Experimental Acute Otitis Media
Aspects on treatment, protection and structural changes

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To Björn, Jonas and Alfred

When someone believes in you, and there are family and friends by your side, what seems impossible can be achieved
ABSTRACT

Acute otitis media (AOM) is a common disease in childhood and is one of the most common causes for outpatient antibiotic treatment. The major aetiological agents of AOM have varied over the decades. Now the three most common pathogens are Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis. The resistance patterns of these organisms have also varied from the beginning of the antibiotic era to the situation we have today with an increasing incidence of penicillin-resistant S. pneumoniae and a moderate to high frequency of beta-lactamase production in H. influenzae and M. catarrhalis. In Sweden we have continued to use the Scandinavian treatment policy of penicillins as the first-line antibiotic treatment of AOM, which has been implemented with good results in the past. The question is if this policy will continue to have acceptable treatment results. In order to investigate aspects of treatment, protection and structural changes in AOM, an animal model was used.

Amoxicillin treatment of AOM caused by H. influenzae was studied. Amoxicillin treatment was shown to shorten the duration of the infection and to reduce the morphological changes normally observed after an untreated AOM. The influence of antibiotic treatment on recurrent AOM was evaluated. Amoxicillin treatment did not lead to less protection against reinfection. Abstaining from antibiotics did not improve the levels of serum IgG antibodies. The IgG levels were significantly higher in treated animals after rechallenge. AOM caused by H. influenzae with a non-beta-lactamase-mediated resistance to beta-lactams was investigated and it was observed that during amoxicillin treatment the chromosomal changes mediating resistance were possibly advantageous for the bacterium. In cultures from children with AOM, there is sometimes growth of several bacteria. The possibility of a sheltering effect of beta-lactamase-producing H. influenzae on a penicillin-sensitive S. pneumoniae in a mixed infection was investigated, and amoxicillin was shown to eradicate the pneumococci from the middle ear despite the presence of beta-lactamase. An increasingly cultured bacterium in nasopharynx and in AOM is M. catarrhalis. It is now beta-lactamase-producing in almost 100% of cases and is thus not eradicated by penicillins. An animal model of AOM caused by beta-lactamase-producing M. catarrhalis was established to study the course of this infection with the possibility of evaluating aspects of virulence between AOM pathogens. The AOM observed was a self-limiting disease.

The results obtained in this study in a rat model support the continuing use of penicillins as first-line drugs in the treatment of AOM. Penicillins are not sufficient to treat all causative agents, but the majority of pathogens including the most virulent bacteria are eradicated from the middle ear.

Key words: acute otitis media, penicillins, rat, treatment, protection, beta-lactamase, Moraxella catarrhalis, Haemophilus influenzae.
ABBREVIATIONS

AOM  acute otitis media
cfu  colony-forming units
ELISA enzyme-linked immunosorbent assay
ET  Eustachian tube
Ig  immunoglobulin
IL  interleukin
LOS lipooligosaccharide
LPS lipopolysaccharide
MBC minimum bactericidal concentration
MEE middle ear effusion
MIC minimum inhibitory concentration
NTHi non-typeable Haemophilus influenzae
OME otitis media with effusion
OMP outer membrane protein
PBP penicillin-binding protein
PCR polymerase chain reaction
RT-PCR reverse transcribed polymerase chain reaction
pcV penicillinV
PMN polymorphonuclear leukocyte
PNSP penicillin non-susceptible Streptococcus pneumoniae
TEM transmission electron microscopy
TGF transforming growth factor
TM tympanic membrane
TNF tumor necrosis factor
ORIGINAL PAPERS

This thesis is based upon the following publications and manuscripts, which will be referred to in the text by their respective Roman numerals:


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INTRODUCTION

1. Acute otitis media in humans

Introduction

Acute otitis media (AOM) is the most common cause of antibiotic prescription for children in the developed world and a disease with a considerable burden in economic costs for society. It causes suffering in young children but has consequences for the whole family. The introduction of antibiotics in the treatment of AOM had a dramatic impact and reduced the number of complications and the overall morbidity.

