identification of a specific epitope of *Haemophilus influenzae* on a 16,600-dalton outer membrane protein. J Infect Dis 1985;152:1300-1307.
2. Murphy TF, Bartos LC, Campagnari AA, Nelson MB, Apicella MA. Antigenic characterization of the P6 protein of nontypeable *Haemophilus influenzae*. Infect Immun 1986;54:774-779.
3. Murphy TF, Kirkham C, Lesse AJ. Construction of a mutant and characterization of the role of the vaccine antigen P6 in outer membrane integrity of nontypeable *Haemophilus influenzae*. Infect Immun 2006;74:5169-5176.
4. Harabuchi Y, Faden H, Yamanaka N, Duffy L, Wolf J, Krystofik D. Nasopharyngeal colonization with nontypeable *Haemophilus influenzae* and recurrent otitis media. Tonawanda/Williamsville Pediatrics. J Infect Dis 1994;170:862-866.
5. Harabuchi Y, Faden H, Yamanaka N, Duffy L, Wolf J, Krystofik D. Human milk secretory IgA antibody to nontypeable *Haemophilus influenzae*: possible protective effects against nasopharyngeal colonization. J Pediatr 1994;124:193-198.
6. Yamanaka N, Faden H. Local antibody response to P6 ofnontypable *Haemophilus influenzae* in otitis-prone and normal children. Acta Otolaryngol 1993;113:524-529.
7. Ishida Y, Abe Y, Yanai M, Kobayashi H, Harabuchi Y. Identification of human T-cell epitopes and highly immunogenic analog peptides on the non-typeable *Haemophilus influenzae* P6 outer membrane protein. Clin Immunol 2006;121:90-99.

Construction and immunogenicity of recombinant adenovirus vectors expressing the HMW or Hia adhesion proteins of nontypeable *Haemophilus influenzae*

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Introduction

Otitis media caused by nontypable *Haemophilus influenzae* (NTHi) is major pediatric health problem and vaccines capable of preventing disease are much needed. Adenoviruses are promising live recombinant vaccine vectors that have the ability to stimulate mucosal immunity in the upper respiratory tract. The HMW1, HMW2 and Hia proteins are all critical adhesins and potential protective antigens expressed by NTHi. The objective of the present study was to assess the immunogenicity of recombinant adenovirus vectors expressing the HMW1, HMW2, or Hia adhesion proteins of NTHi when delivered by a parenteral or mucosal route.

Materials and methods

Plasmids pDC316 and pBHGloxΔE1,3Cre (Microbix Biosystems®) were used for adenoviral vector construction. pDC316 is an E1 shuttle plasmid, derived from the left end of adenovirus 5 genome that contains the human cytomegalovirus immediate early promoter, a polycloning site, and SV40 polyadenylation signals. pBHGloxΔE1,3Cre is a complementary plasmid derived from the nearly full-length adenovirus 5 genome that contains deletions in the adenovirus E1 and E3 regions. Co-transfection of recombinant pDC316, containing either an HMW1, an HMW2, or an Hia gene insert, and pBHGloxΔE1,3 Cre into 293 cells allowed for the generation of infectious recombinant adenovirus vectors by in vivo recombination.

Results

Segments of the *hmw1A* and *hmw2A* structural genes that encode the distal one-half of the mature HMW1 or HMW2 proteins were cloned into the T7 expression vector, pGEMEX-1 (Promega®). These constructs encoded stable HMW1 or HMW2 recombinant fusion proteins that express B-cell epitopes common to most NTHi strains. A segment of the *hia* structural gene that encodes the surface-exposed portion of the mature Hia

protein was cloned into pGEMEX-2. Each of the three GEMEX-adhesin gene fusions was then excised from the respective parent plasmid and cloned into pDC316. Co-transfection of 293 cells with the recombinant pDC316 constructs and pBHGlox resulted in the production of viral plaques from which recombinant adenovirus expressing the GEMEX fusion proteins were recovered. Plaques were purified and high-titer viral stocks were prepared.

Mice were immunized intraperitoneally with a single dose of 10^8 pfu of the HMW1, HMW2 or Hia adenovirus construct. One month after immunization, all mice had developed anti-HMW or anti-Hia serum antibody demonstrable by Western blot analysis and ELISA antibody titers $\geq$400 EU. Chinchillas were also immunized intraperitoneally with 10^8 pfu of the HMW2 or Hia construct. One month after immunization, all chinchillas had developed anti-HMW or anti-Hia serum antibody detectable by Western blot and ELISA titers $\geq$1000 EU. Finally, chinchillas were immunized intranasally with 10^7 to 10^9 pfu of the HMW2 or Hia construct. Two months after immunization, all animals had developed anti-HMW or anti-Hia serum antibody detectable by Western blot and serum ELISA titers $\geq$1000 EU. Higher intranasal immunizing doses were associated with higher serum antibody responses.

Conclusions

Recombinant adenoviruses represent a promising system to achieve mucosal and systemic immunity and protection from mucosal diseases such as otitis media. Recombinant adenovirus vectors expressing the recombinant HMW or Hia proteins will be important new tools in NTHi vaccine development efforts.

Nasal vaccination with new DNA adjuvants elicit long lasting immunity mediated by both Th1- and Th2-type cytokine and NALT dendritic cells

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Background

Nasal administration has been shown to preferentially induce the Ag-specific Ab responses in the upper respiratory tract as well as other mucosal lymphoid tissues, whereas oral immunization is more limited to induction of immunity in the gastrointestinal (GI) tract.[1-2] In order to elicit maximal levels of Ag-specific immune responses in both mucosal and systemic lymphoid tissue compartments, it is essential to employ an appropriate mucosal adjuvant.[2] Although cholera toxin (CT) is an effective mucosal adjuvant in animal models, its innate toxicity limits its usage in humans. In this regard, genetically manipulated non-toxic mutants of CT (mCT) have been developed by using site-directed mutagenesis. Nasal administration of mCTs as adjuvant resulted in Ag-specific S-IgA Ab responses in various mucosal external secretions of mice and non-human primates.[3] Despite these reports, the development of effective and reliable mucosal adjuvants that can be safely co-administered with vaccine Ag is of central importance for new-generation vaccines. Our previous studies showed that a plasmid encoding the Flt3 ligand cDNA (pFL) as nasal adjuvant enhanced both mucosal and systemic antibody (Ab) responses, and were mediated by IL-4 producing CD4+ T cells.[4] In this study, we examined whether a combination of pFL and synthetic CpG ODN as mucosal adjuvants would elicit enhanced mucosal immune responses.

Methods

BALB/c mice were nasally immunized once a week for three consecutive weeks with ovalbumin (OVA) plus pFL and / or CpG ODN. OVA-specific Ab responses in plasma and external secretions were monitored for up to 25 weeks by ELISA. Th1- and Th2-type cytokine responses by OVA-specific CD4-positive (CD4+) T cells were determined by cytokine-specific ELISA and quantitative RT-PCR using a LightCycler. Frequencies of dendritic cell (DC) subsets in nasopharynx associated lymphoid tissue (NALT) were examined by flow cytometry.

Results

Mice immunized with OVA together with both pFL and CpG ODN had significantly higher levels of OVA-specific plasma IgG Ab and S-IgA Ab responses in saliva when compared with mice immunized with CpG ODN only. Although comparable levels of OVA-specific plasma IgG Ab responses were induced by pFL and a combination of pFL and CpG ODN, elevated levels of anti-OVA IgG2a Ab responses were only seen when both pFL and CpG ODN employed as nasal adjuvant. These OVA-specific Ab responses were maintained until 25 week after the last nasal immunization. Furthermore, the frequency of NALT DCs, especially plasmacytoid DCs, was significantly increased by a combination adjuvant.

Conclusions

These results suggest that a combination of pFL and CpG ODN as nasal adjuvant effectively induce long lasting Ag-specific Ab responses mediated by increased NALT DCs in addition to Th1- and Th2-type cytokines produced by CD4+ T cells. This work is supported by NIH grants AG 025873, DE 12242, AI 18958 and AI 043197.

References

1. Mestecky J, Blumberg RS, Kiyono H, McGhee JR. The mucosal immune system. InPaul WE, ed. *Fundamental Immunology*. 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2003: 965–1020.
2. Imaoka K, Miller CJ, Kubota M, McChesney MB, Lohman B, Yamamoto M, Fujihashi K, Someya K, Honda M, McGhee JR, Kiyono H. Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus specific immune responses in reproductive tissues. J Immunol. 1998; 161: 5952-5958.

3. Yamamoto S, Kiyono H, Yamamoto M, Imaoka K, Fujihashi K, Van Ginkel FW, Noda M, Takeda Y, McGhee JR. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. ProcNatl Acad SciUSA 1997; 94: 5267-5272.
4. Kataoka K, McGhee JR, Kobayashi R, Fujihashi K, Shizukuishi S, Fujihashi K. Nasal Flt3 ligand cDNA elicits CD11c(+)CD8(+) dendritic cells for enhanced mucosal immunity. J Immunol 2004; 172: 3612-3619.

Intranasal immunization with phosphorylcholine enhanced the clearance of *S. pneumonia* and nontypeable *H. influenza* from the nasal cavity

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Background

Streptococcus pneumoniae and Haemophilus influenzae are major causative agents of upper airway infections such as acute otitis media (AOM) and paranasal sinusitis. Since the prevalence of antibiotic-resistant bacteria has increased in recent years, the development of vaccines against antibiotic-resistant pathogens is an important goal of public health. Phosphorylcholine (PC) is a structural component of a wide variety of pathogens including Streptococcus pneumoniae and Haemophilus influenzae, and the anti-PC immune responses are known to protect mice against invasive bacterial diseases. Previously, we have demonstrated that intra-nasal immunization with PC increased mucosal as well as systemic immune responses specific to PC in mice, and that the PC-specific antibodies in saliva and sera cross-reacted against several different strains of S. pneumoniae and H. influenzae. In the present study, we analyzed the effect of intra-nasal immunization with PC on the aspect of bacterial clearance from nasal cavity in order to estimate the potentiality of PC as a mucosal vaccine against upper respiratory infection included otitis media.

Methods

The mice were divided into two groups. One group was intranasally immunized with PC-keyhole limpet hemocyanin (KLH) (50 µg/ mouse) and cholera toxin (CT) (1 µg/ mouse) as a mucosal adjuvant. The antigens, diluted in 10 µl of phosphate buffered saline (PBS), were dropped into the nostrils using a pipette. In the other group (the control group), 10 µlof PBS with CT (1 µg / mouse) was instilled intranasally. Inoculations followed a schedule of once per week for three consecutive weeks. Balb/c mice were immunized intranasally three times with PC-keyhole limpet hemocyanin (KLH) together with cholera toxin (CT) as a mucosal adjuvant or CT only. One strain of NTHi was cultured on chocolate agar plates overnight at $37° C$ in a 5% CO_2 incubator, and one strain of S.

pneumoniae on blood agar plates. The cultured bacteria were removed by scraping and suspended in culture medium (10^9 cfu/ml) for nasal challenge. 10-µl aliquots of the live *S. pneumoniae* or NTHi suspension were administered intranasally one week after the last immunization. The mice were sacrificed 12 hours after bacterial inoculation, and nasal washes and nasal passage mucosa specimens were obtained. Nasal mucosa samples were homogenized in 1 ml of culture medium and filtered through a stainless steel screen. Samples of the filtrates and nasal washes were diluted with culture medium in 10-fold steps, and 100-µl aliquots of the diluted samples were spread on blood agar plates or chocolate agar plates. After overnight incubation at $37° C$ in a 5% CO_2, the numbers of colonies were counted.

Results

The numbers of colonies of S. pneumoniae cultured from both nasal washes and nasal passage mucosa were significantly smaller in mice intranasally immunized with PC-KLH than in control mice intranasally immunized with CT alone. The same findings were observed in the clearance of H. influenzae.

Discussion

Surface PC may contribute to the persistence of NTHi in the respiratory airway [1,2]. A gradual selection for variants expressing PC was observed during nasopharyngeal carriage in infant rats, and PC-expressing strains were found to predominate in the human respiratory tract [2]. Another study showed that the presence of PC on the cell surface of NTHi strains promotes enhanced nasopharyngeal colonization and development of AOM in the chinchilla model [1]. In *S. pneumoniae*, PC is necessary for normal growth and cell division [3]. The entry of *S. pneumoniae* into human endothelial cells is dependent upon the interaction between PC on the bacterial surface and the receptor for platelet-activating factor (PAF) [4]. Similarly, a

particular subset of NTHi LOS glycoforms containing PC uses the PAF receptor to mediate the bacterial invasion of bronchial cells [5]. Those findings suggest that expression of PC on bacterial surfaces is associated with bacterial pathogenesis and that PC-specific immune responses might affect invasive pathogens but not strains carried asymptomatically in the respiratory tract. In summary, we demonstrated that bacterial clearance of *S. pneumoniae* and *H. influenzae* from the nasal tract was significantly enhanced in the nasal immunization with PC-KLH and CT. Thus, intranasal vaccination with PC might help to prevent upper airway infections caused by *S. pneumoniae* and *H. influenzae*.

References

1. Tong HH, Blue LE, James MA, Chen YP, DeMaria TF. Evaluation of phase variation of nontypeable *Haemophilus influenzae* lipooligosaccharide during nasopharyngeal colonization and development of otitis media in the chinchilla model. Infect Immun 2000;68:4593-4597.
2. Weiser JN, Pan N, McGowan KL, Musher D, Martin A, Richards J. Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. J Exp Med 1998;187:631-640.
3. Garcia-Bustos JF, Tomasz A. Teichoic acid-containing muropeptides from *Streptococcus pneumoniae* as substrates for the pneumococcal autolysin. J Bacteriol 1987;169:447-453.
4. Cundell DR, Gerard NP, Gerard C, Idanpaan-Heikkila I, Tuomanen EI. *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. Nature 1995;377:435-438.
5. Swords WE, Buscher BA, Ver Steeg Ii K, Preston A, Nichols WA, Weiser JN, Gibson BW, Apicella MA. Non-typeable *Haemophilus influenzae* adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. Mol Microbiol 2000;37:13-27

A mutation on galE gene from *Moraxella catarrhalis* is responsible for the reactivity to a bactericidal anti-LOS rabbit serum elicited from the conjugate vaccine

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Introduction

Moraxella catarrhalis is a gram-negative human respiratory pathogen that causes 15 to 20% of acute otitis media in children.[1] In addition, this bacterium is responsible for 10 to 35% of lower respiratory infections in adults with chronic obstructive pulmonary disease, the fourth leading cause of death in the United States.[2] Since greater than 90% of current *M. catarrhalis* isolates were β-lactamase-positive, the attention to protect human beings from the infection of *M. catarrhalis* has focused on the possibility of vaccination. There have been a number of putative vaccine candidates described for *M. catarrhalis*, One of the most prominent surface components expressed in the outer membrane of this bacterium is the lipooligosaccharide (LOS). The LOS of *M. catarrhalis* is similar to the lipooligosaccharide (LPS) of other gram-negative organism, but it lacks O-specific polysaccharide repeating units. Previous studies have suggested that the LOS is not only a potential virulence factor but also a vaccine candidate since it shows better antigenically conserved among strains than does the LOS of other gram-negative bacteria. We previously synthesized conjugate vaccines from three serotypes A, B, and C.[3-5] The conjugate vaccines elicited high levels of anti-LOS antibodies with bactericidal activity in both mouse and rabbit models. However, the main epitope in LOSs to be recognized by the functional antibodies remains unknown. In this study, an isogenic mutant 26404galE with a specific truncated LOS structure was constructed and investigated for its biological functions.

Materials and methods

1. *Bactericidal anti-LOS rabbit serum.* The serum was elicited from a serotype C conjugate vaccine dLOS-TT plus Ribi adjuvant.[5]

2. *Strains and primers.*

Strains and Primers	Description	Source
M. catarrhalis 26404	Wild-type strain (serotype C)	CCUG *
M. catarrhalis 26404galE	galE knockout mutant strain	This study
galE-F	5'-ctc<u>gtcgac</u>ctgatacaggcgggatttgt-3' (SalI site underlined)	This study
galE-R	5'-ctc<u>gaattc</u>tggcataataggctgcaatg-3' (EcoRI site underlined)	This study
galE-F2	5'-ttgggatagggttgttccag-3'	This study
galE-R1	5'-atcatgtggcactggtcaaa-3'	This study

* Culture Collection of the University of Goteborg

3. *Cloning of galE homologue and construction of the knockout galE mutant.* The putative *galE* homologue was amplified using primers galE-F and galE-R. The PCR products were cloned into pCR2.1 using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) respectively to obtain pCR-galE. The respective insertion was released by EcoRI-SalI digestion and then subcloned into an EcoRI-salI site of SK(+) to form SK-galE. A kanamycin resistance cassette (1,282 bp) was subsequently inserted into the galE gene to form SK-galE-Kan. After verification by sequence analysis, the mutagenic constructs were amplified and used for electroporation. The resulting kanamycin-resistant colonies were selected and confirmed by sequence analysis, and named as 26404galE.

4. *Western blotting.* One mL of each bacterium was adjusted to OD600 = 0.35 in 0.5% DPBSG. After

washing and centrifugation, the bacterial pellets were suspended in 50-µL lysis buffer for SDS-PAGE followed by Western blotting.[5]

5. *Enzyme-linked immunosorbent assay (ELISA).* ELISA was performed for testing the binding activity of the purified LOSs to the bactericidal anti-LOS rabbit serum as described previously with modifications.[5] Briefly, after overnight coating with a 100 µl sample of LOS at 10 µg/ml, the plate was blocked and the rabbit serum was serially diluted and added for 2 hours followed by alkaline phosphatase-conjugated goat anti-rabbit IgG for 1.5 hours. The reactions were read at A_{405} after 1 hour with a substrate.

6. *Flow cytometry.* Logarithmic phase cultures of strain 26404 and 26404galE were washed and resuspended in pre- or anti-LOS rabbit serum and incubated. After washing, incubated with FITC-labeled goat anti-rabbit IgG (Advanced Targeting Systems). The cells were washed again for flow cytometry analysis (Coulter XL-MCL; Beckman Coulter). Approximately 10,000 cells were counted and their relative fluorescence measured.

7. *Bactericidal assay.* Bactericidal anti-LOS rabbit serum was inactivated at 56°C for 30 minutes and both strains 26404 and 26404galE were tested for the bactericidal activity against *M. catarrhalis* with the inactivated serum as described previously.[5]

8. *Mass spectrometric analysis.* LOS was analyzed by MALDI-TOF mass spectrometry using an Applied Biosystems QSTAR® XL System with the MALDI™ 2 source with a high performance quadrupole Time-of-Flight (QqTOF) mass spectrometer. The glycosyl linkage analyses of partially methylated aldiol acetate were carried out using the NaOH method.

Results

Construction of knockout galE mutant 26404galE. Primers galE-F2 and galE-R1 were used to amplify the *galE* gene from kanamycin-resistant colonies. The sequence analysis of PCR products confirmed that the kanamycin resistance cassette was inserted into *galE* gene of 26404 chromosomal DNA at the predicted position. The clone was named as 26404galE. Southern blotting confirmed only one kanamycin resistant gene inserted in the chromosomal DNA of 26404galE (Fig.1).

BLAST searches at Genbank with the deduced polypeptide sequence of strain 26404 revealed 98% or 97% identity when compared with the *galE* amino acid sequence of strain 2951 or 25238 (serotype A).

Structural analysis by MOLDI-TOF MS demonstrated that LOS from 26404galE lacked the terminal galactoses from the 4-linked and 6-linked LOS chains and added an additional glucose to the 4-linked LOS chain (Fig. 2).

The mutant strain 26404galE lost or significantly decreased the binding activity in Western blotting or flow cytometry analyses to the bactericidal anti-LOS rabbit serum (Fig. 3; Fig. 4) than the wild type strain 26404. In ELISA and bactericidal assay, the mutant strain 26404galE showed significant decreased titers than the wild type strain 26404 (Table 1).

Conclusion

The data suggest that the terminal galactoses of serotype C LOS is related to the functional epitope of the serotype C *M. catarrhalis* conjugate vaccine.
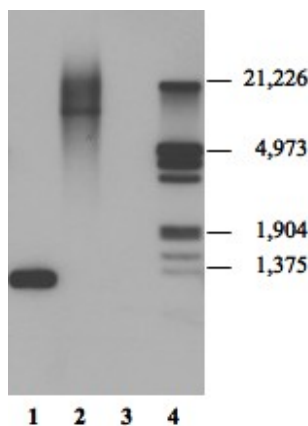
Figure 1. Detection of kanamycin resistant gene inserted into 26404galE chromosomal DNA by Southern blotting. Lane 1, 10 ng of pUC4K (a plasmid with a kanamycin resistance cassette as positive control) plus EcoRI; lane 2, 800 ng of chromosomal DNA from 26404galE plus EcoRV; lane 3, 800 ng of chromosomal DNA from 26404 plus EcoRV; and lane 4, 1 µl of DNA molecular weight marker III, digoxingenin-labeled (Roche). Each digested sample was resolved on a 0.7% agarose gel and Southern blotting was performed using DIG-labeled kanamycin resistance gene probe.
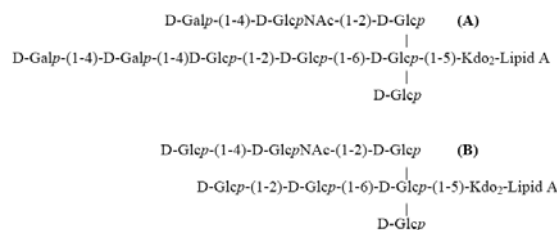
Figure 2. LOS structures from wild type strain 26404 (panel A) or 26404galE (panel B).
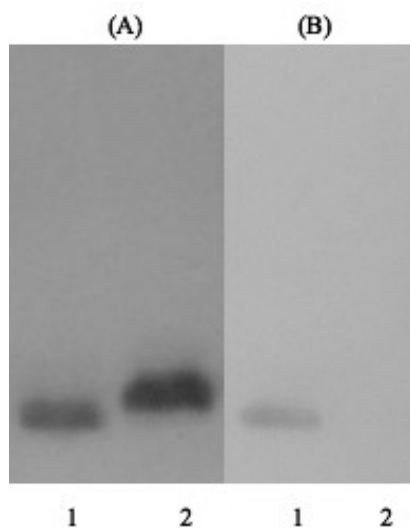
Figure 3. LOS patterns of SDS-PAGE followed by silver staining (A) or Western blot (B) of *M. catarrhalis* wild-type strain 26404 (lane 1) and mutant strain 26404galE (lane 2) and each lane represents 200 ng of purified LOS. A rabbit anti-LOS antibody elicited from a serotype C conjugate dLOS-TT was used at 1:1500 dilution for Western blot.
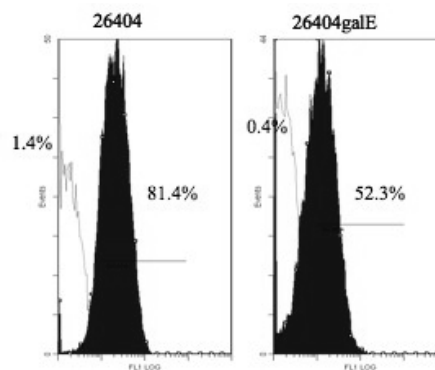
Figure 4. Bacterial binding activity was detected by flow cytometry analysis. Wild type strain 26404 showed very strong signal (81.4%) stained with anti-LOS rabbit serum, however, mutant strain 26404galE showed significant lower signal (52.3%) than wild type strain 26404. Both strains showed very low signals stained with rabbit pre-serum.

Table 1. Comparison of 26404 and 26404galE by ELISA and bactericidal assay

Strains	Type	ELISA titer	Bactericidal titer
26404	Wild type	1:100000	1:80
26404galE	Mutant	<1:500	<1:5

References

1. Verduin CM, Hol C, Fleer A, van Dijk H, van Belkum A. *Moraxella catarrhalis*: from emerging to established pathogen. Clin Microbiol 2002; 15:125-144. Review.
2. Richard K, Anthony C. *Moraxella catarrhalis*: a review of an important human mucosal pathogen. Microbes Infect 2000; 2:547-559.
3. Gu XX, Chen J, Barenkamp SJ, Robbins JB, Tsai CM, Lim DJ, Battery J. Synthesis and characterization of lipooligosaccharide-based conjugates as vaccine candidates for *Moraxella (Branhamella) catarrhalis*. Infect Immun 1998; 66:1891-1897.
4. Gu XX. Synthesis and characterization of lipooligosaccharide-based conjugate vaccines for serotype B *Moraxella catarrhalis*. Infect Immun 2005; 73:2790-2796.
5. Yu S, Gu XX. Biological and Immunological Characteristics of Lipooligosaccharide-Based Conjugate Vaccines for Serotype C *Moraxella catarrhalis*. Infect Immun 2007; 75:2974-2980.

Immune regulation of chemokine and cytokine responses following nontypeable *Haemophilus influenzae* infection

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Background

In a rodent animal model of acute nontypeable *Haemophilus influenzae* (NTHi) in both the lung and middle ear, it has previously been demonstrated that mucosal immunization results in both the enhanced clearance of bacteria[1-5] and modification of the inflammatory response observed as a result of infection.[6-7] Previous experiments in the lung have demonstrated that, following a challenge infection, mucosal immunization results in an increase in the concentration of TNF-α to levels that are approximately four-fold than observed in non-immune animals.[2,5,8] These high concentrations rapidly resolve to levels lower than that observed in non-immune animals.

Challenge with NTHi also induces a more rapid recruitment of neutrophils (PMNs) to the lungs of immune compared with non-immune animals, and these cells rapidly decline to levels lower than that observed in the non-immune controls.[2] The recruitment of macrophages to the lung following challenge with NTHi is bi-modal in immune animals, with an initial peak being observed at approximately 1 hour post-challenge and the second peak observed at 8-12 hours. Both of these peaks are significantly higher than that observed in non-immune animals where macrophages increase in number until 4 hours post-challenge and plateau thereafter. In contrast, in the middle ear, challenge infection following mucosal immunization resulted in a significant suppression of TNF-α levels in the middle-ear fluid[5,8] and enhanced recruitment of white cells.[5] These studies also suggest that immunization modifies the inflammatory response to infection in the middle ear. Clearly, further studies are needed to determine the significance of moderation of the inflammatory response to infection in both the lung and the middle ear in the immune animal and how these observations might impact the development of human vaccines.

The objectives of this study were to further understand the immunoregulatory mechanism of mucosal immunization in the lung and how these might translate to effective host immune responses against infection. mRNA and translated protein for a number of cytokines and chemokines were monitored in NTHi-immunized and non-immunized animals after subsequent challenge infection with NTHi.

Methods and materials

Specific pathogen-free DA rats were immunized with formalin-killed NTHi as previously described.[4,5,9] At 0, 1, 2, 4, 8, 12, and 24 hours post-induction of an acute lung infection, bronchoalveolar lavage (BAL) and lung tissue were collected and processed. IL-1β, IL-6, IL-10, TNFα, MCP-1, and MIP-2 were assayed for mRNA expression (real time reverse transcriptase polymerase chain reaction [RT-PCR]) and protein levels (enzyme-linked immunosorbent assay [ELISA]). RT-PCR and ELISA were conducted using commercially available kits according to the manufacturers' instructions.

Results and discussion

TNF-α. TNF-α is produced by macrophages and monocytes during acute inflammation.[10] This cytokine is responsible for a diverse range of signalling events within cells leading to necrosis or apoptosis. TNF-α directly mediates PMN migration through MIP-2[11] and increased expression of ICAM.[12] In these studies, mRNA for TNF-α peaked at 1-2 hours post-infection and was higher in immune BAL and lung compared with non-immune animals. There was little mRNA in non-immune BAL. The level of TNF-α was higher in the BAL and peaked at 4 hours for both immune and non-immune animals. The levels observed in the immune BAL were greater than those observed in the non-immune BAL for the first 4 hours and fell more rapidly in comparison thereafter. There was very little protein detected in the lung tissue. The high levels of mRNA and protein observed in the immune BAL and mRNA in the immune lung tissue is consistent with the earlier recruitment of macrophages and PMNs and their rapid disappearance we have previously observed in immune compared with non-immune animals. The

differences in the observations between immune and non-immune animals suggest that the recruitment and activation of the macrophages and PMNs is under immune control.

IL-1β. IL-1β is produced by macrophages, monocytes, dendritic cells, as well as other cell types. This cytokine plays an important role in both innate and adaptive immunity and is particularly associated with chronic inflammation at least partly through the inhibition of neutrophil apoptosis.[13] In these studies, most mRNA expression was observed in BAL with a peak at 4 hours for immune animals and two peaks at 2 and 8 hours respectively for non-immune animals. There was little expression observed in the lung. Protein was observed in both the lung and the BAL with higher levels being observed in the lung compared with the BAL. Generally, levels of IL-1β were greater in the immune lung up until 8 hours compared with the non-immune lung. After 8 hours, the levels fell more rapidly compared with non-immune animals. In the BAL, similar levels were observed in both immune and non-immune animals up until 8 hours after which the levels in immune animals fell significantly compared with non-immune animals. The higher levels of IL-1β observed in immune lung may be a result of the higher levels of TNF-α observed in immune animals compared with non-immune animals in the first 4 hours post-infection with NTHi. The persistent levels of IL-1β observed in both non-immune BAL and lung may promote the survival of neutrophils that we have previously observed in the BAL of non-immune animals.

IL-10. IL-10 is an important cytokine for suppressing IL-1 and TNF-α responses.[14] This cytokine also induces CD8+ cell chemotaxis[15] and suppresses CD4+ cell chemotaxis in response to MCP-1. IL-10 has an anti-inflammatory effect on neutrophils and blocks MIP-2 and MCP-1 by promoting degradation of mRNA for these chemokines.[16] A biphasic mRNA response was observed in the BAL with peaks occurring at 4 and 24 hours. There was less expression observed in the immune lung with little or no expression observed in non-immune BAL and lung. A peak in IL-10 was observed at 12 hours with less expression being observed in the immune lung. In the non-immune BAL, there was a steady increase to 8 hours and the levels remained relatively constant over the remainder of the observation period. The increase in IL-10 levels observed in immune BAL corresponded with the decrease that we have previously observed in the TNF-α levels and neutrophil activity.[5-7] The observation of an anti-inflammatory effect on neutrophils fits our early hypotheses of controlled inflammation in the immune state.[6,7] The IL-10 profile in immune animals also

corresponds to our previous observations with respect to CD8+ and CD4+ cell recruitment to the immune lung.[17] An early phase recruitment of CD8+ cells is observed at approximately 1 hour post-challenge with NTHi and again after 8 hours. This profile corresponds to the IL-10 protein profile in the BAL. In contrast, there was no significant recruitment of CD4+ cells to the lung until 24 hours post-challenge with NTHi when the levels of IL-10 in the BAL are decreasing.

IL-6. IL-6 is known to induce the activation of both T cells and macrophages.[18,19]

In these studies, we observed an mRNA peak between 2 and 4 hours in both the lung and BAL. The highest protein levels were observed in the BAL with immune and non-immune BAL having different kinetics with a peak at 4 hours in the immune and 8 hours in the non-immune. In the immune BAL, significant protein levels were observed at 2 and 4 hours post-challenge with NTHi. These levels then fell rapidly to levels lower than that observed in non-immune animals after 8 hours. In contrast, the level of IL-6 in non-immune BAL increased slowly from 2 to 8 hours and then decreased over the remainder of the observation period to levels similar to immune BAL at 24 hours. Similar low levels of protein were observed in immune and non-immune lungs. The increased levels of IL-6 observed earlier in immune animals may be responsible for the increased phagocytic response compared with non-immune animals.

MCP-1 (monocyte chemoattractant protein-1). MCP-1 is a potent and specific chemotactic agent for monocytes and T lymphocytes.[20,21] A triphasic mRNA response was observed in the immune lung at 1, 4, and 12 hours. A biphasic response was observed in the immune BAL with peaks at 2 and 12 hours and non-immune BAL with peaks at 1 and 8 hours. Expression increased in the non-immune lung to 12 hours and remained constant for the remainder of the observation period. The majority of protein was observed in BAL with levels in the immune BAL increasing earlier and peaking at 2 hours compared with the non-immune BAL, which peaked at 4 hours. After the 2-hour peak, the level in immune BAL declined faster compared with non-immune BAL. Lower levels of MCP-1 were observed at 1 hour post-challenge with NTHi, with a significantly lower amount of protein present in the non-immune than immune animals. The phasic mRNA response in immune animals may be significant with respect to immune regulation and could be important for overcoming the suppression of the recruitment of CD4+ cells to the lung.

MIP-2 (IL-8 homolog). MIP-2 is a potent recruitment and activation factor for PMNs.[22-24] This

chemokine also exhibits a number of other inflammatory and immunoregulatory activities. In the non-immune BAL, the level of mRNA expression increased markedly at 2 hours post-challenge with NTHi and remained constant over the remainder of the observation period. A peak of expression was observed at 4 hours in the non-immune lung while there was comparatively less expression in immune BAL and lung. Peak MIP-2 protein levels were observed in immune and non-immune BAL at 2 hours post-challenge with NTHi. The levels in immune animals increased more rapidly and peaked higher compared with the non-immune animals. After the peak at 2 hours, the protein levels in both the immune and non-immune BAL declined rapidly. Despite the persistent presence of mRNA in the non-immune BAL, there was very little protein observed after 12 hours for the remainder of the observation period. The levels of protein observed in the lung were low in both the immune and non-immune animals. The continued elevation of mRNA in the non-immune animals may be an important differentiating factor in the control of inflammatory and immunoregulatory mechanisms between the immune and non-immune state. However, further studies are clearly required to further understand this observation.

Conclusions

The cytokine and chemokine responses generally reflect cellular and inflammatory responses observed in lungs of infected animals.

Mucosal immunization induces alterations in the cytokine and chemokine profiles that are generally consistent with the induction of modified inflammatory responses in the lung.

Observations are not always consistent between the lung and the BAL, suggesting that a differential (partitional) response is possible between cells in the lung and those in the lumen of the airways.

In studies of this nature, there is value in determining both secreted protein as well as messenger expression to fully determine the temporal relationship between immune events.

Similar studies conducted in the middle ear will help understand host responses to bacterial infections of the middle ear and how these may be modified by immunization. Vaccine formulations that are able to protect against infection as well as initiate immune mechanisms that limit inflammatory responses in the middle-ear space would be most desirable.

References

1. Wallace FJ, Clancy RL, Cripps AW. An animal model demonstration of enhanced clearance of nontypeable *Haemophilus influenzae* from respiratory tract after antigen stimulation of gut-associated lymphoid tissue. Amer Rev Respir Dis 1989; 140:311-316.
2. Foxwell AR, Kyd JM, Cripps AW. Characteristics of the immunological response in the clearance of non-typeable *Haemophilus influenzae* from the lung. Immunol Cell Biol 1998; 76:323-331.
3. Kyd JM, Cripps AW. Potential of a novel protein, OMP26, from nontypeable *Haemophilus influenzae* to enhance pulmonary clearance in a rat model. Infect Immun 1998; 66:2272-2278.
4. Kyd JM, Cripps AW, Novotny LA, Verlant V, Lobet Y, Bakaletz LO. Efficacy of the 26-kilodalton outer membrane protein and two P5 fimbrin derived- immunogens to induce clearance of nontypeable *Haemophilus influenzae* from the rat middle ear and lungs as well as from the chinchilla middle ear and nasopharynx. Infect Immun 2003; 71:4691-4699.
5. Cripps AW, Kyd JM. Comparison of mucosal and parenteral immunisation in two animal models of pneumococcal infection: Otitis media and acute pneumonia. Vaccine 2007; 25:2471-2477.
6. Foxwell AR, Kyd JM, Cripps AW. Kinetics of inflammatory cytokines in the clearance of non-typeable *Haemophilus influenzae* from the lung. Immunol Cell Biol 1998; 76:556-559.
7. Foxwell AR, Kyd, JM, Cripps AW. Programmed inflammatory processes induced by mucosal immunisation. Vaccine 2001; 19:2522-2526.
8. Sabirov A, Kodama S, Hirano T, Suzuki M, Mogi G. Intranasal immunization enhances clearance of nontypeable *Haemophilus influenzae* and reduces stimulation of tumor necrosis factor alpha production in the murine model of otitis media. Infect Immun 2001; 69: 2964-2971.
9. Jomaa M, Kyd JM, Cripps AW. Mucosal immunisation with novel *Streptococcus pneumoniae* protein antigens enhances bacterial clearance in an acute mouse lung infection model. FEMS Immunol Med Microbiol 2005; 44:59-67.
10. Berenson C S, Murphy TF, Wrona CT, Sethi S. Outer membrane protein P6 of nontypeable *Haemophilus influenzae* is a potent and selective inducer of human macrophage proinflammatory cytokines. Infect Immun 2005; 73:2728-2735.
11. Tessier PA, Naccache PH, Clark-Lewis I, Gladue RP, Neote KS, McColl SR. Chemokine networks in vivo: involvement of C-X-C and C-C

chemokines in neutrophil extravasation in vivo in response to TNF-α. J Immunol 1997; 159:3595-3602.

12. Myers CL, Wertheimer SJ, Schembri-King J, Parks T, Wallace RW. Induction of ICAM-1 by TNF-α, IL-1 β, and LPS in human endothelial cells after downregulation of PKC. Am J Physiol Cell Physiol 1992; 263:C767-772.

13. Watson RW, Rotstein OD, Parodo J, Bitar R, Marshall JC. The IL-1β-converting enzyme (Caspase-1) inhibits apoptosis of inflammatory neutrophils through activation of IL-1β. J Immunol 1998; 161:957-962.

14. Murray PJ. The primary mechanism of the IL-10-regulated anti-inflammatory response is to selectively inhibit transcription. PNAS 2005; 102:8686-8691.

15. Jinquan T, Larsen CG, Gesser B, Matsushima K, Thestrup-Pedersen K. Human IL-10 is a chemoattractant for CD8+ T lymphocytes and an inhibitor of IL-8-induced CD4+ T lymphocyte migration. J Immunol 1993; 151:4545-4551.

16. Fan J, Heller NM, Gorospe M, Atasoy U, Stellato C. The role of post-transcriptional regulation in chemokine gene expression in inflammation and allergy. Eur Resp J 2005; 26:933-947.

17. Foxwell AR, Kyd JM, Karupiah G, Cripps AW. CD8+ T cells have an essential role in pulmonary clearance of nontypeable *Haemophilus influenzae* following mucosal immunisation. Infect Immun 2001; 69:2636-2642.

18. Atreya R, Mudter J, Finotto S, et al. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: Evidence in Crohn disease and experimental colitis in vivo. Nat Med 2000; 6:583-588.

19. Niemand C, Nimmesgern A, Haan S, Fischer P, Schaper F, Rossaint R, Heinrich PC, Muller-Newen G. Activation of STAT3 by IL-6 and IL-10 in primary human macrophages is differentially modulated by suppressor of cytokine signaling 3. J Immunol 2003; 170:3263-3272.

20. Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M, Moser B. Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human CD4+ and CD8+ T lymphocytes. FASEB J 1994; 8:1055-1060.

21. Huffnagle GB, Strieter RM, Standiford TJ, McDonald RA, Burdick MD, Kunkel SL, Toews GB. The role of monocyte chemotactic protein-1 (MCP-1) in the recruitment of monocytes and CD4+ T cells during a pulmonary *Cryptococcus neoformans* infection. J Immunol 1995; 155:4790-4797.

22. Schmal H, Shanley TP, Jones ML, Friedl HP, Ward PA. Role for macrophage inflammatory protein-2 in lipopolysaccharide- induced lung injury in rats. J Immunol 1996; 156:1963-1972.

23. Shanley TP, Schmal H, Warner RL, Schmid E, Friedl HP, Ward PA. Requirement for C-X-C chemokines (macrophage inflammatory protein-2 and cytokine-induced neutrophil chemoattractant) in IgG immune complex- induced lung injury. J Immunol 1997; 158:3439-3448.

24. Castagliuolo I, Keates AC, Wang CC, et al. Clostridium difficile toxin A stimulates macrophage-inflammatory protein-2 production in rat intestinal epithelial cells. J Immunol 1998; 160:6039-6045.

Role of Toll-like receptors in persistent inflammation of upper respiratory tract and its clinical implication

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Introduction

Bacterial infection and its degradation product such as lipopolysaccharide (LPS) and teichoic acid, has been postulated to induce nasopharyngeal or tubotympanal inflammation, and type-I allergic reaction could coincide as a prolongation factor. Most recently, the immune reaction can be categorized as an innate and acquired immunity, and Toll-like receptors expressed in various cells of mucosal linings play an important role in a defense mechanism against bacterial infection. However, on the other hand, once ostium or tubal blocking is achieved by mucosal swelling, paranasal sinus or middle-ear cleft inflammation might become persistent. Therefore, from this standpoint, we attempted to investigate the distribution of Toll-like receptors in upper respiratory epithelial cells such as human cell lines or mast cells. From the functional aspects, in an in vitro study, the exact role of TLR2 and TLR4 in IL-8 and IL-15 production from epithelial cells was examined when these cells were stimulated with lipoprotein or LPS. Also, we examined in vitro Th2 type cytokine production of murine mast cells stimulated with cross-linking and modulation effects of LPS for cytokine synthesis in a murine allergic rhinitis model. In these protocols, antihistamines are examined to downregulate the cytokine production.

Materials and methods

Cetirizine and tranilast. Cetirizine was manufactured by UCB (Brussels, Belgium) as the test drug. Tranilast was manufactured by Kissei Pharmaceutical Co., Ltd (Matsumoto, Japan).

Cells: Human respiratory epithelial cells CCL30, CCL185 (ATCC). Human monocyte; U937 (ATCC) Medium: DMEM with 10% FCS, PMI1640 with 10% FCS. BMMCs were derived from femoral bone marrow cells of 6-week-old Balb/c mice. After 3 weeks of culture with 10% Walter and Eliza Hall Institute (WEHI)-3-conditioned medium with as a source for IL-3, the cells were harvested for the experiments and consisted of more than 98% mast cells assessed by toluidine blue staining.

Reagents: a-human TLR2, TLR4, and mouse IgG2a(eBioscience). Synthetic Lipid A was provided by Ono Pharmaceutics. Lipoprotein was provided by Bachem.

RNA analysis: Expression of TLR2, 3, 4, 6, and 9, expression of IL-15 and MyD88 was analyzed by Northern blot analysis. Total cellular RNA was prepared using TRIzol reagent. Expression of IL-15 was also analyzed by ABI 7700. IL-15 mRNA load = (value of IL-15/value of GAPDH) $X10^4$. Total cellular RNA was extracted from each cell culture. For RNA blotting, 5-15 mg aliquots of total RNA were electrophoresed in agarose gels. RNAs were transferred to a nylon membrane. After ultraviolet-crosslinking, membranes were soaked in prehybridization solution and then incubated with (32P) aCTP-labeled probe in hybridization solution. The membranes were washed and then exposed to films.

Luciferase assay: CCL185 cells were transiently transfected with 2 mg of pGL3-NF-kB/Luc and 0.2 mg of pRL/SV40 by Lipofectamine according to the manufacture's instruction. Twenty-four hours after the transfection, some cells were pretreated with indicated chemicals for 30 min followed by the addition of Lipoprotein. After 8 hours incubation with lipoprotein, cells were lysed, and the luciferase activity was measured by using the Dual-Luciferase Reporter Assay System (Toyo Ink).

DNA-binding assay: After 0.5 hour incubation with lipoprotein 1mg/ml, cells were lysed. NF-kB activity was measured by using NF-kB p50 Transcription Factor Assay Kits (ACTIVE MOTIF).

Western blot assay: Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and phosphorylation was detected by autoradiography. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and phosphorylation was detected by autoradiography.

