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Clinical and pathogenic aspects of otitis media

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CLINICAL AND PATHOGENIC ASPECTS OF OTITIS MEDIA

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ABSTRACT

The first part of this thesis evaluates two different treatment strategies in tympanostomy tube associated otorrhea. The second explores the tentative relation between the appearance of otitis media with effusion and the immunological activity in the adenoid, focusing on Toll-like receptor (TLR) 4 and TLR7 along with the nitric oxide (NO) inducing enzymes eNOS and iNOS. The third part investigates if TLRs and Nod-like receptors (NLRs) can be found in the middle ear mucosa and if their expression is affected by chronic middle ear disease. Finally, the prerequisites for local NO production in the middle ear during health and disease is studied.

With the duration of otorrhea as primary outcome variable no difference was seen when tympanostomy tube associated otorrhea was treated topically with or without concomitant systemic antibiotics. This implies that topical treatment alone is sufficient in most cases of tube-associated otorrhea, and that a watch-and-wait policy can be recommended vis-à-vis the use of systemic antibiotics. Bacterial cultures showed a clear dominance of typical middle ear pathogens indicating that the otorrhea episodes reflected new episodes of acute otitis media.

TLR4, TLR7, eNOS and iNOS mRNA together with their corresponding proteins could be demonstrated in all adenoids tested. Adenoids from children with otitis media with effusion exhibited higher expression of TLR7 and lower expression of iNOS than controls. Since TLR7 interacts with single-stranded virus RNA it might be that viral infections via this receptor trigger immune reactions in the adenoid that contributes to the development of otitis media with effusion. NO is known to induce anti-microbial effects. A reduced ability to produce NO, reflected in low iNOS levels, might therefore signal an increased risk for developing middle ear disease.

mRNA and protein representing TLR3, TLR4, TLR5, TLR7 along with the Nod-like receptors (NLRs) Nod2 and NALP3 were found in the middle ear mucosa. Patients with chronic middle ear disease exhibited lower expression of TLR4, TLR5, TLR7 and Nalp3 than controls, whereas Nod2 was up-regulated. The finding of TLRs and NLRs represent possible activation sites in what has previously been considered as a compartment low in immunological activity. It opens new perspectives on how the middle ear can be defended against invading micro-organisms. Further, it is not inconceivable that an abnormal set of innate immune receptors can affect the development and outcome of chronic middle ear disease.

eNOS mRNA was detected in the middle ear mucosa whereas iNOS expression was generally low. Mucosa from patients with chronic middle ear disease displayed reduced levels of eNOS in comparison to controls. Gas sampled from the middle ear contained more NO than gas sampled from the ear channel of the contra lateral ear. Together, this indicates that NO might be a natural component of the middle ear gas milieu important for maintaining a healthy local environment.

LIST OF PUBLICATIONS

The present thesis is based on the following papers, which will be referred to by their Roman numerals

I. **Granath A**, Rynnel-Dagöö B, Backheden M, Lindberg K
Tube associated otorrhea in children with recurrent acute otitis media; results of a prospective randomized study on bacteriology and topical treatment with or without systemic antibiotics
International Journal of Pediatric Otorhinolaryngology 2008; 72:1225-1233

II. **Granath A**, Uddman R, Cardell LO
Increased TLR7 expression in the adenoids among children with otitis media with effusion
Acta Oto-Laryngologica 2010; 130: 57-61

III. **Granath A**, Norrby-Teglund A, Uddman R, Cardell LO
Reduced iNOS expression in adenoids from children with otitis media with effusion
Pediatric Allergy and Immunology 2010; in press

IV. **Granath A**, Uddman R, Cardell LO
Deranged Toll- and Nod-like receptor expression in the middle ear mucosa in patients with chronic middle ear disease
Submitted

V. **Granath A**, Weitzberg E, Cardell LO
Nitric oxide production in human middle ear mucosa
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

Abs	Antibodies
TLR	Toll-like receptor
NLR	Nod-like receptor
OME	Otitis media with effusion
AOM	Acute otitis media
rAOM	Recurrent acute otitis media
CMED	Chronic middle ear disease
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acidd
PCR	Polymerase chain reaction
S. pneumoniae	*Streptococcus pneumoniae*
S. pyogenes	*Streptococcus pygenes*
M. catarrhalis	*Moraxella catarrhalis*
H. influenzae	*Haemophilus influenzae*
P. aeruginosa	*Pseudomonas Aeruginosa*
S. aureus	*Staphylococcus aureus*
NTHI	*Non-typable Haemophilus influenzae*
PRR	Pattern recognition receptor
PAMP	Pathogen associated molecular pattern
LPS	Lipopolysaccharide
NO	Nitric oxide
NOS	Nitric oxide synthase

1 THESIS SUMMARY – MAIN SECTION

1.1 BACKGROUND

Different types of otitis media are common clinical problems, especially in children. This thesis addresses novel aspects on the role of innate immunity in acute and chronic middle ear inflammation. In addition it investigates if topical treatment alone is sufficient in cases of tube-associated otorrhea in children with recurrent acute otitis media.

The middle ear

The middle ear is the centre of conductive hearing and consists of a miniature system of recesses located in the temporal bone. The walls are lined with a thin layer of mucosa which is believed to contain only a few immunologically active cells [1]. The tympanic cavity is traditionally considered to be a sterile compartment, whereas other parts of the upper respiratory system are frequently exposed to viruses and bacteria. During healthy conditions the middle ear is gas-containing. The Eustachian tube regulates its air pressure. The drawback of this connection is that microbes and fluids from the nasopharyngeal region might enter into the middle ear this way. The Eustachian tube in children is shorter and less angled than in adults. These anatomical characteristics in children are considered to promote influx of microbes from the nasopharynx to the middle ear [2].

The adenoid

The adenoid, also known as the nasopharyngeal tonsil, is organized lymphatic tissue situated in the nasopharyngeal space. It is anatomically closely related to the nasopharyngeal orifices of the Eustachian tube. Due to its position it is continuously showered with particles and microbiological agents that are ingested or inhaled. It also receives tear liquid from the surface of the eyes which constitutes another gateway for infections. The histology of the human adenoid resembles the structure of lymph nodes and the palatine tonsils in the oropharynx, and they are all part of the same mucosa associated lymphoid tissue system (MALT, NALT in rodents) [3, 4]. Numerous folds increase the surface of the adenoid, which is covered by a viscous secretion that holds

immunologically active lymphocytes, immunoglobulins and other inflammatory mediators [5-7]. Antigen uptake to the lymphoid cells occurs in deep epithelial crypts, and the antigen stimulation enables the activation of B-cells in the germinal centers of the lymph follicles [8]. Lymphocytes derived from adenoids are shown to respond with higher antibody production towards bacteria derived from the nasopharynx compared to lymphocytes from palatine tonsils, blood and spleen stimulated by the same bacteria [9]. IgA and serum IgG can be produced locally in the adenoid. Serum IgG can protect against acute otitis media [10-12]. Locally produced secretory IgA is known to protect from colonization of the adenoid and it may thereby prevent development of acute otitis media [13, 14]. In addition, primed B cells can be seeded out through lymph and blood to secretory tissues like airway mucosa, e.g. in the middle ear, where they can differentiate to Ig-producing plasma cells [7]. As a consequence, local IgA production in the middle ear can be induced by nasal immunization [15].

As discussed above the adenoid has both immune induction and effector properties. The location of the adenoid in close vicinity to the Eustachian tube, and thereby also the tympanic cavity, enables mechanical, microbiological and immunological interactions between the nasopharynx and the middle ear.

Bacteriology of the upper respiratory tract and in ear infections

During healthy conditions in adults and adolescents cultures raised from the nasopharynx mainly show growth of normal respiratory flora and only a few pathogens. In contrast, the majority of young children are almost always colonized with potential pathogens in the nasopharynx (i.e. on the adenoid), without exhibiting signs of infection [16]. The adenoid develops during the first year of life and the colonization is also initiated during this period [17, 18]. It is poorly understood how the colonization equilibrium is maintained and how invasion of pathogens is prohibited.

The bacteria that most frequently colonize the nasopharynx in young children are *S. pneumoniae, H. influenzae, S. pyogenes* and *M. catarrhalis*. These species are also known to induce most bacterial complications of the upper respiratory tract.

S. pneumoniae is the most frequent bacteria in acute otitis media, followed by *H. influenzae and M. catarrhalis*. Several Nordic studies have shown that this order between different middle ear pathogens has been maintained during the last 40 years [19, 20]. *H. influenzae* has both encapsulated and non-encapsulated, or non-typable, subtypes. Vaccination has helped reduce the occurrence of the invasive subtype *encapsulated H. influenzae type B*, so *H. influenzae* is nowadays almost exclusively

non-typable H. influenzae (NTHI), which is considered non-invasive. A link has been demonstrated between bacterial strains harbored in the nasopharynx and strains found in middle ear during acute otitis media [21]. Further it has been shown that nasopharyngeal carriage of middle ear pathogens increases during upper respiratory tract infections and that the quantity of the middle ear pathogens in nasopharyngeal secretions increases during episodes of otitis media compared to healthy periods [17, 22].

Infections in chronic suppurative otitis media and other types of chronic middle ear disease exhibit other types of bacteria [23]. These bacteria are mainly derived from the skin flora, such as *S. aureus,* but Gram-negative species are also common of which *P. aeruginosa* is the most frequent.

