The Role of Endotoxin in the Pathogenesis of Otitis Media with Effusion

Marja Nell

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Trust yourself,

you know more than you think.

Dr. Benjamin Spock

Voor mijn ouders

Contents

AOM	acute otitis media
BPI	bactericidal / permeability – increasing protein
E	endotoxin
ELISA	enzyme – linked immunosorbent assay
ET	eustachian tube
ETO	eustachian tube obstruction
GNB	gram-negative bacteria
IgM	immunoglobulin M
IL	interleukin
LAL	limulus amoebocyte lysate
LM	light microscopy
LPS	lipopolysaccharide
MCS	mucociliary clearance system
MEE	middle ear effusion
MGP	mucus glycoprotein secretion
OME	otitis media with effusion
PAF	platelet activating factor
PBS	phosphate-buffered saline
PMN	polymorphonuclear cell
$rBPI_{21}$	21 kDa recombinant amino-terminal BPI fragment
S-CMC	S-carboxymethylcysteine
SE	standard error of the mean
SEM	scanning electron microscopy
TEM	transmission electron microscopy
TNF	tumor necrosis factor
TP	total protein
URTI	upper respiratory tract infection

Chapter I

General Introduction

1. Otitis Media with Effusion (OME)

Otitis Media with effusion (OME) is very common in young children. By three years of age, approximately 75% of children have had at least one episode of acute otitis media (AOM) and more than 33% of children had recurrent infections, defined as three or more episodes (Klein, 1994). In cases of chronic middle ear infections children may suffer long-term effects with respect to the development of speech and cognitive abilities because of the hearing loss that accompanies otitis media. AOM is defined by the presence of fluid in the middle ear accompanied by signs of acute illness. These signs can be specific to ear disease including pain, otorrhea, and hearing loss as well as systemic signs like fever, irritability, headache, lethargy, anorexia, or vomiting. Uncommon signs of ear infection include tinnitus, vertigo and nystagmus (Klein, 1994). The presence of fluid in the middle ear without signs or symptoms of acute infection is defined as OME. Some children recover from AOM without the formation of middle ear effusion, while others have persistent middle ear effusion for a long period even after the disappearance of the acute inflammation. It is usually difficult to predict whether a particular child with AOM will recover or will have long-lasting middle ear effusion (MEE). A correlation between AOM and OME has been put forward (Mills, 1987). However, AOM is not necessarily preceding the development of OME (Schilder et al., 1992).

After every episode of acute infection effusion persists in the middle ear for weeks to months. Three main types of MEE can be distinguished: serous, mucoid or mucopurulent with varying amounts of inflammatory cells (Grote & Kuijpers, 1980). The serous type appeared to be most frequent in children without infective disease in other parts of the upper airway system. The majority of the mucoid or mucopurulent samples

were from patients with sinusitis. In the first few days of OME polymorphonuclear cells (PMNs) predominate in MEE, after the third to fourth day macrophages are the most predominant finding (Palva et al., 1981). In the chronic effusions macrophages, lymphocytes and plasma cells appear to predominate, but eosinophils and basophils are rare. The presence of effusion in the tympanic cavity is pathognomic in OME. Although middle ear effusion contains beneficial substances, which protect the middle ear from microbial infections, it also possesses many harmful substances. Moreover, these substances itself are able to induce OME (Nakata et al., 1992).

Factors associated with risk for recurrent and severe otitis media include age under 6 year, male, race, season, family history of otitis media, lack of breast-feeding, group day care, exposure to tobacco smoke, viral infections of upper respiratory tract, and sinusitis (Klein, 1994; Shapiro & Bluestone, 1995).

1.1. Role of the Eustachian tube

Infants are predisposed to otitis media because their eustachian tubes are shorter and lie more horizontally than those of older children and because development of the musculature governing opening and closing of the tube is incomplete (Klein, 1994). The eustachian tube has at least three important physiological functions in preventing infection and accumulation of effusion in the middle ear: protection against nasopharyngeal sound pressure and secretions; ventilation of the middle ear to equilibrate gas pressure with atmospheric pressure; and drainage and clearance of middle ear secretions into the nasopharynx (Klein, 1994).

Children with 1) oropharyngeal abnormalities, e.g. cleft palate, 2) craniofacial abnormalities, e.g. Down-syndrome and 3) immotile cilia, e.g. Kartagener's syndrome, and 4) children with mucoviscidosis, often have chronic otitis media because the eustachian tube is unable to clear the middle ear from effusion and to protect the middle ear from microbial pathogens in the nasopharynx (Klein, 1994; Lim & DeMaria, 1988). Ciliary function in the middle ear, especially in the tympanic orifice and the eustachian tube, has an important role in the mucociliary clearance of surplus secretion to the pharynx. Ciliary activity in the tubotympanum is sensitive to a variety of pathological agents including bacteria, bacterial endotoxin, irritant gases and irradiation, resulting in mucociliary dysfunction of the tubotympanum (Ohashi & Nakai, 1991).

It is well known that obstruction of the eustachian tube in mammals, including man, leads to an accumulation of fluid in the middle ear. Therefore, it is clear that eustachian tube obstruction (ETO) is causal in some instances of OME but may be consequential in others (Chole, 1986).

1.2. Treatment

Treatment for OME varies widely and is dependent on the duration and severity of the condition. In most cases mild forms of the disease can resolve spontaneously. However, for treatment of OME there are three possibilities.

1) Most often placement of **ventilation tubes** is performed. Ventilation tubes produce a direct improvement in hearing, but it is unclear if they can modify the course of the disease toward early resolution, prevent long-term otopathological sequelae and developmental consequences of OME (Schilder et al., 1995). In a follow-up study it was shown that

tympanosclerosis and atrophy were the most common sequelae of treatment with ventilation tubes for OME (Lildholdt, 1983).

2) Medication with **antibiotics** can have some disadvantages. The number of patients with OME has increased drastically following the introduction of antibiotics for treating early AOM in the pediatric population, suggesting that antibiotics may be directly or indirectly responsible (Lim & DeMaria, 1982). It is suggested that certain antibiotics, such as penicillin, may interfere with the local IgM production in the middle ear (Howie et al., 1976). Development of local immunity following *Streptococcus pneumoniae* infection in the middle ear is prevented, or interfered with, by administering antibiotics early in infection (Jokipii et al., 1977). These findings reflect intervention in the formation of antibodies due to early elimination of antigen or infecting organisms. It is also possible that certain antibiotics could interfere with the biology of the middle ear mucosa, secretory system or local immune system, thus leading to OME (Lim & DeMaria, 1982). The use of broad-spectrum antibiotics, particularly in the prolonged low doses characteristic of prophylaxis, is thought to play a major role in the evolution and spread of resistance (Shapiro & Bluestone, 1995). Moreover, an important limiting effect of antibiotics is that bacteria can be killed but their toxins still can be effective (Martich et al., 1993).

3) Another possibility for management of otitis media is performance of **adenoidectomy**. Adenoidectomy; removal of nasal lymphoid tissue, has been shown to be beneficial for patients older than 4 years who have recurrent AOM or chronic OME (Gates et al., 1988). The principal benefits of adenoidectomy are the reduction of bacterial reservoir of the nasopharynx and possibly relieve of eustachian tube obstruction by swollen adenoids. Surgical therapy does not cure OME because it is

impossible that a complete removal of the bacterial source can take place (Klein, 1994).

These three ways of management of otitis media are, in most cases, not able to cure chronic OME sufficiently. Therefore, it is important to search for new treatment possibilities, which might be able to break the vicious circle of the pathogenesis of OME.

1.3. Role of infectious organisms

The normal middle ear mucosa is protected by the mucociliary clearance system (MCS) and by surface mucosal immunoglobulins that inhibit bacterial adhesion to the mucosal surfaces. Bacteria mediate adherence to the host cells by adhesive appendages (adhesins or ligands), which interact with host cell receptors (Beachy, 1981). Intratympanical administration of a *Staphylococcus aureus* bacterial suspension resulted in hyperplasia of middle ear epithelium and production of mucus in rats (Grote & van Blitterswijk, 1984). Obstruction of the eustachian tube in rats also resulted in transformation of the epithelium into mucus-producing cells (Kuijpers & Beek, 1984). On the other hand, obstruction of the eustachian tube in germ-free rats never gives rise to glandular development and mucus production (Kuijpers et al., 1984; Beek & Kuijpers, 1984; Kuijpers & Beek, 1987). It was suggested that the most likely explanation for this phenomenon is that obstruction of the normal clearance pathway together with favorable nutritional conditions for bacterial growth of normally harmless inhabitants of the middle ear induces a transformation of squamous cells into secretory cells (Kuijpers & Beek, 1984).

Cultures of MEEs showed the presence of a bacterial pathogen in approximately two-thirds of children with AOM (Klein, 1994). The major pathogens of AOM are *Streptococcus pneumoniae* (25-50%) and *Haemophilus influenzae* (15-30%) followed by *Moraxella catarrhalis* (3-20%). Group A ß-hemolytic Streptococci, *Staphylococcus aureus* and gram negative enteric bacilli are infrequent causes of AOM (Klein, 1994). In the case of chronic OME common pathogenic bacteria include *H. influenzae* (25%), *S. pneumoniae* (9%) and *M. catarrhalis* (5%). Other bacteria that account for the smaller number of cases are *S. aureus*, *Streptococcus pyogenes*, *Escheria coli*, and gram-negative bacilli including Pseudomonas (Lim & DeMaria, 1982). It is clear that gram-negative pathogens are more frequently isolated in cases of chronic OME than AOM.

Respiratory viruses alone or in combination with bacterial pathogens have been identified in approximately one-fifth of MEEs (Klein, 1994). Experimental otitis media could be induced by nasal inoculation of Influenza A virus in chinchillas (Giebink et al., 1980). It appears that after Influenza A infection the middle ear becomes highly vulnerable to a secondary bacterial infection from the nasopharynx. The possible immunosuppressive effects of the respiratory viruses by various alterations of immunocompetent cell function could also have an influence on the pathogenesis of otitis media (Friedman et al., 1984).

1.4. Role of host defense

The middle ear mucosa in the normally healthy state consists of basal cells that give rise to at least three other cell-types, namely non-ciliated cells with or without secretory granules and ciliated cells without secretory granules (Hentzer, 1984). The middle ear is protected by the

mucosal defense system common to all mucus membranes exposed to the external environment. The mucosal defense system comprises:

1) Mechanical defense (e.g. mucociliary clearance system containing the mucus blanket, ciliated cells and secretory cells);

2) Biological defense (e.g. antibacterial enzyme secretion);

3) Immuno-defense (e.g. humoral- and cellular immune system); and

4) Phagocytosis by PMNs and macrophages.

See figure 1.

In the normal middle ear mucosa there is a low secretory cell density and very few if any mucus glands (Bernstein & Ogra, 1980). However, in OME, where viral, bacterial or other antigen is brought into contact with the mucosa of the middle ear cleft, a significant histological and histochemical transformation takes place in the mucosa. Frequently, a metaplasia of the epithelium into a stratified respiratory epithelium with secretory goblet cells and submucosal glands is observed (Sadé, 1971). Viral and bacterial antigens appear to produce factors that may be chemotactic for inflammatory cells that originate from blood vessels lining the middle ear mucosa. This transudation results in an inflammation of the middle ear, bringing in lymphocytes, monocytes, and all of the cells responsible for an immunocompetent response (Bernstein, 1991). As a result many bioactive substances derived from inflammatory cells are produced. The persistence of these mediators leads to tissue injury to the soft tissue as well as to the bony structures in the middle ear cleft, which may lead to permanent damage to the MCS of the middle ear, the tympanic membrane, and the ossicular chain. Furthermore, some evidence exists that these inflammatory mediators may penetrate the round window membrane and lead to permanent sensoneural hearing loss (Bernstein, 1988). Once the inflammation is present, immune

mechanisms may overwhelm the host and cause maintenance of inflammation (Bernstein, 1991). In figure 2 a representation of the different factors playing an important role in the pathogenesis of chronic OME is given.

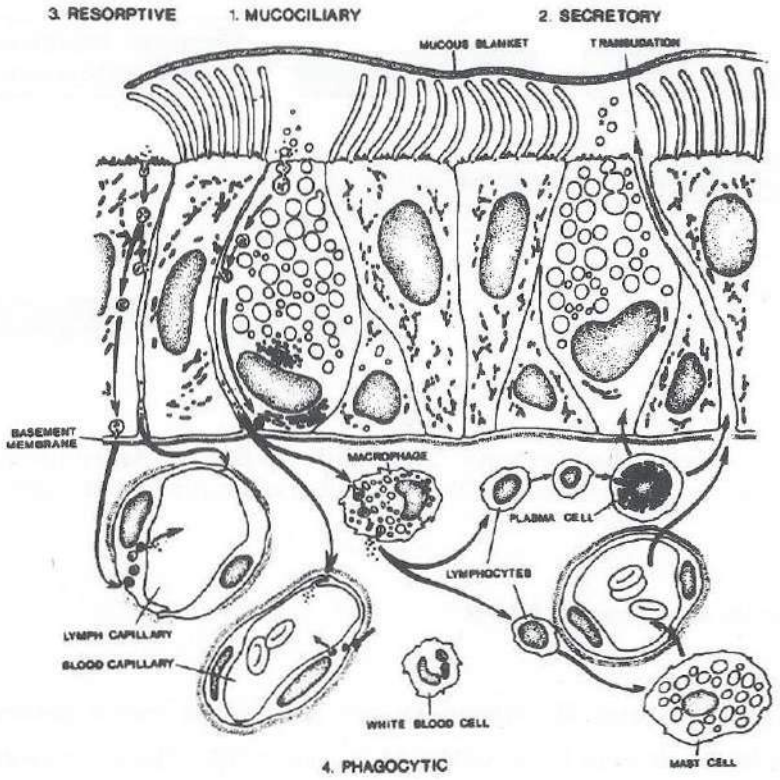

Figure 1: Mucosal defense systems (From Lim & DeMaria, 1988).

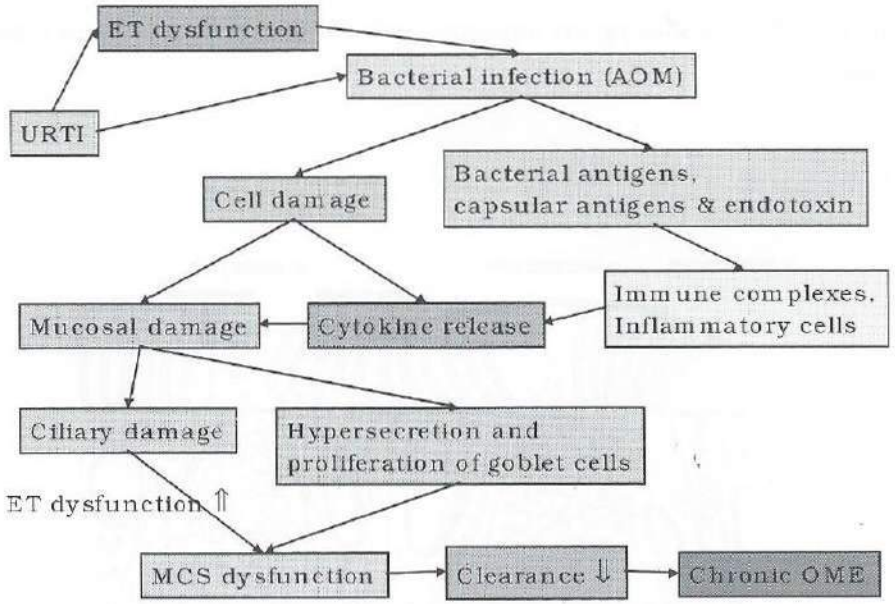

Figure 2: Pathogenesis of chronic OME: Multiple factors contribute to the development and persistence of OME (modified after Juhn et al., 1987).

2. Bacterial endotoxin / LPS

H. influenzae and *M. catarrhalis* are two of the major pathogens known to be involved in both AOM and chronic OME. The gram-negative bacterial cell wall is composed of three layers consisting of an inner membrane, a peptidoglycan layer and an outer membrane. Endotoxin comprises the mayor component of the outer membrane (figure 3). Endotoxins are chemically lipopolysaccharides (LPS) consisting of a sugar part; a polysaccharide, linked covalently to a lipid portion; the lipid A.

Toxic properties of endotoxin are expressed by the lipid A, which is structurally similar and serologically cross-reacting among many gram-negative bacteria (Galanos et al., 1988).

Endotoxin plays an important role in the pathogenesis of otitis media since it has been found in human MEEs (DeMaria et al., 1984; Ovesen & Ledet, 1992), and since injection of viable or non-viable *H. influenzae* or its endotoxin induced inflammatory changes in the middle ear and eustachian tube (Bone, 1992; Meyer et al., 1994). Even when bacteria are no longer viable in the middle ear, bacterial endotoxin can induce OME by direct or indirect action on the humoral and/or cellular host-mediation system (Morrison & Ulevitch, 1978).

The interaction of endotoxin with monocytes and macrophages trigger a broad spectrum of cellular responses, including production of important bioactive factors or mediators, such IL-1, IL-6, IL-8, TNF-α, interferon's, prostaglandin's, and macrophage-derived growth factor, which are implicated in the pathogenesis of septic shock and wound healing (Martich et al., 1993). Endotoxin is also involved in the impairment of the mucociliary function in the tubotympanum, leading to reduced activity of ciliary clearance in the middle ear cavity (Ohashi & Nakai, 1991; Ohashi et al., 1989). Endotoxin enhances leukocyte infiltration into the middle ear, and lysosomal proteases released from leukocytes damage the middle ear mucosa and thereby prolong mucosal inflammation, which may be responsible for delayed recovery from acute OME (Tracey et al., 1988).

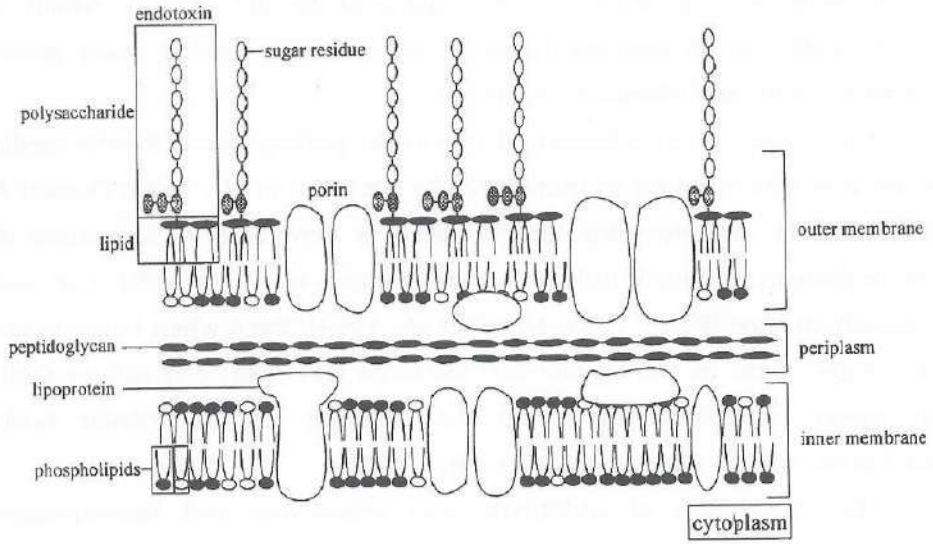

Figure 3: Schematic representation of the *E. Coli* envelope (modified after Raetz, 1990).

2.1. Endotoxin neutralization

While antibiotics can be effective in eradicating the infecting gram-negative organisms, they may induce resistance and they do not inhibit the potentially life-threatening effects of endotoxin (Martich et al., 1993). Therefore, intervention of the pathologic effects of gram-negative bacteria by neutralizing endotoxin would be a good strategy in resolving the inflammation reaction in the middle ear. There have been developed several anti-endotoxin antibodies. Two of these antibodies, HA-1A (a human immunoglobulin M [IgM] monoclonal antibody) and E5 (a murine IgM monoclonal antibody) have been reported to improve survival rates among patients with gram-negative bacteraemia and septic shock

(Romano et al., 1993; Wortel et al., 1992; Ziegler et al., 1991). The effect of HA-1A was also tested *in vitro*, were it was able to suppress the proliferative effects of endotoxin on rat middle ear epithelium (Grote et al., 1995). Unfortunately there are concerns regarding their binding affinity and specificity, as well as their unsatisfactory endotoxin neutralizing capacities *in vitro* and *in vivo* (Warren et al., 1993; Marra et al., 1994). The search for a potent anti-endotoxin has led back to an endogenous lipopolysaccharide regulatory protein: bacterial/permeability-increasing protein (BPI). In a study where endotoxin-binding and -neutralizing properties of both monoclonal antibodies were compared to BPI, it was found that neither monoclonal antibody was as effective as BPI at binding and neutralizing endotoxin (Marra et al., 1994).

3. Bactericidal/Permeability-Increasing Protein (BPI)

BPI, a cationic 55 kDa protein, purified from rabbit and human neutrophil azurophilic granules was first described in 1978 as an antibiotic protein *in vitro* (Weiss et al., 1978), and has been shown to bind avidly to a wide array of endotoxin chemotypes, and to neutralize endotoxin activity (Gazzano-Santoro et al., 1992). Furthermore, it inhibits bacterial growth by a number of discrete outer-membrane alterations, including an increase in the outer membrane permeability (Mannion et al., 1990). BPI also decreases endotoxin-mediated effects as TNF-α production in whole blood and cytokine production by mononuclear cells (Marra et al. 1992; Marra et al., 1990). The target specificity is due to the strong attraction of the cationic BPI for the negatively charged LPS (Weiss et al., 1984). In figure 4 the possible therapeutic potential of BPI is represented. BPI is stored in the azurophilic granules of PMNs, but is also

present on the PMN-cell surface. Due to its specific interaction with LPS, BPI neutralizes LPS and kills gram-negative bacteria (GNB). These actions can be exerted inside the PMN, and by BPI present in the extracellular environment (Dentener, 1996).

A 23 kDa recombinant protein, corresponding to the amino-terminal fragment of human BPI ($rBPI_{23}$) has been shown to bind strongly to lipid A and antagonize some LPS-mediated effects (Mészáros et al., 1993; Kohn et al., 1993; Corradin et al., 1994; Kohn & Kung, 1995). The results from these studies indicate that $rBPI_{23}$ can inhibit the bacterially induced production of certain potentially harmful mediators (e.g. TNF-α, Nitric Oxide) without entirely blocking the host defense, i.e. PMN response against bacteria. It is shown that the amino-terminal recombinant fragment administered as an extracellular agent may possess greater antibacterial range and potencies than the whole protein (Weiss et al., 1992). Also a more recently developed 21 kDa recombinant protein has been shown to possess antibacterial and anti-endotoxin properties, and to reduce the cytokine levels (Horwitz et al., 1996). In a phase II clinical trial $rBPI_{21}$ was well-tolerated and demonstrated a reduction of infections and organ failure in patients with acute traumatic hemorrhage (Demetriades et al., 1999).

The efficacy of BPI in neutralizing endotoxin *in vivo* as well as the potential toxicity of BPI is determined in several animal and human models of bacteraemia and endotoxemia. Infusion of BPI protected CD1 mice (which are relatively endotoxin resistant) against lethal doses of *Staphylococcus abortus* (Marra et al., 1994). When $rBPI_{23}$ was concomitantly administered with endotoxin in CD1 mice (Kohn et al., 1993) and rats (Lin et al., 1994) the cytokine production (TNF, IL-1) was attenuated. However, when $rBPI_{23}$ was administered half an hour after endotoxin infusion, the TNF production was not reduced (Lin et al.,

1994). In experimental endotoxemia in human volunteers the endotoxin-induced changes in cytokine release were reduced and the changes in leukocyte counts and markers for neutrophil activation were blunted by $rBPI_{23}$ (Von der Möhlen et al., 1995).

BPI is thus a very potent endogenous LPS neutralizing and bactericidal protein, contributing significantly to the microbicidal potential present inside the PMN. However, although LPS and TNF are relatively potent inducers of BPI release, the major part of BPI remains inside the PMN (Dentener et al., 1997). Therefore, therapy of gram-negative bacterial infections with BPI may improve the natural working of BPI.

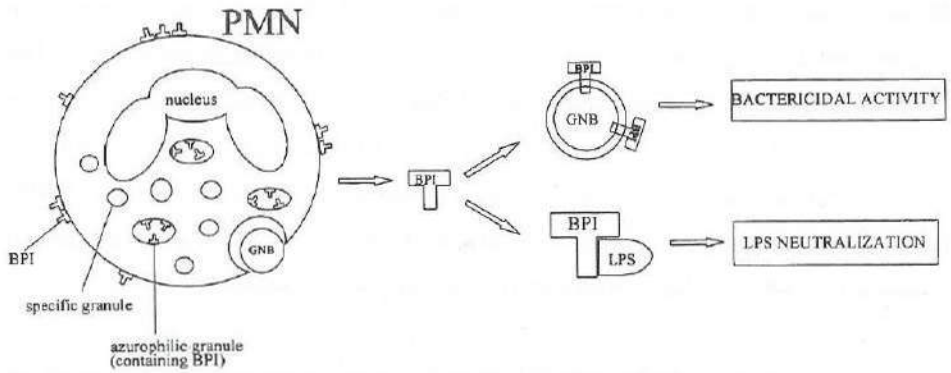

Figure 4: Localization of BPI in PMN and its mode of action (modified after Dentener, 1996).

