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Developing Otitis Media
Experimental Studies in Particular Regarding Inflammatory Changes in the Tympanic Membrane

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To Maria, and our children Agnes, Anton, and Saga
ABSTRACT

Otitis media (OM), one of the commonest of childhood diseases, causes much suffering. OM exists in a variety of forms, two of which are acute otitis media (AOM) and otitis media with effusion (OME). The clinical courses of these conditions differ, AOM usually presenting with earache, fever and/or aural discharge, and the OME usually with hearing impairment. The tympanic membrane (TM) mirrors the events in the middle ear cavity, and pars flaccida (PF) is the initial site of inflammatory changes in the TM. PF is rich in mast cells (MCs), which by releasing various mediators, may trigger TM inflammation.

The aims of the present studies were to investigate early inflammatory changes in the TM in rat models of OM; after mast cell degranulation, in response to AOM, and OME, after myringotomy in AOM and in normal ears. Furthermore, we developed a new rat AOM model, that excludes surgical trauma and resembles the natural route of infection in man.

AOM and OME elicited the first inflammatory response in PF of the TM. The response to OME was discrete, but a slight increase in macrophages was found. During the first 48 hours of AOM, the inflammatory response was intense, following a bimodal pattern. This reaction is similar to that found after MC degranulation. In AOM, macrophages were the predominant cell in PF, while in pars tensa (PT), polymorphonuclear cells (mainly neutrophils) predominated.

When myringotomy was performed in AOM ears, the healing time was shorter than that of myringotomy in normal ears. The highly inflamed lamina propria seemed to promote healing.

During early AOM, as well as following myringotomy, fibrin extravasates into PF and PT. This fibrin deposition may be involved in regulating the inflammatory response.

Repeated nasal challenge with the otitis media pathogen *Streptococcus pneumoniae* provoked AOM and concomitant TM stimulation reduced the number of AOM cases. This new rat AOM model has the advantage of avoiding trauma in the middle ear cavity, while eliciting an intense inflammatory response in the middle ear cavity (MEC).

Keywords: otitis media with effusion, acute otitis media, myringotomy, tympanic membrane, rat, *Streptococcus pneumoniae*, compound 48/80, mast cells
ORIGINAL PAPERS

This thesis is based on the following publications and manuscripts, which will be referred to by their roman numerals:


IV. Eriksson PO, Johansson C, Hellström S. Inflammatory cells during developing otitis media. An experimental sequential study. [manuscript]

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AOM</td>
<td>acute otitis media</td>
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<tr>
<td>CFU</td>
<td>colony-forming units</td>
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<td>EAC</td>
<td>external ear canal</td>
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<td>EM</td>
<td>electron microscopy</td>
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<td>ET</td>
<td>Eustachian tube</td>
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<td>FN</td>
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<td>IR</td>
<td>immunoreactivity</td>
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<td>iv.</td>
<td>intravenous</td>
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<td>LM</td>
<td>light microscopy</td>
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<td>MC(s)</td>
<td>mast cell(s)</td>
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<td>MEC</td>
<td>middle ear cavity</td>
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<td>OM</td>
<td>otitis media</td>
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<td>OME</td>
<td>otitis media with effusion</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PF</td>
<td>pars flaccida</td>
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<tr>
<td>PMN</td>
<td>polymorphonuclear cell</td>
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<td>PnC</td>
<td><em>Streptococcus pneumoniae</em></td>
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<td>PT</td>
<td>pars tensa</td>
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<td>TM</td>
<td>tympanic membrane</td>
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INTRODUCTION

The middle ear cavity (MEC) is located, well hidden, in the temporal bone. The middle ear augments and transmits acoustic energy from the air-filled external ear canal, through the ossicular chain, via the oval window to the fluid-filled inner ear. Pathological processes of the middle ear range from being asymptomatic, as in myringosclerosis and otitis media with effusion without hearing impairment, to life-threatening complications such as meningitis and brain abscesses. However, middle ear disease is seldom fatal nowadays. More often, sequelae of middle ear disease can be recorded as a conductive or sensorineural hearing loss.

Except for the common cold, the most common childhood disease is otitis media (OM). However, OM is not a single disease entity, and the term has therefore been expanded to classify this condition into otitis media without effusion, acute otitis media (AOM), and otitis media with effusion (OME). Also related to this is Eustachian tube (ET) dysfunction, which can cause symptoms similar to those of OM, such as hearing loss, otalgia, and tinnitus, though no middle ear effusion is present [1].

The most common bacteria in AOM are Streptococcus pneumoniae (PnC), non-typeable Haemophilus influenzae, Moraxella catarrhalis and to a lesser extent Streptococcus pyogenes [2-4]. However, other bacteria are also implicated in the pathogenesis, viz. Chlamydia pneumoniae [5, 6]. In addition, viruses are detected in 42 to 82 percent of middle ear effusions by use of PCR techniques [7, 8].

Bacteria can also be cultured in OME (PnC, H. influenzae, M catarrhalis, S. pyogenes), though to a lesser extent, e.g. in 41% of children below the age of two years and in 17% of older children, according to Jero & Karma. [9]. However, when using PCR, bacterial DNA is present in approximately 80% of the effusions [10, 11]. A viral cause of OME has also been suggested, and in 30% of patients viral RNA can be found, in particular human rhinovirus, according to Pitkäranta et al. [12].

A preceding or concomitant viral upper respiratory tract infection increases susceptibility to development of bacterial AOM. Viruses can affect the ET function, via epithelial cell damage, ciliary dysfunction and cause a negative middle ear pressure. Viral infections also increase nasopharyngeal colonization with bacterial AOM pathogens [13-15]. During a viral upper respiratory tract infection it usually takes 2-5 days until the onset of AOM peaks [16, 17].

Aloiococcus otitidis is a Gram positive aerobic coccus, that has been detected in middle ear effusions from children with OME [11, 18]. Whether this bacterium is an OM pathogen or a commensal from the EAC is still unclear [19].

The tympanic membrane (TM) plays a crucial role in OM. Not only is it a target tissue for the pathological process of disease; for clinicians it is a window into the middle ear. In general, various stimuli generating an inflammatory condition in the middle ear elicit the first visible signs of inflammation in pars flaccida (PF) of the TM [20-22].

The distinction between AOM and OME is based on symptoms and on otological
examination of the TM and middle ear. In AOM the TM is inflamed, bulging and/or the MEC is filled with pus. The onset of the condition is rapid. At least one of the following symptoms should be present: otalgia, fever, irritability, anorexia, vomiting, or diarrhoea. In OME, the TM may be retracted, or bulging, and is less mobile. It should be opaque, and it is sometimes difficult to distinguish effusion with certainty. In comparison with AOM, symptoms such as otalgia and fever are absent; instead, conductive hearing loss is a common finding [23].

A considerable body of knowledge has been collected regarding tissue reactions in the middle ear in OM. This knowledge emanates from clinical studies, as well as from experimental studies on OM in animal models. In these studies, various species as well as different methods to elicit OM, have been used [24-26]. However, a common feature of earlier morphological studies is that the data have been collected mainly at weekly intervals [25]. Obviously, data on the early events, during the first hours or days, are less common. We can assume that the processes involved in the early phase of developing OM constitute much more rapid dynamics and, consequently, events can be missed if the intervals between the onset of the condition and collecting of the specimens are too long. However, with a modified experimental design, some of the questions concerning the initial response to OM could be answered.

Over the last 20 years the rat has become the most common species used in OM research. Various models for OME and AOM have been developed. For OME, the Eustachian tube has been blocked at the tympanal orifice [27], but external ligation and cauterization of the tube have been used [28]. The AOM models have so far included surgical trauma either to the MEC [26] or to the TM [29]. Furthermore, by using these models, the natural infectious route for the bacteria is avoided. By developing an animal model that excludes trauma to the MEC, and mimics the natural route by which bacteria enter the MEC, the initial response to bacterial exposure of the nasopharynx, ET and middle ear can be studied.

The present studies were undertaken to answer questions on the early inflammatory cell reactions of the TM in AOM and OME, and to develop a new animal model which excludes trauma to the MEC.

BACKGROUND

Anatomy of the rat middle ear

Middle ear cavity

In many respects the rat middle ear resembles the human middle ear. In both species the middle ear is separated into an epi-, meso-, and hypotympanum. However, certain structures differ in appearance. In contrast to the human, the rat lacks a mastoid system.
with air-filled cells, but has instead a large middle ear bulla.

The epitympanum, or attic space, houses most of the rat middle ear ossicles. The attic is divided into a lateral and a medial compartment, the former being connected with the mesotympanum by the narrow tympanic isthmus. The epi- and mesotympanum are separated by the middle ear ossicles and thin mucosal membranes surrounding the bones, as in the human [30].

On the medial wall of the hypotympanum, anterior to the promontory, the opening of the ET is located and above and anterior to this, the nasal fossa (FN). The round window niche and oval window are located posterior to the promontory (Fig. 1B). The hypotympanum corresponds largely to the bulla of the rodent’s ears [31].