Bacterial cultures from patients with ear infections during tympanostomy tube treatment tend to show airway derived (*S .pneumoniae, H. influenzae, M. catarrhalis*) bacteria in younger children and a bacterial spectrum resembling infections during chronic middle ear disease in older children and adults [24, 25] .

Middle ear disease

Acute otitis media

Acute otitis media (AOM) typically presents with a rapid onset of signs and symptoms such as otalgia, otorrhea (i.e. discharge from the ear canal) and/or fever. The diagnosis also requires the establishment of a middle ear effusion and other visible signs of middle ear inflammation such as bulging of the tympanic membrane, limited or absent mobility of the tympanic membrane or a visible air-fluid level behind the tympanic membrane [26, 27].

To be considered as an episode of AOM the course of infection should, in most cases, be relatively short. The symptoms can be expected to subside within a few days irrespective of whether antibiotic treatment is initiated or not [28]. There is a strong co-variation for the occurrence of AOM with viral infections of the upper respiratory tract [29-31] and most cases occur during the winter season [32]. The viral infection is considered to affect the host-parasite equilibrium of the colonized adenoid. As a consequence of viral infection and inflammation the Eustachian tube temporarily loses its protective function and reflux of bacteria to the tympanic cavity may occur. It has been demonstrated that bacterial invasion alone is often not enough to establish a middle ear infection. A combined viral and bacterial invasion enhances the development of AOM [33]. Most episodes of acute otitis media are of bacterial origin,

even if a minority may be caused by viruses alone [31].The risk of developing AOM increases from 6 months of age and most episodes are seen in children 12-24 months old [34]. A predisposing factor is that infants and toddlers under two years of age have a low ability to produce immunoglobulines due to an immature adaptive immune defence. The capacity for immunoglobulin production increases with age until adolescence [35].

Recurrent acute otitis media

There is a group of young children who are especially at risk for developing frequent episodes of AOM. In 1975 the term "otitis-prone condition" was coined by Howie et al [36]. It has been approximated that about 5% of all children are otitis- prone. Nowadays the term recurrent acute otitis media (rAOM) is more in use. The cause of this condition is multifactorial. It has been demonstrated that children with rAOM have lower levels of IgG_2 than healthy controls, and that there are specific deficiencies in their defense against certain subtypes of *S. pneumoniae* [10, 12]. They also exhibit lower antibody levels directed against the outer membrane protein P6 in *NTHI* [37]. In addition there is a strong genetic influence [38, 39].

Children with rAOM are often treated with insertion of plastic ventilation tubes in the tympanic membrane (also called tympanostomy tubes or grommets). Even though not fully evaluated this treatment seems to improve quality of life [40] and to some extent prevent future episodes of acute otitis media [41].

Otitis media with effusion

Otitis media with effusion (OME) is an inflammatory condition of the middle ear with effusion behind an intact tympanic membrane without acute signs or symptoms [42] . This condition is common during childhood and adolescence [43]. The main clinical characteristic is conductive hearing loss. The effusion dampens the movements of the tympanic membrane and the ossicular chain and causes impaired hearing. The pathogenesis of OME is not fully known but it often occurs after an episode of AOM [44].

Sometimes viable bacteria can be detected in the effusion, but in the majority of cases there is no bacteriological proof of an ongoing infection [45, 46]. OME is also associated with an increased pathogen load in the nasopharynx as compared to controls [47]. Recently evidence of biofilm formation in OME has been presented [48]. Eustachian tube dysfunction has been suggested to promote the development of

longstanding OME [49, 50]. In animal models OME can be evoked by injection of an endotoxin, e.g. lipopolysaccharide (LPS), into the tympanic cavity [51], or by plugging of the Eustachian tube [52]. A hypertrophic adenoid is considered to have a negative influence on the development of OME due to mechanical occlusion of the Eustachian tube orifice in the nasopharynx. It has been clearly demonstrated that removal of the adenoid can have a positive effect on the healing process in OME [53, 54]. Immunological interactions between the adenoid and the middle ear have also been discussed [55]. Longstanding OME with hearing impairment can be treated with tympanostomy tubes or adenoidectomy or both even if the spontaneous resolution over time is high [56].

The role of virus in the pathogenesis of otitis media with effusion is poorly understood. There are data showing a co-variation with viral upper respiratory tract infections [29] and occasional presence of viruses in the middle ear effusions [57].

Chronic middle ear disease

The nomenclature on this subject is often confusing, and there is a lack of consensus in the literature. We have chosen to use the term chronic middle ear disease (CMED) to designate a large group of patients who suffer from continuous ailments of the middle ear. There are several subtypes of CMED. Two of the most frequent are chronic suppurative otitis media and retraction type middle ear disease. The latter can appear with or without cholesteatoma.

Chronic suppurative otitis media is a condition with a chronic perforation of the tympanic membrane together with conductive hearing loss and persistent or recurrent bacterial infection. The perforation can be traumatic or originate from a necrotizing infection. Infection of the middle ear changes the structure of the tympanic membrane and may thereby impair healing of the tympanic membrane [58].

Retraction type middle ear disease designates the formation of retraction pockets and cholesteatoma in the tympanic membrane. Episodes of AOM and longstanding OME change the structure of the tympanic membrane which thereby is predisposed to formation of retraction pockets [52, 58] . Defective function of the Eustachian tube and a negative air pressure in the tympanic cavity are also linked to this disease [59-61]. If the negative pressure fails to equalize the process continues and a cholesteatoma can be formed from the retraction pocket. A cholesteatoma can be described as a cyst lined with epithelial cells that continuously produce keratine. This leads to growth of the cholesteatoma. Inflammatory activity in close vicinity to the cholesteatoma induces the

bone resorption that is a characteristic of this disease [62]. Cholesteatoma can destruct the ossicular chain and temporal bone, in worst case causing complications from the inner ear or even intracranial complications.

Innate immunity

Mechanical and chemical barriers are part of the innate immune system and constitute the first line of defence against invading microbes. An example of this is the combined actions of the mucus layer and the ciliated airway epithelium in the Eustachian tube that protect the tympanic cavity against virus and bacteria. When these barriers fail the innate immune system has a second line of host defence that can act rapidly against microbial threats. These actions are carried out by an evolutionary conserved system of cellular and molecular actors with the ability to directly identify microbes and induce inflammation, which leads to rapid elimination of the invading bacteria or virus. Dendritic cells, macrophages, natural killer cells, granulocytes and epithelial cells are examples of cellular elements included in the innate immune defence. These cell-types can either directly kill invading microbes or produce cytokines which aid elimination of the pathogen [63]. Cells of the innate immune defence carry pattern-recognition receptors (PRRs) that recognise conserved microbial structures called pathogen-associated molecular patterns (PAMPs). Examples of PRRs are the Toll-like receptors (TLRs) and the Nod-like receptors (NLRs). The innate immune system is faster than the adaptive immune defence in activating immune reactions. The innate immune system does not hold an immunologic memory, which is a distinctive feature of the adaptive immune defence. Activation of the innate immune response is also a prerequisite for the onset of adaptive immunity [64].

Cytokines and cell surface markers

Cytokines are small proteins which act as messenger molecules in both adaptive and innate immune responses. They are known to regulate the inflammatory response. Pro-inflammatory cytokines are produced in response to inflammatory danger signals, e.g. presence of bacteria and virus in the tissue. Two important pro-inflammatory cytokines are IL-1β and TNF-α [65, 66]. They are mainly produced in macrophages and their production is linked to the NF-κB pathway [67]. IL-1β can also be produced by epithelial cells and keratinocytes, whereas TNF-α can be produced by activated T lymphocytes. Both cytokines can induce local (e.g. increased blood flow and erythema)

as well as systemical (e.g. fever and muscular pain) inflammatory reactions. Their cellular effects are diverse. In order to facilitate the killing of various microbes they can induce the expression of adhesion molecules and chemotactic factors and also activate macrophages so that intracellular killing of microbes is enhanced. Another action is induction of apoptosis in certain tumour cells. In the present studies, our interest for IL-1β and TNF-α was mainly based on their role in the induction of iNOS in endothelial cells, which subsequently leads to increased production of NO [67-69]. Another central cytokine is IFN-γ which is mainly produced by T-cells and has immune regulatory functions, e.g. activation of macrophages and maturation of cytotoxic T cells [67]. Lymphocytes carry cell surface markers which reflect specific cell characteristics. They have a nomenclature with the prefix CD (cluster of differentiation) followed by a number [70]. These markers can be identified by antibodies. This makes it possible to use immunohistochemistry to differ between lymphocyte subtypes. We have in this thesis surveyed two types of lymphocytes; CD3 positive (mature T-cells) and CD68 positive (macrophages).

Toll-like receptors (TLRs)

During the last decade Toll-like receptors (TLRs) have been subject to intense research. To date there are 10 TLRs (TLR1-TLR10) described in humans [71, 72]. They are all glycoproteins integrated in cell membranes. TLRs have leucine-rich repeat (LRR) motifs in the recognition domain and a Toll IL-1 receptor (TIR) in the cytoplasmic signalling domain [73]. Upon stimulation the TLRs recruit TIR-domain-containing adaptors to the TIR-domain. This activates an intracellular signalling pathway resulting in the induction of inflammatory cytokines. The two most important pathways are the MyD88-dependent and the TRIF-dependent pathways [63]. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are located at the cellular surface, whereas TLR3, TLR7, TLR8 and TLR9 are found intracellularly in endosomal compartments [74]. TLRs are expressed on different immune cells like B cells, macrophages and granulocytes. In addition they are expressed on endothelial and epithelial cells [75, 76]. TLRs identify different pathogen-associated molecular patterns (PAMPs) [77]. TLR1, TLR2 and TLR6 recognize a diverse range of PAMPs of which the some examples are glycolipids and peptidoglycan from *S. aureus*, *S. Pneumoniae* and *S. Pyogenes* [63]. TLR4 mainly reacts to LPS from Gram-negative bacteria. TLR5 recognizes bacterial flagellin. TLR3 is directed against viral double stranded RNA. TLR7, TLR8 and TLR9 can sense microbial nucleic acids [63]. Deficiencies in TLR4 functions have been

demonstrated to predispose for middle ear disease in both experimental models and in humans [78-80].