4. Aim of the Thesis

As discussed in this chapter almost all children suffer from a period with glue-ears and hearing loss due to OME. In most cases these problems cure spontaneously, but 15% of children have chronic problems, which lead to hearing loss for a longer period with sometimes

remaining changes to the eardrum and middle ear ossicles. From these chronic infections gram-negative pathogens, which contain endotoxin in their outer membrane, are often isolated. These children cannot be cured satisfactory with the current therapies. Palliative therapy is the only possibility, often with recurrent placement of ventilation tubes. In this way there are approximately 70.000 ventilation tubes used each year in The Netherlands. Because of the lately reported disadvantages of ventilation tubes and the growing resistance for antibiotics, there is a need for curative therapy for these patients.

Therefore, studies on the functions of endotoxin in the pathogenesis of OME may provide more insight in the development of chronic OME. These studies may also provide a basis for therapeutic experiments in which inflamed middle ear epithelium can be treated with $rBPI_{21}$. The general aim of the present study is twofold: firstly, obtain more insight in the role of endotoxin in the development of chronic OME, and secondly, to develop therapeutic strategies against endotoxin induced chronic OME. As described, there are several factors that play a role in the development of chronic OME of which endotoxin is an important one.

In the present study several specific questions were addressed to investigate the role of endotoxin in the pathogenesis of OME:

1) To what content is endotoxin present in the MEE and is it correlated to the type of effusion, the duration of OME, and the presence of URTI. Furthermore, is the presence of endotoxin related to the presence of TNF-α in the MEE (chapter II).
2) What are the quantitative histological effects of endotoxin on air-exposed cultured human middle ear epithelium (chapter III).
3) Can the *in vitro* induced effects of endotoxin on human middle ear epithelium be inhibited by $rBPI_{21}$ (chapter IV).

4) What are the morphological effects of endotoxin, ETO, and a combination of endotoxin and ETO *in vivo* (chapter V).
5) Can $rBPI_{21}$ prevent the *in vivo* induced effects of endotoxin and endotoxin in combination with ETO when it is applied two days after the induction of OME; when the inflammatory reaction is just started (chapter VI).
6) Can $rBPI_{21}$ restore the *in vivo* induced effects of endotoxin in combination with ETO when it is applied two weeks after the induction of OME; when the inflammatory reaction is already causing damage (chapter VII).

Chapter II

Endotoxin and TNF-α in middle ear effusions: in relation to upper airway infection

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ABSTRACT

Objectives/Hypothesis: This study was performed to elucidate the role of endotoxin and TNF-α in the middle ear effusions (MEE) of children with otitis media with effusion (OME) in relation to the chronicity of the disease and the presence of upper respiratory tract infection (URTI).

Study Design: 140 MEEs were collected from 101 children between 2 and 12 years of age, and evaluated for the presence of cytokine TNF-α and the lipopolysaccharide endotoxin. The amounts were quantified and correlated with the type of MEE, OME duration, and the presence of URTI.

Methods: Endotoxin levels were measured using a Limulus Amoebocyte Lysate (LAL) assay and TNF-α levels were measured with an enzyme-linked immunosorbent assay (ELISA). Means of the different variables were compared using the one-way ANOVA least significance difference test with $P<0.05$

Results: In MEE classified as mucopurulent (22.8%) both endotoxin and TNF-α levels (11.9±3 ng/mg TP and 61.1±21 pg/mg TP respectively) were significant higher compared to serous (23.6%) or mucoid (53.6%) typed effusions. Fifty-five percent of the children who were classified as having chronic OME also had significantly higher amounts of endotoxin and TNF-α. The majority of the children (61%) had no URTI. Whereas children with URTI (36%) did also have significant higher levels of endotoxin and TNF-α in their middle ears.

Conclusions: These results indicate that there is a strong association between the endotoxin- and the TNF-α concentration in the middle ear and the type of MEE, the presence of URTI and the chronicity of the disease.

INTRODUCTION

Otitis media with effusion (OME) is a disease process characterized by the retention of effusion in the middle ear cavity. In general, recovery is spontaneous and immediate treatment is not necessary. However, retention of inflammatory products in the middle ear due to eustachian tube dysfunction with poor drainage, may result in an ongoing chronic inflammatory state, that has the potential for permanent mucosal changes, fibrosis, bone erosion, and hearing loss (Chole, 1986).

In the middle ear effusion (MEE) many different substances, like endotoxin and cytokines, have been identified (Yellon et al., 1995). Endotoxin is a component of the outer membrane of gram-negative bacteria and possesses various biological activities that can cause inflammation. It is also released in a biologically active form during cell growth or death of the organism due to antibiotic treatment or host immune mechanisms (Gu et al., 1995). Endotoxin is not readily eradicated by local host defence mechanisms and has been shown to persist in the middle ear, even after effective antibiotic treatment, for up to 3 months (Willett et al., 1998). Furthermore, it has been shown to induce mucosal inflammation with accumulation of effusion in the middle ears of rats (Nell & Grote, 1999) and guinea pigs (Nonomura et al., 1986). Several investigators have reported that the endotoxin concentration in MEE might be an important parameter reflecting the chronicity of OME in children (Willett et al., 1998; Nonomura et al., 1986).

Cytokines are glycoproteins produced by neutrophils, macrophages, lymphocytes, and other cells. They are potent mediators of inflammation, and regulators of the immune response (Yellon et al., 1991). An important cytokine found in MEE is tumor necrosis factor-alpha (TNF-α). It is

produced mainly by macrophages in response to microbial or endotoxin stimulation (Morrison & Ryan, 1987). TNF-α activates polymorphonuclear cells (PMNs), promotes fibroblast proliferation, inhibits vascular endothelial- and B-lymphocyte proliferation, and stimulates cartilage and bone resorption (DeMaria & Murwin, 1997). A high level of TNF-α production by middle ear macrophages correlates with the persistence of chronic OME (Yellon et al., 1991).

The middle ear cavity and the eustachian tube are part of the upper respiratory airway system (Kuijpers & Beek, 1984). A number of studies have suggested a correlation between the initiation of OME and upper respiratory tract infection (URTI). Therefore, we studied a possible relationship between the occurrence of MEE and infection in other parts of the upper respiratory tract in a large series of children who underwent tympanostomy tube placement. Furthermore, a possible relationship between endotoxin- and TNF-α concentration in MEE, and chronicity of the disease was investigated.

Materials and Methods

Study group

140 MEEs were collected from 101 children ranging in age from 2-12 years, who were diagnosed as having OME. The samples were collected during routine tympanostomy tube placement. They were diluted to a total volume of 1 mL with sterile pyrogen-free saline (Sigma Chemical Co., St. Louis, MO, USA), and frozen at -20°C until assayed. The effusions were classified in three different types: serous, mucoid, or mucopurulent, according to Grote & Kuijpers (1980). Medical files were searched through

whether the children have had previous upper respiratory tract infections, and how long they were suffering from OME.

Assays

Endotoxin- and TNF-α concentration were determined in the MEE with commercially available kits. Endotoxin by a LAL assay (BioWhittaker Inc., Walkersville, Maryland, USA), and TNF-α with ELISA (Predicta, Genzyme Diagnostics, Cambridge, UK). The detection limit for endotoxin was 5 pg/ml, and for TNF-α 10 pg/ml. MEE samples were serially diluted (10^{-1} to 10^{-3}) with pyrogen-free distilled water (NPBI BV, Emmer-Compascuum, The Netherlands) to bring the levels within the range of the standard curve (Prior & Spagna, 1979). The standard curves were generated from known concentrations of endotoxin or TNF-α provided by the manufacturer. They were then used to predict the quantity based on the level of spectrophotometric absorbance for each sample using regression analysis (Molecular Devices, SOFTmaxPRO, Sunnyvale, Ca., USA). The absorbance was measured with a Microplate reader (Molecular Devices, SPECTRAmax 250, Sunnyvale, Ca., USA). All samples were run in duplicate.

Total protein (TP) concentrations of the MEEs were determined by the Bradford technique with the Bio-Rad protein microassay (Bio-Rad, Richmond, Ca., USA). Exact determination of original MEE volumes was difficult because of the viscosity of the effusions. Therefore, all MEEs were diluted to a total volume of 1 mL. Since the dilution of the samples varied all levels of endotoxin and TNF-α were corrected as milligrams total protein (mg TP).

Statistical analysis

Endotoxin concentrations presented in ng/mg TP and TNF-α concentrations in pg/mg TP were analysed as a function of middle ear effusion type, OME duration, and presence of URTI. To compare means of the different variables, a one-way ANOVA least significance difference test with a significance level of $P<0.05$, was performed using the Statistical Package for the Social Sciences (SPSS). Results are reported as mean concentration ± standard error of the mean (SE) per mg of TP.

RESULTS

Middle ear effusions

A total of 140 MEEs were obtained from 101 patients with a gender distribution of 42 females and 59 males, whose age ranged from 2 to 12 years. During the routine tympanostomy the MEE were classified into serous, mucoid or mucopurulent, according to the appearance of the MEE and the aspect of the tympanic membrane (Grote & Kuijpers, 1980). The majority of the MEEs (53.6%) were classified as mucoid (75 effusions), 23.6% of the collected MEEs were of the serous type (33), and only 22.8% were classified as mucopurulent (32).

Endotoxin and TNF-α in MEE

The concentrations of endotoxin and TNF-α were examined in all the MEE as a function of the effusion type (Fig. 1). The mean concentration (± SE) of endotoxin in the mucopurulent classified MEEs was 11.9 ± 3.0 ng/mg TP. This concentration was significant higher compared to the endotoxin levels in the serous typed effusions (0.3 ± 0.1 ng/mg TP) and the mucoid typed effusions (2.8 ± 0.9 ng/mg TP). Additionally, the mean

levels of TNF-α determined in the different types of MEE revealed low concentrations in the serous typed effusions (7.0 ± 4.1 pg/mg TP), as well as in the mucoid typed effusions (13.9 ± 3.3 pg/mg TP). Whereas in the mucopurulent type of MEE a concentration of 61.1 ± 21.0 pg/mg TP was found, which was significant higher than the TNF-α level in the serous typed effusions.

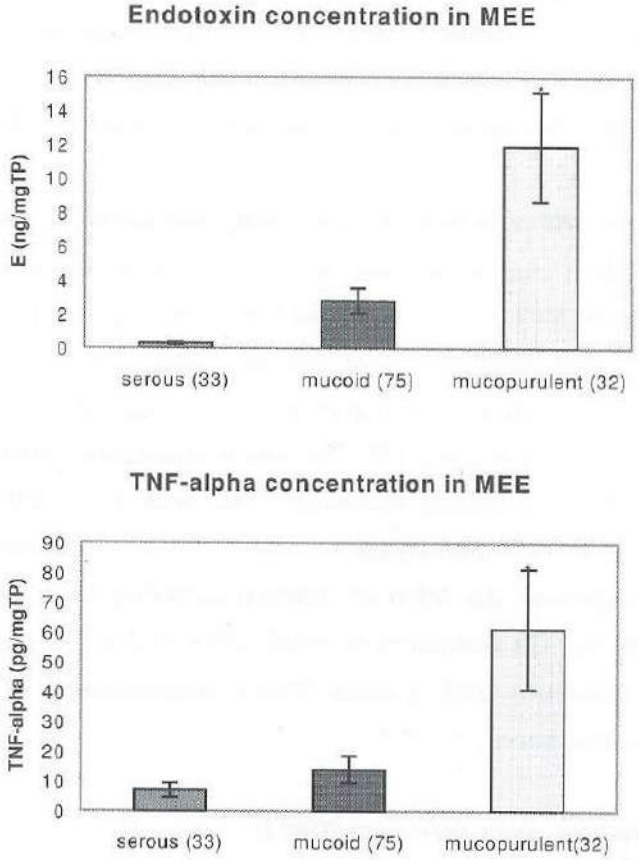


Figure 1: Endotoxin- and TNF-α concentrations in the MEE per mg TP. Both graphs show the mean concentrations in the different types of middle ear effusion ± SE. The mucopurulent MEEs contained significantly higher (*) amounts of endotoxin and TNF-α compared to both the serous and the mucoid typed effusions ($P<0.05$).

OME duration

Medical files of the children were searched for information about the duration of the preceding period of OME. The OME duration was classified into three different phases. Acute OME was identified for children having a period of less then 3 months of OME before the MEE was collected. 23% of the children in our study were suffering from acute OME. 18.5% of the children were classified as having subacute OME: lasting 3 to 6 months. Subsequently, chronic OME, identified for children having a period of more than 6 months of OME, was found in 55% of the patients. In only 3.5% no duration of the OME could be established.

Endotoxin and TNF-α in relation with OME duration

In all children the endotoxin and the TNF-α concentration was determined in the MEE as a function of the OME duration (Fig. 2). In the children who were suffering from chronic OME the mean endotoxin concentration was significantly higher (6.5 ± 1.5 ng/mg TP) compared to acute OME (0.9 ± 0.2 ng/mg TP). The mean endotoxin concentration in children, classified as having subacute OME, was 2.7 ± 1.0 ng/mg TP. Like the endotoxin concentration, the TNF-α concentration was significantly higher in the MEE of children suffering from chronic OME (35.7 ± 9.6 pg/mg TP) compared to acute OME (4.7 ± 1.9 pg/mg TP). In the cases of subacute OME a mean TNF-α concentration of 12.9 ± 4.4 pg/mg TP was measured.

Upper respiratory tract infection (URTI)

In 3% of the cases it was not possible to establish to presence of URTI before tympanostomy. 36% of the children in our study with MEE were also suffering from URTI, whereas 61% of the children had no major problems with URTI. However, the children with URTI did have significant

higher concentrations of endotoxin in their MEE (9.0 ± 2.2 ng/mg TP), compared to children without URTI (1.6 ± 0.5 ng/mg TP). Furthermore, these children did also have significant higher amounts of TNF-α in their MEE (48.9 ± 13.7 pg/mg TP) compared to children without URTI (8.7 ± 2.5 pg/mg TP) (Fig. 3).

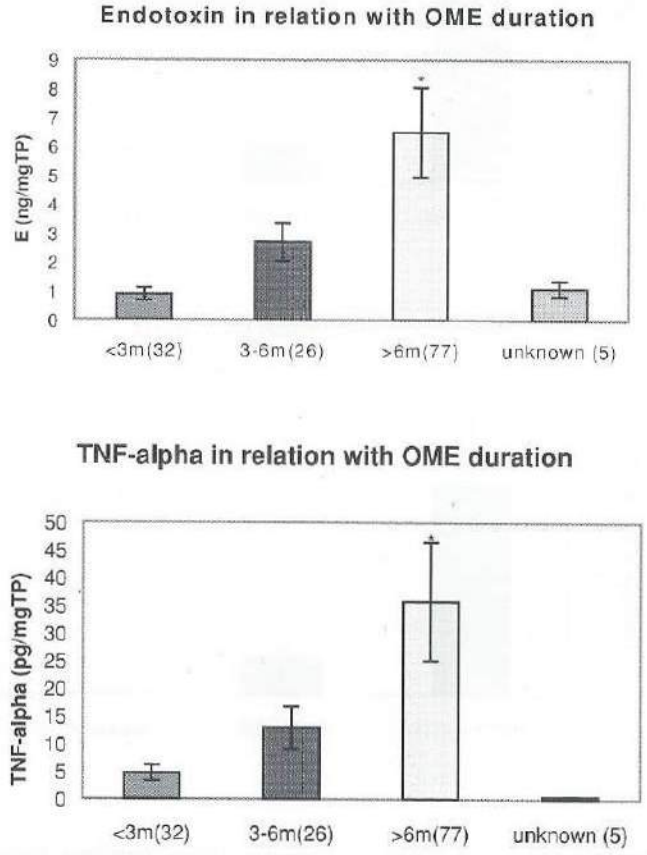
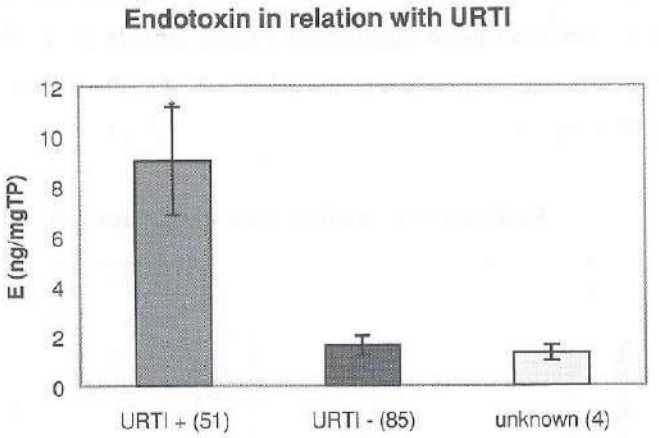


Figure 2: Endotoxin- and TNF-α concentrations per mg total protein in relation with OME duration. Both graphs show the mean concentrations ± SE in the different phases of OME; acute (<3 months); subacute (3-6 months); and chronic (>6 months). During the chronic phase both endotoxin- and TNF-α concentrations are significantly higher (*) compared to the acute phase ($P<0.05$).



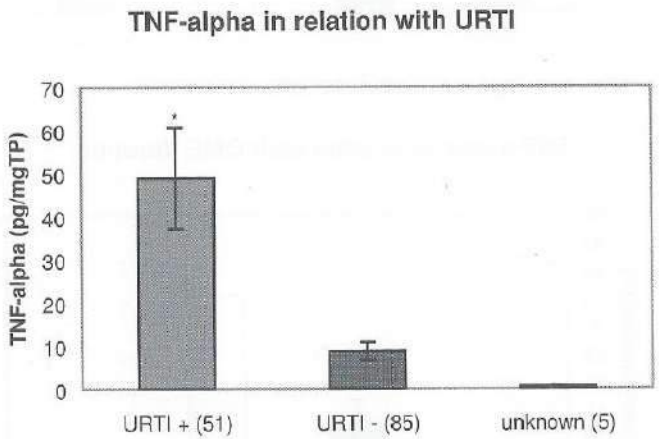


Figure 3: Endotoxin- and TNF-α concentrations per mg total protein in relation with upper respiratory tract infection. Both graphs show the mean concentrations ± SE. Presence of URTI showed significantly higher (*) amounts of endotoxin- and TNF-α concentrations compared to no URTI ($P<0.05$).

Discussion

Retention of MEE and eustachian tube obstruction seem to play a major role in the pathogenesis of chronic OME. It is well known that obstruction of the eustachian tube leads to an accumulation of serous fluid in the middle ear (Kuijpers & Beek, 1984). Therefore, it is clear that eustachian tube dysfunction is causal in some instances of OME. However, eustachian tube dysfunction may also be a sequela of OME (Chole, 1986). Younger children have smaller eustachian tubes, which lie more horizontally and therefore are believed to be more susceptible to obstruction from mucosal swelling caused by bacterial inflammation (Sando et al., 1991). This relates well to our previous data, which showed that a combination of eustachian tube obstruction and endotoxin injection in the rat induced a persistent mucosal inflammation (Nell & Grote, 1999).

The last two decades middle ear effusions have been studied extensively to gain insight into the local inflammatory process. Certain cytokines act as mediators of inflammation and as regulators of the immune response. However, while their control of inflammation represents a beneficial response to infection and injury, cytokines also can cause pathologic changes, including mucosal hyperplasia, bone erosion, fibrosis, and hearing loss (Yellon et al., 1995). An important cytokine in the case of OME is TNF-α. It is a polypeptide hormone secreted by macrophages in response to microbial or endotoxin stimulation (Yellon et al., 1991; Morrison & Ryan, 1987). Not only TNF-α but also other cytokines, notably IL-1b, and numerous other inflammatory mediators are induced as a consequence of endotoxin stimulation of the middle ear (DeMaria & Murwin, 1997). The diversity of potent chemicals in MEE suggests that inflammation is initiated and

sustained by the activation of mediator cascades in response to infection. In the case of chronic OME, residual endotoxin trapped in the middle ear may promote continued production and secretion of cytokines, and thereby may provide a mechanism for sustaining inflammation indefinitely.

The present study suggests that TNF-α is associated to the presence of endotoxin in the middle ear effusion, and that they are both correlated with the duration of OME. A significant correlation between endotoxin and TNF-α, however, could not be measured, which may be due to large variation in the data. Presence of endotoxin in MEE may contribute to the induction of mucosal edema, vasodilatation, suppression of mucociliary transport, and hyperproliferative changes in the middle ear epithelium (DeMaria, 1988). Retention of bacterial components and inflammatory mediators causes persistent inflammation of the middle ear, and higher levels of endotoxin and TNF-α will accumulate in the MEE. Additionally, endotoxin and TNF-α are found to be correlated with the type of MEE. They were both found mainly in the mucopurulent type effusion, whereas in the serous and the mucoid type effusion endotoxin- and TNF-α levels were significant lower.

The mucosal lining of the middle ear and eustachian tube is in connection with the upper respiratory tract. Infections of the upper respiratory tract have been demonstrated to result in the local release of a variety of inflammatory substances (DeMaria & Murwin, 1997). Furthermore, URTI can provoke obstruction of the eustachian tube, which in turn results in accumulation of fluid in the middle ear (Fireman, 1997). In addition, *H. influenzae* is documented as a common cause of otitis media, sinusitis, and pneumonia (Klein, 1997). Therefore, it is

suggested that occurrence of OME is related to the presence of URTI. In our study, the children with OME who were also suffering from URTI did have significant higher levels of endotoxin and TNF-α. Therefore, we suggest that URTI plays indeed a role in the pathogenesis of OME. Earlier clinical studies have also indicated that OME occurs more frequently during URTI (Casselbrandt et al., 1985). Otten & Grote (1990) found that children with chronic infections of the upper respiratory tract have a poor tendency to recover from OME. Several studies have demonstrated that URTI could affect eustachian tube function (Fireman, 1997; Takahashi et al., 1995), which is probably the most important factor in the correlation between URTI and OME.

These new data support our hypothesis that the persistent presence of endotoxin in the middle ear stimulates the inflammatory reaction. Endotoxin can remain in the middle ear cavity after the elimination of viable bacteria by either antibiotic treatment or host defence mechanisms, particularly when there is either a poor tubal function or obstruction of the eustachian tube. Inflammatory mediators together with endotoxin contribute to the pathogenesis of OME. Although, the cause of otitis media is multifactorial; eustachian tube dysfunction; bacterial or viral infection; nasal inflammation or URTI, endotoxin seems to play a mayor role in this. Therefore, the development of pharmacological intervention strategies to block endotoxin might contribute to a more effective treatment of OME.

CONCLUSION

In conclusion we have demonstrated the relationship between the endotoxin- and the TNF-α concentration in middle ear effusions of children with OME and 1) the type of MEE, 2) the duration of the preceding OME, and 3) the presence of URTI. Children with mucopurulent MEE are highly susceptible to develop chronic OME especially when they have URTI. High endotoxin concentrations in MEE are an indication for the chronicity of the disease. Therefore we conclude that endotoxin plays an important role in the pathogenesis of OME.

Chapter III

Effect of endotoxin on cultured human middle ear epithelium

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Abstract

This study was performed to investigate the quantitative histological effects of endotoxin *in vitro*. The effect of endotoxin was investigated on air-exposed cultured human middle ear epithelium. Concentrations of 0, 1 and 100 ng/ml endotoxin were used. Complete differentiation of the cells was not reached at 12 days. After 21 days endotoxin had induced an increased proliferation of the epithelial layer. Furthermore, an increase in the number of secretory cells and in the amount and length of microvilli was observed at this time. There were no significant morphological differences between the high and the low endotoxin concentrations, which supports our hypothesis that endotoxin, induces an all-or-nothing reaction. These findings are in agreement with our previous results on serially submerged cultured rat middle ear epithelium. From these results we conclude that endotoxin is an important factor in the disturbance of the morphology of the middle ear epithelium, which may lead to chronic otitis media with effusion. In addition, our tissue culture method proved to be a good model for further studies on human middle ear mucosa.

Introduction

Endotoxin from gram-negative bacteria like *Haemophilus influenzae* and *Moraxella catarrhalis* is frequently associated with otitis media with effusion (OME). Animal experiments have shown that transbullar injection of endotoxin, present on either viable or non-viable bacteria or in an isolated form, induced marked inflammatory effects (Nell & Grote, 1999; Okazaki et al., 1984; Nonomura et al., 1986). Because endotoxin is also found in middle ear effusions obtained from patients treated for OME with tympanostomy tubes (DeMaria et al., 1984) it is likely that endotoxin

plays an important role in the development of OME. However, the precise mechanism by which endotoxin causes OME is at present unknown.

In previous studies we demonstrated on serially cultured rat middle ear epithelium that endotoxin strongly stimulates the proliferation and the formation of tracks of cells with deviating morphology (van Blitterswijk et al., 1989; Hesseling et al., 1994). Recently we have developed an air-exposed culture method for middle ear epithelium on a collagenous underlayer (Hesseling et al., 1993). This new method is more comparable to the *in vivo* situation. For example, a collagenous underlayer is usually present *in vivo*, and might play an essential role in general differentiation. Furthermore, the middle ear mucosa *in vivo* is also air-exposed.

In the present study we investigated the effects of endotoxin on air-exposed cultured human middle ear epithelium.