But now, approximately 50 years later, we are asking ourselves whether we should continue with our treatment policies or not. This is a consequence of the increasing resistance in AOM pathogens that has occurred worldwide and has led to the increasing use of broad-spectrum antibiotics which seem to further promote the development of bacterial resistance. More recent studies of treatment of AOM, which often focus on the relief of symptoms and not the eradication of bacteria from the middle ear, have only shown a limited benefit of antibiotic treatment. After antibiotic treatment the bacteria can be eradicated from the middle ear, but the inflammation and the concurrent viral infections may still continue to give symptoms. A confounding factor in this research area is the poor definitions of AOM that exist and the varying diagnostic accuracy observed.

Differences occur between countries concerning incidence, prevalence of AOM pathogens, prescription patterns and use of antibiotics. Resistant bacteria occur with a remarkable variation between countries which implies that treatment policies play a role.

1.1 Definitions and diagnosis

Definitions

A definition of AOM that is accepted and used all over the world does not exist. One definition of AOM that has been widely used is the presence of middle ear effusion (indicated by abnormal mobility and/or position of the tympanic membrane[TM]) and acute symptoms and signs of an infection (earache, irritability, fever, poor appetite, vomiting and/or diarrhoea). Acute discharge through perforation of the TM or through a tympanostomy tube is also defined as AOM (Karma et al 1987, Rosenfeld & Bluestone 2003).

Otitis media with effusion (OME) is an inflammation of the middle ear with middle ear effusion (MEE) but without the acute symptoms and signs accompanying AOM and there is no perforation of the TM (Rosenfeld & Bluestone 2003).

Diagnosis

Making a correct diagnosis of AOM is often difficult, particularly in young children, and over-diagnosis of AOM is common (Garbutt et al 2003). Paediatricians from several countries were evaluated for diagnostic accuracy of AOM and OME and it was evident that they often misdiagnosed OME as AOM (Pichichero 2003). Use of
a pneumatic otoscope and tympanometry may reduce the number of diagnosed AOM by >30 % (Blomgren & Pitkaranta 2003). In a recent review it was found that a cloudy, bulging, or clearly immobile TM is highly suggestive of AOM and a distinctly red TM also increases the likelihood (Rothman et al 2003). The review also suggests that ear pain may be an important symptom but that other symptoms are not reliable. For proper diagnosis of AOM or OME, visual examination of the TM is essential.

2.2 Epidemiology

The incidence of AOM varies and is dependent on the age of the child and the season of the year. The highest peaks occur during the fall, winter and spring (Ruuskanen et al 1989, Pukander et al 1982). At least one episode of AOM is experienced by approximately 50% of all children before their first birthday (Ruuskanen & Heikkinen 1994, Alho et al 1991, Sipilä et al 1987), and the peak incidence of AOM occurs between the ages of 6 to 18 months (Pukander et al 1982, Lundgren et al 1983, Sipilä et al 1987, Teele et al 1989, Alho et al 1991, Paradise et al 1997). Recurrent episodes of AOM are common, and two or more episodes were reported in 20% of infants by 6 months of age (Daly et al 1999).

In a study in Finland the occurrence of AOM increased by 68% from the 1970s to the 1990s. The clinical picture of AOM has in this period become milder, with fewer febrile patients and a decreased rate of spontaneous otorrhea, but children are more often treated with broader spectrum antibiotics (Joki-Erkkilä et al 2000).

Risk factors

Several risk factors for AOM have been identified and these vary in different studies depending on the environment. Host-related factors are young age (Jero & Karma 1997), male sex (Teele et al 1989) and genetic predisposition (Casselbrant et al 1999, Kvaerner et al 1997). Other factors are attendance at day care (Alho et al 1990), parental smoking (Stenström et al 1993), not being breast-fed (Duncan et al 1993, Duffy et al 1997), siblings (Varon et al 2000) and the use of pacifiers (Niemelä et al 1995, Uhari et al 1996).