ELISA assay: Concentration of IL－15 in the culture supernatants of respiratory epithelial cells were measured by commercial ELISA kit (GT) according to the manufacture's instruction. Cytokines in culture supernatants were measured individually by an ELISA (R&D Systems).

Flow cytometric analysis: The cells were stained with FITC-and PE conjugated mAb.FITC-aTLR4, PE-aTLR2mAb and mice IgG2a were used. The stained cells were analyzed by a FACSCalibur (Becton Dickinson).

Statistical analysis: The statistical significance of data was determined by Student's *t*-test. A value of p<0.05 was taken as significant. The statistical significance of the data was determined by Student's *t* test. *p* <0.05 was taken as significant.

Results

An analysis of TLRs in URT epithelial cells. Respiratory epithelial cells constitutively expressed mRNA for TLR2, 3, 6, but not for TLR4. Lipoprotein induced IL-15 and IL-8 production of respiratory epithelial cells, which strictly depend on TLR2 (Fig. 1). Lipoprotein-induced IL-15 production of respiratory epithelial cells was abolished by NF-kB inhibition (Fig. 2). Lipoprotein-mediated IL-8 production in respiratory epithelial cells was abolished with NF-kB inhibition by Oxatomide (Fig. 3).

Inhibitory effect of antihistamine on cytokine production from mast cells in vitro with cross-linking with IgE and Antigens. In vitro culture of bone marrow-derived mast cells (BMMCs) indicated that allergen-induced IL-5 production from mast cells was downregulated by cetirizine pretreatment (Fig. 4), but was not influenced by tranilast pretreatment. Cetilizine did not suppress IL-5 production from mast cells, if anti-DNP IgE on BMMCs was crosslinked with a high dose of DNP antigens.

Effect of LPS on murine allergic rhinitis model at the eliciting phase. Murine allergic rhinitis model was constructed as follows. Mice were presensitized twice with immunization of ovalbumin (OVA) and Al (OH)$_3$ and thereafter intranasally challenged with OVA. As a result, as shown in Fig. 5, LPS aggravated the eliciting phase of type-I allergic reaction, in a murine allergic rhinitis model, possibly by way of Th2 cytokine upregulation from mast cells via TLR4.

Discussion and future goals

In the present study, Toll-like receptors expressed on epithelial cells, mast cells, and macrophages residing in upper respiratory tract mucosae, are demonstrated to have an important role on the pathogenesis of persistent inflammation in the nasopharyngeal cavity and middle-ear cleft. Therefore, paranasal sinus or persistent middle-ear effusion might be explained by such an interaction between bacterial degradation product and Toll-like receptors on resident epithelial cells and/or recruited inflammatory cells. Innate immunity is highly evaluated to non-specifically evacuate nasopharyngeal or middle-ear pathogens via Toll-like receptors on epithelial cells and/or recruited inflammatory cells into the paranasal sinus or middle ear. To this end, our results may lead us new therapeutic strategy (H1 receptor antagonists, signal transduction inhibitors, anti-sense therapy) to downregulate the stagnant inflammation in the paranasal sinuses or the tubotympanum.

Figure 1

Lipoprotein Induces IL-15 from Respiratory Epithelial Cells
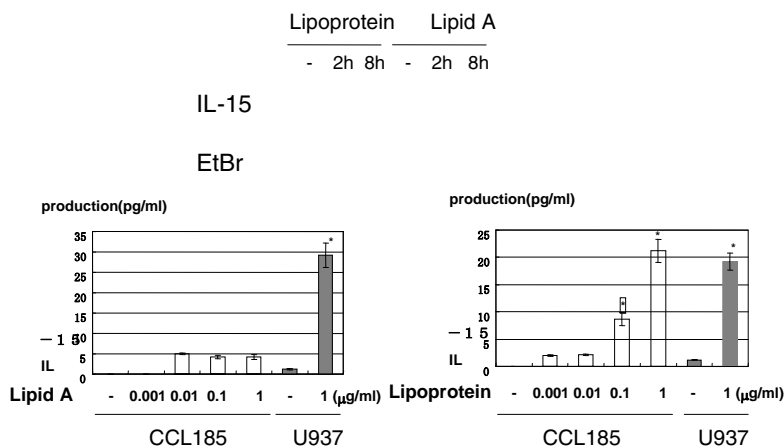
Figure 2

Lipoprotein-mediated IL-15 mRNA induction is impaired by NF-B inhibitor
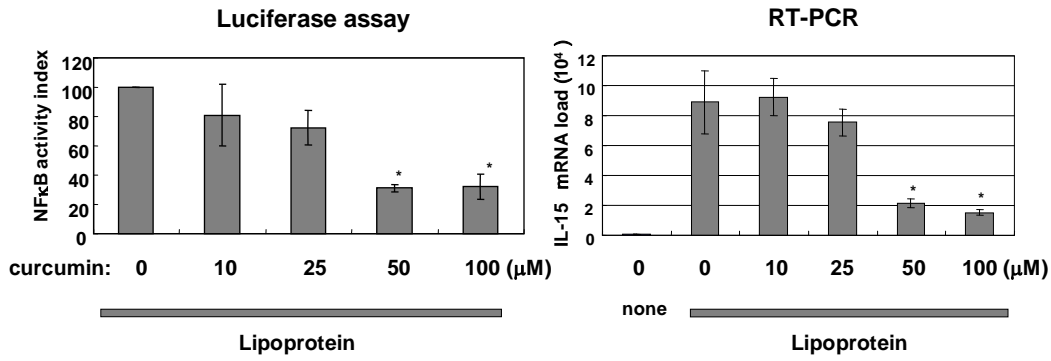
Figure 3

Effect of oxatomide on IL 8 production in respiratory epithelial cells

* *p*<0.05 **p*<0.01
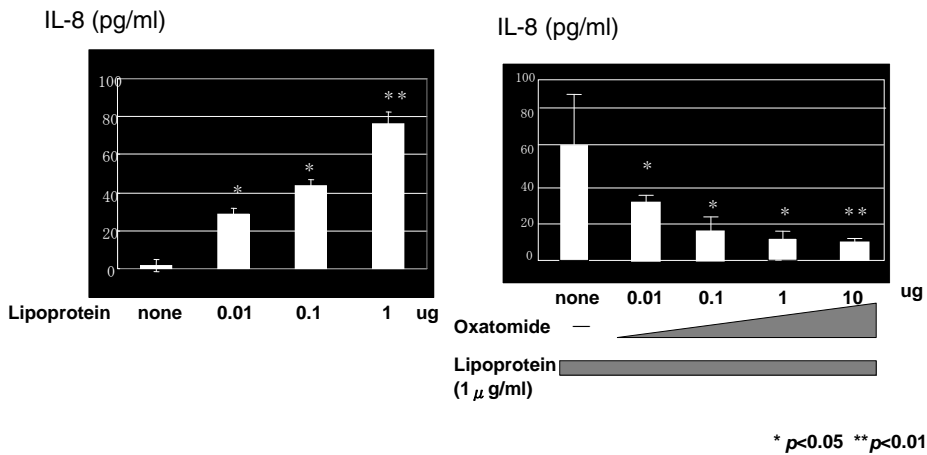
Figure 4

Effect of Cetirizine on cytokine production from Mast cell

IL-4 (pg/ml)

IL-6 (pg/ml)　　IL-13 (pg/ml)　　TNF-α (pg/ml)
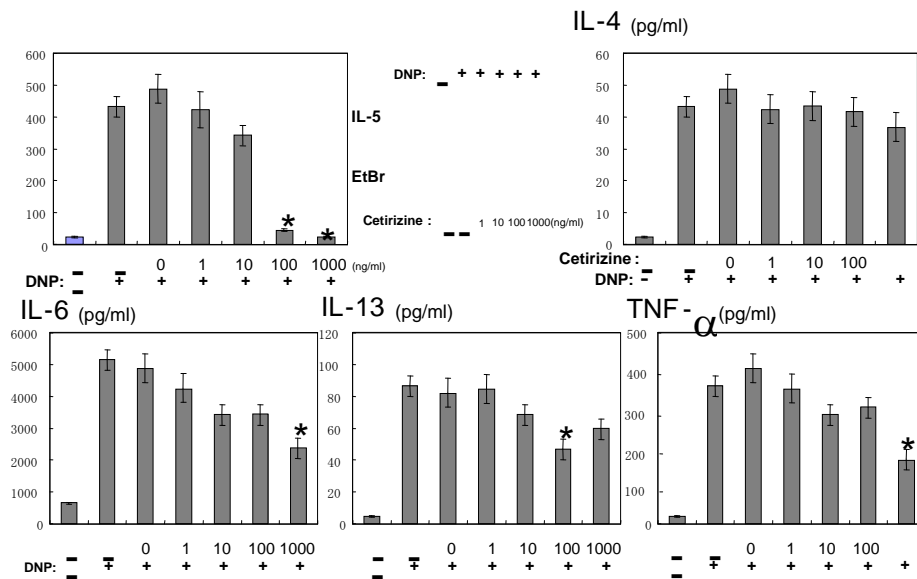
Figure 5

Effect of LPS on nasal symptoms (Balb/c mice) –eliciting phase-
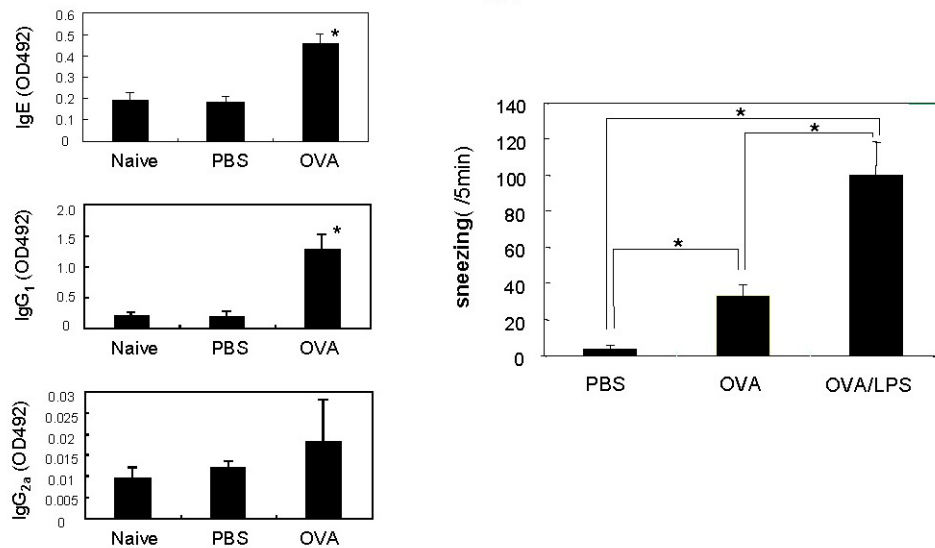
CD14, MyD88 and Toll-like receptor-2 and -4 polymorphisms do not predispose to chronic active otitis media in children

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Objective

To evaluate whether TLR2, TLR4, CD14, and MyD88 gene polymorphisms predispose to chronic active otitis media (COM).

Material and methods

In a case-cohort study of 90 children with COM and 375 controls TLR2 single nucleotide polymorphisms (SNPs) (-16934; Pro631His; Arg753Gln), TLR4 SNPs (Thr399Ile), CD14 SNPs (-651; -260), and MyD88 SNPs (1944) genotype and haplotype frequencies were compared. Genotyping was carried out with sequence-specific polymerase chain reaction (SSP-PCR). Haplotypes for the SNP positions of the TLR2 gene and the CD14 gene were estimated from unphased genotype data using the Baysian statistical method. Subgroup analysis were performed for the children with COM according to age ($\leq$3/>3 years).

Results

The frequency of the wild type and mutant SNP alleles of the TLRs, CD14, and MyD88 genes as well as the genotype frequencies and haplotype frequencies of CD14 and TLR2 did not differ between the patient and the control group. Subgroup analysis according to age did not influence the results.

Conclusions

Polymorphisms of the TLR2 (-16934; Pro631His; Arg753Gln), TLR4 (Thr399Ile), CD14 (-651; -260), or MyD88 (1944) genes do not appear to be associated with an increased susceptibility to COM.

Outer membrane vaccine candidate targets against NTHi

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Objective

Vaccine development against nontypeable *Haemophilus influenzae* (NTHi) has often focused on surface-exposed outer membrane proteins (OMPs). However, to date, vaccine candidates targeting NTHi have typically failed to be protective against heterologous isolates due to the variable nature of most outer membrane proteins. We describe a strategy to identify and characterize potential conserved outer membrane vaccine candidates that rely on identifying conserved genes with signal sequences indicative of membrane association.

Design

(i) identification and determination of the level of conservation of genes in the NTHi genome that encode surface-exposed proteins, through DNA sequencing and alignment, using a set of 35 isolates strategically selected to be representative of the genetic diversity of NTHi[1,2], (ii) cloning, expression, and purification of candidate proteins, and (iii) generation of polyclonal antisera to candidate proteins to evaluate antibody accessibility and functionality.

Results

Genes encoding OMPs were identified and selected using the PSORT prediction server. To date, six genes have been sequenced amongst 35 NTHi isolates that span the population diversity of NTHi. Levels of conservation range from 96.5% to 99.1% at the DNA level, and 95.4% to 98.7% at the protein level. Two genes have been chosen for cloning, expression, and purification.

Conclusions

This strategy appears successful for the identification of potential OMP candidates. Studies of immunogenicity and function of elicited antibodies will be necessary to determine if these conserved proteins elicit cross-protective antibodies.

References

1. Bolduc GR, Bouchet V, Jiang RZ. Variability of outer membrane protein P1 and its evaluation as a vaccine candidate against experimental otitis media due to nontypeable *Haemophilus influenzae*: an unambiguous, multifaceted approach. Infect Immun 2000; 68:4505-4517.
2. Bouchet V, Hood DW, Li J, et al. Host-derived sialic acid is incorporated into *Haemophilus influenzae* lipopolysaccharide and is a major virulence factor in experimental otitis media. Proc Natl Acad Sci (USA) 2003; 100:8898-8903.

A single nasal dose of flt3 ligand enhances mucosal immune responses in the nasopharynx

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Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is a major pathogen of otitis media (OM) and other upper respiratory tract diseases.[1] Due to the increase of antibiotic-resistant strains of NTHi in recent years, the development of vaccine(s) against NTHi is considered an important goal for public health. One of the outer membrane proteins of NTHi, P6, is a common antigen (Ag) among all strains and is considered as a candidate for a mucosal vaccine.[2]

Nasal immunization is the most effective therapeutic regimen to induce mucosalsecretory-IgA (S-IgA) responses and systemic IgG responses.[3] To elicit maximal levels of Ag-specific immune responsesin both mucosal and systemic compartments, itis necessary to use an appropriate mucosal adjuvant.[3,4] To date, cholera toxin (CT) has been one of the most potentmucosal adjuvants for the enhancement of Ag-specific immune responses when co-administered with protein Ag via either the oral or nasal routes. However, CT is toxic in humans.[4] Consequently, an alternative adjuvant would be necessary for the development of mucosal vaccine.

Dendritic cells (DCs) are essential for the induction of Ag-specific immune responses; DC-targeted vaccination might be an effective strategy for the induction of the specific immune responses. Fms-like tyrosine kinase receptor-3 ligand (Flt3L) mobilizes and stimulates myeloid and lymphoid progenitor cells, and DCs.[5,6] Here, we have investigated the efficacy of nasal Flt3L application for the induction of protective immunity against NTHi in the nasopharynx with the ultimate purpose of developing a mucosal vaccine for preventing OM.

Materials and methods

Animals and immunization

Specific pathogen-free BALB/c mice, 6 weeks of age, were used in all experiments. P6 outer membrane protein was purified from NTHi (strain 76) in our laboratory as described previously.[7] Mice were administered with recombinant human Flt3L 10 μg intranasally on day 0. After the Flt3L application, mice were further immunized with P6 10 μg on day 6, 13, and 20, and mice were killed on day 27 (Flt3L+P6 group). Control was immunized with P6 without Flt3L application (P6 group). Mice, administered phosphate buffer saline (PBS) without antigen, were used for negative control.

Flow cytometry

After Flt3L application, mononuclear cells (MNCs) were isolated from nasal-associated lymphoid tissue (NALT), and the number of CD11c$^+$ DCs in NALT was analyzed by flow cytometry.

P6-specific antibody assays

P6-specific antibody titers in nasal wash and serum were determined with enzyme-linked immunosorbent assay (ELISA), as previously described.[7,8]

For quantification of Ig-producing cells, MNCs were isolated from the nasal passage (NP), NALT, and the spleen (SP) and the numbers of P6-specific IgA-, IgG-, and IgM-producing cells were determined with enzyme-linked immunospot (ELISPOT) assay, as previously described.[7,8]

P6-specific CD4$^+$ T cell responses and real-time RT-PCR

CD4$^+$ T cells were isolated from SP by sorting with MACS (Miltenyi Biotec). Feeder cells were prepared from T cell-depleted splenocytes from naive (non-immunized) mice subjected to 30 Gyγ ray-irradiation. Purified CD4$^+$ T cells ($2x10^6$ cells/ml) and feeder cells ($2x10^6$ cells/ml) were incubated in the presence of 10 μg/ml P6 for 96 hours at 37°C in 5% CO_2. Total RNA was extracted from cultured cells, and then reverse transcription (RT) was performed. Quantification of mRNA levels was performed using the ABI PRISM 7700 Sequence Detector (Applied Biosystems). Target cDNA levels were quantified and relative gene expression was determined.

Nasal challenge with live NTHi

On day 27, a 10 μl (10^8 CFU) aliquot of the live NTHi suspension was injected into the nose. Twenty-four hours after the nasal challenge, mice were killed and nasal wash samples were collected. Ten μl aliquots of the diluted samples were spread on chocolateagar plates to determine the number of live bacteria. After overnight incubation at 37^oC in 5% CO_2, NTHi colonies were identified by standard bacteriologic techniques. The numbers of colonies were then counted.

Statistics

Mann-Whitney U-test was used to determine the significance of the data. P-values < 0.05 were considered significant

Result

The number of DCs in NALT

Six days after Flt3L application (day 6), $CD11c^+$ DCs markedly increased in NALT after nasal Flt3L administration (day 0, 1.5×10^3; day 6, 5.3×10^3 cells per mouse).

P6-specific antibody responses

P6-specific immune responses were induced by nasal immunization with P6. The levels of IgA antibodies in nasal wash were significantly elevated in Flt3L+P6 group. The serum IgG antibodies were also elevated (Fig. 1). The specific antibodies were detected at a low level in P6 group. P6-specific immune responses were not induced by nasal administration with Flt3L without P6 (data not shown).

P6-specific IgA antibody-producing cells increased markedly in NP from Flt3L+P6 group (Fig. 2). Fewer IgA-producing cells were detected in P6 group. Fewer antibody-producing cells were evident in NALT and SP compared with NP. P6-specific antibody-producing cells were not detected in PBS-administered control mice (Fig. 2).

P6-specific Th1 and Th2 cytokine mRNA expression

Th1 and Th2 cytokine mRNA expression in P6-specific SP $CD4^+$ T cells was determined by quantitative RT-PCR. The levels of expression of IFN-y, IL-4, 5, 6, 10 were examined, and their relative expression to the calibrator, RNA preparations from cells from P6-immunized mice, are detected. Elevated levels of each cytokine expression were detected in cells from Flt3L+P6 group without skewing the Th1/Th2 polarization. The expression of these cytokines was not detected in PBS-administered mice (data not shown).

NTHi clearance from the nasopharynx

Bacterial clearance was examined after nasal immunization. Enhanced NTHi clearance was observed in Flt3L+P6 group as indicated by the reduced number of live NTHi in nasal washes (Fig. 3). Although P6-specific antibodies were detected in nasal washes and serum from P6 group, these were insufficient to account for the protection against NTHi challenge. Nasal administration with Flt3L without P6 did not enhance NTHi clearance (data not shown).

Discussion

Nasal immunization is now considered as an effective vaccination route for the induction of Ag-specific S-IgA responses in the upper respiratory tract.[3,4] Nasal vaccination is considered to be an effective therapeutic regimen for preventing OM. Our previous study has demonstrated that nasal immunization with P6 and CT, or P6 and CpG oligonucleotide induced NTHi-specific protective immunity in the middle ear and the nasopharynx.[8,9] The focus of the present study was to investigate whether nasal Flt3L application combined with P6 immunization would induce NTHi-specific protective immunity in the upper respiratory tract. The present study demonstrated that nasal Flt3L application enhanced P6-specific immune responses and enhanced the clearance of NTHi from the nasopharynx.

The stimulatory effect of Flt3L on DC maturation is well documented.[10] Recently, nasal[11], or tracheal applications[12] of Flt3L were reported. Mucosal administration with Flt3L with protein Ag induced Ag-specific systemic and mucosal immune responses, indicating that Flt3L might be a new mucosal adjuvant.[11,12] In the present study, nasal Flt3L application resulted in a significant increase of DC in NALT, which might have a stimulatory effect on local antibody production. The elevated levels of IgA titer in the nasal washes of Flt3L+P6 group were associated

with a specific, local B cell immune response, as indicated by a marked increase of P6-specific IgA producing cells in the NP. The sustainedIgA-producing cellular response may be due to the activation of T cells, showing enhanced expression of Th1 and Th2 cytokines. Thus, nasal Flt3L application expanded DCs, resulting in T-cell activation and then the enhancement of antibody production in the nasal mucosa.

It has been well established that CD4$^+$ T cells and their derived Th1 and Th2 cytokine responses are essential for the induction of Ag-specificS-IgA responses. In this regard, we have compared Th1 and Th2 cytokine expression by P6-specific CD4$^+$ T cells from mice with/without nasal Flt3L application. Interestingly, enhanced levels of cytokine mRNA expression were detected in mice with nasal Flt3L application. Nasal immunization with P6 predominantly induced expression of IFN-y and IL-6, which is necessary for the development of mucosal IgA.[13,14] Both IL-4 and IL-5 were expressed at a low level. Nasal Flt3L application enhanced both Th1 and Th2 cytokine mRNA expression but did not skew Th1/Th2 polarization.

In conclusion, a single nasal dose of Flt3L induced differential increase of dendritic cells and enhanced antibody production in the nasal mucosa but did not induce tolerance. These findings suggest that nasal application of Flt3L might be a new strategy for nasal vaccination to prevent OM.

Figure 1

The levels of P6-specific antibody titer in nasal wash (a) and serum (b) were determined by ELISA. Nasal immunization with P6 combined with nasal Flt3L administration induced high levels of both mucosal IgA and systemic IgG responses. These results are expressed as the mean ± SE and were obtained from three separate experiments. n.d.; not detected.
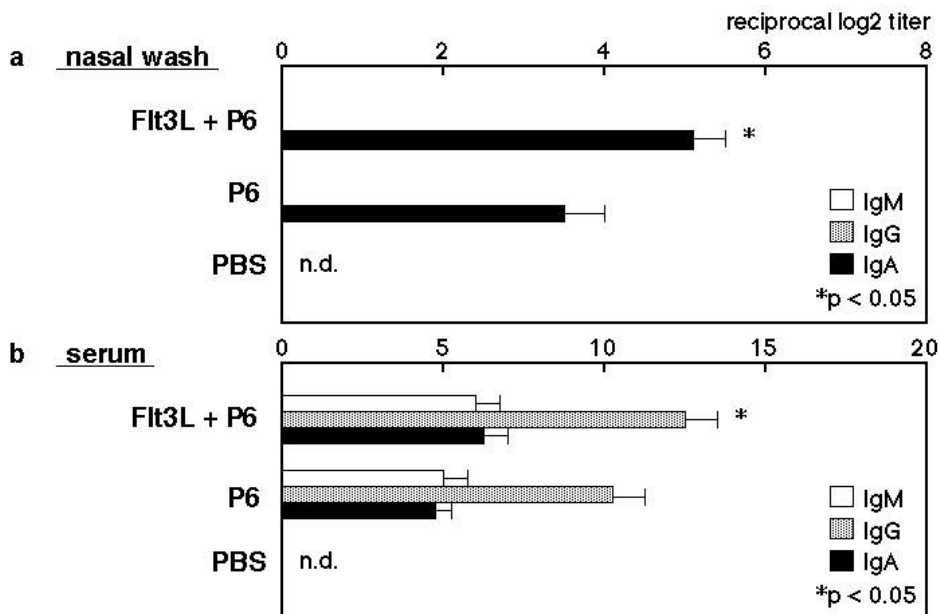
Figure 2

The number of P6-specific antibody producing cells in nasal passage (a) and spleen (b) were determined by ELISPOT assay. P6-specific IgA-producing cells significantly increased in nasal passage by nasal immunization with P6 combined with nasal Flt3L administration. These results are expressed as the mean ± SE and were obtained from three separate experiments. AFC; antibody-forming cells, n.d.; not detected.
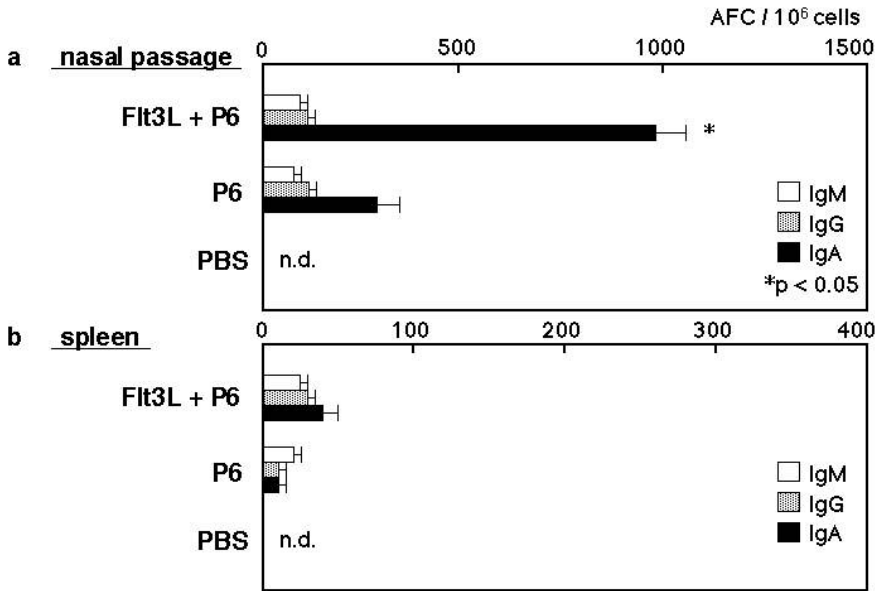
Figure 3

Bacterial clearance from nasopharynx was determined by counting the number of live NTHi in nasal washes. The concentration of NTHi was expressed as log10 of colony forming unit (cfu) per milliliter of nasal wash. NTHi clearance was enhanced by nasal immunization with P6 combined with nasal Flt3L administration. ns; no significance.
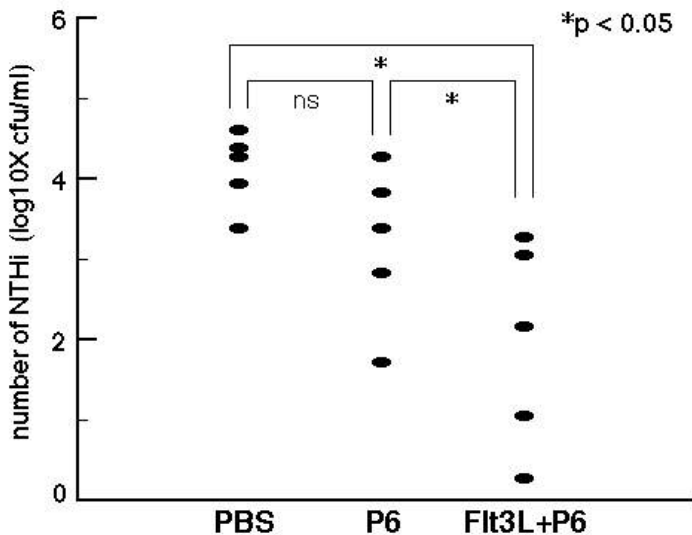
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References

1. Murphy TF. Respiratory infections caused by non-typeable *Haemophilus influenzae*. Curr Opin Infect Dis 2003; 16:129-134.
2. Poolman JT, Bakaletz L, Cripps AW, Denoel PA, Forsgren A, Kyd J, Lobet Y. Developing a nontypeable *Haemophilus influenzae* (NTHi) vaccine. Vaccine 2000; 19 Suppl:S108-S115.
3. Kiyono H, Fukuyama S. NALT versus Peyer's–patch-mediated mucosal immunity. Nat Rev Immunol 2004; 4:699-710.
4. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005; 11 suppl:S45-S53.
5. Marakovsky E, Brasel K, Teepe M, Roux ER, Lyman SD, Shortman K, McKenna HJ. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. J Exp Med 1996; 184:1953-1962.
6. Brasel K, De Smedt T, Smith JL, Maliszewski CR. Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. Blood 2000; 96:3029-3039.
7. Kodama S, Suenaga S, Hirano T, Suzuki M, Mogi G. Induction of specific immunoglobulin A and Th2 immune responses to P6 outer membrane protein of nontypeable *Haemophilus influenzae* in middle ear mucosa by intranasal immunization. Infect Immun 2000; 68:2294-2300.
8. Abe N, Kodama S, Hirano T, Eto M, Suzuki M. Nasal vaccination with CpG oligodeoxynucleotide induces protective immunity against nontypeable *Haemophilus influenzae* in the nasopharynx. Laryngoscope 2006; 116:407-412.
9. Sabirov A, Kodama S, Hirano T, Suzuki M, Mogi G. Intranasal Immunization enhances clearance of nontypeable *Haemophilus influenzae* and reduces stimulation of tumor necrosis factor alpha production in the murine model of otitis media. Infect Immun 2001; 69:2964-2971.
10. Williamson E, Westrich GM, Viney JL. Modulating dendritic cells to optimize mucosal immunization protocols. J Immunol 1999; 163:3668-3675.
11. Kataoka K, McGhee JR, Kobayashi R, Fujihashi K, Shizukuishi S, Fujihashi K. Nasal Flt3 ligand cDNA elicits CD11c$^+$CD8$^+$ dendritic cells for enhanced mucosal immunity. J Immunol 2004; 172:3612-3619.
12. Pabst R, Luhrmann A, Steinmetz I, Tschernig T. A single intratracheal dose of the growth factor fms-like tyrosine kinase receptor-3 ligand induces a rapid differential increase of dendritic cells and lymphocyte subsets in lung tissue and bronchoalveolar lavage, resulting in an increased local antibody production. J Immunol 2003; 171:325-330.
13. Finkelman FD, Holmes J, Katona IM, Urban JF Jr, Beckmann MP, Park LS, Schooley KA, Coffman RL, Mosmann TR, Paul WE. Lymphokine control of in vivo immunoglobulin isotype selection. Annu Rev Immunol 1990; 8:303-333.
14. Ramsay AJ, Husband AJ, Ramshaw IA, Bao S, Matthaei KI, Koehler G, Kopf M. The role of interleukin-6 in mucosal IgA antibody responses in vivo. Science 1994; 264:561-563.

Intranasal immunization with phosphorylcholine reduced type I allergic responses in mice

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Introduction

Mucosal vaccine is a promising strategy to prevent acute otitis media (AOM). Phosphorylcholine (PC) is a structural component of a wide variety of pathogens such as *Streptococcus pneumoniae* and *Haemophilus influenzae* and is considered one of the candidates of mucosal vaccine.[1] Mouse and human antibodies (Ab) specific to PC protect mice against lethal challenge with *S. pneumoniae*.[2,3] Moreover, intranasal immunization of mice with PC induces specific mucosal and systemic immune responses and enhances the clearance of *S. pneumoniae* and *H. influenzae* from the nasal cavity.[4] Those findings suggest that intranasal immunization with PC might be effective to prevent AOM. However, since intranasal immunization induces Th2-type mucosal immune responses, the occurrence of type I allergy is an issue in the clinical application of intranasal vaccine.

In the present study, we examined the influence of intranasal immunization with PC on the induction of type I allergy.

Materials and methods

BALB/c mice were used in all experiments. The mice were divided into four groups. In Group A and B, the mice were immunized with PC conjugated with bovine serum albumin (PC-BSA) intra-peritoneally or intra-nasally three times/week. Then, ovalbumin (OVA) conjugated with alum (OVA-alum) was administered intra-peritoneally three times/week, followed by intra-nasal administration with OVA-alum every day for one week. The mice of Group C were sensitized with OVA-alum but not previously immunized with PC. Group D was a control group with no administration of PC or OVA-alum. On day 49, nasal symptoms were examined just after nasal administration with OVA-alum, and serum IgE levels were determined by enzyme-linked immunosorbent assay (ELISA).

In another experiment, CD4+ T cells were isolated from nasal-associated lymphoid tissue (NALT) and spleen of each group of mice, and IL-4 levels produced from those CD4+ T cells in response to the stimulation with OVA were determined by ELISA. Further, in order to examine the effect of IL-12 on OVA-specific IgE production, CD11c+ dendritic cells were isolated from NALT of mice. The cells were cultured with PC, and the levels of IL-12 were determined by ELISA.

Results

The symptoms of nasal allergy such as nasal rubbing and sneezing were compared among the four groups. In Group A and B that were immunized with PC-BSA intra-peritoneally or intra-nasally before sensitization with OVA-alum, the number of nasal rubbing was significantly reduced compared to Group C mice sensitized with OVA-alum but not immunized with PC-BSA. There was no significant difference in the OVA-induced nasal symptoms between the groups of A and B immunized with PC-BSA and the control group D. The same findings were observed in the number of sneezes. Total IgE levels in serum were determined by ELISA in each period after the sensitization with OVA-alum. In Group A, B, and C, total IgE levels were increased after each sensitization with OVA-alum. However, the levels were significantly lower in Group A and B immunized with PC-BSA when compared to Group C having no previous immunization with PC-BSA. OVA-specific IgE was also detected after intra-peritoneal sensitizations with OVA-alum and remarkably increased after intra-nasal sensitization with OVA-alum. The levels were also significantly reduced in Group A and B immunized with PC-BSA compared to Group C having no previous immunization with PC-BSA.

IL-4 production from NALT and splenic CD4+ T cells were significantly reduced in mice having intra-peritoneal or intra-nasal immunization with PC-BSA compared to mice having no previous immunization with PC-BSA. Moreover, IL-12 production from NALT dendritic cells was remarkably increased by the stimulation with PC-BSA in a dose-dependent manner.

Discussions

The present study clearly demonstrated that intra-nasal immunization with PC reduced the production of total as well as antigen-specific IgE induced by systemic and topical sensitization with OVA-alum. The nasal symptoms caused by the provocation with OVA were correlated with the IgE levels and reduced in mice immunized with PC-BSA. To investigate the mechanism of how total and OVA-specific IgE levels were decreased by the immunization with PC-BSA preceding the sensitization with OVA-alum, IL-4 production from NALT CD4+ T cells and IL-12 production from NALT dendritic cells were examined. IL-4 is a Th2-type cytokine associated with IgE class switch, and IL-12 is known to be produced by dendritic cells and decrease the production of Th2-type cytokines such as IL-4. The results showed that intra-nasal immunization with PC-BSA reduced IL-4 production from NALT CD4+ T cells and enhanced IL-12 production from NALT dendritic cells. Recently, it was demonstrated that PC is a ligand of TLR4 of matured dendritic cells and IL-12 produced by dendritic cells is associated with the induction of Th1-type immune responses.[5] Collecting those findings, it can be speculated that intranasal immunization with PC as well as bacterial infection activates dendritic cells to produce IL-12 via TLR4, which reduces the production of IL-4 from CD4+ T cells and shifts the Th2-type immune responses induced by OVA to Th1-type immune responses.

Conclusions

Intranasal immunization with PC reduced the symptoms of nasal allergy and the production of IgE induced by the following sensitization with OVA. PC reduced IL-4 production from CD4+ T cells and enhanced IL-12 production from dendritic cells. Those findings suggest that PC might be an effective and safe nasal vaccine that is capable of inducing mucosal immune responses and reducing the induction of IgE-mediated type I allergy.

References

1. Harnett MM. Phosphorylcholine: friend or foe of the immune system? Immunol Today 1999; 20: 125-129.
2. Briles DE, Forman C, Crain M. Mouse antibody to phosphocholine can protect mice from infection with mouse-virulent human isolates of Streptococcus pneumoniae. Infect Immun 1992; 60:1957-1962.
3. Goldenberg HB, McCool TL, Weiser JN. Cross-reactivity of human immunoglobulin G2 recognizing phosphorylcholine and evidence for protection against major bacterial pathogens of the human respiratory tract. J Infect Dis 2004; 190:1254-1263.
4. Tanaka N, Fukuyama S, Fukuiwa T, et al. Intranasal immunization with phosphorylcholine induces antigen specific mucosal and systemic immune responses in mice. Vaccine 2007; 25:2680-2687.
5. Goodridge HS, Marshall FA, Else KJ, et al. Immunomodulation via novel use of TLR4 by the filarial nematode phosphorylcholine-containing secreted product, ES-62. J Immunol 2005; 174:284-293.

Innate immune signaling in nontypeable *Haemophilus influenzae-*induced otitis media

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Introduction

Major features of otitis media (OM) include mucosal hyperplasia and leukocyte infiltration caused by pathogenic microorganisms such as the gram-negative bacterium nontypeable *Haemophilus influenzae* (NTHi), followed by bacterial clearance and recovery.[1,2] These responses are mediated by the activation of specific responses by the cells of the middle ear mucosa and its vasculature. How these responses are elicited is not known, but cell surface receptors and linked intracellular transduction pathways seem certain to play a major role.

Many infectious models have shown that Toll-like receptor (TLR)-mediated recognition of microbial molecules (PAMPs, pathogen associated microbial patterns) is a critical first step in innate immunity, and plays a crucial role in the response to and elimination of pathogens.[3] In the present study we evaluated the contributions of TLRs and the TLR signaling to pathogenesis and bacterial clearance in NTHi-induced OM.

Materials and methods

The middle ears (MEs) of MyD88-, TLR2- and TLR4-null mice, wild-type (WT) mice (C57BL/6 and WB/B6 F1) as well as rats were exposed surgically and inoculated with NTHi or sham operated. Animals were sacrificed at different time points from 0 hours to 21 days (21d). ME were evaluated histologically to determine mucosal thickness and leukocyte infiltration. ME effusion was cultured to identify colony forming units (CFU) as a measure of bacterial clearance. MEgene expression was evaluated by cDNA microarray in WT mice (NTHi vs sham), while protein levels were determined by Western blot in rats.

Results

TLR null mice evidenced a normal initial inflammatory response to NTHi. The mucosal thickness and cellular infiltration in NTHi-inoculated TLR-deficient mice were similar to the response in WT mice from day 0 to day 3. However, while WT mice recovered rapidly thereafter, TLR- (particularly TLR2-) and MyD88-deficient mice displayed much more chronic OM, with delayed and incomplete recovery of mucosal thickness and leukocyte infiltration.

TLR2 mRNA expression showed a 7-fold upregulation at 24 hours with subsequent recovery to baseline in NTHi-inoculated WT mice, while TLR4 and MyD88 upregulation was more modest. The protein level of TLR2 in rat ME showed a substantial peak at 3d, which decreased to baseline by 10d. TLR4 showed little increase, and was detected up to 10d. Similar MyD88 protein levels were observed at every time point until day 10. All knockout mice remained culture positive far longer than WT mice. However, TLR2 KO mice showed a lower number of colony-forming unit (CFU) at all times, when compared to TLR4 and MyD88 gene deletion mice.

Discussion

TLR and MyD88 signaling do not appear to be required for the mucosal hyperplasia that occurs during the initial phases of NTHi-mediated OM in the mouse. This suggests that other signaling mechanisms are responsible for mediating hyperplasia. This could include TLR signaling via the alternative MyD88-related adaptor molecule TRIF,[4] or TLR-independent PAMP signaling pathways such as NOD-like receptors[5] or C-type lectins TGFa.[6] Additional studies will need to be performed to explore such potential mechanisms.

In contrast, TLRs and MyD88 contribute to the infiltration of leukocytes into the ME lumen. Infiltration was delayed, although not eliminated, in mice null for these molecules. Moreover, TLR signaling appears to be crucial for ME recovery and bacterial clearance. This is especially true for TLR2 and MyD88.This critical role for TLR signaling implicates the TLRs in OM susceptibility.

Acknowledgements

References

1. Lim DJ, Birck H. Ultrastructural pathology of the middle ear mucosa in serous otitis media. Ann Otol Rhinol Laryngol1971; 80:838-853.
2. Palacios S, Pak K, Rivkin A, Kayali A, Austen D, Aletsee C, Melhus Å, Webster N, Ryan AF.Role of p38-Mitogen-Activated Protein Kinase in middle ear mucosa hyperplasia during bacterial otitis media. Infect Immun 2005; 72:4662-4667.
3. Akira S. TLR signaling. Curr Top Microbiol Immunol2006; 311:1-16.
4. Watters TM, Kenny EF, O'Neill LA. Structure, function and regulation of the Toll/IL-1 receptor adaptor proteins. Immunol Cell Biol 2007; 85:411-419.
5. Delbridge LM, O'Riordan MX. Innate recognition of intracellular bacteria. Curr Opin Immunol 2007; 19:10-16.
6. McGreal EP, Martinez-Pomares L, Gordon S. Divergent roles for C-type lectins expressed by cells of the innate immune system. Mol Immunol 2004; 41:1109-1121.

Induction of innate immune molecule, human beta defensin 2 by NTHi requires TLR2 mediated MyD88 and IRAK-TRAF6 signaling pathway in human middle-ear epithelial cells

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Introduction

The respiratory mucosal epithelia, including the middle-ear mucosa, are directly exposed to the environment and serve as an effective first line of defense against a variety of microorganisms. Nontypeable *Haemophilus influenzae* (NTHi)[1] is one of the major otitis media (OM) pathogens and is also a causing agent for sinusitis and chronic obstructive pulmonary disease (COPD).[2] In the past three decades, there has been a dramatic worldwide increase in antibiotic resistance in respiratory pathogens. There is, thus, an urgent need to develop new and innovative, non-antibiotic approaches to prevent and manage this disease.[3] The pathogenesis of OM is multi-factorial. One group of factors that may be of particular relevance to OM susceptibility consists of the antimicrobial innate immune molecules (AIIMs)[4,5,6,7], as well as the pathogen recognition receptors such as the Toll-like receptors (TLR).[2,8]

Innate immune molecules such as lysozyme, lactoferrin, palate, lung, and nasal epithelium clone (PLUNC) and defensins are produced by the mucosal epithelial cells and provide the host with a constitutive or immediately inducible innate defense system that is capable of effectively dealing with the continuous attacks of a variety of pathogens at the mucosal epithelial surfaces.[9] Among the AIIMs, the β-defensin family is one of the most potent innate immune molecules.[10,11] We have recently shown that both human β-defensin 1 and 2 (HBD-1 and -2) have bactericidal/bacteriostatic activity against NTHi.[9] Moreover, in a previous study, we demonstrated that IL-1α can up-regulate the transcription of HBD-2 in human middle-ear epithelial cells (HMEEC), mediated by the Src dependent Raf-MEK1/2-ERK signaling pathway.[12] Innate immune system has been known to utilize the TLRs to recognize and bind to pathogen-associated molecular patterns (PAMPs) leading to the activation of the MAPK or the NF-kB-dependent cell signaling cascades ultimately resulting in a fast, full-blown proinflammatory response.[13]

Induction of HBD-2 in the human middle ear is not well understood. In this study, we show that NTHi12-induced HBD-2 up-regulation mainly takes place through the TLR2-MyD88-IRAK1-TRAF6-MKK3/6-p38 MAPK pathway. This result may also explain the significant synergistic effects of NTHi and IL-1α co-stimulation.[14]

Materials and methods

Bacterial culture and preparation of whole cell lysate. The NTHi strain 12 used in this study is a clinical isolate. The preparation procedure was described in a previous paper.[9] The cleared whole cell lysate (WCL) was prepared by sonication and centrifugation and stored at -80°C and the precipitated pellet (P) was resuspended in PBS.