Materials and Methods

Tissue culture

Middle ear epithelium was air-exposed cultured on a collagenous underlayer as described by Smola et al. (1993). Human middle ear epithelium biopsies were obtained from patients, without middle ear pathology, undergoing middle ear surgery at the ENT-department of the Leiden University Medical Center. The biopsies were placed in a sterile phosphate-buffered saline solution (PBS) and stored at 4°C until they were explanted, the same day, on a collagenous underlayer. After complete outgrowth of the explants, approximately after 7 days, the tissue was divided over 6 new collagenous underlayers. Two served as control, 2 were cultured with low endotoxin concentration (1 ng/ml) and

2 with high endotoxin concentration (100 ng/ml). Medium [DMEM (Gibco-BRL, UK) /Ham's F12 (ICN Pharmaceuticals, USA) 3:1, supplemented with 5% Hyclone bovine calf serum (Greiner) 0.4 µg/ml hydrocortisone, 1 µM L-isoproterenol (Sigma Chemical Co, USA) 1 ml ITS (5 µg/ml insulin, 5 µg/ml transferrin and 5 µg/ml selenous acid; Collaborative Biomedical Products, USA) 50 µg/ml L-ascorbic acid (Sigma), 1 ng/ml epidermal growth factor (Sigma), 50 µg/ml gentamycin (Gibco-BRL) and 2.5 µg/ml fungizone (Gibco-BRL)] was changed twice a week, and endotoxin was added. After 12 or 21 days, tissue was fixed for light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Endotoxin

The endotoxin used for this study was prepared from *Salmonella typhimurium* (Sigma, catalogue no. L-6511). It was chosen because of its biological similarity to endotoxin derived from non-typeable *H. influenzae* (Nakamura et al., 1992), which is one of the major pathogens in OME. Endotoxin was used in two different concentrations: 1 and 100 ng/ml. These concentrations are comparable with endotoxin concentrations found in MEE (DeMaria et al., 1984)

Morphological analysis

The tissue specimens were fixed with a solution of 1.5% glutaraldehyde in cacodylate buffer (0.14 M, pH 7.4) and subsequently dehydrated in a graded series of ethanol. For LM the specimens were subsequently embedded in paraffin. Sections were stained with Haematoxyline-Eosine (HE) for histological studies and for glycoprotein histochemistry with alcian blue-PAS.

For SEM the tissue specimens were critical point dried (Balzers CPD020) using liquid CO_2. After mounting and coating with gold in a Balzers MED010 sputtercoater, the distribution of the epithelial cells was studied with a Philips 525M scanning electron microscope at 15 kV. For TEM the tissue specimens were post-fixed in 1% osmium tetroxide in aqua dest for 30 minutes, rinsed with PBS and subsequently dehydrated in a graded series of ethanol. The samples were embedded in Epon. Following polymerization, ultra-thin sections were cut on a LKB ultratome (LKB, Bromma, Sweden), stained with uranyl acetate and lead citrate, and examined with a Philips EM410 transmission electron microscope at 80 kV.

Statistical analysis

Using LM, the thickness and the cell size were measured at 15 different spots for each endotoxin concentration. The cell size was measured along the length of the cell. Using SEM, 100 epithelial cells were counted in four different areas. The numbers of ciliated and goblet cells were expressed as a percentage. To compare means of the different variables, a one-way ANOVA least significance difference test with a significance level of $P<0.05$ was performed using the Statistical Package for the Social Sciences (SPSS).

RESULTS

Using low magnification LM, it was observed that cultures of 12 days were still not confluent. SEM confirmed this observation. Furthermore, it was clear that differentiation of the cells at this time was incomplete. Only low numbers of microvilli were observed, whereas ciliated or secretory cells were absent. Cultures of 21 days, on the other hand, were

confluent and well differentiated. Therefore, only the effect of endotoxin on tissue cultured for 21 days was evaluated.

Mucosa biopsies were obtained from the epitympanic part of the middle ear cavity and specimens cultured in the absence of endotoxin showed large, flat non-ciliated epithelium with few secretory cells (Fig. 1A). In contrast to this, the same cells cultured in the presence of endotoxin were thicker, i.e. multilayered (Fig. 1B), and a significant increase in the thickness of the mucosal layer was measured (Fig. 2). Both in the epithelium, the basal area and the supra-basal area, large vacuolar structures were visible. Observations after staining according to the PAS reaction showed that numerous secretory cells were present in the tissues cultured in the presence of endotoxin.

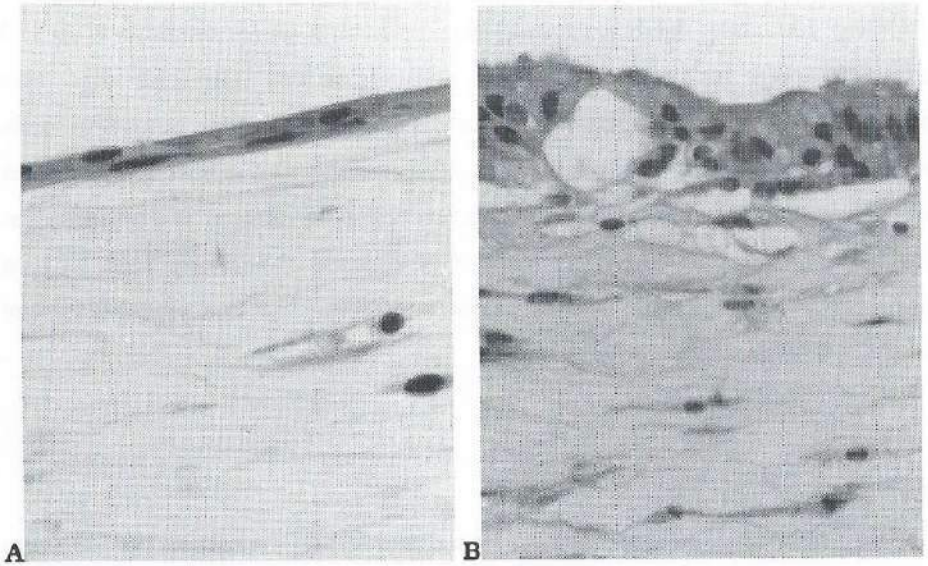

Figure 1: Light micrographs of human middle ear mucosa, air-exposed cultured for 21 days. **A)** 0 ng/ml endotoxin and **B)** 1 ng/ml endotoxin added twice a week to the culture medium (original magnification X200, HE staining). Endotoxin induced an increased proliferation of the epithelial layer with large vacuolar structures and large amounts of secretory cells.

SEM of the same cultures revealed the presence of small microvilli on the mucosal surface and sharp borders between the large, flat epithelial cells (Fig. 3A). In the absence of endotoxin, secretory (3.3%) and ciliated cells (11.5%) were infrequently observed. On the contrary, tissue cultured in the presence of endotoxin showed an increased number of secretory cells (fig. 3B). The percentage of goblet cells increased from 3 to 13% whereas no change in the amount of ciliated cells was measured (Fig. 4). Although the epithelial cells appeared to be smaller and more spherical, the average size of the cells was not significantly different when tissue was cultured with endotoxin. Furthermore, the borders between the cells were less sharp, and on the surface of the cells cultured with endotoxin more microvilli were present.

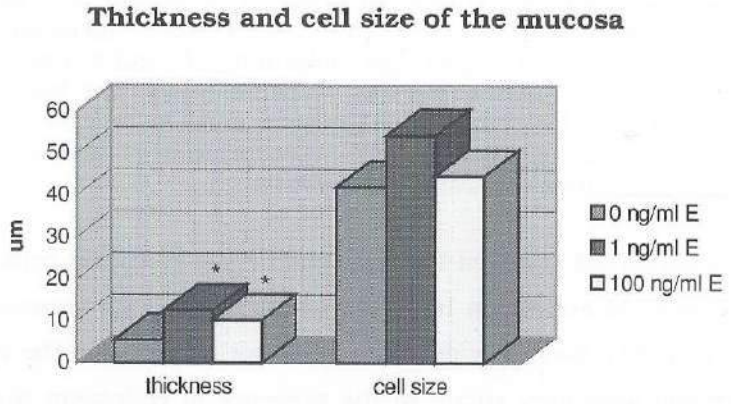

Figure 2: Thickness and cell size measured in the human middle ear mucosa cultured with different endotoxin (E) concentrations. Both endotoxin concentrations induced a significant (*) increase in the thickness of the epithelial layer compared to the control without endotoxin. No significant difference in cell size was found.

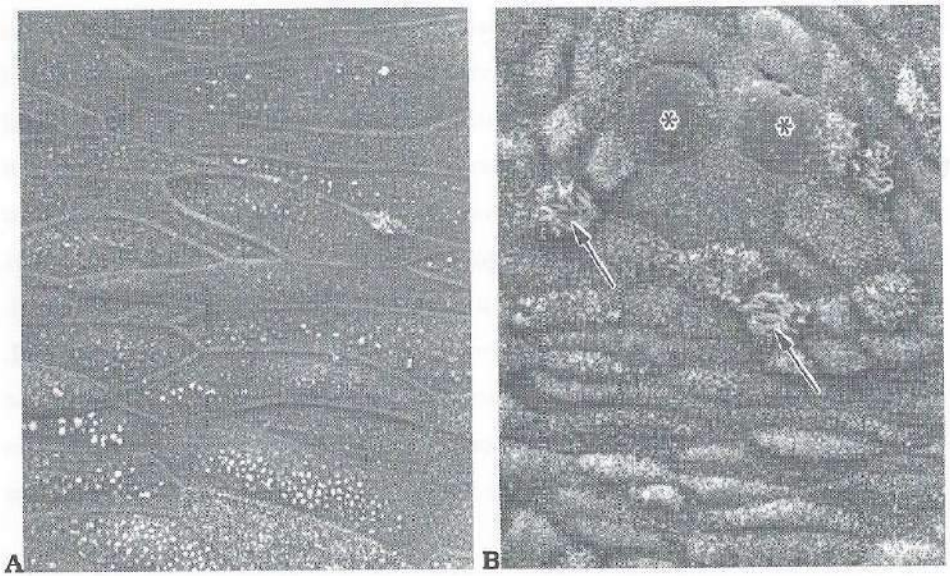

Figure 3: Scanning electron micrographs of human middle ear mucosa, air-exposed cultured for 21 days. **A)** 0 ng/ml endotoxin, and **B)** 1 ng/ml endotoxin added twice a week to the culture medium (original magnification X625). Due to the addition of endotoxin, increased numbers of secretory cells (asterisks) were observed. The borders of the epithelial cells were less sharp, furthermore, some cells were smaller and more spherical. Numerous microvilli were present on the cell surface, which were sometimes relatively long (arrows).

In addition, TEM revealed that the middle ear epithelial cells cultured in the absence of endotoxin had very small intercellular spaces between the cells (Fig. 5A). Secretory granules were not observed in the cytoplasm and microvilli were very small. In the presence of endotoxin the amount and length of the microvilli was increased (Fig. 5B). In the cytoplasm of the cells an abundant amount of granules was present.

Statistically significant changes were not observed between the high and the low endotoxin concentrations. Morphological changes observed using a concentration of 1 ng/ml endotoxin were also seen using 100 ng/ml: an increased thickness of the epithelial layer, an increased amount of secretory cells and an increase in amount and length of

microvilli. However, the intercellular space between the epithelial cells was more increased using 100 ng/ml endotoxin. In this intercellular space also an increased amount of large microvilli was present.

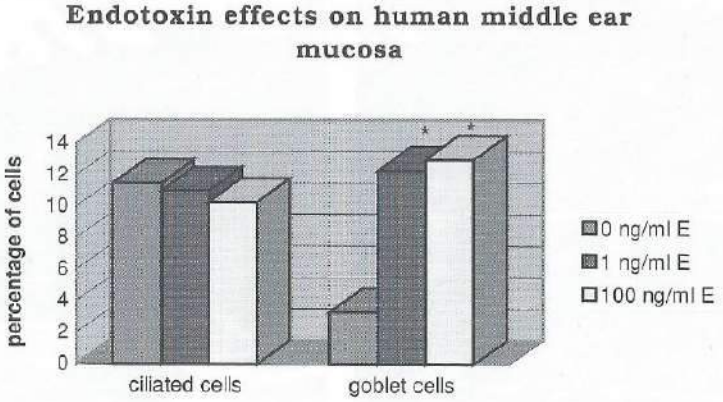

Figure 4: Percentage of ciliated- and goblet cells counted after 21 days of culturing human middle ear mucosa with 0, 1 or 100 ng/ml endotoxin (E). No significant differences in the percentages of ciliated cells were found. However, both endotoxin concentrations induced a significantly higher (*) percentage of secretory cells compared to no endotoxin ($P<0.05$).

Discussion

The middle ear mucosal surfaces are protected by complex biological defense mechanisms: immune response, secretory activity and mucociliary clearance (Lim & DeMaria, 1988; Park & Lim, 1993). Dysfunction of the mucociliary clearance system is considered to be an important mechanism in the development of OME. Middle ear effusion in OME, induced due to increased vascular permeability and increased

secretory activity, will accumulate in the middle ear cavity when ciliary activity is diminished (Ohashi et al., 1989).

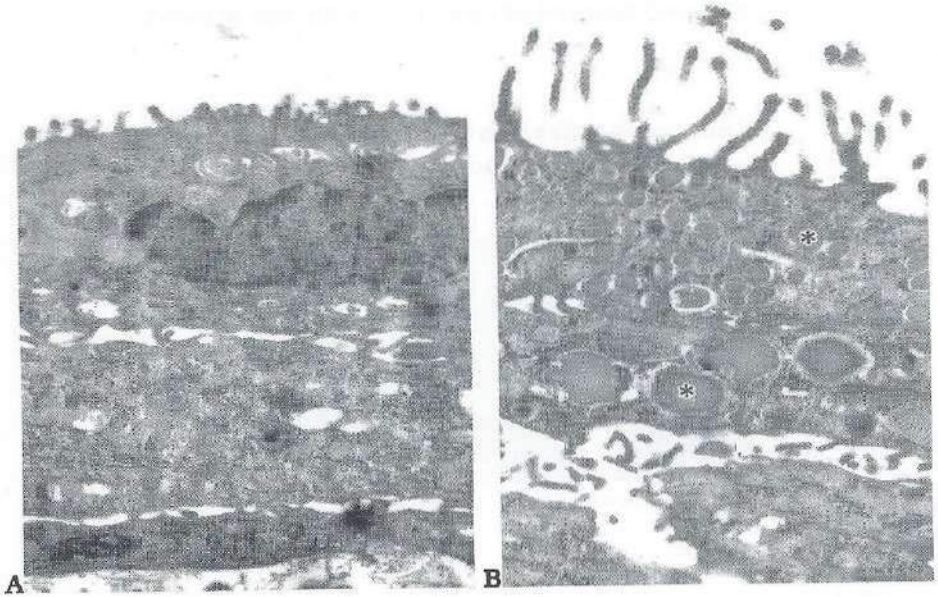

Figure 5: Transmission electron micrographs of human middle ear mucosa air-exposed cultured for 21 days. **A)** 0 ng/ml endotoxin and **B)** 100 ng/ml endotoxin added twice a week to the culture medium (original magnification X5900). Large intercellular spaces were present in cultures with high endotoxin concentrations. Furthermore, endotoxin induced granules (asterisks), which are probably secretory granules. The microvilli on the cell surface are relatively long compared to the cultures without endotoxin.

The results of our present study suggest that endotoxin has a direct effect on the middle ear epithelium. The significant increase in thickness of the mucosal layer and the presence of smaller, more spherical cells strongly suggest an increased proliferation. Addition of endotoxin to the culture medium influenced not only the proliferation, but also the formation of secretory epithelium. These findings correspond well with those described in other tissue culture experiments (Nakamura et al.,

1992) and animal experiments (Okazaki et al., 1984; Nonomura et al., 1986) in which endotoxin was added to the culture medium or to the middle ear cavity. Nonomura et al. (1986) found "ballooning" of ciliated cells and increased secretion by goblet cells due to inoculation of *H. influenzae* type b endotoxin in guinea pigs. Transformation of squamous epithelium into ciliated/secretory epithelium in the middle ear of conventionally raised animals after obstruction of the eustachian tube was observed by Kuijpers & Beek (1984). They concluded that these effects were due to the presence of micro-organisms.

In our tissue culture experiments endotoxin did not induce an increase in ciliated cells. However, the increased amount of microvilli and the increased microvillus length observed, suggests an increased involvement of the epithelium in fluid transport (Kuijpers & Beek, 1987). The low amount of ciliated cells is probably due to the fact that the tissue specimens were obtained from the epitympanum where normally almost no ciliated cells are present. On the other hand, in some animal experiments degeneration of cilia was observed. Ohashi et al. (1989) found approximately 40% degenerated ciliated cells seven days after injection of 10 µg/ml endotoxin. Degeneration of cilia in vivo is probably a result of increased concentrations of inflammatory mediators and endotoxin in the middle ear cavity, which can induce tissue damage.

We did not observe significant differences between both endotoxin concentrations used. A dose response relationship was not evident in a study by DeMaria et al. (1989) either. These authors found that, although a concentration of 0.1 ng endotoxin per ear did not result in any inflammatory changes in the middle ear, the same intensity of response was observed at all other dosage levels (0.01 - 100 µg/ear). Nakamura et al. (1992) found that a concentration of 100 µg/ml resulted in cell death of serially cultured chinchilla middle ear epithelium. In our previous

experiments (van Blitterswijk et al., 1989; Hesseling et al., 1994) with serially submerged cultured rat middle ear epithelium the number of cells increased with the endotoxin concentration from 1 ng to 100 µg. However, with rat meatal epidermis no relation between the concentration and the intensity of the reactions was evident (Hesseling et al., 1994).

The presented results suggest that the development of middle ear disease may be influenced by changes in the mucosal layer due to endotoxin leading to a disturbance of the mucociliary clearance system The tissue culture model used seems very suitable for further investigation of the effects on middle ear epithelium.

Chapter IV

Inhibition of Endotoxin effects on cultured human middle ear epithelium by Bactericidal/Permeability - Increasing Protein

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ABSTRACT

Hypothesis/Background: Endotoxin can induce morphological changes to middle ear epithelium, which can disturb the mucociliary clearance system (MCS) and lead to otitis media with effusion (OME). The Bactericidal/Permeability-Increasing (BPI) protein is a major component of neutrophil granules, and binds with high affinity to endotoxin. In this study, the capacity of $rBPI_{21}$, a recombinant amino-terminal analog derived from BPI, was investigated to inhibit the effects of endotoxin on cultured human middle ear epithelium using light microscopy, scanning electron microscopy and transmission electron microscopy.

Methods: Human middle ear epithelium was air-exposed cultured on a collagenous underlayer with different additions of endotoxin and BPI to the culture medium. The tissue specimens were examined after 4 weeks for the number of ciliated and secretory cells, thickness of the mucosal layer and cell size.

Results: Morphological changes induced by endotoxin were increased thickness of the mucosal layer and increased number of secretory cells. These changes were significantly diminished or even not present when endotoxin was added together with $rBPI_{21}$ to the culture medium.

Conclusion: $rBPI_{21}$ can inhibit morphological changes in the middle ear epithelium due to endotoxin. Hence, we believe that $rBPI_{21}$ can be a new therapeutic agent in the treatment of OME.

INTRODUCTION

Endotoxin is suspected to be an important mediator in the pathogenesis of otitis media with effusion (OME). In animal experiments transbullar injection of endotoxin, present on viable and non-viable bacteria or in an isolated form, caused marked inflammatory effects (DeMaria & Lim, 1985). Furthermore, endotoxin induced morphological changes on cultured middle ear epithelium (Hesseling et al., 1994; Nell et al., 1999). These morphological changes result in a disturbance of the mucociliary clearance system (MCS) of the middle ear, which plays an important role in elimination of substances from the middle ear toward the upper respiratory tract. Therefore, endotoxin may cause the accumulation of middle ear effusion and enhance the risk of concurrent infection. Recovery of the middle ear morphology may well be an important step in the recovery from chronic OME.

Bactericidal/permeability-increasing protein (BPI) is a naturally occurring molecule present in the granules of polymorphonuclear cells (PMNs), and it has been implicated in the host defensive response to gram-negative bacterial infections (Weiss et al., 1978; Elsbach & Weiss, 1998). In addition to having bactericidal properties, BPI binds with high affinity to the highly conserved lipid A portion of endotoxin and can inhibit its actions both *in vitro* (Marra et al., 1990) and *in vivo* (Ooi et al., 1991; Jin et al., 1995). Previous investigations have shown that a 21 kDa recombinant amino-terminal fragment of BPI ($rBPI_{21}$) protects animals against the effects of gram-negative bacteria and endotoxin (Ammons et al., 1996). Furthermore, in man, $rBPI_{21}$ appears safe and non-immunogenic and is in Phase II/III clinical trials with apparent therapeutic benefit (Elsbach & Weiss, 1998; Giroir et al., 1997).

This report examined whether $rBPI_{21}$ can inhibit morphological changes to the middle ear epithelium due to endotoxin.

Materials and Methods

Tissue culture

The human middle ear epithelium used in this study was obtained from patients undergoing middle ear surgery. The biopsies were air-exposed cultured on a collagenous underlayer as described by Smola et al. (1993). After complete outgrowth of the explants (approximately after 7 days) the tissue was divided over 7 new collagenous underlayers and cultured for 4 more weeks. Twice a week, medium was changed with different additions of endotoxin and/or $rBPI_{21}$ (see Table 1). These experiments were performed in duplicate with tissue obtained from 2 different patients.

Endotoxin / BPI

Lipopolysaccharide from *Salmonella typhimurium* (Sigma, L-6511) was used as endotoxin. It was used because earlier studies proved its reactivity with middle ear tissues and because of its biological similarity to endotoxin derived from non-typeable *Haemophilus influenzae* (Nakamura et al., 1992). The final endotoxin concentration in the culture medium was 1 ng/ml. $rBPI_{21}$ was obtained from XOMA (US) LLC. (Berkeley, Ca., USA), and was used at a final concentration of 1 µg/ml $rBPI_{21}$; to ensure complete neutralization of endotoxin (Marra et al., 1994). Three groups served as control: CI) without addition of endotoxin or $rBPI_{21}$ to the medium, CII) with 1 ng/ml endotoxin, and CIII) with 1 µg/ml $rBPI_{21}$. Four others were used as experimental group: EI) 1 week endotoxin addition followed by 3 weeks $rBPI_{21}$, EII) 2 weeks endotoxin addition followed by 2 weeks $rBPI_{21}$, EIII) 1 week endotoxin addition followed by 3 weeks endotoxin premixed with $rBPI_{21}$, and EIV) 4 weeks addition of endotoxin premixed with $rBPI_{21}$.

Microscopy

After four weeks, tissue specimens were fixed in 1.5% glutaraldehyde in 0.14 M cacodylate buffer, pH 7.4, at 4°C for two days, and for transmission electron microscopy (TEM) post-fixed in 1% osmium tetroxide for 30 minutes, and rinsed with phosphate-buffered saline (PBS). The samples were subsequently dehydrated in a graded series of ethanol. For light microscopy (LM) the specimens were embedded in paraffin. Sections were stained with Haematoxyline-Eosine (HE) for histological studies and with alcian blue-PAS for glycoprotein histochemistry. For scanning electron microscopy (SEM) the samples were critical point dried using liquid CO_2, gold coated in a Balzers MED010 sputtercoater, and studied with a Philips 525M scanning electron microscope. The TEM samples were embedded in Epon. After polymerization, ultra-thin sections were cut on an LKB ultratome (LKB, Bromma, Sweden), stained with uranyl acetate and lead citrate, and examined with a Philips EM410 transmission electron microscope at 80 kV.

Quantitative Analysis

The tissues were evaluated by LM, SEM and TEM. Using LM the thickness of the mucosal layer was measured. The size of the epithelial cells in diameter was measured using SEM. Furthermore, the percentages of ciliated- and secretory cells were counted in at least five different areas using SEM. To compare means of the different variables, a one-way ANOVA least significance difference test with a significance level of $P<0.05$, was performed using the Statistical Package for the Social Sciences (SPSS). Results are reported as mean concentration ± standard error of the mean (SE).

RESULTS

Untreated

The middle ear epithelium cultured without addition of endotoxin or $rBPI_{21}$ to the culture medium was predominantly composed of flat and usually thin large polygonal epithelial cells with varying numbers of microvilli (Figs. 1A, 2A). The mucosal layer consisted of only one or two cell-layers with a thickness of 15.5 ± 2.4 µm. Occasionally some ciliated cells (11.5 ± 1.2 %) and very few secretory cells (3.3 ± 0.6 %) were present. The average cross-section of the cells was 30.6 ± 3.0 µm (Table 1). Furthermore, with TEM it was observed that epithelial cells contained some electron dense inclusions, presumably residual bodies, and that the microvilli had a normal appearance (Fig. 3A).

Endotoxin addition

Addition of 1 ng/ml endotoxin to the culture medium induced hyperproliferation of the mucosal layer (Fig. 1B). The thickness of the mucosal layer significantly increased to 29.4 ± 5.6 µm and was multi-layered. Diameter and percentage of ciliated cells were not significantly different from the untreated control group (Table 1). However, in some areas the thickness of the mucosal layer was dramatically increased. In these areas, cilia were abundantly present and were disorganized (Fig. 2B). The epithelial cells also possessed an increased amount of microvilli on their surface, which were irregularly formed and generally longer compared to the untreated cells (Fig. 3B). Furthermore, endotoxin induced a significant increase in the number of secretory cells (12.3 ± 0.5 %, Fig. 2C).