Fig. 1 Schematic drawing of the rat middle ear cavity. A) Lateral wall showing middle ear ossicles, tympanic membrane with its vascular supply and mast cells. B) Medial wall showing middle ear ossicles, tracts of ciliated epithelium, mast cells.

Middle ear mucosa
The middle ear mucosa resembles that of the human middle ear. Most of the middle ear is covered by a simple squamous/cuboidal epithelium [32-34]. The ciliated cells are more or less restricted to two tracts, the anterior and inferior [32, 34, 35](Fig. 1B). In the rat the tracts are covered with a pseudostratified columnar epithelium of ciliated and secretory cells. The anterior tract continues from the tubal opening and ends on the anterior superior part of pars tensa (PT) of the TM. This anterior tract appears to connect the medial epitympanic compartment with the ET. The inferior tract runs below the promontory, posterior to the round window niche, and ends on the posterior part of the TM, close to the tympanic isthmus [34].
Tympanic membrane

The TM constitutes the lateral limit of the MEC. Its obvious functions are sound transmission and as a barrier against external infectious and irritating agents. It also plays an important diagnostic role in middle ear conditions. The human TM was first described by Hippocrates [36]. In 1832 Shrapnell described it as comprising two parts; the PT and the PF [37] (Fig. 1B).

Pars tensa

In the rat, as in the human, the PT consists of three layers. The rat PT is 5-10 µm thick, compared with 64-95 µm in man. The outer layer is a keratinizing, stratified squamous epithelium, 2-3 cell layers thick. The middle layer, lamina propria, consists mainly of an outer radial and an inner circular layer of densely packed collagen fibres surrounded by thin subepidermal and subepithelial layers of loose connective tissue [38] (Fig. 2B). Fibroblasts and nerve fibres are present in the connective tissue layers [39, 40]. These nerve fibres exhibit neuropeptide immunoreactivity, for e.g. substance-P and calcitonin gene-related peptide, neurokinin A, and in the fibrocartilaginous ring of the PT, catecholamine containing nerve fibres can be detected [41, 42].

Vessels in the PT, originating from the external carotid artery, are present along the handle of the malleus, beneath the outer epithelium, and along annulus fibrosus. In the annulus area the vessels are located directly beneath the inner epithelium emanating from the MEC [43] (Fig. 1A). Under normal conditions, the central portion of the PT is devoid of vessels [44]. Mast cells (MCs) are present in the PT, though few and can be found along annulus fibrosus and along the malleus handle [34].

The main function of the PT is to mediate sound wave transmission to the middle ear ossicles and inner ear.

Pars flaccida

The PF is contiguous with the EAC skin. It is thicker than the PT, in the rat measuring approximately 30 µm [45] compared with 30-230 µm in man [46]. Like the PT, the PF consists of an outer epidermal layer, a middle lamina propria layer and an inner mucosal layer (Fig. 2A). However, in contrast to PT the lamina propria of the PF is made up of loose connective tissue with fibroblasts, collagen fibres, elastic fibres, and nerve fibres [47]. The blood vessels of the PF emanate from the external carotid artery and are found in an inner and outer network, mainly beneath the outer keratinized epithelium but also directly beneath the inner epithelium [43]. Numerous MCs are present in both the human and the rat PF [45, 48].

The physiological role of the PF is still not fully understood. Experimental studies have shown that the PF responds before the PT when subjected to an inflammatory stimulus, irrespective of the causative agent [20-22].

The elasticity of the PF is remarkable. The rat PF responds to a minor change in negative middle ear pressure by retracting and to a positive middle ear pressure by bulging [49]. It has also been suggested that the PF acts as a baroreceptor, detecting small changes in middle ear pressure [50]. Under pathological conditions the retracted PF is associated
with a mastoid with less pneumatization [51]. Whether or not the mobility of the PF serves as a pressure regulator to maintain the functionality of the sound conductive capacity of the middle ear, or is a sign of disease, is an open question.

Fig. 2 Electronmicrograph of A) normal pars flaccida and B) normal pars tensa. Outer keratinized epithelium top of figure.

**Eustachian tube**

Bartolomeus Eustachius described the pharyngo-tympanic tube in 1563 in his *Epistola de Auditus Organis*. It was renamed the Eustachian tube by Antonio Valsalva about 150 years later.

In general, the anatomy of the rat ET resembles that of the human. The distance from the nasopharyngeal orifice to the tympanic orifice of the tube in the rat measures about 4.5 mm [52]. In the rat the tube is longer than e.g. that of the gerbil [53]. When the soft palate is removed the mucosal lips of the nasopharyngeal orifice can be visualized. The tympanic orifice is located in the nasal part of the MEC, above the floor of the middle ear bulla. The surface of the ET is covered by a pseudostratified respiratory epithelium with goblet cells, ciliated cells, non-secretory/non-ciliated cells and basal cells. Numerous serous and mucous glands are present in the lamina propria [52]. In contrast to the guinea-pig [54], there are few MCs in the mucosa of the rat ET [55]. It has been found that the opening pressure of the rat ET is comparable to that of the human [49]. In contrast, the ET of the chinchilla is (semi-)patulous [56].

The functions of the ET involve pressure regulation of the middle ear, protection of the middle ear from nasopharyngeal sound pressure and secretions, and clearance of secretions from the MEC. Thus, ET dysfunction plays a central role in the pathogenesis
of OM [57].

For pathogens to enter the ET, the defence system has to be overcome. The first line of defence harbours the mucociliary apparatus and molecules of the innate immune-system. Glands and epithelial secretory cells have the capacity to secrete mucins, aquaporins, and surfactant. Secreted anti-microbial proteins and peptides, suggested to be important in the defence, are lysozyme, lactoferrin, defensins and collectins [58].

**Mast cells**

MCs are distributed throughout the body and in close proximity to epithelial surfaces of the skin, the respiratory tract, and in the gastrointestinal mucosa [59]. Interestingly, the PF of the TM contains an extremely large number of MCs, in the human and in the rat [45, 48]. Rat PF contains approx. 18000 MCs per mm$^3$, which is fourfold greater than the peritoneum [45].

**Mast cell heterogeneity**

MCs are a heterogeneous group of cells. The first evidence of histochemically and functionally different MCs was described during the 1960s when Enerbäck classified rodent MCs as connective tissue mast cells or mucosal mast cells [60-63]. These two types differ structurally but also in their content of mediators. For instance, the connective tissue MCs contain more histamine and serotonin than do mucosal MCs. In the connective tissue MC the proteoglycan heparin predominates. In the mucosal MC, the predominant proteoglycan is chondroitin sulphate B [59]. There is also a difference in protease content.

Human MCs are often classified according to their content of neutral proteases, chymase and tryptase [64].

**Mast cell mediators**

One unique feature of the MCs is their ability to release a wide variety of mediators. These may be preformed and/or synthesized de novo. They are categorized into preformed secretory granule-associated mediators, lipid-derived mediators, and cytokines.
Table 1. Examples of the MC mediators [59].

<table>
<thead>
<tr>
<th>Preformed granule-associated mediators</th>
<th>Lipid-derived mediators (synthesized de novo)</th>
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<tbody>
<tr>
<td>Amines (histamine, serotonin (in rodents))</td>
<td>Platelet activating factor</td>
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<tr>
<td>Proteoglycans (heparin, chondroitin sulphates)</td>
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<td>Neutral proteases (chymase, tryptase)</td>
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<td><strong>Neutral proteases</strong> (synthesized de novo)</td>
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<td>Lipid-derived mediators (synthesized de novo)</td>
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<td><strong>Lipid-derived mediators</strong> (synthesized de novo)</td>
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<td><strong>Lipo-oxygenase products</strong></td>
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<td><strong>Cyclo-oxygenase products</strong></td>
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<tr>
<td>Prostaglandins</td>
<td>Leukotrienes</td>
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<tr>
<td>Thromboxanes</td>
<td>Hydroperoxyeicosatetraenoic acids</td>
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<td>Hydroxyeicosatetraenoic acids</td>
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<tr>
<td>Cytokines and Chemokines</td>
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<tr>
<td>Metabolites of arachidonic acid:</td>
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<tr>
<td>Protein or glycoprotein molecules</td>
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<tr>
<td>Interleukins, interferons, colony-stimulating factors (i.e.; Il-2, -3, -4, -5, -6, -8, IFN-γ, GM-CSF, TNF-α, MIP-1α, MIP-1β, TCA-3, MCP-1, RANTES, lymphotactin)</td>
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**Mast cell activation**

MCs can be activated by different mechanisms. IgE-receptors, expressed on the surface of MCs, bind allergens with IgE antibodies attached, and the mediators are released. Furthermore, binding of various peptides (such as substance P and calcitonin gene-related peptide), cytokines, anaphylatoxins, dextrans, lectins, to low affinity IgG receptors can activate MCs. Basic compounds, such as compound 48/80 and polymyxin B, can also activate MCs [59]. Compound 48/80, a well-known MC secretagogue, results from condensation of p-methoxyphenethylmethylamine with formaldehyde [65, 66]. It activates only connective tissue MCs [60] after an assumed direct activation of G-proteins [59].