Nod-like receptors (NLRs)

The NLRs are intracellular PRRs located in the cytosol where they sense bacterial proteins and danger signals. To date 23 NLRs have been discovered. The NLRs have a tripartite structure; C-terminal LRR for recognition of agonists, a central Naip, CIITA, HET-E and TP-1 containing domain called NACHT and a N-terminal responsible for signalling. There are three types of N-terminals: a caspase recruitment domain (CARD), a pyrin domain (PYD) and a baculovirus inhibitor of apoptosis protein repeat domain (BIR). Based on the nature of the N-terminal the NLRs have been divided into subfamilies; CARD-containing NODs, NACHT domain-, LRR- and PYD domain containing proteins (NALPs) and BIR containing neuronal apoptosis inhibitor proteins (NAIPs) [81]. NLRs are expressed in cells and tissues that have a role in immunity, of which granulocytes are one [82]. Nod1, Nod2 and Nalp3 are also found in epithelial cells [83] for example in nasal airway epithelium [84]. Only a few NLR ligands are known so far and most of the NLR pathways still have to be described. Nod1 and Nod2 are the most studied NLRs and they react to bacterial peptidoglycans, which are degradation products of bacterial cell wall components released by intracellular or phagocytosed bacteria. They can in this way initiate innate responses through the NF-κB pathway. Mutations in Nod1 and Nod2 have been linked to inflammatory disorders like sarchoidosis, asthma and inflammatory bowel disease [81, 85]. Some NLRs, e.g. Nalp3, can react upon danger signals by forming cytoplasmic multi-protein complexes called inflammasomes. These complexes activate caspase-1, an event that in turn leads to the proteolytic activation of pro-inflammatory cytokines like IL-1β [81, 83]. In addition, Nalp3 was recently shown to be activated by the adjuvant aluminium hydroxide [86] and recent findings also indicates a role in allergy [84]. Mutations in NALPs have been linked to auto-immune disorders, possibly as a result of aberrant IL-1β regulation [81, 83].

Nitric oxide (NO)

NO is a colourless gas radical that is reactive due to its chemistry with an unpaired electron. NO has been shown to be a mediator produced in a number of tissues, with local effects in many organs, including the airways, and it exerts a number of diverse effects throughout the human body [87]. NO is involved in regulation of blood flow, platelet function, neurotransmission, immunity, bone resorption and inflammation [88-91]. In 1991 NO in exhaled air was discovered in an experimental model [92]. Locally produced NO has been sampled from both the upper and the lower human airways, and the paranasal sinuses have been implied as a major site for its production [93]. NO is a small molecule that readily diffuses through cell membranes in order to perform its actions. This means that NO cannot be stored in tissues, but has to be produced de novo when need arises. It has a very short half-life in tissues (seconds) and is rapidly oxidized into nitrate and nitrite by oxygen and water. In gas phase it is much more stable, enabling measurements to be performed.

The mucociliary system is dependent on NO for its function [94], and decreased NO levels are reflected in a low beat frequency of the airway cilia [95]. NO can also exert direct effects on cells, e.g. neutrophils and macrophages, in the immune system [88]. It also exhibits bactericidal, fungicidal and antiviral effects, thereby helping the immune system in fending off invading micro-organisms [96].

1.2 OBJECTIVES

The overall aim of this thesis was to examine immunological processes in the human adenoid and in the middle ear mucosa in relation to different types of otitis media, focusing on local defense mechanisms. In addition, different treatment strategies in tympanostomy tube associated otorrhea were evaluated.

Specific aims were to:

- Establish if there is a clinically significant difference in the outcome between topical treatment combined with systemic antibiotics and topical treatment alone for tube associated otorrhea in young children with rAOM. A secondary aim was to determine if the otorrhea reflected new episodes of acute otitis media.

- Analyze differences in the expression of TLR4 and TLR7 in adenoids from children with OME and healthy controls.

- Compare the expression of eNOS and iNOS in adenoids from children with OME with the expression found in hypertrophic adenoids from children without middle ear disease.

- To map and quantify cytokines in different compartments of the adenoid with special emphasis on their potential role in the iNOS activation pathways.

- To study the presence of TLRs and NLRs of the human middle ear mucosa and explore if their expression rates are affected by chronic middle ear disease.

- Investigate the prerequisites for local nitric oxide production in the human middle ear during health and disease.

1.3 METHODS

All projects were approved by the Ethical Review Board in Stockholm and an informed consent was obtained from the patients or from their parents.

Study outlines and sampling procedures

Paper I is a prospective randomized study comparing topical treatment and systemic antibiotics to topical treatment alone in cases of tube associated otorrhea. In addition the bacteriology of the ear discharge was evaluated. Fifty children under three years of age were divided into two treatment groups by a computerized randomization process, including stratification by gender to exclude possible gender-related differences in treatment outcome. All children were included at the date of surgery with insertion of tympanostomy tubes in both ears due to rAOM. At least three episodes before 12 months of age or six episodes before 18 months of age were required in order to meet the inclusion criteria. Inclusion was done during the winter season when the risk of acute otitis media is increased.

Papers II and III are primarily based on adenoids obtained from 26 children 2-13 years old. Twelve children suffered from significant hearing loss (confirmed by audiometry) due to otitis media with effusion. The remaining14 children had no signs or symptoms of middle ear disease or hearing impairment, but were subject to adenoidectomy due to adenoid hypertrophy.
The adenoid tissue was taken out using standard surgical procedure , snap frozen and stored in -80°C until use. Prior to the surgical procedure nasopharyngeal secretions were collected by careful suction. A bacteriological swab directly from the surface of the adenoid was taken. Imprints of surface cells were collected by gently pressing a sterile plastic foam swab against the adenoid surface and then printing the swab to adhesion glass slides.

Paper IV and V are in part built on analysis of mucosal biopsies from the middle ear cleft (middle ear cavity or mastoid cell system) from patients (1-82 years old) undergoing middle ear surgery. The specimens were immediately placed in a RNA-stabilization reagent (RNA-later,Qiagen) and stored in -80°C until use. Forty-eight patients suffered from different types of chronic middle ear disease and 45 underwent cochlear implant surgery and had healthy middle ears. The limited amount of mRNA that could be derived from the middle ear mucosa made it necessary to pool the material for quantitative analysis. It is therefore not possible to link specific phenotypic disease information with individual profiles. Specimens obtained separately were used for immunohistochemical analysis.

In paper V the presence of NO in middle ear gas samples was assessed in 11 patients between 18-56 years of age. Patients with a perforation due to chronic middle ear disease and patients with a tympanostomy tube inserted as a result of either chronic middle ear disease or Menières disease were included in this study. The outer ear canal was blocked for 5 minutes after which the gas was obtained by slow aspiration with a microsurgical suction tube connected to a 5 ml syringe. The syringe was sealed immediately and analysed at the lab within 20 minutes. Control samples were aspirated close to the intact tympanic membrane of the contra lateral ear, using the same procedures.

Clinical monitoring and data collection (Paper I)

Each patient was monitored for six months after receiving tympanostomy tubes. During this period patients were seen by the research team in case of otorrhea or other symptoms of ear infection. All patients regardless of treatment group had topical treatment in case of symptoms with otorrhea. The topical treatment consisted of ear drops containing a combination of topical antibiotics and a mild steroid (oxitetracykline + polymyxine B + hydrocortisone, Terracortril with Polymyxine B®), and a simple rinsing procedure with sterile saline solution. The rinsing procedure was designed to clear the outer ear canal from discharge that could interfere with the application of the ear drops. In addition, patients in the systemical antibiotics group had orally administrated amoxicillin, or an equivalent drug in case of penicillin allergy. The parents kept a diary on signs and symptoms of infection and a bacterial swab was taken from the ear discharge in case of otorrhea early in the course of the episode. Data for the analysis were obtained from the patients medical records, from the diary kept by the parents and from the bacteriological results.

Bacteriological cultures (Paper I and II)

Routine bacteriological cultures were performed at the Department of Clinical Microbiology at Karolinska University Laboratory. The samples were inoculated within 3 hours on horse blood, sheep blood or hematin agar plates. All plates were incubated aerobically (hematin and sheep blood plates with 5% CO_2) overnight at 37°C. *S. pneumoniae* was identified by its typical colony morphology and optochin sensitivity. The requirement of X (haemin) and V (nicotinamide-adenine dinucleotide) factor for growth were used to determine the presence of *H. influenzae*. *M. catarrhalis* was diagnosed by the typical morphology of its colonies, gram staining and a positive oxidase reaction.