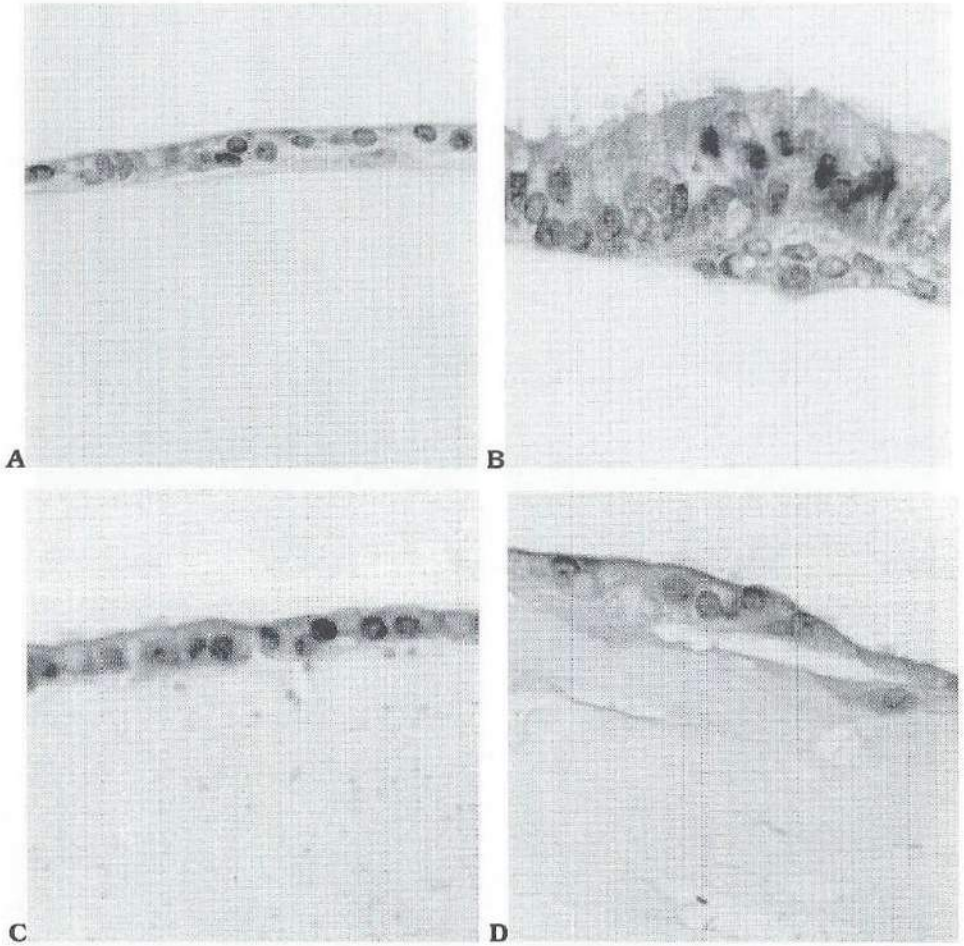
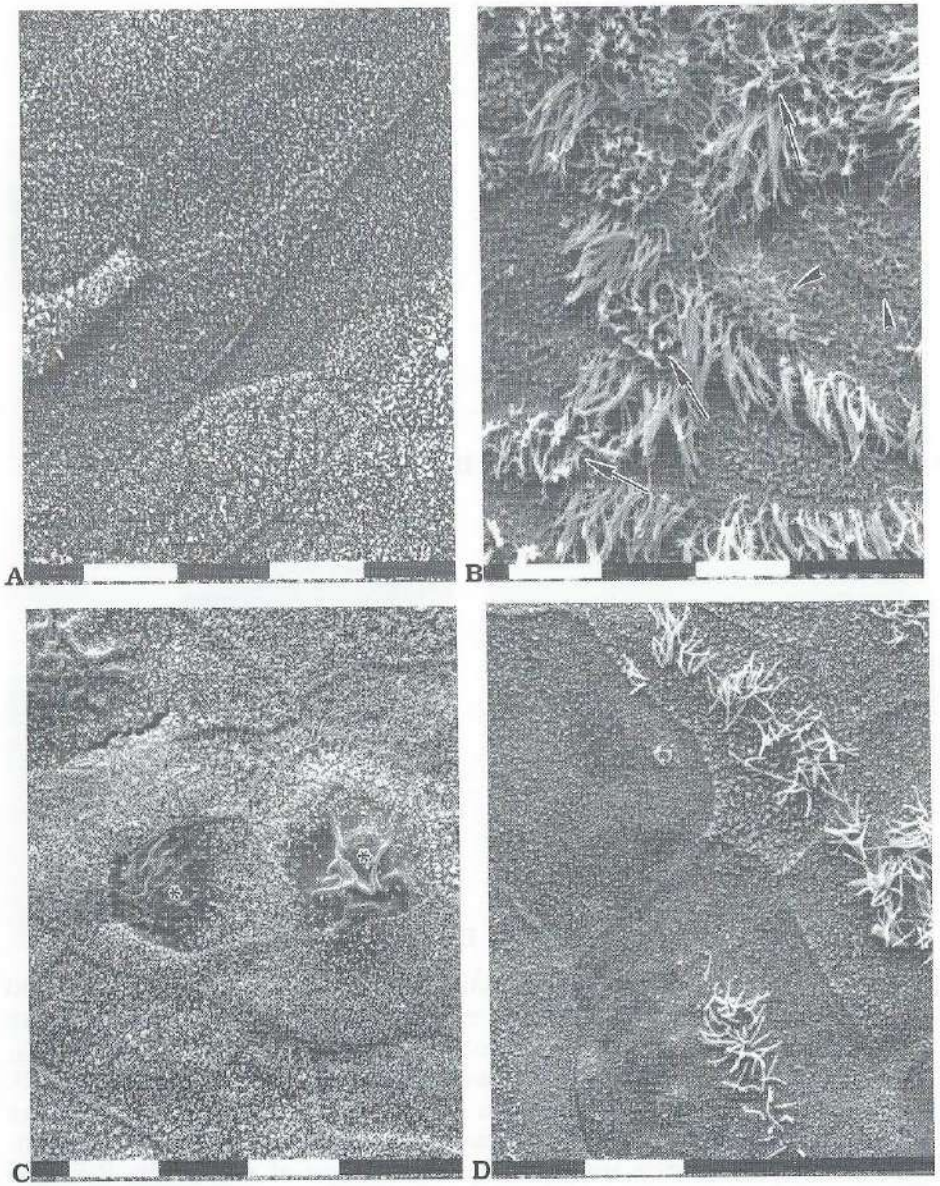


Figure 1: LM micrographs of human middle ear mucosa, air-exposed cultured for 4 weeks. **A)** Without endotoxin or $rBPI_{21}$ in the culture medium (CI), **B)** with 1 ng/ml endotoxin (CII), **C)** with 1 µg/ml $rBPI_{21}$ (CIII), and **D)** 1 week endotoxin + 3 weeks $rBPI_{21}$ (EI). HE staining, X200. Endotoxin induced hyperproliferation of the mucosal layer. In some areas cilia were abundantly present and were disorganized due to endotoxin. $rBPI_{21}$ did not affect the mucosal layer. Addition of $rBPI_{21}$ for 3 weeks after one week culturing with endotoxin showed a normalized mucosal layer with some cilia, which appeared not to be disorganized.



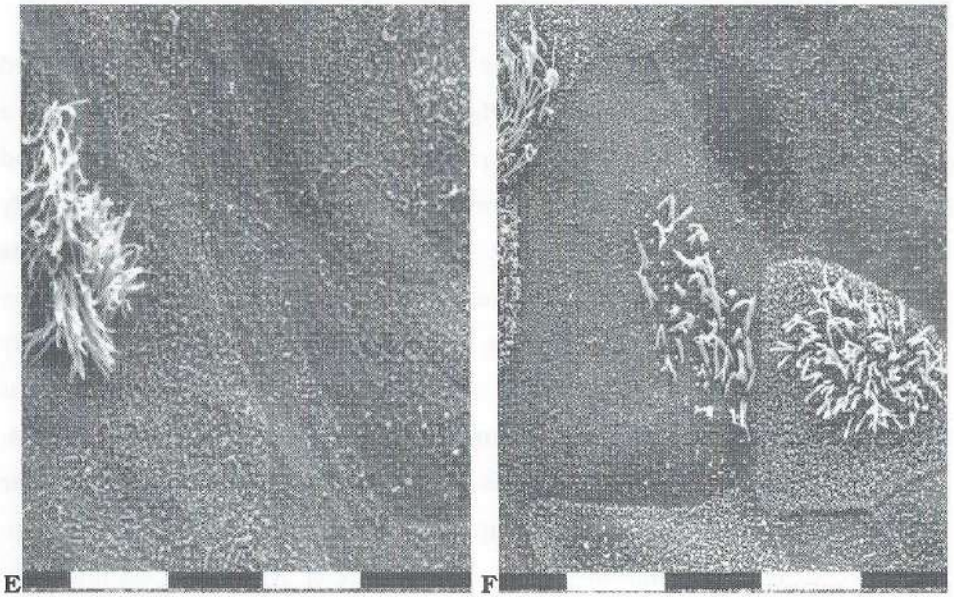


Figure 2: SEM micrographs of human middle ear mucosa, air-exposed cultured for 4 weeks. **A)** Without endotoxin or $rBPI_{21}$ in the culture medium (CI), **B/C)** with 1 ng/ml endotoxin (CII), **D)** with 1 µg/ml $rBPI_{21}$ (CIII), **E)** 1 week endotoxin + 3 weeks E/BPI (EIII), and **F)** 4 weeks E/BPI (EIV) (original magnification X1250, bar = 10 µm. Endotoxin induced an increase in the number of secretory cells (asterisks), disorganized cilia (arrows), and anomalous microvilli (arrowheads). Cilia seen after addition of $rBPI_{21}$ had a normal appearance.

BPI addition

Addition of $rBPI_{21}$ to the culture medium resulted in more cubical epithelial cells. The epithelial layer consisted of only one or two cell-layers with a thickness of 15.2 ± 2.8 µm, which was not significantly different from the untreated group (Fig 1C). The percentage of ciliated cells was not significantly increased (Table 1) and the cilia had a normal appearance (Fig. 2D). Furthermore, no increase in the number of secretory cells was observed. With TEM some residual bodies were observed and microvilli appeared to be normal.

Endotoxin / BPI addition

In the experimental groups we can separate those which received first endotoxin followed by $rBPI_{21}$, from those which received a combination of endotoxin and $rBPI_{21}$ together (see table 1). In both EI and EII experimental groups, the thickness of the mucosal layer was slightly but not significantly increased (Fig. 1D). The percentage of ciliated cells was slightly increased in the EI group and significantly increased in the EII group, whereas the percentage of secretory cells was significantly increased in both groups (Table 1). These results were not very different from endotoxin addition to the culture medium for 4 weeks. Nevertheless, in the last two experimental groups, the thickness of the mucosal layer was not significantly increased compared to the control untreated group. The percentages of ciliated- and secretory cells were not significantly changed in the EIII group. In the EIV group, on the other hand, the percentage of ciliated cells was significantly increased. However, no disorganized cilia were observed (Fig. 2F). Finally, the percentage of secretory cells was not significantly changed in both last experimental groups (EIII and EIV). Microvilli appeared normal in all experimental groups and with TEM residual bodies were also observed (Fig. 3C).

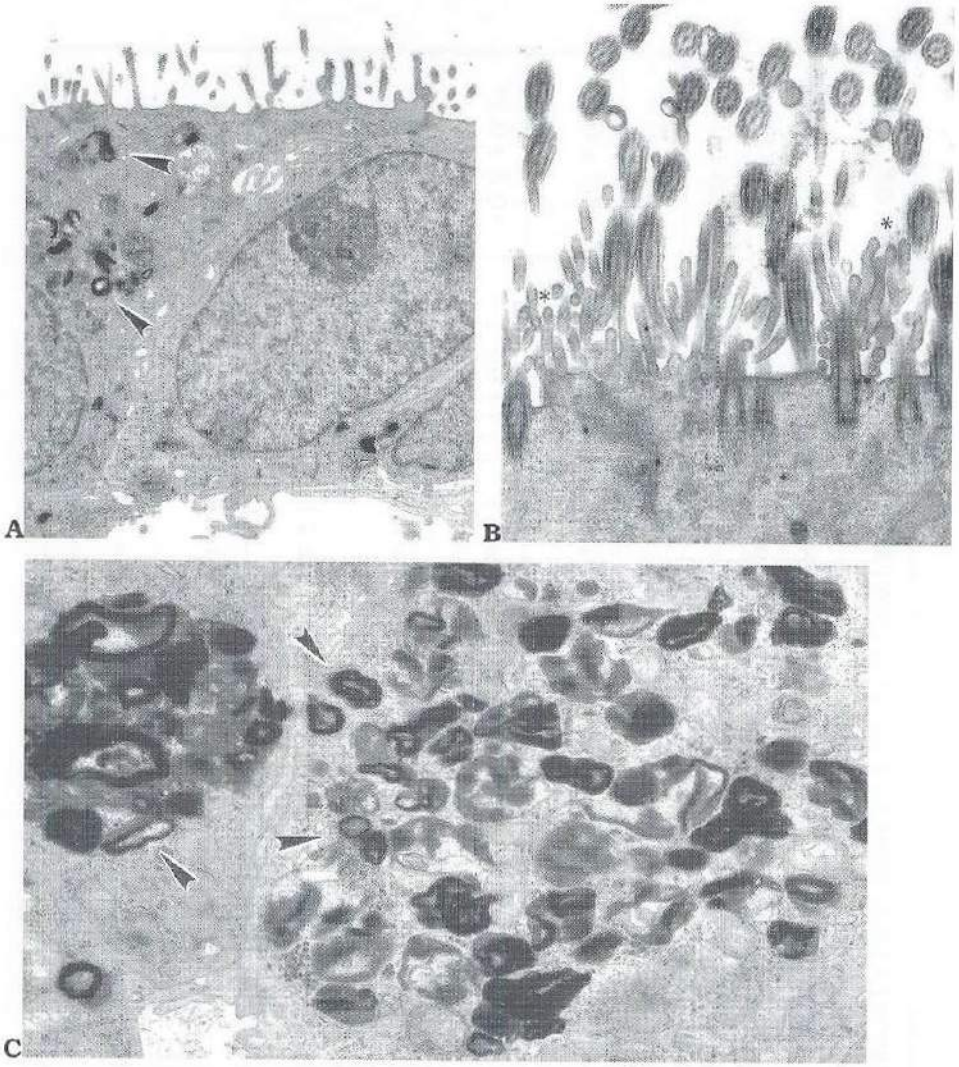


Figure 3: TEM micrographs of human middle ear mucosa, air-exposed cultured for 4 weeks. **A)** Without endotoxin or $rBPI_{21}$ in the culture medium (CI) original magnification X4400, **B)** with 1 ng/ml endotoxin (CII) original magnification X10400, and **C)** 1 week endotoxin + 3 weeks E/BPI (CIII) original magnification X5900. Endotoxin induced some very outsized microvilli, which were sometimes anomalous (asterisks). In some experimental groups lots of residual bodies were present, however, also the untreated middle ear epithelium contained some residual bodies (arrowheads).

TABLE 1: QUANTITATIVE RESULTS OF THE CONTROL AND EXPERIMENTAL GROUPS

Group	*Additions*	*Thickness* (µm)	*Diameter* (µm)	*Ciliated cells* (%)	*Secretory cells* (%)
CI	-	15.5 ± 2.4	30.6 ± 0.3	11.5 ± 1.2	3.3 ± 0.6
CII	1 ng/ml E	29.4 ± 5.6*	28.4 ± 2.9	11.0 ± 2.7	12.3 ± 0.5*
CIII	1 µg/ml $rBPI_{21}$	15.2 ± 2.8	24.7 ± 2.7	12.0 ± 0.7	3.7 ± 0.7
EI	1 wk E + 3 wk $rBPI_{21}$	19.0 ± 2.7	31.5 ± 3.1	14.0 ± 1.8	10.8 ± 0.9*
EII	2 wk E + 2 wk $rBPI_{21}$	17.7 ± 2.3	31.4 ± 2.3	16.0 ± 0.9*	11.0 ± 0.9*
EIII	1 wk E + 3 wk E/$rBPI_{21}$	15.5 ± 2.1	26.5 ± 2.8	12.5 ± 1.3	3.5 ± 0.6
EIV	4 wk E/$rBPI_{21}$	13.2 ± 2.5	25.2 ± 2.6	18.5 ± 1.9*	1.5 ± 0.6

C = control groups, E = experimental groups, wk = weeks, E = endotoxin (1ng/ml), $rBPI_{21}$ = 1 µg/ml, * significantly different from CI with $P<0.05$

DISCUSSION

In the current study we used a newly developed culture method for human middle ear epithelium (Nell et al., 1999; Smola et al., 1993). With this method it is now possible to study the effects of different incubation conditions on human middle ear epithelium, which is cultured under air-exposed conditions. Due to this air-exposed culture technique, middle ear epithelium can proliferate and differentiate to secretory and ciliated cells like in the *in vivo* situation. This combination of secretory and ciliated cells forms the MCS of the middle ear, which is responsible for the clearance of the middle ear cavity. To maintain an appropriate clearance activity of this system, the rheologic properties of the mucus and its coupling with ciliary bundles are considered to be critical (Takasaka & Kawamoto, 1985).

In our study we found that 1 ng/ml endotoxin induced hyperproliferation of the epithelial layer and a significant increase in the number of secretory cells. Furthermore, cilia were disorganized and microvilli were anomalous. In previous studies by our group, Grote et al. (1995) found clustering of microvilli due to addition of endotoxin to the culture medium of submerged tissue cultures, which could not be explained. Kuijpers & Beek (1987) found an increase in microvillus-length after eustachian tube obstruction in rats. They suggested that an increased microvillus-length would implicate an increased involvement of the epithelium in fluid transport. Ohashi et al. (1987) found that endotoxin *in vitro* caused dysfunction of cilia in the tympanic cavity. Furthermore, in a morphological study it was found that endotoxin induced degeneration of ciliated cells and formation of compound cilia 7 days after inoculation of endotoxin into the middle ears of guinea pigs (Ohashi et al., 1989). In our study, however, we did not observe compound cilia. On the other hand, we did observe disorganized cilia,

which probably have less beating activity. Diminished activity of cilia in the middle ear together with the increased secretory activity is in all probability responsible for the accumulation of fluid in the tympanic cavity. Therefore, we believe that improvement of the MCS may result in clinical benefit.

In this study we investigated whether neutralization of endotoxin could inhibit morphological changes to the middle ear epithelium. To bind and to clear endotoxin, several monoclonal antibodies directed against bacterial lipopolysaccharide have been developed. In an earlier study we evaluated the effects of HA-1A, a human monoclonal antibody against endotoxin, on rat middle ear epithelial cells stimulated by endotoxin (Grote et al., 1995). The results of that study showed that HA-1A significantly inhibited the proliferative effects induced by endotoxin. However, the morphological effects of endotoxin on these cells were, although weaker, still present. Furthermore, these antibodies have shown unsatisfactory endotoxin-neutralizing capacities (Marra et al., 1994; Warren et al., 1993).

Bactericidal/permeability-increasing protein, an endogenous anti-infective molecule was first described in 1978 by Weiss et al. as an antibiotic protein, and has been shown to bind avidly to a wide array of endotoxin chemotypes and neutralize endotoxin activity. In a prospective, randomized, placebo-controlled laboratory study Marra, et al. (1994) showed that no monoclonal antibody used in their study was as effective as BPI at binding or neutralizing endotoxin *in vitro* or *in vivo*. Therefore, we studied the effects of $rBPI_{21}$, a recombinant amino-terminal analog fragment of BPI, on neutralization of endotoxin-induced morphological changes on air-exposed cultured human middle ear epithelium.

We found that $rBPI_{21}$ did not induce significant changes to the middle ear morphology. However, the morphological changes induced by

endotoxin were significantly inhibited by $rBPI_{21}$. Particularly when $rBPI_{21}$ was added simultaneously with endotoxin to the culture medium, morphological changes were reduced to an increase in the number of ciliated cells, which had a normal morphology. When $rBPI_{21}$ was added after endotoxin addition, morphological changes were, although weaker, still present. This suggests that $rBPI_{21}$ is most effective when endotoxin is still present and that $rBPI_{21}$ is able to restore the MCS of the middle ear. From a review on drugs affecting the clearance of middle ear secretions, it was clear that very few drugs are available that can be used in management of OME (Blumer, 1998; Giebink, 1992). Lin et al. (1996) studied the effect of a Platelet Activating Factor (PAF) receptor inhibitor on *in vitro* mucus glycoprotein secretion (MGP). They found that this inhibitor did not completely eliminate MGP secretion induced by PAF. In an *in vivo* study, Hori et al. (1994) demonstrated that S-carboxy-methylcysteine (S-CMC) in chinchillas with immune-mediated OME induced a reduction of the damage to ciliated cells and reduced goblet cell hyperplasia. However, S-CMC did not act on the infiltrating inflammatory cells to prevent the release of chemical mediators such as histamine and prostaglandin E2. In other studies, anti-inflammatory drugs were able to block some inflammatory mediators or secretion by local mast cells (Goldie et al., 1993; Takahashi et al., 1997). However, they will not neutralize endotoxin. Furthermore, the antibacterial effects of antibiotics may include increased shedding of endotoxin by damaged bacteria, thereby actually aggravating clinical symptoms (Elsbach & Weiss, 1995).

In conclusion, our study strongly suggests that the most important morphological changes induced by endotoxin that lead to a disturbance of the MCS are inhibited by $rBPI_{21}$ when endotoxin is still present. Therefore, we believe that $rBPI_{21}$ might be of clinical benefit in the management of OME.

Chapter V

Structural changes in the rat middle ear mucosa due to endotoxin and eustachian tube obstruction

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A preliminary report of this was study was presented at the 7th International Congress of Pediatric Otorhinolaryngology, June 7-10, 1998, Helsinki, Finland, and published on CD-ROM (*Advance in Pediatric Otorhinolaryngology* 1999, article 106)

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Abstract

This study was performed to investigate the *in vivo* effects of endotoxin on the morphology of the middle ear epithelium. The middle ears of 48 rats were used to examine the effects of endotoxin injection, eustachian tube obstruction (ETO) or a combination of ETO and endotoxin injection. Animals were killed after 1, 2, 4 or 12 weeks and the middle ears were processed for light microscopy and scanning electron microscopy. Compared to the normal middle ear mucosa, the epithelial layer was more pseudostratified, cuboidal or cylindrical after endotoxin injection or ETO. In the early phase, numerous ciliated cells occurred in areas originally almost devoid of these cells. At 3 months, degeneration of ciliated cells was observed. The combination of ETO and endotoxin injection also induced a more pseudostratified, cuboidal or cylindrical epithelium with an increased number of goblet cells. However, an early decrease occurred in the number of ciliated cells in the tympanic orifice. Furthermore, inflammatory cells, mainly PMNs, macrophages and lymphocytes, invaded the subepithelial layer after ETO and endotoxin injection. These structural changes resulted in an impairment of the mucociliary transport system for clearance of the middle ear cavity. For this reason we believe that both endotoxin and eustachian tube obstruction or dysfunction play an important role in inducing persistent mucosal changes in the middle ear cavity, thereby prolonging otitis media with effusion.

Introduction

Bacterial infection of the middle ear and obstruction or dysfunction of the eustachian tube are believed to be the major causative factors for otitis media with effusion (OME) in children. Experimental tubal occlusion causes an effusion of serous fluid into the middle ear cavity and promotes the pathogenic behavior of micro-organisms normally present in germ-carrying rats (Kuijpers et al., 1979; Kuijpers et al., 1984). OME can also be induced by inoculation of non-viable *Haemophilus influenzae* (DeMaria et al., 1984). A part of the outer membrane of this organism, and of most other gram-negative bacteria, is the lipopolysaccharide endotoxin (Morrison & Ulevitch, 1978). Endotoxin is a strong inducer of inflammation and a modulator of the immune response and has been shown to induce a disturbance of the mucociliary clearance system (MCS) (Ohashi et al., 1989). Previous results from our laboratory have also shown that endotoxin has a prominent effect on serially cultured rat middle ear epithelium (van Blitterswijk et al., 1989).

Since probably both tubal occlusion and an inflammatory reaction in the middle ear are involved in OME, it is conceivable that both contribute to the chronicity of OME. To the best of our knowledge, the long-term effects of this occlusion combined with inflammation remain to be investigated. Therefore, this study was devised to elucidate the structural changes in the middle ear mucosa after endotoxin injection alone, ETO alone, and endotoxin injection in combination with ETO, and to obtain a better understanding of the factors that are important in the development of chronic OME.

MATERIALS AND METHODS

Animals

Forty-eight healthy 10-week-old female Wistar rats (bodyweight, about 200 g) were used in this study. During anesthesia with nitrous oxide each eustachian tube (ET) was exposed by a ventral approach, medial to the posterior belly of the digastric muscle, and obstructed by plugging a small piece of Gelfoam (Upjohn Co., USA) into the tube. When used, endotoxin was injected through the tympanic membrane until the solution overflowed. The endotoxin employed was prepared from *Salmonella typhimurium* (Sigma; L-6511) and was chosen because of its similarity to endotoxin derived from non-typeable *H. influenzae* (Nakamura et al., 1992). Injection of 50 µl of a solution of 100 µg/ml endotoxin resulted in a final concentration of approximately 5 µg endotoxin per ear.

After 1, 2, 4 or 12 weeks, the animals were killed with CO_2 gas and subsequently decapitated. The middle ear was dissected from the skull, denuded of adhering tissues and further processed for light microscopy (LM) and scanning electron microscopy (SEM). For each time period 6 ears served as a control (untreated or PBS injected), 6 ears served as endotoxin experimental group, 6 as ETO experimental group, and 6 as ETO+E experimental group. These 6 ears were divided for LM and SEM examination (see Table 1).