Mammalian epithelial cell culture. The human middle-ear epithelial cell line (HMEEC) used in this study was immortalized with the E6/E7 genes of human papilloma virus type 16.[15] HMEEC cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc, Gaithersburg, MD) and Bronchial Epithelial Basal Medium (BEBM) (Clonetics, Walkersville, MD) supplemented with bovine pituitary extract (52 µg/ml), hydrocortisone (0.5 µg/ml), hEGF (0.5 ng/ml), epinephrine 0.5 (µg/ml), transferrin (10 µg/ml), insulin (5 µg/ml), triiodothyronine (6.5ng/ml), retinoic acid (0.1 ng/ml), gentamycin (50 µg/ml) and amphotericin-B (50 ng/ml). All cells were cultured in a humidified atmosphere of 5% CO2 and 95% air. A549 (human lung carcinoma) cell lines were cultured in DMEM supplemented with 5% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD).

Blocking TLR2/TLR4 with monoclonal-Antibody. HMEEC cells were cultured until 80% confluency. The cells were treated with 10 µg/ml of

human TLR2 or TLR4 blocking antibodies (eBioscience, San Diego, CA) for 30 minutes at room temperature followed by stimulation of 5 µg/ml NTHi WCL for 4 hours. For the control, isogenic antibody (IgG2a, k) was used. All experiments were conducted as triplicates.

Plasmids transfection and chemical inibitors. The HMEEC cells were cultured on 12 well plates for 24 hours and then transiently transfected with the dominant-negative mutant (DN) plasmids. For the chemical signal blocking study, the HMEEC cells were cultured for 2 days to 80% confluency. The cells were starved with basic medium without supplement overnight, then pre-treated with 5 µM chemical inhibitors, SB203580, PD98059, U0126 or vehicle for 1 hour before treating with NTHi WCL. The chemical inhibitors were purchased from Calbiochem (La Jolla, CA).

Animal studies. Male, 10 week-old C57BL/6 mice were used for the *in vivo* kinetic of NTHi-induced Defb2 expression. All aspects of animal handling were performed according to approved HEI IACUC protocol. The mice were transtympanically inoculated with 10 µl of the NTHi WCL after being anesthetized with ketamine (5 mg/100 g), for the no-NTHi control, blank vehicle, 1X PBS was inoculated. At 0, 6, 9, 12, and 24 hours post-inoculation, the middle-ear mucosal RNA of three mice were harvested by irrigation of the bulla with three, 3.5 µl Trizol (Invitrogen). For the comparison study of Debf2 expression in TLR2 KO and wild type animals, the right ear served as NTHi WCL-treated and left side served as PBS-treated control. The middle-ear mucosal RNA was collected at 4 hours post-inoculation and analyzed by real-time quantitative PCR.

RNA extraction and real-time quantitative RT-PCR analysis: RNA from cell lines or middle-ear mucosa was extracted using the Trizol (Invitrogen, Carlsbad, CA) and cDNA was synthesized with SuperScript II RNase H⁻ reverse transcriptase according to the manufacturer's protocol (Life Technologies, Inc, Gaithersburg, MD). Applied Biosystems Assays-on-Demand primer and probe sets were then used to perform real-time quantitative PCR on the samples using an ABI 7700 sequence detector (ABI, Foster City, CA) according to the manufacturer's protocol. Cyclophilin served as the internal standard.

Western blot analysis and kinase assays. Cells were lysed with buffer containing 25 mM TrisCl, 1 mM EDTA, 5 mM MgCl 2, 1 mM DTT, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF, and 5 µg/ml each of leupeptin, aprotinin, and pepstatin. The homogenate was centrifuged at 13,000 rpm for 10

min and supernatant was collected. Protein concentration was measured with the BCA protein assay kit (Bio-Rad, Inc., Richmond, CA). Cell lysates containing 50 µg of protein were boiled for 5 minutes in reducing SDS-PAGE sample buffer (0.125 Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.2% bromophenol), subjected to SDS-PAGE, and transferred to a 0.2-µm pore nitrocellulose membrane (Schleicher & Schuell) in 20% methanol, 25 mM Tris, and 192 mM glycine, pH 8.3. The membrane was blocked with 5% milk, prior to addition of the primary antibodies, followed by secondary antibody coupled to horseradish peroxidase (1:10,000; Amersham Biosciences). Bound antibody was detected by enhanced chemiluminescence (NEN Life Science Products). Antibodies against p38 α/β and phospho-p38 α/β were purchased from Cell Signaling (Beverly, MA).

siRNA Transfection and ELISA. The small interfering RNA (siRNA) was used for a transient gene knock-down study. All transient transfections were carried out in triplicate using *NeoFX* reagent (Ambion, Austin, TX) to final concentration of 10 nM following the manufacturer's instructions. After 20 hours, the transfected cells were changed with fresh growth medium and cultured further for 2 days. The cells were then stimulated with NTHi WCL either for 4 hours for gene expression study or for 24 hours to measure protein. For the protein measurement, the culture supernatant was harvested and stored at -80º C until ELISA (Phoenix Pharmaceuticals, Inc., Belmont, CA). For the gene analysis, cells were processed to harvest total RNA and used for real-time quantitative PCR.

Results and conclusions

1. NTHi up-regulates DEFB4 expression in HMEEC.
2. NTHi up-regulates DEFB4 expression in A549 cell.
3. TLR2 is the major receptor of NTHi for DEFB4 induction in HMEEC.
4. MyD88-IRAK-TRAF6 is the downstream pathway for DEFB4 induction.
5. p38 MAP Kinase is involved in the up-regulation of HBD2 by NTHi.
6. NTHi up-regulates Defb2 expression in mouse middle-ear epithelial cells.

In conclusion, there was no significant difference of HBD2 induction in HMEEC that were treated with various cell membrane mutants of NTHi. Also, we observed that NTHi WCL signal through TIR domain was the same as IL-1α, but the downstream of the two stimulants are different. These different signaling pathways may contribute the synergistic

induction of HBD2 in real infection. Last but not least, there may exist intracellular pattern recognition receptors, and those receptors act as auxillary receptors for the induction of HBD2.

References

1. Bluestone JA, Khattri R, Sciammas R, Sperling AI. TCR gamma delta cells: a specialized T-cell subset in the immune system. Annu Rev Cell Dev Biol 1995; 11:307-353.
2. Shuto T, Imasato A, Jono H, et al. Glucocorticoids synergistically enhance nontypeable *Haemophilus influenzae*-induced Toll-like receptor 2 expression via a negative cross-talk with p38 MAP kinase. J Biol Chem 2002; 277:17263-17270.
3. Rosenfeld RM, Bluestone CD. Evidence-Based Otitis Media. Hamilton, Ontario: BC Decker Inc.; 1999.
4. Boe R, Silvola J, Yang J, Moens U, McCray PB Jr., Stenfors LE, Seljfelid R. Human beta-defensin-1 mRNA is transcribed in tympanic membrane and adjacent auditory canal epithelium. Infect Immun 1999; 67:4843-4846.
5. Lim DJ, Chun YM, Lee HY, Moon SK, Chang KH, Li JD, Andalibi A. Cell biology of tubotympanum in relation to pathogenesis of otitis media - a review. Vaccine 2000; 19(Suppl 1):S17-25.
6. Park K, Lim DJ. Development of secretory elements in murine tubotympanum: lysozyme and lactoferrin immunohistochemistry. Ann Otol Rhinol Laryngol 1993; 102:385-395.
7. Suzuki T, Somekawa Y, Yamanaka N, Niida Y, Kataura A. Lactoferrin in middle ear effusion. Auris Nasus Larynx 1985; 12(Suppl 1):S154-5.
8. Takeda K, Akira S. TLR signaling pathways. Semin Immunol 2004; 16:3-9.
9. Lee HY, Andalibi A, Webster P, et al. Antimicrobial activity of innate immune molecules against *Streptococcus pneumoniae, Moraxella catarrhalis* and nontypeable *Haemophilus influenzae*. BMC Infect Dis 2004; 4:12.
10. Ganz T, Lehrer RI. Antimicrobial peptides of vertebrates. Curr Opin Immunol 1998; 10:41-44.
11. Selsted ME, Ouellette AJ. Mammalian defensins in the antimicrobial immune response. Nat Immunol 2005; 6:551-557.
12. Moon SK, Lee HY, Li JD, et al. Activation of a Src-dependent Raf-MEK1/2-ERK signaling pathway is required for IL-1alpha-induced upregulation of beta-defensin 2 in human middle ear epithelial cells. Biochim Biophys Acta 2002; 1590:41-51.
13. Froy O. Regulation of mammalian defensin expression by Toll-like receptor-dependent and independent signalling pathways. Cell Microbiol 2005; 7:1387-1397.
14. Moon SK, Lee HY, Pan H, et al. Synergistic effect of interleukin 1 alpha on nontypeable *Haemophilus influenzae*-induced up-regulation of human beta-defensin 2 in middle ear epithelial cells. BMC Infect Dis 2006; 6:12.
15. Chun YM, Moon SK, Lee HY, Webster P, Brackmann DE, Rhim JS, Lim DJ. Immortalization of normal adult human middle ear epithelial cells using a retrovirus containing the E6/E7 genes of human papillomavirus type 16. Ann Otol Rhinol Laryngol 2002; 111:507-517.

Measuring cellular immune responses to pneumococcal proteins in young children with otitis media

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Background

Several bacteria are important in otitis media (OM) pathogenesis including *Streptococcus pneumoniae* (Pnc), nontypeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis* (Mc).

Currently only pneumococcal vaccines based on capsular type are available. These have had a limited impact against OM due to:

- No coverage of other OM pathogens like NTHi and Mc
- Limited number of Pnc serotypes covered
- Replacement of vaccine serotypes
- Immunogenic properties of polysaccharides
- Role of mucosal immunity vs systemic immunity

Development of a protein-based vaccine covering multiple pathogens will be important to prevent OM. However, to be successful at this, a better understanding of the nature of protective immune responses against protein antigens is essential. This requires optimization of culture systems, for which several key issues need to be addressed like:

1. Which bacterial antigens to use in these assays and how to assure purity of these proteins
2. At which cell concentration to culture, especially when using cells of infants and young children
3. How long to culture to obtain informative results
4. What culture media to use
5. Do age and OM status of subjects influence the results

The specific aims of our study were to develop a robust cell culture system to assess T-cell memory responses in peripheral blood mononuclear cells from young children with and without OM and characterize the type of T-cell memory responses against protein vaccine candidates in children with and without OM.

Methods and materials

Peripheral blood mononuclear cells (PBMCs) were isolated from children between 0 and 6 years of age. Data on ear disease history was recorded using a questionnaire. Cells were stimulated in vitro with pneumococcal proteins (CbpA, PspA, Pneumolysin). Different cell concentrations ($1x10^6$ and $2x10^6$ cells/ml), timing of culture (48, 72 or 96 hours), culture media (AIM, AIM+5%AB serum or RPMI+5%AB serum), and subjects (grouped for age and OM disease status) were tested. Culture supernatants were assessed for cytokines (IFN-γ, IL6, IL10, IL13, TNF-α) using time-resolved fluorometry.

Results

Antigens. Bacterial proteins used in cell culture assays are often produced in *Escherichia coli* and can therefore be contaminated with lipopolysaccharide (LPS). It is very important to clean and test antigens for LPS contamination before use in culture systems. We cultured PBMCs from 18 children between 0 and 2 years of age using pneumococcal proteins CbpA and PspA for 72 hours in RPMI+5%AB serum with and without polymyxin B (PMX), which inhibits cytokine responses due to LPS contamination. TNF-α CbpA responses were inhibited by PMX, indicating LPS contamination, whereas PspA responses were not (Fig. 1). We therefore focused on PspA in further experiments.

Cell concentration. The cell concentration used in culture may influence cytokine levels detected. However, using higher cell numbers will limit the amount of antigens that can be studied. We cultured PBMCs of 8 children between 0 and 2 years of age with pneumococcal pneumolysin for 48 hours in RPMI+5%AB using either $1x10^6$ and $2x10^6$ cells/ml and measured IL6, IL10, and IL13 in culture supernatants. Culturing with a higher cell concentration results in higher cytokine levels (Fig. 2).

Duration of cell culture. How long the culture is continued for will influence the amount and types of

cytokines measured at that certain time point. Kinetics should be determined for each cytokine individually. In general, early cytokines are IL6, TNF-α, and IL10, whereas IL5, IL13, and IFN-γ levels tend to be higher at later time points. Figure 3 shows IL10 (early cytokine) and IL13 (late cytokine) levels in culture supernatants of PBMCs cultured for 48 and 96 hours with pneumococcal pneumolysin in RPMI+5%AB.

Culture media. The culture media used may influence the level of cytokines measured in culture supernatants. AIM-V medium was developed to culture in a serum-free system and to promote T-helper type 2 responses. RPMI is the more standard medium used in cell culture systems. Figure 3 shows IL6, IFN-γ, and IL13 levels in 48-hour culture supernatants when stimulating with pneumococcal PspA or not (control), using AIM, AIM+5%AB, or RPMI+5%AB. We found that serum is necessary to elicit T-cell cytokine responses in infants to Pnc PspA. RPMI+5%AB seems to induce a T-helper type 1 skewed response with higher IFN-γ levels, whereas AIM+5%AB seems to induce a more T-helper type 2 skewed response with higher IL13 levels.

Age and disease status of subjects. Using the optimal culture conditions, we aim to characterize the T-cell memory response typical of children with recurrent OM, by culturing PBMCs of children with and without recurrent ear infections. Figure 5 shows IL6, TNF-α, and IFN-γ levels in 48-hour culture supernatants when stimulating with pneumococcal PspA or not (negative control) in AIM+5%AB using cells from 0-2 and 4-6 year old healthy controls (HC) and 0-2 and 4-6 year olds with recurrent ear disease (ear). Indeed, different cytokine patterns are observed according to age and disease status when analyzing this preliminary data. IL6 and TNF-α levels are higher in children with recurrent OM. Compared to healthy age-matched controls, INF-γ responses are lower in young children with ear disease, but increased in the older children with recurrent OM.

Conclusions

Assessing cellular responses to pneumococcal proteins in young children is not straightforward; however, we believe it is very important. Our preliminary data suggests that culturing with a higher cell number ($2x10^6$ cells/ml) would be ideal when assessing responses to bacterial proteins. Whether this is feasible depends on the number of cells available per individual. Serum should be used in this culture system and culture supernatants should be collected at both 48 and 96 hours to obtain information on the specific cytokines of interest.

A similar cytokine response pattern is found when using either AIM or RPMI (high responders with one are also high responders with the other medium); however, RPMI seems to induce a slightly more Th1 skewed response, whereas AIM seems to incite a Th2 type of response profile.

Our preliminary data of pneumococcal protein-specific PBMC cytokine responses showed that children with recurrent OM tend to display a more pro-inflammatory cytokine pattern with increased IL6 and TNF-α levels compared to age-matched controls. This pro-inflammatory cytokine profile might be important in contributing to the inflammation and disease in recurrent OM. Bacterial Th1 immunity, as measured by PspA- specific INF-γ responses, seems to be initially deficient in young children with recurrent OM, but this is no longer the case in 4- to 6-year old children with recurrent OM, possibly reflecting increased exposure through ongoing carriage and infection. We are currently carrying out further in-depth investigations into adaptive T-cell responses to pneumococcal proteins in young children with and without recurrent and severe AOM to confirm these preliminary findings that may have implications for future vaccine design.
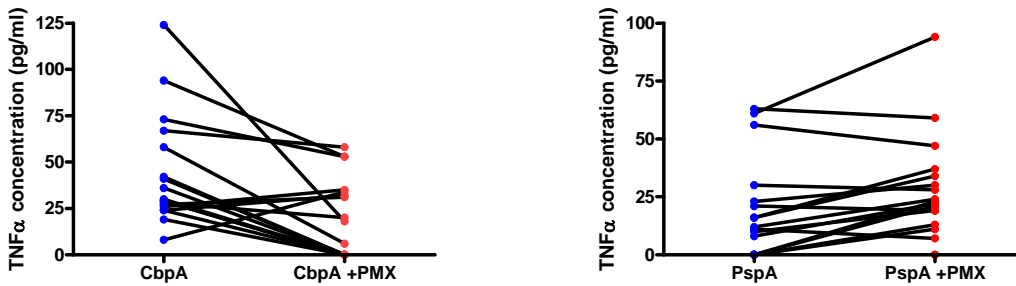
Figure 1. TNF-a concentration in culture supernatants after culturing for 72 hours in RPMI+5%AB with Pnc proteins CbpA and PspA with and without PMX.
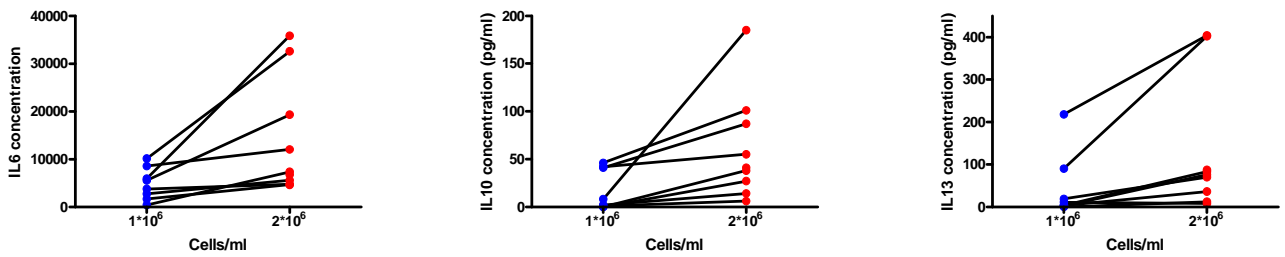
Figure 2. IL6, IL10, and IL13 responses to Pnc protein Ply after culturing for 48 hours in RPMI+5%AB using either $1x10^6$ or $2x10^6$ cells/ml
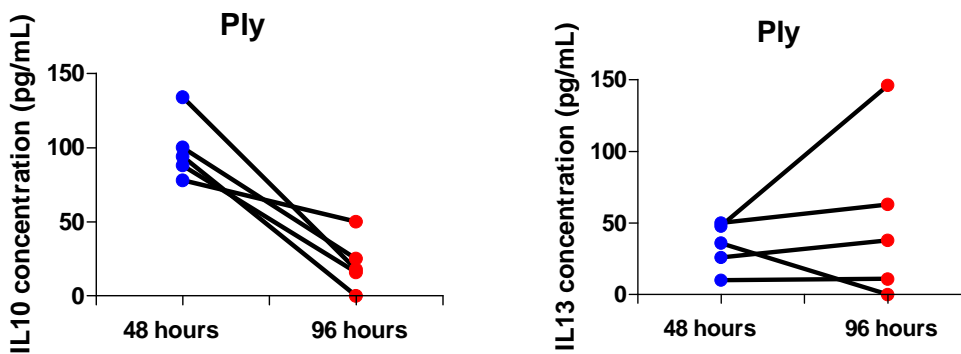
Figure 3. IL10 and IL13 responses to Pnc protein Ply after culturing in RPMI+5%AB for 48 and 96 hours.
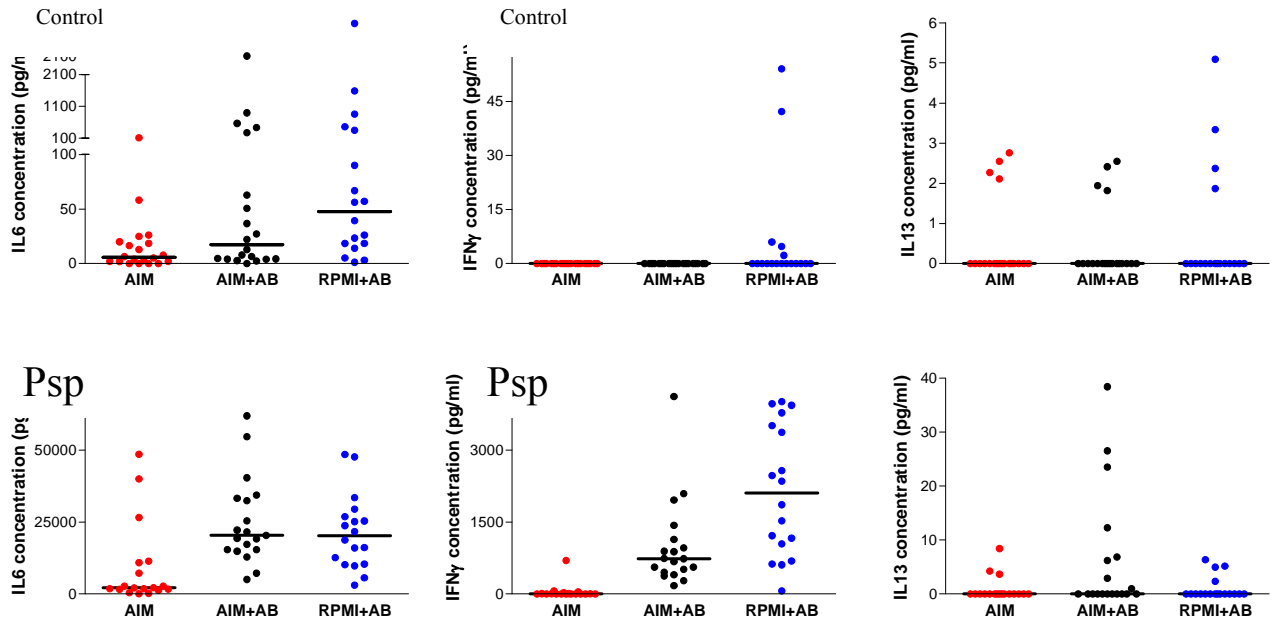
Figure 4. IL6, IFN-y and IL13 concentrations in culture supernatants after culturing for 48 hours without stimulus (control, top panels) and with pneumococcal PspA (lower panels) in AIM, AIM+5%AB or RPMI+5%AB.
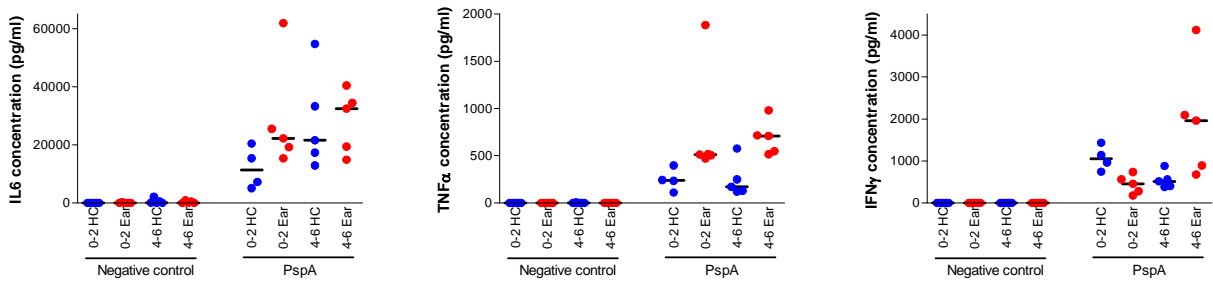
Figure 5. IL6, TNF-α and IFN-y levels in supernatants after 48 hours in culture without stimulus and with Pnc protein PspA in AIM+5%AB using cells from 0-2 or 4-6 year old healthy controls (HC) and 0-2 or 4-6 year olds with ear disease (Ear).

Contribution of lysozyme to the host defense against otitis media with *Streptococcus pneumoniae* in mouse

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Respiratory mucosa, including those of the eustachian tube (ET) and middle ear, are known to secrete multiple antimicrobial molecules such as Lysozyme (Lz), whose role in otitis media (OM) still remains speculative. We assessed the antimicrobial properties of the Lz in the ET against the respiratory pathogen *Streptococcus pneumoniae* 6B by using Lz knock-out (Lz $M^{-/-}$) mice. In wild-type (WT) animals, higher levels of Lz mRNA expression and Lz activity were detected in the ET than in the lung. Liquid broth assays showed that the ET protein extracts of Lz $M^{-/-}$ mice have significantly lower antimicrobial activity against *S. pneumoniae* 6B than that of WT mice. Moreover, the low antimicrobial activity of ET protein extracts from Lz $M^{-/-}$ mice are confirmed by Western blot and gel overlay assay. The results support the hypothesis that the antimicrobial activity defect in Lz $M^{-/-}$ mice is caused by the defect of Lz M. Live bacterial studies revealed that 3 days after challenge with live *S. pneumoniae* 6B, 88% of animals developed culture-positive OM in Lz $M^{-/-}$ mice, whereas WT mice did not. Otomicroscopic examination of the tympanic membrane at day 3 demonstrated significant differences in the severity of infection between Lz $M^{-/-}$ and WT mice. These results indicate that depletion of Lz M in the ET may lead to the persistence of *S. pneumoniae* in the middle-ear cavity and development of *S. pneumoniae*-induced OM; it also suggests that Lz is an important component of innate host defense and that Lz deficiency is a risk factor for OM.

An immunological study in Chinese adult patients with otitis media with effusion

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Otitis media with effusion (OME) is a major health issue and one of the most common reasons for surgery in children. The precise etiology remains unclear. To characterize the relation of allergy or infection to OME, we carried out immunological studies in patients with OME. Middle-ear effusion blood sera were obtained from 36 Chinese adults with OME aged 18-69 years. The samples were assayed for IgE, IgA, IgG, and IgM. The antibody titers were measured by means of an enzyme-linked immunosorbent assay (ELISA) and Agar Gel Immuno Diffusion (AGID) and compared with normal standard values. The incidence of circulating T lymphocytes (ET-RFC) and Tsen were monitored in the blood of OME patients. A significantly higher level of IgE was found in the sera of OME patients and approximately 66.7% (24/36) of them were high in TR/Tsen. Furthermore, significantly higher middle-ear effusion IgA and IgG levels were detected in 36.1% (13/36) and 69.4% (25/36) of patients, respectively. Our results indicated that most of the effusion was the result of local secretion and suggested that the middle-ear mucosa is capable of mounting a specific local immunological response. Most of the patients with middle-ear effusion had hypersensitiveness and abnormality of the immunoregulation. This may play a significant role in the etiology of recurrent middle-ear effusion.

Relationship of effusion bacteria, serum immunoglobulin, and effusion immunoglobulin in otitis media with effusion

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Purpose

Bacterial infection and immunity are important in the development of otitis media with effusion (OME) in children who have not developed eustachian tube function. We have evaluated the relationships of bacterial presence in effusion fluid and immunoglobulin (Ig) concentrations in effusion fluid and serum.

Materials and methods

Middle-ear effusion and blood samples were collected from 58 OME patients who had undergone ventilation tube insertion. Bacteria in effusion fluid were detected by standard bacterial culture and polymerase chain reaction (PCR). Serum and middle-ear fluid Ig concentrations were compared with serum Ig concentration in 64 control children.

Results

Bacteria were detected in 24/58 (41.4%) effusion fluid samples by PCR and in 12/58 (20.6%) by standard culture. There was no correlation between effusion Ig concentration and the presence of bacteria or between serum and effusion Ig concentrations, but serum Ig concentration was related to the presence of effusion bacteria ($p < 0.05$). Serum IgG, IgA and IgM in patients with OME were lower than in control patients ($p < 0.05$).

Conclusions

These results suggest that the presence of effusion bacteria in OME may be related to systemic immunity, but that the concentration of Ig in effusion fluid may not be affected by the presence of effusion bacteria.

Toll-like receptor 2 and 4 in otitis media with effusion

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Purpose

Toll-like receptors (TLRs) detect microbial infection and can directly induce innate host defense responses. Toll-like receptor 2 was shown to be involved primarily in the recognition of peptidoglycans and lipoteichoic acid of Gram-positive bacteria. Toll-like receptor 4 recognizes lipopolysaccharides and lipoteichoic acids from Gram-negative and -positive bacteria. Both mutations lead a reduced capacity to elicit inflammation and a risk for Gram-positive and -negative infections. This study was performed to investigate the expression of TLR 2 and 4 and its mutations in otitis media with effusion (OME).

Materials and methods

Middle-ear fluids were collected from 50 OME patients who had ventilation tube insertion. Bacteria in effusion fluids were detected by standard bacterial culture. Secreted IgG, IgA, and IgM were measured by enzyme-linked immunosorbent assay (ELISA). TLR 2 and 4 were assessed by reverse transcriptase-polymerase chain reaction (RT-PCR). Genomic DNA from each patient was isolated and the presence of mutations was determined by restriction digestion and DNA sequencing analysis.

Results

Detected bacteria were *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, MRSA, coagulase-negative *Staphylococcus* and *Streptococcus pyogenes*, *Bacillus* and Gram-negative rods. The amounts of IgM, IgA, and IgG were 145.4±11.9 ng/ml, 21.9±2.1, and 10.7±1.7, respectively. TLR 2 and 4 were expressed in middle-ear fluids and the expression of TLR 2 was higher than that of TLR 4. However, there was no correlation between the expression of TLR 2 and 4 and concentration of immunoglobulin or presence of bacteria ($p>0.05$). There were no mutations of TLR 2 (arg 753 Gln, Arg 677 Trp) and TLR 4 (asp 299 Gly, Thr 399 Ile).

Conclusion

LR 2 and 4 were expressed in all middle-ear fluids of OME, but mutations of TLR 2 and 4 did not exist. TLR 2 and 4 may play a vital role in immunological responses in OME.

Epitope-mapping the immune response of children with otitis media and adults with chronic obstructive pulmonary disease to the PilA protein of nontypeable *Haemophilus influenzae* type IV pilus

Leanne Adams, Laura Novotny, B.A., Sanjay Sethi, M.D., Timothy Murphy, M.D., Lauren Bakaletz, Ph.D.

Nontypeable *Haemophilus influenzae* (NTHi) is an opportunistic pathogen that causes multiple respiratory tract diseases, including otitis media (OM) and exacerbations of chronic obstructive pulmonary disease (COPD). Previously, our laboratory has demonstrated that NTHi express Type IV pili, which facilitate colonization of the mammalian airway, the first step in initiation of mucosal disease. To better understand the immune response to the Type IV pilus, we epitope-mapped the PilA subunit using clinical specimens and four sequential synthetic peptides that represent an N-terminally truncated, mature pilin protein.

We assayed serum and sputum samples from adult COPD patients as well as serum and middle-ear effusions from OM-prone children for the presence of antibodies reactive with the PilA-derived peptides via biosensor. Fifty-five percent of serum samples obtained pre- and post-exacerbation from adults with COPD demonstrated greater overall recognition of the N-terminus of the PilA protein, whereas the reactivity of sputum samples was more varied. Similar to that observed with sera from adult COPD patients, 55% of serum samples from OM-prone children showed greater overall recognition of the N-terminal PilA peptide. Interestingly, 73% of middle-ear effusions from OM-prone children demonstrated greatest recognition of the C-terminal peptide.

Collectively, our data demonstrated that healthy children colonized with NTHi, OM-prone children with active OM, and adults with COPD who were either experiencing an exacerbation or not, recognized the PilA protein of NTHi Type IV pilus immunologically. However, whereas the response to colonization by NTHi appears to be skewed towards the N-terminus of PilA, frequent induction of disease resulted in a shift toward recognition of additional epitopes. This response was particularly notable in middle-ear effusions from OM-prone children wherein the C-terminal domain appeared immunodominant. We will rely on these data for the design of future vaccine candidates against NTHi-induced diseases of the respiratory tract wherein the PilA protein is targeted.

Detection of bacteria using PCR in middle-ear effusion: Correlation with presence of allergy by MAST

Jeong-Hoon Oh, M.D., Min Kyo Jung, M.D.

Introduction

Otitis media with effusion (OME), the persistence of fluid in the middle-ear cavity greater than 3 months, has been variously attributed to infectious, allergic, and anatomic causes.[1] Recently, it has been widely accepted that dysfunction of the eustachian tube, unresolved bacterial and/or viral infections in the middle-ear cleft, and local allergic responses are the most important contributing factors although the exact pathogenic mechanism of OME is not clearly understood.[2-4] Allergy has been suggested as a potential predisposing cause of OME, and there have been attempts to reveal the mechanism of increased susceptibility to otitis media with the presence of allergy. However, it has been demonstrated that allergic rhinitis produces eustachian tube blockage and middle-ear underpressure, but rarely does middle-ear effusion (MEE) occur unless accompanied by either a viral or bacterial infection.[5-6] Furthermore, previous study showed the reaction to combined bacteria and allergen in sensitized mice was more persistent than the response to either alone and suggested that an interaction between allergic and infectious middle-ear reactions might play a role.[7]

The aim of the present study was to evaluate whether allergy can influence the bacterial detection rates by polymerase chain reaction (PCR) in MEEs of OME patients. The detection rates of common bacterial pathogens including *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* were compared between the positive and the negative groups in MAST in this study.

Materials and Methods

Study design. Fifty-four ears of 34 children between ages 3 and 10 years undergoing tympanostomy tube placement for OME at the Department of Otolaryngology, Daejeon St. Mary's Hospital, College of Medicine, the Catholic University of Korea between January 2004 and December 2006 were prospectively enrolled. Participation was by parental informed consent, and the study obtained ethical approval from the institutional review board. All patients had MEE persisting more than 3 months unresponsive to medical treatment.

Diagnosis of allergy. Prior to undergoing tympanostomy tube placement, MAST-CLA test was performed to semi-quantify the total IgE within blood serum of patients and specific IgE for 35 types of allergens in the Korean panel. Then, each allergen was classified from class 0 to class 6, and more than class 2 was diagnosed as positive for allergy.

Sample collection and polymerase chain reaction. Prior to fluid collection, the external auditory canal was irrigated with 70% alcohol. Middle-ear fluid was collected in a suction collector (Storz, Germany). Effusions collected under sterile conditions were transferred immediately to the -70°C. refrigerator for later check. The genomic DNA was extracted by mixing 50 L of the stored MEE with 900 L cell lysis solution, followed by a 10-minute centrifugation at 15,000 rpm at room temperature. DNA was extracted using PCR premix (Bioneer®, Daejeon, Korea). For the purpose of PCR, P6 protein was used as a primer for *H. influenzae*, PBP 2B for *S. pneumonia* and M46 clone for *M. catarrhalis*. Thirty-five cycles of denaturation at 95°C, annealing at 55°C and extension at 70°C was carried out using a DNA thermal cycler. Electrophoresis was used for 30 minutes in 2% agarose gel for the detection of amplified product. Specimens of this study with consistent PCR results were used as positive controls and distilled water for a negative control. The detection rates of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* between the MAST-positive and the MAST-negative groups were compared by using chi-square test.

Results

Of total 54 ears, 30 ears were found positive in MAST, while 24 were negative. The detection rate by conventional culture method was only 9.0% (5/54), and the cultured species were *S. pneumoniae*, *Staphylococcus epidermidis, Staphylococcus capitis,* and α-hemolytic *Streptococcus*, respectively. The overall detection rate of bacterial DNA using the PCR method was 70.3% (38 of 54 ears), and there was no significant difference between the positive and

negative groups in MAST (Table 1). In terms of detection rate of *S. pneumoniae* (total 50.0%), the positive group had 13 ears (43.3%) and the negative group had 14 ears (58%), showing no significant difference between the two groups. Detection rate of *H. influenzae* (total 31.4%) also did not show a significant difference between the groups with 11 ears in the positive group and 6 ears in the negative group. *M. catarrhalis* was detected in 9 ears (total 16.6%), 8 ears from the positive group and 1 from the negative group, showing a significant difference between the two groups (p<0.05) (Table 1 and Fig. 4). Meanwhile, more than two types of bacteria were detected 14 of 38 ears (36.8%), and there was no significant difference between the two groups (p>0.05).

Discussion

Allergy was first reported to be a cause of OME in 1929, and numerous studies have been performed to determine the relationship between allergy and OME.[8] Analysis of the effusion content have consistently revealed significantly elevated levels of allergy-related mediators (IL-4, IL-5, IL-6, RANTES, ECP, tryptase, IgE), as well as differences between atopic and nonatopic patients with OME.[9] There is now clear evidence that MEEs can reflect a Th1 or Th2 pattern of inflammation. However, the link between allergy and OME remains controversial. Epidemiological studies on OME have been showing increased,[10,11] or not affected prevalence of allergic diseases in OME patients [12,13], and this variability is partly due to the discrepancies in the definition of allergy among different studies, nonhomogeneous patient populations, and noncontrolled study design.[9] Some controlled studies have failed to show any increase in the prevalence of an atopic history or positive skin prick tests in children with OME compared to normal children.[14]

There have been attempts to reveal the mechanism of increased susceptibility to otitis media with the presence of allergy. If allergy plays any role in OME, it is probably in the form of allergic rhinitis delaying the resolution of a MEE by affecting the eustachian tube function. Although it is uncertain whether IgE sensitization or clinical allergic diseases associated with mechanical eustachian tube dysfunction, the mechanism has been hypothesized to be eustachian tube dysfunction caused by inflammation and edema. However, stimulation of the middle ear with allergens has been found to induce inflammation in the middle ear, but not OME, suggesting that the middle-ear mucosa does not act as the shock organ of allergy.[15] Most studies do not

support the notion that middle-ear mucosa serves as the target organ for allergy.[14] A Pittsburgh group demonstrated that allergic rhinitis produces eustachian tube blockage and middle-ear underpressure, but rarely does MEE occur unless accompanied by either a viral or bacterial infection.[5,6]

Bacterial infection is also known as an important factor in the pathogenesis of OME. It seems that OME could develop when other protective barriers (mucociliary system, immune system, and eustachian tube) are not yet functioning properly or not offering enough resistance to pathogenic bacteria.[14] *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* have been reported as the most common bacterial pathogens. High detection rates of up to 90% in MEE using PCR, which has a superior ability in detecting bacterial species, have been reported.[16] In the present study, the overall detection rate of bacterial DNA using the PCR method was 70.4%, which is consistent with previous studies.

Alteration of normal eustachian tube function may be an important factor in the development of MEE result from both allergy and bacterial infection. Ebmeyer et al. found that the reaction to combined bacteria and allergen in sensitized mice was more persistent than the response to either alone and suggested the possibility of interaction between allergic and infectious middle-ear reactions.[7] In the present study, we could not find the significant difference of bacterial detection rate between allergic and non-allergic group except in the detection of *M. catarrhalis*. This significant difference is partly due to the relatively small size of the study population. The findings of *S. pneumonia* and *H. influenza* suggest that allergy does not increase the susceptibility to bacterial infection in OME. However, it is still controversial whether there is an interaction between allergic and infectious middle-ear reactions. Further study will be necessary.

Conclusion

The bacterial detection rate in the MEE samples by conventional culture method was only 9.0%. High bacterial detection rate (70.4%) in OME fluids using the PCR method was observed and the detection rates of *S. pneumoniae, H. influenzae,* and *M. catarrhalis* were 50.0%, 31.5%, and 16.7% respectively. It is conceivable that bacteria, which have biofilm properties, are equally important in the pathogenesis of OME in atopic children. Whether there is an interaction between allergic and infectious middle-ear reactions is still controversial.
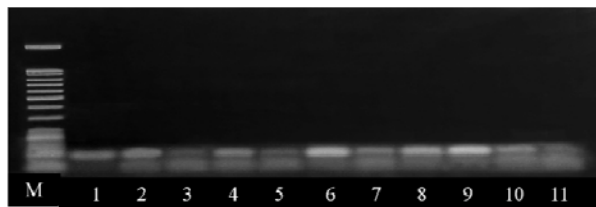
Figure 1. Electrophoresis of PCR products for *S. pneumoniae*. Left lane: 100bp marker. Lane 1: positive control. Lane 2-11: PCR product from effusion. Positive bands are shown in all lanes.
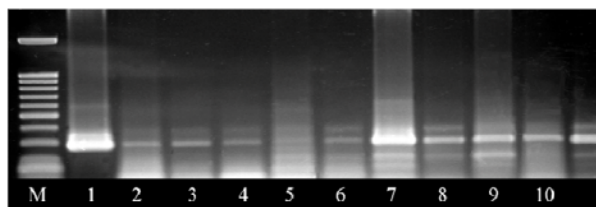
Figure 2. Electrophoresis of PCR products for *H. influenzae*. Left lane: 100bp marker. Lane 1: positive control. Lane 2-10: PCR product from effusion. Positive bands are shown in all lanes.
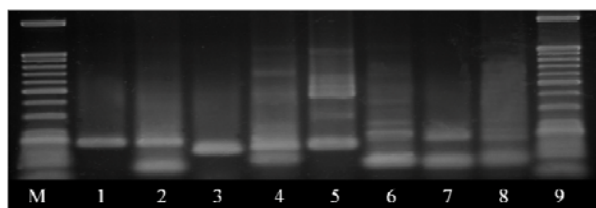
Figure 3. Electrophoresis of PCR products for *M. catarrhalis*. Left lane and Lane 9 : 100bp marker. Lane 1: positive control. Lane 2-8: PCR product from effusion. Positive bands are shown in lane 2 and 5.
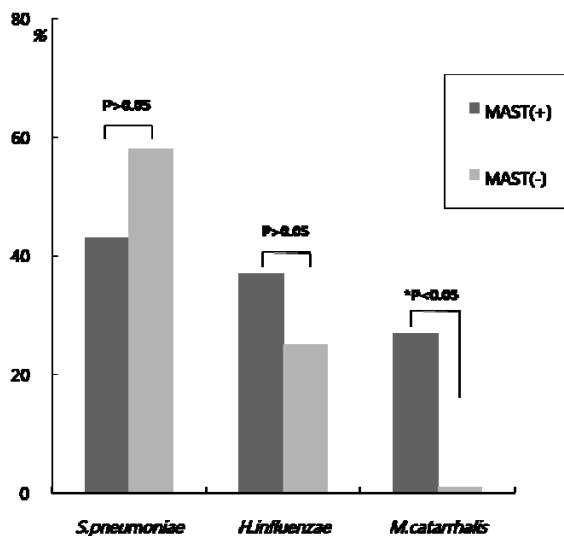
Figure 4. Comparisons of detection rates of *S. pneumoniae, H. influenza,* and *M. catarrhalis* in middle-ear effusions. (*: P<0.05*)

Table 1. The PCR results from the 54 pediatric middle-ear effusions analyzed for *H. influenza*, *S. pneumonia,* and *M. catarrhalis.* (*: P<0.05*)

	MAST (+) (n=30)	MAST (-) (n=24)	Total (n=54)
S. pneumoniae	13 (43.3%)	14 (58.3%)	27 (50.0%)
H. influenzae	11 (36.7%)	6 (25.0%)	17 (31.5%)
M. catarrhalis	8* (26.7%)	1* (4.2%)	9 (16.7%)
Positive bacterial DNA	22 (73.3%)	16 (66.7%)	38 (70.4%)

References

1. Aul JJ, Anderson KW, Wadowsky RM, et al. Comparative evaluation of culture and PCR for the detection and determination of persistence of bacterial strains and DNAs in the Chinchilla laniger model of otitis media. Ann Otol Rhinol Laryngol 1998; 107:508-513.

2. Schousboe LP, Ovesen T, Pedersen CB. Middle ear epithelium has inflammatory capacity. Acta Otolaryngol 2002 (Suppl); 543:89-91.

3. Moyse E, Lyon M, Cordier G, et al. Viral RNA in the middle ear mucosa and exudates in patients with chronic otitis media with effusion. Arch Otolaryngol Head Neck Surg 2000; 126:105-110.

4. Yellon RF, Doyle WJ, Whiteside TL, et al. Cytokines, immunoglobulins, and bacterial pathogens in middle ear effusions. Arch Otolaryngol Head Neck Surg 1995; 121:865-869.