Immunohistochemistry (Paper II - IV)

Immunohistochemistry is a staining technique that utilizes monoclonal or polycolonal antibodies (Abs) for the visualization of specific proteins in cells and tissue. Expression of pro-inflammatory cytokines in adenoid tissue and imprints as well as TLRs, NLRs, and NOSs in adenoid tissue and middle ear mucosa were assessed with immunohistochemistry. Specimens were fixed in formaldehyde, sectioned (3-8μm) and mounted on glass slides. Blocking agents were applied to the slides to prevent endogenous enzyme activity that could obscure the staining. Cell membrane permeability was increased with the use of saponin or Triton X. Antibodies directed against the protein to be assessed were applied to the slides. The antibodies were either monoclonal or polyclonal. In these papers monoclonal antibodies were used for the assessment of cytokines and cell surface markers, and polyclonal antibodies for pattern recognition receptors and nitric oxide synthases. Non-specific binding of the antibodies was blocked by incubating the slides with a serum (goat or calf). HRP-labelled or biotinylated secondary antibodies directed against the animal species used for raising the primary andibodies (mouse, rat or rabbit) were applied. Avidin peroxidase was added to the slides with biotinylated secondary antibodies, but for specimens with HRP-labelled antibodies this step could be excluded. A staining could thereafter be visualised by the use of 3,3´-diaminobenzidine. Some slides were also counter stained with haematoxylin. A positive staining in cells or tissue was seen as brown colour. Light field microscopy was used to evaluate and analyse the stainings. Visual scoring was performed by counting the amount of positive cells per 1000 cells (ICC results). In some analysis when positive cells were scarce a semiquantitative scale was used (+, ++, +++) and related to the size of the biopsy. For IL-1β in tissue, computerized image analysis was used with a Quantimet 550 IW image analyzer (Leica). With this technology positive and negative tissue areas were defined, and then the biopsy was scanned with camera and microscope connected to the computer equipment. This process results in a computer calculated value of the percentage positively stained area relative the total surveyed cell area of the biopsy.

Luminex (Paper III)

A Luminex100^{TM} **(**Luminex, Austin, TX**)** analyzer with Fluorokine MAP assay (R&D systems, Minneapolis, MN) was used to analyse cytokines in secretions from adenoids and middle ear effusions. The principle of the assay is that antibodies directed against the protein you wish to analyse are attached to micro beads which contain a colour. The colour is specific for the desired analyte. The beads are allowed to bind to the analyte (e.g. cytokines), followed by addition of biotinylated detection antibodies that are specific to the analyte of interest and streptavidin conjugated to the fluorophore phycoerythrin (PE). Washing and resuspension procedure is performed in between. The solution with the labelled Abs attached to the analyte is quantified in the Luminex machine by two different lasers. One of the lasers detects the bead colour and determines the analyte that is being detected, whereas the second laser identifies the magnitude of the PE signal and consequently the amount of bound analyte. The concentration of each analyte can be derived from the standard curve that is computer calculated by a statistical method. Luminex technology allows for simultaneous analysis of several proteins in small samples.

Real-time polymerase chain reaction (Paper II - V)

Quantitative real-time PCR is a method that monitors amplification of DNA instantly during the amplification process. Before quantitative real-time PCR is performed RNA is extracted from the samples. This is done with RNeasy mini kit (Qiagen). The samples are initially stored in RNA-later (Qiagen). They are placed in RLT lysis buffer (Qiagen) and homogenized. RNA extraction is performed by adding a high salt buffer and allowing purified RNA to bind to a membrane after which it is eluted in water. RNA concentration and purity are measured by spectrophotometry. RNA is then converted to cDNA using a reverse transcriptase kit with oligo-dT primer.
The real-time PCR process is rendered possible by use of a cyclic heating and cooling procedure. High temperatures (95°C) are used to dissociate dsDNA into two single strands. The mixture is cooled in the presence of sequence-specific oligonucleotide primers representing the DNA you wish to quantify. The primers attach to the correct site of the now single stranded template DNA and they can now direct the polymerase to elongation and completion of a new DNA strand, dsDNA. The dsDNA is then separated by reheating and the reaction starts over. The process is repeated for a set number of cycles. The fluorescence is proportional to the amount of product formed.

The number of amplification cycles required to obtain a particular amount of DNA molecules is registered as the cycle threshold value (Ct).
Assuming certain amplification efficiency (typically a doubling of the number of molecules per cycle) it is possible to calculate the number of DNA molecules that were initially present in the sample. This enables quantification of the starting amount of RNA in cell and tissue samples. The quantification process is based on measurements of the emitted fluorescence from the applied dyes (*Figure 1* and *2*). The results reflect gene expression rates. Several proteins can be assessed simultaneously, and the method can manage very small sample sizes. The fluorescent reactions used in this thesis are of two types. In paper V we used a fluorescent dye (SYBR green) that binds to dsDNA (the amplification product) and fluorescence is emitted when the association to dsDNA occurs (figure 1). Melting curve analysis was used to determine the specificity of the primers. The primers used in this assay were custom made and unlabeled. In paper II-IV we used ready-made unlabeled primers together with a reporter probe labelled with fluorophore .The probe also has a quencher fluorophore attached, that absorbes the excitation energy from the reporter fluorophore. It can for instance be a second fluorescent dye. During PCR amplification the polymerase (Taq) cleaves the probe. The reporter is liberated from the quencher and thereby measurable fluorescence from the reporter probe is emitted (*Figure 2*).The gene expression of the analyte is assayed using the comparative cycle threshold (Ct) method where the relative amount of the analyte mRNA is determined by subtracting the Ct value for the gene of interest with the Ct value for the housekeeping gene β-actin (Δ Ct). Data are expressed in relation to 100,000 molecules of β-actin as $100{,}000 \times 2^{-\Delta Ct}$.

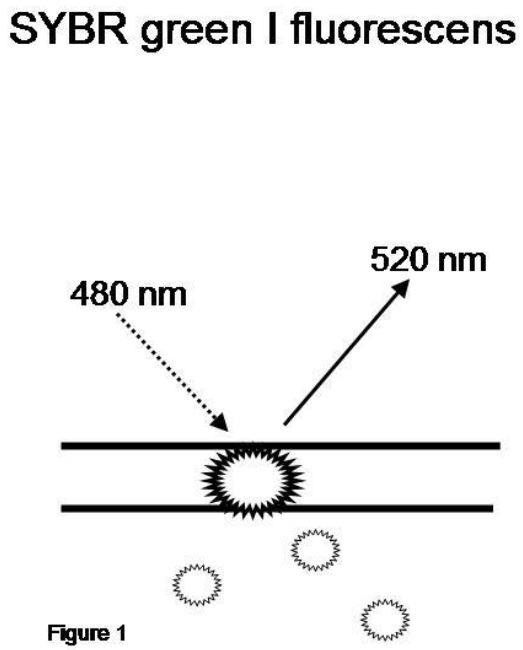

Figure 1 This picture shows in a schematic way how fluorescence is emitted when the double-stranded DNA is completed in the SYBR I green fluorescence reaction

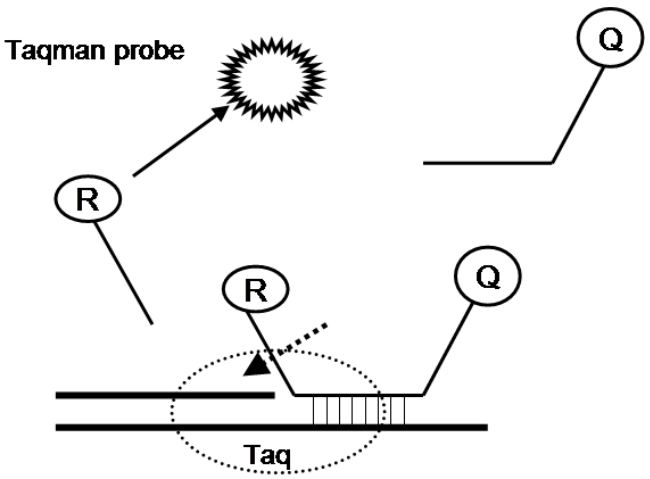

Figure 2 A schematic picture of the Taqman polymerase reaction. When the double-stranded DNA is completed the Taq polymerase cleaves the binding to the reporter probe. The liberation of the reporter from the quencher results in the emission of measurable fluorescence.

Nitric oxide measurements (Paper V)

NO in middle ear gas samples were measured by chemiluminescence. This method is based on the reaction of NO with ozone to produce excited-state nitrogen dioxide. When it returns to the normal state light is emitted (about 600 nm wavelength). The light reaction can be detected and enables measurements of the NO concentration in gas mixtures. Gas phase chemiluminescense detection has both high specificity and sensitivity. NO concentrations as low as parts per billion (ppb) can be measured by this method. To detect background contamination room air was sampled and NO measured. If concentrations of NO in room air exceeded 5 ppb the measurements were omitted.

Statistical methods

In paper I statistical analysis was performed by a co-author at the Department of Learning, Informatics, Management and Ethics at Karolinska Institutet. Log rank test was used along with a multivariate analysis (Statistica software) and a power analysis for the log rank test (nQuery 4.0 software).

In paper II-V the non-parametric Mann-Whitney test was used along with Bonferroni corrections when applicable. For the paired observations (paper V) Wilcoxons test for non-parametric data was used. All calculations were performed using commercially available software (GraphPad Prism 4.0).

1.4 RESULTS AND COMMENTS

Tube-associated otorrhea reflects new episodes of acute otitis media and can be treated with topical agents (paper I)

The main purpose of the study was to evaluate two common treatment strategies for tube associated otorrhea in children with recurrent acute otitis media.