Light microscopy

Specimens were fixed with a solution of 1.5% glutaraldehyde in 0.14 M cacodylate buffer at pH 7.4, decalcified with a solution of 10% EDTA and 1.5% glutaraldehyde in 0.14 M cacodylate buffer at pH 7.4 and subsequently dehydrated in a graded series of ethanol. Tissues were then

embedded in glycol methacrylate (JB4, Brunschwig Chemie, Amsterdam, The Netherlands). Sections were stained with toluidine blue for histological studies and alcian blue-PAS for glycoprotein histochemistry.

Scanning electron microscopy

Part of the specimens, prepared as described for LM, were processed for SEM. For this purpose, after fixation specimens were dehydrated in a graded series of ethanol and critical point dried using liquid CO_2. The distribution of the epithelial cells was studied with a Philips 525M scanning electron microscope after mounting and coating with gold in a Balzers MED010 sputtercoater.

TABLE 1: ***Distribution of rat middle ears***

	1 week		2 weeks		4 weeks		12 weeks		Total ears
	LM	SEM	LM	SEM	LM	SEM	LM	SEM	
No injection	1	1	1	1	1	1	1	1	8
PBS	2	2	2	2	2	2	2	2	16
E	4	2	4	2	4	2	4	2	24
ETO	4	2	4	2	4	2	4	2	24
ETO + E	4	2	4	2	4	2	4	2	24
Total	24		24		24		24		96

Schedule of the distribution of rat middle ears following eustachian tube obstruction and/or exposure to endotoxin (LM=light microscopy, SEM=scanning electron microscopy, PBS=phosphate-buffered saline, E=endotoxin, ETO=eustachian tube obstruction, ETO+E=eustachian tube obstruction + endotoxin injection; 5 µg/ear)

Results

Light microscopy demonstrated the presence of extremely small, one-layered squamous epithelium in the hypotympanum, containing very few ciliated cells (Fig. 1A). Compared to no injection, PBS did not induce structural changes in the middle ear mucosa. Injection of endotoxin induced thickening of the middle ear mucosa due to vasodilatation, edema, and infiltration of polymorphonuclear cells (PMNs), macrophages and lymphocytes into the subepithelial layer. The epithelial layer had become more pseudostratified and cuboidal while numerous ciliated cells occurred in areas originally almost devoid of these cells (Fig. 1B).

Eustachian tube obstruction induced similar changes in the hypotympanum as endotoxin injection. The combination of ETO and endotoxin injection also induced a thickening of the middle ear mucosa due to vasodilatation, edema and infiltration of PMNs, macrophages and lymphocytes into the subepithelial layer. However, the amount of infiltrated cells, particularly PMNs, was much higher than after endotoxin injection or ETO alone. During the first 2 weeks mainly PMNs were observed, later macrophages and lymphocytes were also present.

Compared to PBS injection, fewer ciliated cells were observed after ETO + E (Fig. 1C). Three months after endotoxin injection, ETO or ETO + E, the epithelial layer in the hypotympanum was still more pseudostratified and cuboidal, and some new bone formation could be seen. However, at this time, less vasodilatation, edema or cell infiltration was observed (Fig. 1D).

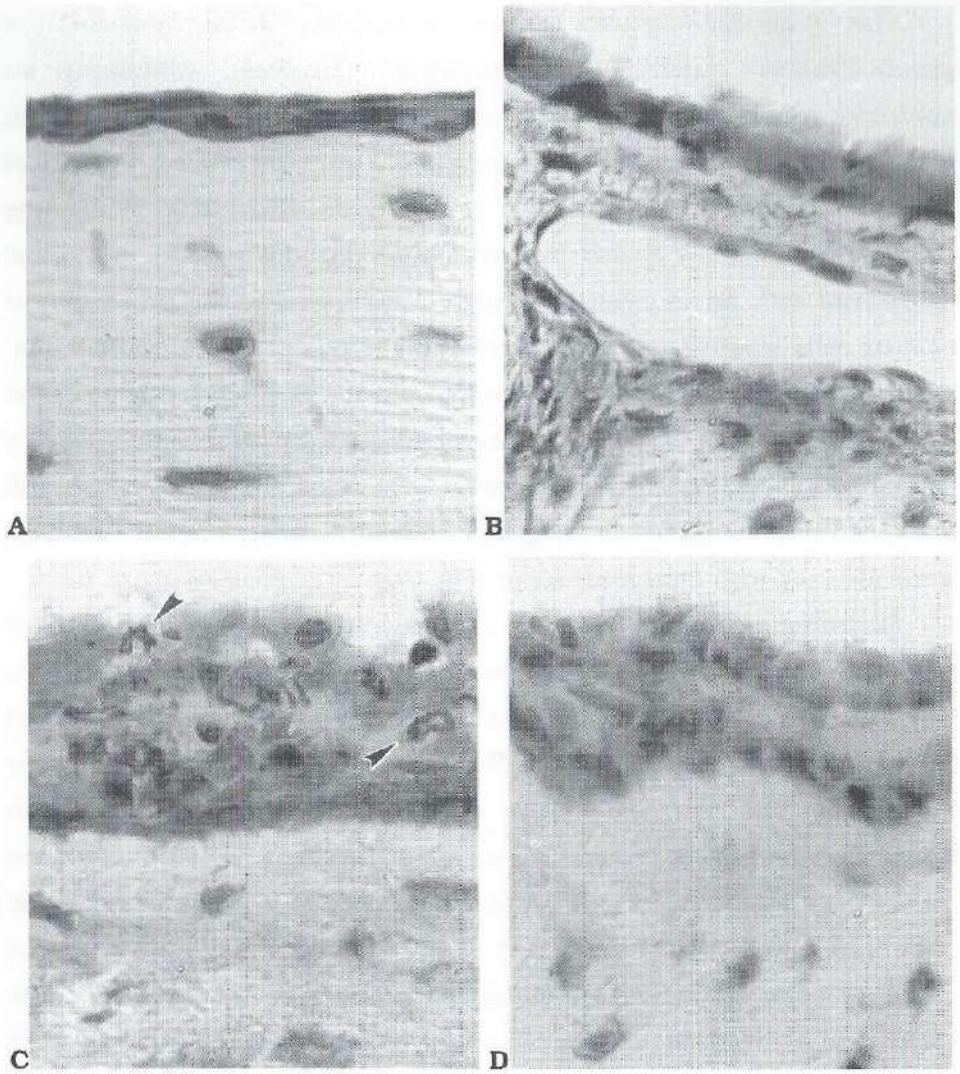

Figure 1: Light micrographs of the middle ear mucosa in the hypotympanum after: **A)** PBS injection; **B)** endotoxin (5 μg/ear) injection, 2 weeks; **C)** ETO + E, 1 week; and **D)** endotoxin injection, 12 weeks. (Toluidine blue staining, original magnification X400). Endotoxin induced an increase in ciliated cells, 2 weeks after application. Twelve weeks after application of endotoxin the mucosal layer was still thickened, cells were more cuboidal but hardly any ciliated cells were observed. ETO+E induced a large infiltration of inflammatory cells (arrowheads).

The tympanic orifice of the eustachian tube consisted of pseudostratified, cuboidal or cylindrical epithelium, containing an abundant number of ciliated cells and a few goblet cells (Fig. 2A). Both endotoxin injection and ETO induced a more pseudostratified, cylindrical epithelium with an increase in goblet cells (Fig. 2B). While the combination of ETO + E also induced a more pseudostratified, cylindrical epithelium with an increased estimate of goblet cells, the proportion of ciliated cells was lower after 1 week. Furthermore some vasodilatation, edema and cell infiltration were observed but not as abundantly as in the hypotympanum (Fig. 2C). Three months after endotoxin injection, ETO or endotoxin injection in combination with ETO, the epithelial layer was still thickened and contained many goblet cells, but hardly any ciliated cells were observed with light microscopy (Fig. 2D).

Scanning electron microscopy demonstrated the presence of a few microvilli on the squamous epithelium in the hypotympanum, which contained few ciliated cells (Fig. 3A). While endotoxin injection induced the formation of new ciliated cells between other flat epithelial cells after 1 week (Fig. 3B), the combination of ETO + E induced an increase in microvilli, which were preferably located at the borders of the epithelial cells (Fig. 3C). One month after endotoxin injection or ETO the epithelial cells were swollen and contained many ciliated cells (Fig. 3D). The epithelial cells contained an abundant number of microvilli and the surface was irregular 3 months after endotoxin injection, ETO or ETO + E.

The epithelial layer of the tympanic orifice contained numerous ciliated cells and some goblet cells (Fig. 4A). Endotoxin injection induced an increase in ciliated cells after 1 week (Fig. 4B), whereas ETO and the combination of ETO + E induced a decrease in the proportion of ciliated cells relative to the goblet cells (Fig. 4C). However, after 1 month an

increase in goblet cells and a decrease in ciliated cells were observed in all experimental groups; this was even more prominent after 3 months. Severely swollen squamous epithelium (i.e., cobblestone-like cells) was induced beside the tracks of ciliated cells in the tympanic orifice 3 months after ETO + E (Fig. 4D).

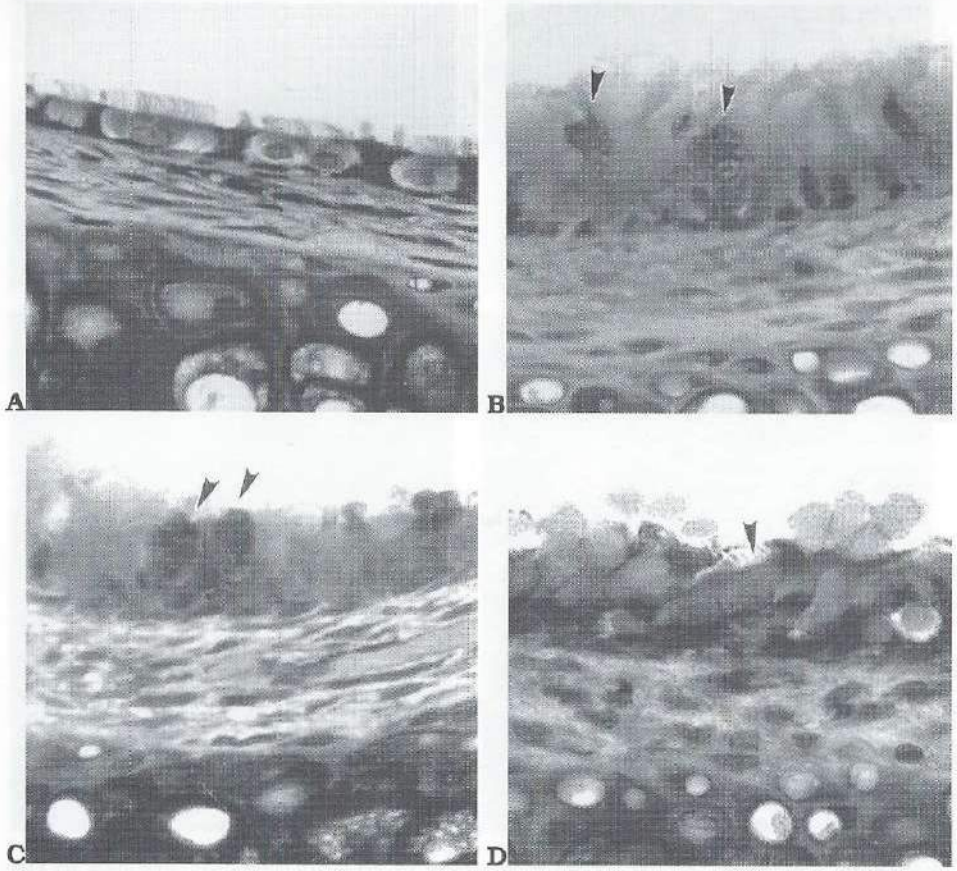

Figure 2: Light micrographs showing the middle ear mucosa near the tympanic orifice of the eustachian tube after: **A)** PBS injection; **B)** endotoxin injection (5 µg/ear), 1 week; **C)** ETO + E, 1 week; and **D)** endotoxin injection, 12 weeks. (Toluidine blue staining, original magnification X400). Compared to PBS injection all the experimental groups showed an increase in secretory cells (arrowheads). ETO+E induced a direct decrease in ciliated cells. Twelve weeks after endotoxin application hardly any ciliated cells were seen.

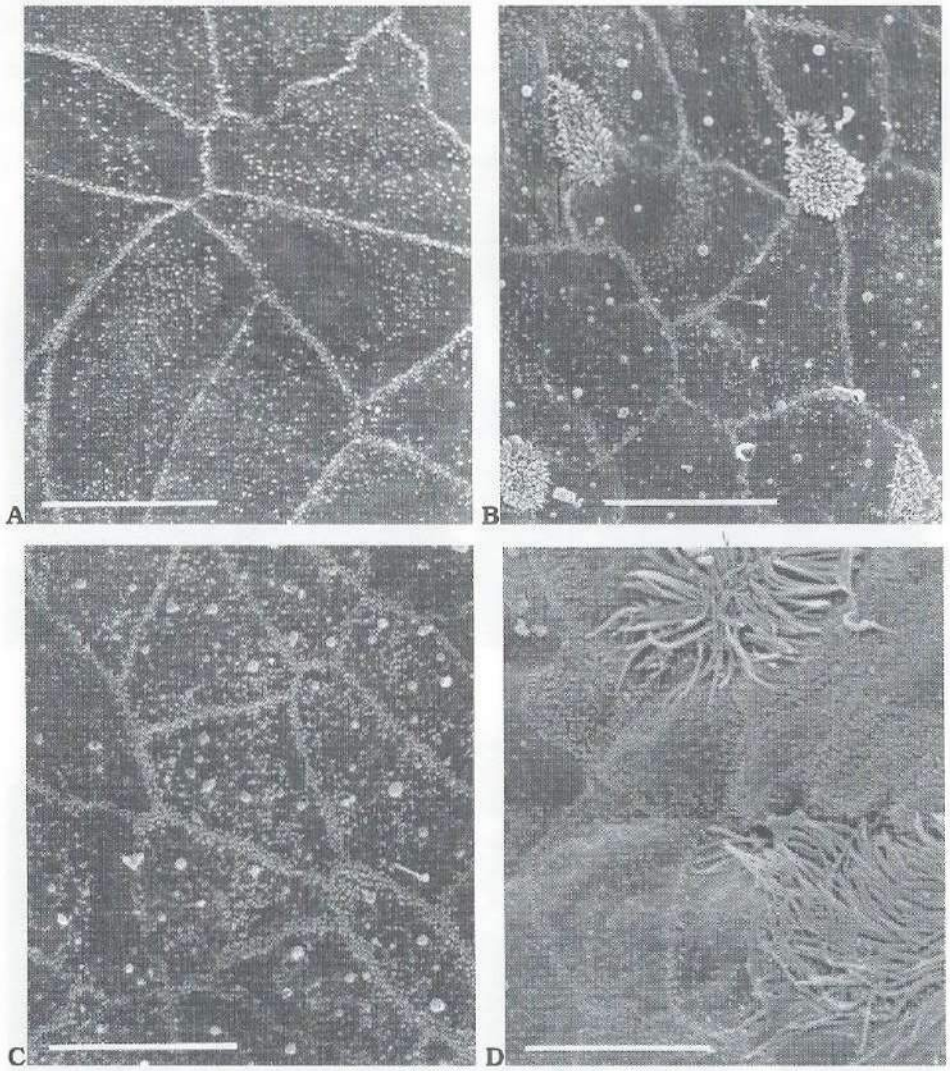

Figure 3: Scanning electron micrographs of the middle ear mucosa in the hypotympanum after: **A)** PBS injection; **B)** endotoxin injection (5 µg/ear), 1 week; **C)** ETO + E, 1 week; and **D)** endotoxin injection (5 µg/ear), 4 weeks. One week after endotoxin application formation of ciliated cells was observed and after 4 weeks the epithelial cells were swollen. ETO + E induced an increase in microvilli particularly on the borders of the cells. (Original magnification X2500, bar = 10 µm)

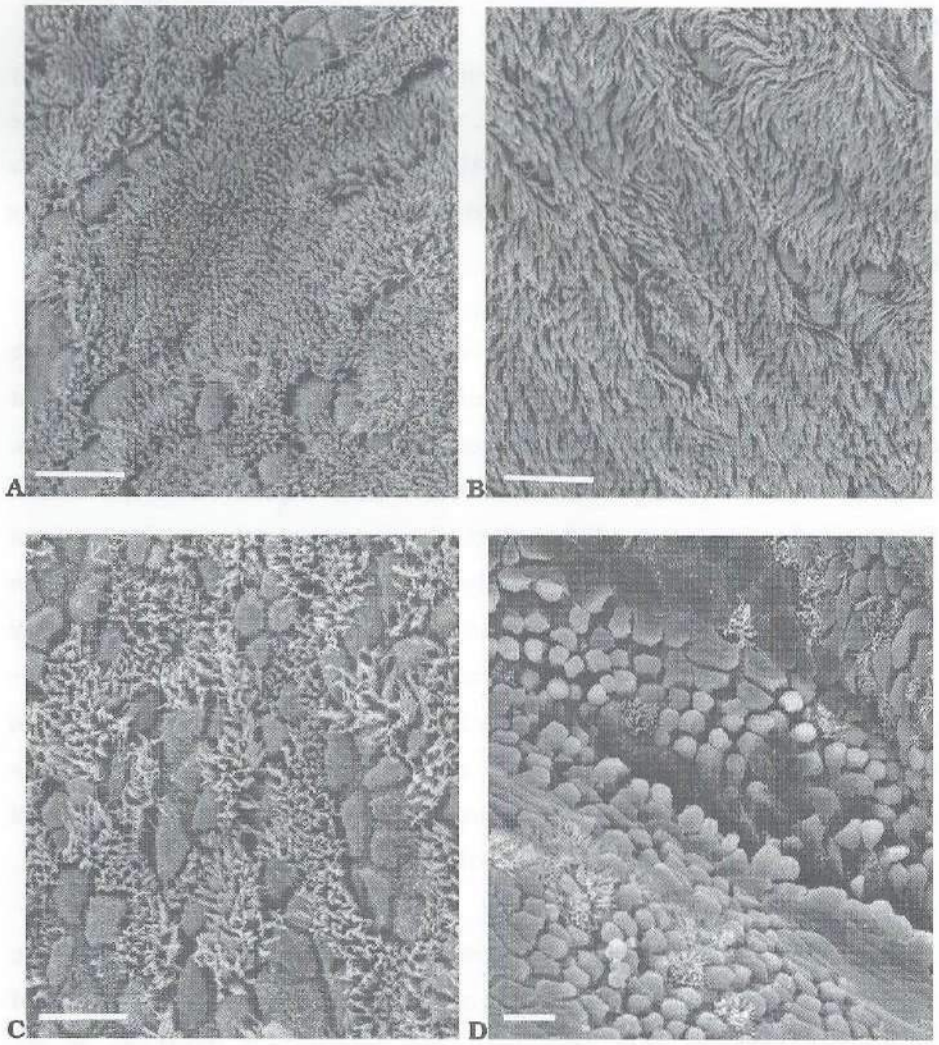

Figure 4: Scanning electron micrographs showing the middle ear mucosa near the tympanic orifice of the eustachian tube after: **A)** PBS injection; **B)** endotoxin injection (5 µg/ear), 1 week; **C)** ETO + E, 1 week; and **D)** ETO + E, 12 weeks. The MCS consists of ciliated cells interspersed with some secretory cells. Endotoxin first induced some increase in ciliated cells, which was followed by a decrease. ETO + E induced an increase in secretory cells, later degeneration of ciliated cells and formation of cobblestone-like cells occurred. (Original magnification: **A-C** X1250, **D** X680, bar = 10 µm)

DISCUSSION

The middle ear epithelium is an important defense system, especially the MCS close to the tympanic orifice, which provides the clearance of the middle ear cavity (Ohashi et al., 1989). Blockade of this system is supposed to be an important factor in the development of secretory otitis media (Kuijpers et al., 1984).

Our study demonstrated that injection of endotoxin into the middle ear or obstruction of the eustachian tube could induce a more pseudostratified, cuboidal epithelium in the hypotympanum. Light micrographs showed a temporal increase in the number of ciliated cells. Moreover, SEM clearly showed that flat squamous epithelium differentiated into a more ciliated epithelium. As a result the clearance of the middle ear cleft will be temporally increased. In the tympanic orifice differentiation into a more pseudostratified, cylindrical, secretory epithelium was observed. This activation of the secretory activity, together with the formation of ciliated cells, can be considered as an attempt to fortify the defense system (Kuijpers et al., 1984). Goblet cell hyperplasia is believed to be a result of noxious agents of the inflammatory process or of toxic capacities of the infective micro-organisms (Cayé-Thomasen et al., 1995).

Most of our results are consistent with those of others who have shown that endotoxin or obstruction of the eustachian tube is capable of inducing an inflammatory process in the middle ear (DeMaria et al., 1989; Kuijpers et al., 1979; Kuijpers et al., 1984; Nonomura et al., 1986). However, the initial increase in ciliated cells observed in our present study differs from some other reports. Ohashi et al. (1989) found 40% degeneration of ciliated cells due to endotoxin (10 µg/ml) after 7 days. No explanation is found for this difference. Basal cells in the epithelial layer can differentiate into ciliated- or non-ciliated cells, including goblet cells

(Hentzer, 1984). It is conceivable that a low amount of endotoxin stimulates the formation of ciliated cells, whereas high concentrations cause degeneration or dysfunction.

The combination of ETO + E also induced a more pseudostratified, cuboidal or cylindrical epithelium. While endotoxin injection alone induced an increase in the number of ciliated cells in the early phase, the combination of ETO + E did not. However, an increase in microvilli, preferably at the border of the squamous epithelial cells, was observed. SEM also showed tracks of cobblestone-like cells beside the tracks of ciliated cells in the tympanic orifice. Kuijpers et al. (1984) also observed this phenomenon after ETO in germ-carrying rats.

In the subepithelial layer infiltration of PMNs, macrophages and lymphocytes was observed. Interaction of endotoxin with these inflammatory cells causes the release of inflammatory mediators (Morrison & Ulevitch, 1978), which can contribute to tissue damage (Tracey et al., 1988).

Using a combination of ETO and endotoxin injection likely resulted in higher concentrations of endotoxin remaining for a longer period in the middle ear cavity. Therefore, endotoxin was able to induce persisting structural changes. Presumably due to the toxic effects of endotoxin and the inflammatory mediators released, degeneration of ciliated cells occurs. The differentiation into secretory ciliated epithelium, followed by a degeneration of cilia, induced a disturbance of the MCS for a lengthy period.

Our present study strongly suggests that endotoxin from gram-negative bacteria that are trapped in the middle ear cavity because of poor tubal function can promote recurrent or chronic OME.

Chapter VI

Bactericidal/Permeability-Increasing Protein prevents mucosal damage due to endotoxin and eustachian tube obstruction

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Abstract

Bactericidal/permeability-increasing protein (BPI) is a major component of neutrophil granules, and binds with high affinity to the gram-negative bacterial lipopolysaccharide endotoxin (LPS) and can inhibit its actions. In this study, the efficacy of a recombinant 21 kDa amino-terminal LPS binding fragment of BPI ($rBPI_{21}$) was assessed in a rat model of chronic otitis media with effusion (OME). OME was induced by a combination of eustachian tube obstruction (ETO) and endotoxin injection or endotoxin injection alone. Injection of $rBPI_{21}$ directly into the middle ear two days after OME induction protected the middle ear mucosa from morphological changes, which may disturb the normal clearance of the middle ear. Hence, it is postulated that $rBPI_{21}$, when it is given during the acute phase of the inflammation, can be a new therapy for recurrent or chronic OME.

Introduction

Chronic or recurrent otitis media with effusion (OME) is a frequent disease during childhood, and its complications and sequelae often persist into the adult years (Giebink, 1992). Two important factors in the development of OME are obstruction or dysfunction of the eustachian tube and bacterial infection. Gram-negative bacterial infection is a growing cause of otitis media in children (Blumer, 1998). Endotoxin or lipopolysaccharide (LPS) is a component of gram-negative bacteria (GNB); it can be released upon bacterial infection and possesses various biological activities that can cause inflammation. Endotoxin alone has been shown to induce mucosal inflammation with accumulation of effusion in the middle ears of chinchillas (DeMaria et al., 1984) and guinea pigs (Nonomura et al., 1986). Furthermore, endotoxin has been

detected in middle ear effusions (MEE) (DeMaria et al., 1984; Iino et al., 1987; Iino et al., 1985) where it was found to be significantly higher in children with chronic OME compared to children with acute OME. Finally, endotoxin is also thought to be cytotoxic to ciliated epithelial cells (Johnson & Inzana, 1986) and to be one of the major factors that cause chronic MEE even when bacteria are no longer viable (DeMaria et al., 1984; Nonomura et al., 1986; Tanimura et al., 1987; Gu et al,. 1995).

We recently developed an animal model of chronic OME using a combination of eustachian tube obstruction (ETO) and endotoxin injection (Nell & Grote, 1999). This procedure induces an increase in secretory cells of the epithelium and degeneration of cilia, which results in a disturbance of the mucociliary clearance system (MCS) of the middle ear. Endotoxin injection or ETO alone also disturbs the MCS but induces less mucosal damage. As long as this disturbance of the MCS is present in the middle ear, OME continues. Therefore, re-establishment of the MCS may be an important step to break the vicious circle and recover OME.