**Mast cell functions**

For decades, MCs have been regarded as immediate reacting cells in anaphylactic reactions and therefore as potentially harmful cells. In allergy, MCs set in motion a series of inflammatory events that elicit an immediate response. However, MCs have diverse roles, and are related to other pathophysiological events such as fibrosis, late phase reactions, autoimmune disease, angiogenesis, neoplasia, inflammatory bowel disease [59], and tissue repair [67]. It is interesting that MCs have been suggested to be important for immune defence. They are strategically located in the interface tissues between the external environment and the host, and come into contact with invading infectious agents at an early stage. The release of mediators initiates an inflammatory reaction, an important response for an adequate immune defence [68].
Middle ear mast cells

That MCs are present in the middle ear mucosa has been known since the late 1950s [69] and further studies showed them to be a common component of the middle ear mucosa [34, 70, 71]. MCs have also been found in the ET mucosa of guinea pigs [54]. Related to this it is of interest that histamine reduces the patency of the ET [72, 73]. Furthermore a low dose histamine induces a ciliostimulatory effect, whereas a high dose deteriorates ciliary activity of tubotympanal mucosa [74]. As mentioned earlier, PF contains an extremely large number of MCs, located in the loose connective tissue layer beneath the outer keratinized epithelium.

The MCs of PF are activated by various stimuli, and an increased histamine content is found in the effusion material of the MEC after mechanical stimulation of the external auditory canal (EAC) [75], and after application of compound 48/80 [76]. After performing these procedures, effusion material accumulates in the attic region of the MEC and it is suggested that MCs are involved in the pathogenesis of OME. Significant amounts of histamine have also been found in human middle ear effusions [77]. Furthermore MCs have also been suggested to play a role in bone resorption in chronic otitis media [78, 79].

In experimental AOM conditions MCs degranulate at an early stage [21]. It is conceivable that they are of importance to initiate an adequate and early immune response of the TM and MEC.

Experimental OM models

To our knowledge, the first study on experimentally evoked OM was published by Ludwig Hayman in 1913. As experimental animal, he used the guinea pig, inoculating various bacteria through the nose, through the TM or through the bulla to elicit an infectious condition. He also tried to intervene in the course of the infection, when he perforated the TM prior to nasal inoculation of bacteria [80-83].

The species used in experimental OM research have varied over the years. On entering the PubMed and covering January 2000 to August 2003 and searching for animal studies, otitis media, we find the rat to be the most common species used in OM research. The rat is used in more than half of the experimental studies, and is three times more commonly used than the chinchilla in second place. Other species utilized are guinea pig, rabbit, gerbil, mouse, and monkey. Twenty years earlier, the rat was only the third most common species (~15%) in OM research, lagging behind the chinchilla (~50%) and the guinea pig (~20%).

The established rat model of AOM allows us to study the various OM pathogens during the course of infection [26, 84, 85]. However, when inoculating live bacteria into the MEC, this procedure bypasses the natural route of infection and will entail a surgical trauma. Animal models using intranasal inoculation of bacteria have been described for other species, e.g. guinea pig and chinchilla [80, 86], but in the rat not without perforation of the TM [29].
Inflammatory cells

In the rat TM and MEC, few types of immunocompetent cells are found under normal conditions. The exception is the presence of MCs in the mucosal tracts with ciliated and secretory cells [34] and in the PF [87] and macrophages and dendritic cells in the MEC mucosa [88], and ET [89]. Only a few NK cells, T- and B-lymphocytes, and scarcely any granulocytes can be found [89]. These findings are consistent with those in the MEC mucosa of the mouse [90].

During pneumococcal AOM, early infiltration of inflammatory cells is found in the rat TM [21]. Three days after bacterial inoculation, macrophages predominate over T- and B-cells [88]. However, pneumococcal cell wall products and non-viable PnC also elicit an inflammatory response, with infiltration of polymorphonuclear cells (PMNs) to the middle ear mucosa [91, 92]. The inflammatory response in experimental OME differs from that of experimental AOM. In OME, the inflammatory cells in the effusion peak 3-4 weeks after initiating the event and neutrophils and macrophages predominate. In AOM neutrophils predominate, and are most abundant one week after bacterial inoculation [93].

Myringotomy

Myringotomy has been performed since the 18th century, originally as a treatment for deafness [94].

Myringotomy can be used as a therapeutic measure in AOM and OME. However in the treatment of uncomplicated AOM, myringotomy with or without antibiotic treatment has not proved to be more effective than antibiotics alone, concerning relief from symptoms or earlier resolution of disease. For the treatment of OME, myringotomy and ventilation tubes are superior to myringotomy alone [95].

When myringotomy is performed in the rat TM, an immediate and early inflammatory reaction is triggered. The inflammatory reaction occurs in the PF earlier than that in the PT [22]. After performing myringotomy in the rat TM, it usually heals within 9-11 days. In the healing of a PT perforation, a keratin spur advances ahead of an ingrowing and remodelling epithelium and connective tissue [96]. When myringotomy is performed in experimental AOM, the infection resolves more slowly and the connective tissue structure does not return to normal. However, in AOM the closure time of the perforation is reduced [97].
AIMS OF THE STUDY

The aim of the present thesis was to characterize the early inflammatory changes in the TM in response to MC degranulation, OME, and AOM with or without myringotomy, and to establish a rat model for AOM which mimics the natural route of infection.

Against this background, experiments were designed to:

- characterize and compare the early inflammatory cell response in AOM caused by *Streptococcus pneumoniae*, with that in OME, caused by blocking of the ET;

- describe the morphological changes in the TM after degranulation of MCs with the secretagogue 48/80;

- compare the early inflammatory response in the TM to myringotomy in an AOM condition versus normal condition;

- develop a rat AOM model, mimicking the natural route of infection, with minimal trauma to the animal;

- elucidate whether inflammatory reactions in the TM affect the development of AOM during intranasal challenge of bacteria.
MATERIALS AND METHODS

Animals
Male Sprague-Dawley rats weighing 200-350 g were used in the studies. All investigations were approved by the University of Umeå Ethical Committee for Animal Studies (A83/97, A120/00). The animals were kept under standard laboratory conditions with free access to food and water.

Anaesthesia

Paper I: For otomicroscopy and TM treatment, anaesthesia was induced with a short-acting barbiturate, sodium methohexital (Brietal® Eli Lilly and Co, Ind., USA) administered by injection through a tail vein. To prolong the period of anaesthesia, an intraperitoneal bolus dose of pentobarbital sodium (Apoteksbolaget Umeå, Umeå, Sweden) (40 mg/kg) was added.

Papers II, III and IV: For otomicroscopy and the surgical procedures the animals were anaesthetized by injecting of sodium methohexital via a tail vein.

Paper V: For the bacterial inoculation, TM treatment and otomicroscopy, the animals were anaesthetized with propofol (Diprivan®, AstraZeneca, Södertälje, Sweden) via a tail vein.

All animals were sacrificed with an intraperitoneal overdose of pentobarbital sodium.

Experimental procedures

Paper I: Thirty-five rats were used in the study. Two were kept as untreated controls, while 22 were treated with compound 48/80 (Sigma-Aldrich Sweden AB, Stockholm, Sweden), dissolved in physiologic saline at a concentration of 2 mg/ml. Approximately 0.1 ml of the solution was instilled into each right and left EAC to cover the entire TM. The remaining 11 rats were treated with physiologic saline. A cotton tamponade, soaked in the same solution, was then placed in all EACs.

The rats were treated once daily for 4 consecutive days with the same solution. Two compound-treated and one saline-treated animal were sacrificed at 3, 6, 9, 12, 18, 24, 36, and 48 hours and 4, 6, and 8 days after treatment. TM status was examined and documented at treatment and sacrifice. The 2 untreated rats were sacrificed at the end of the experiment.

Paper II: The animals were divided into two groups. In both, a ventral neck incision was performed, and the left middle ear bulla was reached and opened. In one group (n=28), experimental AOM was initiated by inoculating of PnC, type 3, suspended in Todd-Hewitt broth (Difco®, ~50 µl, 10⁷ CFU/ml) [26]. In the other group (n= 28), a gutta-percha plug was inserted into the tympanic orifice of the ET to initiate OME [27]. The right middle ear was left as control. Another 4 rats were kept as untreated controls.
After otomicroscopic examination and documentation, 4 animals from the AOM group and 4 from the OME group were sacrificed at 3, 6, 9, 12, 18, 24 and 48 hours. The 4 control animals were killed at the end of the experiment.

Paper III: In 36 rats, experimental AOM was initiated in the left middle ear as described above. After 2 days (day 0), all ears which had been exposed to bacteria had developed signs of AOM. Four animals selected at random were immediately sacrificed. In the remaining animals, bilateral myringotomy was performed in 26 and unilateral myringotomy in 6. The TM perforation occupied the posterior superior quadrant of the PT. At 3, 6, 9, 12, 24 and 48 hours, and at 4 and 7 days, 4 animals were sacrificed after otomicroscopic examination of the TM status.