Results

Fifty children were included in the study. During the six month long study period 36 children (76%) exhibited one or more episodes of otorrhea. In total, 50 episodes were registered of which 41 were treated according to the protocol. The analysis was performed both according to intention to treat and per protocol. In the per protocol analysis only episodes that were treated according to the allocated treatment protocol were included. In the intention to treat analysis all episodes were referred to their original treatment group irrespective of the actual given treatment. Only the first episode of each child was included in the statistical calculations. The reason for this was that some children had repeated episodes of otorrhea, and by adding these repetitive episodes you could obscure the treatment outcome data due to the proneness to ear infection in these particular children. A majority (88%) of the episodes healed within seven days irrespective of the chosen treatment. In the topical treatment group the median duration of otorrhea was somewhat longer (4.5 days) as compared to the median duration in the group that was treated with systemic antibiotics (3.5 days). However, this difference was not statistically significant. The results were the same both when analysis was performed per protocol or by intention to treat. In the topical treatment group the protocol stated that systemic antibiotics should be added if the child's general condition deteriorated with fever > 38.5°C, severe pain and general malaise. This was done in seven episodes in four children.

Thirty-five bacteriological cultures from 27 children showed an overall dominance of airway derived bacteria, *H. influenzae* being the most common species (found in 17 cultures) followed by *S. pneumoniae* (in 13 cultures). In eight cultures more than one pathogenic bacteria species was found, whereas five cultures were negative. In two cases *S. aureus* was identified together with *H. influenzae*. There were no findings of *P. aeruginosa* in otorrhea cultures during the study period.

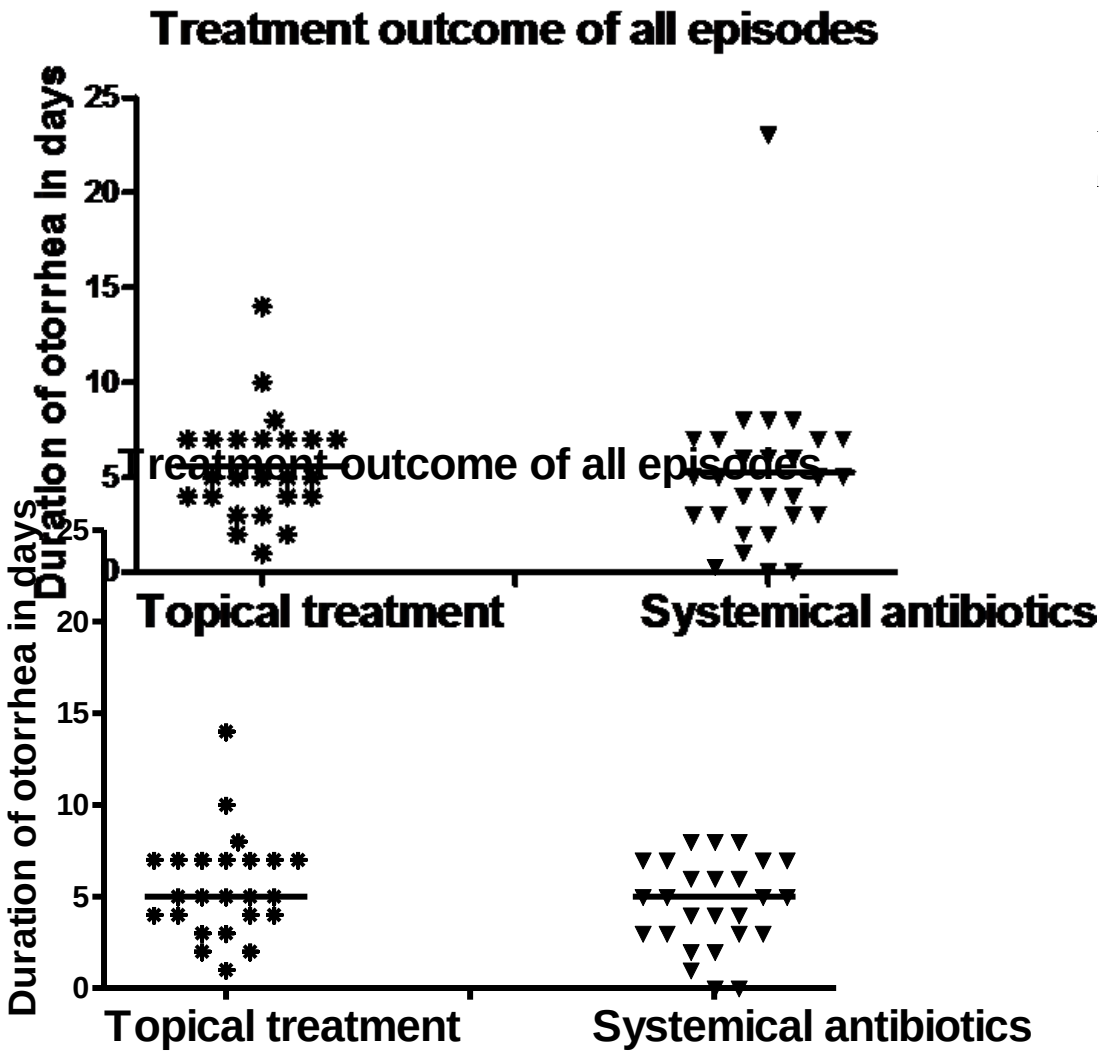

Figure 3 Topical treatment alone (n=24) was compared to topical treatment and systemic antibiotics (n=26) in children with recurrent acute otitis media in a prospective randomized study. The primary outcome variable was duration of otorrhea measured in days. Each child was monitored for six months. The picture is a plot of the duration of all the recorded episodes in each group. The horistontal line is the group median.

* represents episodes in the group with topical treatment alone and ▼ the group with topical and systemical antibiotic treatment .

Comments

The addition of systemic antibiotics to the topical treatment did not improve the patient's outcome. From a clinical point of view this is not surprising. Even though there is no documentation on the frequency of different treatment regimes for tube-associated otorrhea, the use of topical agents is the most common way to treat this ailment. Many clinicians also use a watch-and-wait policy with no treatment at all, although the knowledge on the natural course of untreated tube otorrhea is limited.

In consistence with previous reports [25, 97] we predominantly found airway derived bacteria. We did not find any treatment related differences in the outcome, but when considering the occurrence of these bacteria systemic treatment might have a theoretical advantage over topical treatment. This has also been suggested in a previous study [98]. The frequency of otorrhea episodes after tube placement was close to the results in a previous study with similar inclusion criteria [99] so we concluded that our study group and design rendered a representative sampling. The results support the notion that otorrhea in young children with tubes due to rAOM represents new episodes of AOM, rather than otorrhea due to external contamination from the outer ear canal.

The patients in our study were infants under 3 years of age. At the time of the study systemic antibiotics were recommended to children under two years of age in cases of AOM [26]. Nonetheless, our data indicate that it would be safe to treat acute tube associated othorrhea with topical agents alone. This assumption seems not to be affected by the fact that the otorrhea probably represents "regular" AOM episodes. In fact, the similar treatment outcome could reflect that systemic antibiotics had no effect, even though the study design does only allow us to speculate on this.

The lack of difference between the study groups could in theory also be a result of both treatments being ineffective and that we in fact observed the natural course of otorrhea. There are however previous observations that contradict this.

In our study the overall duration of the otorrhea episodes was shorter compared to previous reports [100]. The duration of otorrhea in the topical treatment group of the present study, was also shorter than the duration for the placebo group in another study on systemic treatment of tube associated otorrhea [98]. This can be related to the protective effect of topical treatment, since local treatment prevents external contamination and formation of granulation tissue. The absence of *P. aeruginosa* and low frequency of *S. aureus* support such an assumption since these bacterial species are considered to represent infections primary derived from the skin. Both groups had the same instructions regarding topical treatment, including the washing procedure with sterile saline solution. Ear toilet with different rinsing procedures is sometimes advocated as an effective treatment for ear discharge in an array of middle ear conditions including tube treatment.

Data from studies on external otitis have shown that topical steroids without topical antibiotics are effective [101, 102]. It would therefore be logical that the combined action of topical antibiotics and a mild steroid in the eardrops we used exercised the major effect. We do not know if the ear drops entered the tympanic cavity.

Experimental studies have shown that a pre-existing ear discharge might facilitate the leakage of topical solutions from the outer ear canal to the middle ear via a tympanostomy tube [103]. Since the ear drops always were applied during otorrhea in the present study, it can not be ruled out that a small amount of topical antibiotics may have entered the middle ear and thereby exerted a direct effect on middle ear pathogens.

We do believe that the rinsing procedure was an important step in our goal to achieve an effective topical treatment. One could speculate on the importance of giving topical treatment (eardrops or rinsing with saline solution or both) at an early stage as it was done in the present study. Topical treatment could prohibit contamination from skin bacteria to emerge to a clinical infection and possible biofilm formation that could result in more longstanding and complicated otorrhea episodes.