Bactericidal/permeability-increasing protein (BPI), a 55 kDa cationic protein present in the granules of polymorphonuclear neutrophils (PMNs), is a naturally occurring molecule that has been implicated in the host defensive response to gram-negative bacterial infection (Elsbach & Weiss, 1993). In addition to having bactericidal properties, BPI binds to the highly conserved lipid A portion of LPS with high affinity and can inhibit its actions (Marra et al., 1994). The anti-endotoxin properties of BPI reside predominantly in the 25 kDa amino-terminal portion of the molecule (Ooi et al., 1991). Previous investigations have shown that a 21 kDa recombinant amino-terminal fragment of BPI ($rBPI_{21}$) protects animals against the effects of GNB and endotoxin (Elsbach & Weiss, 1998). Furthermore, in man, $rBPI_{21}$ appears safe and non-immunogenic

and is in Phase II/III clinical trials with apparent therapeutic benefit (Elsbach & Weiss, 1998; Giroir et al., 1997; Demetriades et al., 1999).

In the present study we aimed to assess the in vivo capacity of $rBPI_{21}$ to prevent mucosal damage in chronic OME.

Materials and Methods

Animals

Sixty-four female Wistar rats (bodyweight about 200 g, 10 weeks old) were used in this study. They were divided into four control groups; no-injection, sterile pyrogen free PBS injection, 2 mg/ml $rBPI_{21}$ injection (BPI), and injection of 2 µg/ml endotoxin premixed (1:1) with 2 mg/ml $rBPI_{21}$ (E/BPI). And four experimental groups; 2 µg/ml endotoxin injection (E), E with 2 mg/ml $rBPI_{21}$ injection after two days (E+BPI), eustachian tube obstruction (ETO) in combination with 2 µg/ml E injection (ETO+E), and ETO+E with 2 mg/ml $rBPI_{21}$ injection after two days (ETO+E+BPI). It was chosen to applicate BPI after two days to investigate the effect of BPI during an acute inflammation reaction.

During anaesthesia with nitrous oxide, the solutions were injected through the tympanic membrane until the solution overflowed. The endotoxin used was prepared form *Salmonella typhimurium* (L-6511, Sigma, Zwijndrecht, The Netherlands), which is similar to endotoxin derived from non-typeable *Haemophilus influenzae* (Nakamura et al., 1992). Injection of 50 µl of a solution of 2 µg/ml endotoxin results in a final concentration of approximately 100 ng endotoxin per ear. $rBPI_{21}$ was obtained from XOMA (US) LLC. (Berkeley, CA., USA), to ensure complete neutralization, an amount equal to 1000 times the endotoxin concentration of $rBPI_{21}$ was used (Marra et al., 1994). The eustachian

tube was reached by a ventral approach, medially to the posterior belly of the digastric muscle, and obstructed by plugging a small piece of Gelfoam (Upjohn Co., USA) into the tube, additionally some tissue glue (Historesin®, Braun, Melsungen, Germany) was used to keep the Gelfoam in the tube.

After 1, 2, 4 or 12 weeks, the animals were sacrificed with CO_2 gas and subsequently decapitated. The middle ear was dissected from the skull, denuded of adhering tissues and further processed for light microscopy (LM) and scanning electron microscopy (SEM).

Light microscopy

The specimens were fixed with a solution of 1.5% glutaraldehyde in cacodylate buffer (0.14 M, pH 7.4), decalcified with a solution of 10% EDTA in 1.5% glutaraldehyde in cacodylate buffer (0.14 M, pH 7.4) and subsequently dehydrated in a graded series of ethanol and embedded in glycol methacrylate (JB4, Brunschwig Chemie, Amsterdam, The Netherlands). Sections were stained with toluidine blue for histological studies and with alcian blue-PAS for glycoprotein histochemistry.

Scanning electron microscopy

Two middle ears of each period, prepared as described for LM, were processed for SEM. For this purpose, the specimens were after fixation, dehydrated in a graded series of ethanol and critical point dried using liquid CO_2. The distribution of the epithelial cells was studied with a Philips 525M scanning electron microscope at 15 kV after mounting and coating with gold in a Balzers MED010 sputtercoater.

Statistical analysis

The absolute numbers of ciliated and goblet cells were counted in duplicate in each ear in two standardized areas of the same size in the tympanic orifice. Statistical comparisons were made by Tukey - HSD test with a significance level of $P<0.05$, using the Statistical Package for the Social Sciences (SPSS).

RESULTS

Histopathological findings in the control groups

By LM and SEM, control middle ears remained apparently normal during the whole period. The hypotympanum of the middle ear consisted of thin, one-layered squamous epithelium, containing very few ciliated cells (Fig. 1A). In the tympanic orifice of the eustachian tube a more pseudostratified, cuboidal or cylindrical epithelium was observed which contained an abundant number of ciliated cells and few secretory cells (Fig. 2A). This part represents the mucociliary clearance system. Inoculation of $rBPI_{21}$ induced some infiltration of PMNs in the middle ear cavity, but these cells were not present in the subepithelial layer and were disappeared within two weeks. Furthermore, no morphological changes to the epithelial layer were observed due to $rBPI_{21}$ injection. Compared to no injection, the premixed solution of endotoxin and $rBPI_{21}$ did not induce any significant mucosal alterations to the middle ear.

Histopathological findings due to LPS

Injection of endotoxin induced a thickening of the middle ear mucosa in the hypotympanum due to vasodilatation, oedema and infiltration of PMNs, macrophages and lymphocytes into the subepithelial layer. The

epithelial layer had become more pseudostratified, cuboidal and numerous ciliated cells occurred in areas originally almost devoid of these cells. Three months after endotoxin injection the epithelial layer was still thickened (Fig. 1B). In the tympanic orifice an increase in ciliated and secretory epithelium was observed after two weeks. After three months, however, the tympanic orifice contained many secretory cells but only few ciliated cells were observed with LM. SEM showed that cilia could indeed be found but they were deformed (Fig. 2B).

Histopathological findings after treatment with $rBPI_{21}$

Injection of $rBPI_{21}$ two days after the induction of OME by endotoxin injection prevented the deformation of cilia and the increase in secretory cells. Using LM, less thickening of the epithelial layer and no infiltration of inflammatory cells in the subepithelial layer was observed after one week (Fig. 1C). In the tympanic orifice an abundant amount of ciliated cells were present which had a normal appearance (Fig. 2C).

Histopathological findings due to ETO+E

As could be expected, the combination of tube obstruction and endotoxin injection induced thickening of the middle ear mucosa due to vasodilatation, oedema and infiltration of PMNs, macrophages and lymphocytes in the hypotympanum (Fig. 1D). However, the amount of infiltrated cells, particularly PMNs, was much higher than after endotoxin injection alone. Compared to PBS injection, less ciliated cells were observed in the tympanic orifice and with SEM severely swollen squamous epithelium; i.e. formation of cobblestone-like cells was observed after three months (Fig. 2D). Furthermore, an increase in secretory cells was observed.

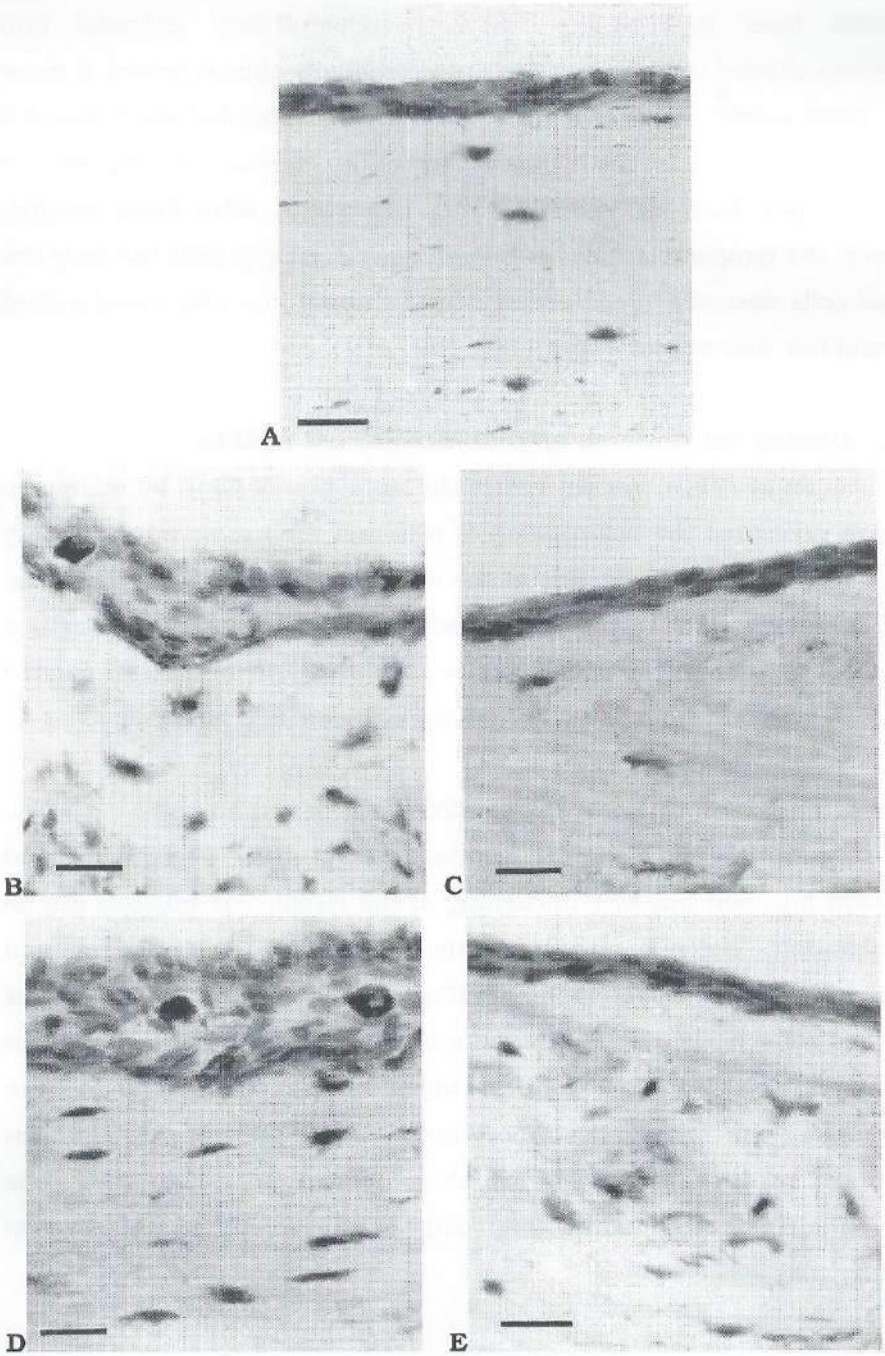

Figure 1: Light micrographs of the hypotympanum of the middle ear at 12 weeks after: **A)** PBS injection; **B)** endotoxin injection (100 ng/ear); **C)** endotoxin injection with $rBPI_{21}$ injection after two days; **D)** ETO+E; and **E)** ETO+E with $rBPI_{21}$ injection after two days. Endotoxin and ETO+E both induced increased proliferation of the epithelial layer and influx of inflammatory cells. These changes were not observed after application of $rBPI_{21}$ (original magnification X200, bar = 10 μm).

Histopathological findings after treatment with $rBPI_{21}$

$rBPI_{21}$ injection prevented the thickening of the middle ear mucosa in the hypotympanum after ETO in combination with endotoxin injection (Fig. 1E). No infiltration of inflammatory cells in the subepithelial layer was observed. In the tympanic orifice an abundant amount of ciliated cells were present and no increase in secretory cells was seen. Furthermore, with SEM no formation of cobblestone-like cells was observed (Fig. 2E).

Quantitation of mucosal damage

The number of ciliated cells, counted in a standardized part of the tympanic orifice, are represented in figure 3 for the different control- and experimental groups. The control groups PBS, BPI and E/BPI were not significantly different from each other. Endotoxin injection first induced a small increase in ciliated cells; nevertheless this was followed by a significant decrease after three months. Injection of endotoxin followed by $rBPI_{21}$, on the other hand, did not induce any significant change in the number of ciliated cells. Compared to PBS injection, the number of ciliated cells significantly decreased after induction of OME by ETO and endotoxin injection. In this case injection of $rBPI_{21}$ also prevented the decrease in the number of ciliated cells.

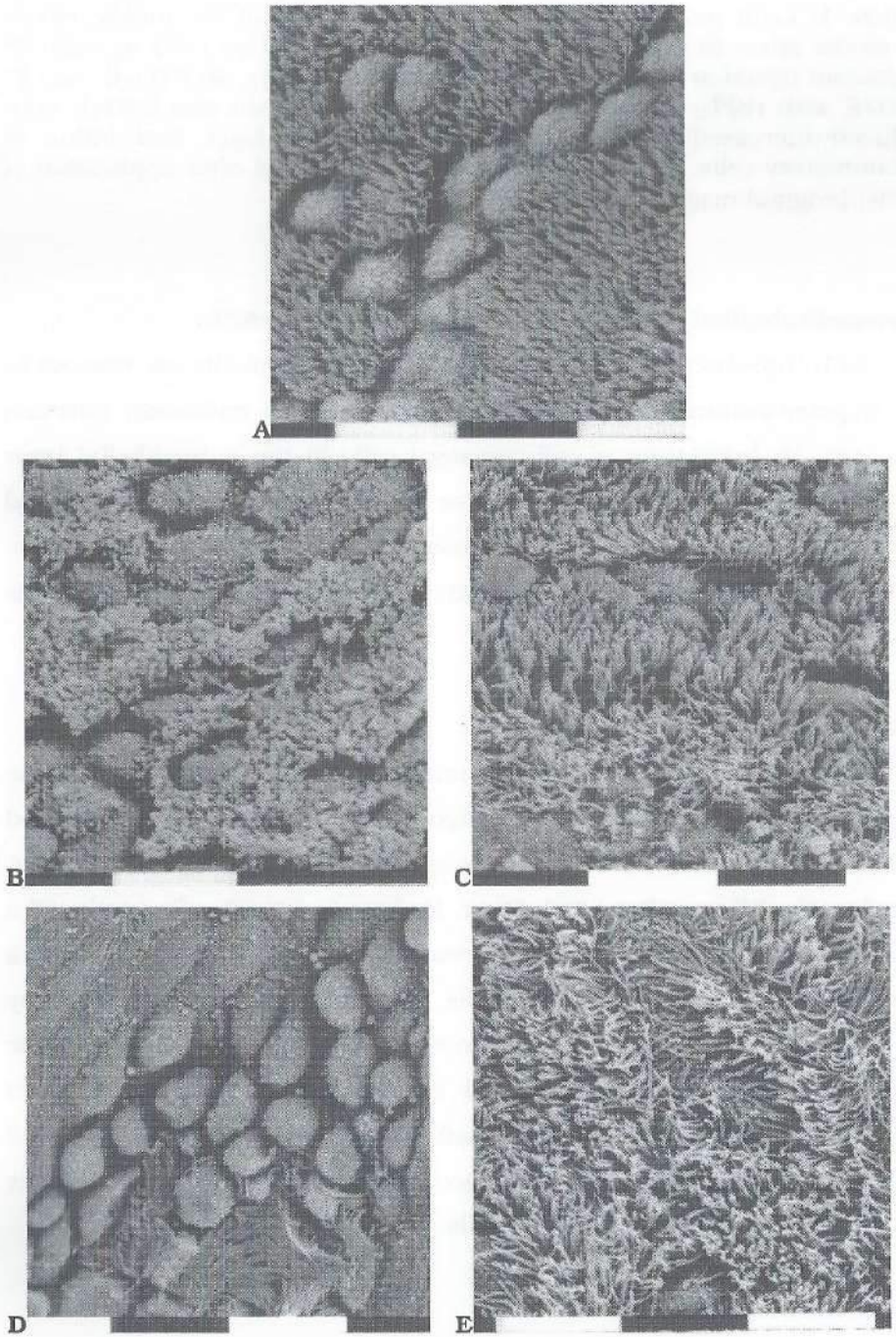

Figure 2: Scanning electron micrographs of the tympanic orifice at 12 weeks after: **A)** PBS injection; **B)** endotoxin injection (100 ng/ear); **C)** endotoxin injection with $rBPI_{21}$ injection after two days; **D)** ETO+E; and **E)** ETO+E with $rBPI_{21}$ injection after two days. Endotoxin induced deformation of cilia, whereas ETO+E induced degeneration of cilia and formation of cobblestone-like cells. This was not observed after $rBPI_{21}$ injection and cilia had a normal appearance (original magnification X1250, bar = 10 μm).

In the same part of the tympanic orifice the numbers of secretory goblet cells, stained with alcian blue-PAS, were counted and are represented in figure 4. Compared to PBS, BPI injection induced a small but significant increase in goblet cells after one and twelve weeks. However, BPI premixed with endotoxin did not induce a significant increase of goblet cells. Injection of endotoxin induced a significant increase in goblet cells during the whole period, whereas E+BPI did not. The combination of ETO+E, finally, also induced an increase in goblet cells, which was prevented by BPI injection.

Discussion

In cases of chronic OME, gram-negative pathogens are frequently isolated. Endotoxin, the lipopolysaccharide outer membrane constituent of GNB, has been recognised as an important factor in the development of OME (DeMaria et al., 1984; Nonomura et al., 1986). Endotoxin is a strong inducer of inflammation and a modulator of the immune response, and has been shown to induce a disturbance of the MCS (Ohashi et al., 1989). This MCS is considered to be an important defense system of the middle ear cavity, and disturbance of this system is suspected to be an important factor in the development of chronic OME.

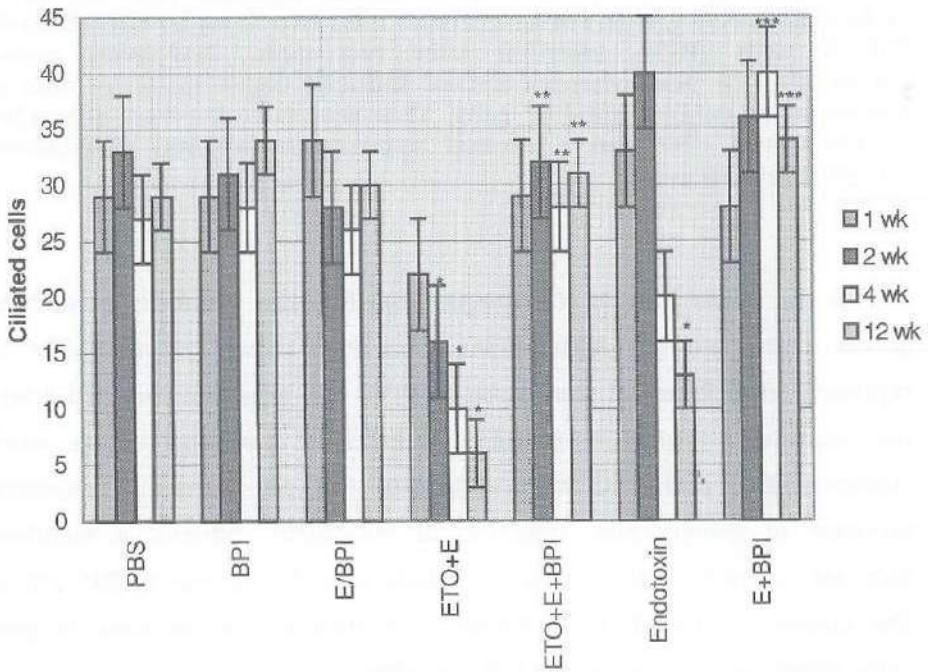

Figure 3: Numbers of ciliated cells ± standard error of the mean, counted in the tympanic orifice after PBS injection, $rBPI_{21}$ injection, Endotoxin premixed with $rBPI_{21}$ (E/BPI), eustachian tube obstruction with endotoxin injection (ETO+E), ETO+E with $rBPI_{21}$ injection after two days (ETO+E+BPI), endotoxin injection (E), and endotoxin injection with $rBPI_{21}$ injection after two days (E+BPI). Statistical comparisons were made by Tukey - HSD test with a significance level of $P < 0.05$, * represent values significant different from PBS, ** represent values significant different from ETO+E of the same week, and *** represent values significant different from E of the same week. The application of $rBPI_{21}$ after two days prevented the decrease in the numbers of ciliated cells induced by OME induction.

The present study and our previous study (Nell & Grote, 1999) demonstrated that endotoxin injection in the middle ear cavity of rats, in combination with obstruction of the eustachian tube, induced a disturbance of the MCS. The induced secretory cell hyperplasia is thought to result from inflammatory mediators induced by LPS or from the toxic capacities of endotoxin itself (Cayé-Thomasen et al., 1995). Endotoxin injection alone first induced an increase in ciliated cells, which was followed by a decrease and deformation of the cilia. This dysfunction of cilia by endotoxin is responsible for the accumulation of surplus fluid in the tympanic cavity (Ohashi et al., 1988). Using a combination of ETO and endotoxin injection, a degeneration of cilia and formation of cobblestone-like cells was induced. It is likely that this combination gave rise to higher concentrations of endotoxin, which remain for a longer period in the middle ear cavity. Furthermore, it is conceivable that a low amount of endotoxin stimulates the formation of ciliated cells, whereas high concentrations cause degeneration or dysfunction of the cilia (Nakamura et al., 1992; Ohashi et al., 1989). The differentiation into secretory/ciliated epithelium, followed by a degeneration of cilia, induced a disturbance of the MCS for a lengthy period, promoting the development of recurrent or chronic OME.

In a previous *in vitro* study, we reported that the proliferative and morphological effects of endotoxin on cultured rat middle ear epithelium could be suppressed by a human monoclonal antibody against endotoxin; HA-1A (Grote et al., 1995). It is been established that BPI can bind to the lipid A part of endotoxin and neutralizes its activity, whereas HA-1A binds but does not neutralize endotoxin (Marra et al., 1994).

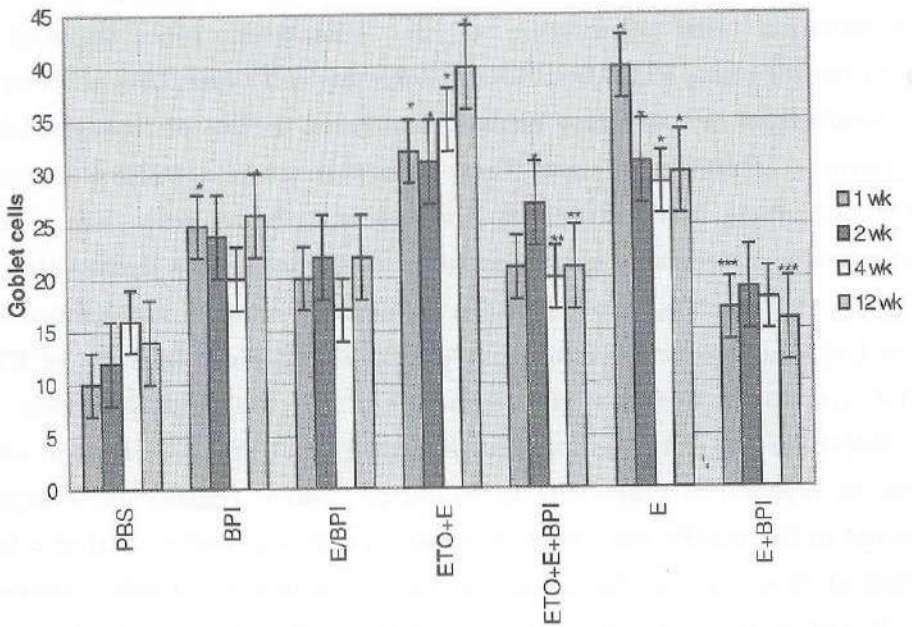

Figure 4: Numbers of goblet cells ± standard error of the mean, counted in the tympanic orifice after PBS injection, $rBPI_{21}$ injection, Endotoxin premixed with $rBPI_{21}$ (E/BPI), eustachian tube obstruction with endotoxin injection (ETO+E), ETO+E with $rBPI_{21}$ injection after two days (ETO+E+BPI), endotoxin injection (E), and endotoxin injection with $rBPI_{21}$ injection after two days (E+BPI). Statistical comparisons were made by Tukey - HSD test with a significance level of $P < 0.05$, * represent values significant different from PBS, ** represent values significant different from ETO+E of the same week, and *** represent values significant different from E of the same week. $rBPI_{21}$ induced some increase in the number of goblet cells; however, application of $rBPI_{21}$ after OME induction inhibited significantly the increase in the numbers of goblet cells.

BPI, isolated from PMNs, is bactericidal only toward GNB. This target cell specificity is attributable to the strong affinity of BPI for LPS (Elsbach & Weiss, 1993). The amino-terminal fragments of BPI contain the determinants of LPS binding, and exhibit a 50 to 70-fold higher binding affinity than the whole BPI molecule. In animal experiments, $rBPI_{21}$ protected against the effects of GNB and endotoxin (Elsbach & Weiss, 1995). In man, intravenously administered $rBPI_{21}$ appears to be safe and non-immunogenic and inhibited endotoxin-induced cytokine release (Von der Möhlen et al., 1995). In Phase II/III clinical trials involving severe pediatric meningococcemia and hemorrhagic trauma, $rBPI_{21}$ proved to have apparent therapeutic benefit (Elsbach & Weiss 1998; Giroir et al., 1997; Demetriades et al., 1999). Furthermore, $rBPI_{21}$ apparently acts synergistically with some antibiotics (Elsbach & Weiss, 1995; Lin et al., 1996) and it may also overcome or reduce bacterial resistance to selected antibiotics (Lin et al., 1996). In this study, we found that $rBPI_{21}$ prevented the induction of morphological changes in the rat middle ear due to endotoxin.