Paper IV: The paraffin-embedded specimens in paper II were used. Experimental AOM and OME had each been initiated unilaterally in 14 rats. Animals were sacrificed at 3, 6, 9, 12, 18, 24 and 48 hours. Two untreated rats served as controls.

Paper V: Twenty-two rats were used for the study. Twenty rats underwent bacterial inoculation into the nasopharynx via a polyethylene catheter (O.D. 0.965 mm) via the left nostril. The tip of the catheter was placed at the level of the ET, approximately 33 mm from the nostril. Through the catheter, PnC type 3, suspended in Todd-Hewitt broth (Difco® 100 µl, ~10^7 CFU/ml) was instilled into the nasopharyngeal space. The rats were inoculated in the same manner, once daily for 5 consecutive days. To elicit MC degranulation in the TM, 10 rats were treated with the MC secretagogue compound 48/80, dissolved in physiologic saline at a concentration of 2 mg/ml. Under the otomicroscope, approximately 0.1 ml of the compound solution was instilled into the external ear canal to cover the TM. Five rats received this treatment bilaterally and 5 rats unilaterally. Another, 10 rats were treated, 5 unilaterally and 5 bilaterally, with physiological saline instead of compound 48/80. The contralateral TM was left untreated in all unilaterally treated animals.

Both compound 48/80 and saline were applied 12 h prior to the first inoculation of PnC and then each time the animals were anaesthetized for inoculation of bacteria. Two animals were kept as untreated controls. The middle ear status was examined and documented at the treatment of the TM and when the rats were sacrificed on day 6.

**Plastic-embedded material**

After sacrifice by an overdose of pentobarbital, all the animals were decapitated. The middle ear bulla was dissected free from soft tissue and opened. The MEC was filled with a fixative containing 3% glutaraldehyde in 75 mmol/l sodium cacodylate buffer with 4% polyvinylpyrrolidone and 2 mmol/l CaCl₂ added. The TM and surrounding bony rim was cut free from the temporal bone and the specimens were immersed in the fixative (paper I); in papers II (n=30), III (n=18), and V the whole skull was immersed in the fixative after opening of the bulla.

After dissection, all specimens were post-fixed overnight in 1% OsO₄ and dehydrated in
a graded series of alcohol. The specimens were then embedded in an epoxy resin. Semithin sections, 0.5 - 1.0 µm were prepared for light microscopy (Reichert’s Ultracut S; Leica, Austria), and, ultrathin sections 70 - 85 nm were contrasted with uranyl acetate and lead citrate for electron microscopy (papers I, II, III). The PFs and the PTs were cut in the horizontal plane. The ETs were sectioned longitudinally.

**Paraffin-embedded material**

Papers II (n=30) and IV (n=30): The heads with the middle ear bullas opened were fixed in 4% buffered formalin solution containing 0.0027 M KCl, 0.0015 M KH₂PO₄, 0.1369 M NaCl, 0.0090 M Na₂HPO₄. The skulls were immersed in the same fixative for one week. The specimens were dissected and rinsed in phosphate buffer and dehydrated in a graded series of ethanol. The specimens were embedded in paraffin wax and 5 µm thick sections, cut in the horizontal plane, were prepared for light microscopy.

Paper III (n=18): After decapitation and opening of the middle ear bulla, a fixative containing 4% buffered formalin in physiological saline (pH 7.0) was instilled into the MEC. The heads were then irradiated up to 45°C in a microwave oven (M-696; Miele, Gütersloh, Germany) set at 700 W [98]. Thereafter, the temporal bones were transferred to 100 mM PBS (pH 7.35) and immersed for 24 hours before dissection of the specimens.

**Histochemistry**

Toluidine blue staining was used on all plastic-embedded sections. Paraffin-embedded sections were stained with eosin-haematoxylin, van Gieson (papers II and IV)

**Immunohistochemistry**

Paper IV: The sections were dewaxed in xylene and rehydrated in a graded series of ethanol. Endogenous peroxidase activity was blocked with 0.3% H₂O₂ (3% H₂O₂ ED1) and the slides were then rinsed in PBS. Antigen retrieval was performed for all antibody stainings, except for CD45RA (Table 2). After rinsing in PBS, non-specific binding sites were blocked with normal rabbit serum (X0902, Dako A/S, Glostrup, Denmark) or normal swine serum (X091, Dako A/S, Glostrup, Denmark), diluted 1:20 for 30 min at room temperature. The sections were incubated with the primary antibody for 60 minutes in room-temperature as defined in Table 2. After rinsing in PBS, a biotinylated rabbit anti-mouse IgG antibody (EO464, Dako), or a biotinylated swine anti-rabbit IgG antibody (E0353 Dako), diluted 1:300, was applied for 30 min. The slides were washed in PBS, followed by incubation with an avidin-biotin horseradish peroxidase complex (Vectastain Elite ABC-kit, Vector Laboratories, Immunkemi, Järfälla, Sweden). The peroxidase activity was visualized by diaminobenzidine (DAB) (Vector Laboratories, Immunkemi, Järfälla, Sweden) and the brown precipitate was then examined in a light microscope after counterstaining with Mayer’s haematoxylin and coverslipped.

To confirm a positive staining, a spleen processed similar to the experimental specimens, was used.
<table>
<thead>
<tr>
<th>Clone</th>
<th>Specificity</th>
<th>form</th>
<th>Dilution</th>
<th>Source</th>
<th>Antigen retrieval</th>
<th>Normal serum</th>
<th>Biotinylated secondary ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED1</td>
<td>Tissue macrophages</td>
<td>Purified</td>
<td>1:100</td>
<td>Serotec, Novakemi, Enskede, Sweden (MCA341R)</td>
<td>0.1% trypsin, 8 min, 37°C</td>
<td>Rabbit</td>
<td>Rabbit anti-mouse IgG ab</td>
</tr>
<tr>
<td>ED2</td>
<td>Resident macrophages</td>
<td>Purified</td>
<td>1:100</td>
<td>Serotec, Novakemi, Enskede, Sweden (MCA342R)</td>
<td>0.1% trypsin, 8 min, 37°C</td>
<td>Rabbit</td>
<td>Rabbit anti-mouse IgG ab</td>
</tr>
<tr>
<td>OX33</td>
<td>CD45RA</td>
<td>Supernatant</td>
<td>1:100</td>
<td>Harlan Sera-lab Ltd, Loughborough, England (MAS-258b)</td>
<td>None</td>
<td>Rabbit</td>
<td>Rabbit anti-mouse IgG ab</td>
</tr>
<tr>
<td>OX34</td>
<td>CD2</td>
<td>Purified</td>
<td>1:80</td>
<td>Cedarlane Laboratories Ltd, Hornby, Ontario, Canada (CL34AP)</td>
<td>0.01M citrate buffer, pH 6.0, microwave oven, 630 W, 101°C, 9 min</td>
<td>Rabbit</td>
<td>Rabbit anti-mouse IgG ab</td>
</tr>
<tr>
<td>PMN</td>
<td>PMN cells</td>
<td>Polyclonal</td>
<td>1:2000</td>
<td>Cedarlane Laboratories Ltd, Hornby, Ontario, Canada (CLAD5140)</td>
<td>0.01M citrate buffer, pH 6.0, microwave oven, 630 W, 101°C, 15 min</td>
<td>Swine</td>
<td>Swine anti-rabbit IgG ab</td>
</tr>
</tbody>
</table>

Table 1. The different antibodies used for immunohistochemical detection of macrophages (ED1, ED2), B-cells (OX33), T-cells (OX34), and polymorphonuclear cells (PMN).
Slides incubated with only normal serum, excluding the primary antibody, were used as negative controls.

**Morphometry/Statistical analysis**

Paper I: The variations in thickness of the PF at the different time points were measured by means of a millimetre scale under the light microscope. From a section cut at the same level in all PFs, the thickness was measured at three different points. Statistically significant mean values were determined by one-way ANOVA. By use of Dunnet’s post hoc test, the mean control value was compared with each of the other mean values. Differences were considered significant at $p \leq 0.05$.

Paper IV: Morphometry was performed using a point-counting technique according to Weibel [99], to determine the invasion of inflammatory cells in the PF for ED1, ED2 and PMN cells. Statistical differences were calculated with the Poisson regression test, and differences were considered significant at $p \leq 0.05$.

Paper V: The statistical significance for the development of AOM when comparing the different treatment groups was calculated with Fisher’s exact test. Differences were considered significant at $p \leq 0.05$.

**Otomicroscopy**

During the experiments all animals underwent otomicroscopic examination. The TM status was depicted on a protocol sheet and four different parameters were noted: occurrence of vessels, retractions, fluid in the MEC, and development of myringosclerosis. These parameters were graded semiquantitatively on a four-grade scale (normal to massive). The occurrence of perforations was also documented.