Increased TLR7 expression and reduced iNOS activity in adenoids from children with otitis media with effusion (papers II + III)

These studies were undertaken in order to evaluate a possible relation between the appearance of OME and the immunological activity in the adenoid, focusing on NO activity, two selected TLRs and various cytokines

Results

Two groups of children were studied: one group of 12 children with OME and one control group with 14 participants. The group characteristics are shown in *table 1*.
Real-time PCR analysis was used to assess the mRNA expression levels of TLR4, TLR7, eNOS and iNOS in adenoid tissue samples. The corresponding protein expressions were confirmed using immunohistochemistry. Children with OME exhibited higher levels of TLR7 and lower values of iNOS as compared to the controls. No such difference was seen for TLR4 or eNOS. The amount of remaining material did however not allow for a visual quantification of the TLRs or the NOSs in all tissue samples.
There was no obvious difference in the bacterial colonization between the study groups. Both were dominated by *H. influenzae* followed by *S. pneumoniae* (*Table 1*).
Pro-inflammatory cytokines were assessed in nasopharyngeal secretion, imprints and tissue sections. Our analysis focused on IL-1β and TNF-α, due to their well known relation to iNOS. The levels of IL-1β and TNF-α in secretion from the adenoid surface were assessed by a Luminex assay and did not differ between the two study groups. Scoring of IL-1β, TNF-α and IFN-γ in imprints was performed by counting amount of positive cells out of 1000 cells totally counted. These results are displayed in *Figure 4*.
The out-come of the cell imprint analysis indicated that the spread of results in the control group was more marked than in the otitis group. Some children exhibited high scores on all analyzed cytokines, but these cases could not be correlated to the bacteriological outcome, age or hearing levels. Cells positive for the lymphocyte cell surface markers CD3 and CD68 were also quantified in imprints. CD3 rates were relatively high and CD68 counts were generally low, but no differences were seen between the groups (*Figure 4)*.
IL-1β in tissue sections was quantified by computerized image analysis. IL-1β positive cells were mainly found around areas containing epithelium, near the adenoid surface

and intersected crypts. A tendency towards a higher expression in the OME group was seen, but it did not reach significance. The expression of TNF-α and IFN-γ was sparse compared to IL-1β. Positive cells were also found in all tissue compartments in a more random pattern as compared to IL-1β.

A semi quantitative (+, ++, +++) overview of tissue sections regarding these two cytokines did not indicate any difference between the two study groups.

Photographs of stained cells in imprints and tissue are shown in *figure 5*. Some individuals reached much higher scores than the mean in the group, and the majority of those were in the control group. It was in some cases a co-variation so that one child had high scores in all the assessed cytokines/surface markers, but not in all cases. The two groups seemed to have different characteristics but there was no statistically significant difference. We did not find any apparent correlation of cytokine levels to the bacteriological outcome, age or hearing levels.

Table 1

	Controls (n=14)	**OME (n=12)**
Gender (boy/girl)	9/5	11/1
Age range (months)	22-111	23-159
Age mean/median	51/43.5	62/59.5
Hearing level mean/median (dB)		31/34
Negative culture	1	2
H.influenzae	10	10
S.pneumoniae	4	6
M.catarrhalis	1	5
S.pyogenes	2	2
1 species in culture	6	5
2 species in culture	4	3
3 species in culture	1	4

Cytokines in imprints

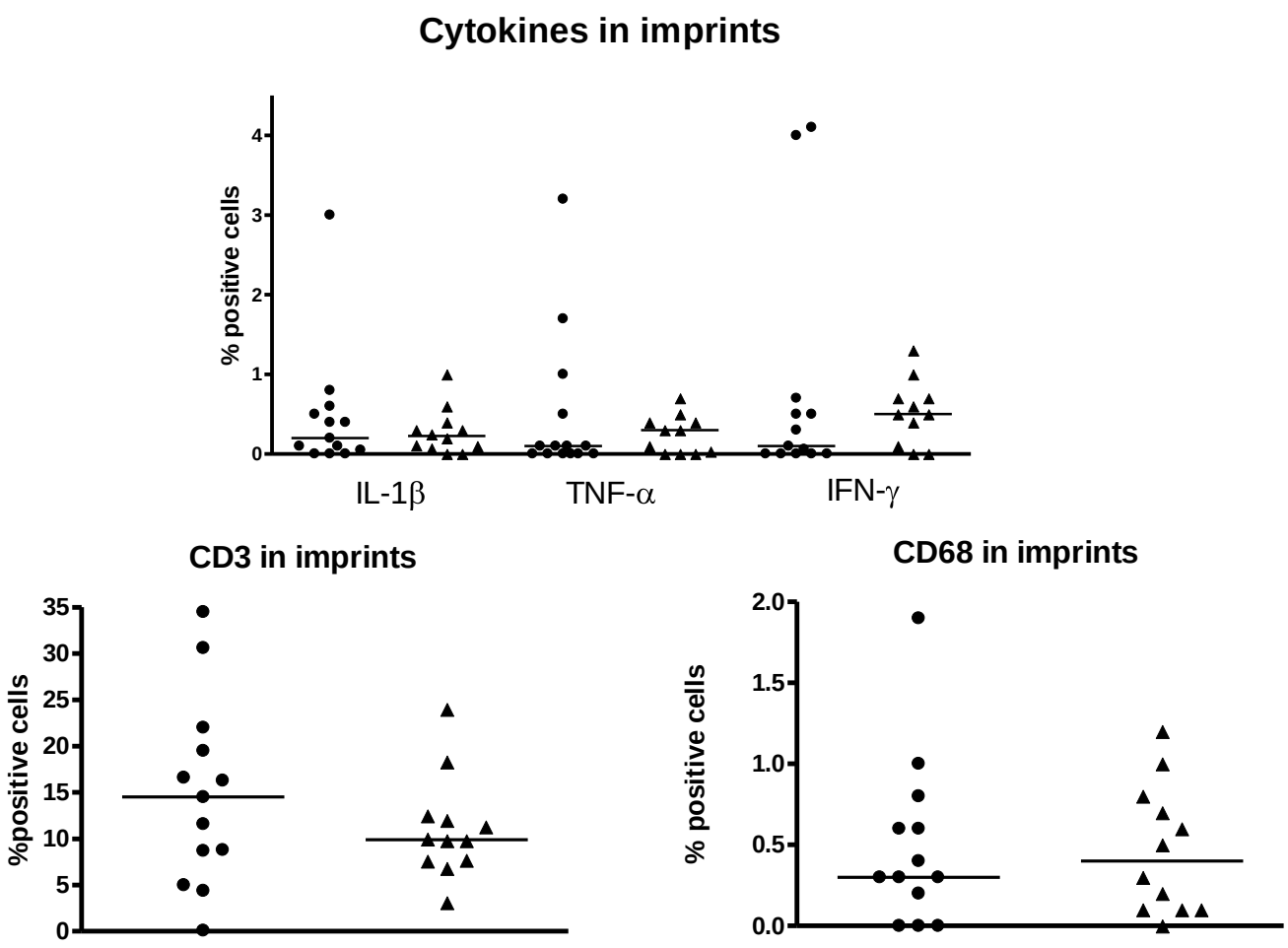

Figure 4 Imprints from the adenoid surface of children with otitis media with effusion (n=12) (▲) and controls (n=14) (●) with healthy middle ears were collected. Assessment of the immunoactivity was performed by immunohistochemistry. Visual scoring was performed during regular light field microscopy by counting the frequency of positively stained cells (positive cells/1000 counted cells) for the cytokines IL-1β, TNF-α, IFN-γ, and the cell surface markers CD3 and CD 68. The horizontal line represents the group median.

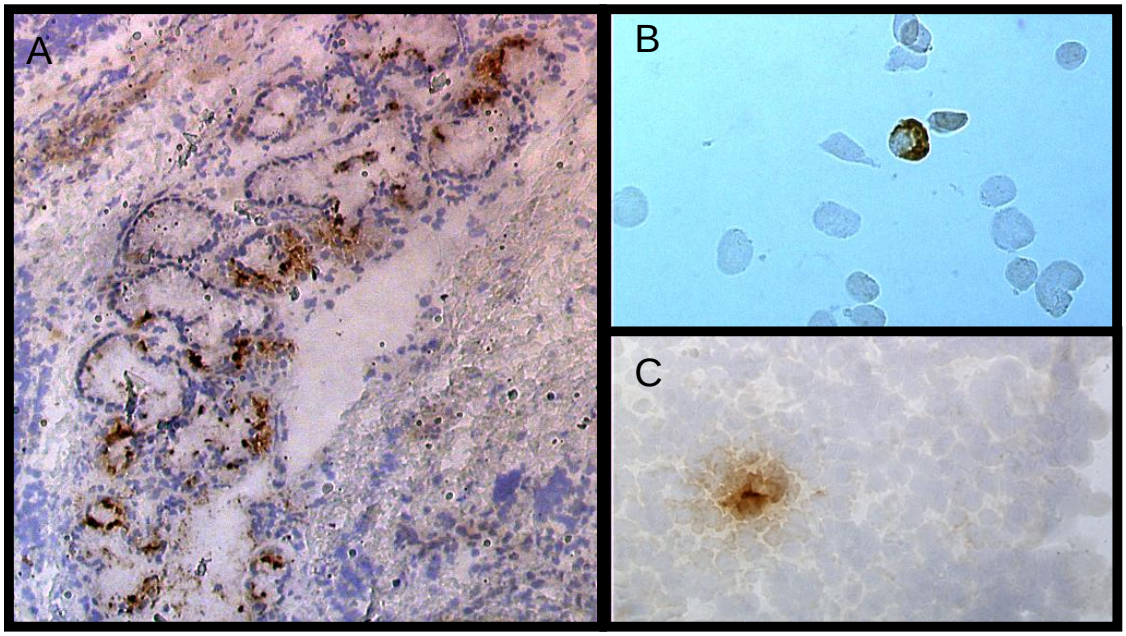

Figure 5 Positively stained cells from the assessment of cytokines by immunohistochemistry. A) IL-1β positive cells adjacent to the epithelia of an intersected crypt. B) a cell positive for TNFα in an imprint slide and C) a cell positive for IFN-γ in an imprint slide Magnification A)25X and B+C 63X.