Complications

The acute complications of AOM may be intratemporal or intracranial. Intratemporal complications include mastoiditis, facial paralysis, petrositis and labyrinthitis, while intracranial complications include meningitis, extradural abscesses, subdural empyema, focal otitic encephalitis, brain abscess, dural sinus thrombosis and otic hydrocephalus (Rosenfeld & Bluestone 2003). The frequency of complications due to AOM was higher in the early antibiotic era than is reported today.

In the 1950s, 17 % of children who were not treated with antibiotics developed mastoiditis (Rudberg 1954). In a Danish study the clinical picture of mastoiditis from 1977 to 1996 had changed. During the last 10-year period there was fewer cases of AOM in the childrens background, shorter duration of hospitalisation, and the more frequent presence of Streptococcus pyogenes (Petersen et al 1998). The most common organisms recovered from cultures in mastoiditis are S. pneumoniae
and *S. pyogenes* (Spratley *et al* 2000, Luntz *et al* 2001). It is interesting to note that a multinational survey showed that the incidence rate of acute mastoiditis in the Netherlands, where a restrictive antibiotic policy is advocated for AOM, is slightly higher than in many countries with higher antibiotic prescription rates (Van Zuijlen *et al* 2001).

### 1.3 Microbiology in AOM

#### 1.3.1 The role of viruses

A viral respiratory infection often precedes AOM episodes. In particular, respiratory syncytial-virus, influenza A and B, and adenovirus infections confer an increased risk of AOM in the following 2 weeks (Heikkinen *et al* 1995). Viruses have been isolated from the MEE in 40% of cases (Canafax *et al* 1998) but as a sole pathogen only in 6% (Heikkinen 2000). The detection of viruses has conventionally ranged between 30% and 50%, but a recent study using antigen detection and PCR for several viruses could document viral infection in the nasopharyngeal specimens of 90% of the children with AOM (Heikkinen & Chonmaitree 2003).

Although AOM in many children is diagnosed concurrently with upper respiratory tract infection, the development of AOM only occurs after a certain time interval after the onset of the viral infection. After onset of upper respiratory infection in children attending day care the highest incidence of AOM was observed on day 3 and 75% were diagnosed during the first week after the onset of respiratory symptoms (Ruuuskainen & Heikkinen 1994). During respiratory infection the balance of normal bacterial flora in the nasopharynx is altered with an increase in AOM pathogens and a decrease in the non-pathogen bacterial flora (Faden *et al* 1990).

#### 1.3.2 Bacteria

The most common method for obtaining bacterial cultures is nasopharyngeal swabs. The ideal is to be able to culture from the MEE directly, and that may be possible in the draining ear but is more difficult with an intact TM. Today tympanocentesis is not done routinely and nasopharyngeal swabs are thus the method available in routine practice. Bacteria cultured in the nasopharynx are correlated to the pathogens found in AOM in MEE, but the nasopharynx is not a sterile location. The human nasopharynx is a natural reservoir for AOM pathogens (Stenfors & Raisanen 1990) and these could be acquired from 31% of healthy infants at 2 months, from 49% at 6 months and from 62% at 10 months of age (Aniansson *et al* 1992). Colonisation is a dynamic process with a change in bacterial strains over time (Faden *et al* 1995). Bacterial species such as *Streptococcus viridans* and anaerobic streptococci, which may have inhibitory activity against potential pathogens, also colonise the nasopharynx (Brook & Gober 1998, Tano *et al* 2000).

In the first half of the twentieth century the most frequently isolated bacterium in AOM was *S. pyogenes* but since the 1950s it has become less common (Haugsten & Lorentzen 1980). Now the major pathogens in AOM are *S. pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* (Jacobs *et al* 1998). Of these *S. pneumoniae* and *H. influenzae* have been isolated as major causative agents all over the world, while the occurrence of *M. catarrhalis* shows considerable differences between geographic regions (Kamme *et al* 1971, Del Castillo *et al* 1996, Bluestone *et al* 1992, Del Beccaro *et al* 1992, Kilpi *et al* 2001).
In varying degrees two or more bacterial species are isolated from middle ears in AOM and this can occur in a small percentage to 29% in a recent study (Ruohola et al. 2003). The duration of MEE was longer in children with two or more bacterial types, compared to those with only one pathogen or in whom no pathogen was detected. One-quarter to one-third of bacterial cultures in AOM yield no growth or display only non-pathogens such as *Staphylococcus epidermidis* or diphteroides, but the detection of pathogens depends on culture methods (Del Beccaro et al. 1992).