5. Doyle WJ, Boehm S, Skoner DP. Physiologic responses to intranasal dose response challenges with histamine, methacholine, bradykinin and prostaglandin in adult volunteers with and without nasal allergy. J Allergy Clin Immunol 1991; 86:924-935.

6. Skoner D, Doyle W, Boehm S, Fireman P. Priming of the nose and eustachian tube during natural pollen exposure. Am J Rhinol 1989; 3:53-57.

7. Ebmeyer J, Furukawa M, Pak K, et al. Role of mast cells in otitis media. J Allergy Clin Immunol 2005; 116:1129-1135.

8. Yeo SG, Park DC, Eun YG, et al. The role of allergic rhinitis in the development of otitis media with effusion: effect on eustachian tube function. Am J Otolaryngol 2007; 28:148-152.

9. Chantzi FM, Kafetzis DA, Bairamis T, et al. IgE sensitization, respiratory allergy symptoms, and heritability independently increase the risk of otitis media with effusion. Allergy 2006; 61:332-336.

10. Borge P. Atopy and secretory otitis media. Immunological studies and responses to topical corticosteroid therapy. J Laryngol Otol 1983; 97:117–129.

11. Corey JP, Adham RE, Abbass AH, Seligman I. The role of IgE-mediated hypersensitivity in otitis media with effusion. Am J Otolaryngol 1994; 15:138–144.

12. Saim A, Saim L, Saim S, Ruszymah BH, Sani A. Prevalence of otitis media with effusion amongst pre-school children in Malaysia. Int J Pediatr Otorhinolaryngol 1997; 41:21–28.

13. Caffarelli C, Savini E, Giordano S, et al. Atopy in children with otitis media with effusion. Clin Exp Allergy 1998; 28:591–596.

14. de Ru JA, Grote JJ. Otitis media with effusion: disease or defense? A review of the literature. Int J Pediatr Otorhinolaryngol 2004; 68:331-339.

15. Doyle WJ, Takahara T, Fireman P. The role of allergy in the pathogenesis of otitis media with effusion. Arch Otolaryngol 1985; 111:502-506.

16. Park CW, Han JH, Jeong JH, et al. Detection rates of bacteria in chronic otitis media with effusion in children. J Korean Med Sci 2004; 19:735-738.

NOD2-dependent NF-kB activation is involved in NTHi-induced DEFB4 up-regulation of the middle-ear epithelial cells as a TLR2-independent signaling pathway

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Introduction

In addition to serving as a physical barrier, lining epithelial cells of body surfaces and lumens actively produce antimicrobial molecules in response to invading pathogens. DEFB4, previously known as human ß-defensin 2, is a small cationic antimicrobial peptide, and is produced by a variety of epithelial cells.[1, 2] It predominantly kills Gram-negative bacteria such as *E. coli* and nontypeable *Haemophilus influenzae* (NTHi)[3] by increasing permeability of the bacterial membrane. While DEFB1 is expressed constitutively, DEFB4 is only expressed in response to bacterial molecules of NTHi[4] and *E. coli*[5] as well as cytokines such as IL-1.[2] It is known that the gene regulatory region of the DEFB4 locus contains several NF-kB binding motifs and the proximal NF-kB DNA binding site is mainly responsible for DEFB4 up-regulation.[5-7]

Our preliminary data showed that NTHi-induced DEFB4 up-regulation requires the TLR2-dependent signaling in epithelial cells (unpublished data). However, blocking of TLR2 did not completely inhibit NTHi-induced DEFB4 up-regulation, indicating the presence of an alternative, TLR2-independent signaling pathway. In the quest for this pathway, we focused on the cytoplasmic pathogen-associated pattern recognition receptors such as nucleotide-binding oligomerization domain 1 (NOD1) and NOD2, which are known to be involved in DEFB4 up-regulation induced by muramyl dipeptide (MDP) and *Helicobacter pylori*, respectively.[8, 9]

In the present study, we show NTHi-induced DEFB4 up-regulation requires NOD2-dependent NF-kB activation mediated by the RICK- IκKα/β-IκBα in epithelial cells. Furthermore, we first demonstrate that the distal NF-kB binding site of DEFB4 functions as a NTHi-specific enhancer.

Materials and methods

Reagents-CAPE (caffeic acid phenetyl ester, an inhibitor of NF-kB activation), JSH-23 (an inhibitor of NF-kB activation), Wedelolactone (an IκK inhibitor), and MG132 (a cell-permeable proteasome inhibitor) were purchased from Calbiochem (San Diego, CA). Taqman primers and probes for human DEFB4 (NM_004942: ABI assay number Hs00175474_m1), TLR2 (NM_003264: ABI assay number Hs00152932_m1), NOD1 (NM_006092: ABI assay number Hs00196075_m1), NOD2 (NM_022162: ABI assay number Hs00223394_m1) and cyclophilin (NM_005729: ABI assay number 4326316E) were purchased from Applied Biosystems (Foster City, CA).

Bacterial culture and preparation of bacterial lysate-NTHi strains isolated from the middle ear (strain 12 and 9274) and from the lung (strain 1479 and 2019) were used in this study. The NTHi lysate was prepared as described.[4] Briefly, a single colony of NTHi was harvested from a chocolate agar plate, inoculated into 30 ml of brain heart infusion broth supplemented with nicotinamide adenine dinucleotide (3.5 µg/ml) and placed in a shaking incubator overnight. The supernatant was discarded after centrifugation at 10,000xg for 10 minutes. The pellet was resuspended in 10 ml of phosphate-buffered solution (PBS) and sonicated to lyse the bacteria. The lysate was then centrifuged at 10,000xg for 10 min and the supernatant was collected. The protein concentration of the NTHi lysate was determined using the BCA™ protein assay kit (Pierce Biotechnologies, Rockford, IL).

*Animals.*10 week-old male C57BL/6 mice (Charles River Laboratories, Inc., Wilmington, MA) were used in immunohistochemistry. All aspects of animal handling were performed according to the approved HEI IACUC guidelines. Animals were anesthetized with intraperitoneal injection of Ketamine (80 mg/kg) and xylazine (10 mg/kg). 10^5 cfu of live NTHI (5 µl) was transtympanically inoculated to the middle ear cavity. As a control, 5 µl of sterile normal saline was inoculated. Animals were euthanized 2 days after inoculation and the temporal bones were dissected for immunolabeling. For primary culture of middle ear epithelial cells, 10 week-old male C57BL/6 mice and TLR2-/- mice (provided by Dr. Jian-Dong Li, University of Rochester, Rochester, NY) were

euthanized and decapitated. The bulla was isolated after sterile dissection, and the bony capsule was carefully removed, preserving the middle-ear mucosa. Explants of the middle-ear mucosa were plated onto the collagen-coated petri dishes in DMEM supplemented with 10% FBS.

Plasmid construction, transfection and Luciferase assay. The 5'-flanking region (-2625 to +1) of the DEFB4 gene was subcloned to the pGL3-basic (Promega, Madison, WI), a promoterless vector expressing luciferase as previously described [2], which was named as pDEFB4/-2625/luc. Nested deletions of pDEFB4/-2625/luc (1.7 kb, 1.1 kb and 0.7 kb) were obtained using the Erase-a-Base® System (Promega) in which exonuclease III was used to specifically digest DNA from a blunt restriction site of BstZ17I (New England Biolabs, Ipswich, MA) and the opposite end was protected from digestion by a 4-base 3' overhang restriction site of KpnI (New England Biolabs). 200 bp-sized segments of the DEFB4 5'-flanking region (-2503 to -2301, -2324 to -2121, -2131 to -1919, -1938 to -1732 and -1751 to -1642) were subcloned upstream of pTAL-luc, a vector containing the luciferase gene with a TATA-like promoter region from the Herpes simplex virus thymidine kinase (TK) promoter (Clontech, Mountain View, CA). All identification of the vector constructs were confirmed by DNA sequencing. Cells were seeded into six-well plates at a density of 1.5×10^5 cell/well and transfected at ~60% confluence. Transfection was performed using the Transit®-LT1 Transfection Reagent (PanVera, Madison, WI) according to the manufacturer's instructions. pRL-TK vector (Promega) was co-transfected to normalize for transfection efficiency. Transfected cells were then starved overnight in serum-free DMEM, followed by exposure to the NTHi lysate for 10 h before harvesting. All transfections were carried out in triplicate. The cells were then washed with phosphate-buffered saline, dissolved in 250 µl of cell culture lysis reagent (Promega), and harvested by scraping. Luciferase activity was measured using a luminometer (Pharmingen, La Jolla, CA) after adding the necessary luciferase substrate (Promega). Results were expressed as fold-induction of luciferase activity, taking the value of the non-treated group as 1.

Results

NTHi induces DEFB4 in the epithelial cells. To determine whether NTHi up-regulates Defb2, a mouse orthologue to DEFB4 (www.informatics.jax.org/searches/homology_report.cgi?_Marker_key=40831) *in vivo*, we transtympanically inoculated live NTHi into the mouse middle ear cavity and performed immunolabeling of the middle ear mucosa using polyclonal anti-human ß-defensin 2 antibody. The results showed that Defb2 expression was up-regulated with infiltration of inflammatory cells and mucosal edema in the NTHi-inoculated group, compared to a saline-inoculated control.

Like TLR2, NOD2 is required for NTHi-induced DEFB4 up-regulation. Denstinogram of RT-PCR shows that NTHi-induced DEFB4 up-regulation is preserved despite of depleting TLR2 (~2 folded) compared to the wild type control (~7 folded). Surprisingly, our results showed that NOD2 exhibited more significant involvement in NTHi-induced DEFB4 up-regulation compared to NOD1. Interestingly, there was no difference between NOD1-specific siRNA and NOD2-specific siRNA in inhibiting NTHi-induced IL-8 up-regulation (data not shown), suggesting that NOD1 and NOD2 are differentially involved depending on the target genes involved. Real time quantitative PCR showed that NTHi-induced DEFB4 up-regulation was almost completely inhibited when both TLR2 and NOD2 were simultaneously silenced, indicating that NOD2 is required for NTHi-induced DEFB4 up-regulation and is the alternative signaling pathway to TLR2-mediated signaling.

NTHi up-regulates DEFB4 through NF-κB activation mediated by IκKα/β̃IκBα signaling pathway. The result showed that NF-kB p65 was translocated to the nucleus upon exposure to NTHi lysate. Moreover, NTHi-induced NF-kB translocation was blocked by CAPE (an inhibitor of NF-kB activation) and Wedelolactone (an IκK inhibitor). To investigate the NTHi-induced NF-kB activation in other cells, pNFκB-luc (a vector containing multiple copies of the NF-kB consensus sequence fused to pTAL-luc) was transfected into HMEEC, A549 and HeLa cells. Luciferase assay showed that NF-kB activity was up-regulated upon exposure to NTHi lysate in all three cells. Moreover, NTHi-induced NF-kB activation was inhibited by transfection with either NOD2-specific or RICK-specific siRNA. To determine if NF-kB activation is necessary for NTHi-induced DEFB4 up-regulation, cells were pre-treated with CAPE and JSH-23 (chemical inhibitors of NF-kB activation) before exposure to NTHi lysate. Real time quantitative PCR showed that NTHi-induced DEFB4 up-regulation was inhibited more than 70% by either CAPE or JSH-23. We also explored the involvement of IκKα/βIκBα signaling in NTHi-induced DEFB4 up-regulation by exposing cells to NTHi lysate after pre-treatment with either an IκK inhibitor (wedelolactone) or an IκBα inhibitor (MG132). Real

time quantitative PCR showed that NTHi-induced DEFB4 up-regulation was inhibited by wedelolactone and MG132 in a dose-dependent manner.

Distal NF-kB binding site of DEFB4 functions as an enhancer responding to NTHi. Luciferase assay showed that deletion of the distal NF-kB binding site (NF-κB1) surprisingly led to a >60% reduction in NTHi-induced DEFB4 levels, indicating an enhancer activity (3 to 4 folded) of NF-κB1. Since this result disagreed with the previous reports [6], we prepared heterologous constructs containing segments of the DEFB4 promoter region in order to define the NTHi-response enhancer more precisely. 0.2 kb-sized segments of the DEFB4 5' flanking region were subcloned in the upstream of the pTAL-luc vector, a vector containing the firefly luciferase gene with a TATA-like promoter region from the Herpes simplex virus thymidine kinase promoter. Luciferase assay showed that -2324/-2121 segment containing the NF-κB1 binding site has an enhancer activity (>2 folded), suggesting a NTHi-response region. The EMSA showed that the nuclear extract of NTHi-treated cells bound to the wild type NF-κB1, but not to the mutated NF-κB1 (data not shown). To explore the *in vivo* binding capacity of NF-κB1 binding site to NF-kB p65 of NTHi-treated cells, we next performed ChIP assays using an anti-NF-kB p65 antibody and the primers spanning either NF-κB1 or NF-κB2. NTHi induced protein/DNA binding in the DNA segment spanning either NF-κB1 or NF-κB2 (a positive control), but not in the negative control segment lacking the NF-kB consensus sequences

Discussion

In the present study, we demonstrated that NTHi up-regulates DEFB4 expression through a NOD2-dependent NF-kB activation mediated by RICK-IκKα/β-IκBα signaling in the epithelial cells, alternative to TLR2-dependent signaling (Fig. 1). Our results showed that once the p65 domain of the NF-kB complex is translocated to the nucleus upon exposure to NTHi lysate, it binds to the distal NF-kB binding site of the DEFB4 promoter region, acting as an enhancer responsible for NTHi-induced DEFB4 up-regulation.

The gene regulatory region of DEFB4 contains several NF-kB binding motifs and in particular, the proximal NF-kB binding site is known to be a major contributor for DEFB4 up-regulation.[6] It was reported that the distal NF-kB binding site of DEFB4 does not function as an enhancer for LPS-induced DEFB4 up-regulation, but our results demonstrate that it responds to NTHi and/or NTHi-

specific molecules. We believe that the discrepancy can be explained by differences in ligand-receptor specificities. LPS, a cell wall component of Gram-negative enterobacteria, requires TLR4-dependent signaling, while NTHi molecules mainly work through the TLR2 even though NTHi is Gram-negative. The distal NF-kB binding site of DEFB4 is believed to respond to the presence of NTHi molecules, but not to LPS, suggesting that the enhancers of DEFB4 respond differentially to different ligands.

ß-defensins are secreted by host epithelial cells lining the body surfaces in response to challenging pathogens. Multiple signaling pathways are known to orchestrate DEFB4 expression, such as toll/IL-1 receptor (TIR)-dependent NF-kB activation, TIR-dependent MAPK signaling, IL-17-dependent JAK signaling, protease-activated receptor 2 (PAR2)-dependent NF-kB activation and NOD2-dependent NF-kB activation. This study shows that NOD2-dependent NF-kB activation is required for NTHi-induced DEFB4 up-regulation in epithelial cells, as one of the signaling networks regulating DEFB4 expression. In addition, NOD2-dependent signaling is tightly regulated to avoid over-stimulation. Activated CARD12 interferes with the activation of NOD2, functioning as a negative feedback loop, further regulating NOD2 activity. Our results demonstrated that inhibition of CARD12 results in the enhancement of NTHi-induced DEFB4 up-regulation, indicating the existence of a CARD12-mediated inhibitory regulator in NOD2-dependent DEFB4 up-regulation. Further studies are needed to reveal the interaction among these signaling networks for regulating DEFB4 expression.

Taken together, we have successfully demonstrated that NTHi up-regulates DEFB4 through NOD2-dependent NF-kB activation as the alternative TLR2-independent signaling pathway, implicating the signaling networks for regulating DEFB4 expression tightly. Moreover, our study shows that the distal NF-kB binding site of DEFB4 functions as an enhancer responding to NTHi, suggesting that gene regulatory regions of DEFB4 differentially respond to the specific ligands.
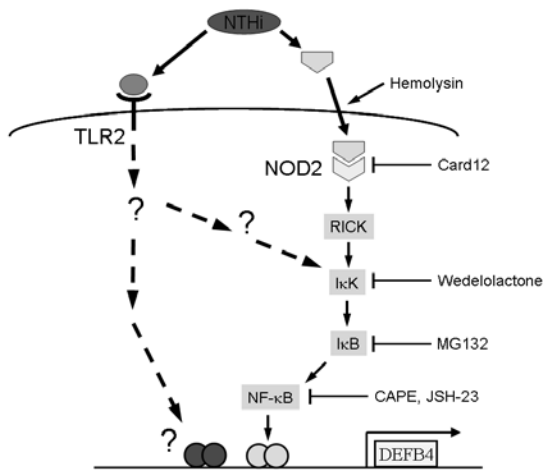
Figure 1. The illustration showing a TLR2-independent NOD2-dependent signaling pathway involved in NTHi-induced DEFB4 up-regulation in epithelial cells.

References

1. Harder J, Bartels J, Christophers E, Schroder JM. A peptide antibiotic from human skin. Nature 1997; 387:861.
2. Moon SK, Lee HY, Li JD, et al. Activation of a Src-dependent Raf-MEK1/2-ERK signaling pathway is required for IL-1alpha-induced upregulation of beta-defensin 2 in human middle ear epithelial cells. Biochim Biophys Acta 2002; 1590:41-51.
3. Lee HY, Andalibi A, Webster P, et al. Antimicrobial activity of innate immune molecules against Streptococcus pneumoniae, Moraxella catarrhalis and nontypeable Haemophilus influenzae. BMC Infect Dis 2004; 4:12.
4. Moon SK, Lee HY, Pan H, et al. Synergistic effect of interleukin 1 alpha on nontypeable Haemophilus influenzae-induced up-regulation of human beta-defensin 2 in middle ear epithelial cells. BMC Infect Dis 2006; 6:12.
5. Wehkamp J, Harder J, Wehkamp K, et al. NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium. Infect Immun 2004; 72:5750-5758.
6. Tsutsumi-Ishii Y, Nagaoka I. Modulation of human beta-defensin-2 transcription in pulmonary epithelial cells by lipopolysaccharide-stimulated mononuclear phagocytes via proinflammatory cytokine production. J Immunol 2003; 170:4226-4236.
7. Wang X, Zhang Z, Louboutin JP, Moser C, Weiner DJ, Wilson JM. Airway epithelia regulate expression of human beta-defensin 2 through Toll-like receptor 2. Faseb J 2003; 17:1727-1729.
8. Boughan PK, Argent RH, Body-Malapel M, et al. Nucleotide-binding oligomerization domain-1 and epidermal growth factor receptor: critical regulators of beta-defensins during Helicobacter pylori infection. J Biol Chem 2006; 281:11637-11648.
9. Voss E, Wehkamp J, Wehkamp K, Stange EF, Schroder JM, Harder J. NOD2/CARD15 mediates induction of the antimicrobial peptide human beta-defensin-2. J Biol Chem 2006; 281:2005-2011.

Toll-like receptor 2-dependent NF-kß activation is involved in nontypeable *Haemophilus influenzae*-induced MCP-1 upregulation in the spiral ligament fibrocytes of the inner ear

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Introduction

Inner ear dysfunction secondary to chronic otitis media (OM) is not uncommon, including high-frequency sensorineural hearing loss or vertigo. Although chronic middle-ear inflammation is believed to cause inner ear dysfunction by entry of OM pathogen components or cytokines from the middle ear into the inner ear, the underlying mechanisms are not well understood.[1, 2]

Preliminary studies of human temporal bones with labyrinthitis showed the infiltration of lysozyme-positive round cells with a monomorphic nucleus into the spiral ligament (unpublished data). Also, spiral ligament spirocyte (SLF) cell lines[3] showed an induction in MCP-1 expression after treatment with nontypeable *Haemophilus influenzae* (NTHi) lysate, one of the most common OM pathogens. Moreover, it has previously been shown that monocytes can infiltrate the cochlea, exhibiting chronic middle-ear inflammation or acoustic trauma. These results led us to focus on MCP-1 as an SLF-derived proinflammatory chemokine, attracting effector cells and causing inner ear dysfunction. NTHi is a small Gram-negative bacterium, existing as a commensal organism in the human nasopharynx. Although NTHi is a Gram-negative bacterium, it is believed to express molecules that activate not only toll-like receptor 4 (TLR4) but also TLR2.[4, 5] The interactions of NTHi antigens with specific host molecules are likely to be involved in the transition of NTHi from a commensal to a pathogenic organism.

Here we show that NTHi induces MCP-1 up-regulation in the SLFs via TLR2-dependent activation of NF-κB, which in turn, is mediated by IκKß-dependent IκBα phosphorylation. Furthermore, we demonstrate that the binding of NF-κB to the enhancer region of MCP-1 is involved in this up-regulation. In addition, we have also identified a potential NF-κB motif, responsive and specific to certain NTHi molecules or ligands. These results may provide us with new therapeutic strategies for prevention of inner ear dysfunction secondary to chronic middle-ear inflammation.

Materials and methods

Reagents. CAPE (caffeic acid phenetyl ester, a NF-κB inhibitor) and MG132 (a cell-permeable proteasome inhibitor) were purchased from Calbiochem (San Diego, CA). Taqman primers and probes for rat MCP-1 (**Rn00580555_m1**), mouse MCP-1 (Mm00441242_m1) and rat GAPDH (4352338E) and mouse GAPDH (4352339E) were purchased from Applied Biosystems (Foster City, CA).

Cell culture. The rat spiral ligament cell line (SLF), immortalized with Adeno12-SV40 hybrid virus[3], was maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, and penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Epidermal fibroblasts of Sprague-Dawley rat (CRL-1213) were purchased from ATCC (Manassas, VA).

Bacterial culture and preparation of bacterial lysate. NTHi strain 12, originally a clinical isolate from the middle-ear fluid of a child with acute otitis media, was used in this study. The NTHi lysate was prepared as described.[6]

Real-time quantitative polymerase chain reaction (PCR). Real-time quantitative PCR was performed as described.[7] Briefly, Three hours after treatment with NTHi lysate, total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA), and cDNA synthesized using the TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA). Multiplex PCR was performed using the ABI 7500 Real Time PCR System (Applied Biosystems) with gene-specific primers and a FAM™-conjugated probe for MCP-1 and a VIC®-conjugated probe for GAPDH. The cycle threshold (CT) values were determined according to the manufacturer's instructions. The relative quantity of mRNA was also

ear dysfunction secondary to chronic middle-ear inflammation.

determined using the $2^{-\Delta\Delta CT)}$ method. CT values were normalized to the internal control (GAPDH), and the results were expressed as fold-induction of mRNA, with the mRNA levels in the non-treated group set as 1.

Plasmids, Transfection and Luciferase assay. The vectors expressing a dominant-negative mutant TLR2 (TLR2_DN), a wild type TLR2 (TLR2_WT), a dominant-negative mutant TLR4 (TLR4_DN), a dominant-negative mutant IκKß [IκKß (K49A)] and a transdominant mutant IκBα [IκBα (S32/36A)] were previously described. The luciferase-expressing vectors with 5' flanking regions of rat MCP-1 were kind gifts from Dr. Eizirik (Brussels University, Brussels, Belgium).

Electrophoretic mobility shift assays and Transcription factor assay. The following are 5'-biotin labeled double-stranded oligonucleotide probes ordered from Integrated DNA Technologies, Inc. (Coralville, IA): rat MCP-1 enhancer NF-κB site 1, 5'-AAGGGTCTGGGAACTTCCAAT-3'; NF-κB site 2, 5'-AGAATGGGAATTTCCACACTCTT-3'. In vitro binding of NF-κB to the MCP-1 enhancer was determined using the LightShift® chemiluminescent EMSA kit (Pierce Biotechnologies) according to the manufacturer's instruction. For a transcription factor assay, activated transcription factors were analyzed using ELISA-based TransFactor Kit (Clontech, Mountain View, CA) according to the manufacturer's instruction.

Statistics. All experiments were carried out in triplicate. Results are expressed as mean $\pm$ standard deviation. Statistical analysis was performed using Student's *t*-test, with significance considered to be $p<0.01$ or $p<0.05$.

Results

NTHi highly induces MCP-1 in the spiral ligament fibrocytes. To determine whether NTHi up-regulates MCP-1 in SLFs of animal models, we transtympanically inoculated live NTHi in the mouse middle ear and performed immunolabeling of the cochlear lateral wall. The results showed up-regulation of MCP-1 expression in the NTHi-treated group, compared to a control.

Toll-like receptor 2 and MyD88 are involved in NTHi-induced MCP-1 up-regulation. To evaluate if TLR2 is required for NTHi-induced MCP-1 up-regulation, dominant-negative mutant constructs of TLR2 and TLR4 were transfected in SLF. The results show that NTHi-induced MCP-1 up-regulation was inhibited by transfection of SLF with a dominant-

negative mutant construct of TLR2 by 80-90%, but not by TLR4. Knocking out the TLR2 blocks NTHi-induced MCP-1 up-regulation by more than 90%. Interestingly, in the case of MyD88-targeted spiral ligament fibrocytes, MCP-1 remained unchanged when exposed to the NTHi lysate, indicating that MyD88 may be involved in TLR2-independent signaling pathways.

Activation of NF-κB via IκKß-dependent IκBα phosphorylation is required for NTHi-induced MCP-1 up-regulation. Immunolabeling of SLFs showed that NF-κB translocates to the nucleus upon exposure to the NTHi lysate. Real-time quantitative PCR demonstrated that NTHi-induced MCP-1 up-regulation was inhibited, in a dose-dependent manner, by 30%-40%, in cells pretreated with CAPE or by 80%-90%, in cells pretreated with MG-132. Moreover, NTHi-induced MCP-1 up-regulation was inhibited by transfection of SLF with a dominant-negative mutant construct of IκKß [IκKß (K49A)] or a transdominant mutant form of IκBα [IκBα (S32/36A)], by 85-95% or 70-90%, respectively.

Binding of NF-κB to the enhancer region of MCP-1 is involved in NTHi-induced MCP-1 up-regulation. The promoter constructs were transfected in SLF and the luciferase activity was measured after treatment with the NTHi lysate. The promoter construct with the enhancer region induced luciferase activity more than five-fold with NTHi treatment, whereas the promoter construct without the enhancer region showed no significant response to NTHi treatment. In order to identify potential NF-κB binding site(s) *in vitro*, electrophoretic mobility shift assays were performed using complementary single stranded DNA probes spanning two of the NF-κB motifs, NF-κB site 1 (-2,272 to -2,297) and NF-κB site 2 (-2,242 to -2,266). Two complexes were apparent upon exposure to the NTHi lysate in the presence of NF-κB site 1 probe, but none were formed with NF-κB site 2 probe. This was unexpected since based on a previous report,[8] which used IL-1ß as a stimulant, NF-κB site 2 is a potential NF-κB binding motif, responsive to NTHi treatment. NTHi treatment activated the p65 subunit, but not the other NF-κB subunits (p50 or c-Rel) or control transcription factors.

Discussion

In this study, we demonstrated that SLFs up-regulate MCP-1 upon exposure to NTHi lysate. We have shown that this up-regulation requires the TLR2-dependent activation of NF-κB, mediated by IκKß-dependent IκBα phosphorylation (Fig. 1). We also showed the

presence of a potential NF-κB promoter motif that is sensitive to NTHi specific molecules/ligands. Additionally, NF-κB binding to the enhancer region of MCP-1 is required in NTHi-induced MCP-1 up-regulation. Considering that epithelial-derived IL-1 synergistically enhances NTHi-induced DEFB4 up-regulation,[7] it is also possible that NTHi-induced secondary molecules of the host (i.e. cytokines) may be involved in the activation of TLR2-independent signaling pathways. These results point to the complexity of the signaling pathways involved in controlling the expression of MCP-1.

In conclusion, our studies demonstrate that NTHi, a common human pathogen of OM and obstructive pulmonary disease, induces up-regulation of MCP-1 via a TLR2-dependent NF-κB activation pathway in the SLFs of the cochlea. As far as we know, this is a first report elucidating the involvement of TLR2 in MCP-1 up-regulation by bacterial molecules in the SLFs of the cochlea. Our findings may lead to the development of a new model for molecular inner ear defense mechanism and inner ear dysfunction. TLR activation by the specific ligands entering the inner ear, may result in up-regulation of SLF-derived pro-inflammatory chemokines, leading to attraction of effector cells and causing inner ear dysfunction. Further studies are needed to better understand the molecular pathogenesis of the inner ear dysfunction secondary to OM, such as sensorineural hearing loss and dizziness.
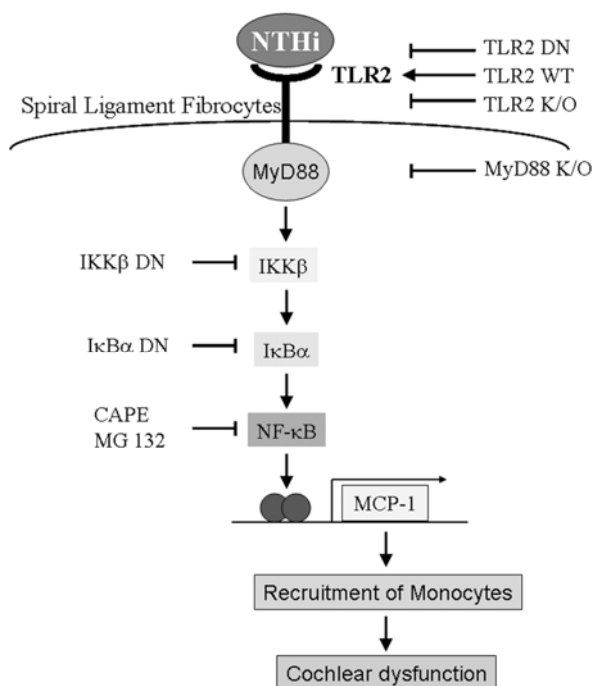
Figure 1. Schematic representation of the signaling pathways involved in NTHi-induced MCP-1 up-regulation. As indicated, NTHi up-regulates MCP-1 expression via TLR2-dependent NFκB activation. It is hypothesized that the up-regulation of MCP-1 results in recruitment and attraction of effector cells, leading to cochlear dysfunction and subsequent sensorineural hearing loss.

References

1. Engel F, Blatz R, Kellner J, Palmer M, Weller U, Bhadki S. Breakdown of the round window membrane permeability barrier evoked by streptolysin O: possible etiologic role in development of sensorineural hearing loss in acute otitis media. Infect Immun 1995; 63:1305-1310.
2. Lim DJ, Kawauchi H, DeMaria TF. Role of middle ear endotoxin in inner ear inflammatory response and hydrops: long-term study. Ann Otol Rhinol Laryngol Suppl 1990; 148:33-34.
3. Yian C, Moon SK, Jin S, et al. Characterization of rat spiral ligament cell line immortalized by Adeno12-SV40 hybrid virus. Annals Otol Rhinol Laryngol 2006; 115:930-938.
4. Shuto T, Imasato A, Jono H, et al. Glucocorticoids synergistically enhance nontypeable Haemophilus influenzae-induced Toll-like receptor 2 expression via a negative cross-talk with p38 MAP kinase. J Biol Chem 2002; 277:17263-17270.
5. Wieland CW, Florquin S, Maris NA, et al. The MyD88-dependent, but not the MyD88-independent, pathway of TLR4 signaling is important in clearing nontypeable haemophilus influenzae from the mouse lung. J Immunol 2005; 175:6042-6049.
6. Lee HY, Andalibi A, Webster P, et al. Antimicrobial activity of innate immune molecules against Streptococcus pneumoniae, Moraxella catarrhalis and nontypeable Haemophilus influenzae. BMC Infect Dis 2004; 4:12.
7. Moon SK, Lee HY, Pan H, et al. Synergistic effect of interleukin 1 alpha on nontypeable Haemophilus influenzae-induced up-regulation of human beta-defensin 2 in middle ear epithelial cells. BMC Infect Dis 2006; 6:12.
8. Kutlu B, Darville MI, Cardozo AK, Eizirik DL. Molecular regulation of monocyte chemoattractant protein-1 expression in pancreatic beta-cells. Diabetes 2003; 52:348-355.

Lysozyme M deficiency increases susceptibility to pneumococcal otitis media

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Introduction

Otitis media (OM), or middle-ear infection, is one of the most common pediatric infectious diseases, second only to the common cold, and is the most common cause of hearing impairment in children.[1] OM occurs when OM pathogens of the nasopharynx enter the middle ear via the eustachian tube (E-tube). The E-tube is covered by mucociliary epithelium as a part of the upper respiratory tract, connecting the middle-ear cavity to the nasopharynx. Under certain circumstances, when microorganisms colonize nasopharyngeal mucosal surface as commensal bacteria, they may gain access to the middle-ear cavity through the E-tube, resulting in OM.[2] Defense of the E-tube and middle ear (tubotympanum) against invading pathogens is provided by numerous factors, including the mucociliary system and the antimicrobial molecules of the innate and adaptive immune systems.[3] Lysozyme, extensively studied as one of the antimicrobial innate immune molecules (AIIMs), is ubiquitously synthesized and secreted by glandular serous cells, surface epithelial cells, and macrophages in the human airway.[4] In the tubotympanum, lysozyme is found in specialized epithelial cells, including those of the serous glands of the E-tube and middle-ear mucosa. Mice have two lysozyme genes: 1) lysozyme M predominantly expressed in macrophage, bone marrow, and lung tissue, and 2) lysozyme P expressed mostly in the small intestine (Paneth cells). Only lysozyme M gene-targeted animals are available as a model of lung and skin infection. We aimed to assess the antimicrobial property of lysozyme in the E-tube of lysozyme M-/- mice in order to evaluate the role of lysozyme in OM pathogenesis. In this study, we show for the first time that depletion of lysozyme results in delayed clearance of *Streptococcus pneumoniae* from the middle-ear cavity.

Materials and methods

Bacterial culture. S. pneumoniae serotype 6B was purchased from American Type Culture Collection (Manassas, VA). The bacteria were plated on a chocolate agar plate and incubated overnight at 37ºC in 5% CO_2. A single colony was inoculated in 10 ml of Todd-Hewitt broth and incubated overnight. One ml of this overnight culture was then transferred to 9 ml of fresh medium and incubated for a further 3 hours. The bacteria were washed twice with 10 mM sodium phosphate and the optical density (O.D.) at 620 nm was determined after being resuspended in sterile saline.

Animals and the middle-ear infection model. Lysozyme M-/- mice were generated by insertion of functional EGFP gene to target lysozyme M gene. Eight-week old C57BL/6 and lysozyme M-/- mice were anesthetized intraperitoneally, with a cocktail of ketamine-HCl and xylazine. The middle ear was transtympanically inoculated with 50 CFU of *S. pneumoniae* 6B in sterile saline. The tympanic membrane was observed on the days 1, 3, 5, and 7 after inoculation using an otoscopic digital imaging system. Tympanic membrane inflammation was graded on a scale of 0 to 2 as follows; Grade 0: normal, Grade 1: mild inflammation with air-fluid level and Grade 2: severe inflammation with massive middle-ear effusion. Middle-ear lavage was collected on day 3 and day 7 by washing with 20 μl of sterile saline and was subsequently plated on chocolate agar plates. Colonies were counted after overnight incubation.

Results

Deficiency of lysozyme M increases susceptibility to middle-ear infection with *S. pneumoniae* 6B. To determine if lysozyme M deficiency increases susceptibility to pneumococcal middle-ear infection, live *S. pneumoniae* 6B was transtympanically inoculated to the mouse middle ear. After otoscopic observation and photography of the tympanic membrane, the middle-ear lavage was plated on chocolate agar plates and colonies were counted after overnight incubation at 37º C in 5% CO_2 in order to quantify live bacteria. Within one day after inoculation, most of the mice began to show inflammation, characterized by a small amount of turbid and yellow fluid in the middle-ear cavity. Compared to wild type mice, bacterial clearance from

the middle ear of lysozyme M-/- mice was significantly delayed on day 3, but not on day 7 (Table 1). Eight out of 9 lysozyme M-/- mice showed positive bacterial culture on day 3, but none of 10 wild type mice. On day 7, bacterial culture was positive in only one out of 10 lysozyme M-/- mice. The otoscopic findings showed that the middle-ear inflammation was severe one day after inoculation of live *S. pneumoniae* 6B in lysozyme M-/- mice, compared to wild-type mice. On day 1, Grade 0 (no inflammation) was seen in 13 (68.4%) out of 19 wild type mice, but only in 4 (21.1%) out of 19 lysozyme M-/- mice. Grade 2 (severe inflammation with abundant effusion) was not seen in wild type mice, but in 7 (36.8%) out of 19 in lysozyme M-/- mice. However, the later clinical courses (on day 3, 5, and 7) were not different between wild type and lysozyme M-/- mice. Taken together with bacterial clearance data, this indicates that lysozyme M plays an important protective role at the early phase of infection.

Discussion

In this study, we showed for the first time that lysozyme M-/- mice were more susceptible to pneumococcal OM with severe inflammation compared to wild type mice, especially in the early phase. It is likely that 3 days during lysozyme M-/- mice's susceptibility may be too short for the host adaptive immune system to respond effectively, thus the depletion of lysozyme M may be the underlying reason for this susceptibility to infection. AIIMs are considered as a possible alternative therapeutic approach, which could overcome current trends of increasing antibiotic resistance among OM pathogens. The evidence of bacterial countermeasures to AIIMs, such as covalent modification of cell wall or membrane and expression of membrane proteases, has been known. However, it is likely that bacteria have not generally developed effective resistance to AIIMs, considering AIIMs have been around for a long time in the animal kingdom.

In mammals, lysozyme can be found in most tissues and is an important component of the innate defense system of all mucosal surfaces, including the digestive tract, genitourinary tract, the respiratory tract, as well as the E-tube and the middle ear. Lysozyme over-expression leads to an increased survival rate after streptococcal and pseudomonal lung infection,[5] while lysozyme deficiency results in a decrease in pseudomonal clearance from the airways[6] and an increase in micrococcal skin inflammation.[7] Consistent with these findings, our result also showed that

lysozyme deficiency results in an increased susceptibility to pneumococcal OM in mice.

Conclusions

It is evident that lysozyme M plays a critical role in protecting the middle ear against invading pathogens, especially in the early phase. We suggest that the exogenous lysozyme could be used as an adjuvant therapeutic agent for treating OM.

Table 1. Bacterial clearance from the middle ear.

Post-inoculation day	Positive Bacterial Culture*		
	Wild type	Lysozyme M[-/-]	Fisher's Exact Test
3	0/10	8/ 9	$p < 0.01$
7	0/ 9	1/10	NS

*The middle-ear lavage was collected at 3 and 7 days after inoculation of live *S. pneumoniae* 6B to investigate the presence of live bacteria. Values are given as the number of culture-positive animals/total number of animals. NS: no significant difference.

References

1. Bluestone CD. Studies in otitis media: Children's Hospital of Pittsburgh University of Pittsburgh progress report--2004. The Laryngoscope 2004; 114(11 Pt 3 Suppl 105):1-26.
2. Tonnaer EL, Rijkers GT, Meis JF, et al. Genetic relatedness between pneumococcal populations originating from the nasopharynx, adenoid, and tympanic cavity of children with otitis media. J Clin Microbiol 2005;43:3140-3144.
3. Lim DJ, Chun YM, Lee HY, et al. Cell biology of tubotympanum in relation to pathogenesis of otitis media--a review. Vaccine 2000; 19 Suppl 1:S17-25.
4. Ganz T. Antimicrobial polypeptides. Journal of leukocyte biology 2004; 75:34-38.
5. Akinbi HT, Epaud R, Bhatt H, Weaver TE. Bacterial killing is enhanced by expression of lysozyme in the lungs of transgenic mice. J Immunol 2000; 165:5760-5766.
6. Cole AM, Thapa DR, Gabayan V, Liao HI, Liu L, Ganz T. Decreased clearance of Pseudomonas aeruginosa from airways of mice deficient in lysozyme M. J Leukoc Biol 2005; 78:1081-1085.
7. Ganz T, Gabayan V, Liao HI, et al. Increased inflammation in lysozyme M-deficient mice in response to Micrococcus luteus and its peptidoglycan. Blood 2003;101:2388-2392.

Intranasal vaccination of infant mice induces protective immunity in the absence of nasal-associated lymphoid tissue

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Introduction

Intranasal (IN) immunization is an effective regimen to induce secretory IgA antibodies in upper respiratory tract. Murine nasal-associated lymphoid tissue (NALT) has been considered to be the major inductive site for initiation of secretory IgA immune responses in the respiratory tract by IN vaccine.[1] In addition, cervical lymph nodes (CLN), which do not belong exclusively to the mucosal immune system, also share immune inductive properties with NALT and serve to amplify the specific responses generated within NALT. Recent studies have emphasized the importance of enhancing mucosal immunity in early life by IN immunization[2]; however, the ability of NALT to function as a mucosal inductive site during early life is largely unknown. In the present study, we assessed the need for NALT and CLN in induction of protective immunity in the middle ear (ME) and nasopharynx (NP) following IN vaccination of infant mice.

Material and methods

NALT and CLN were removed in infant (8-day-old) mice. IN immunization was performed in untreated mice, in mice that received NALT or sham NALT surgery, and in mice that received CLN or sham CLN surgery. Immunization was performed beginning on day 10, i.e., 2 days after surgery. 2.5 µg of pneumococcal polysaccharide conjugate vaccine (Wyeth Vaccines, Pearl River, NY) was administered IN on day 10 together with 0.2 µg of interleukin-12 (IL-12, Wyeth Vaccines) IN on days 10-13. Another group of mice received 2.5 µg of conjugate vaccine on day 10 without IL-12. The conjugate vaccine consisted of serotype 14 pneumococcal polysaccharide (PPS14) covalently linked at a ratio of 2:1 to CRM_{197} diphtheria toxoid. Mice were boosted IN on day 17 with 5 µg of PPS14 with or without IL-12 in a volume 10 µl, and sera and NP wash samples were collected for analysis one week later. Control mice received phosphate buffered saline (PBS) vehicle only. Anti-PPS14 antibody levels in NP washes and serum after IN vaccination of infants were measured by enzyme-linked immunosorbent assay (ELISA). For NP samples, end-point titers were expressed as the reciprocal $\log_2$ of the last dilution that had an optical density at 405 nm (OD_{405}) of >0.1 OD unit above the OD_{405} value of negative control samples obtained from unimmunized mice. Dilutions corresponding to 25% maximal binding were used as the titers for serum antibody.

Pneumococcal colonization studies were performed using a mouse model of NP and ME infection that was established after IN administration of pneumococci. This model mimics the natural route of pneumococcal infection and does not bypass the innate and specific immune mechanisms that are present at NP mucosa surfaces, in contrast to infections established by direct inoculation of bacteria into the ME.[3] One week after the booster immunization, mice were inoculated for 5 consecutive days (days 24-28) IN with 10^6 CFU of type 14 *S. pneumoniae* and sacrificed on day 31. NP and ME washes were plated onto blood agar to determine the concentrations of live bacteria. The mice were monitored by otomicroscopic observation to confirm tympanic membrane changes (vessel dilation, increased thickness, and reduced translucency).

Results

In the present study, we developed a technique to destroy NALT in 8-day-old mice. On day 17 after surgery, there was no presence of organized lymphoid tissue in the nasal cavity and only sparse numbers of lymphocytes in the area previously occupied by the NALT. Similarly, after CLN surgery, there was no evidence of lymphoid tissue in the location typical for CLN location.

Levels of nasal IgA and serum total antibodies were significantly greater in the vaccinated group treated with IL-12 (IgA: 3 ± 0.5; Total: 424 ± 109) than in the mice treated with vaccine only (IgA: 0; Total: 138 ± 52), PBS (IgA: 0; Total: 7 ± 3), or IL-12 only (IgA: 0; Total: 9 ± 3) ($P < 0.05$). Titers of serum antibody were significantly lower in the PBS and IL-12–only groups than in the mice treated with vaccine only ($P < 0.05$).