Comments

When looking at the results of the TLR-assessment it is interesting to note that the bacteriological cultures did not differ between the groups. TLR-responses against *H.influenzae* and *S.pneumoniae* are commonly linked to TLR4, so the similar levels of TLR4 mRNA expression between the groups was to be expected given the similarities in bacterial colonization. TLR7, on the other hand, responds to viral infection. Unfortunately, viral mRNA in adenoid tissue could not be assessed in this study. The difference in TLR7 mRNA expression rates that was revealed makes it tempting to speculate on a possible viral influence in the development of OME.

The expression of iNOS mRNA was lower in adenoids obtained from children with OME. This could reflect a defect function in the induction of iNOS and subsequently impaired local production of nitric oxide (NO). We did not find any difference in the expression of IL-1β and TNF-α, cytokines known to activate iNOS.

NO is known to have anti microbial effects, and is also involved in regulation of blood flow and other important functions involved in inflammation [104-106]. Children with an inability to initiate iNOS expression properly could therefore suffer from an

inappropriate local inflammatory response and a poor defense against microbial agents derived from the upper airways. A defective local NO production in the nasopharyngeal space might also be involved in the pathogenesis of OME, possibly along with viral infection as mentioned above.

The similar outcome regarding cytokine levels between the two study groups raises the question whether the observed down-regulation of iNOS in OME occurs on some other level than cytokine induction. Cytokines are released during a brief period after which there is a peak in cytokine expression. It is possible that we in this study did not capture these peak events. The pattern of cytokine release in peaks could also explain the outlier rates of cytokine expression that a few patients exhibited, mainly in the control group (*figure 4*).

A previous study of cytokines in nasopharyngeal secretions by Lindberg et al [107] demonstrated low levels of IL-1β in children with rAOM. With reference to those results the similarities in cytokine levels between the two groups in the present study indicate that the inflammatory response in OME differs from the one found in children with rAOM.

The clear differences found regarding TLR7 and iNOS imply that adenoid tissue in children with otitis media with effusion have an altered innate immune response compared to adenoid tissue in children with healthy middle ears. These findings further support the theory that there is a connection between middle ear disease and the adenoid. This connection is not merely of a mechanical type with a large adenoid obstructing the Eustachian tube, but also of an immunological nature.

A deranged expression of TLR4, TLR5, TLR7, Nod2 Nalp3 and eNOS is found in conjunction with chronic middle ear disease (papers IV+V)

The study was designed to investigate if a set of TLRs and NLRs, representing possible activation sites of an innate immune response, could be found in the middle ear mucosa. We also wanted to know if NO could be found and produced in the middle ear cavity. The final aim was to explore whether the TLR, NLR and NOS expression rates were affected by ongoing chronic middle ear disease.

Results

Samples of the middle ear mucosa were obtained from patients with CMED. Patients with healthy middle ears receiving cochlear implants were used as controls. Since the amount of mucosa that could be obtained from each patient was small, samples were pooled in order to allow analysis. This limited the number of receptors that could be investigated and omitted a more detailed phenotypic analysis.

mRNA expression of TLR3, TLR4, TLR5, TLR7, Nalp3 and Nod2 could be detected in all pools of mucosal samples. Corresponding proteins were demonstrated in tissue sections by immunohistochemistry. Patients with CMED exhibited lower expression rates of TLR4, TLR5, TLR7 and Nalp3 as compared to healthy controls. On the contrary, Nod2 was up-regulated in patients with middle ear disease. No difference was seen regarding TLR3 expression.

The assessment of mRNA representing NOS revealed down regulated expression rates of eNOS in the CMED group, whereas iNOS expression was low in both groups. Concentrations of NO in gas sampled from middle ear were higher than in gas samples taken close to the intact tympanic membrane of the contra lateral ear.

Comments

The presence of pattern-recognition receptors in the mucosa from both diseased and healthy middle ears opens new perspectives on how the middle ear can be defended against invading micro- organisms. It has been demonstrated that vascular leakage makes it possible for agents of the adaptive immune defense to reach the middle ear upon infection [108] . Further, stimulation of an immune induction site in the MALT system, presumably the adenoid in humans, may induce local IgA production in the middle ear mucosa [15, 109]. Based on these findings it has often been presumed that the middle ear is inert when not stimulated as described above. The present study

revealed that the human middle ear mucosa has a repertoire of TLRs and NLRs that can react to infections. You could describe these pattern-recognition receptors as watch dogs, ready to act when intrusion occurs. A recent study has revealed the presence of bacteria and even biofilm in clinically healthy ear from children during cochlear implantation [110]. These findings could mean that the paradigm of the middle ear being sterile and lacking of immunoreactivity during clinically healthy conditions may change. If bacteria in fact are present in the middle ear cavity every now and then a normal function in PRRs might be crucial. Impaired antigen recognition by e.g. TLRs could possibly allow an intrusion of small amounts of bacteria to cause an infection, even without the adjuvance of viral infections.

The importance of TLRs in the immune defence of the middle ear has already been demonstrated in experimental studies, since mice who have a deficiency in TLR4 are prone to develop spontaneous chronic otitis media [79].

The results of our study are consistent with these experimental findings, and there are also human genetic studies that stresses the importance of TLRs in middle ear infections [80]. We were only able to perform the investigations on a group level, and can therefore not correlate the results with individual phenotypes. In future studies on innate immunity of the middle ear it would be of interest to correlate the expression of innate immunity to different phenotypes, or even gene expression. So far, we can only speculate on the background of the down regulated TLRs and Nalp3 in patients with chronic middle ear disease. It is known that there is a genetic predisposition for having recurrent episodes of otitis media [38, 39], and it is not far-fetched to assume that certain individuals may have inborn defects of the local innate immune defense of the middle ear. It is also possible that repeated infections and a pathological middle ear milieu could influence the local expression of innate immune receptors.

The expression of iNOS and eNOS in the middle ear mucosa has been demonstrated in previous studies [111, 112], but a clear difference in expression rates, with a down regulation of eNOS in chronic middle ear disease, is a novel finding. This could mean that NO is present in the middle ear gas mixture and that NO is necessary for maintenance of a healthy middle ear environment. To assess this further, we attempted to measure NO concentrations in middle ear gas samples from tympanic membranes with a preformed opening (a chronic perforation or a tympanostomy tube). The higher concentrations of NO in middle ear gas samples as compared to gas sampled from the outer ear canal implies that NO is in fact present in the middle ear gas.

In one patient with ongoing otitis media with effusion, the obtained NO concentrations in gas aspirated from the middle ear after paracenthesis was very low (i.e. lower than NO concentration in ambient air). This could theoretically be explained by the overall impaired gas exchange over the mucosa, and to malfunction of the Eustachian tube in that particular case. The highest NO concentrations were obtained from a patient who had an infection during tympanstomy tube treatment. It is not possible to draw definitive conclusions on these findings. Nonetheless, it is tempting to speculate that patients with an ongoing OME might have an impaired ability to produce NO or that it is difficult to measure NO when the middle ear is obstructed by effusion. On the contrary an increased NO-production, possibly via iNOS induction, could occur during infection and inflammation with a ventilated middle ear cavity (in this case due to tube treatment).

The overall low iNOS expression in both groups could be a reflection of the fact that most patients, even in the chronic middle ear disease group, lacked signs of infection during surgery (described in paper IV). It could also be a consequence of a defect in the ability to induce iNOS, which may be a part of the pathogenesis of chronic middle ear disease. In healthy middle ear mucosa a low iNOS expression would be anticipated due to the undisturbed middle ear milieu.

1.5 CONCLUSIONS

- The duration of otorrhea, measured in days from the start of treatment, was used as primary outcome variable comparing systemic antibiotics in combination with topical treatment and topical treatment alone for treatment of tube associated otorrhea. Close to 90% of all children were cured within 7 days regardless of treatment regime and there was no difference in the duration of otorrhea between the study groups. This implies that topical treatment alone is sufficient in most cases of tube-associated otorrhea, and that a watch-and-wait policy can be recommended vis-à-vis the use of systemic antibiotics. Bacteriological cultures from the ear discharge revealed growth of airway bacteria like *S. pneumoniae, H. influenzae* and *M. catarrhalis*. This strongly suggests that otorrhea during these conditions is a manifestation of acute otitis media, rather than a sign of non-specific inflammation of the upper airway, or of external otitis media of the ear canal.

- Presence of TLR4 and TLR7 mRNA along with their corresponding proteins could be demonstrated in all tested adenoids. Children with a clinically significant hearing loss due to otitis media with effusion exhibited higher expression levels of TLR7 than children with no signs of middle ear disease. No such difference was seen for TLR4. TLR7 interacts with single-stranded virus RNA. It is not inconceivable that that a virus infection, via TLR7, might trigger immune reactions in the adenoid that contributes to the development of otitis media with effusion.

- Adenoids from children with otitis media with effusion exhibited lower levels of iNOS mRNA than controls. No such difference was seen for eNOS. Corresponding eNOS and iNOS protein could be found in and around the surface epithelium. It is previously known that various microbes can induce immune reactions in the nasopharyngeal space and thereby trigger inflammatory reactions in the middle ear The presently suggested NO

production might be an important part of this interaction. If so, children with low ability to activate iNOS in the adenoid and thus produce NO might be at risk for developing middle ear disease.

- Using various techniques, an array of cytokines could be demonstrated in different compartments of adenoids obtained from healthy children and children with continuous otitis media with effusion. A detailed analysis of IL-1β and TNF-α that was performed due to their role in the iNOS activation pathways did not reveal any differences between the study groups. This suggests that the low iNOS expression in children with otitis media with effusion might not be the result of down regulation on the cytokine level.