**Streptococcus pneumoniae**

*S. pneumoniae* was identified as an AOM pathogen in the late 1800s. It was roughly as common as *S. pyogenes* up to the 1950s, after which it has been the most frequent bacteria associated with AOM (Nielsen 1945, Del Beccaro et al. 1992). In the 1990s pneumococci have been isolated from middle ear fluid samples obtained during AOM in 30-50% of cases (Jacobs et al. 1998).

*S. pneumoniae* is an encapsulated gram-positive diplo-coccus, and the capsule is the most important virulence factor. The serotypes, 3, 6, 9, 14, 18, 19 and 23 have been the most common in AOM over decades (Nielsen 1945, Orange & Gray 1993). Today *S. pneumoniae* is the microorganism most often associated with severe and fatal complications in AOM (Barry et al. 1999, Goldstein et al. 1998, Klein 1994). Spontaneous clearance from the middle ear is approximately 20% (Klein 1993, Rosenfeld et al. 1994, Howie & Ploussard 1972), the lowest of the three common pathogens.

**Non-typeable Haemophilus influenzae**

*H. influenzae* was first classified as a pathogen causing AOM (Wirth 1928) in 1928 but more regularly after 1945 (Nielsen 1945) at least partly because of failure to detect the bacterium with special growth requirements. Now it is found in variable proportions of AOM from 10-40% (Jacobs et al. 1998). In the 1950s it was also shown that in the majority of cases, *H. influenzae* is non-encapsulated (Bjuggren & Thunevall 1950).

Non-typeable *H. influenzae* (NTHi) strains are small gram-negative coccobacilli, which neither produce nor have the genetic material to code for a polysaccharide capsule. Lacking a capsule, *H. influenzae* is thus less virulent than *S. pneumoniae*. Lipooligosaccharide (LOS) is a major component of the outer membrane. This endotoxin is responsible for cytokine activation in an infection and contributes to the interaction with the host. Eight biotypes of *H. influenzae* have been identified (Kilian 1991) and the most frequently occurring in AOM are biotypes II and III.

In a recent study of children up to 2 years of age (Kilpi et al. 2001), the bacterium was associated with recurrent AOM and with older age in the child. The carriage rate of NTHi is high and may be up to 70% in children (Kuklinska & Kilian 1984), and the bacteria occur as obligate parasites on the mucous membranes of humans.

Children with AOM caused by NTHi developed serum bactericidal activity following infection. However, when a new episode of AOM caused by NTHi followed, bactericidal activity against the first isolate was not effective in vitro against the second isolate (Bernstein et al. 1992). The spontaneous clearance
rate of NTHi from the middle ear is approximately 50% (Klein 1993, Rosenfeld et al 1994, Howie & Ploussard 1972).

*Moraxella catarrhalis*

*M. catarrhalis* (earlier Branhamella and Neisseria) was for many years regarded as a non-pathogenic organism. The organism was first described in 1896 (Frosch & Kolle 1896, Verduin et al 2002). It has been recognised as a specific pathogen in AOM since 1927 (Hart 1927) but not until the 1980s (Kovatch et al 1983) was it fully recognised as an AOM pathogen. Since then the proportion of AOM associated with *M. catarrhalis* has increased up to as much as 23% of AOM cases (Kilpi et al 2001).