**Light microscopy**

The specimens were examined with a Zeiss Axiophot light microscope and images were recorded digitally using a Dage-MTI DC-330 3CCD colour camera (Parameter AB, Stockholm, Sweden) connected to a personal computer with an Image-Pro plus software (Parameter AB). Photomicrographs and figures were made using software Adobe Photoshop 6.0 and Corel Draw 9 and 10, with adjustment of contrast and brightness in individual images.

**Electron microscopy**

Electron microscopy was performed with a JEOL 1200EX, Tokyo, Japan. Electron micrographs were made using of negative film, developed, and processed to photo paper.
RESULTS

The early inflammatory changes in the tympanic membrane in AOM, evoked by inoculating Streptococcus pneumoniae type 3 into the middle ear (papers II and IV).

AOM developed after inoculation of the pneumococcal suspension and pus started to fill the MEC. The TM’s responded early with increased vascularity and bulging. All TM’s perforated spontaneously within 24 hours after instilling of bacteria.

At a structural level, the first inflammatory changes appeared in the PF of the TM. At 3 hours there was a slightly increased number of polymorphonuclear cell immunoreactivity (PMN-IR) cells. Monocytes/macrophages were also present and these were more abundant than PMN-IR cells. Monocytes/macrophages were also found in the control specimens. The inflammatory cells increased in a bimodal pattern and in the PF the maximum infiltration of cells was found at 48 hours (Table 3). The number of T-cells in the PF were sparse at these early time points. However, they were slightly more common than B-cells.

Epithelial changes appeared early, starting with a swelling of the inner epithelium of the PF at 3 hours. Inflammatory cells leaked into the MEC from gaps in between epithelial cells (Fig. 3). At 48 hours the inner epithelium was double-layered.

MCs degranulated early. A metachromatic staining representing MC granules were observed extracellularly. From 9 hours onwards, a filamentous material with an ultrastructurally typical band pattern of fibrin started to accumulate in the PF (Fig. 4).

The infiltration of inflammatory cells in the PT was delayed, compared with that of the PF. At 6 hours inflammatory cells started to accumulate in the area between the annulus and the skin of the external ear canal. The inflammatory cells started to infiltrate the PT from the periphery and at the handle of the malleus. At 9 hours the complete PT was infiltrated with inflammatory cells. In contrast with the PF, PMN-IR cells were the more abundant than monocytes/macrophages. The PMN cells, mainly neutrophils, migrated initially upon the collagenous fibres, to disperse among the collagenous fibres and at 24 hours the PT were perforated. A few T-cells could be found in the infected PT’s, but B-cells did not occur. Interestingly, B-cells accumulated in the skin of the EAC.

As in the PF, fibrin was deposited in the PT.

Obviously the PF is the part of the TM that reacts first. Moreover, the cells responding to the inflammatory stimulus caused by AOM differ between the PF and the PT. In the former, macrophages predominate, whereas in the latter, PMNs predominate. Fibrin is also deposited in the TM during early inflammatory development.
<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>AOM ED1</th>
<th>AOM ED2</th>
<th>AOM PMN</th>
<th>OME ED1</th>
<th>OME ED2</th>
<th>OME PMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.35</td>
<td>0.551</td>
<td>1.27</td>
<td>0.616</td>
<td>-</td>
<td>0.91</td>
</tr>
<tr>
<td>6</td>
<td>1.88</td>
<td>0.186</td>
<td>0.67</td>
<td>0.440</td>
<td>0.44</td>
<td>0.000</td>
</tr>
<tr>
<td>9</td>
<td>5.41</td>
<td>0.000</td>
<td>1.66</td>
<td>0.297</td>
<td>1.11</td>
<td>0.545</td>
</tr>
<tr>
<td>12</td>
<td>2.94</td>
<td>0.019</td>
<td>1.36</td>
<td>0.494</td>
<td>0.40</td>
<td>0.000</td>
</tr>
<tr>
<td>18</td>
<td>7.42</td>
<td>0.000</td>
<td>2.06</td>
<td>0.128</td>
<td>0.42</td>
<td>0.000</td>
</tr>
<tr>
<td>24</td>
<td>8.70</td>
<td>0.000</td>
<td>3.64</td>
<td>0.004</td>
<td>0.81</td>
<td>0.237</td>
</tr>
<tr>
<td>48</td>
<td>12.60</td>
<td>0.000</td>
<td>16.53</td>
<td>0.000</td>
<td>0.19</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 3. The relative increase in the different inflammatory cells in PF during AOM and OME. RR = relative risk. Statistical significant differences were calculated with the Poissons regression test and differences were considered significant at $p \leq 0.05$. 
Fig 3. Light micrograph of a sectioned rat pars flaccida (PF) of the tympanic membrane, 9 hours after inoculating *Streptococcus pneumoniae* into the middle ear cavity (MEC). Top of figure outer surface of PF. Bar = 25 µm (toluidine blue staining, original magnification x 20). Note the leakage of inflammatory cells into the MEC.

Fig 4. Electronmicrograph of area of connective tissue layer in PF of AOM specimen. Blood vessel with inflammatory cells in lumen is seen. Interstitial accumulation of filamentous material has occurred (arrow). Higher magnification of filamentous material, with characteristic striation of fibrin (inset).
The early inflammatory changes in the tympanic membrane in OME, evoked by blocking of the tympanal orifice of the Eustachian tube (papers II and IV).

Three hours after the blocking of the tympanal orifice of the MEC, fluid started to accumulate in the attic region. MCs displayed a metachromatic staining. After 2 days the MEC was almost completely filled with effusion material. The PF was retracted, with dilated vessels.

Similar to the otomicroscopical status, the structural changes in the TM were almost exclusively restricted to the PF. A slight increase in monocytes/macrophages was found (Table 3). This increase was statistically significant at 12 hours for ED1-IR cells and at 6 to 24 hours for ED2-IR cells. Few PMN-IR cells were seen in the PF sections. Neither T- nor B-cells occurred in the PFs. At 6 hours onwards, MCs showed metachromatic staining. The inner epithelium became thickened.

The PTs from the ears with OME showed only slight changes. At 12 hours a few specimens showed a few inflammatory cells. In general, the occurrence of inflammatory cells of the PTs resembled that in the control specimens.

It is concluded that although the MEC is completely filled with effusion, there is only a minor inflammatory response from the TM. The serous OME condition in this model must thus be assumed to reflect a passive accumulation of normally secreted middle ear mucosal fluid caused by ET obstruction.

The role of pars flaccida mast cells as initiators of tympanic membrane inflammation (paper I).

An inflammatory response was elicited when the MC secretagogue, compound 48/80, was applied onto TM. Three hours later, MCs had reacted metachromatically or were degranulated and inflammatory cells started to infiltrate the PF. At 6 hours the PFs were thickened and opaque, with dilated vessels. At the light microscopical level the PF showed edema but still few inflammatory cells. Thereafter the edema decreased but the number of inflammatory cells of the PF increased, peaking at 24 and 48 hours respectively, coinciding with increased PF thickness. Fluid accumulated in the attic at 48 hours. Both the outer and the inner epithelium of the PF showed progressive changes, first at 6 hours after application of compound 48/80. The inner epithelium was double layered and partly ciliated after 48 hours. Four days after the last treatment, the epithelia were almost normalized.

In PT, inflammatory cells started to invade at the fibrocartilaginous ring 12 hours after applying compound 48/80, continuing to spread beneath the keratinized squamous epithelium and the connective tissue layer. These cells had disappeared at 4 days. Apparently the PF MCs have an inherent capacity to provoke an intense inflammatory reaction, resembling that in AOM. It is tempting to suggest that the MCs are involved and act as initiators of a developing inflammatory condition of the MEC, such as AOM.

The early inflammatory response to myringotomy with a pre-existing AOM condition (paper III).

The ears that had been subjected to bacterial inoculation 2 days earlier showed typical signs of AOM, with bulging and discoloured TMs prior to the myringotomy. After
myringotomy, the EACs filled with pus and the TM status was difficult to ascertain during
the first 24 hours after myringotomy.

After 2 days the EACs had partly dried up and the TMs showed vasodilatation and
patent perforations. The PFs were retracted, with fluid in the attic region. At day 4, a crust
covered the perforation in three out of four cases and the inflammatory reaction seemed
to have diminished. By the end of the experiment, day 7, all perforations had healed and
the inflammation had abated. Dilated vessels persisted along the handle of the malleus, in
the annulus, and in the wounded quadrant.

The inoculation of bacteria caused a massive infiltration of inflammatory cells into the
TM, degranulated MCs, and profound changes in outer and inner epithelia. Six hours
after myringotomy, keratinocytes of the outer squamous epithelium of the PT
accumulated at the perforation border. At 12 hours, the epidermal layer showed deep
indentations into the basal lamina. Twenty-four hours after myringotomy, the
inflammatory cells started to decrease in number in the PFs. In PT too, inflammation
started to decrease, especially in the non-perforated areas. After 2 days, MCs seemed to
regain their granules and the mucosal epithelium lost its goblet-like appearance. After 4
days the PF still showed an increased number of lymphocytes and macrophages. At this
time point, 3 out of 4 specimens were closed, but only one of these had regained a distinct
triple-layered structure. After one week, the epithelia of the PF were normal, though the
connective tissue layer still showed hypervascularity. In the PT all perforations were
healed, though the lamina propria continued to be thickened. Sparse myringosclerotic
deposits were noted in the untouched quadrant of the PT as well as in the submucosal
layer of the PF.