Injection of $rBPI_{21}$ into the middle ear cavity two days after the induction of OME prevented the induction of morphological changes to the MCS. Within the first two weeks, $rBPI_{21}$ injection alone induced the infiltration of inflammatory cells, mainly PMNs, into the middle ear cavity. However, except for a slight increase in secretory cells, no morphological changes to the epithelial layer were observed. Nevertheless, $rBPI_{21}$ prevented the significant increase in secretory cells after OME induction, and $rBPI_{21}$ also prevented the hyperproliferation of the epithelial layer and the infiltration of inflammatory cells into the subepithelial layer. Furthermore, no deformation or degeneration of the cilia was observed after application of $rBPI_{21}$. Finally, the formation of cobblestone-like cells induced by ETO+E was inhibited by $rBPI_{21}$.

In cases of ear infections complicated by eustachian tube obstruction, bacterial products, including endotoxin, can be trapped in the middle ear. The uncleared bacterial products and endotoxin can perpetuate the inflammation, even after viable bacteria have been killed with antibiotics, and further compromising the MCS. In these situations, continued treatment with antibiotics alone is not likely to be effective. Moreover, widespread use of antibiotics is unwise due to growing bacterial resistance. Therefore, agents that can inhibit the inflammatory activity of endotoxin could help break the inflammatory cycle and re-establish an effective MCS.

In our previous *in vitro* experiments, the monoclonal antibody HA-1A was able to significantly diminish the proliferation of cultured rat middle ear epithelium. The morphological effects of endotoxin on these cells were, although weaker, still present (Grote et al., 1995). In addition, Polymyxin B, a polypeptide antibiotic, has been shown to bind and inactivate endotoxin (Darrow & Keithley, 1996). In a guinea pig model, cellular infiltrate, effusion volume, and mucosal oedema in response to endotoxin were reduced, but not eliminated, when endotoxin was premixed with Polymyxin B before installation into the middle ear (Darrow & Keithley, 1996). $rBPI_{21}$ has stronger endotoxin-binding and -neutralization properties than HA-1A (Marra et al., 1994) and appears to be more effective than Polymyxin B in a similar model. Our results suggest that $rBPI_{21}$ is a potential therapy for preventing the occurrence of recurrent or chronic OME.

Chapter VII

Efficacy of Bactericidal/Permeability-Increasing protein in experimental Otitis Media with Effusion induced by eustachian tube obstruction and endotoxin

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ABSTRACT

We investigated the therapeutic effect of a recombinant endotoxin-binding protein, bactericidal/permeability-increasing protein ($rBPI_{21}$), on experimentally induced otitis media with effusion (OME) in rats. OME was induced by obstruction of the eustachian tube in combination with endotoxin injection. A single dose of $rBPI_{21}$ was administered directly into the middle ear cavity two weeks after the induction of OME. Histological examination of the middle ear mucosa at 4 and 12 weeks after OME induction showed that morphological changes, like an increase in secretory cells and loss of ciliated cells, were restored by $rBPI_{21}$ treatment. These results demonstrate that the middle ear mucosa recovers from inflammatory changes associated with OME after treatment with $rBPI_{21}$. This suggests that $rBPI_{21}$ may be useful in the treatment of OME as well as mucosal infections of the respiratory tract.

INTRODUCTION

Almost all children suffer from at least one period with glue-ears and hearing loss due to otitis media with effusion (OME). In most cases these problems resolve spontaneously, but approximately 15% of children have chronic problems with conductive hearing loss for a longer period and sometimes remaining changes of the eardrum and middle ear ossicles (Klein, 1994). The two most important factors in the development of OME are gram-negative bacterial infection and dysfunction of the eustachian tube. Gram-negative bacteria (GNB) contain the lipopolysaccharide endotoxin (LPS) in their outer membrane. The toxic properties of endotoxin are represented by the lipid A part, which is structurally similar and serologically cross-reacting among many GNB (Gazzano-Santoro et al., 1992). Moreover, endotoxin has been found in human

middle ear effusions (DeMaria et al., 1984; Ovesen & Ledet, 1992) and injection of viable or non-viable *Haemophilus influenzae* or its endotoxin induced inflammatory changes in the middle ear and eustachian tube (DeMaria & Lim, 1985). Obstruction of the eustachian tube disables the normal clearance of the middle ear. Together with the inflammatory changes in the middle ear, which leads to dysfunction of the mucociliary clearance system (MCS), an accumulation of fluid in the tympanic cavity occurs. For these reasons we previously developed a model for chronic OME where both eustachian tube obstruction (ETO) and endotoxin injection were used to induce dysfunction of the MCS which persisted for 12 weeks (Nell & Grote, 1999).

Up till now children with OME are treated with recurrent placement of ventilation tubes or with antibiotics. However, because of the reported disadvantages of ventilation tubes (Schilder et al., 1995; Lildholdt, 1983) and the growing resistance for antibiotics (Shapiro & Bluestone, 1995) it is important to find alternatives which can prevent the occurrence of chronic OME. Furthermore, tube insertion will only temporarily remove the middle ear effusion, and antibiotics can be effective in eradicating the infecting bacteria, yet they do not inhibit the effects of the infecting bacteria and their endotoxin.

Bactericidal/permeability-increasing protein (BPI), a cationic 55 kDa protein, was purified from rabbit and human neutrophil azurophilic granules, and first described in 1978 as an antibiotic protein (Weiss et al., 1978). BPI has been shown to bind avidly to a wide array of endotoxin chemotypes and to neutralize its activity (Gazzano-Santoro et al., 1992). Furthermore, it inhibits bacterial growth by a number of discrete outer-membrane alterations, including an increase in the outer membrane permeability (Mannion et al., 1990).

In this study, $rBPI_{21}$, a 21 kDa recombinant amino-terminal analog derived from BPI, was examined as a possible new therapeutic agent in the treatment of OME.

Materials and Methods

Animals

Twenty-four female Wistar rats (bodyweight about 200 g, 10 weeks old) were used in this study. They were divided into three control groups: without injection (untreated), injection of 2 mg/ml $rBPI_{21}$, and injection of BPI-formulation buffer (BPI-buffer). And two experimental groups; eustachian tube obstruction (ETO) in combination with injection of 2 µg/ml endotoxin (ETO+E), and ETO+E with injection of 2 mg/ml $rBPI_{21}$ after two weeks (ETO+E+$rBPI_{21}$).

Induction of Otitis Media with Effusion

During anaesthesia with nitrous oxide the eustachian tube was reached by a ventral approach, medially to the posterior belly of the digastric muscle, and obstructed by plugging a small piece of Gelfoam (Upjohn Co., USA) into the tube. Additionally some tissue glue (Historesin®, Braun, Melsungen, Germany) was used to keep the Gelfoam in the tube. This was directly followed by injection of endotoxin through the tympanic membrane. The endotoxin used was prepared form *Salmonella typhimurium* (Sigma, L-6511, The Netherlands). It was chosen because of its similarity to endotoxin derived from non-typeable *H. influenzae* (Nakamura et al., 1992). Injection of 50 µl of a solution of 2 µg/ml endotoxin results in a final concentration of approximately 100 ng endotoxin per ear.

Bactericidal / Permeability - Increasing protein (BPI)

The recombinant amino-terminal fragment $rBPI_{21}$ (2 mg/ml) was obtained from XOMA (US) LLC. (Berkeley, CA., USA). It was injected directly into the middle ear cavity two weeks after the induction of OME in a final concentration of approximately 100 µg per ear, which is 1000 times the endotoxin concentration used. This concentration was used to create the possibility of complete neutralization of endotoxin in the middle ear (Marra et al., 1994). In this study, $rBPI_{21}$ was applied after two weeks to investigate the effect of $rBPI_{21}$ in an inflammation reaction that is already going on.

Light microscopy (LM)

After 4 or 12 weeks, the animals were sacrificed with CO_2 gas and subsequently decapitated. The middle ear was dissected from the skull, denuded of adhering tissues and fixed with a solution of 1.5% glutaraldehyde in cacodylate buffer (0.14 M, pH 7.4). For LM, the specimens were decalcified with a solution of 10% EDTA in 1.5% glutaraldehyde in cacodylate buffer (0.14 M, pH 7.4) and subsequently dehydrated in a graded series of ethanol and embedded in glycol methacrylate (JB4, Brunschwig Chemie, The Netherlands). Sections were stained with toluidine blue for histological studies and with alcian blue-PAS for glycoprotein histochemistry.

Scanning electron microscopy (SEM)

For SEM two middle ears of each period, prepared as for LM, were after fixation, dehydrated in a graded series of ethanol and critical point dried using liquid CO_2. The distribution of the epithelial cells was studied with a Philips 525M scanning electron microscope at 15 kV after mounting and coating with gold in a Balzers MED010 sputtercoater.

Statistical analysis

The numbers of ciliated- and secretory cells were counted in duplicate in each ear in two standardized areas of the tympanic orifice. The numbers of macrophages were counted in duplicate in each ear in the submucosal layer of the epitympanum and the hypotympanum of the middle ear bulla. To compare means of the different variables, one-way ANOVA Tukey's - HSD test with a significance level of $P < 0.05$, was performed using the Statistical Package for the Social Sciences (SPSS). This test uses the studentized range statistic to make all of the pairwise comparisons between groups and sets the experimentwise error rate at the error rate for the collection for all pairwise comparisons. Results are reported as mean cell numbers ± standard error of the mean (SE).

RESULTS

Untreated rat middle ear mucosa

The hypotympanum of the untreated rat middle ears consisted of thin, squamous epithelium with few microvilli (Fig. 1A). The tympanic orifice of the eustachian tube, on the other hand, consisted of pseudostratified, cuboidal or cylindrical epithelium, containing an abundant number of ciliated cells and a few secretory cells (Fig. 2A). With LM the number of secretory cells and ciliated cells were counted in two standardized areas of the tympanic orifice. At 12 weeks 14 ± 2 secretory cells and 24 ± 3 ciliated cells were counted (Table 1). In the subepithelial layer of the hypotympanum and the epitympanum the number of macrophages were counted; at 12 weeks 13 ± 4 macrophages were present (Table 1).

TABLE 1: *Quantitative results presented ± standard error of the mean*

	Cilia		Secretory cells		Macrophages	
	4 wk	12 wk	4 wk	12 wk	4 wk	12 wk
Untreated	26±4	24±3	15±3	14±2	10±4	13±4
$rBPI_{21}$	23±4	21±3	19±2	16±3	17±5	21±4
BPI-buffer	23±3	22±3	19±3	15±2	11±6	20±9
ETO+E	8±3*	6±4*	27±4*	31±4*	25±2*	27±3*
ETO+E+$rBPI_{21}$	28±4**	26±6**	18±3**	18±4**	11±5**	18±3

* Represents values significantly different from untreated ears, ** represents values significantly different from ETO+E of the same week

BPI control

Injection of $rBPI_{21}$ or BPI-formulation buffer in the middle ear cavity did not induce histological changes to the middle ear mucosa. Furthermore, no significant changes in the numbers of secretory cells nor in the numbers of ciliated cells were measured (Table 1). The numbers of macrophages increased to 21 ± 4 at 12 weeks after $rBPI_{21}$ injection, however this was not significantly different from the untreated ears.

Experimentally induced OME

Obstruction of the eustachian tube in combination with endotoxin injection induced thickening of the middle ear mucosa. In the subepithelial layer a significant number of macrophages (27 ± 3 at 12 weeks) were counted (Table 1).

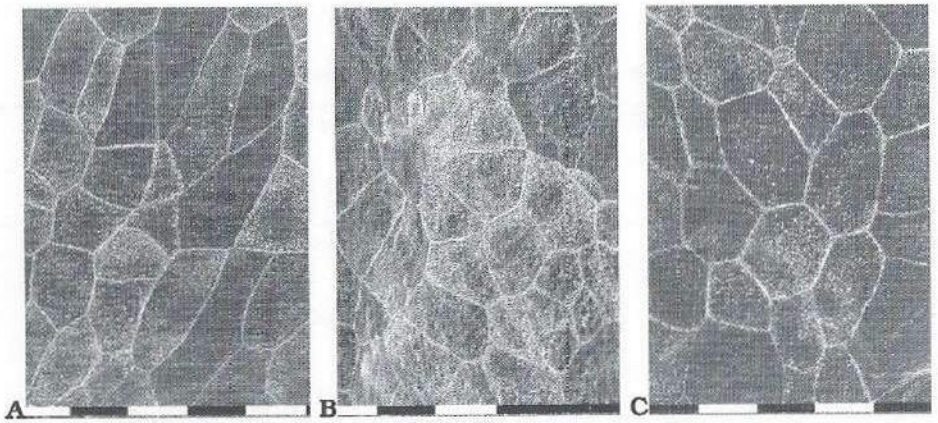

Figure 1: Scanning electron micrographs of the hypotympanic part of the rat middle ear cavity at 4 weeks. **A)** Untreated ear, **B)** ETO+E, and **C)**. ETO+E+rBPI$_{21}$. The untreated ears showed a normal thin squamous epithelium. OME induction with ETO+E induced an abundant number of microvilli and swelling of the epithelial layer. Injection of rBPI$_{21}$ two days after ETO+E showed again a normal thin squamous epithelium (original magnification X1250, bar = 10 μm).

Furthermore, in the tympanic orifice, a significant increase in the number of secretory cells (31 ± 4) and a significant decrease in the number of ciliated cells (6 ± 4) were measured at 12 weeks (Table 1). With SEM it was observed that the epithelial cells in the hypotympanic part of the middle ear cavity contained an abundant number of microvilli and the surface was irregular and swollen (Fig. 1B). In the tympanic orifice, on the other hand, abnormalities in the MCS were observed, including large areas of secretory cells with sporadic mucus deposits and separation of some epithelial cells (Fig. 2B).

BPI treatment

Injection of rBPI$_{21}$, an amount equal to 1000 times the endotoxin concentration, two weeks after the induction of OME resulted in a normal

thin squamous epithelium in the hypotympanum, which was not thickened and contained few microvilli (Fig. 1C). The number of macrophages was significantly decreased at 4 weeks; 11 ± 5 compared to 25 ± 2 for ETO+E. At 12 weeks, however, they were decreased but not significantly different from ETO+E (Table 1). Furthermore, in the tympanic orifice the numbers of secretory cells were significantly decreased at 4 and 12 weeks (18 ± 4) compared to ETO+E. The numbers of ciliated cells were significantly increased at 4 weeks (28 ± 4) and 12 weeks (26 ± 6) compared to ETO+E. Both numbers of ciliated and secretory cells, and the number of macrophages, were not significantly different from the untreated ears (Table 1). Finally, with SEM it was observed that cilia in the tympanic orifice had a normal appearance and no mucus deposits or separation of epithelial cells were observed (Fig. 2C).

DISCUSSION

OME is one of the most common chronic pathological conditions encountered in pediatric otologic practice. This disease is characterized by the accumulation of fluid in the middle ear, which frequently can cause deafness at a critical time in a child's development and can interfere with speech development and social interaction. In the majority of patients, resolution of the effusion may occur spontaneously. There will remain, however, a significant number of children in whom effusion persists even after the acute infection has resolved (Klein, 1994).

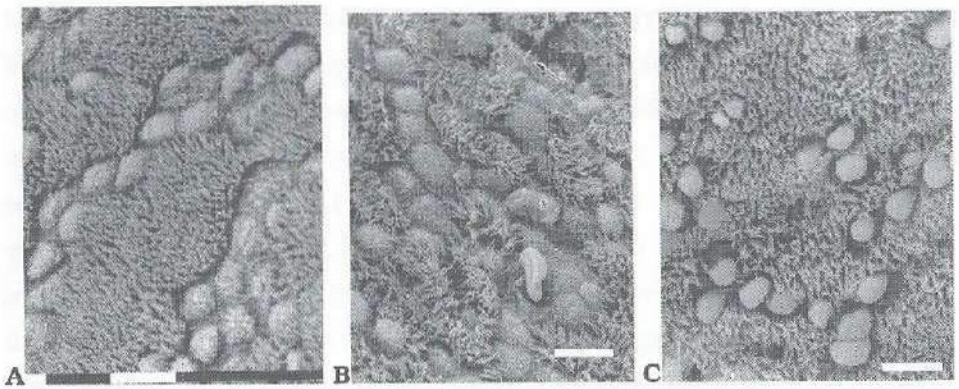

Figure 2: Scanning electron micrographs of the tympanic orifice of the rat middle ear cavity at 4 weeks. **A)** Untreated ear, **B)** ETO+E, and **C).** ETO+E+rBPI$_{21}$. Showing a part of the mucociliary clearance system of untreated ears with few secretory cells and an abundant number of ciliated cells. After induction of OME with ETO+E, increased numbers of secretory cells, with mucus deposits (arrows) and separation of epithelial cells (asterisks) were observed. Injection of rBPI$_{21}$ two weeks after OME induction showed a normal mucociliary clearance system (original magnification X1250, bar = 10 μm).

Persistent effusion, infection, or epithelial damage promotes the release of a number of potent inflammatory mediators. These, together with bacterial toxins and enzymes, are stimuli for metaplasia of the middle ear epithelium to a more secretory type, similar to that found in the lower respiratory tract (Dentener et al., 1997). Any effusion formed in the tympanic cavity will be removed to the pharynx, provided that the MCS is functioning normally. However, if the eustachian tube is blocked or the MCS is damaged or inflamed, the effusion accumulates until the middle ear cavity is filled. Kuijpers et al., (1984) found that obstruction of the eustachian tube in germ-carrying rats resulted in transformation of the epithelium into mucus-producing cells, which was not observed in germ-free rats. They suggested that the most likely explanation for this

phenomenon was that obstruction of the normal clearance pathway together with favorable nutritional conditions for bacterial growth of normally harmless inhabitants of the middle ear induces a transformation of squamous cells into secretory cells.

Since the MCS is composed of ciliated cells as well as secretory cells and a mucus blanket, impaired mucociliary function is due not only to mucus abnormalities but also to lowered ciliary activity (Ohashi et al., 1989). The MCS propels the mucus towards the eustachian tube by means of beating cilia. However, increased mucus production, with sometimes altered consistency, may affect seriously ciliary beat and its coordination by penetrating the intraciliary spaces (Dentener et al., 1997). Furthermore, Ohashi et al. found that ciliary function is sensitive to a variety of pathological agents including bacteria and bacterial endotoxin, resulting in mucociliary dysfunction (Ohashi & Nakai, 1991; Ohashi et al., 1989). They showed the presence of compound cilia, vacuolation of ciliated cells and approximately 40% degeneration of ciliated cells, 7 days after inoculation of 10 μg/ml LPS in the guinea pig middle ear cavity.

For these reasons, our model for inducing OME in rats is based on obstruction of the eustachian tube together with introducing endotoxin in the middle ear cavity. This study and our previous study (Nell & Grote, 1999) demonstrated the formation of persisting changes to the middle ear epithelium after ETO and endotoxin injection. These changes comprehended hyperplasia of secretory epithelium and degeneration of ciliated cells in the tympanic orifice. Furthermore, a significant increase in the number of macrophages in the subepithelial layer was observed. These changes of the middle ear epithelium are resulting in dysfunction of the MCS. We believe that an improvement of the MCS will clear the effusion from the middle ear cavity and will result in an aerated middle

ear. Therefore, the aim of the present study was to elucidate the effect of $rBPI_{21}$ treatment on the MCS in our model of OME.

The human neutrophil granule protein BPI is part of the normal mechanism for dampening an otherwise overwhelming response to bacteria and their endotoxin. BPI binds to LPS near the lipid A domain, and formation of the LPS-BPI complex abrogates harmful host responses to LPS. For example, BPI blocks LPS-mediated stimulation of both neutrophils and macrophages (Marra et al., 1992). Furthermore, BPI has been shown to neutralize LPS-mediated cytokine production (Arditi et al., 1994; Von der Möhlen et al., 1995) and it has cytotoxic activity against GNB (Elsbach & Weiss, 1993; Weiss et al., 1992; Elsbach & Weiss, 1993). Finally, BPI directs its potent toxicity exclusively toward gram-negative bacteria (Elsbach & Weiss, 1992; Weiss et al., 1978).

The amino-terminal fragment of natural BPI has been shown to exhibit all biological activities of the whole protein and was recently cloned and expressed (Gazzano-Santoro et al., 1992; Elsbach & Weiss, 1998). BPI acts as an agent with dual actions that includes inhibition of the proliferation of a range of gram-negative bacterial species and also potent inhibition of host responses to LPS. We demonstrated that injection of a 21 kDa recombinant amino-terminal modified fragment of BPI, in an amount equal to 1000 times the endotoxin concentration, inhibited the morphological changes in the MCS. In our experimental model of chronic OME the number of macrophages in the submucosal layer of the middle ear cavity was significantly higher than in the untreated ears at both 4 and 12 weeks. Injection of $rBPI_{21}$ or BPI-buffer as a control induced a small but not significant increase in the number of macrophages. Whereas, treatment with $rBPI_{21}$ two weeks after the induction of chronic OME reduced the increase in the number of macrophages.

Hori et al. (1994) demonstrated that S-carboxymethylcysteine (S-CMC) in chinchillas with immune-mediated OME induced a reduction of the damage to the ciliated cells and reduced goblet cell hyperplasia. However, S-CMC did not act on the infiltrating inflammatory cells to prevent the release of chemical mediators such as histamine and prostaglandin E2. Hydroxyzine, an antihistamine, was investigated in children with secretory otitis media by Theoharides et al. (1994). They found that the rate of relapse was significantly reduced using hydroxyzine, and so was the amount of histamine present in middle ear effusions. Takahashi et al. (1997) showed that endotoxin-induced hypertrophic and metaplastic changes of goblet cells in rat nasal respiratory epithelium could be inhibited by intraperitoneal injection of anti-inflammatory drugs. Goldie et al. (1993) studied anti-inflammatory drugs and their effect on the arachidonic acid metabolites. They found that Indomethacin inhibited the accumulation of middle ear effusion.

Anti-inflammatory drugs can block some inflammatory mediators or secretion by local mast cells, however, they will not neutralize bacteria or their endotoxin. Therefore, endotoxin will still be present in the middle ear cavity and if the clearance is still not functioning properly endotoxin can start the inflammation reaction again. BPI, on the other hand, neutralizes endotoxin and also inhibits gram-negative bacterial proliferation. $rBPI_{21}$ injection in the middle ear two weeks after induction of OME, when the inflammation reaction is already causing damage, inhibited the secretory cell hyperplasia, the ciliated cell degeneration and prevented the influx of macrophages. This will promote the function of the MCS and clearance of the middle ear can be improved. Therefore, based on our results, we believe that an important contributory role for $rBPI_{21}$ in the treatment of OME as well as (upper) respiratory tract infections may be anticipated.

Chapter VIII

General Discussion and Summary

Factors in OME development

The retention of inflammatory products in the middle ear cavity due to bacterial infection and poor drainage of the eustachian tube results in an ongoing inflammatory reaction (Chole, 1986). Whether obstruction of the eustachian tube is the cause of dysfunction of the tube itself or caused by the inflammatory reaction is a matter of debate. However, it has been shown that children with shorter eustachian tubes that are more horizontally positioned, are more susceptible for an obstruction of the eustachian tube after excessive production of middle ear secretion, bacterial products, inflammatory cells and mucosal swelling (Bluestone & Doyle, 1985).

Inflammatory cells in the middle ear, attracted upon bacterial infection, produce all kinds of cytokines. These cytokines are potent mediators of inflammation and regulators of the immune response (Yellon et al., 1995; Yellon et al., 1991). However, while their control of inflammation represents a beneficial response to infection and injury, cytokines can also cause pathological changes, including mucosal hyperplasia, bone erosion, fibrosis and hearing loss (Yellon et al., 1995). The diversity of potent chemicals in MEE is indicative for the fact that in an inflammatory reaction, once initiated, appropriate conditions can sustain the activation of mediator cascades, assuming that the conditions remain appropriate. In the case of chronic OME, residual endotoxin released from gram-negative bacteria that is trapped in the middle ear can initiate and promote continued production of cytokines and thereby provide a mechanism for sustaining the inflammatory reaction.

For example, TNF-α, an important cytokine found in MEE, is suggested to activate PMNs, promote fibroblast proliferation, inhibit vascular endothelial cell- and B-lymphocyte proliferation, and stimulate cartilage and bone resorption (DeMaria & Murwin, 1997). In chapter II it

was shown that there is an association between the endotoxin- and the TNF-α concentration in MEEs of children with OME. However, no statistical correlation was found. In addition, it was found that children with mucopurulent MEE are more susceptible to develop chronic or recurrent OME especially when they also suffer from an URTI.

Bacterial infection of the upper respiratory tract is therefore suggested to correlate with the initiation of OME (Kuijpers & Beek, 1984; Doyle et al., 1994; Fireman, 1997; Klein, 1997; Miura et al. 1997; Takahashi et al., 1995). Persistence of chronic URTI proved to be a negative prognostic factor for curing OME (Otten & Grote, 1990). Moreover, URTI has a negative influence on tubal compliance in children with OME (Miura et al., 1997). It was suggested that the compliance of the eustachian tube might depend not only on the property of the cartilaginous framework of the tube but also upon the mucosal condition (Brandtzaeg et al., 1997).