On days 24-28 after birth, vaccinated mice were inoculated IN with type 14 pneumococci. It was found that the number ($\log_{10}$ CFU) of pneumococci recovered in NP washes (5.9 ± 0.09) as well as the incidence of ME colonization (78%) was not reduced in mice that had received vaccine alone. In contrast, treatment with vaccine plus IL-12 caused markedly lower numbers of NP CFU (5.3 ± 0.1) ($P<0.05$) and a decreased incidence of ME colonization (20%) ($P< 0.05$). There were no significant differences in numbers of bacteria recovered from animals treated with vehicle or IL-12 only (NP: 5.9 ± 0.1; ME: 78%).

We next sought to determine whether the presence of NALT or CLN is a strict requirement for the induction of mucosal and serum antibody responses in neonates. Sham surgeries did not impair the ability of mice to produce specific mucosal or serum antibodies, when compared to mice without surgery. No antibody was detected in the nasal washes or serum of sham surgery mice that were treated with PBS vehicle alone. In vaccinated animals, it was found that both the sham surgery (3 ± 0.5) and NALT-deprived groups (2.1 ± 0.2) had significant elevations in nasal IgA antibody levels ($P < 0.001$) that were not significantly different from each other. However, only low levels of mucosal IgA antibody responses were induced in mice after surgical removal of CLN (0.8 ± 0.4) as compared to the sham surgery group (3.5 ± 0.7) ($P<0.05$). Serum antibody responses were also assessed in NALT- and CLN-deficient mice. Interestingly, IN immunization of mice after removal of NALT resulted in increases in the levels of serum antibodies (1727 ± 513), much greater than those observed in the mice that were subjected to sham NALT surgery (353 ± 54) ($P>0.05$). In contrast, the levels of serum antibodies induced in CLN-deficient mice (175 ± 35) were lower than those after sham CLN surgery (329 ± 52) ($P< 0.05$). In fact, the levels of serum antibodies in CLN-deficient mice were comparable to those seen in intact mice that received IN vaccine alone.

We next evaluated the roles of NALT and CLN in protection of the respiratory tract. Sham surgeries did not impair the ability of mice to control NP and ME pneumococcal colonization following IN bacterial inoculation, when compared to mice without surgery. NALT-deficient or sham NALT surgery mice demonstrated similar decreases in NP (NALT-deficient: 5.5 ± 0.1; sham surgery: 5 ± 0.1) and ME colonization (NALT-deficient: 33%; sham surgery: 17%) following IN pneumococcal challenge. In contrast, CLN-deficient mice demonstrated higher numbers of bacteria in NP washes ($6 + 0.1$) as well as a higher incidence of ME colonization (83%)

compared to sham CLN surgery mice (NP: $4.3 + 0.8$; ME:17%) ($P<0.05$). There were no decreases in NP or ME colonization in NALT- or CLN-deficient mice that were administered vaccine only or PBS (data not shown).

Discussion

This study used an infant IN immunization protocol with PPS14 conjugate vaccine and IL-12 as adjuvant, and demonstrated the importance of CLN but not NALT for induction of protective mucosal antibody responses against pneumococcal infection in young animals.

There is substantial evidence that in the adult respiratory tract, NALT is a major inductive site for antigen-specific IgA antibody responses. Recently, several reports showed that specific secretory IgA responses can be induced in the absence of selected mucosal inductive sites, including NALT.[4-6] Based on the significant mucosal IgA antibody responses obtained in mice without NALT, it was important to determine the source of those responses. Thus, we addressed the possibility that CLN are required for the induction of antigen-specific nasal responses. Surgical removal of the CLN that drain both NALT (central CLN) and nasal mucosa (superficial CLN), was found to significantly impair the ability of the host to generate nasal IgA antibodies following IN immunization. A similar decrease in levels of nasal IgA antibodies was observed when mice lacking only superficial CLN were IN immunized (data not shown). Others have likewise reported that the absence of lymph nodes that drain the respiratory or gastrointestinal tracts causes a significant impairment in the ability to generate mucosal IgA antibody responses following IN or oral immunization, respectively.[4,5] These findings suggest that lymph nodes that drain mucosal tissues are the primary inductive sites for mucosally administered vaccines. It is possible that following IN immunization, lymphocytes in CLN migrate into the respiratory lamina propria for subsequent IgA immune responses. Moreover, it is possible that CLN can compensate for defects in immunological inductive functions if NALT is absent.

The present study further showed that removal of CLN significantly impaired expression of both mucosal and serum antibodies following IN immunization with vaccine plus IL-12, suggesting that CLN play an important role not only for mucosal IgA antibody expression but also for systemic antibody responses. The unexpected finding was the observed

increase of serum antibody levels in NALT-deficient mice (~ 4.8 fold increase). One of the possible explanations for this increase is that NALT surgery facilitated the diffusion of IN administered vaccine from the mucosa into the blood.

In addition to measuring antibody responses, the roles of NALT and CLN in protection of the respiratory tract from pneumococcal colonization was also evaluated. Elimination of NALT did not impair the ability of vaccinated hosts to control NP and ME pneumococcal colonization following IN bacterial inoculation. In contrast, protection against pneumococcal colonization was impaired in mice that underwent surgical removal of CLN prior to immunization. These findings indicate that CLN are essential to confer protection in the NP and ME after vaccination, whereas NALT plays only the minor role in that protection.

Several factors such as the physical nature of antigen (soluble versus particulate), the use of adjuvant, and the maturity of the lymphoid tissues could contribute to the present findings. In general, soluble antigens are thought to be readily absorbed through the nasal mucosa, whereas NALT is more adept at taking up particulate antigens.[7,8] However, the ability of soluble vaccines to interact with NALT may be increased by the use of adjuvants.[9,10] Although the exact mechanism of action of IL-12 as a mucosal adjuvant is largely unknown, it is possible that IL-12 enhances penetration of antigen through the nasal epithelia. Another potentially relevant factor is the differential organogenesis of NALT and CLN in young mice. Initiation of NALT formation begins soon after birth[1], and this lymphoid tissue is present in 8-day-old mice. Although NALT of 8-day-old mice has detectable T- and B-cell areas, its cellular composition and chemokine/adhesion molecule expression is decreased compared to adult mice.[6,11] In contrast, CLN development begins during embryogenesis, and 8-day-old mice have fully developed CLN. Considering this, it is possible that in neonates, NALT is less capable than CLN in functioning as an immunologic inductive tissue.

In humans, the surgical removal of tonsils and adenoids, which are thought to be the functional equivalent of NALT, does not appear to compromise protection of the upper respiratory tract nor result in significant immunodeficiency.[12] The observations discussed above provide additional evidence that surgical removal of NALT tissue, at least in a mouse model, does not affect the ability to respond to subsequent IN vaccination.

The results presented in this study indicate that in young mice, NALT plays a minor role in the generation of mucosal IgA antibodies following IN immunization with PPS conjugate vaccine plus IL-12. In contrast, CLN appear to play an essential role in induction of respiratory IgA responses. In the absence of NALT, it is possible that CLN provide a compensatory immune response, and that response is sufficient to reduce NP and ME bacterial colonization.

References

1. Kuper CF, Koornstra PJ, Hameleers DM, Biewenga J, Spit BJ, Duijvestijn AM, van Breda Vriesman PJ, Sminia T. The role of nasopharyngeal lymphoid tissue. Immunol Today 1992; 13: 219-224.
2. Bjarnarson SP, Jakobsen H, Del Giudice G, Trannoy E, Siegrist CA, Jonsdottir I. The advantage of mucosal immunization for polysaccharide-specific memory responses in early life. Eur J Immunol 2005; 35:1037-1045.
3. Sabirov A, Metzger DW. Intranasal vaccination of neonatal mice with polysaccharide conjugate vaccine for protection against pneumococcal otitis media. Vaccine 2006; 24:5584-5592.
4. Wiley JA, Tighe MP, Harmsen AG. Upper respiratory tract resistance to influenza infection is not prevented by the absence of either nasal-associated lymphoid tissue or cervical lymph nodes. J Immunol 2005; 175:3186-3196.
5. Yamamoto M, Rennert P, McGhee JR, Kweon MN, Yamamoto S, Dohi T, Otake S, Bluethmann H, Fujihashi K, Kiyono H. Alternate mucosal immune system: organized Peyer's patches are not required for IgA responses in the gastrointestinal tract. J Immunol 2000; 164: 5184-5191.
6. Ying X, Chan K, Shenoy P, Hill M, Ruddle NH. Lymphotoxin plays a crucial role in the development and function of nasal-associated lymphoid tissue through regulation of chemokines and peripheral node addressin. Am J Pathol 2005; 166:135-146.
7. Balmelli C, Demotz S, Acha-Orbea H, De Grandi P, Nardelli-Haefliger D. Trachea, lung, and tracheobronchial lymph nodes are the major sites where antigen-presenting cells are detected after nasal vaccination of mice with human papillomavirus type 16 virus-like particles. J Virol 2002; 76:12596-12602.
8. Balmelli C, Roden R, Potts A, Schiller J, De Grandi P, Nardelli-Haefliger D. Nasal immunization of mice with human papillomavirus type 16 virus-like particles elicits neutralizing antibodies in mucosal secretions. J Virol 1998; 72:8220-8229.

9. Kodama S, Suenaga S, Hirano T, Suzuki M, Mogi G. Induction of specific immunoglobulin A and Th2 immune responses to P6 outer membrane protein of nontypeable Haemophilus influenzae in middle ear mucosa by intranasal immunization. Infect Immun 2000; 68:2294-2300.

10. Wu HY, Nikolova EB, Beagley KB, Russell MW. Induction of antibody-secreting cells and T-helper and memory cells in murine nasal lymphoid tissue. Immunology 1996; 88:493-500.

11. Hameleers DM, van der Ende M, Biewenga J, Sminia T. An immunohistochemical study on the postnatal development of rat nasal-associated lymphoid tissue (NALT). Cell Tissue Res 1989; 256:431-438.

12. Ikinciogullari A, Dogu F, Ikinciogullari A, Egin Y, Babacan E. Is immune system influenced by adenotonsillectomy in children? Int J Pediatr Otorhinolaryngol 2002; 66:251-257.

EP2E (SPAG11): Inducible beta-defensin-like antimicrobial innate immune molecules in the human middle ear and eustachian tube

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Our body is protected by antimicrobial innate immune molecules including alpha- and beta-defensin. Beta-defensins are cationic peptides produced by epithelial cells that are thought to play an important innate immune function at mucosal surfaces. EP2E is a novel member of the beta-defensin family of antimicrobial innate immune molecules. We hypothesized that: 1) The middle ear and eustachian tube is protected by a highly effective innate immune system consisting of epithelial cell-derived bactericidal molecules as well as complement and phagocytes and 2) Members of the beta-defensin family are expressed by the tubotympanum epithelial cells and are among nature's most potent antimicrobials. Using these hypotheses, the objective of the study was to determine the antimicrobial activity of EP2E, the effect of bacterial infection on its expression, and its distribution in the middle ear and eustachian tube. In addition, we propose an ultramicroscopic EP2E action mechanism.

Materials and methods

Quantitative polymerase chain reaction. EP2E (SPAG11) mRNA expression was assayed by quantitative polymerase chain reaction (Q-PCR) using cDNA derived from each organ of the ear. Expression of EP2E(SPAG11) was also evaluated using Q-PCR in tissues obtained from cultured human middle-ear epithelial cells 4 hours after incubation with lipopolysaccharide (LPS).

Peptides, refolding and antimicrobial assays. Based on the sequence deduced from EP2E(SPAG11) cDNA, a 43-amino acid peptide was assembled using automated [n-(9-fluorenyl)methoxycarbonyl] solid-phase synthesis. EP2E(SPAG11) and EP2E, each of which was homogeneous by electrophoretic and mass spectrum analysis, were quantified by UV absorbance at 280 nm. They were tested for antimicrobial activity against bacterial strains *(Escherichia coli* ML35p, *Moraxella catarrhalis*, NTHi, and *Streptococcus pneumoniae* T3) in a CFU assay as described previously.[2] Culture densities were measured spectrophotometrically at 600 nm then resuspended to a final concentration of 10 6 CFU/ml. An A600 reading of 1 corresponds to 1.5×10^9 CFU/ml for the NTHi, 4.0×10^8 CFU/ml for *M. catarrhalis*, 1.0×10^8 CFU/ml for *S. pneumoniae*, and 2.5×10^8 CFU/ml for *E. coli*. The organisms were incubated with various concentrations of peptide at 37 °C with constant shaking for 1 hour. Surviving microbes were plated in triplicate on trypticase soy broth plates (*E. coli*) or on chocolate agar plates (NTHi, *M. catarrhalis*, and *S. pneumoniae*).

Immunohistochemistry.
Immunohistochemistry was carried out using human and rat temporal bone sections using a specific antibody raised against human EP2E synthetic peptide. For the human temporal bones, the sections were decelloidinized and antigen retrieval was performed by placing the samples into citrate buffer (0.01 M, pH 6.0) for 30 minutes. Three percent hydrogen peroxide solution was applied on the samples for 10 minutes and the slides were washed in phosphate buffered saline (PBS) three times for 5 minutes each. After incubation with blocking serum for 1 hour, specimens were incubated with rabbit polyclonal anti-EP2E antibody (1:100 dilution, 10ug/ml) was used as a primary antibody for overnight at 4˚C. After washing three times in PBS for 5 minutes each, primary antibody labeling was detected with AEC kit (Histostain-SP; Zymed Laboratories Inc., South San Francisco, CA). Rabbit IgG was used for negative controls. Samples were viewed and photographed by using a Zeiss Axiovert 135 TV microscope and Axio Cam software system (Zeiss, Germany).

Results

The results showed that rat EP2E (SPAG11) is expressed in the eustachian tube as well as the middle ear. Using a rat model, its expression was upregulated upon challenge with the bacterial endotoxin LPS. The eustachian tube as well as the middle ear showed higher expression than the cochlea and vestibule. The sequence was confirmed from the PCR product.

A specific antibody has been raised against synthetic human EP2E peptides and was used to detect EP2E in sections of human archival temporal bones. EP2E peptide was detected in the epithelial cells of the middle ear in a patient with otitis media (OM). A positive signal has been found only in the OM case. The EP2E labeling was localized in the apical and basal layers of epithelial cells.

Human EP2E peptide was synthesized chemically and refolded by oxidative refolding.[3] Synthetic EP2E (SPAG11) inhibited the growth of *E. coli*, NTHi*, S. pneumoniae* and *M. catarrhalis* on radial assay. The antimicrobial activity has been confirmed in solution against the OM pathogen NTHi. Almost complete bactericidal activity was found at a concentration of 2.5 - 5.0 ug/ml. The antimicrobial activity was comparable to rat beta-defensin 2, which is one of the most potent antimicrobial molecules.

Discussion

A member of the defensin family, Bin1b in the rat and EP2E in humans, has been found to be expressed in eustachian tube and middle-ear epithelial cells. EP2E (SPAG11) is inducible peptide with limited tissue expression during bacterial infection. EP2E expression was upregulated in a patient with OM. Ultrastructural studies indicated that EP2E (SPAG11) indeed disturbs the bacterial membrane. Because it exhibits antimicrobial activity against bacterial pathogens, this peptide may serve as an innate defense against microbial invasion in the middle ear and eustachian tube in rats and in humans.

References

1. Li P, Chan HC, He B, et al. An antimicrobial peptide gene found in the male reproductive system of rats. Science 2001; 291:1783-1785.
2. Lee H-Y, Andalibi A, Webster P, et al. Antimicrobial activity of innate immune molecules against Streptococcus pneumoniae, Moraxella catarrhalis and nontypeable Haemophilus influenzae. BMC Infect Dis 2004; 4:12.
3. Singh RR, Rao AGA. Reductive unfolding and oxidative refolding of a Bowman-Birk inhibitor from horsegram seeds (Dolichos biflorus): evidence for 'hyperreactive' disulfide bonds and rate-limiting nature of disulfide isomerization in folding. Biochim Biophysica Acta 2002; 1597:280-291.

Pseudomonas aeruginosa lipopolysaccharide-mediated osteoclastogenesis, a MyD88- and Toll-like receptor 4-dependent process

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Chronic middle-ear infections such as chronic otitis media (COM) and infected cholesteatoma are biofilm infections that are invariably associated with bony destruction of the temporal bone. Bony erosion is the sole consequence of osteoclast activation and most commonly leads to conductive hearing loss. Although these biofilm infections likely harbor multiple species, the Gram-negative bacterium, *Pseudomonas aeruginosa* (PA), is the most common organism isolated from COM and cholesteatoma specimens. Virulence factors from Gram-negative bacteria such as lipopolysaccharide (LPS) have been shown to both promote osteoclast formation/activation and to be present in higher concentrations in patients undergoing bony erosion. Although LPS is known to promote bone resorption, little is known regarding underlying mechanisms, and even less is known regarding the osteoclastogenic potential of PA LPS. To this end, we examined the role of direct interactions of PA LPS with osteoclast precursors in terms of osteoclast differentiation and bone resorbing ability.

We found that both purified murine bone marrow mononuclear cells and RAW 264.7 cells (a murine hematopoietic cell line) were capable of robust osteoclastogenesis after exposure to PA LPS only if previously primed with suboptimal levels of receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL), a cytokine critical to the process of osteoclast formation. Furthermore, the osteoclasts derived from this method were capable of bone erosion on hydroxyapatite-coated vessels, establishing clinical relevance to this phenomenon.

These experiments were then repeated in mice with targeted knockouts for the putative LPS receptors, toll-like receptor 4 (TLR4) and TLR2, as well as in mice with targeted disruption of myeloid differentiation factor 88 (MyD88), a TLR adaptor protein critical to signaling downstream from both TLR4 and TLR2. We found that PA-LPS–mediated osteoclast formation was TLR4- and MyD88-dependent and independent of TLR2. To further characterize the process of LPS-mediated osteoclastogenesis, concentrations of tumor necrosis factor alpha (TNF-α) and interferon beta (IFN-β) were determined in precursors undergoing LPS-mediated osteoclastogenesis. These two cytokines are accepted as specific to the MyD88-dependent and MyD88-independent pathways, respectively, and both were significantly elevated compared to precursors undergoing RANKL priming. Furthermore, levels of osteoprotegerin, a soluble RANKL decoy receptor and osteoclast differentiation inhibitor, were unchanged. This established that changes in concentrations of this cytokine are not involved in LPS-mediated osteoclast formation.

In summary, we have established for the first time that LPS from PA is capable of promoting the formation of bone-resorbing osteoclasts, and this process depends upon intact TLR4 and MyD88 signaling. Furthermore, levels of TNF-α and IFN-β are elevated in osteoclast precursors undergoing osteoclast formation. This likely reflects an autocrine mechanism by which LPS drives robust osteoclast formation involving both MyD88-dependent and MyD88-independent pathways.

Incidence of acute otitis media and sinusitis complicating upper respiratory tract infection: the effect of age

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Introduction

Acute otitis media (AOM) and sinusitis are two of the most common bacterial complications of upper respiratory tract infections (URI) in children. It has been found that 29- 50%[1,2] of all URIs develop into AOM and 5-10% develop into sinusitis.[3] On average a child younger than 5 years of age has 2-7 episodes of URI per year[4,5], a child attending daycare may have up to 14 episodes per year.[6] By age three, 80% of children have had at least one episode of AOM[7] and approximately 13% have had sinusitis.[8] The peak age incidence of AOM is between ages 6-18 months, compared to 2-6 years of age for sinusitis.[8] Despite the frequency of these infections and their close association with URI, there has been no study to date that determines the age specific incidence of AOM and sinusitis following a URI. This study is an analysis of the age incidence of AOM and sinusitis following URI in a subgroup of children enrolled in an ongoing long-term study of the pathogenesis of virus-induced AOM.

Methods

Healthy children aged 6 to 35 months were enrolled from January 2003 to March 2006 in a prospective, longitudinal study of the pathogenesis of virus-induced AOM (Chonmaitree et al., unpublished study). The study was designed to capture all URI episodes occurring during the one-year follow-up period to study the rate and characteristics of AOM following URI. Children were seen by a study physician as soon as possible after the onset of URI symptoms and then followed again a few days later (days 3-7 of the URI) for URI complications. Each URI episode was studied and monitored closely for at least 3 weeks for the development of AOM or sinusitis. AOM complicating URI was considered when the episode occurred within 21 days of the URI. AOM and sinusitis diagnosis followed established guidelines published by the American Academy of Pediatrics (AAP)[9,10] and the Joint Task Force on Practice Parameters for Allergy and Immunology.[8]

AOM was defined by acute onset of symptoms (fever, irritability, or earache), signs of inflammation of the tympanic membrane and presence of fluid in the middle ear documented by pneumatic otoscopy and/or tympanometry.[10] Sinusitis complicating URI was considered when children had persistent URI symptoms for more than 10 days without improvement or an abrupt increase in severity of symptoms, fever, or purulent nasal discharge prior to day 10 of illness.[9] Children diagnosed with AOM or sinusitis were given antibiotic therapy consistent with standard of care.

All URI, AOM, and Sinusitis were included in the analysis. Data were analyzed by chi square using STATA 9.0 © (Stata Corporation, College Station, TX). Rate ratios were calculated by Episheet 2001, Spreadsheets for the Analysis of Epidemiologic Data by Rothman.[12]

Results

This report consists of data from 112 patients who completed the study as of October 2005. Fifty-five (49%) were male, 25 (22%) were Caucasian, 34 (30%) were African American, 47 (42%) were Hispanic and 6 (5%) were other races. The mean and median ages of the children at enrollment were 15 and 13 months, respectively. Sixty-four percent of the children were fully immunized with pneumococcal conjugate vaccine (PCV7) according to the Advisory Committee on Immunization Practices schedule. The mean and median number of weeks of breastfeeding was 16 and 23, respectively; 37% of the children were breastfed for longer than 2 weeks. Thirty percent of the children were enrolled in day care; 18% percent of children 6-11 months old, 40% of 12-23 month old and 44% of 24-35 month olds were in day care. Twenty-nine percent of the children were exposed to cigarette smoke.

The children were followed for a total of 1231 patient months during which time a total of 623 URI episodes occurred; the URI episodes resulted in 188 AOM and 52 sinusitis episodes. There were 17 episodes of AOM that did not follow a URI. The overall incidence of URI was 0.51/patient month (6.12 episodes/ patient year); AOM=0.15 / patient month (2.01 episodes/ patient year); and sinusitis=0.04/

patient month (0.48 episodes/ patient year).Thirty percent of all URIs were complicated by AOM and 8% by sinusitis, these include fifteen URI episodes (2%) that were complicated by both AOM and sinusitis (47% concomitantly and 53% sequentially, most children were diagnosed AOM before sinusitis). The peak days of diagnosis for AOM were on days 3, and 5 (14% and 13% respectively). Fifty-seven percent of AOM episodes were diagnosed in the first week of URI. Fifteen (29%) of the sinusitis episodes were diagnosed because of abrupt increase in severity of symptoms, fever, or purulent nasal discharge prior to day 10 of illness the remainder were diagnosed based on persistence of symptoms beyond 10 days.

Children in the 6-11 month old and 12-23 month old age groups had the same incidence of URI (0.51/ pt-month). Children in the third year of life had 0.48 URI/pt-month. Children who attended day care had a URI rate of 0.59 URI episodes/ pt-month, compared to 0.47 URI episodes/ pt-month in children who did not attend daycare (rate ratio: 1.2, CI: 1.1-1.4 p=0.01). AOM occurred after URI in 36% of episodes in children 6-11 months old (Figure), 29% of episodes in children in the second year of life, and 15% of episodes in children in the third year of life (P < 0.001 by fishers exact test). Older children tended to have fewer AOM episodes following URI (P <0.001 by Cochrane-Armitage trend test). Sinusitis complicated URI in 7%, 10%, and7% of episodes in children from 6-11, 12-23, and 24-35 months, respectively.

Discussion

It is well established that the peak age for AOM is between 6 and 18 months.[7,12,13] More recent data have suggested that AOM generally occurs as a complication of viral URI.[14] In this study, we clearly demonstrate that 30% of URI episodes in children result in AOM and the disease occurs most often in children between 6 and 11 months of age even though these children are as equally susceptible to URI as children in the second year of life. We also found sinusitis following URI occurs less frequently than AOM (8%) and the disease was more commonly diagnosed in children from 12-23 months of age.

For otitis media to occur, bacteria colonized in the nasopharynx must enter the middle ear via the Eustachian-tube. Normally, bacteria are prevented from entering the middle ear by the ciliated epithelium lining the Eustachian-tube. Respiratory virus infection disrupts the mucociliary system impairing the ear's primary mechanical defense from bacterial invasion. In addition, Eustachian-tube dysfunction can lead to reduced middle ear pressure, forcing mucus,

nasopharyngeal secretions, and bacteria into the middle ear. This creates an ideal milieu for bacterial super-infection.[15]

We found the highest incidence of AOM following URI to be between 6 and 11 months of age and these data parallel what was found by Teele et al[7]. The increased susceptibility to AOM in younger children has been postulated to be secondary to inadequate immunologic response, and shorter, straighter and narrower Eustachian tube.[16] It stands to reason that the longer children are protected from exposure to known avoidable otitis media risk factors, the later the onset of AOM, and the lower their life time incidence.

The pathophysiologic processes that occur in paranasal sinuses during a viral URI are similar to those that occur in the middle ear. The ciliated epithelium in the sinuses also loses its ability to move debris from the nasal cavity. When a child sniffs or blows his nose negative pressure is formed within the sinus cavity drawing in bacteria and debris, once again creating a model environment for bacteria to proliferate and cause sinusitis. Nevertheless, sinusitis is still a disease with a much lower incidence than AOM.

We found that the incidence of sinusitis was lower in the 6-11 month age group and in children over 24 months when compared to children 12-23 months but the difference did not reach statistical significance. We postulate that the incidence of sinusitis may peak in the 12-23 month old children because these children are less likely to develop AOM, thus less likely to have received antibiotic. Therefore, any low grade bacterial infection in the sinuses would go unnoticed until cleared by the child's own natural defense or progress to clinical sinusitis requiring subsequent initiation of antibiotic therapy. Children in the third year of life may have a lower incidence of AOM and sinusitis possibly because these children have already developed partial immunity to many microbial pathogens and subsequently do not have a strong inflammatory response to infection. This study was not powered to determine a difference in the incidence of sinusitis; different studies targeted to sinusitis may further clarify these differences.

In summary, we found that children from 6-11 months of age were at highest risk for developing AOM after URI. Although older children were more likely to be in day care, they developed fewer episodes of AOM and sinusitis. Delaying entry into group day care until the second year of life could help reduce incidence of AOM in infants and young children.

Acknowledgement
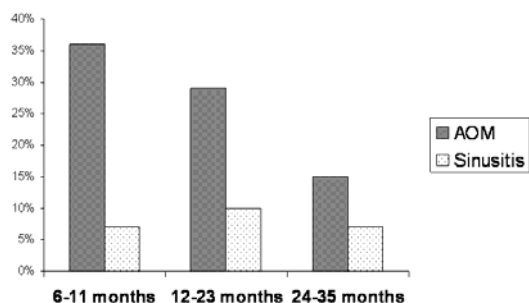
Figure 1: AOM and Sinusitis Incidence by Age. AOM occurred more often following URI in younger children (P <0.001 by Ffishers exact test). AOM incidence decreased with increasing age (Cochrane-Armitage trend test). N= number of URI episodes

References

1. Heikkinen T, Ruuskanen O. Temporal development of acute otitis media during upper respiratory Tract infection, Pediatr Infect Dis J.1994;13:659-661.
2. Kontiokari T, Neimelä M, et al. Time to development of acute otitis media during an upper respiratory tract infection in children. Pediatr Infect Dis J. 1999;18:303-305.
3. Cherry JD, Shapiro NL, Deville JG. Sinusitis In: Feigin RD, Cherry JD, Demmler GJ, Kaplan SL, eds. Textbook of Pediatric Infectious Diseases. 5th ed. Philadelphia: Saunders, 2004: 201-212.
4. Monto AS, Ullman BM. Acute respiratory illness in an American community: The Tecumseh study. JAMA. 1974;227:164-169.
5. Fox JP, Hall CE, Cooney MF, et al. The Seattle virus watch. II. Objectives, study population and its observation, data processing and summary of illness. Am J Epidemiol. 1972;96;270-285.
6. Stahlberg MR. The influence of form of day care on occurrence of acuter respiratory tract infections among young children. Acta Pediatr Scand. 1980;282:1-87.
7. Teele DW, Klein JO, Rosner B.Epidemiology of otitis media during the first seven years of life in Children in greater Boston:A prospective, cohort study. J Infect Dis.1989;160:83-94.
8. Salvin RG, Spector SL, Bernstein IL. The diagnosis and management of sinusitis: A practice parameter update. J Allergy Clin Immunol. 2005;116:S13-47.
9. American Academy of Pediatrics. Subcommittee on Management of Sinusitis and Committee on Quality improvement. Clinical practice guideline: management of sinusitis. Pediatrics. 2001;108:798-808.
10. American Academy of Pediatrics. Subcommittee on Management of Acute Otitis Media Diagnosis and Management of Acute Otitis Media. Pediatrics 2004; 113:1451-1465.
11. Rothman, K. Spreadsheets for the analysis of Epidemiological Data 2004. Accessible at: http://members.aol.com/krothman/episheet.xls.
12. Daly KA, Giebink GS.Clinical epidemiology of otitis media. Pediatr Infect Dis J. 2000;19:S31-6.
13. Paradise JL, Rockette HE, Colborn DK et al. Otitis media in 2253 Pittsburgh-area infants: prevalence and risk factors during the first two years of life. Pediatrics. 1997;99:318-333.
14. Heikkinen T, Chonamitree.Importance of respiratory iruses in acute otitis media. Clin Microbiol Rev. 2003;16:230-241.
15. Bakaletz LO. Viral Potentiation of bacterial superinfection of the respiratory tract. Trends Microbiol 1995;3:110-114.
16. Klein JO, Bluestone CD. Otitis media. In: Feigin RD, Cherry JD, eds. Textbook of Pediatric Infectious Diseases. 5th ed. Philadelphia: Lippincott Williams & Wilkins, 1999:215-234.

Mastoiditis before and after new treatment recommendations for AOM

Karin Stenfeldt, M.D., Ph.D., Ann Hermansson, M.D, Ph.D.

Introduction

Acute mastoiditis is an uncommon but serious complication of acute otitis media (AOM) that mostly affects young children. The symptoms are AOM with concurrent redness, tenderness and swelling over the mastoid process behind the ear, lowering of the roof of the external ear canal, often associated with general illness. The incidence has declined when compared to the first half of the 20th century, due to the routine use of antibiotics in the treatment of AOM. In a consensus document in the year 2000, recommendations regarding treatment of AOM were changed in Sweden. Instead of prescribing antibiotics routinely for AOM, watchful waiting was recommended as an alternative for children over the age of 2 years. It has been inferred that these recommendations might lead to an increased rate of complications of AOM, e.g., mastoiditis.

Aim of the study

This is a retrospective study of patients from University Hospital of Lund and Ängelholm Hospital in southern Sweden during the period 1996 to 2005. The aims of the study were (a) to map the occurrence and clinical course of mastoiditis before and after the new treatment recommendations for AOM, (b) to define persons at risk of developing mastoiditis, and (c) to define diagnostic criteria for mastoiditis.

Materials and methods

All patients with the diagnoses code for mastoiditis were selected. In order to analyse patients who were admitted to hospital with an initial suspicion of mastoiditis, patients with the diagnoses codes for AOM and external otitis media were identified. In all, 800 charts were reviewed. In 117 cases there had been an initial suspicion of mastoiditis and these patients were included in the study.

A protocol including many variables was used for systematic analysis of the medical charts. These included age in months, sex, medical history, previous ear diseases, tube treatment, history of AOM, age at AOM, number of AOM episodes, previous ear surgery, laboratory findings including findings in bacterial cultures, computed tomography findings, complications, and sequelae.

Results

Using the final diagnoses that were given in the medical charts, 32 patients were diagnosed with mastoiditis, and 12 were judged as incipient mastoiditis (sub-acute mastoiditis), for a total of 44 mastoiditis patients in all. Seventy-three of the patients never developed mastoiditis, and were found to have AOM or external otitis media.

It was very difficult to distinguish the groups. Patients with similar clinical courses seemed to have been given different diagnoses. Therefore, all charts were reviewed to find common diagnostic criteria. Diagnoses were changed in 12 patients to fit the criteria of the present study. Some patients were not so easily defined, but were "borderliners."

Criteria for mastoiditis in this study were: Cases where mastoidectomy was performed with findings of osteitis and pus were of course judged as mastoiditis. In other cases, when a patient did not undergo mastoidectomy, but where the clinical course was characteristic for mastoiditis with AOM, redness and tenderness over the mastoid, protruding ear, and/or lowering of the ear canal roof, and general illness, were also judged as mastoiditis.

Patients with AOM who displayed redness over the mastoid or a protruding ear, but who had a benign course with fast healing, were considered to have incipient mastoiditis.

A case of draining tube with secretion in combination with redness over the mastoid and a protruding ear was classified as AOM with secondary external otitis media.

Primary external otitis media accompanied by redness over the mastoid or a protruding ear was classified as external otitis media.

After analysis and changing of the diagnoses according to the criteria given above, acute mastoiditis was present in 31 patients, and incipient mastoiditis was found in 11 patients, 42 mastoiditis cases in all. Other diagnoses were set in 75 cases.

Sixteen cases of acute mastoiditis were diagnosed during the period 1996 to 2000, compared to 15 cases during 2001 to 2005. When the incipient mastoiditis cases are included, 24 cases of mastoiditis were identified in the first period 1996 to 2000, and 18 cases in the second period 2001 to 2005 (Fig.1).

The number of patients undergoing mastoidectomy because of suspicion of mastoiditis

was 15 for the first period, and 8 patients underwent mastoidectomy during the second period (Fig. 2).

The mean age of mastoiditis patients (including incipient mastoiditis) was 6 years, median 2 years and 6 months. (The mean age for mastoiditis was 6 y 8 m, median 1 y 11 m; incipient mastoiditis, mean 3 y 11 m, median 3 y). The mean age of patients with AOM and external otitis was 13 y 1 mos and the median age was 7 y and 2 mos.

The mean time from the first symptom to in-hospital care was 6.7 days for mastoiditis patients and median time was 4 days. For the incipient mastoiditis patients, the mean time was 3.7 days, and the median time was 2.5 days. For the whole mastoiditis group the mean time from first symptom to in-hospital care was 6 days and median time 4 days.

The mean time for first symptom to in-hospital care for patients with external otitis or AOM was 7.2 days and median time was 5.5 days.

Patients were given antibiotics prior to hospital care in 12 (40%) of the mastoiditis patients and in 4 (36%) of the incipient mastoiditis patients. On the other hand, 18 (60%) mastoiditis patients and 7 (64%) incipient mastoiditis patients were not given antibiotics prior to hospital care. Information about antibiotic treatment was not found in one patient. In all 16 (39%) patients in the total mastoiditis group were given antibiotics before hospital care while 25 (61%) did not receive antibiotics.

Sixty-one patients (82%) in the group of AOM and external otitis were given general antibiotic treatment before hospital admittance, while 13 (18%) were not under antibiotic treatment. Two of these had finished an antibiotic course within 2 days prior to admittance. Two were given local treatment with topical steroid and antibiotics (Polymyxin B). No information about antibiotics was found in one patient.

The findings of bacterial cultures at mastoidectomy in patients with mastoiditis were *Streptococcus pneumoniae*, Group *A Streptococci*, and *Staphylococci*. None of the cultured bacteria were resistant to penicillin.

Discussion

The present study shows that the number of patients with mastoiditis did not increase after the new treatment recommendation of watchful waiting was introduced. Other authors have reported an increase in the number of mastoiditis patient during recent years (Benito 2007), but the cause of this increase remains undetermined. It has been speculated that the increase in penicillin resistant bacteria could be one reason, or new recommendations of not treating otitis media with antibiotics in order to stop the increasing threat of antibiotic resistant bacteria.

Predisposing factors in mastoiditis were low age and rapid onset of infection as indicated by the short time interval from first symptom to admittance to hospital. The patients with a more severe mastoiditis had a low median age (1 year, 11 months) compared to those with a milder, incipient mastoiditis. Patients with incipient mastoiditis had a shorter duration from first symptom to in- hospital care and one can speculate that because these patients received proper care early, the development of a more fulminant disease was stopped. The number of patients undergoing mastoidectomy declined over the years (Fig 2). The reason is unclear, but may be a result of changed treatment traditions over time in favour of antibiotic treatment in combination with myringotomy.

The number of patients who received antibiotics was higher in acute and external otitis media compared to the patients with mastoiditis. The patients with external otitis had a longer duration of symptoms before hospital care and had often obtained treatment before admission to hospital. In a retrospective, multi-centre study by Luntz et al (Luntz 2001), 54% of patients had received antibiotics prior to the mastoiditis episode compared to 40 % in the present study.

The number of mastoiditis patients who received antibiotics before hospital care did not decline after the new treatment recommendations of the year 2000. The patients who did not receive antibiotics had not been to a doctor before admittance to hospital care. None of the mastoiditis patients in the present study was under watchful waiting in connection with AOM. This does not imply doctors generally do not follow the new guidelines. The mastoiditis patients constitute a very small proportion of the huge number of otitis media patients, and it could be that these patients were sicker and were therefore given antibiotics according to the guidelines.

Conclusions

Most of the patients in the present study who were admitted to hospital with suspicion of mastoiditis were finally diagnosed with AOM or external otitis media. Predisposing factors in mastoiditis were low age and rapid onset of infection.

It was shown that the proportion of patients with mastoiditis who received antibiotics before hospital admittance did not decrease since new guidelines were introduced in 2000. The study does not show whether AOM patients who developed

mastoiditis waited longer before receiving antibiotic treatment of AOM.

General criteria for the diagnosis of mastoiditis must be established to enable comparisons between centres and over time. It is important to follow up the consequences when treatment recommendations for AOM are changed.
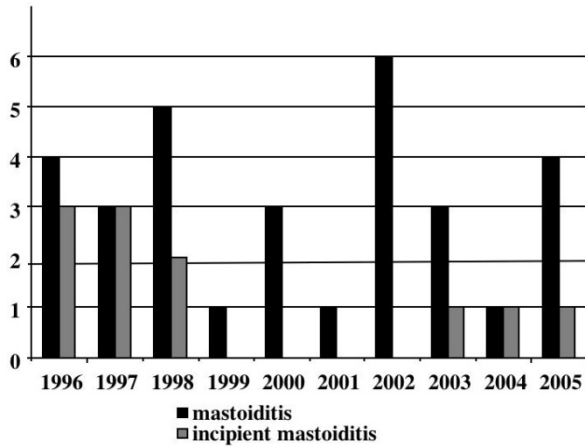
Figure 1.

The number of mastoiditis patients over time. Mastoiditis cases did not increase after the new treatment recommendations were introduced. There were 16 cases of acute mastoiditis in the first period, and 15 in the second. When incipient mastoidits cases are included the numbers were 24 and 18 respectively.
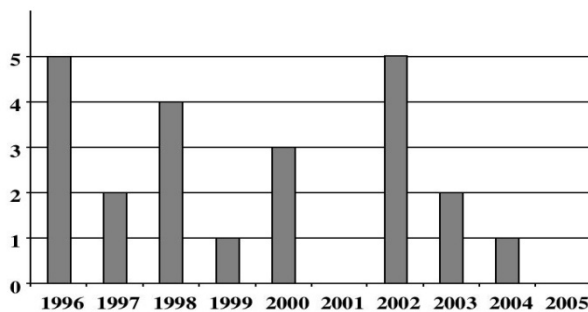
Figure 2.

The number of patients who underwent mastoidectomy over time

References

1. Benito MB, Gorricho BP. Acute mastoiditis: Increase in the incidence and complications. Int J Pediatr Otorhinolaryngol 2007; 71:1007-1011.

2. Luntz M, Brodsky A, Nusem S et al. Acute mastoiditis –the antibiotic era: a multicenter study. Int J Pediatric Otolaryngol 2001; 57:1-9.

Retraction pockets of the pars tensa in children: Evolution and treatment

Desiderio Passali, M.D., Ph.D., Pasquale Cassano

Forty-two children, aged 4-14 years, with retraction pockets variously located submitted to medical or surgical treatment, compared to a control group of 20 children who were not treated but observed for many years. In younger children, grade I and II retraction pockets were treated with medical therapy or with ventilation tube insertion. Those with grade III or IV underwent pocket excision and tympanic reinforcement with temporalis fascia or cartilage grafting, eventually associated to ossiculoplasty. In older children, the latter treatment was also done in some of the grade II cases, characterized by long-lasting pathology or serious conductive hearing loss. Results were followed-up for at least 24 months.

In patients with grade I or II retractions (16 cases; 38%), medical treatment or ventilation tube insertion cured the pathology in 15 (94%) cases; one case evolved to grade III. In 26 (62%), subjects with more serious retraction pockets (grade III or IV), anatomic successes were obtained in 22 (85%) and good functional results in 19 (73%). Retraction pocket recurrence was verified in posterior-superior (2 cases) and anterior-superior (1 case) areas; in one case a tympanic post-operative perforation occurred. The poorest functional results were verified in posterior-superior retractions, especially in cases requiring additional ossiculoplasty. In the control group, only 7 (35%) grade I or II retractions spontaneously disappeared; 6 (30%) evolved into a cholesteatoma, 3 (15%) developed more serious retraction pockets, and 4 (20%) developed extensive tympanosclerosis with grave conductive hearing loss.

Developmental impact of OME: real but restricted

Mark Haggard, Ph.D., Helen Spencer

Background

The literature on developmental impact of OME is unsatisfactory, due to much confounding, samples containing few with truly severe and persistent disease, and weak causal inference.

Aim

To locate where any correlation of disease severity with impact severity may lie.

Method

Over 4000 ORL ear-related referrals of 3-8 year olds in 12 districts of the UK's highly gate-kept healthcare system were monitored. Children meeting consent and entry criteria for an RCT, including B tympanograms, otoscopic evidence of fluid and $>/= 20$dBHL on the better ear, gave a database of 655 cases aged 42-84 months. These had questionnaire scores on physical health, plus 3 months later, the same plus a 49-item parentally completed developmental impact "outcome" score (behaviour, speech & language, parent quality of life). The residual (degree of discrepancy) from the regression between reported hearing difficulties and a binaural tympanometric score, was fitted, as adjustment covariate for parental reporting bias (over-/under- rating).

Results

On 483 cases with complete data, multiple regression with 7 highly significant influences (all in predicted direction) explained 40 % of the variance. These were in decreasing order of strength, from strongest down: bias, age, eardrum status from otoscopy, recent URTI history, preceding hearing level (HL, 3 months before), recent RAOM history, and socioeconomic status. Influences were additive, not synergistic. The concurrent HL was deleted as redundant (p=0.645). Bias-adjustment weakened most influences, but its error-reducing effect left them as reliable (i.e. in p-value) as unadjusted.

Discussion

The disease and demographic influences on developmental impact make pathophysiological and developmental sense. The specific lag with which HL influences development strongly supports causality, not mere association. By controlling for any consultation bias (i.e. the milder referred cases acting as "controls"), the regression design shows that the more severe and persistent cases do suffer developmental impacts, as in the clinical lore, but these must be rare in the population, nominally 5-10 % of within-age ear referrals, even in the restrictive UK system.

Conclusions

Many facets of OME, in addition to the evidently causal preceding HL, contribute to its developmental impact, so assessment needs to be multi-aspect as well as multi-occasion. This powerful model offers a stratification framework for example when equating sample severity in international standardization or examining further influences. Case finding and intervention strategies aiming to reduce OM disease impact must, via proper assessment, select the few cases severe and persistent enough to show real impact.

What is the diagnostic value of parental information about language development in children with otitis media with effusion

Maj-Britt G Lauritsen, Ph.D., Margareta Soderstrom, M.D., Per Jensen, M.D., Jorgen Lous, M.D.