The early inflammatory response to myringotomy without a pre-existing AOM condition (paper III).

After myringotomy of healthy middle ears, the PFs showed an early increase in
vascularity. Concomitant with a retracted PF, fluid had accumulated in the attic region.
Within the experimental period, 7 days, the PT perforations were reduced in size, but had
not sealed. Furthermore, gradually increasing sclerotic deposits were noted in the TM.
The earliest histological changes in PF appeared after only 3 hours. Increasing edema,
infiltration of inflammatory cells and degranulated MCs were found. Fibrin was deposited
in the PF. The inflammatory changes in PF peaked at 24 hours. After 2 days the MCs
seemed to regain their granules. At day 7 the PFs appeared normal. The inflammatory
response of the PTs lagged behind that of the PF. During the first 24 hours only a minor
invasion of inflammatory cells was noticed. After 48 hours, keratinocytes reached the
manubrial portion of the perforation and at day 7 there was an intense accumulation these
cells at the edge of the perforation.

Myringosclerosis was seen after only 12 hours, subsequently increasing in extension and
density. The sclerotic deposits were more extensive than in the myringotomized PTs of
the AOM ears.

It is concluded from paper III that a TM perforation heals faster during AOM, than in
the healthy middle ears.
In the non-infected ears, MC degranulation and an inflammatory response from PF occurred soon after myringotomy. Simultaneously fibrin was deposited in PF. The inflammation of PT showed a lag whereas in the infected ears, the inflammatory response of the TM was intense already at the time of myringotomy. Early accumulation of active keratinocytes at the perforation border of PT, supported by a highly inflamed lamina propria, seemed to promote healing.

**Development of an AOM model by intranasal challenge with Streptococcus pneumoniae (paper V)**

Rats challenged intranasally with the AOM pathogen *Streptococcus pneumoniae*, serotype 3, developed AOM. Bacterial challenge on 5 consecutive days caused 23 of 40 ears to developed AOM (Fig. 5). Middle ear samples were taken from all ears and altogether 21 of 40 cultures confirmed growth of pneumococci.

Ears with AOM showed an opaque TM, with increased vascularity. The MECs appeared completely or partly filled with pus. In PF, inflammatory cells were found infiltrating the connective tissue, together with degranulated MCs and changes in the epithelia. These pronounced changes were also seen in the mucosa of the FN. In the areas of ET with a ciliated epithelium, the cilia were lacking in places or showed an altered structure and irregular length. In the non-ciliated epithelium, inflammatory cells had infiltrated between the epithelial cells. Inflammatory cells were present in the tubal lumen.

It is concluded that it is possible to induce AOM in the rat, in a manner that resembles the natural route of infection in man. Furthermore, this AOM condition provokes an inflammatory response in the ET as well as in the MEC. The latter resembles that occurring after intrabullar inoculation of bacteria.

**Manipulation of the tympanic membrane affects the development of AOM (paper V)**

In the ears in which the MC secretagogue compound 48/80, or saline, was applied to the TM, the development of AOM was reduced (Fig. 5). AOM developed in 8 out of 15 and in 6 of 15 ears respectively, which should be compared with the untreated TMs, where 9 out of 10 developed AOM.

It is concluded that pro-inflammatory mediators released from the TM, especially PF, may be beneficial in the defence against AOM.
Fig. 5. Development of acute otitis media in untreated ears and ears with the different topical tympanic membrane treatments (percentage of ears) from the first days of inoculation of Streptococcus pneumoniae, type 3, (day 1) until sacrifice (day 6). Statistical differences were calculated with Fisher’s Exact test and considered significant as follows: * = $p \leq .05$ and ** = $p \leq .01$. 

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**Example Diagram Description:**

The diagram illustrates the percentage of ears with acute otitis media (AOM) over different days. The percentage is represented for untreated ears and ears treated with Compound 48/80 or saline. Statistical significance is indicated with symbols: * for $p \leq .05$, ** for $p \leq .01$, and NS for non-significant. The data shows a decrease in AOM percentage over time, with statistical significance noted on certain days.

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DISCUSSION

Earlier studies concerning middle ear inflammation have often been less focused on the initial events. In this thesis my intention has been to design experiments that investigate the first hours of a developing inflammatory condition of the middle ear. In this respect AOM, OME, myringotomy (with or without concurrent AOM) and degranulation of PF MCs were investigated. In addition, we have developed a rat-AOM model that avoids surgical trauma and resembles the natural route of infection in man.

The experiments regarding early inflammatory changes in AOM and OME have been focused on those that take place in the TM. The TM mirrors events in the middle ear cavity and is also a focus of inflammation. Moreover, the rat TM is easy to visualize, access, and manipulate under experimental conditions. In this respect, the intact TM may in the future also become important for drug delivery to the middle ear.

The tympanic membrane in developing AOM

The otomicroscopic appearance of the TM reflects the pathological conditions of the middle ear.

In AOM, an early inflammatory response was noticed. The first signs of inflammation were observed in PF. This is consistent with earlier findings by our research group [21]. Within 3 hours of inoculating bacteria into the MEC, PF responded with increasing edema and infiltration of inflammatory cells. The inflammatory changes followed a bimodal pattern with two peaks within the first 48 hours. In PT, the first signs of leaking cells were found 6 hours after bacterial inoculation.

PF and PT have differing characteristics and diverse functions. PT plays an obvious role in hearing physiology, whereas the physiological role of PF is more difficult to establish. However, several studies have shown that PF plays a unique role in a developing inflammatory condition of the TM:

1. Irrespective of stimulus, PF reacts before PT [20-22].
2. The first sign of middle ear effusion is found in the attic region, concomitant with increased vascular leakage from PF vessels [20].
3. A neurogenic inflammatory mechanism is probably activated in PF, whereas neurotransmitters, such as substance-P and acetylcholine, increase vascular leakage in PF [41, 100].
4. PF contains an abundance of MCs [87] in close contact with vessels and nerves [41].

In AOM, early edema was found in PF. MCs were degranulated. These cells have the capacity to release mediators such as serotonin and histamine which increase the vascular leakage [41, 100, 101], permitting leukocyte adhesion and extravasation into the TM.
We were able to show for the first time that the cells responding to the inflammatory stimulus caused by AOM differed in the two parts of the eardrum (paper IV). In PF, macrophages predominated, whereas in PT PMNs (mainly neutrophils) were found. Interestingly, macrophages were also seen in normal PFs, whereas they were absent in PT. Macrophages are cells found in practically all tissues in the body. Although important in immune defence, they also participate in various pathophysiological processes, such as wound healing [102] and bone remodelling [103]. They can detect, ingest and destroy infectious agents, and by antigen presentation initiate T-cell responses [104]. Macrophages can act pro-inflammatorily when there is an uptake of infectious agents, but when apoptotic cells are phagocytosed, they can suppress the release of inflammatory cytokines [105]. During early AOM, monocytes/macrophages increased bimodally in PF, which could mean that whereas the first increase depended on the release of MC mediators, the second one could be dependent on mediators released from the invading inflammatory cells.

Numerous PMNs also infiltrated the TM after bacterial exposure. In PT, PMNs predominated, while invading monocytes/macrophages were relatively sparse. Neutrophils, as well as macrophages, are important for the initial innate immune defence, by phagocytosing particles and releasing potent inflammatory stimuli [106].

The first signs of inflammatory cells invading the PT were found in the space between the EAC and annulus fibrosus. This space showed abundant vessels and MCs that had degranulated in an early stage. Inflammatory cells were noted in the periphery of PT and near the malleal vessels. These two spaces coincide with the described auditory epidermal cell generation centre in mouse [107] and man [108]. It is conceivable that this space plays a role in inflammation when cells leak into PT, and may also contribute to nutrition of the avascular areas of PT under normal conditions.

It is interesting that the PMNs, mainly neutrophils, can migrate into the avascular areas of the PT. Extravascular migration of neutrophils depends on a complicated interplay with migration in chemoattractant concentration gradients, and an interplay with the abundance of matrix proteins, such as collagen, which express integrins to which the neutrophils attach [109].

It is plausible that the early defence of the TM and MEC during AOM depends on an immediate activation of the innate immune system by MC and macrophage activation. This initiates an invasion of newly recruited monocytes/macrophages and some hours later also neutrophils which enhance further inflammatory response.

Fibrin was deposited in the TM during early AOM. The initial increased vascular leakage allowed fibrinogen to extravasate and become converted into fibrin by thrombin. The fibrin was identified by its typical band pattern when analysed at the ultrastructural level [110, 111].