*M. catarrhalis* is a gram-negative diplococcus that expresses the endotoxin LOS but lacks a capsule and is thus less virulent than *S. pneumoniae*. Severe complications after AOM are rare, and the organism seldom causes serious systemic disease (Marchant 1990, Murphy 1996). Studies indicate that the severity of symptoms and number of bacteria during an AOM episode appear to be lower for *M. catarrhalis* than for *S. pneumoniae* or *H. influenzae* (Faden et al 1992, Van Hare et al 1987).

Studies to date are in agreement that *M. catarrhalis* is a genetically heterogeneous species from which successful clones occasionally proliferate (Enright & McKenzie 1997, Verduin et al 2002). The carriage rate of *M. catarrhalis* is high in children, up to 75% (Verduin et al 2002, Faden et al 1994), and is higher in winter and autumn than in spring and summer (Van Hare et al 1987). A relationship between colonisation of *M. catarrhalis* and the development of AOM has also been shown (Faden et al 1994). The spontaneous clearance rate from the middle ear is high, approximately 80% (Klein 1993, Rosenfeld et al 1994).

1.4 Treatment of AOM

1.4.1 Background

The management of AOM has recently been much debated. In part this has been a consequence of the continued escalation in incidence of antibiotic-resistant upper airway pathogens. Antibiotics have been routinely used for the treatment of AOM in most countries since the early 1950s. Recently this practice has been under scrutiny and several studies are conducted to measure the benefit of antibiotic treatment. The practice of antibiotic treatment for AOM has been questioned because of a high spontaneous resolution and wide diagnostic criteria for AOM (van Buchem et al 1981, Mygind et al 1981, Del Mar et al 1997). When evaluating the natural history of untreated AOM, about 60% of children with AOM initially managed without antibiotics are symptom free in 24 hours. By 2-3 days 80% are without residual symptoms, excluding MEE. Residual MEE after AOM is common, with 65% occurrence at 2 weeks, 40% at 1 month and 25% at 3 months (Rosenfeld & Bluestone 2003). Three months after AOM no difference between the penicillin and the placebo groups with regard to the results of otoscopy and tympanometry were observed (Mygind et al 1981).

Several recently published meta-analyses (Rosenfeld et al 1994, Takata et al 2001, Glasziou et al 2000) support the conclusion that antibiotics exert only a modest benefit compared with placebo for children with AOM and that there has
been no demonstrable superiority of any antibiotic over amoxicillin in the treatment of this condition. The meta-analysis made in Evidence-based Otitis Media (Rosenfeld & Bluestone 2003) found that initial antibiotic therapy did not relieve AOM symptoms by 24 hours, but provided 4% greater relief by 2-3 days. Antibiotic-treated children had 9% greater symptom relief by 4-7 days. However, in children with pneumococcal AOM, the penicillin-treatment was shown to reduce pain (Mygind et al 1981), but not in children with AOM caused by $H. influenzae$. The benefit of antibiotic treatment can possibly be related to bacterial species.

Several of the studies included in the meta-analyses excluded children under 2 years of age, including all studies that reported outcomes at 24 hours, and the diagnosis of AOM varied. Nearly all trials excluded children with immune deficiencies, cleft palate, craniofacial anomalies, pre-existing OME, complicated AOM, and concurrent bacterial infections (sinusitis, bronchitis), and some trials excluded children under 2 years of age, those with recurrent AOM, or those with severe symptoms. All children with irregular clinical courses received antibiotics. Damoiseaux et al found a greater antibiotic benefit at 4-7 days for children under 2 years of age compared with studies limited to older children (van Buchem et al 1981, Burke et al 1991, Damoiseaux et al 2000, Rosenfeld & Bluestone 2003).

When scrutinising randomly controlled trials, the children are not a random sample of children with AOM but seem to represent a select group with less severe symptoms. Consequently, results can maybe not be broadly extrapolated to all children with AOM, especially not to children with severe symptoms and young age.