Background

Otitis media with effusion (OME) is common in children. The indication for treatment has been discussed for decades. The question is: Which children would benefit most from tympanostomy tubes? Special attention is needed for children with delayed language development. How do we find these children? Is the parents' concern about their child's language development of value or is other parental information more predictive of delayed language development?

Objective

To evaluate the diagnostic accuracy of parents' concern about their child's language development as a diagnostic method for identifying language difficulties in preschool children with OME and to explore the usefulness of other key questions.

Methods and materials

After written consent from parents of preschool children attending a day care center, the parents completed a questionnaire with seven questions about their perceptions of their child's language development, hearing, and history of otitis media. A speech therapist examined the child within two weeks with the Reynell test of verbal comprehension and a physician examined ear status with tympanometry.

We used multiple linear regressions in analysing the different predictors of language delay measured by the Reynell test of verbal comprehension.

Results

All 513 parents of children ages 3, 4, or 5 years old were asked to participate. Parental consents were obtained for 388, and 12 were excluded because inclusion criteria were not met. Parents of 354 children returned a completed questionnaire and all were included. Mean age was 4.1 years; 51% were female, 9% were bilingual, and 10% received or had received speech therapy. A total of 26% had had treatment with tympanostomy tubes at the time of inclusion.

Some 31% of the parents expressed concern about their child's language development. Concern was defined as a positive answer to the question: "Are you in any way concerned about the language of your child?"

In a univariate model, we found an association between Reynell score and: age, sex of the child, maternal educational level, bilingualism, all seven parental questions of language development, ear or hearing problems before the age of 3 years, treatment with tympanostomy tubes, and tympanometry.

In the multivariate analysis, significant associations were found between low Reynell score and parents' ratings of delay in child vocabulary and sentence construction (Table 1). Parental concern was not an independent predictor (Table 1), and bilingualism; ear problems before the age of 3 years; and questions about pronunciation problems, misunderstanding, problems in telling a story, listening to a story, ever having been treated with tympanostomy tubes, or pathological tympanometry within 2 weeks were not independent predictors for low Reynell score.

Diagnostic accuracy of parent-rated delay in vocabulary or sentence construction against a Reynell score below the 10th percentile showed positive likelihood ratios of 7.48 (95% CI: 3.94-14.19), and 5.44 (95% CI: 3.21-15.31) respectively, but modest negative likelihood ratios of 0.62 (95% CI: 0.46-0.83) and 0.58 (95% CI: 0.42-0.82).

Conclusions

Parents' rating of delay in child vocabulary or sentence construction provides better information about language difficulties, measured by the Reynell test, than parents' expression of concern.

Table 1. Predictors of low Reynell score in 3-5 year-old children

Covariate	Beta	95% confidence interval	p
Age of child (years)	6.77	5.85 to 7.69	<0.001
Female	1.30	0.08 to 2.50	0.036
Education of mother, high school or higher	2.29	0.67 to 3.91	0.006
Vocabulary delayed rated by parents	-4.50	-7.14 to -1.86	0.001
Sentence construction delayed rated by parents	-4.83	-7.34 to 2.32	<0.001
Parents concerned about language	0.38	-1.10 to 1.86	0.61
Time with suspected hearing loss before the age of 3 years	-0.15	-0.28 to -0.01	0.035

Otitis media, an indication for dentofacial growth abnormality assessment

Peyman Nejatbakhsh Azadani, M.D., Elnaz Jafarimehr, M.D., Aman Ghoujeghi, M.D., Amir Naghdi Sedeh, M.D., Shahab Mohammadian, M.D.

Introduction

Otitis media (OM) is the most frequent diagnosis recorded for infants and children who visit physicians because of illness.[1] Although far less frequently than in the past, serious complications of OM still occur.[2] Fifty percent (n = 7 100 000) of the burden of hearing impairment caused by chronic suppurative otitis media (CSOM) was in Eastern Mediterranean countries with the population of 473,644,000 in 1999.

Among genetic and environmental factors, dysfunction of the Eustachian tube (ET) is recognized as the principal cause of otitis media. The ET connects the upper respiratory tract to the middle ear and has at least three physiologic functions: pressure regulation of the middle ear, protection the middle ear from nasopharyngeal sound pressure and secretions, and clearance of its secretions into the nasopharynx.[4]

It has been shown that a shorter, wider, and straighter ET predisposes a child to OM because it disturbs the ET function.[5] Craniofacial factors, i.e., cleft palates, high palatal vaults[6], and long facial growth patterns[7] are pointed out to be related to otitis media. Moreover, neuromuscular factors, such as the effect of a deep bite on changing the function of cranial nerve V, the tensor veli palatini, and the soft tissue surrounding the temporomandibular joint[8-10] have been shown to be linked to Eustachian tube dysfunction (ETD).

The ET is not just a tube but is part of a system of contiguous organs including the nasal cavity, palate, pharynx, and the middle ear; and ET structure is the culmination of years of development and growth.[1] Since 90% of the child's facial growth is achieved by 5 years[11], preventing dentofacial growth abnormalities in these ages could stop ETD and consequently OM. The aim of the present study was to examine whether malocclusion as a component of dentofacial growth abnormality could cause OM.

Materials and methods

Between January 4, 2005 and January 23, 2006, with the approval of the ethics community of the university and after obtaining parental informed consent, 132 children in the Department of Otolaryngology or in its clinic at Taleghani Hospital, Tehran, Iran participated in a case-control study. The indication for all participants was the presence of a complete dental eruption. These children were of a low socioeconomic status according to their family educational level, parental occupations, and total monthly family income. Children with OM were defined as case subjects. The definition of children with OM in this study was based on having tympanotomy tubes in place indicated by OM with abnormal tympanometry. A control group, matched with age and sex from the same population, was defined as children with neither history of recurrent acute or chronic middle ear disease (MED) nor having tubes. History of MED was determined by chart review of the patient's otolaryngology record.

Parents were interviewed regarding their child's age, sex, family history of otitis media, daycare attendance, non-breast-feeding, upper respiratory infection, passive smoker, snoring history, seasonal allergy, pacifier history, and thumb sucking habit.

Exclusion criteria included craniofacial syndromes, incomplete lip or palate closure, Down syndrome, any chronic medical conditions, non-Farsi-speaking parents, or not being able to cooperate for the examination.

All procedures, including occlusal measurement were performed under the direction of a single investigator and recorded on a structured data collection form. These occlusal relationships were measured using a digimatic caliper. All measurements were performed when the patient was in a complete occlusion (maximum intercuspation). Each distance was measured at least three times and its mean was brought into our analyses. The occlusal assessment was performed both vertically as overbite and horizontally as overjet, canine, and molar relationships (Fig. 1).

The crown height of mandibular central incisor referred to as the total height of the lower incisor (THLI); and the amount of the lower central incisor, not covered by the upper teeth, referred to as visible height of the lower incisor (VHLI). So, we can simply calculate the depth of bite by the following formula: ((THLI - VHLI)/THLI) which shows the covered part of the lower central incisors by the upper

teeth. If there is no overlapping of teeth, it would be considered an open bite. Between 0 and 2/3 of coverage of the lower incisor was defined as normal overbite and more than 2/3 of coverage was defined as deep bite.

The distance from the tip of the upper incisor perpendicular to surface of the lower incisor defined as overjet. We categorized the anterior/posterior relationship of maxillary and mandinular primary molars and canines in three groups: mesial step, edge to edge, or distal step terminal plane. Because of a few number of children with flush terminal plane and distal step, we put them all in one group named non-mesial step.

Descriptive analyses were performed. The regression test was used to assess connections between the variables. Those variables suggested by the literature and those related to both depth of bite and OM in the univariate analyses were considered as potential confounders. All independent potential confounders entered in to the first multivariate model. Variables with p values greater than .05 were removed sequentially until only those variables with the values equal or smaller than cutoff remained in the final model. All analyses were realized using SPSS, version 15 for Windows.

Results

Included in the study were 132 children (2 to 6 years of age) who met the inclusion criteria. Of the enrolled patients 52 children (40%) were in the case subjects (mean age: 56 ± 17 months), while the remaining 80 children (60%) made up the control group (mean age: 52 ± 13 months). No association between these two mean ages was detected.

Deep bite seems to play a major role in OM. Children with a deepbite are at an increased risk (approximately 10 times) of developing OM (P = .01) in univariate analysis, compared with 16 in multivariate full model analysis and 12 in multivariate final model analysis.

The results of the analyses are summarized in Table 1. In univariate analysis it was found that genetic factors, recurrent upper respiratory infection, and snoring history were risk factors for malocclusion.

We controlled for all identified confounders in multivariate logistic regression analysis whereas in the final model those variables with the value of less than 0.05 were included (Table 1). Risk factors for dentofacial malocclusion in the final model were family history of OM (odds ratio (OR) = 4.6*)* and seasonal allergy (OR = 3.1).

It was found that 118 of 132 children (89%) had a mesial step terminal plane relationship of the primary second molars. 8 of them (6%) were with a flush terminal plane and 6 of them (5%) were with a distal step relationship. Deep bite was detected in 26 of 132 children (20%) whereas overjet > 3mm was found among 36 of all children (27%).

Discussion

To the best of our knowledge, this is the first time that a controlled study in a developing country has described the relationship between malocclusion and OM. This study suggests that dentofacial growth abnormality should be investigated in patients with OM because based on our results, children who have deep bites are more likely to have OM compared with normal children (p value = 0.01).

These results are supported by other investigations with similar findings.[12] The explanation for the difference in clinical outcome measures between the deep dental overbite and OM is likely multifactorial. Genetic factors, such as different races, and environmental factors, such as low socioeconomic status, should play major roles. Infectious diseases which are important in otitis media are more common in developing countries. So, these children would be expected to have more OM than those in developed countries. There are some other supporting reports[13,14] suggesting that manipulating the primary dental occlusion could be an effective method to exclude the primary dentition and reduce or eliminate OM in young children between the ages of two to six years of age. Villano[15] also proposed that the auditory function in patients with conductive hearing loss may be corrected through correction of the palatal anatomy (maxillary expansion), which influences the muscular function of the eustachian tube.

In contrast, Watase[16] did not find any relation between malocclusion and OM. In that study 112 children younger than 6 years who had OM were examined for their occlusion. Although no relationship between these two factors was detected, children in this study have not been compared with a control group and the results of an uncontrolled study would not be convincing.

The 1996 World Development Report estimated that about 2.163 million disability-adjusted life-years (DALYs) were lost due to OM, 94% of which comes from developing countries.[3] It shows also that DALYs from OM in the Eastern Mediterranean region is 270,000 which is very high compared with the Americas and the European region at about 86,000. There are many factors especially in the developing

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world which can cause OM. Deep bite is not the only cause of ETD. Considering that all children had a low socioeconomic status, this demonstrated association between ETD and deep overbite may be influenced by this factor. Finally, we believe that dentofacial growth assessment should be performed routinely in all children with OM because it allows the cause of ETD to be identified and we might be able to decrease the incidence of OME by improving the function of the ET via controlling for dentofacial growth.

overbite = (THLI - VHLI)/THLI
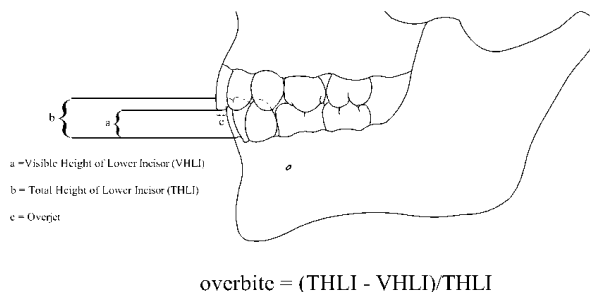
Fig. 1. Dental overbite measurement and calculation.

TABLE I

Univariate and Multivariate Logistic Regression Analyses of Children With or Without Malocclusion Who Have the characteristics on the Left Column

Characteristic	Univariate analysis			Multivariate Analyses			
	Malocclusion			Full model		Final model	
	Yes (26)	No (106)	p		p	OR (95% CI)	p
OR(95% CI) p							
Otitis media	81%	29%	0.01	16 (2.9, 52.4)	0.01	12.0(3.7, 34.1)	0.01
Characteristics							
Male	50%	51%	0.93	0.8 (0.3, 2.4)	0.72	—	—
Family history	35%	13%	0.01	4 (1.0, 14..6)	0.04	4.6 (1.4 14.9)	0.01
Daycare attendance	42%	36%	0.54	0.9 (0.3, 2.7)	0.99	—	—
Bottle feeding (0-6 mo)	23%	19%	0.63	2.3 (0.6, 8.9)	0.23	—	—
URI >5 per year	81%	43%	0.01	1.7 (0.4, 8.6)	0.49	—	—
Cigarette smoke exposure	27%	28%	0.89	1.0 (0.3, 2.8)	0.98	—	—
Snoring history	69%	36%	0.01	0.3 (0.1, 2.1)	0.23	—	—
Seasonal allergy history	8%	18%	0.20	9.7 (1.7, 36)	0.02	3.1 (0.9, 9.6)	0.05
Pacifier use	23%	20%	0.71	1.4 (0.3, 5.8)	0.60	—	—
Thumb or finger habit	15%	12%	0.67	1.8 (0.4, 8.1)	0.42	—	—

References

1. Bluestone CD. Otitis media and eustachian tube dysfunction. In: Bluestone CD, Stool SE, eds. *Pediatric Otolaryngology*. 4th ed. Philadelphia, PA: Saunders; 2002: 474-501.
2. Kenna MA. Otitis media with effusion. In: Bailey BJ, Calhoun KH, eds. *Head and Neck Surgery-Otolaryngology, vol. 1*. 2nd ed. Philadelphia, PA: Lippincott-Raven; 1998: 1297-1310.
3. Acuin J. Chronic suppurative otitis media. Burden of illness and management option. WHO 2004 (serial online)
4. Bluestone CD. Anatomy and physiology of the eustachian tube. In: Cummings CW, Haughey BH, eds. *Otolaryngology Head and Neck Surgery*. 4th ed. Elsevier-Mosby; 2005: 3003-3025.
5. Todd NW. Otitis media and eustachian tube caliber. Acta Otolaryngol Suppl1983; 404:1-17.
6. Kitajiri M, Sando I, Hashida Y, Doyle WJ. Histopathology of otitis media in infants with cleft and high-arched palates. Ann Otol Rhinol Laryngol 1985; 94:44-50.
7. Mann W, Hones I, Munker G. Growth influences on tubal function. Acta Otolaryngol 1979; 87:451-457.
8. Costen JB. A syndrome of ear and sinus symptoms dependent upon disturbed function of the temporomandibular joint. Ann Otol Rhinol Laryngol 1934; 43:1-15.
9. Stack BC, Funt LA. Temporomandibular joint dysfunction in children. J Pedod 1977; 1:240-247.
10. Loudon M. Recent advancements in vertical dimension: primary molar buildups. Funct Orthod 1990; 7:10-16.
11. Stool SE, Vig KL, Petrone JA, Hymer B. Postnatal craniofacial growth and development. In: Bluestone CD, Stool SE, eds. *Pediatric Otolaryngology, Vol. 1*, 4th ed. Philadelphia: PA, Saunders, 2002: 22-36.
12. Mcdonnell JP, Needleman HL, Charchut S, Allred EN, Roberson DW, Kenna MA, Jones D. The relationship between dental overbite and Eustachian tube dysfunction. Laryngoscope 2001; 111:310-316.
13. Marasa FK, Ham BD. Case reports involving the treatment of children with chronic otitis media with effusion via craniomandibular methods. J Craniomandibular Pract 1988; 6:256-270.
14. Branam SR, Mourino AP. Minimizing otitis media by manipulating the primary dental occlusion. J Clin Pediatr Dent 1998; 22:203-206.
15. Villano A, Grampi B, Fiorentini R, Gandini P. Correlations between rapid maxillary expansion (RME) and the auditory apparatus. Angle Orthod 2006; 76:752-758.
16. Watase S, Mourino AP, Tipton GA. An analysis of malocclusion in children with otitis media. Pediatr Dent 1998; 20:327-330.

Quality of life and psycho-social development in children with O.M.E.

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Background/objectives

Otitis media with effusion (OME) is primarily a paediatric disease that is a concern to both parents and teachers as they regard the health status of their children. For this reason it's important to evaluate not only the typical symptoms of OME (fullness, hearing loss) but also the emotional distress, the daily activity limitation and the caregiver concerns that can result. Quality of life (QoL) is not an objective parameter, but a subjective one, influenced by the personality of the patient (experiences, psychological conditions, habits) and caregivers. Therefore QoL is an important parameter to evaluate in patients with OME.

The aim of the present study is to correlate the results from a survey on Otitis Media (OM), and the State-Trait Anxiety Inventory (S.T.A.I.) study. The survey investigated the prevalence of otitis media in our territory, the influence on the development of language and personality and its social cost. The S.T.A.I is a test suitable for differentiating the state of anxiety caused by a specific event (in this case OME) from a trait anxiety (anxious personality) in parents and caregivers.

Methods

The retrospective survey was carried out in two primary public schools in Colle Val d'Elsa (Siena-Italy) on 252 children, ages 6-11 years. Parents and caregivers were administered questions concerning demographic data and clinical history (age, sex, number and kind of OME episodes, mouth breathing, other upper airways diseases), the development of language, the number of school days lost by the children, or working days lost by the parents due to otitis media. The children's teachers were administered questions about: speech impairment, learning skills and social activity. The S.T.A.I. test was administered to the parents or caregivers of 20 paediatric outpatients (4-12 years, mean 6.8 years) affected by OME.

Results

The results obtained from the survey showed that the incidence of OM was 41.26% (104/252 children) with a percentage of serous otitis media (SOM) of 43.26% (45/104).

A correlation between speech impairment and otitis media was indicated in 14 children, mostly in the first three classes: four children in the first, four children in the second, four children in the third, two children in the fourth and zero children in the fifth class. Speech impairment was probably caused by transmissive hearing loss. Otitis media and its sequelae also caused absence from school: the children lost a mean of 51 school days (19.77% of cases), but only 3.57% of children lost more than 10 days. Their parents lost a mean of 24 working days (9.52% of cases) with 5.95% of cases less than 10 days. However it is important to mention that a lot of mothers were housewives, so the absence from work is probably underestimated.

We divided the population of our study in two groups - otitis positive and otitis negative. We analysed some interesting parameters using the chi-square statistical test. Significant correlation was demonstrated between otitis media and difficulties in speech (p=0.019), limited vocabulary (p=0.055), and difficulties in reading (p=0.010). A high significant correlation was demonstrated between otitis and delayed answering (p=0.009), need to have questions repeated (p=0.005) and distraction (p=0.004). All of these problems were mostly present in the first three classes and improved with children growing up.

The psychosocial development appeared to be more problematic even in the fourth and fifth class, mostly because of persistent attention disturbances. In the S.T.A.I. test 50% (10) of the parents or caregivers had a high state-anxiety score, thus they were mostly concerned with the health status of their children. The other 50% (10) of the parents or primary caregivers had a high trait-anxiety score, showing an anxious personality. These findings could be helpful in understanding the real gravity of symptoms: in fact, parents with an anxious personality are expected to exaggerate their child's symptoms and problems.The two proposed tests could provide complementary data to evaluate children with OME: the OM survey can be used as a screening test for detecting children with asymptomatic OME, to hypothesize if the delayed language development can be associated with OME. The S.T.A.I. can be used to point out a state or a trait anxiety in parents for the purpose of better understanding their point of view. The parents and caregivers' personalities are very influential regarding the impact of OME on a child's quality of life. For example, anxious parents are expected to put more restrictions on a child's activities (i.e. sports, parties, friends), thereby decreasing their quality of life.

Conclusions

In conclusion, the OME impact on children cannot be based only on the parent's rating of hearing, the medical history, audiometry, tympanometry or the appearance of the tympanic membrane. We need to also ask specific questions of parents in order to assess the quality of life which should be considered one of the most important parameters to be taken into consideration because of its possible correlation with developmental problems. We think that the combination of the results yielded by the tests proposed by us could provide a useful contribution to this view.

Tympanic membrane closure (TMC) after tympanocentesis

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Background

Tympanocentesis is a common procedure in the management of acute otitis media (AOM), allowing middle ear pressure alleviation, pain relief and microbiologic sampling for pathogen identification. Limited data on TM healing following tympanocentesis is available.

Objective

To evaluate the relationship between age, tympanocentesis, bacteriology and pathogen eradication from MEE on TM closure (TMC).

Patients and Methods

Study population included 111 children aged 3-36 mo. (mean 14±6m, 93 %<2y, 35% without previous episodes) enrolled during 2001-2004 in 4 studies evaluating the efficacy of antibiotics by the double-tympanocentesis method. After tympanocentesis, one of the following antibiotic drugs was administered: oral amoxicillin/clavulanate (90/6.4 mg/kg/day bid for 10 days), oral levofloxacin (20 mg/kg/day bid for 10 days),oral cefdinir (25 mg/kg once daily for 10 days) and oral cefprozil (15 mg/kg/day bid for 10 days).[1-4] Only the files of patients examined by the same otolaryngologist were included. TMC was diagnosed by otomicroscopic examination. Demographic data were recorded, along with information on tympanic membrane findings (redness, bulging and opacity). In addition, the microbiologic results of the MEF cultures were recorded.

Results

Ninety-three out of 111 (84%) patients underwent a second tympanocentesis on Day 4-6; 103 (93%) and 95 (86%) were evaluated on Day 11-14 (end of therapy) and 22-28, respectively. 173 ears underwent tympanocentesis on Day 1 and 139 on Day 4-6. Positive MEF cultures were recorded on Day 1 in97 of113 (86%) patients. A total of 128 pathogens were isolated: *H. influenzae*, 76; *S. pneumoniae*, 44; *M. catarrhalis*; 7 and *Streptococcus pyogenes*, 1 (Table 1). A single pathogen was recovered from cultures performed in 119 (69%) ears and a mixed infection with 2 pathogens was found in 31 (18%) ears. TMC on Day 4-6 was observed in 153/173 (88%) tympanocenteses performed at enrollment. TMC was not influenced by age, previous AOM history, MEE culture positivity, specific pathogens and bacterial eradication on Day 4-6. TMC occurred on Day 11-14 in all 20/173 (12%) unhealed ears on Day 4-6. 116/127 (91%) evaluated ears tapped on Day 4-6 showed TMC on Day 11-14. 9/10 not healed on Day 11-14 showed TMC on Day 22-28.

Discussion

Data on the outcome of incisional myringotomy or tympanocentesis procedures in children with AOM are unavailable. In the present study, we evaluated the closure rates of 1 or 2 tympanocentesis procedures performed as part of 4 double–tympanocentesis studies evaluating the microbiologic and clinical efficacy of various antibiotic regimens for the treatment of AOM in a large number of children. We analyzed the timing of tympanic membrane closure after double tympanocentesis related to disease etiology and history, previous tympanocentesis procedures, patient age and bacterial eradication. We collected data only from those patients in whom the tympanocentesis was performed by the same 2 otolaryngologists, in order to assure the consistency of the technique and continuity of the follow-up. In this study, we showed that the closure of the tympanostomy after tympanocentesis procedures occurs early in most cases (> 90% of cases on day 4-6 after the procedure) and is independent of disease etiology and history, age and bacterial eradication. We conclude that: 1) TM perforation healed in most cases within a few days regardless of patient age, AOM etiology, previous AOM history and bacterial eradication; 2) 2 consecutive tympanocenteses performed at short-time intervals are associated with high TMC rates.

Table 1. MEF cultures at enrollment: 128 isolates from 173 ears in 99 patients with culture-positive AOM

PATHOGEN	No. patients (n = 113)	Ears (n = 173)
Haemophilus influenzae	50	88
Streptococcus pneumoniae	16	26
Moraxella catarrhalis	3	4
Streptococcus pyogenes	1	1
Haemophilus influenzae &Streptococcus pneumoniae	25	26
Haemophilus influenzae & Moraxella catarrhalis	1	1
Streptococcus pneumoniae & Moraxella catarrhalis	3	4
No growth	14	23

References

1. Dagan R, Hoberman A, Johnson C et al. Bacteriologic and clinical efficacy of high-dose amoxicillin/ clavulanate (90/6.4 mg/kg/day) in children with acute otitis media, including penicillin-resistant *Streptococcus pneumoniae.* Pediatr Infect Dis J 2001;20:829-837.
2. Arguedas A, Dagan R, Pichichero M et al. An open-label, double tympanocentesis study of levofloxacin in the treatment of children with, or at high risk for, recurrent or persistent acute otitis media. Pediatr Infect Dis J 2006; 25:1102-1109
3. Arguedas A, Dagan R, Leibovitz E, Hoberman A, Pichichero M, Paris M. A multicenter, open-label study of cefdinir in children with acute otitis media at high risk of persistentor recurrent infection. Pediatr Infect Dis J 2006;25:211-218.
4. Pichichero M, Dagan R, Arguedas A et al. A multi-center, open-labeled study of cefprozil in children with persistent and recurrent acute otitis media (Abstract G-819). 45th Annual Interscience Conference on AntimicrobialAgents and Chemotherapy, December 16-19, 2005, Washington, DC.

International differences in reported hearing difficulties in OME for a given HL: use of tympanometric/audiometric relation and bias-adjustment to overcome these in the international standardization of OM8-30 and its two measures of impact

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Introduction

Clinical instruments beyond those using physiological/biological measures require good measurement properties and international standardization, especially in the developmental and quality-of-life domains that are of concern in otitis media (OM). The Eurotitis 2 project is translating the OM8-30, a 32-item questionnaire giving domains scores for physical health and developmental status/outcome relevant to OM, then standardising the scores by fitting a model of determinants of impact to samples of feasible size from each nation. The analyses have benefitted from the availability of a linking objective determinant of OM impact (HL or tympanometry) and from the bias-adjustment technique OM8-30 offers, both as expressed in the Haggard-Spencer OM impact model (this symposium). This study further illustrates robustness against a likely data shortcoming in all types of low-cost multi-center study: absence of a relatively resource-intensive clinical measure (HL).

Method

Three regressions from the UK TARGET trial database generated three optimal formulae for children aged 42 to 84 months. Regression (a) predicted HL from binaural modified-Jerger tympanogram types to roughly equivalence these with HL (N=3058). Regression (b) related parentally reported hearing difficulties (RHD score from the 4 scaled hearing items in OM8-30) to this predicted HL (N=1664), to obtain the discrepancies (residuals) between objective measure and report to form bias estimates, and (c) did the same as (b) but for true HLs. In modest convenience samples (N= 57, 30) of referred children with OME diagnosis in outpatient clinics in two major Italian cities, parents filled out the translation of OM8-30, which was scored according to formulae developed on large reference samples. In clinic1, HLs were mostly not feasible, so tympanometry-based predicted HLs (pseudo-HLs) were substituted for true HLs.

Results

Overall, the Italian children had similar objective severity to the UK reference sample (Mean 30 dB in HL or predicted HL), but they had considerably worse RHD (0.43 SD units; $p < 0.001$). This is interpreted as stronger parental awareness after hearing/ear status is controlled for, which can in certain contexts be considered a bias. Clinic 2 children had slightly worse developmental impact (raw data, +0.2 SD units), but these differences were reduced to triviality with opposite sign by bias-adjustment (−0.07 SD units for developmental impact). This adjustment is justified because bias as calculated entered the models significantly and more strongly than other determinants. Pooling the Italian data as their homogeneity permits, and with adjustment for such parental bias, Italian data become congruent with those from UK, NZ, NL, France, Belgium and Finland.

Discussion

HL (or tympanometry-based predicted HL) did not relate to either physical or developmental impact, again confirming the need for separate assessment of these other domains of impact. The physical health data could have been pooled without adjustment as it showed lesser bias and little clinic difference, but, like the developmental impact averages, clinic averages became more similar when adjusted. As in TARGET and other Eurotitis samples, fitting a bias term based on an objective anchor variable reduced variability in developmental impact, hence it can clarify real differences and reduce spurious differences.

Conclusions

The two Italian datasets are similar enough to each other to combine, and, with adjustment for bias, the model-based international standardization achieved so far holds up. Although rough, the equivalence between tympanometry and HL in OME, as derived from a very large sample, enables small/medium samples to be linked for pooling or contrast when either but not both is present.

Classification of posterior pars tensa retraction

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Background

Previous classifications of pars tensa retractions have included posterior retraction, which may lead to sinus cholesteatoma, and total retraction of pars tensa, which may lead to tensa cholesteatoma. To understand the pathogenesis of these two types of cholesteatoma, separate classifications of the two different retractions are needed.

Objective

We propose a new classification for posterior retraction, which is based on our long-term epidemiological studies on secretory otitis and eardrum pathology, as well as studies on the pathogenesis of cholesteatoma:

Stage 1:   Atrophy and slight retraction of the posterosuperior quadrant of the eardrum. Retraction does not touch the incus.

Stage 2:   Retraction progressed to the long process of the incus, but without ossicle fixation. The retraction can be elevated by Valsalva`s manoeuvre or with Siegle`s otoscope.

Stage 3:   Myringo-incudopexi. Retraction is adherent and fixated to the long process of the incus and the lenticular process. This stage indicates irreversibility of the retraction.

Stage 4:   Myringo-stapediopexi. Long process of the incus and the lenticular process is resorbed and the retraction is adherent to the head of the stapes.

Stage 5:   Myringocruropexi, with resorbed stapes head and neck. This stage can be subdivided in stage 5 a with intact stapedial arch and in stage 5 b with partial defects of stapes crura.

Stage 6: Myringoplatinopexi, with total resorption of the crura and the retraction membrane covering the entire footplate.

Tympanostomy tube complications and sequelae in children with otitis media with effusion: A follow-up study of three years

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Background

Otitis media with effusion (OME) is an inflammation of the middle ear in which there is fluid collection behind an intact tympanic membrane but no signs or symptoms of acute infection.[1] Often considered a continuation of the inflammation process found in prolonged or recurrent episodes of acute otitis media (AOM)[2], OME is a normal consequence of AOM, and its resolution occurs in 3 to 6 months in most children.[3] Since Armstrong reintroduced the use of tympanostomy tubes (TT) in 1954[4], myringotomy with tube insertion has been established as an effective treatment of OME that does not resolve spontaneously. TT insertion is one of the two most frequent pediatric surgeries in the United States and the main reason why children require general anesthesia.[5] Although it is a simple procedure with significant benefits, TT insertion may have unwanted sequelae. Sequelae in the tympanic membrane and the middle ear of children with OME treated with TT were reported in several studies in the international literature, but no data about Brazilian children are available. It is often difficult to separate sequelae caused by the disease from those caused by the treatment. Some of the complications of TT insertion are otorrhea, tympanosclerosis, tympanic membrane perforation, retractions, and cholesteatoma.[6,7]

Previously inexistent tympanic membrane abnormalities (incidence) can only be detected in prospective follow-up studies. Incidence data are necessary to estimate the risk of developing sequelae during a certain period of time in individuals who did not have any previous abnormalities.

Objectives

To determine types and incidence of sequelae and complications of TT insertion in a group of Brazilian children with OME who had recurrent otitis media or chronic OME, underwent myringotomy and TT insertion, and were regularly followed up.

Methods and materials

This prospective longitudinal study enrolled 75 children (150 ears) with a diagnosis of OME seen in a pediatric otolaryngology clinic from June 2002 to October 2003 and followed up until July 2005. Patients were included if they met the following criteria: age between 11 months and 10 years; effusion in the middle ear for 6 weeks or longer (OME); an associated diagnosis of either recurrent AOM (3 or more episodes of AOM in 6 months) or chronic otitis media with effusion (COME) characterized by effusion for more than 3 months; and surgical indication for TT insertion. One of the authors followed up all patients for at least 6 weeks before surgery. Immittance audiometry was performed when necessary to confirm effusion in the cases of recurrent acute otitis media (rAOM), and all patients with a diagnosis of COME underwent audiometry and immittance audiometry. Pneumatic otoscopy using videoendoscopy was performed in all patients 24 hours before surgery to confirm the absence of signs and symptoms of acute infection. Patients with AOM, other upper airway infections, use of antibiotics, pre-existent complications of otitis media (OM) and anatomic anomalies that predispose to OM (Down Syndrome, craniofacial anomalies) were excluded.

Surgeries were performed under general anesthesia. Short-term 1.2 x 2.6 mm silicone tubes (Donaldson type; Medicone®, Pomp Produtos Hospitalares, Cachoeirinha, Brazil) were inserted in the lower anterior quadrant of the tympanic membrane (TM).

Patients were examined using video otoscopy 7 and 30 days after surgery and every 45 days thereafter or whenever any complication occurred. The following variables were evaluated: time to TT extrusion; frequency of otorrhea, perforations, and other structural TM changes after TT extrusion; and number of AOM and OME episodes after TT extrusion.

Quantitative variables (age, follow-up time, time to TT extrusion) were recorded as means ± standard deviations; qualitative variables (surgical

indication, type of surgery, otorrhea, tympanosclerosis; retraction, perforation, AOM and OME after TT extrusion) were recorded as absolute and relative (percent) frequencies.

The Kolmogorov-Smirnov (K-S) test was used to check normality of data. Of all quantitative variables, only time to TT extrusion had to undergo logarithmic transformation to be evaluated by analysis of variance because of its asymmetry. Analysis of variance (ANOVA) was used for the comparisons of means, and the chi-square test for the comparisons of proportions. Level of significance was set at $\alpha = 0.05$.

Excel® 2002 was used to store data in a database, and SPSS® version 11.0 was used for statistical analyses.[8]

This study was approved by the Ethics Committee of Hospital de Clinicas de Porto Alegre, Porto Alegre, Brazil. Written informed consent was obtained from the parents or guardians of all children. Surgery was indicated before inclusion of patients in the study, and was one of the criteria for inclusion in the study sample.

Results

All 75 children (150 ears) underwent bilateral TT placement. Ages ranged from 11 months to 9 years and 4 months (mean ± standard deviation = 34.7 ± 18.5 months); 60% were boys and all were white. Two patients were lost to follow-up after the first postoperative evaluation; one had undergone surgery because of rAOM and the other, because of COME. Therefore, 146 ears of 73 children were followed up.

rAOM was diagnosed in 69.3%, and COME in 30.7% of the patients. Patients with rAOM had a mean 5.3±1.4 episodes of otitis per semester, and those with COME had effusion in the middle ear for a mean time of 4.8±1.1 months.

Family history of AOM was found in 45.3% of the patients, and 89.3% of the children attended a daycare center.

Patients were followed up for up to 38 months after TT insertion, and mean follow-up time was 23.16±8.46 months. Mean number of follow-up visits was 17.04±9.72 per patient. Mean time to TT extrusion was 12.13±6.06 months (Table 1).

Tables 1 and 2 show absolute and relative frequencies and means and standard deviations for the variables under study. Otorrhea appears in both tables because its occurrence is described in the literature both by ear and by patient. Therefore, 61.6% of the patients had otorrhea at some time during follow-up;

31.5% of the ears had one episode of otorrhea, and 15.8%, two or more episodes.

Mean age at initial TT placement of patients who did not need additional surgery was 35.9±19.1 months, whereas mean age for those that underwent additional TT placement was 25.6±12.5 months, and this difference was statistically significant (P=0.04).

The analysis of a possible association between surgical indication for TT insertion (ROM or COME) and perforation, retraction, tympanosclerosis, additional surgery, and time to TT extrusion did not yield any statistically significant results.

Because otorrhea is a frequent and recurrent finding in patients with TT, its occurrence was analyzed according to other variables, and results are shown in Table 3. Time to TT extrusion was significantly longer in the ears with more episodes of otorrhea (P=0.01). The analysis of type of first surgery showed that children who underwent adenoidectomy at the same time of TT insertion (TT+Ad) had a significantly lower number of episodes of otorrhea (P=0.02).

Discussion

The comparison of the results of this study with findings in the literature is limited because of different definitions of OME, rAOM, and COME as surgical indications for TT placement.

Regular follow-up of a child by the same investigator to identify the type of OM and to record the time that effusion persists is a difficult task to be conducted in healthcare institutions. Therefore, one of the advantages of this study was the fact that it included only patients seen at one of the authors' pediatric otolaryngology clinic. This investigator conducted all initial evaluations and postoperative follow-up, which included otoscopy using videoendoscopy at each visit. This fact may also explain the excellent adherence to postoperative follow-up: only 2 of the 75 children were lost to follow-up.

Mean time to TT extrusion (12.13 months) was similar to that reported by Collete et al.[9] (13.8 months) and Casselbrant et al.[10] (12 months). The short-term Donaldson-type tube used in this study has an estimated time to extrusion of 6 to 18 months, and our results corresponded to expected time to extrusion.

Tympanic membrane perforations after extrusion were found in 2.1% of the ears, which is similar to the 3% found by Daly et al.[6] and the 2.2% found in both the meta-analysis conducted by Kay et al.[5] and in the study by Golz et al.[11] These data

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confirm that the incidence of perforations is low when short-term tubes are used. The two studies mentioned above report perforation rates of 16.6% and 14.5% for long-term tubes. Although Golz et al.[11] reported a statistically significant difference between the incidence of perforations according to type of surgical indication (COME = 1.56%; rAOM = 16.4%), their findings were not confirmed in our study.

The incidence of tympanosclerosis (23.3% of the ears) was not significantly different from the 32% reported by Kay et al.[5], but was lower than the 40%, 48%, and 53% found by Daly et al.[6], Schilder et al.,[12] and Sederberg-Olsen et al.[13]

Cholesteatomas were not detected in the ears of patients followed up in our study, which may probably be explained by the fact that the follow-up time was relatively short (mean 23.16 months) and because this complication has a low incidence rate: 0.7% in the meta-analysis conducted by Kay et al.[5] and 0.6% in the study by Giebink.[14]

TM retraction was found in 39.7% of the ears in this study whereas values found by other authors range from 26% found by Maw and Bawden[15] and 28.1% by Kay et al.[5] to 37% by Tos et al.[16] and 42% in a long-term follow-up study conducted by Daly et al.[6] The fact that our follow-up was shorter than that of studies conducted in other countries may have affected the validity of data about retraction because retraction may develop later as a consequence of eustachian tube dysfunction. Moreover, the lack of a uniform definition of retraction by the authors reviewed also limits comparisons: some authors analyzed retraction pockets and pars flaccida and pars tensa retractions individually, whereas other authors analyzed them as a group.

Episodes of AOM and OME may recur after TT extrusion in some children. In this study, 64.4% of the children had at least one episode of AOM, and 26.0% had OME after tube extrusion. Tympanostomy tubes act as substitutes for the eustachian tube, but normal eustachian tube functioning may take years to resume. Therefore, new episodes of otitis may be expected, and some authors, such as Muenker[17], do not even list it as a complication. In our series, most episodes of AOM and OME were clinically controlled, but 16.4% of the children needed the placement of a second set of TT, a frequency that was similar to the 19.9% reported by Boston et al.[18] In the present study, children who received a second set of TT had a significantly lower mean age (25.6 months) at the time of initial TT placement than those who did not undergo additional surgery (35.9 months). Boston et al.[18] also found that the need for a second set of TT was almost two times greater in the group of children who were

younger at the time of the first surgery. These data seem to be in agreement with the fact that the peak ages for AOM and OME are 1 and 2 years, and that immunologic maturation and development of the middle ear and craniofacial structures play an important role in the decrease of these diseases. It is still unclear whether early surgical intervention prevents anatomic sequelae or delays in the development of language and speech; therefore, the risk of placement of a second set of TT should be evaluated in children with an early indication of myringotomy and TT insertion.

Nine of the 12 children who underwent placement of a second set of TT also underwent adenoidectomy; the others had already undergone adenoidectomy at the time of the first surgery. The benefits of adenoidectomy in the treatment of COME and rAOM are not clearly defined and not all mechanisms that may explain its positive effects are well known. Paradise et al.[19] recommended adenoidectomy for children who had rAOM after TT extrusion, and Valtonen et al.[20] performed adenoidectomy every time that a second set of TT was inserted. Current guidelines on OME report that each eight adenoidectomies avoid one second TT placement surgery, but stress that each additional surgery avoided by adenoidectomy reflects a decrease in the incidence of AOM and OME.[3]

Otorrhea is one of the most common complications after TT placement. In our series, 61.6% of the patients had otorrhea at some time during follow-up; 31.5% of the ears had one episode of otorrhea, and 15.8%, two or more episodes. Collete et al.[9] reported an incidence of otorrhea in 74.8% of the children whose TT were still in place after 12 to 18 months. The frequency of otorrhea reported in the literature varies substantially: Kalcioglu et al.[7] reported an extremely low rate (1.25% of 239 children), whereas the meta-analysis conducted by Kay et al.[5] found 42%.

In this study, a significantly longer time to TT extrusion was found for the ears with two or more episodes of otorrhea (P=0.01). This is in agreement with the concept that TT takes longer to be extruded when an abnormality of the middle-ear mucosa persists. A significant decrease in the number of otorrhea episodes was found when adenoidectomy was performed at the time of initial TT placement (P=0.02). The benefits of adenoidectomy may be associated with the removal of potentially infected nasopharyngeal tissue, as it is known that the bacteria found in the nasopharynx are similar to those found in the middle ears of children with OME.[21,22]

Conclusions

Otorrhea had the greatest incidence rate in the analysis of complications and sequelae in a cohort of Brazilian children who underwent TT insertion due to OME. Children who underwent adenoidectomy at the time of initial TT placement had fewer otorrhea episodes.

Time to TT extrusion was longer in the ears that had more episodes of otorrhea.

Lower age at the time of initial surgery for TT placement was associated with greater need for additional TT insertion.

The analysis of results suggests that one in each six patients who undergo TT placement will need additional surgery for a second pair of tubes.

Children who undergo TT placement should be followed up adequately and regularly even after TT extrusion, because sequelae may frequently occur in these cases.