It is of interest regarding the development of the early inflammatory reaction that fibrinogen can interact and bind to integrins on leukocytes and endothelial cells [112] and consequently participate in starting inflammation when the inflammatory cells extravasate.
We found the fibrin located close to the vessels and we speculate whether it acts after the initial leakage of plasma and invasion of cells in order to seal vascular gaps and hinder this reaction. Furthermore, the formation and degradation of fibrin leads respectively to the formation of proinflammatory fibrinopeptides and fibrin degradation products. Fibrinopeptides are potent neutrophil chemoattractants [113-115].

Fibrin is degraded by plasmin. Interestingly, when we investigated the healing of PT perforations in plasminogen deficient mice, we found an abnormal inflammatory reaction and healing of the TM with an accumulation of fibrin and neutrophils, even 143 days after the myringotomy (unpublished observation). Without this mechanism, the wounded TM cannot heal. Obviously there must be a mechanism which arrests the deposition of fibrin at a certain time point.

During the first 48 hours of AOM, there was little response in T- and B-cells in TM, though B-cells accumulated in adjacent EAC skin, close to the glands of the EAC. In the epithelial layers of human EAC skin, IgA- and IgG-IR have been detected and in surrounding sebaceous and ceruminous glands, IgA, IgM, and also IgG have been localized [116]. In developing AOM the potential source of antibody production in the EAC increased. An increased amount of Ig in epithelia and cerumen during infection may explain the clinical observation in AOM of an otomicroscopically normal ear canal skin, yet a bulging and inflamed TM.

The tympanic membrane in developing OME

The sterile serous OME condition following blockage of the ET resulted in discrete inflammatory changes, though the middle ear was filled with effusion. We suggest that this phenomenon, at least in this model, is mainly a reflection of passive accumulation of a normally secreted mucosal fluid caused by ET blockage [117].

A limited increase in macrophages was noticed in the PF, statistically significantly at 12 hours after provoking the OME. Furthermore, there seemed to be a differentiation of macrophages because of an increase in ED-2 positive cells compared with ED-1 positive cells. In this sterile OME condition, an increase in macrophages did not seem to be of importance for the early resolution of middle ear effusion.

PMNs were sparse at all early points in time in both PT and PF. The numbers of T- and B-cells were comparable to those in normal TMs.

It is worth noting that the use of specific antibodies showed that macrophages were the predominant inflammatory cell, while PMNs were less abundant. This explains the contrasting results presented in papers II and IV.

The discrete inflammatory changes in the OME model correspond well to the less pronounced symptoms observed in this clinical disease entity.
The tympanic membrane mast cells in middle ear disease

Earlier studies showed that MCs are activated under various inflammatory conditions in the middle ear.

1. MC degranulation with compound 48/80 causes an accumulation of fluid in the attic region, and histamine release to the middle ear [75].
2. Stimulation of EAC causes histamine release to the MEC [20].
3. MCs are degranulated during experimental AOM [21].
4. MCs are degranulated after myringotomy [22].
5. MCs are present in chronic OM, with or without cholesteatoma [78, 79, 118].
6. MCs are a potential source of inflammatory mediators found in effusion material during experimental (as well as in human) AOM and OME [119].

MCs constitute a group of cells that puzzles. Ehrlich, who is the acknowledged discoverer of the MC (although Recklinghausen had characterized them about 15 years earlier [120]) described them as overnourished connective tissue cells, consequently naming them *Mastzellen* in German [121]. It was later shown that their granules were secretory granules released by stimulation.

The MCs release their mediators in various patterns. Classical exocytosis involves single granule extrusion, while in compound exocytosis an intracellular granule – granule fusion precedes their secretion. A third mechanism is represented by piecemeal degranulation, in which there is a selective release of granule mediators [122].

The PF MCs are strategically located at the interface of the EAC and MEC. It is tempting to suggest that the MCs of PF carry out a variety of functions, depending on the existing condition. They may release mediators that act both pro- and anti-inflammatory [123].

When the MC secretagogue compound 48/80 was applied to the TM, an intense inflammatory reaction was provoked. This reaction resembled that during developing AOM. In both conditions, MCs were found to be degranulated at an early stage. Edema developed early, followed by an invasion of inflammatory cells due to an increased vascular leakage. This invasion was intense in both conditions, with inflammatory cells occurring later in PT than in PF.

It has been shown that MCs are of importance for an appropriate immune defence in other organ systems. MC-deficient mice are more susceptible to infection because the influx of neutrophils to the infectious site is impaired. This is suggested to depend on the MC’s capacity to release proinflammatory and chemotactic mediators, such as TNF-α [124]. MCs can also express MHC (major histocompatibility complex) class I and activated murine MCs can express MHC class II, thus presenting bacterial antigens to T-cells via the T-cell receptor (TCR) [125]. It has also been shown that MCs can recognize bacteria by virtue of specific receptors present at the MC surface [126]. These data, though not yet complete, suggest the importance of MCs as initiators of defence in infectious conditions.
We suggest that in a developing AOM, MCs release mediators, such as histamine and serotonin [101], followed by a release of arachidonic acid metabolites and cytokines/chemokines which have chemoattractant properties for inflammatory cells [59], thus initiating an immune defence reaction.

After myringotomy an early degranulation of MCs was found, as well as an intense edema of PF. The PT was invaded by inflammatory cells after a time lag. This experimental condition showed obvious similarities to the inflammatory course seen after application of compound 48/80 to the TM. By virtue of their arsenal mediators, MCs been suggested to play an important part in the early process of wound healing, as well as in scar formation [67]. In a recent study on MC-deficient mice, the healing time of skin wounds was comparable to that in normal mice, though the recruitment of neutrophils to the wounded area was impaired [127].

It is conceivable that MCs are important in triggering PMN recruitment, found in PF during developing inflammation following perforation of the TM.

One may conclude that there is evidence of the involvement of MCs in the pathophysiology of middle ear diseases, such as AOM, OME, and tympanic membrane healing. However, the exact role of the mast cells in these processes is still uncertain.

**Myringotomy of normal and infected tympanic membranes**

Interestingly, when comparing the healing of PT perforations in infected and normal ears, the infected TMs heal faster. Wound healing in general is a dynamic process that involves inflammation, new tissue formation, and tissue remodelling, which leads to reconstruction of the wounded area [102].

On comparing the initial inflammatory phase in the perforation models, differences were noticed. In the AOM ears, the PTs were already heavily invaded by inflammatory cells, and vessels were abundant at the time of myringotomy. In the normal middle ears, the inflammatory response of PT was delayed and the first signs of inflammation were noted in PF, concomitant with MC degranulation.

Thus various cytokines, growth factors and other serum components, important for the healing process, may already be present in the infected TMs at myringotomy, whereas in the normal TMs there is a considerable time lag after perforation before these factors are recruited. After myringotomy of normal ears, an accumulation of fibrin was noted in both PF and PT. An increased vascular leakage allows fibrinogen to extravasate and to be converted into fibrin by thrombin. In skin wounds, a fibrin clot forms, which serves as a barrier against invading microorganisms, as a provisional matrix for invading cells, and also as a reservoir of growth factors important for the healing process [128]. Furthermore, one may speculate that, during the AOM condition, a pus-filled ear serves as a provisional matrix that promotes a more rapid healing of the PT perforation.

During AOM, proliferation and activation of the outer and inner epithelia were found before myringotomy. The outer epithelium showed increased number of cell layers and the keratinocytes were swollen. The epidermal layer showed indentations in the basal lamina. The lamina propria was highly inflamed. These findings seemed to promote healing and confirm the importance of the proliferating epithelium in TM healing.
The healing of TM perforations can be affected by growth factors \[129\], and the expression of endogenous basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), keratinocyte growth factor (KGF), and transforming growth factors α and β (TGF) are induced in TM perforations \[130-132\]. Information on expression of growth factors during AOM is sparse. However, TGF-β is found to be upregulated during pneumococcal OM \[133\] and concomittant ET blockage \[134\]. In animals subjected to anti-inflammatory treatment with glucocorticoids, the induction of growth factors is reduced, concomittant with the healing of TM perforations \[135\].

The complex process of wound healing involves cell – cell and cell – matrix interactions and is affected by various growth factors and cytokines \[128\]. It seems plausible that infection by PnC affects these mechanisms and the course of healing. From a clinical point of view, there seems to be little risk of persistent TM perforations following diagnostc tympanocentesis, whereas the use of myringotomy can be questioned because of the rapid closure of the incision.

**A new AOM model, using intranasal challenge with Streptococcus pneumoniae**

The rat is well established as an AOM model, using the common OM pathogens \[26, 84, 85\]. In paper V it was shown that intranasal challenge with PnC induced AOM in the rat middle ear. The number of ears developing AOM increased after repeated challenge. As one instillation caused only a moderate AOM response (unpublished observations), it appears necessary to repeat the nasopharyngeal inoculation in order to cause the middle ear infection in this model. Repeated exposure to the bacterial suspension, containing both viable and degraded bacteria, may amplify an inflammatory reaction in the nasopharynx at the ET orifice. Consequently, this may hamper the mucociliary defence and contribute to ET dysfunction and AOM development.