### 1.4.2 Antibiotics and antibiotic resistance

#### Antibiotic resistance promotion

The major cause of antibiotic resistance seems to be the use of antibiotics. A study in Iceland showed that mainly co-trimoxazole and erythromycin selected penicillin non-susceptible $S. pneumoniae$ (PNSP) in children who had received three or more courses of treatment (Arason et al 1996). In another study co-trimoxazole was reported to be the only antibiotic associated with a significantly increased risk of carriage of PNSP (Melander et al 1998). Other data obtained in Europe show that when comparing antibiotic sales and PNSP, the antimicrobial resistance of $S. pneumoniae$ to penicillins is correlated with use of beta-lactam antibiotics and macrolides (Bronzwaer et al 2002).

The role of antibiotic treatment of AOM is to facilitate healing of infection and avoid complications and sequelae. Antibiotics vary in their type of interaction with the pathogens. Eradicating bacteria from the middle ear increases clinical improvement by about 30% compared with failure to eliminate pathogens (Carlin et al 1991, Dagan et al 1998, Marchant et al 1992).

Bacteria have a remarkable array of tools at their disposal to overcome antibiotics. A single genetic mutation may lead to resistance with only slight alterations of the pathogenicity or viability of the bacterial strain. The extent to which bacteria develop resistance to antimicrobial drugs vary, but so far resistance has developed to all antimicrobial drugs.
Penicillins

The story begins in 1928 when Alexander Fleming described a substance he called penicillin that had the ability to eradicate bacteria (Fleming 1929). Several years passed until the research began in earnest during the Second World War. In 1953 a biosynthetic penicillin fenoximetylpenicillin, or penicillinV (pcV) was constructed which was an important step forward for oral treatment opportunities. The core of the penicillin molecule was synthesised in 1959 and a number of semi synthetic penicillins were thereafter manufactured, among them ampicillin and amoxicillin.

Penicillins belong to the beta-lactam class. These drugs act by a time-dependent killing mechanism; that is, they must reach concentrations in the MEE that are above the MIC of the pathogen and remain above this concentration for at least 40-50% of the dosing interval (Craig & Andes 1996).

Since the beginning of the antibiotic era, pcV has been a first-line drug in treatment of AOM in Scandinavia and continues to be. Motives to keep pcV have been low cost, bactericidal effect, few adverse effects and a low tendency for inducing bacterial resistance. The narrow antibacterial spectrum seems to be of importance in not promoting PNSP, even though several studies have shown a correlation with beta-lactams and development of bacterial resistance (Bronzwaer et al 2002, Nasrin et al 2002). The disadvantages with the use of pcV are the low absorption from the gastro-intestinal tract (ca 50%), the high degree of protein binding (80%) resulting in a low free microbiologically active part, the limited antibacterial spectrum against gram-negative bacteria and the bad taste of the oral mixtures.

In the 1970s, ampicillin and amoxicillin were found to be effective against S. pneumoniae and H. influenzae. Amoxicillin displaced ampicillin due to the lower incidence of diarrhoea. Amoxicillin has been the recommended drug for initial routine empiric therapy of uncomplicated AOM for 25 years in the United States because of its clinical efficacy and long record of safety. It is highly effective against S. pneumoniae and displays the best pharmacodynamic profile against PNSP of any of the commonly available oral antimicrobial agents (Craig & Andes 1996). Pharmacodynamic studies indicate that with higher doses of amoxicillin (70-90 mg/kg/day vs. 40-45 mg/kg/day), higher middle ear concentrations are achieved and these are sufficient to eliminate PNSP (Lister et al 1997, Canafax et al 1998). When comparing pcV and amoxicillin, amoxicillin is better absorbed from the gastro-intestinal-tract and reaches higher middle ear concentrations. The half-life of amoxicillin is longer and the protein binding is lower (Block 1995, Craig 1996). If eradication of bacteria from the middle ear is to be achieved in AOM caused by H. influenzae, amoxicillin is a better choice than pcV (Howie 1985). Amoxicillin, however, has more adverse effects and a broader anti-bacterial spectrum.