Table 1. Results for a total of 146 ears

Variables	Frequency	%
Otorrhea		
None	77	52.7
1 episode	46	31.5
2 or more episodes	23	15.8
Perforation	3	2.1
Retraction	58	39.7
Tympanosclerosis	34	23.3
Cholesteatoma	0	0
Time to TT extrusion (months)*	12.13(± 6.06)	
Duration of follow-up (months)*	23.16(± 8.46)	
Number of visits *	17.04(± 9.72)	

* Variables presented as mean (± standard deviation)
TT: tympanostomy tube

Table 2. Results for 73 patients (*)

Variables	Frequency	%
Otorrhea	45	61.6
OME after TT extrusion	19	26.0
Right ear	1	1.4
Left ear	2	2.7
Both	16	21.9
AOM after TT extrusion		
No episode	26	35.6
1 to 3 episodes	36	49.3
> 3 episodes	11	15.1
Surgery for second set of TT		
Not performed	61	83.6
TT	3	4.1
TT+Ad	9	12.3

(*) 2 patients lost at follow-up
OME: otitis media with effusion   AOM: acute otitis media
TT: tympanostomy tube
TT + Ad: tympanostomy tube + adenoidectomy

Table 3. Comparison of variables according to occurence of otorrhea per ear

| | Otorrhea | | | |
Variables	No episodes	1 episode	2 or more episodes	P
Time to TT extrusion (in months)*	11.06(±5.44)	12.35(±5.97)	15.26(±7.26)	0.01
Age (in months)*	35.6(±13.97)	34.91(±24.20)	28.57(±18.31)	0.26
Surgical indication (ears)**				
rAOM	55(52.9%)	29(27.9%)	20(19.2%)	
COME	22(52.4%)	17(40.5%)	3(7.1%)	0.11
Type of first surgery (ears)**				
TT	27(40.9%)	24(36.4%)	15(22,7%)	
TT + Ad	50(62.5%)	22(27.5%)	8(10.0%)	0.02

TT: tympanostomy tube;   TT + Ad: tympanostomy tube + adenoidectomy
rAOM: recurrent acute otitis media; COME: chronic otitis media with effusion
* Variables presented as mean (± standard deviation) (ANOVA)
** Variables presented as frequency (percentage) (chi-square test)

References

1. Bluestone CD, Gates GA, Klein JO, et al. Recent advances in otitis media. 1. Definitions, terminology, and classification of otitis media. Ann Otol Rhinol Laryngol Suppl 2002; 188: 8-18.
2. Giebink GS. Progress in understanding the pathophysiology of otitis media. Pediatr Rev 1989; 11:133-138.
3. American Academy of Family Physicians, American Academy of Otolaryngology-Head and Neck Surgery, American Academy of Pediatrics Subcommittee on Otitis Media with Effusion. Clinical practice guideline: otitis media with effusion. Pediatrics 2004; 113:1412-1429.
4. Armstrong BW. A new treatment for chronic secretory otitis media. Arch Otolaryngol 1954; 59: 653-654.
5. Kay DJ, Nelson M, Rosenfeld RM. Meta-analysis of tympanostomy tube sequelae. Otolaryngol Head Neck Surg 2001; 124:374-380.
6. Daly KA, Hunter LL, Lindgren BR, Margolis R, Giebink GS. Chronic otitis media with effusion sequelae in children treated with tubes. Arch Otolaryngol Head Neck Surg 2003; 129: 517-522.
7. Kalcioglu MT, Cokkeser Y, Kisilay A, Ozturan O. Follow-up of 366 ears after tympanostomy tube insertion: why is it draining? Otolaryngol-Head Neck Surg 2003; 128:560-564.
8. SPSS (Statistical Package for the Social Sciences) for Windows, Rel. 11.0.1.2001. Chicago: SPSS Inc.; 2001.
9. Collette A, Paradise JL, Colborn DK. Otorrhea in young children after tympanostomy-tube placement for persistent middle-ear effusion: prevalence, incidence, and duration. Pediatrics 2001; 107:1251-1258.
10. Casselbrant ML, Kaleida PH, Rockette HE, et al. Efficacy of antimicrobial prophylaxis and of tympanostomy tube insertion for prevention of recurrent acute otitis media: results of a randomized clinical trial. Pediatr Infect Dis J 1992; 11:278-286.
11. Golz A, Netzer A, Joachims HZ, Westerman T, Gilbert LM. Ventilation tubes and persisting tympanic membrane perforations. Otolaryngol-Head Neck Surg 1999; 120:524-527.
12. Schilder AG, Zielhuis GA, van den Broek P. Long term effects of otitis media with effusion--otologic findings. In: Lim DJ, Bluestone CD, Klein JO, Nelson JD, Ogra PL, eds. *Proceedings of the 5th International symposium on recent advances in otitis media.* Philadelphia: BC Decker; 1993:585-588.
13. Sederberg-Olsen JF, Sedelberg-Olsen AE, Jensen AM. Late results of treatment with grommets for middle ear condition. In: Lim DJ, Bluestone CD, Klein JO, Nelson JD, eds. *Proceedings of the 4th International symposium on recent advances in otitis media.* Toronto: BC Decker; 1988:269-271.
14. Giebink GS. Epidemiology of otitis media with effusion. In: Bess FH, ed. Hearing impairment in children. Parkton, MD: York Press Inc; 1988:75-90.
15. Maw AR, Baden R. Tympanic membrane atrophy, scarring, atelectasis and attic retraction in persistent, untreated otitis media with effusion and following ventilation tube insertion. Int J Pediatr Otorhinolaryngol 1994; 30:189-204.
16. Tos M, Stangerup S-E, Larson P. Dynamics of eardrum changes following secretory otitis: a prospective study. Arch Otolaryngol Head Neck Surg 1987; 113:380-385.
17. Muenker G. Results after treatment of otitis media with effusion. Recent advances in otitis media with effusion. Ann Otol Rhinol Laryngol 1980; suppl 68:308-311.
18. Boston M, McCook J, Burke B, Derkay C. Incidence of and risk factors for additional tympanostomy tube insertion in children. Arch Otolaryngol Head Neck Surg 2003; 129:293-296.
19. Paradise JL, Bluestone CD, Rogers KD, et al. Efficacy of adenoidectomy for recurrent otitis media in children previously treated with tympanostomy-tube placement. J Am Med Assoc 1990; 263:2066-2073.
20. Valtonen H, Qvarnberg Y, Nuutinen J. Tympanostomy in young children with recurrent otitis media. A long-term follow-up study. J Laryngol Otol 1999; 113:207-211.
21. Stewart IA. Evaluation of factors affecting outcome of surgery for otitis media with effusion in clinical practice. Int J Pediatr Otorhinolaryngol 1999; 49:S243-S245.
22. Linder TE, Marder HP, Munzinger J. Role of adenoids in the pathogenesis of otitis media: a bacteriologic and immunohistochemical analysis. Ann Otol Rhinol Laryngol 1997; 106:619-623.

Case-control study of chronic/recurrent otitis media sequelae

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Background

Tympanic membrane sequelae have been reported among children who are treated with tympanostomy tubes, both in randomized controlled trials and observational studies. However, little is known about risk factors for these sequelae (atrophy/monomer/high static admittance, tympanosclerosis, and retraction) among siblings with histories of otitis media (OM).

Objective

To investigate relationships between OM history, tympanostomy tube treatment, known OM risk factors, and otitis media sequelae among siblings enrolled in a family study of chronic/recurrent OM.

Methods

We conducted an age-matched case-control study of siblings to determine predictors for sequelae. Cases had one or more sequelae, and controls had no sequelae. Multivariate analyses, including generalized estimating equation (GEE1) analyses were used with a sandwich estimator of variance to account for correlations between family members, controlling for gender, age, allergies, asthma, recurrent OM, OME >2 months, number of smokers in the household, exclusive breastfeeding, and daycare attendance.

Results

Among 120 cases and 118 controls, history of tube treatment significantly predicted atrophy/ monomer/high static admittance and tympanosclerosis (p<.0001 for both). Results were consistent when the tube variable was defined as tube surgery yes/no or number of tube surgeries. Male gender significantly increased the risk of retraction (p = 0.03), while age (p=.08), tube treatment (p =0.06), and history of OME (p = 0.09) showed borderline relationships with this sequela.

Conclusion

Treatment with tympanostomy tubes in this cohort of siblings was highly predictive of tympanic membrane sequelae.

Taste damage from tonsillectomy or otitis media may lead to overweight children: The U.S. National Health Examination Surveys (NHES), 1963-1970

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Background

Recent studies suggest overweight/obesity may be associated with suspected damage to the taste nerve in the middle ear from otitis media (OM). Damage to taste nerves from the anterior tongue with OM, or posterior tongue with tonsillectomy, may change oral sensations that affect food preferences and intakes, resulting in increased body mass index (BMI). We examine nationally representative data from an era when tonsillectomy was more common.

Methods

The analysis is based on NHES-Cycle 2, 1963–1965, children aged 6–11 years (n=7,119) and NHES-Cycle 3, 1966–1970, adolescents aged 12–17 years (n=6,768). Home interviews with parents included socio-demographic and medical history questions. In mobile exam centers, technicians recorded standardized height/weight measurements, and physicians examined children's tonsils and ear canals. Using empirical sex- and age-specific norms, "overweight" is defined as $\geq 85^{th}$ percentile BMI.

Results

Based on physicians' exams, younger children's tonsils were: "removed" (23.4%), "present/normal" (41.5%), or "enlarged" (35.0%); adolescents: 38.3%, 33.6%, and 28.1%, respectively. Younger children's OM history was: "ever runny ears/eardrum opened" (13.6%), "earaches past year" (18.0%), or "neither problem" (68.4%); adolescents: 11.0%, 11.4%, and 77.6%, respectively. Adjusting for age, sex, race, income, and education with logistic regression, overweight risk in younger children increased with tonsillectomy (odds ratio [OR]=1.4; 95% confidence interval: 1.2-1.7). Overweight risk in adolescent girls increased with tonsillectomy (OR=1.3; 1.0-1.7) and OM history (OR=1.9; 1.3-2.8), while overweight risk in adolescent boys increased with enlarged tonsils (OR=1.4; 1.1-1.8).

Conclusion

The risk of overweight increased among younger children and adolescents with tonsillectomy and among adolescents with OM history.

An animal model for the study of otitis media

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Introduction

Otitis media (OM) is primarily an infectious disease of the middle ear. There has long been a perceived association between OM and allergy, but the evidence for this association has been mixed. Mast cells, the central cell in allergic processes, are one of the few resident leukocytes of the middle-ear mucosa.[1] The incidence of OM in atopic children is somewhat higher than that seen in non-allergic children in some, but not all studies, while anti-allergy therapy has generally been ineffective for this condition.[2] Allergic sensitization of animals followed by allergen challenge of the middle ear provokes OM[3], but whether this occurs in patients is unclear. We investigated the ability of allergic challenge to interact with OM induced by bacterial infection in a murine model. We also evaluated middle-ear infection in mice lacking mast cells, a critical mediator of allergy and of innate immune responses.

Materials and methods

Genetically mast cell-deficient (W/Wv) mice lacking the *Kit* receptor required for mast cell differentiation, as well as congenic wild-type control mice were used. Mice were either naïve or immunized with ovalbumin employing an alum adjuvant. The mice were challenged in the middle ear with ovalbumin and/or non-typeable *Haemophilus influenzae* (NTHi) in the middle ear, using a ventral approach and injection into the bulla. Control animals were injected with saline. Some mast cell-deficient mice were reconstituted with cultured mast cells derived from the bone marrow of control mice prior to middle-ear challenge with NTHi. Middle ears were fixed, decalcified, and histologically evaluated for OM.

Results

As noted by others[3,4], antigenic challenge of the middle ear in sensitized animals resulted in acute OM in wild type mice. In mast cell-deficient animals, ovalbumin produced a response similar to that seen with saline injection in WT animals. Also as noted previously[5], NTHi inoculation of the WT ME resulted in acute OM, with significant mucosal hyperplasia and leukocytic infiltration. In mast cell-deficient animals, both mucosal hyperplasia and leukocyte infiltration induced by NTHi were significantly blunted. If mast cell-deficient animals had been reconstituted previously with cultured WT mast cells, the normal ME response to NTHi was restored. When middle-ear antigenic challenge and NTHi inoculation were combined in WT mice, OM was more rapid and long-lasting than with either stimulus alone, but was no more intense.

Discussion

The results described above confirm that both antigenic challenge and bacterial infection lead to OM that is similar in character. However, in combination, these stimuli can alter the time course of OM, enhancing both the rapidity of onset and the duration of inflammation. This suggests that allergy and infection recruit separate but overlapping inflammatory response in the middle ear. The data also suggest that allergy can be either a cause or contributor to OM, and that co-existence of allergy and infection could potentially alter the course of OM in children.

The observation that bacterial OM is reduced in mast cell-deficient animals is intriguing. The mast cell not only mediates allergic responses, but is also a major effector of innate immunity. Our data implicate this cell in the initial defense of the middle ear against infection.

Acknowledgements

References

1. Lim DJ. Functional morphology of the lining membrane of the middle ear and Eustachian tube. Ann Otol Rhinol Laryngol 1974; 83 (Suppl 11):1-22.
2. Tewfik TL, Mazer B. The links between allergy and otitis media with effusion. Curr Opin Otolaryngol Head Neck Surg 2006; 14:187-190.
3. Miglets A. The experimental production of allergic middle ear effusions. Laryngoscope 1973; 83:1355-1384.
4. Ryan AF, Catanzaro A, Wasserman SI, Harris JP. Secondary immune response in the middle ear: immunological, morphological, and physiological observations. Ann Otol Rhinol Laryngol 1986; 95:242-249.
5. Melhus A, Ryan A. A model of otitis media in the mouse. APMIS 2003; 111:989-994.

Elucidating genetic pathways for chronic otitis media – the utility of mouse genetic models

Professor Steve Brown

Otitis media (OM), inflammation of the middle ear, is the most common cause of hearing impairment and surgery in children. Recurrent (ROM) and chronic (COME) forms of otitis media are known to have a strong genetic component, but nothing is known of the underlying genes involved in the human population. Mouse models will be key to elaborating the genetic pathways involved and providing candidate genes for genetic studies in the human population.

Firstly, genetic susceptibility to OM varies amongst mouse inbred strains, providing a resource to identify and study loci involved with middle-ear inflammatory disease. A second source of mouse models is to undertake screens for conductive deafness due to chronic middle-ear inflammatory disease in ongoing mutagenesis programs. It will be important as the worldwide mouse knock-out project comes to fruition and large-scale phenotyping of mouse mutants gets underway that appropriate screens are applied to identify mouse mutants with OM. ENU mutagenesis in the mouse is a phenotype-driven mutagenesis approach where large cohorts of mouse mutants carrying randomly induced ENU mutations around the genome are subjected to a variety of phenotype screens to identify diverse disease models. Screens for deaf mouse mutants are already a feature of many ENU programs.

From a deafness screen as part of the Harwell mouse mutagenesis program, we have identified two novel dominant mutants, Jeff and Junbo, which develop a conductive deafness due to a chronic suppurative otitis media. Both these mutants represent models for chronic forms of middle-ear inflammatory disease in humans. The Jeff mutant carries a mutation in an F-box gene, Fbxo11, a member of a large family of proteins that are specificity factors for the SCF E3 ubiquitin ligase complex.[1] Junbo carries a mutation in the Evi-1 transcription factor[2], a gene previously implicated in myeloid leukemia. Evi-1 represses the TGF-β signalling pathway by the binding of Smad3. The Junbo mutation provides *in vivo* evidence implicating this pathway in the development of OM. Models such as Jeff and Junbo provide entry points for dissecting the genetic pathways in both the mouse and human. Initial studies of FBXO11 SNPs in human OM families have uncovered nominal evidence of association, indicating the genetic involvement of human FBXO11 in chronic otitis media with effusion and recurrent otitis media.[3] It will be important to characterize further mouse models and identify the underlying genes in order to build a more comprehensive picture of the genetic pathways involved and the ensuing pathophysiological mechanisms.

References

1. Hardisty-Hughes RE, Tateossian H, Morse SA, et al. A mutation in the F-box gene, Fbxo11, causes otitis media in the Jeff mouse. Hum Mol Genet 2006; 15:3273-3279.
2. Parkinson N, Hardisty-Hughes RE, Tateossian H, et al. PLoS Genet 2006; 2:e149.
3. Segade F, Daly KA, Allred D, et al. Association of the FBXO11 gene with chronic otitis media with effusion and recurrent otitis media: the Minnesota COME/ROM Family Study. Arch Otolaryngol Head Neck Surg 2006; 132:729-733.

Genetic pathways for chronic otitis media – identification of mouse models and characterisation of the underlying genes

Professor Steve Brown, Rachel Hardisty-Hughes, Hilda Tateossian, Ph.D., Jiewu Yang, Ph.D., Susan Morse

Otitis media (OM), inflammation of the middle ear, is the most common cause of hearing impairment and surgery in children. Recurrent (ROM) and chronic (COME) forms of otitis media are known to have a strong genetic component, but nothing is known of the underlying genes involved in the human population. From a deafness screen as part of the mouse mutagenesis programme we have identified two novel dominant mutants, Jeff and Junbo, which develop a conductive deafness due to a chronic suppurative otitis media. Both these mutants represent models for chronic forms of middle ear inflammatory disease in humans. The Jeff mutant carries a mutation in an F-box gene, Fbxo11, a member of a large family of proteins that are specificity factors for the SCF E3 ubiquitin ligase complex (Hardisty-Hughes R. et al. 2006 Hum. Mol. Genet. 15, 1-7). Fbxo11 is expressed in the mucin secreting cells of the middle ear epithelia during the period at which otitis media develops in Jeff. Initial studies of FBXO11 SNPs in human OM families have uncovered nominal evidence of association, indicating the genetic involvement of human FBXO11 in chronic otitis media with effusion and recurrent otitis media (Segade et al., 2006). Jeff homozygotes show cleft palate, facial clefting and perinatal lethality and Fbxo11 is also expressed in the epithelial palatal shelves during development. Junbo carries a mutation in the Evi1 transcription factor (Parkinson et al. 2006 PLoS Genetics 2: e149), a gene previously implicated in myeloid leukaemia. Evi1 represses the TGF-? signalling pathway by the binding of Smad3. The Junbo mutation provides in vivo evidence implicating this pathway in the development of OM. Interestingly, we have investigated interacting partners to Fbxo11 and find that it interacts with spectrin beta II that itself is known to interact with Smad3 to mediate the effects of TGF-B signalling. The evidence suggests that both Fbxo11 and Evi1 cause OM through effects on signalling via Smad3 and provide a common mechanistic route for the development of chronic OM. Fbxo11 and Evi1 are two of the first molecules to be identified contributing to the genetic etiology of otitis media.

References

1. Hardisty-Hughes RE, Tateossian H, Morse SA, et al. A mutation in the F-box gene, Fbxo11, causes otitis media in the Jeff mouse. Hum Mol Genet 2006; 15:3273-3279.
2. Parkinson N, Hardisty-Hughes RE, Tateossian H, et al. PLoS Genet 2006; 2:e149.
3. Segade F, Daly KA, Allred D, et al. Association of the FBXO11 gene with chronic otitis media with effusion and recurrent otitis media: the Minnesota COME/ROM Family Study. Arch Otolaryngol Head Neck Surg 2006; 132:729-733.

Molecular pathogenesis of otitis media in a mouse model with the hypophosphatemia-Duke mutation (PhexHyp-Duk)

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Although otitis media (OM) is still a common disease in children and adults, the pathogenesis and the underlying genetic pathways are not yet fully understood. We have discovered that mice with the hypophosphatemia-Duke mutation (PhexHyp-Duk) present with a high incidence of OM. PhexHyp-Duk/Y hemizygous males are mildly growth-retarded in overall body size, show elevated hearing thresholds, and some exhibit circling behavior. The incidence of OM in PhexHyp-Duk/Y mice was 64.7%. The goblet cells presented metaplasia and hyperplasia in the middle-ear epithelia of PhexHyp-Duk/Y males. Increased proliferating nuclear cell antigen (PCNA) expression indicated proliferation of some ciliated cells, nonciliated cells, basilar cells, and fibroblasts in PhexHyp-Duk/Y adult males. In addition, increased expression in the ear of Muc5ac, Muc5b, and Fgf23 was found in PhexHyp-Duk/Y mutant ears compared to X+/Y wild type littermate control ears. The PhexHyp-Duk mutation was previously shown to cause elevated levels of fibroblast growth factor 23 (Fgf23), which in turn is known to increase mouse prostaglandin E2 (PGE2) production. PGE2 is considered to be a mediator of inflammation because of its potent vascular permeability-increasing activity. High PGE2 expression may be responsible for the increased Muc5ac and Muc5b gene expression observed in our study, and be a contributor to OM. We hypothesize that upregulation of PGE2 and mucin genes represent a new signal transduction pathway for OM.

Experimental otitis media with effusion in the mouse

Patricia Hebda, Ph.D., Sancak Yuksel, M.D., Juliane Banks, B.S., Per Olof Eriksson, M.D., Mark Barsic, Joseph Dohar, M.D., M.S.

Background

Otitis media (OM) with effusion (OME) represents a significant clinical problem especially in pediatric patients. This condition can occur following acute OM even after successful antibiotic therapy, but it can also arise de novo, presumably due to Eustachian tube dysfunction.[1] *Hydrops ex vacuo* is a valid explanation for the development and persistence of otitis media with effusion (OME) under certain conditions.[2] Eustachian tube obstruction (ETO) in animals induces middle ear (ME) pressure dysregulation reflected as underpressures, causing increased vasculature permeability and ME effusion. However, the mechanism(s) responsible for transducing the biological signals associated with the underpressure that lead to ME mucosal inflammation are not known. Previous work in our laboratory using the rat model of ETO has shown that some inflammatory cytokines are upregulated over a prolonged time course and may be contributing to the pathophysiology of OME.[3,4] In order to further pursue these studies, our group determined to establish the model of ETO in the mouse, in order to take advantage of the many genetically engineered phenotypes and gene expression probes available for this species.

Objective

To establish and characterize a mouse model of OME via ETO and measure changes in structure and in expression of key inflammatory mediators.

Methods

Unilateral ETO was induced in C57BL/6 mice by electrocautery of the Eustachian tube (ET). Weekly otoscopy assessed clinical indicators of OME. Histology and molecular analyses determined morphologic and inflammatory changes.

Results

Following ETO, clinical signs of OME appeared within 1 week and persisted for at least 8 wks, with retraction of the tympanic membrane and ME effusion. Clinical assessment is summarized in Figure 1, and indicates that ME effusion occurred within 1 week following ET obstruction, and progressed from serous to mucoid over the 8 week period of study. Some effusion was also detected in the contralateral ears of the ETO group (ETO-c), but these tended to remain serous and were not accompanied by significant retraction of the tympanic membrane. Therefore, it was concluded that the ETO-c ears did not experience ME pressure dysregulation.

Histology revealed that with ETO there was frank ME effusion with some inflammatory cells present (Figure 2). There were also morphologic changes, shown graphically in Figure 3) with significant thickening of the ME mucosa (about 3.1x normal), and the underlying thin bone of the bulla tympanica (about 1.8x normal). As noted in Figure 2, a few contralateral ears also presented with mild serous effusion which eventually resolved; but these lacked other changes induced by ETO (refer to Figure 3). This observation of effusion without ETO is attributed to surgical manipulation.

Molecular analysis of the ME tissues, focusing on the expression of key inflammatory mediators, also support an inflammatory component being present with ETO. These results will be fully reported in another manuscript (Hebda et al., in preparation).

Conclusions

In the absence of infection, *hydrops ex vacuo* is a valid explanation for the development and persistence of otitis media with effusion (OME) under certain conditions. The proposed central role of ME pressure dysregulation in maintaining persistent mucosal inflammation and effusion has important implications to designing effective treatments for OME. This mouse model of OME is both feasible and effective in our hands and will be useful for our mechanistic studies of ME pressure dysregulation, because of the availability

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of molecular probes and primers for mouse proteins, and transgenic knockout mouse strains with deficits in the inflammatory response.

Acknowledgments
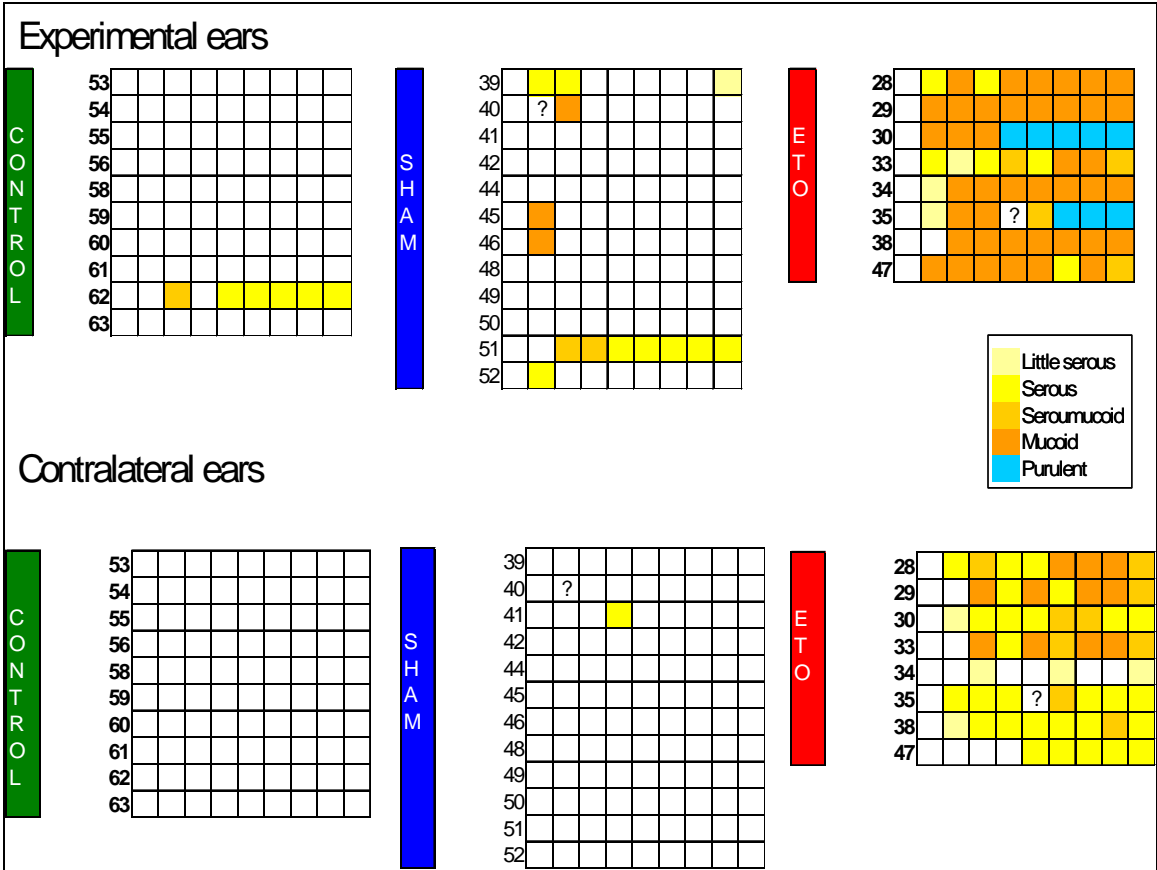
Figure 1: Weekly clinical assessment of ME status. Otoscopy was performed by a single trained and evaluated observer (SY) who was blinded to animal treatment groups. The grids display results for individual animals (animal numbers indicated in columns along the left of each grid) from 0 to 8 weeks following the surgical procedure to create ETO (each box along a row represents one week). The results show a persistent ME fluid in the ETO group that was present at 1 week and progressed over time from serous to mucoid. The contralateral ears in the ETO group (ETO-c) exhibited ME fluid in the absence of ET obstruction, attributed to a regional inflammatory response. However, retraction of the TM was lower or absent in the ETO-c group, indicating that negative pressure was not a contributing factor in this instance.
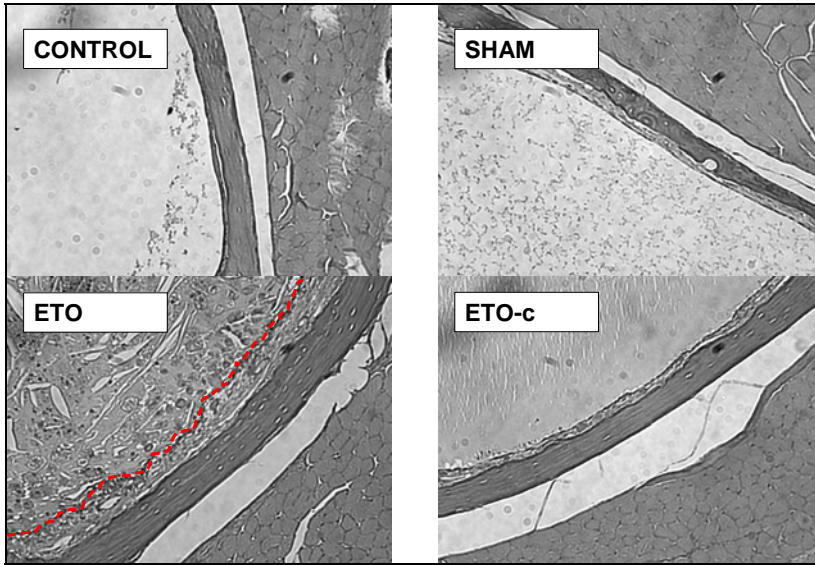
Figure 2: Histology of mouse middle ears at 8 weeks, stained with hematoxylin and eosin. Control animals were untreated. Sham animals underwent the surgical procedure without disruption of the ET.ETO animals had the left ET obstructed by electrocautery (ETO), with no obstruction of the right contralateral ear (ETO-c).Note that there was fluid accumulation in the ME of the ETO animals on both sides, but only the ET obstructed side (ETO) had inflammatory cells present and showed thickening of the ME mucosa and bulla tympanica. The dashed line in the lower left panel marks the luminal margin of ME mucosa.
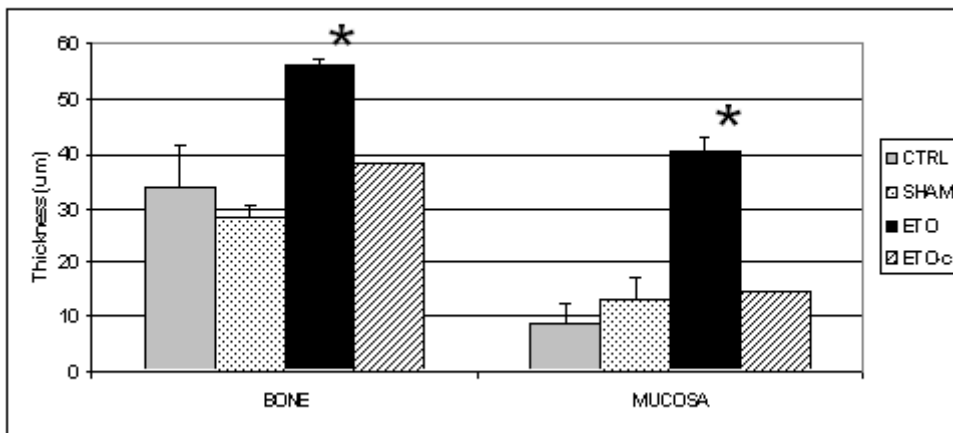
Figure 3: Measured thicknesses of the ME bone (bulla tympanica) and mucosa after 8 weeks of ETO. The 4 groups are the same as described in Figure 2.The bars indicate mean values for each group with error bars showing standard errors of the mean (SEM).Only the ETO ears showed significant thickening compared to the untreated control ears (* = $p<0.05$), with the bone increasing by 80% and the mucosa by 210%.

References

1. Bluestone CD and Klein JO. Otitis media in infants and children. Philadelphia, PA: WB Saunders Co.; 1998.
2. Bluestone CD and WJ Doyle. ET Function: Physiology and role in otitis media. Ann Otol Rhinol Laryngol 1985; 94(suppl. 20):1-60.
3. Piltcher OB, Swarts JD, Magnuson K, Alper CM, Doyle WJ, Hebda PA. A rat model of otitis media with effusion caused by Eustachian tube obstruction with and without *S. pneumoniae* infection: Methods and disease course. Otolaryngol Head Neck Surg. 2002;126:490-498.
4. Hebda PA, Piltcher OB, Swarts JD, Alper CM, Zeevi A, Doyle WJ. Cytokine profiles in a rat model of otitis media with effusion caused by Eustachian tube obstruction with and without *S. pneumoniae* infection. Laryngoscope. 2002; 112:1657-1662.

Otitis media in a mouse with viable motheaten mutation

Heping Yu, B.S., Jiang Ping Zhang, M.D., Christopher McCarty, M.S., Qing Zheng, M.D.

Otitis media (OM) is the most common infectious disease among children, accounting for as many as 30 million office visits annually. OM results from interaction between microbial load (viral and bacterial) and immune response. Mice homozygous for the *viable motheaten* (*me*^v) mutation develop severe autoimmune disease and spontaneous otitis media. Hearing loss in OM-afflicted *viable motheaten* mice was determined by auditory brain stem response (ABR) to board band clicks, and 8, 16 and 32 kHz pure tone stimuli. We have examined the incidence of OM in these mice, and characterized OM by histological pathology, Fluorescence Activated Cell Sorting (FACS) analysis, toluidine blue staining, Mayer's mucicarmine staining, anti-PCNA and anti-TNF-α immunohistochemical staining. In sum, 66.7% of the *viablemotheaten* mice presented with suppurative otitis media. Inflammatory cells accumulated in the middle ear cavity, and expression of TNF-α was increased in the affected middle ear. There were few mucosal mast cells, and low numbers of B and T cells in the lymph node, spleen and blood. Our results show that *viable motheaten* mice have a high incidence of spontaneous OM and are a promising genetic mouse model for investigating development of OM and the inflammatory response without prior exposure to exogenous pathogens.

Otitis media in a mouse with a mutation in Ptpn6

Jiang Ping Zhang, M.D., Christopher M. McCarty, M.S., Qing Yin Zheng, M.D.

Otitis media (OM) is the most common infectious disease among children, accounting for as many as 30 million office visits annually. It results from interplay between microbial load (viral and bacterial) and immune response. Mice homozygous for the viable motheaten spontaneous mutation (Ptpn6me-v) develop severe autoimmune disease. We have examined the incidence of OM in mice homozygous for this mutation (me-v mice), and characterize this OM by histological pathology, FACS analysis, toluidine blue staining, Mayer's Mucicarmine staining, and anti-PCNA and anti TNF-alpha antibody immunohistochemistry staining. Most Ptpn6me-v mutant mice presented suppurative OM. The incidence of OM was 66.7%. In the Ptpn6me-v mutant mice, the Goblet cells presented metaplasia, hyperplasia, inflammatory cells accumulation, and high expression of TNF-alpha in the middle ear. The mucosal mast cells were few; B- and T-cells were absent in the lymph node, spleen, and blood. Our results show that the Ptpn6me-v mutant mice present a high incidence of OM and are good genetic mouse models to research OM and the immune response.

Histomorphometric estimation of air cell development in experimentally-induced otitis media

Kristianna Mey, M.D., Mads Sølvsten Sørensen, M.D., D.R.Mc., Preben Homøe, M.D.

Objectives

The objective of this study were to examine the effect of acute otitis media on the bony development of the growing middle ear air cell system with a reliable and concise stereological histomorphometric method and to generate a standard baseline growth plot for the middle ear of the rat.

Study design

The authors conducted a histomorphometric analysis af bullar volume during the entire growth period after early induction of unilateral otitis media.

Methods

The authors conducted undecalcified processing of 27 rat skulls sectioned horizontally at various ages from 20 to 83 days after unilateral trans-tympanic injection of *streptococcus pneumoniae* at 19 days postpartum. Stereological estimation of bullar volume was performed in the experimental and the control ear with Cavalieri's principle. Body mass and air cell volume were plotted as functions of subject age using standard development equations on the experimental results.

Results

Volumes of the middle ear air cell system were consistently smaller in the otitis ears. Bullar growth was completed at 60 days regardless of otitis history, but body mass was increasing throughout the experimental period.

Conclusions

A single incident of otitis media introduced early in life is sufficient to significantly reduce the final volume of the bulla in rats. This finding may mimic the effect of otitis media contracted early in childhood on the development of the mastoid air cells. The standard growth plot provides a timeframe for studies of signaling molecules responsible for bone modeling in pneumatization.

Glucocorticoid and mineralocorticoid suppression of acute otitis media in the mouse

Carol MacArthur, M.D., Beth Kempton, Ph.D., Jacqueline DeGagne, B.S., Dennis Trune, Ph.D.

Abstract

The middle ear innate immune response to bacteria leads to acute inflammation consisting of fluid accumulation, infiltration of inflammatory cells, and mucosal thickening. Although inflammation from otitis media generally subsides after 5-7 days, suppression of this response would help alleviate suffering and minimize risk to the inner ear. To investigate the role of systemic corticosteroids in middle ear inflammation from otitis media, both glucocorticoid and mineralocorticoid steroids were investigated for their ability to reduce inflammatory symptoms. Clearance of middle ear fluid has long been ascribed to the function of the Eustachian tube (ET). There is now evidence that the middle ear epithelium plays a major role in fluid absorption, while the role of the ET is more to allow gas pressure equilibration. The immunoregulatory actions of glucocorticoids reduced the severity of the middle ear innate immune response. Interestingly, mineralocorticoids were also effective in reducing the inflammatory response at 5 days. Steroid control of middle ear disease may be useful in alleviating symptoms faster and reducing risk to the inner ear.

Introduction

Otitis media (OM) is one of the most prevalent inflammatory diseases in the pediatric population.[1] The only effective primary treatment for acute otitis media (AOM) is antibiotics. However, a consequence of antibiotic therapy is bacterial death and release of bacterial inflammatory products (lipopolysaccharide, peptidoglycan, and DNA), which can exacerbate and prolong inflammation in the middle ear.[2,3,4] Alleviation of symptoms can be accomplished by oral anti-inflammatory agents, oral antibiotics or the infection can resolve on its own, up to 70% of the time.[5] Recent efforts to withhold the use of antibiotics for this disease entity have been put forward to decrease the number of antibiotic prescriptions written every year. Decreasing the use of antibiotics is hoped to decrease bacterial resistance to antibiotics, an emerging problem in many parts of the world. Therefore, new strategies are needed for alleviating the pain and hearing loss that accompany AOM. These symptoms result from the inflammatory reaction to the bacteria present in the middle ear. Thus strategies to decrease fluid and inflammation would be potentially beneficial in the treatment of AOM. The lack of therapeutic options beyond antibiotics has led to considerable animal research efforts to better understand the mechanisms of AOM and develop new strategies for its prevention or treatment.

Steroids are known to impact inflammation. Glucocorticoids lessen inflammation and mineralocorticoids are known to act on fluid homeostasis. It has been reported that steroids may have an impact on Na^+ and fluid transport through the middle ear mucosal epithelium.[6,7] The fluid homeostasis function of the middle ear epithelium is likely to have a major role in removing fluid that accumulates during inflammation such as seen with acute and chronic OM. If the fluid were controlled, hearing would be restored and pain from inflammation would be alleviated. Using an animal model, the possibility that both glucocorticoids and mineralocorticoids could both impact middle and inner ear disease in OM was investigated. The goal of the study was therefore to sort out which steroid role, fluid homeostasis (mineralocorticoids) or immune suppression (glucocorticoids), would experimentally be best in controlling middle ear fluid and cellular infiltration in this model of acute OM.

Design

Balb/c mice (n = 60) were bilaterally inoculated transtympanically with 3.5 µl heat-killed *Streptococcus pneumonia* (10^9 organisms per ml) and examined after 3 days or 5 days. Control mice (N=20) received no steroid treatment and 10 were killed at each time point. Oral steroid treatments given in the drinking water[8], consisting of either the mineralocorticoid aldosterone (15 µg/kg) or the glucocorticoid prednisolone (5 mg/kg), were begun the day before inoculation and continued until sacrifice. Ten mice from each treatment were examined at each time point (3 and 5 days). The animals were euthanized by an overdose of anesthetic (ketamine and xylazine), fixative was intracardially perfused (1.5% glutaraldehyde - 3% paraformaldehyde in 0.1M

phosphate buffer), and the dissected skulls immersed in fixative overnight. The middle and inner ears were left intact and connected to each other by the skull base so both ears were processed together for histology and sectioning. Tissues were microwave decalcified in EDTA, embedded in glycol methacrylate plastic, sectioned in the horizontal plane at 5 μm, serially mounted on glass slides, stained, and coverslipped. Slides were examined with the Leica DMLB microscope.

Middle ears were assessed at 10x power at a standard middle ear section (level of the stapedial artery) for fluid area, number of inflammatory cells, and tympanic membrane (TM) thickness to determine if steroid treatments suppressed middle ear disease. The fluid area was measured with a calibrated micrometer grid in the eyepiece and the cells within the fluid area were counted. The thickness of mucosa and membranes was measured with a micrometer scale within the opposite eyepiece. Three sections were measured and the three individual measures for each middle ear parameter were averaged to derive one mean value for each parameter for each ear. Statistical analyses (t-tests) were then done on each averaged histologic measurement and each treatment group and endpoint, comparing treatment groups to controls.

Results

Histologic Measures:

Prednisolone significantly reduced histologic measures of inflammation compared to controls at 3 days (cell number $p < 0.009$; TM thickness $p < 0.015$) and at 5 days (fluid area $p < 0.013$; cell number $p < 0.009$; TM thickness $p < 0.004$).Figure 1A, B, and C show each treatment group at 3 and 5 days, with averages for middle ear fluid area, cell number and TM thickness. Significant p values (<0.05) are shown on the figure. Aldosterone did not suppress disease at day 3, but did significantly suppress disease at day 5 (fluid area $p < 0.032$; cell number $p < 0.006$).

Histologic Sections:

Control ears often had significant inflammation of the middle ear at days 3 and 5 on histologic analysis. Features of inflammation, such as accumulation of middle ear inflammatory exudate, hyperplasia of the middle ear mucosa, thickening of the round window membrane and TM, were commonly seen (Figure 2A,B,C). Note extensive middle ear fluid with inflammatory cells, TM reaction, and a clear inner ear.

By contrast, Prednisolone-treated ears had significantly lower measures of inflammation. Figure 2d is an example of a prednisolone-treated ear that completely cleared the typical inflammation from the heat-killed bacterial injections to the middle ear.

Discussion

While systemic steroids have been used extensively for ear diseases such as sudden sensorineural hearing loss and in earlier trials for treatment of otitis media with effusion (OME), animal research on the use of various steroids for OM has not been conducted. Specifically, investigation of the use of steroids to clear the middle ear fluid that accompanies OM has not been explored. Middle ear fluid present during OM causes conductive hearing loss by virtue of decreasing transmission of sound through the middle ear. Therefore, earlier resolution of this fluid would alleviate troubling sequelae of OM such as pain and decreased hearing. Currently, no good therapy exists to clear fluid with the exception of myringotomy or tympanostomy tube placement. While clearance of middle ear fluid has long been ascribed to the function of the Eustachian tube (ET), there is now evidence that the middle ear epithelium plays a major role in fluid absorption, while the role of the ET is more to allow gas pressure equilibration.[6] Other laboratories have reported that fluid regulation in the middle ear is via the Na^+ channel dependant process through the middle ear mucosal epithelium. The use of mineralocorticoids offers some appeal as use of such an agent would avoid the systemic morbid sequelae of glucocorticoids.

The glucocorticoid, prednisolone, was effective in our study in reducing middle ear inflammation in response to bacterial challenge to the middle ear at both 3 and 5 days. Presumptively, this is due to the strong glucocorticoid action of this steroid. Interestingly, prednisolone also has a modest mineralocorticoid effect, one-fifth that of the glucocorticoid effect. One could speculate that the mineralocorticoid action of prednisolone could also be acting in a positive way to decrease middle ear fluid. Aldosterone, basically a pure mineralocorticoid (10,000:1), was also found to reduce middle ear inflammatory response to bacterial injections, but only at the 5 day endpoint. Thus, while the glucocorticoid agent was effective in decreasing fluid and cell count in the middle ear, the mineralocorticoid was also effective. This lends support to the concept that sodium transport of middle ear fluid is an important function in clearing fluid and thus in controlling inflammation in the middle ear.

Conclusions

The immune-suppressive actions of glucocorticoids reduced the severity of the middle ear innate immune response. Interestingly, mineralocorticoids were also effective in reducing the inflammatory response at 5 days. Thus, steroid control of middle ear disease may be useful in alleviating symptoms faster and reducing risk to the inner ear. Further investigations are ongoing into various steroids to assess the efficacy on clearance of middle ear inflammation in comparison to prednisolone.