The present AOM model has advantages over the previous rat AOM model, since the route of infection resembles that in man. When the ET is bypassed, experiments intended to elucidate factors such as bacterial adherence and the local immune defence system of the tube, are difficult. In a recent study it was shown that AOM can be induced by intranasal inoculation of bacteria in the rat. In that study, however the rat TM was perforated to allow instillation of histamine into the middle ear in order to facilitate bacterial entry into the MEC \[29\]. This method interferes with the course of inflammation in the TM when it is perforated. Release of histamine can induce an inflammatory response in the MEC which should affect both the opening and also mucociliary dysfunction of the ET \[72-74, 136\].

The morphological appearance of the TM and of the middle ear mucosa in the present study reflected an inflammatory reaction comparable to that in the earlier AOM model \[137\]. Moreover, inflammatory cells were found in the ET lumen.

Otomicroscopy revealed AOM in 23 out of 40 ears; in 4 of these 23, viable bacteria could not be cultured. We suggest that the lack of positive cultures may have been due to the culture technique, or else a sterile effusion was caused by non-viable bacteria/bacterial fragments or impaired ET function \[91, 92\].
Manipulation of the tympanic membrane affects the development of AOM

When either saline or the mast cell secretagogue compound 48/80 was applied to the TM, the development of AOM was inhibited. Compound 48/80 provokes an intense inflammatory reaction, especially in PF (paper I), and histamine is released into the MEC [76]. It is remarkable that saline also reduced the number of ears with AOM.

In both conditions we hypothesize that an effusion production is elicited, particularly from PF [75, 76]. The middle ear effusion containing components of the innate immune system [27, 58] is suggested to prevent bacteria from initiating growth in the MEC.

Clinical considerations

The TM is a target for the middle ear disease processes, and reflects the middle ear pathology. In the present studies the early inflammatory changes in the TM were investigated during inflammatory conditions of the middle ear.

The spontaneous healing rate of clinical AOM disease is approximately 80% and critically applied evidence-based medicine shows that the use of antibiotics in uncomplicated AOM can be questioned [138, 139]. In this respect, “watchful waiting” is suggested as an alternative option in a child with AOM [140]. Similarly, antibiotics have not shown any convincing advantages for long-term resolution of OME [138]. These statements, which to some will seem quite nihilistic, may be interpreted as “no point in attempting anything”. However, in the clinical situation the patient will benefit from alleviation of symptoms and a shortened duration of the condition. Continuing research which hopefully will reveal the various events involved in the development of middle ear inflammation may in the future provide us with new treatment modalities for OM.

We have shown that AOM in particular provoked an early and massive inflammatory reaction in the TM. The clinical consequence is an inconsolable child suffering from earache. The extravasation of inflammatory cells was not limited to the TM, but was also found in the skin of the EAC, close to the annulus. The sterile OME model revealed a minor inflammatory response which may reflect a serous transudate, secreted from the normal middle ear epithelium and accumulated due to ET dysfunction.

Looking ahead it is tempting to suggest that TM could become an early treatment approach to such middle ear diseases as AOM and OME. Transdermal drug delivery has been successfully applied for pharmacological treatment with drugs such as nitroglycerine, estradiol, and fentanyl. As TM shows distinct similarities to the skin, transtympanic membrane drug delivery would, in comparison with systemic administration, have advantages of site-specific administration, diminished variability in systemic drug concentration, and would avoid the initial gastrointestinal and hepatic passage and metabolism. Future research may provide us with drugs that can be applied locally and early, as ear-drops for OM condition, which will penetrate the TM and have an anti-inflammatory/antibacterial effect in the TM and MEC, and promote ET function or alleviate the pain. These drugs may affect the unspecific immune system, which we showed to be activated early during AOM (paper IV).

Other studies have shown that middle ear effusion contains various inflammatory mediators believed to be important during AOM [119]. However, when steroids and
antihistamines have been used in clinical trials to reduce the inflammation, these drugs did not seem to affect the long-term course of either AOM or OME [138, 141]. Instead of blocking the inflammatory response it might be of interest to enhance it in a controlled manner – eg. regulate mast cell functions – in order to eradicate bacteria. One indication of this was that when we stimulated the TM, the developing AOM was inhibited (paper V).

The new rat AOM model presented here is so far the best to mimic the natural route of infection during AOM. It ought to be possible to use this model to test and develop vaccines against OM pathogens. In addition, the role of the ET in developing AOM can be further evaluated.
Summary and General conclusions

- AOM induced by intrabullar inoculation of *Streptococcus pneumoniae*, elicits an intense inflammatory bimodal inflammatory response of the TM, in which PF reacts earlier than PT.

- During AOM, the cellular inflammatory response of the TM differs in PF and PT; in the former, macrophages predominate; and in the latter, PMNs. This indicates the importance of an unspecific immune defence during early AOM, and that PF and PT can exert activity differently.

- During early AOM, as well as following myringotomy, fibrin extravasates into PF and PT. This fibrin deposition may be involved in regulating the inflammatory response.

- OME initiates a discrete early inflammatory response, in which macrophages seem to be the most active cell type.

- The accumulation of effusion during OME may reflect, at least in this experimental model, a passive accumulation of fluid normally secreted from the middle ear mucosa.

- Healing after myringotomy differs in normal vs. infected middle ears. TM in infected ears healed faster and with a more rapid decrease in inflammation than TM in control ears. Diagnostic tympanocentesis will thus involve little risk of creating persistent perforations, but will also mean a weaker/modest therapeutic effect.

- AOM can be provoked in the rat by repeated intranasal challenge with the OM pathogen *Streptococcus pneumoniae*.

- TM stimulation during concomitant intranasal challenge with bacteria hinders the development of AOM.
Olika typer av öroninflammation – akut otit och sekretorisk otit (otosalpingit, ”vätska bakom trumhinnan”) – är vanliga sjukdomar hos barn såväl som hos vuxna.


Vid sekretorisk otit kan trumhinnan vara indragen eller buktande, samtidigt som den kan vara något förtjockad och ofta en gulskimrande vätska i mellanörat. Till skillnad mot akut otit saknas öronvärk och feber vid sekretorisk otit och det dominerande symtomet är hörselnedsättning.

Trumhinnan speglar vad som händer i mellanörat och är därför viktig för diagnostik av mellanöronsjukdom. Den består av en större akustisk del, pars tensa, och en mindre övre mer elastisk del, pars flaccida. Oavsett orsak till inflammation i mellanörat ser man de första förändringarna i pars flaccida. Pars flaccida är mera hudlik än pars tensa och är således rikligt vaskulariserad och har ett stort antal mastceller som kan frisätta inflammatoriska substanser vid olika stimuli.

I avhandlingsarbetet har syftet varit att kartlägga tidig inflammationsutveckling i trumhinnan vid olika typer av öroninflammation och vid perforation av trumhinnan med eller utan pågående infektion. Mastcellernas roll vid inflammationsutveckling har studerats och en ny experimentell modell för akut öroninflammation utvecklats. Den senare efterliknar mer utvecklingen av akut öroninflammation hos människa än tidigare modeller.

Undersökningarna har utförts i modeller för experimentell akut otit, sekretorisk otit och trumhinneperforation åstadkomna på råtta. Vävnaderna har analyserats med olika morfologiska tekniker.

Vi har funnit att när mastceller i pars flaccida aktiveras med en substans – compound 48/80 – som degranulerar mastceller, sker en kraftig inflammationsutveckling, först i pars flaccida och senare i pars tensa. Den inflammatoriska reaktionen liknar den vid akut otit hos människa då pars flaccida reagerar före pars tensa. Den tidiga inflammatoriska reaktionen vid akut otit skiljer sig mellan pars flaccida och pars tensa avseende cellsvar, i den förra överväger makrofager och i den senare neutrofiler. En pågående inflammation i trumhinnan påverkar också läkningsförloppet av en trumhinneperforation. Vid akut otit då trumhinnan är fylld av inflammatoriska celler och mellanörat av pus läcker hålet i trumhinnan snabbare. Detta talar för att paracentes av trumhinnan i den kliniska situationen har en mättligt behandlande effekt och att nyttan av ett sådant ingrepp i kliniken framför allt ska ses som diagnostiskt när man vill ta en representativ odling från mellanöronsekret.

Vid både den akuta otiten och efter perforation av en normal trumhinna sker en ansamling av fibrin i bindvävslagret i trumhinnan. Det ökade kärläckaget vid inflammationen bidrar till att fibrinogen läcker ut i vävnaden och där omvandlas till fibrin. Fibrinet bidrar sannolikt till reglering av inflammation i trumhinnan och därigenom även till utläkning av otit och läkning av trumhinneperforationer.
I den nya modellen för akut otit åstadkoms öroninflammation efter det att bakterier inokulerats i näsan, d.v.s. genom en jämförbar spridningsväg som vid öroninflammation hos människa. I denna modell kan man också visa att otitutvecklingen kan minskas genom att stimulera inflammationsutveckling i trumhinnan.
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