Role of the mucosal defense

The MCS is an important defense mechanism. Especially in the middle ear cavity, which is a small and isolated body compartment, clearance is very important. The MCS consists of ciliary epithelium interspersed with secretory cells and a mucus blanket. Impaired mucociliary function is not only due to mucus abnormalities but also due to disturbed ciliary activity (Ohashi et al., 1989). Increased mucus production, sometimes with altered consistency, may affect ciliary beat and its coordination (Brown et al., 1985). Furthermore, ciliary function is sensitive to a variety of pathological agents including bacteria and bacterial endotoxin (Ohashi et al., 1989; Ohashi & Nakai, 1991). Therefore, hyperplasia of the mucosa, massive secretion of mucus by increased numbers of goblet cells, and degeneration or disorganization of

cilia, are the most important factors inducing dysfunction of the MCS. To study middle ear mucosa in a culture model it is important that the mucosa differentiates into the appropriate epithelial cell-types. For that reason, an air-exposed human middle ear culture model was developed, using a collagenous underlayer, which is comparable to the *in vivo* situation. In chapter III the effects of endotoxin on air-exposed cultured human middle ear mucosa were studied. The results suggested that endotoxin has a direct effect on middle ear mucosa. Endotoxin induced: hyperproliferation of the epithelial layer, an increase in the numbers of secretory cells, and an increase in the amount of microvilli. In contrast to Ohashi et al. (1995), we did not observe compound cilia although cilia seen in our study were sometimes very disorganized. Furthermore, Ohashi et al. (1989) found that endotoxin *in vitro* affected the ciliary activity. *H. influenzae* is found to cause degeneration of cilia (Mylotte et al., 1985) and epithelial damage (Read et al., 1991). The morphological effects of endotoxin as described in chapter III did not significantly alter after the application of the two different endotoxin concentrations used (1 and 100 ng/ml). These concentrations resulted in similar morphological changes. Whether there is an all-or-nothing reaction or a dose-response reaction due to endotoxin is, therefore, still not clear. For the induction of morphological changes of the middle ear mucosa a small amount of endotoxin is apparently sufficient to initiate a reaction, whereas high endotoxin concentrations cause cell death (Nakamura et al., 1992). Sufficient amounts of endotoxin will probably start an inflammatory reaction, and under appropriate conditions the inflammation will continue. A dose response relationship was also not evident in an *in vivo* study by DeMaria et al. (1989). These authors found that, a concentration of 0.1 ng endotoxin per ear did not result in any quantitative cytological or histological changes in the middle ear whereas, the response to other

dosage levels (0.01 - 100 µg/ear) resulted all in similar inflammatory effects. In previous studies of our group (van Blitterswijk et al., 1989; Hesseling et al., 1994) with serially submerged cultured rat middle ear epithelium the total number of epithelial cells, counted after trypsinization using a Bürker chamber, increased during 14 days with the endotoxin concentration (from 1 ng to 100 µg). However, with rat meatal epidermis no relation between the concentration and the number of epidermal cells was evident (Hesseling et al, 1994).

In chapter V, the endotoxin effects *in vivo* were studied. It was found that 5 µg endotoxin per ear induced a more pseudostratified, cuboidal epithelium with a temporarily increase in the number of ciliated cells in the rat hypotympanum. Furthermore, an influx of inflammatory cells in the subepithelial layer was observed. In the tympanic orifice, differentiation into a more pseudostratified, cylindrical, secretory epithelium was observed. This activation of the secretory activity, together with the formation of ciliated cells, can be considered as an attempt to fortify the defense system (Kuijpers et al., 1984). Low concentrations of endotoxin probably induce an increase in ciliated cells. Secretory cell hyperplasia is believed to be a result of noxious agents of the inflammatory process or of toxic capacities of infective micro-organisms (Cayé-Thomasen et al., 1995). However, when endotoxin was inoculated into the middle ear after obstruction of the eustachian tube, secretory cell hyperplasia, degeneration of ciliated cells, infiltration of abundant numbers of inflammatory cells, and hyperproliferation of the epithelial layer were observed. Due to the obstruction, endotoxin and various inflammatory mediators will remain in the middle ear cavity. The presence of endotoxin in combination with obstruction of the eustachian tube can result in damage of the MCS. Therefore, it is clear that children with a gram-negative bacterial infection and an obstructed eustachian

tube, caused by either the infection or by dysfunction of the tube, are more susceptible to develop recurrent or chronic OME.

Restoration of the MCS

To treat chronic or recurrent OME it is important to clear the middle ear from the endotoxin, the inflammatory cells and the inflammatory mediators. Treatment with ventilation tubes will only produce a direct improve in hearing but it is probable that they will not solve the problems permanently. It also should be borne in mind that the use of ventilation tubes involves a risk of complications and sequelae which may result in chronic middle ear disease (Lildholdt, 1983). Antibiotics will kill the bacteria but leave the endotoxin in the middle ear cavity. Under normal conditions, any effusion formed in the middle ear cavity will be removed to the nasopharynx, provided that the MCS is functioning properly. However, when the eustachian tube is blocked e.g. due to inflammation, or if the MCS is damaged, the effusion will remain and accumulate in the middle ear cavity. In this thesis it was described that both obstruction of the eustachian tube and the presence of endotoxin induce a permanent mucosal damage which disturbs the clearance. Furthermore, endotoxin *in vitro* induces secretory cell hyperplasia and disorganized functioning of cilia. This will have a negative effect on the clearance as well. Therefore, it is very important to restore the damaged MCS so that the clearance of the middle ear can be re-established. In chapter IV, the Bactericidal/permeability – increasing protein was examined for its inhibition of the effects of endotoxin on cultured human middle ear epithelium. It was found that addition of $rBPI_{21}$ together with endotoxin to the culture medium inhibited or even blocked the formation of morphological changes to the middle ear mucosa by endotoxin. Only some increase in the number of ciliated cells was observed, however, after

treatment with $rBPI_{21}$ no disorganized cilia were present. In chapters VI and VII the effects of $rBPI_{21}$ were studied in an OME model, as was described in chapter V. First inoculation of $rBPI_{21}$ two days after the induction of chronic OME with endotoxin and ETO was studied. $rBPI_{21}$ induced some increase in the number of goblet cells. No disorganization of the cilia was seen. Moreover, application of $rBPI_{21}$ prevented the significant increase in secretory cells, the hyperproliferation of the epithelial layer, and the infiltration of inflammatory cells into the subepithelial layer due to ETO + E. When $rBPI_{21}$ was inoculated two weeks after the induction of OME it was able to restore the numbers of ciliated and secretory cells in the tympanic orifice of the middle ear. However, the infiltration of macrophages in the subepithelial layer was not completely inhibited.

Diverse antibiotics have also shown to inhibit mucus production of rat nasal epithelium after endotoxin installation (Takahashi et al., 1997). However, in spite of the beneficial effects of antibiotics, we have to be careful with their use because of the still growing resistance of certain micro-organisms to antibiotics. Moreover, a major disadvantage of antibiotics is that they can kill the bacteria, but do not neutralize endotoxin. Furthermore, anti-inflammatory drugs have shown to block some inflammatory mediators or their secretion by local mast cells, however, they will not neutralize endotoxin. Thus, current therapies are not sufficient in clearing endotoxin from the middle ear. Endotoxin will remain present in the middle ear cavity, and when the MCS is damaged, endotoxin can keep the inflammatory reaction active. BPI, on the other hand, proved to be able to neutralize endotoxin and also to restore the MCS. Therefore, BPI can be a good alternative in the treatment of chronic OME. In conclusion, this thesis shows that endotoxin *in vitro* as well as in animal experiments has an important influence on the development of

OME and that local neutralization of endotoxin by BPI is able to restore the damage of the middle ear mucosa.

Concluding remarks

The question how long endotoxin actually remains active in the middle ear cavity is still not answered. From the results described in this thesis we have seen that endotoxin can induce morphological changes that persist for 12 weeks. It is also uncertain whether the middle ear mucosa will restore from the damage.

The changes due to endotoxin alone as seen in the present studies were less severe after 12 weeks compared to endotoxin combined with ETO after the same period. Due to the obstruction not only endotoxin but also the inflammatory cells and cytokines remained active in the middle ear and consequently, more damage to the middle ear mucosa could occur. Even when endotoxin is absorbed by macrophages, there will probably be enough bacterial antigens and inflammatory mediators, which persist in the middle ear fluid. Most likely this fluid will be present and active as long as the clearance system is not functioning properly.

BPI seems an ideal therapeutic agent because it is able to neutralize not only endotoxin, but also to inhibit the endotoxin induced responses in the middle ear. This is a promising observation. It is evident that intensive testing of its effect on epithelium and hearing capacity is required before BPI can be applied clinically.

Final conclusions:

1) Endotoxin and TNF-α are present in high concentrations in mucopurulent MEEs of children with a chronic OME (more than six months) and with URTI.

2) Endotoxin induces increased proliferation of human middle ear epithelium *in vitro* with an increased number secretory cells, disorganized cilia, and large amounts of outsized microvilli.
3) The effects of endotoxin *in vitro* can be significantly diminished by addition of $rBPI_{21}$, particularly when endotoxin is still present.
4) *In vivo* endotoxin induces a more secretory and ciliated epithelium, however, 12 weeks after application cilia were found to be disorganized or degenerated. Endotoxin in combination with ETO induces secretory epithelium with degeneration of cilia, and a large influx of inflammatory cells in the subepithelial layer.
5) The middle ear epithelium can be protected from the effects of endotoxin and eustachian tube obstruction by application of $rBPI_{21}$ two days after the induction of OME.
6) The middle ear epithelium can also be restored from damage due to endotoxin and eustachian tube obstruction when $rBPI_{21}$ is applied two weeks after the induction of OME, however, increased numbers of macrophages were still present in the subepithelial layer.

Future directions

The *in vitro* model for human middle ear epithelium as described in chapter III, may be relevant for studies on the role of endotoxin on other tissues: e.g. nasal epithelium or lung tissue. It is also possible to use this model to examine other factors that play a role in the development of chronic infections. The described *in vivo* model (chapter V) it may also be used to study the behaviour of endotoxin in the middle ear: Is it neutralized or is it still active in the middle ear cavity? Is it taken up by macrophages? Do epithelial cells take it up? Or is it passing the epithelial layer towards the bloodstream? Furthermore, the investigation on the influence of BPI on gram-positive bacteria may be of interest. Finally, the

prescription of BPI has to be established in more detail: When should it be prescribed? What dosage should be used? And in which form, are questions that have to be answered.

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Nederlandse Samenvatting

Bijna 75% van alle jonge kinderen heeft minstens een keer middenoorontsteking met ophoping van vocht (effusie) in het middenoor (otitis media met effusie; OME). Dit gaat soms gepaard met een tijdelijk gehoorsverlies. Meestal gaat de ontsteking vanzelf weer over, maar als de effusie aanwezig blijft krijgt ongeveer 15% van deze kinderen een chronische vorm van middenoorontsteking. Hierbij kunnen langdurige gehoorproblemen ontstaan. Tevens kan hierdoor blijvende schade aan het trommelvlies of de gehoorbeenketen optreden. Chronische OME kan zelfs doofheid tot gevolg hebben.

De twee belangrijkste factoren bij het ontstaan van middenoorontsteking zijn gram-negatieve bacteriële infectie en slecht functioneren van de buis van eustachius. Gram-negatieve bacteriën (GNB) bevatten het lipopolysaccharide endotoxine (LPS) in hun buitenste membraan. De toxische eigenschappen van endotoxine worden vertegenwoordigd door het lipide A, wat bij alle verschillende GNB identiek is.

Endotoxine is aangetoond in middenooreffusies en is tevens in staat gebleken inflammatoire reacties in het middenoor en in de buis van eustachius op te wekken. Wanneer de buis van eustachius slecht functioneert of verstopt is kan overtollig vocht niet meer uit het middenoor verwijderd worden. Dit gebeurt normaal gesproken door het mucociliaire clearance systeem (MCS), wat bestaat uit slijmcellen en cellen met ciliën. Door beweging van de ciliën kan het slijm met daarin eventueel aanwezige bacteriën of bacterie producten verwijderd worden uit het middenoor. Als door GNB-infectie en/of obstructie van de buis van eustachius het MCS ontregeld raakt blijven deze producten in het middenoor aanwezig en kunnen zo de ontstekingsreactie opnieuw induceren zodat een chronische OME ontstaat. Zo ontstaat er een vicieuze cirkel. Om deze te doorbreken was het idee om endotoxine te

neutraliseren in het middenoor zodat het MCS weer zou kunnen herstellen.

In dit proefschrift is daarom allereerst de rol van endotoxine bij het ontstaan van chronische OME onderzocht. Vervolgens is gekeken of de effecten van endotoxine op het middenoorweefsel geneutraliseerd kunnen worden met behulp van een endotoxine neutraliserend eiwit (BPI). Dit eiwit is een endogeen eiwit van 55 kDa en is gezuiverd uit granula van witte bloedcellen van konijn en mens. Het is voor het eerst beschreven als antibiotisch eiwit in 1978. BPI bindt sterk aan de verschillende endotoxine chemotypes van GNB en neutraliseert zijn activiteit. Tevens remt BPI de groei van GNB. BPI bindt aan LPS nabij het lipide A deel en vormt zo het LPS-BPI complex dat de schadelijke respons tegen het LPS blokkeert, zoals de LPS-gemedieerde stimulatie van witte bloedcellen. Tevens wordt de door LPS-gemedieerde productie van cytokines (onstekingsmediatoren) geneutraliseerd. BPI heeft verder ook een toxische activiteit tegen GNB.

Tot op heden worden kinderen met middenoorontsteking vooral behandeld met trommelvliesbuisjes, antibiotica of met het verwijderen van de neusamandelen. Vanwege de nadelen van trommelvliesbuisjes (voortijdige uitval, verkleving trommelvlies etc.), de groeiende resistentie van bacteriën voor antibiotica en het niet volledig kunnen verwijderen van de ontstekingshaard, is het belangrijk nieuwe therapeutische mogelijkheden voor middenoorontsteking te ontwikkelen.

In hoofdstuk 2 is onderzocht in welke hoeveelheid endotoxine aanwezig is in middenooreffusies van kinderen met middenoorontsteking. Verder is onderzocht of de endotoxine concentratie gerelateerd is met de TNF-α concentratie. TNF-α is een cytokine geproduceerd door macrofagen als reactie op een ontsteking. Voor dit experiment zijn 140 effusies van

101 kinderen verzameld en geanalyseerd. Het merendeel van deze effusies (53,6%) werd getypeerd als mucopurulent (slijmerig + bacteriën), 23,6% van deze effusies werd getypeerd als sereus (waterig), en slechts 22,8% als muceus (slijmerig). 55% van de kinderen had een chronische middenoorontsteking (langer dan 6 maanden), 18,6% tussen de 3 en 6 maanden (sub-acuut), en 22,8% had acute middenoorontsteking (minder dan 3 maanden). Bij 61% van de kinderen werd tevens bovenste luchtweg infectie vastgesteld. De hoogste concentratie endotoxine werd gemeten in mucopurulente effusies (12 ± 3 ng/mg Totaal Eiwit). Ook de TNF-α concentratie was het hoogst in de mucopurulente effusies (61 ± 21 pg/mg Totaal Eiwit). Kinderen met chronische OME en bovenste luchtweg infectie hadden significant hogere endotoxine en TNF-α concentraties. Deze resultaten wijzen op een relatie tussen endotoxine en TNF-α in middenooreffusies en 1) het type effusie, 2) de duur van de voorafgaande middenoorontsteking, en 3) de aanwezigheid van bovenste luchtweg infectie. Kinderen met mucopurulente effusie hebben waarschijnlijk grote kans op ontwikkeling van chronische OME, zeker als ze ook een bovenste luchtweg infectie hebben. Hoge endotoxine concentraties in middenooreffusies zijn een indicator voor de ernst van de ziekte. Endotoxine speelt dus een belangrijke rol in de pathogenese van middenoorontsteking.

In hoofdstuk 3 is het effect van endotoxine onderzocht op humaan middenoorweefsel, gekweekt volgens de air-exposed methode. Endotoxin concentraties van 1 en 100 ng/ml zijn 2 keer per week toegevoegd aan het kweekmedium. Vervolgens zijn de histologische veranderingen kwantitatief onderzocht met behulp van licht microscopie (LM), scanning electronen microscopie (SEM) en transmissie electronen microscopie (TEM). Onder invloed van endotoxine was er na 21 dagen een toename in de proliferatie van de epitheellaag zichtbaar. Tevens was er een toename

van het aantal slijmcellen en van het aantal microvilli, welke ook in lengte waren toegenomen. Uit deze resultaten kunnen we concluderen dat endotoxine een belangrijke factor is in de verstoring van het middenoorepitheel. Deze verstoring kan verantwoordelijk zijn voor het ontstaan van chronische OME. Deze kweekmethode is tevens een goed model gebleken voor verdere studies met humaan middenoorepitheel.

In hoofdstuk 4 is onderzocht of de effecten van endotoxine op humaan middenoorepitheel geneutraliseerd kunnen worden door een recombinant fragment van BPI ($rBPI_{21}$). Humaan middenoorepitheel werd hiertoe air-exposed gekweekt gedurende 4 weken in kweekmedium waaraan gedurende verschillende periodes endotoxin (1 ng/ml) en/of $rBPI_{21}$ (1ug/ml) was toegevoegd. Endotoxine induceerde een toename in de proliferatie van de epitheellaag en een toename van het aantal slijmcellen. Wanneer eerst endotoxine en daarna rBPI21 werd toegevoegd aan het medium was er geen verdikking van de epitheellaag, maar het aantal slijmcellen was nog steeds toegenomen. Ook was er onder invloed van rBPI21 een toename van het aantal ciliën, maar deze waren niet afwijkend. Wanneer endotoxine gelijktijdig met rBPI21 werd toegediend was er alleen nog een toename van het aantal cellen met ciliën. Het lijkt erop dat rBPI21 in staat is de veranderingen in het middenoorepitheel door endotoxin te remmen.

In hoofdstuk 5 zijn de effecten van endotoxine en obstructie van de buis van eustachius (ETO) bij ratten onderzocht. Hiertoe is met behulp van LM en SEM na 1, 2, 4 en 12 weken de histologie van het middenoor bekeken. Vergeleken met een onbehandeld middenoor bleek dat de epitheellaag door endotoxine (5 ug/oor) of door ETO veranderd was naar meerlagig, cubisch of cylindrisch epitheel met een toename van het aantal slijmcellen per strekkende oppervlakte eenheid. Eerst werd een toename van het aantal cellen met ciliën gezien, maar na 12 weken was er een

duidelijke afname van deze cellen. Een combinatie van ETO en endotoxine injectie induceerde dezelfde veranderingen behalve dat er een directe afname van het aantal cellen met ciliën werd waargenomen. Tevens was er een toename van het aantal ontstekingscellen (macrofagen, lymfocyten en neutrofielen) die in de subepitheliale laag aanwezig waren. Deze veranderingen resulteerden in een beschadiging van het MCS. Hieruit blijkt dat een combinatie van endotoxine injectie en ETO in staat is om OME bij ratten te induceren. Zowel endotoxine als ETO spelen dus een belangrijke rol bij het ontstaan van langdurige veranderingen in het middenoorepitheel waardoor de middenoorontsteking voortduurt.

In hoofdstuk 6 is onderzocht in hoeverre $rBPI_{21}$ in staat is om de bij de rat geïnduceerde effecten van endotoxine en ETO te voorkomen. Hiertoe werd $rBPI_{21}$ twee dagen na inductie van middenoorontsteking rechtstreeks in het middenoor toegediend. De histologie werd bestudeerd na 1, 2, 4 en 12 weken. Het bleek dat toediening van $rBPI_{21}$ het verlies van ciliën kon voorkomen. Tevens werd door $rBPI_{21}$ de significante toename van het aantal slijmcellen voorkomen. Uit deze resultaten blijkt dus dat $rBPI_{21}$ in staat is om het middenoorepitheel te beschermen tegen verstoringen van het MCS, en wellicht een therapie kan zijn ter preventie van chronische OME.

In hoofdstuk 7, tenslotte, is gekeken of $rBPI_{21}$ ook in staat is om geïnduceerde effecten van endotoxine en ETO te herstellen. Hiertoe is $rBPI_{21}$ twee weken na de inductie van middenoorontsteking bij ratten rechtstreeks in het middenoor toegediend. Histologie werd vervolgens na 4 en 12 weken bestudeerd. Het bleek dat $rBPI_{21}$ in staat was om het verlies van ciliën en de toename van het aantal slijmcellen te herstellen. Na 12 weken was er slechts een lichte toename van het aantal macrophagen in de subepitheliale laag. Uit de resultaten blijkt dat de geïnduceerde veranderingen in het MCS hersteld kunnen worden door

$rBPI_{21}$ behandeling. $rBPI_{21}$ kan wellicht een belangrijke rol gaan spelen bij de behandeling van middenoorontsteking en andere ontstekingen van de (bonveste) luchtwegen.

Samengevat heeft dit onderzoek aangetoond dat endotoxine een belangrijke rol speelt bij het ontstaan van middenoorontsteking en tevens bij de continuering van de ontstekingsreactie. Het blijkt vooral in combinatie met obstructie van de buis van eustachius in staat om langdurige verstoring van het mucociliaire clearance systeem te veroorzaken waardoor ontstekingsmediatoren en bacterie producten in het middenoor aanwezig kunnen blijven en de ontstekingsreactie kunnen blijven activeren. Het endotoxine neutraliserende eiwit BPI is in staat gebleken om histologische veranderingen, zowel in het kweekmodel als in het rattenmodel voor OME, te voorkomen en zelfs te herstellen. Dit kan betekenen dat behandeling met $rBPI_{21}$ in de toekomst een nieuwe therapie voor middenoorontsteking zal zijn.

Na vier jaar in de gelegenheid te zijn geweest om onderzoek te mogen doen op de afdeling keel- neus- en oorheelkunde, is dit boekje tot stand gekomen. Met soms wat zweet, hard werken, weer verhuizen, inzicht, doorzettingsvermogen, en niet al teveel stress is het eigenlijk allemaal op rolletjes gegaan. Maar, de resultaten waren natuurlijk niet tot stand gekomen zonder de bijdrage van anderen die mijn onderzoek mogelijk hebben gemaakt. Een aantal wil ik hier noemen.

Alle medewerkers van de afdeling KNO en van het Audiologisch Centrum. Brenda voor het steeds maar weer moeten verhuizen van het lab en het opstarten van alle werkzaamheden. Bart van der Lans voor het meedenken met het maken en afdrukken van de vele lichtmicroscopische foto's. Jeroen voor het helpen ontwerpen van de voorkant.

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Marijke von der Möhlen heeft me in contact gebracht met XOMA (US) LLC. en geholpen met de aanvraag voor BPI. Zonder dat zou het onderzoek niet zijn geworden tot wat het nu is.

De contacten met de afdeling KNO in Nijmegen, waar ik de nodige kennis heb opgedaan op het gebied van de middenoor-histologie en het opereren van de ratten.

Verder alle familie en vrienden die gezorgd hebben voor de nodige afwisseling van het onderzoek met andere leuke dingen. Mijn tennismaatjes: René, Jan Willem en Diana voor de nodige inspanning en ontspanning op de baan en daarnaast.

Tenslotte mijn ouders voor de mogelijkheid die jullie mij gegeven hebben om te kunnen studeren en de richting op te gaan die ik leuk vond. Wiebe, voor alle steun, liefde en vertrouwen in mijn kunnen.

Marja

De schrijfster van dit boekje werd op 20 juni 1968 geboren te Amersfoort. In 1984 behaalde zij haar Mavo-diploma, waarna ze naar de middelbare laboratoriumschool in Amersfoort ging. Na het behalen van haar diploma in de klinisch-chemische richting werd de studie voortgezet aan de hogere laboratorium school. Na het behalen van de propedeuse nam ze de overstap naar de Universiteit Utrecht waar ze met de studie medische biologie begon in 1989. Tijdens deze studie deed ze onderzoek naar cel-gemedieerde immuniteit bij katten met AIDS, bij de vakgroep Infectieziekte & Immunologie van de faculteit Diergeneeskunde in Utrecht onder leiding van dr. Claire Boog. Daarna onderzocht zij drug-targeting met immunoliposomen bij ovarium carcinoma's, bij de vakgroep pathologie van het RIVM onder leiding van dr. Peter Steerenberg en Monique Vingerhoeds. Tenslotte ging ze 6 maanden onderzoek doen naar *Leishmania infantum* infectie bij honden, bij de vakgroep pathologie van de Universidad Complutense in Madrid, onder leiding van prof. dr. José Maria Alunda. In 1994 studeerde ze af, waarna ze in 1995 bij de vakgroep KNO in Leiden met haar promotieonderzoek begon, waarvan de resultaten staan beschreven in dit proefschrift. Binnenkort hoopt ze als post-doc het onderzoek met het bactericidal/permeability-increasing protein (BPI) voort te zetten op het Leids Universitair Medisch Centrum.

Stellingen

behorende bij het proefschrift:
The role of endotoxin in the pathogenesis of otitis media with effusion.

1. Het voorkomen van chronische middenoorontsteking gaat meestal gepaard met de aanwezigheid van taai slijm in het middenoor en infecties van de bovenste luchtwegen. *Dit Proefschrift.*

2. De toename van het aantal slijmcellen in het middenoor en de vorming van cilia door endotoxine is bedoeld voor een verbetering van de clearance van het middenoor, maar lijdt vaker tot een obstructie. *Dit Proefschrift.*

3. BPI beschermt niet alleen tegen systemische effecten van endotoxine, maar kan ook locaal in het middenoor bescherming bieden. *Dit Proefschrift.*

4. Kinderen met middenoorontsteking zijn onder te verdelen in twee groepen: zij die binnen een paar maanden beter worden, en zij die dat niet worden.
R.M. Rosenfeld. *Int. J. Pediatric Otorhinolaryngol.* 1998;43:189-192.

5. Toename van antibiotica-gebruik bij middenoorontsteking heeft weliswaar de ernst van de ziekte vermindert maar niet het aantal gevallen.
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