Acknowledgement

Figure 1:Histologic measures of inflammation at 3 and 5 days of treatment.
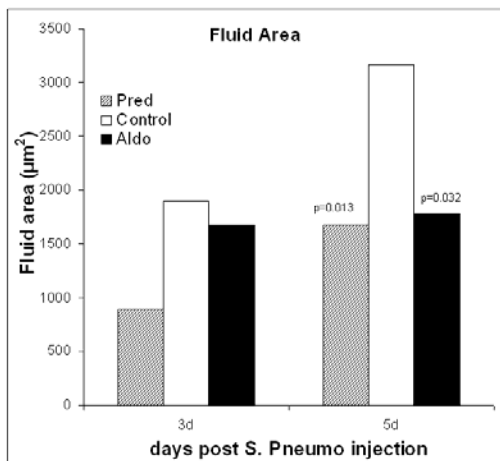
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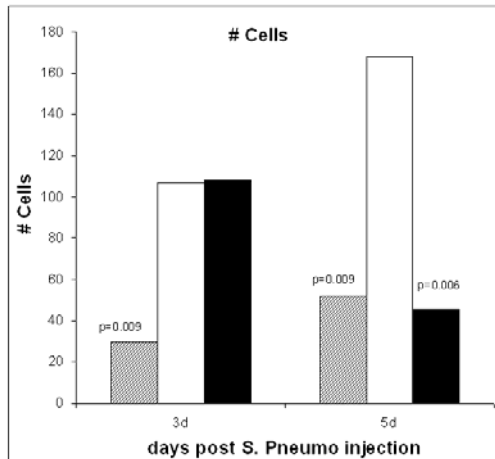
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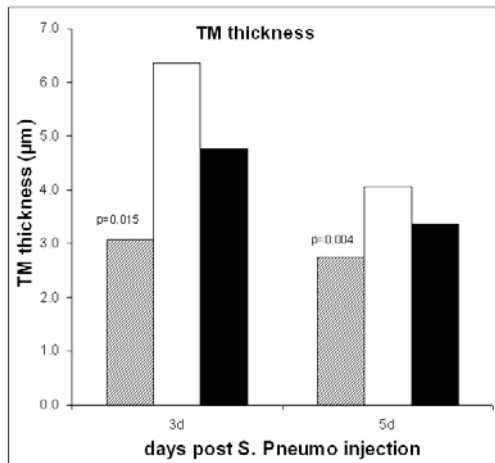
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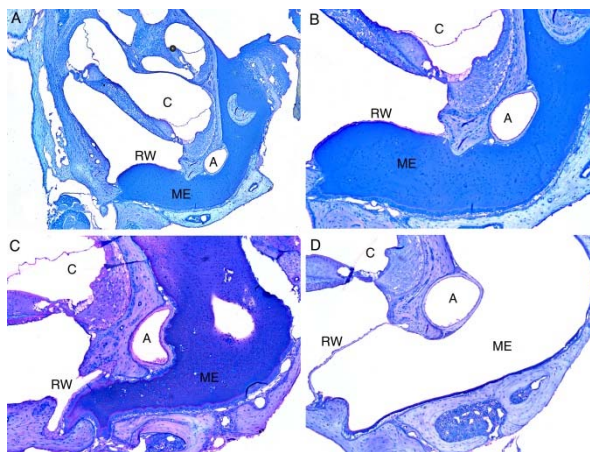
Figure 2:A: Control ear 3 day. TM thickened, fluid in middle ear (ME).B:Higher magnification of control ear

3 day. ME fluid and inflammatory cells seen. C:Control ear 5 day. Note increased inflammatory cell infiltrate and mucosal hypertrophy. D:Prednisolone-treated ear5 day. Note clearance of typical inflammatory changes seen at 5 days.

*ME = middle ear, C = cochlea, A = stapedial artery, RW = round window

References

1. Bluestone CD, Klein JO. Otitis Media in Infants and Children, 3rd edition.Saunders, Philadelphia, 2001.
2. Melhus A. Effects of amoxicillin on the expression of cytokines during experimental acute otitis media caused by non-typeable *Haemophilus influenzae*. J Antimicrob Chem 2001; 48:397-402.
3. Hunter SE, Singla AK, Prazma J, Jewett BS, Randell SH, Pillsbury HC.Mucin production in the middle ear in response to lipopolysaccharides. Otolaryngol Head Neck Surg 1999; 120:884-888.
4. Jewett BS, Prazma JP, Hunter SE, Rose AS, Clark JM, Sartor BR, Pillsbury HC. Systemic reactivation of otitis media with effusion in a rat model. Otolaryngol Head Neck Surg 1999; 121:7-12.
5. Rosenfeld RM and Bluestone CD. Evidence-Based Otitis Media, 2nd edition. BC Decker, Inc, London, 2004
6. Li J-P, Kania R, Lecain E, et al.In vivo demonstration of the absorptive function of the middle ear epithelium. Hear Res 2005; 210:1-8.
7. Choi JY, Son EJ, Kim JL, et al. ENaC- and CFTR-dependent ion and fluid transport in human middle ear epithelial cells. Hear Res 2006; 211:26-32.
8. Trune DR, Kempton JB, Gross ND. Mineralocorticoid receptor mediates glucocorticoid treatment effects in the autoimmune mouse ear. Hear Res 2006; 212:22-32.

Effects of low-intensity focused ultrasound on the mouse middle ear

Kanako Noda, M.D., Takashi Hirano, M.D., Ph.D., Masashi Suzuki, M.D.

Recently, ƒÀ-lactamase-negative ampicillin-resistant *Haemophilus influenzae* and penicillin resistant *Streptococcus pneumoniae* have increased among children with acute otitis media. These resistant bacteria were becoming less susceptible to commonly prescribed antibacterial drugs. We tested the hypothesis that low-intensity focused ultrasound (LIFU) increases vessel permeability in the mouse middle ear and increases local antibacterial drug activity. We made a murine model of acute otitis media by inoculation with *H. influenzae* into the middle ear cavity in 80 mice. At day 3 after the inoculation, 60 mice were given ampicillin (50mg/kg or 10mg/kg or 2mg/kg) intraperitoneally as one daily dose for 3days with or without LIFU (1.0W/cm2 with a 20% duty cycle for 30 seconds). Twenty mice were given phosphate buffered saline (PBS), and served as a control. At day 10 after the inoculation, samples of middle ear effusions (MEEs) were obtained by myringotomy at the time of decapitation. We counted the number of inflammatory cells and bacteria in MEEs, and compared these results with the histological changes of the middle ear by hematoxylin-eosin staining. The number of inflammatory cells and bacteria in MEEs in the LIFU group were lower than that in the control group. And the inflammatory changes of the middle ear, such as mucosal thickening, inflammatory cell infiltration, became weak in the LIFU group when compared with the control group. So the application of the LIFU along with administration of ampicillin may be an effective regimen for acute otitis media.

Efficacy of a novel oral carbapenem, Tebipenem pivoxil (TBM-PI), against experimental otitis media caused by penicillin resistant *Streptococcus pneumonia* in chinchilla

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Introduction

Acute otitis media (AOM) is one of the most common bacterial infectious diseases during childhood. Clinical improvement of AOM is closely correlated with the eradication of pathogens from the middle ear cavity. Amoxicillin (AMX) is the drug of the first choice against AOM.[1] However, penicillin resistant *S. pneumoniae* (PRSP) with reduced susceptibility to ß-lactams have been observed in recent years.[2] As the prevalence of PRSP has increased, microbiological and clinical failures are reported in the treatment of pediatric AOM.[3,4] The development of alternative agents that have broad coverage with respect to the various antimicrobial resistant pathogens including PRSP are strongly required if the prevalence of the antimicrobial resistant pathogens continues to be increased.

Carbapenems are well recognized to have broad and strong antibacterial activities. Tebipenem pivoxil (TBM-PI) is a novel oral carbapenem and is active against various respiratory pathogens especially against *S. pneumoniae*.[5,6] In this study, we evaluated the efficacy and pharmacokinetic profiles of TBM-PI against experimental otitis media.

Materials and Methods

Development of experimental AOM models in chinchilla

Prior to infections, both ears of chinchillas were confirmed as normal healthy middle ear status using otomicroscopy. The mid-log phase of *S. pneumoniae* strains grown in Todd-Hewitt broth (Becton Dickinson Microbiology Systems, NJ, USA) with 0.5 % yeast extracts (Difco Laboratories, NJ, USA) were diluted to a appropriate concentration of 100 to 1000 CFUs/ml in sterilized saline. Ten to 100 CFUs of pneumococcal strains in 100 µl saline were inoculated directly into the right middle ears of the chinchilla via the transbullar approach by a 25-gauge needle and tuberculin syringe under intra-peritoneal anesthesia with pentobarbital sodium. Middle ear washes with 0.2 ml of sterilized saline were collected through a 3 mm hole in the superior bulla on day 2, 5 to 6, and 9.

Evaluation of efficacy of antibiotics against experimental AOM

The development of AOM was confirmed by otomicroscopy 24 h after the inoculation. Then, chinchillas were randomly assigned into three groups for treatments with AMX, TBM-PI, and without antibiotics (controls). The chinchillas were treated orally with a 5-day course of TBM-PI (25 mg/kg/dose twice daily), amoxicillin (AMX) (25 mg/kg/dose twice daily) or controls.

Results

The outcomes of treatments were evaluated by bacteria culture and improvement of AOM.

Quantitative cultures in middle ear washes at day 2 of TBM-PI yielded 3.84 ± 2.00 Log_{10} CFUs/ml which was significant compared to AMX yielded 6.75 ± 0.82 Log_{10} CFUs/ml and control yielded 6.60 ± 0.77 Log_{10} CFUs/ml (p<0.001) (Fig.1). Those at day 5 of TBM-PI yielded 3.53 ± 2.39 Log_{10} CFUs/ml which was significant compared to AMX yielded 6.04 ± 1.31 Log_{10} CFUs/ml and control yielded 6.88 ± 0.78 Log_{10} CFUs/ml (p<0.05).And those at day 9 of TBM-PI yielded 4.66 ± 2.25 Log_{10} CFUs/ml which was not significant compared to AMX yielded 6.51 ± 0.33 Log_{10} CFUs/ml.

Clinical outcomes of experimental AOM after treatment were defined on the results of otomicroscopic examinations. Existence of middle ear effusions, redness and bulging of tympanic membrane, purulent ear discharge were evaluated under the

otomicroscope. The clinical outcomes were designated as marked improved, slight improved, no improvement, and progressive disease. TBM-PI showed markedly improvement of experimental AOM (Fig. 2).

Discussion

Because of *S. pneumoniae* as the most frequent causative pathogen responsible for AOM, antimicrobial therapies against AOM have been targeted against this microorganism. Penicillin resistant *S. pneumonia* (PRSP) represented a greater proportion of treatment failures in AOM.[3,4] To control the intractable AOM caused by PRSP, a variety of options including increasing the dose of AMX at the initial therapies or alternative antibiotics for primary treatment failures are suggested in the antimicrobial treatment of AOM.[7,8] The chinchilla experimental models of AOM caused by PRSP that mimics the human condition are useful for evaluating the clinical efficacies of various antimicrobial treatment options.

TBM-PI is the most recently developed oral carbapenem susceptible to PRSP (MIC=0.06 µg/ml) and is a promising antimicrobial agent against PRSP. The in vivo efficacies data in the current study showed TBM-PI were equally effective to high dose AMX in clinical outcomes of AOM. Treatments with TBM-PI significantly reduced the numbers of *S. pneumoniae* in the middle ear, while the numbers of *S. pneumoniae* in the middle ear of the chinchilla treated with high dose of AMX increased on day 9 again.

In conclusion, the current study showed the chinchilla offers a reasonable animal model for evaluating clinical efficacies of antimicrobial agents against AOM caused by PRSP.
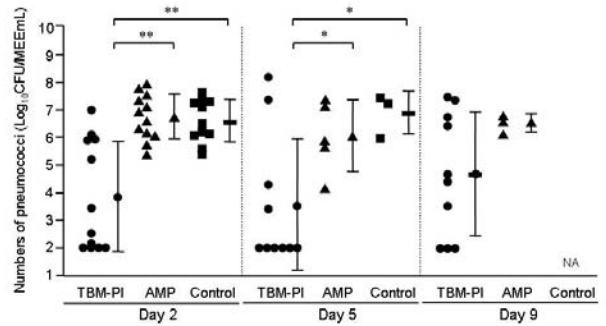
Fig. 1

Fig. 1 CFUs of *S. pneumoniae* identified in middle ear fluids.

Bar: mean±SD, Dot: CUFs of *Streptococcus pneumoniae* identified in the middle ear washes.

Fig. 2

Fig. 2 The changes in tympanic membrane findings after treatments with antibiotics.

-: marked improvement, +: slight improvement, ++: no improvement, +++: progressive disease.*: p<0.001 compared with other groups on day 2 and p<0.05 compared with other groups on day 5 and day 9.NA: Data not available.

References

1. RosenfeldRM, VertreesJE, Carr J, Cipolle RJ, Uden DJ, Giebink GS, Canafax DM. Clinical efficacy of antimicrobial drugs for acute otitis media: Metaanalysis of 5400 children from thirty-three randomized trials. J Pediatr1994; 124:355-367.
2. Mason EO Jr, Laplan SL, Lamberth LB, Tillman J. Increased rte of isolation of penicillin-resistant *Streptococcus pneumoniae* in a children's hospital and in vitro susceptibilities to antibiotics of potential therapeutic use. Antimicrob Agents Chemother 1992; 36:1703-1707.
3. Hotomi M, Yamanaka N, Samukawa T, Suzumoto M, Sakai A, Shimada J, Ikeda Y, Faden H. Treatment and outcome of severe and non-severe acute otitis media. Eur J Pediatr 2005; 164:3-8.
4. Hotomi M, Yamanaka N, Shimada J, Ikeda Y, Faden H. Factors associated with clinical outcomes in acute otitis media. Ann Otol Rhinol Laryngol 2004; 113:846-852.
5. Kobayashi R, Konomi M, Hasegawa K, Morozumi M, Sunakawa K, Ubukata K. In vitro activity of tebipenem, a new oral carbapenem antibiotic, against penicillin-nonsusceptible *Streptococcus pneumoniae*. Antimicrob Agents Chemother 2005; 49:889-894.
6. Hikida M, Itahashi K, Igarashi A, Shiba T, Kitamura M. In vitro antibacterial activity of LJC 11,036, an active metabolite of L-084, a new oral carbapenem antibiotic with potent antipneumococcal activity. Antimicrob Agents Chemother 1999; 43:2010-2016.
7. Barry B, Muffat-Joly M, Gehanno P, Pocidalo JJ. Effect of increased dosages of amoxicillin in treatment of experimental middle ear otitis due to penicillin resistant *Streptococcus pneumoniae*. Antimicrob Agents Chemother 1993; 37:1599-1603.
8. Faden H, Bernstein JM, Brodsky L, Stanievich J, Ogra PL. Effect of prior antibiotic treatment on middle ear disease in children. Ann Otol Rhinol Laryngol 1992; 101:87-91.

Regulation of TGF-β signalling by Fbxo11 – the gene mutated in the Jeff otitis media mouse

Hilda Tateossian, Ph.D., Rachel Hardisty-Hughes, Susan Morse, Professor Steve Brown

The N-ethyl-N-nitrosurea (ENU)-induced mouse mutant *Jeff* was previously described as having chronic proliferative otitis media.[1] Homozygote *Jeff* mice are born with their eyelids open and die within a few hours after birth.[2] They have also respiratory problems, facial cleft, and cleft palate. Using an antibody against Fbxo11 (the gene mutated in *Jeff*) for immunohistochemistry, we examined the expression of the protein on sections of palates and eyelids at the time of their development.

The transforming growth factor β (TGF-β) superfamily is a large family of cytokines involved in a number of cellular processes such as proliferation, differentiation, epithelial mesenchymal transformation, and apoptosis. They mediate their effects from membrane to nucleus through combinations of type I and type II serine/threonine kinase receptors and their downstream effectors, Smad proteins. Certain Smads became phosphorylated by activated type I receptors and form heteromeric complex with a common-partner Smad4, which translocates into the nucleus to control gene transcription. TGF-β signalling is controlled by many mechanisms, one of which being E3 ubiquitin ligase-mediated ubiquitination. Fbxo11 is a member of the F-box family and by similarity with other Fbox proteins probably functions as part of a SCF (SKP1-cullin-F-box) protein ligase complex, recognizing and binding to some phosphorylated proteins and promoting their ubiquitination and degradation.

There is considerable evidence supporting the role of members of the TGF-β family in palate development. We have used different antibodies to study the expression of some of the members of the TGF-β family on sections of wild type and *Jeff* homozygote palates at different stages of palate formation. The results suggest an increased level of phosphorylated Smad2, but not Smad2 on sections of E15.5 homozygote palates. We have also demonstrated that the mutation in Fbxo11 results in accumulation of receptor-phosphorylated Smad2 in the nucleus. The mutation also results in resistance to TGF-β induced cell growth arrest and reduced TGF-β induced programmed cell death. We have applied the same panel of antibodies to sections of eyelids to see if the same processes (proliferation and apoptosis) are affected in the development of the eyelids in the *Jeff* mice.

References

1. Hardisty RE, Erven A, Logan K, et al. The deaf mouse mutant *Jeff* (*Jf*) is a single gene model of otitis media. J Assoc Res Otolaryngol 2003; 4:130-138.
2. Hardisty-Hughes RE, Tateossian H, Morse SA, et al. A mutation in the F-box gene, Fbxo11, causes otitis media in the Jeff mouse. 2006 Hum Mol Genet 2006; 15:3273-3279.

Fbxo11, mutated in the OM mouse mutant model Jeff, is a novel apoptosis inhibitor and binds spectrin beta II

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The *Jeff* mutant carries a mutation in the F-box gene, *Fbxo11*, a member of a large family of proteins that are specificity factors for the SCF E3 ubiquitin ligase complex.[1] *Jeff* develops a conductive deafness due to a chronic suppurative otitis media and represents an important model for chronic forms of middle-ear inflammatory disease in humans.

We have transfected both wild type and F-box deletion (delF11) forms of Fbxo11 into cos7 cells. We demonstrated that over-expression of delF11 causes severe growth inhibitory effects in contrast to overexpression of wild type Fbxo11. Treatment of untransfected cos7 cells with MG132, a proteosomal inhibitor, results in 33% cell death after 5 hours' incubation. In comparison, MG132 treatment of cos7 cells overexpressing wild type Fbxo11 causes only 10% cell death. Further analysis of apoptosis among transfected and untransfected cos7 cells strongly indicates that Fbxo11 has anti-apoptotic effects. These results agree with recent findings which demonstrate that Fbxo11 neddylates and inhibits p53 which itself is pro-apoptotic.

In addition, we have proceeded to identify interacting partners to Fbxo11 and to establish potential substrates for ubiquitination mediated by Fbxo11. We have performed co-immunoprecipitations with Fbxo11 from both cos7 and mouse tissue and identified spectrin beta II as an interacting partner to Fbxo11. Spectrin beta II is known to interact with Smad3 mediating the TGF-β signalling pathway. The interaction of Fbxo11 with spectrin beta II provides *in vivo* evidence for the pivotal nature of this signalling pathway in mediating otitis media and suggests that Fbxo11 may mediate otitis media through two routes – the TGF-β signalling pathway and via the pro-apoptotic effects of p53.

Reference

1. Hardisty-Hughes RE, Tateossian H, Morse SA, et al. A mutation in the F-box gene, Fbxo11, causes otitis media in the Jeff mouse. 2006 Hum Mol Genet 2006; 15:3273-3279.

Impact of various therapeutic steroids on C3H/HeJ mouse chronic otitis media

Carol MacArthur, M.D., Beth Kempton, B.S., Jacqueline DeGagne, B.A., Dennis Trune, Ph.D.

Introduction

Chronic otitis media (COM) not only causes significant middle-ear pathology, but can also impact the inner ear. Movement of cytokines, inflammatory cells, and bacterial products through the round window membrane places the cochlea at significant risk for sensorineural hearing loss, tissue remodeling, and other permanent changes. Although inner-ear pathology in COM has been described in clinical reports and temporal bone studies[1,2,3], we have little insight into the pathologic mechanisms due to lack of an appropriate animal model for spontaneous and chronic middle-ear disease. We have recently reported that the C3H/HeJ mouse spontaneously develops middle-ear disease that does not clear.[4] C3H/HeJ mice possess a gene defect in their TLR4 receptor that renders them unable to respond to Gram-negative infections.[5,6] As a result, approximately 50% develop spontaneous COM.[7] The bacteriology of this spontaneous infection is now known to be a Gram-negative infection. *Klebsiella oxytoca* was the primary organism cultured from the C3H/HeJ mouse in recent work from our lab.[8] Because the inner ear is at risk in COM, mice were evaluated to determine the impact of middle-ear disease on the inner ear. Also, mice with COM were treated with various therapeutic steroids to determine if such steroid treatments can control either middle- or inner-ear pathology.

It has been reported that steroids may have an impact on Na^+ and fluid transport through the middle-ear mucosal epithelium.[9,10] The fluid homeostasis function of the middle-ear epithelium is likely to have a major role in removing fluid that accumulates during inflammation, such as seen with acute otitis media (AOM) and COM. If the fluid was controlled, hearing would be restored and pain from inflammation would be alleviated. The goal of the study was therefore to differentiate which steroid role--fluid homeostasis (mineralocorticoids), immune suppression (glucocorticoids), or both (steroid with both actions)-- would experimentally be best in controlling middle-ear fluid and cellular infiltration in this model of COM (Table 1).

Design

C3H/HeJ (n=62) mice with COM were tested for baseline auditory brainstem response (ABR) thresholds and then given steroids with differences in their anti-inflammatory (I) and fluid absorption (F) functions: prednisolone (26), dexamethasone (8), fludrocortisone (14), and aldosterone (14). Prednisolone, fludrocortisones, and aldosterone were all given in the drinking water[11] at the following doses: prednisolone 5 mg/kg/day, fludrocortisone 10 µg/kg/day, aldosterone 15 µg/kg/day. Dexamethasone was given as a subcutaneous injection daily at 0.75 mg/kg/day. Untreated mice (10) served as controls and were given plain drinking water without any steroid. ABR thresholds were measured before treatment and at 2 and 4 weeks after treatment. Histologic examination was made of middle-ear and inner-ear pathology after the last ABR test.

ABR electrophysiology. ABR thresholds were measured at 4, 8, 16, and 32 kHz according to our standard protocol[12] on all ears to determine levels of cochlear function and middle-ear status at 2 and 4 weeks of treatment. To determine the impact of treatment (or no treatment) on a particular ear, the total ABR threshold shift at four frequencies (4K, 8K, 16K, 32K) was derived.[11] If the threshold across all four frequencies was better by 20db or more, then the hearing in that ear was considered improved. If the total threshold shift was worse by 20 dB or more, the hearing in that ear was considered worse. Cochlear function was judged as unchanged if the threshold shift was +/- 15 dB or less. Thus, each ear was classified as improved, unchanged, or worse for its respective treatment (steroid or no treatment). The proportion of ears falling into each treatment outcome was then compared by the Chi-Squared nonparametric test.

Middle-ear histopathology. The animals were euthanized by an overdose of anesthetic (ketamine and xylazine), fixative was intracardially perfused (1.5% glutaraldehyde - 3% paraformaldehyde in 0.1M phosphate buffer), and the dissected skulls immersed in fixative overnight. The middle and inner ears were left intact and connected to each other by the skull base so both ears were processed together for histology and sectioning. Tissues were microwave decalcified in

EDTA, embedded in glycol methacrylate plastic, sectioned in the horizontal plane at 5 μm, serially mounted on glass slides, stained, and coverslipped. Slides were examined with the Leica DMLB microscope. Inflammation was qualitatively evaluated for presence of middle-ear fluid, middle ear inflammatory cell infiltration, and changes in the mucosa of the tympanic cavity and round window membrane. The eustachian tube was also examined for epithelial cell changes, cellular infiltrates, or anatomic changes.

Inner-ear histopathology. The inner ear was assessed for pathology of the organ of Corti, auditory nerve, perilymphatic spaces, and vestibular organs. Of particular interest was the presence or absence of inflammatory cell infiltrates, fibrillar deposition, vascular changes, and intercellular edema.

All animal procedures were approved by OHSU IACUC.

Results

Physiology

Control mice. The group receiving no treatment showed significant worsening of the ABR thresholds at both 2 and 4 weeks (Fig. 1A,B). Forty percent (4/10) of control ears had no change in ABR thresholds at 4 weeks and 60% (6/10) of control ears had worsening ABR thresholds at 4 weeks. No animals had improved ABR thresholds in the control group at 4 weeks.

Treatment mice: prednisolone. Hearing was either improved or unchanged in the prednisolone-treated group (Fig. 218-1A, B). Forty-three percent of ears (7/16) actually had improvement of ABR thresholds at 2 and 4 weeks, while 56% (9/16) were unchanged (Fig. 1A,B). None had worsened ABR thresholds.

Dexamethasone: Dexamethasone treatment was less effective than prednisolone. ABR thresholds improved in 12.5% (1/8) of ears, while 75% (6/8) were no change, and 12.5% worse at 4 weeks (Fig. 1A,B).

Fludrocortisone: Most fludrocortisones-treated ears (93%) showed no change in the ABR thresholds at 4 weeks, but a few improved ABR thresholds at 4 weeks (7%) (Fig. 1A, B).

Aldosterone: Aldosterone-treated mice showed 50% unchanged at 4 weeks, 14% improved, and 35% worse at 4 weeks (Fig. 1A, B).

Average ABR threshold change from baseline at 2 and 4 weeks was also evaluated. Prednisone-treated animals were by far the group that experienced improved thresholds (seen as a negative change in Fig. 2A, B). Dexamethasone and fludrocortisone also had some improvement in thresholds at 4 weeks (Fig. 2B).

Histopathology

Twelve percent of prednisolone-treated mice were able to clear the middle-ear inflammation seen on histopathology, while no controls did. Control histopathology revealed extensive inflammatory cell infiltrate filling the middle ear with mucosal inflammation and hypertrophy. On the other hand, middle ears that were able to clear the OM often showed resolution of such changes (Fig. 3B). Also, 38% of prednisolone-treated mice had middle-ear inflammation only (without inner ear) compared to 10% of controls. No control, dexamethasone-, fludrocortisones-, or aldosterone-treated animals cleared the middle-ear process during the study period. All aldosterone-treated animals had middle-ear inflammation at the end of the study period. Seventy-nine percent had both middle- and inner-ear inflammation on histopathology, while 21% had middle-ear inflammation only. Dexamethasone- and fludrocortisones-treated mice all had both middle- and inner-ear inflammation at the end of the study period. Dexamethasone-, fludrocortisones-, and aldosterone-treated mice showed ear pathology similar to untreated mice.

Histologic examination of the middle and inner ears evaluated the amount and character of cellular infiltrates in both control and treatment animals. The cellular infiltrate in the infected middle ears was dense, highly cellular (macrophages, neutrophils), and often filled the entire middle-ear space (Fig. 3A). This degree of inflammation is commonly seen in this TLR4-deficient animal model.[4]

Many animals with ongoing OM also had a remarkable amount of inner-ear inflammation. This was characterized by bacteria and/or inflammatory cells in the scala vestibuli and/or scala tympani. Inner-ear disease often was seen ipsilateral to the untreated OM ears, indicating the significant impact of OM on inner-ear pathology (Fig. 3A).

Conclusion

Treatment of mice with the glucocorticoid prednisolone was the most effective of the various steroids used in the study in controlling the hearing loss associated with otitis media. Treatment improved hearing in as many as 43% of the treatment animals, whether they had middle-ear disease or not, also

suggesting a direct effect on inner-ear inflammation. Less effective, but still having some improvement over no treatment was the glucocorticoid dexamethasone. Up to 12% of dexamethasone-treated animals showed improved ABR thresholds at 4 weeks, while no control animals had improved ABR thresholds. The mineralocorticoids, aldosterone and fludrocortisone, were slightly better than the no treatment group, with 14% and 7%, respectively, improving the ABR thresholds at 4 weeks of treatment.

Histologic changes were most evident in the prednisolone-treated animals, with 12% showing actual clearing of the middle-ear inflammation and infection. No other treatment groups had clearing of the middle-ear process. Prednisolone-treated animals also showed the largest percentage of clear inner ears (50%), suggesting that this glucocorticoid was able to impact inner-ear inflammation better than the other steroids studied.

The combined immunosuppressive and fluid absorption actions of prednisolone were the most effective in reducing the severity of chronic middle-ear disease and improving cochlear inflammation. These preliminary studies offer some insight into the potential steroid control of middle- and inner-ear disease during COM.

Figure 1. Hearing results (ABR) by treatment group and length of treatment. Results are grouped by better (total threshold shift across 4 frequencies better than 20 dB), no change (threshold shift +/- 15 dB or less), or worse (threshold shift worse by 20 dB or more)
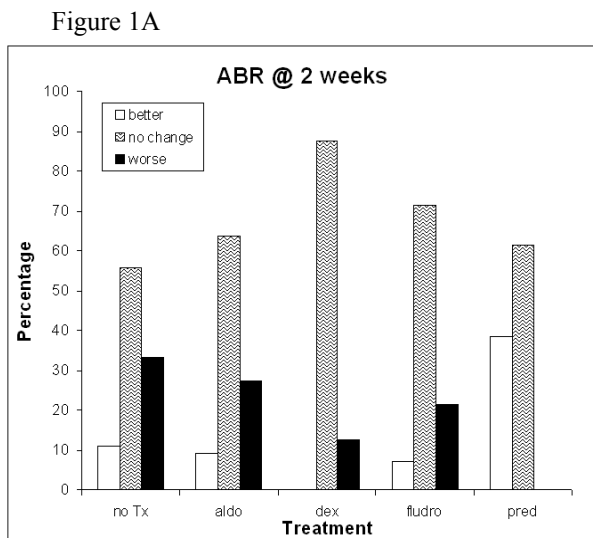
Figure 1A
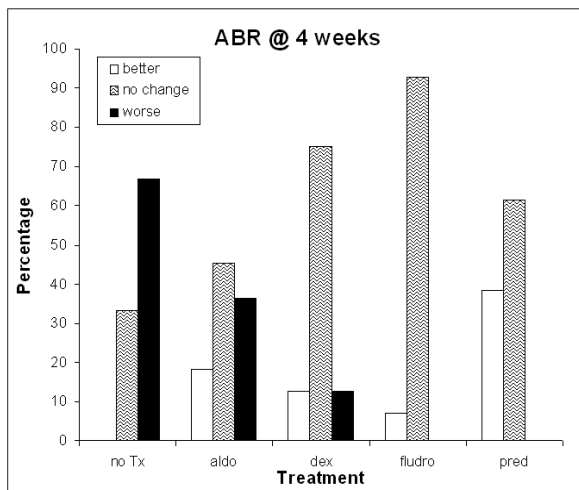
Figure 1B

Figure 2. Average ABR threshold change from baseline at 2 and 4 weeks of treatment.

Figure 2A
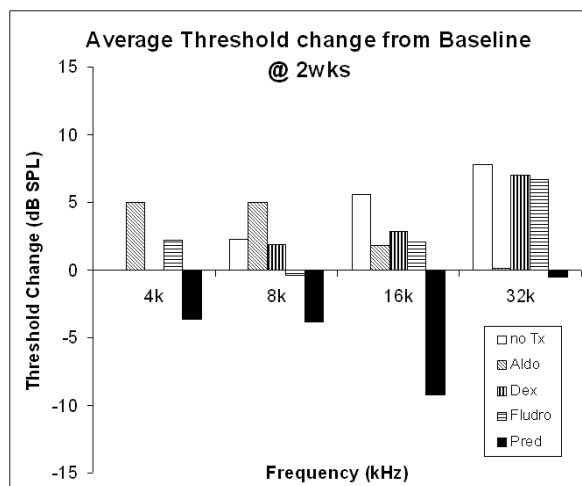
Figure 2B
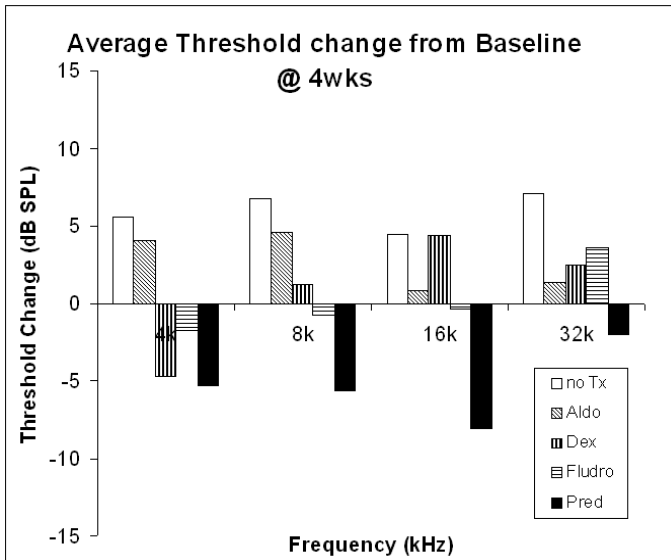
Figure 3
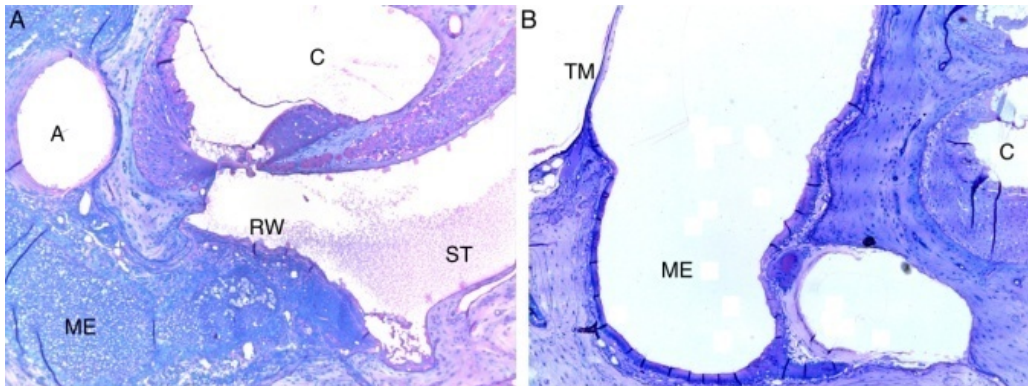
A. Dexamethasone-treated ear. Extensive inflammation seen of middle and inner ear. Note thickening of round window membrane and middle ear mucosa, and inner ear debris in scala tympani. B. Prednisone-treated ear. Note absence of middle and inner ear inflammation. *A – artery, C = cochlea, ME – middle ear, RW – round window, TM – tympanic membrane, ST – scala tympani

Table 1. Steroid Treatments to Control Inflammatory Sequelae of Otitis Media	Glucocorticoid Effect (Immunosuppression)		
		+	-
Mineralocorticoid Effect (Fluid Homeostasis)	+	Prednisolone	Fludrocortisone Aldosterone
	-	Dexamethasone	Water

References

1. Cureoglu S, Schachern PA, Paparella MM, Lindgren BR. Cochlear changes in chronic otitis media. Laryngoscope 2004; 114:622-626.
2. Ichimiya I, Suzuki M, Hirano T, Mogi G. The influence of pneumococcal otitis media on the cochlear lateral wall. Hear Res 1999; 131:128-143.
3. Schachern PA, Paparella MM , Hybertson R, Sano S, Duvall AJ. Bacterial tympanogenic labyrinthitis, meningitis and sensorineural damage. Arch Otolaryngol Head Neck Surg 1992; 118:53-57.
4. MacArthur CJ, Hefeneider SH, Kempton JB, Trune DR. C3H/HeJ Mouse Model for spontaneous chronic otitis media. Laryngoscope 2006; 116:1071-1079.
5. Qureshi ST, Lariviere L, Leveque G, et al. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). J Exp Med 1999; 189:615-625.
6. Poltorak A, He X, Smirnova I, Liu MY, et al. Defective LPS signaling in C3H/HeJ and C57/10ScCr mice: Mutations in Tlr4 gene. Science 1998; 282:2085-2088.
7. Mitchell CR, Kempton JB, Scott-Tyler B, Trune DR. Otitis media incidence and impact on the ABR in lipopolysaccharide-nonresponsive C3H/HeJ mice. Otolaryngol Head Neck Surg 1997; 117:459-464.
8. MacArthur CJ, Pillers D, DeGagne J, Kempton JB, Trune DR. Gram-negative pathogen *Klebsiella oxytoca* is associated with spontaneous chronic otitis media in Toll-like receptor 4 deficient C3H/HeJ mice. Acta Otolaryngol 2008; 128:132-138.
9. Li J-P, Kania R, Lecain E, et al. In vivo demonstration of the absorptive function of the middle ear epithelium. Hear Res 2005; 210:1-8.
10. Choi JY, Son EJ, Kim JL, et al. ENaC- and CFTR-dependent ion and fluid transport in human middle ear epithelial cells. Hear Res 2006; 211:26-32.
11. Trune DR, Kempton JB, Gross ND. Mineralocorticoid receptor mediates glucocorticoid treatment effects in the autoimmune mouse ear. Hear Res 2006; 212:22-32.
12. Mitchell CR, Kempton JB, Creedon TA, Trune DR. Using a 56-stimulus train for the rapid acquisition of auditory brainstem responses. Audiol Neurotol 1999; 4:80-87.

Three *Moraxella catarrhalis* strains differed in their ability to cause acute otitis media in the rat

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Moraxella catarrhalis is now known as a cause of acute otitis media (AOM). It has been difficult to study AOM caused by *M. catarrhalis* in animal models as the bacteria often fails to cause infection in the middle ear. Three strains of *M. catarrhalis* were inoculated into the middle ears of 60 Sprague-Dawley rats to study whether there was a difference in their ability to cause AOM and to observe the morphological changes in the middle ears. The strains used had been characterized by endonucleases supplemented by ribotyping and were kindly provided by Dr. Brygge, Rigshospitlet, Copenhagen, Denmark. The rats were followed for 2 months with otomicroscopy and were sacrificed at various time intervals for histological samples. At day 4, all rats inoculated with one of the strains had AOM otomicroscopically, whereas the other strains caused AOM in five out of 12 and three out of 11 rats. Inflammatory changes were seen in the histological samples from all three strains. The three *M. catarrhalis* strains differed in their ability to cause AOM in the rat.

A mouse model of middle-ear infection after intranasal administration of pneumococci

Albert Sabirov, Ph.D., Dennis Metzger, Ph.D.

Introduction

The pathogenesis of otitis media (OM) involves nasopharyngeal (NP) bacterial colonization followed by retrograde ascension of the pathogen up the eustachian tube into the middle ear (ME). Whereas there are various established mouse models of NP carriage, the actual spread of bacterial or viral infection into the eustachian tube and ME has not been extensively studied. Previously, we demonstrated the applicability of a mouse model of OM that utilizes direct intrabullar challenge to assess the protective efficacy of intranasal (IN) vaccination.[1,2] A disadvantage of this technique is the fact that direct ME inoculation is an artificial route of infection as it bypasses NP colonization. The aim of the present study was to establish a model of ME infection following IN administration of pneumococci. This model has an advantage in that it replicates the natural route of ME infection and could be used for testing the efficacy of mucosal vaccines.

Material and methods

Bacteria

Streptococcus pneumoniae strain TJO983, which expresses PPS14, was grown overnight on blood agar plates and cultured at 37Cº in Todd-Hewitt broth supplemented with 0.5% yeast extract. The identity of the pneumococci was confirmed by colony morphology on blood agar plates and by sensitivity to optochin. Bacteria were harvested by centrifugation and washed twice in sterile phosphate buffered saline. The bacteria were resuspended in Todd-Hewitt broth containing 0.5% yeast extract and 15% glycerol, and stored in aliquots at –80ºC.

IN challenge with *S. pneumoniae* and induction of NP and ME colonization

Infant BALB/c mice (24 day old) were inoculated for five consecutive days (days 1-5) IN with 10^6 CFU of type 14 *S. pneumoniae*. For time-course studies, the mice were sacrificed on days 2 to 13, and assayed for the presence of pneumococci in NP and ME wash fluids. For dose-course studies, infant mice were inoculated IN with various doses (10^4-10^7 CFU) of *S. pneumoniae* type 14 for 1 or 5 consecutive days. Mice were sacrificed on day 8 and assayed for the presence of pneumococci in ME and NP wash fluids. NP and ME washes were collected, diluted 10-fold, and 50 µl of the diluted samples were plated onto blood agar to determine the concentrations of live bacteria. The mice were monitored by otomicroscopic observation to confirm tympanic membrane changes (vessel dilation, increased thickness, and reduced translucency).

Results

All mice showed the presence of pneumococci in NP washes on days 2 to 13. ME infection developed at various time points after IN inoculation of pneumococci (10^6 CFU) for 5 consecutive days (days 1-5). The infection in the ME and tympanic membrane changes were not seen until day 6. The number of mice with ME infection gradually increased from day 6 (50%) until it peaked on day 8 (100%) suggesting that pneumococcal ME colonization is the consequence of sustained bacterial growth. Similarly, the number of mice with tympanic membrane changes increased from day 6 (25%) to day 8 (71%). By day 8, most of the mice (71%) exhibited bilateral ME infection. The number of pneumococci in the ME was proportionate to that in the NP. A decrease in IN inoculation dose ($<10^6$) significantly affected the probability of ME colonization (10^4: 0%; 10^5: 28%). Similarly, the number of inoculations (<5 times) also affected the probability of ME colonization by day 8 postchallenge (1 inoculation: 14%; 3 inoculations: 43%).

Discussion

We have established a murine model of OM following IN administration of pneumococci. In this model, the portal of pathogen entry into the ME would thus resemble the disease process in humans. Previously, the mouse model[3,4] was utilized to demonstrate the progression of acute OM following single IN

inoculation with pneumococci. Malley et al.[3] demonstrated that following IN inoculation with pneumococci, the frequency of OM varied from <50% (by serotype 14) to 100% (by serotypes 6B and 23F). McCullers et al.[4] confirmed the presence of pneumococci in NP and their subsequent expression in the ME by visualization of the bioluminescent bacteria (type 19F); however, the extent of inflammatory changes in the ME was not demonstrated. We found progression of OM in normal mice following five daily IN inoculations of pneumococci serotype 14. The infection correlated with the presence of ME effusions and tympanic membrane changes. Time-course studies showed that the optimal sampling time for measurement of bacterial carriage in NP and ME was day 8 after initial IN challenge. The possible mechanism responsible for ME infection could be induction of an inflammatory reaction at the eustachian tube orifice in the NP following repeated exposure to *S. pneumoniae*. In addition, we also demonstrated the utility of this acute OM model for the evaluation of IN vaccine efficacy against pneumococcal disease.[5]

References

1. Sabirov A, Metzger DW. Intranasal vaccination of neonatal mice with polysaccharide conjugate vaccine for protection against pneumococcal otitis media. Vaccine 2006; 24: 5584-5592.
2. Sabirov A, Kodama S, Hirano T, Suzuki M, Mogi M. Intranasal immunization enhances clearance of nontypeable *Haemophilus influenzae* and reduces stimulation of tumor necrosis factor alpha production in the murine model of otitis media. Infect Immun 2002; 69:2964-2971.
3. Malley R, Morse SC, Leite LC, Areas AP, Ho PL, Kubrusly FS, Almeida IC, Anderson P. Multiserotype protection of mice against pneumococcal colonization of the nasopharynx and middle ear by killed nonencapsulated cells given intranasally with a nontoxic adjuvant. Infect Immun 2004; 72:4290-4292.
4. McCullers JA, Karlstrom A, Iverson AR, Loeffler JM, Fischetti VA. Novel strategy to prevent otitis media caused by colonizing Streptococcus pneumoniae. PLoS Pathog 2007; 3:28.
5. Sabirov A, Metzger D. Intranasal vaccination of infant mice induces protective immunity in the absence of nasal-associated lymphoid tissue (abstract). 9th International Symposium on Recent Advances in Otitis Media. St. Petersburg, Fla., 2007:132.

AUD review of otitis media grant applications

Edwin Clayton, Ph.D.

Recent changes in CSR policy may impact the manner in which otitis media applications are assigned and reviewed at NIH. I will discuss how these policy changes affect the Auditory System (AUD) study section. Some of the topics I will address include: restrictions on the number of ad hoc reviewers, the usage of phone and mail reviews, reviewer workloads, future CSR initiatives, and the subject matter most appropriate for AUD versus other NIH study sections.

Research opportunities update from NIDCD/NIH

Bracie Watson, Ph.D.

This presentation will provide an opportunity to hear updates and learn about recent activities at NIDCD/NIH. Topics include the status of otitis media research in the NIDCD portfolio, the NIDCD R03 program, and the new NIH Pathway to Independence Award (K99/100).

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