- mRNA for TLR3, TLR4, TLR5, TLR7, Nod1 Nod2 and Nalp3 mRNA and corresponding protein were found in the human middle ear mucosa. The amount of TLR4, TLR5, TLR7 and Nalp3 mRNA was markedly down-regulated in patients with chronic middle ear disease, whereas Nod2 mRNA was increased. The limited amount of mRNA that could be derived from the middle ear mucosa made it necessary to pool the patients for the quantitative analysis. It was therefore not possible to link specific phenotypic disease information to individual TLR/NLR profiles. However, it appears that the innate immune function of the middle ear might be down regulated in patients with chronic infections. Further, it could not be excluded that the derangement of pattern recognition receptors might play a part in the pathogenesis of chronic middle ear disease.

- eNOS mRNA was detected in the middle ear mucosa of all tested samples, whereas the mRNA expression of iNOS was generally low. Patients with chronic middle ear disease displayed reduced levels of eNOS in comparison to controls. Gas sampled from the middle ear contained more NO than gas sampled from the ear canal close to the intact tympanic membrane of the contra lateral ear. These results indicate that local production of NO might be of importance in maintaining a healthy middle ear environment. Our findings warrant further studies of NO as a natural component of the middle ear gas milieu.

1.6 SAMMANFATTNING PÅ SVENSKA

I denna avhandling studeras nya aspekter på det medfödda immunsystemets roll vid uppkomst och utveckling av akut och kronisk mellanöreinflammation. Vidare undersöks olika sätt att behandla öronflytning hos barn som rörbehandlats med anledning av upprepade akuta öroninflammationer.

Bakgrund

Mellanörat är ett gasfyllt hålrum i tinningbenet som kommunicerar med omgivningen via örontrumpeten. Örontrumpeten mynnar i näshålans bakre del invid den sk adenoiden (polypen bakom näsan). I motsats till luftvägarna i övrigt har den tunna mellanöreslemhinnan ingen direkt kontakt med lymfkörtlar eller annan immunkompetent vävnad. Trots detta kan många infektioner i mellanörat ändå läka spontant. Hur detta sker är fortfarande till stor del okänt. Immunologiska reaktioner i adenoiden och antikroppar som når mellanörat via blodet tros dock vara av betydelse för detta förlopp.

Barn som drabbas av tätt återkommande akuta öroninflammationer kan ofta framgångsrikt behandlas med inoperation av ett plaströr i trumhinnan. Samma behandling kan tillgripas vid tillstånd med långvarigt kvarstående vätskebildning i mellanörat (öronkatarr).

Immunförsvaret brukar indelas i två samverkande delar, en medfödd och en förvärvad. Den medfödda delen aktiveras mycket snabbt då olika mikrober (smittämnen) detekteras i syfte är att förhindra infektionsutveckling. Den förvärvade delen svarar under loppet av dagar genom att utveckla specifika cellbaserade verktyg för att effektivt bekämpa invaderande mikrorganismer. I denna avhandling kartläggs förekomsten av två typer av sk mönster igenkännande receptorer (pattern recognition receptors), Toll-lika och Nod- lika receptorer (TLRs och NLRs) i adenoid och mellanöreslemhinna. TLRs och NLRs som utgör en relativt nyupptäckt del av det medfödda immunsvaret har förmågan att känna igen och reagera på vissa specifika delar mikrober som ex v virus och bakterier.

Kväveoxid (NO) är en gas som bildas genom en enzymatisk reaktion i slemhinnan. Det är välkänt att stora mängder NO kan bildas i såväl lunga som i näs- och bihålesystemet.

Produktionen av NO stiger vid inflammation och i många sammanhang har NO tillskrivits en förmåga att hämma mikrobiell tillväxt. Om NO också finns som en del i mellanörats gas blandning har dock förblivit okänt.

Mål

Syftet med föreliggande 5 delvis integrerade studier har varit att ta reda på om:

- Barn med plaströr som drabbas av akut öronflytning behöver ha peroral antibiotikabehandling eller om det räcker med enbart lokal behandling .
- TLRs och enzymer för NO produktions finns i adenoiden och om deras uttryck förändras vid långdragen öronkatarr.
- TLRs och NLRs återfinns i mellanöreslemhinna och om deras uttryck påverkas av kronisk mellanöreinflammation
- NO utgör en del av gas blandningen i mellanörat och om denna gas kan ha någon betydelse för utveckling av mellanöresjukdom

Metoder

Studie 1: För utvärdering av behandling av rörflytning studerades en grupp yngre barn som fått plaströr inopererade. I samband med att de drabbades av öronflytning behandlades hälften enbart med lokala örondroppar medan den andra hälften fick lokal behandling i kombination med peroral antibiotika. Bakterieodlingar togs från öronsekret innan behandlingen startades.

Studie 2 och 3: Adenoider tillvaratogs i samband med operation från barn med hörselnedsättning (bekräftad med hörselmätning) pga öronkatarr. Som jämförelsematerial användes adenoider från barn helt utan symptom eller kliniska tecken på öronbesvär. Förekomst av två TLRs, TLR 4 och TLR7 samt två enzymer ansvariga för NO produktion, eNOS och iNOS undersöktes med hjälp av real-time PCR (teknik för bestämning av mängden mRNA för ett visst protein, det så kallade genuttrycket) och immunhistokemi (infärgning av vävnad eller celler för ett visst protein med hjälp av antikroppar). Vidare togs bakterieodlingar från adenoiden och en rad inflammatoriska mediatorsubstanser, sk cytokiner, i vävnad, sekret och ytceller analyserades.

Studie 4 och 5: Små provbitar av slemhinna togs från mellanörat under öronoperationer på patienter med kroniska öronsjukdomar. Som jämförelsematerial togs provbitar från friska mellanöron hos patienter som opererades med insättning av cochlea-implantat. Immunaktivitet i proverna undersöktes med hjälp av real-time PCR och immunhistokemi. Utöver detta mättes koncentrationen av NO i gasprover från mellanörat hos patienter med hål i trumhinnan eller som hade fått plaströr inopererade.

Kommenterade resultat

- De bakterier som återfanns vid odling på öronsekret var av samma slag som de som vanligen ses vid akut öroninflammation, vilket talar för att flytningen kan betraktas som ny akut öroninflammation. Den tid det tog för en episod av rörflytning att läka ut (d v s tid tills flytningen upphörde) förkortades inte genom tillägg av peroral antibiotika. Lokalbehandling med örondroppar torde därför vara tillräcklig vid behandling av de flesta fall av plaströrsflytning.

- TLR4, TLR7, eNOS och iNOS påträffades i alla insamlade adenoider. Ingen skillnad i TLR4 uttrycket sågs mellan grupperna, däremot var uttrycket av TLR7 högre och iNOS lägre i de adenoider som kom från barn med långdragen öronkatarr. Dessa fynd talar för att det medfödda immunförsvaret i adenoiden kan vara av betydelse för skyddet mot utveckling av mellanöreinflammation. Då TLR7 känner igen virus kan de högre nivåerna av denna receptor tyda på att virusinfektioner bidrager till utvecklingen av långdragen öronkatarr. Vidare kan de låga iNOS nivåerna reflektera en lokalt nedsatt förmåga till NO produktion med nedsatt bakteriellt skydd som följd. Trots att många cytokiner (t.ex. IL-1β, TNF-α och IFN-γ) mättes i både vävnad och sekret sågs ingen skillnad mellan grupperna. Detta kan tala för att långdragen öronkatarr i vissa stycken tycks skilja sig från andra typer av luftvägsinflammation. Det var heller ingen skillnad mellan grupperna beträffande bakterieväxt på adenoidens yta, vilket tolkades som att skillnaderna mellan grupperna ej var bakteriellt orsakade.

- Sju medfödda immunoreceptorer undersöktes i prov på mellanöreslemhinna. I samtliga återfanns TLR3, TLR4, TLR5, TLR7, Nod1 Nod2 and Nalp3. De tre senare är exempel på sk NLRs. Nivåerna av TLR4, TLR5, TLR7 och Nalp3 var nedreglerade medan nivån av Nod2 var uppreglerad hos patienter med anamnes på kronisk mellanöreinflammation. Av tradition har mellanörat betraktats som immunologiskt ”fattigt” med begränsade möjligheter att försvara sig själv. Fyndet av immunoreceptorerna med dess sjukdomsrelaterade förändringar som här rapporterats kan komma att förändra vår syn på mellanörats försvar. Förklaringar till hur mellanörat under normala förhållanden kan upprätthålla sin sterilitet och orsaker till varför akuta öroninflammationer ofta läker ut spontant skall kanske i framtiden sökas i det lokala medfödda immunförsvarets uppbyggnad.

- Analys av mellanöreslemhinnan bekräftade också fynd rörande närvaron av eNOS och iNOS. Uttrycket av iNOS var generellt lågt i vår studie. Hos patienter med pågående kronisk öroninflammation var mängden eNOS lägre än i kontrollgruppen. För första gången rapporteras också fynd talande för att gasformigt NO verkligen finns i den gasblandning som utfyller mellanörat. Detta är inte förvånande då lokal NO produktion tidigare visats i såväl de övre som i de nedre luftvägarna. Om fyndet står sig torde de få långtgående konsekvenser för hur vi ser på såväl mellanöras lokala immunförsvar som dess gasinnehåll och regleringen därav.

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