Adding the beta-lactamase inhibitor clavulanate to amoxicillin is a method to neutralise the beta-lactamases produced by H. influenzae and M. catarrhalis. Data support the efficacy of amoxicillin-clavulanate to eradicate beta-lactamase producing H. influenzae from the middle ears. For M catarrhalis the data are limited, and it is not possible to conclude that amoxicillin-clavulanate is superior to amoxicillin (Dagan et al 2001), in part due to the high spontaneous clearance rates.
The Scandinavian treatment policy

A roundtable conference in Helsingborg in 1966 was the starting point in the process leading to the Swedish or Scandinavian policy of treatment with pcV. The conference led to an article (Juhlin et al 1967) on the question of the adequate dose of penicillin with the recommendation from the conference that the dose should be at least double the earlier recommended dose. Research followed and the dosage of pcV was tested in clinical studies (Lundgren et al 1967, Kamme et al 1969). The results were in concordance with the conference recommendation. The doubled dose, 52 mg/kg/day, meant a treatment outcome of 88%, independent of bacteriological aetiology. Further studies showed later that it was possible to reduce the treatment period from 10 days to 5 days and from administration 3-4 times per day to 2 times daily with the same treatment outcome (Rundcrantz & Sundfors 1974, Ingvarsson et al 1980, Ingvarsson & Lundgren 1982). With the higher dose regime the MICs for S. pneumoniae, S. pyogenes and M. catarrhalis were cleared by the penicillin concentration in the middle ear with a broad margin and the MICs cleared for H. influenzae in 90% of cases. The treatment policy recommends pcV as the first-line therapeutic choice, in a high dose regime (50mg/kg/day) twice or three times daily to children and adults, with a treatment period of 5 days in AOM. This policy is still being used despite the increasing bacterial resistance.

Pneumococcal resistance

The mechanism of pneumococcal resistance to penicillin G and other beta-lactam antibiotics involves alterations in one or more of the penicillin-binding proteins (PBPs) causing their lowered affinity to beta-lactam antibiotics. All beta-lactams exert their effect by binding to enzymes in the bacterial cell wall so that the building of the cell wall is stopped.

The prevalence of resistant strains of the common AOM pathogens is increasing. One of the most alarming changes has been observed among strains of S. pneumoniae. In the 1940s, all S. pneumoniae strains were susceptible to penicillin. It was not until the 1960s that reports of strains of pneumococci with intermediate levels of penicillin resistance began to appear (Appelbaum 1992). Now the prevalence of S. pneumoniae with reduced susceptibility for penicillin varies with high prevalence (30-40%) in some regions, notably southern Europe, and low prevalence in other regions (Gehanno et al 2001, Hoban et al 2001). In Sweden the prevalence of PNSP is <10% according to STRAMA (Swedish Strategic Programme for the Rational use of Antimicrobial agents and Surveillance of Resistance, www.strama.org, www.srga.org).

Resistance among gram-negative bacteria

Beta-lactamases are enzymes capable of hydrolysing the beta-lactam ring of penicillins, and related antimicrobial drugs, rendering them inactive. There are dozens of beta-lactamases which vary in substrate specificity, host range and difficulty to treat.

In M. catarrhalis two types of beta-lactamases can be found (Verduin et al 2002), and recent studies have suggested that one of these beta-lactamases may have a gram-positive origin which makes M. catarrhalis the first gram-negative bacterial species possessing such a beta-lactamase (Bootsma et al 1999). The beta-lactamases
from *M. catarrhalis* have also been shown to be able to shelter concomitant bacteria by inactivating penicillin therapy (Hol *et al* 1994). In the beginning of the antibiotic era this pathogen was penicillin susceptible but has since then acquired beta-lactamase producing capability. Today 90% or more of all isolates are beta-lactamase producing (Manninen *et al* 1997).

*H. influenzae* has two different mechanisms of antibiotic resistance. The most common mechanism of resistance is the production of beta-lactamase (Brunton *et al* 1986, Medeiros *et al* 1986). Another mechanism for resistance to ampicillin is a structural alteration of the PBPs (Clairoux *et al* 1992) that is chromosomally mediated. Beta-lactamase production is increasing in the world but varies between countries. In Finland beta-lactamase production increased from 8% to 24% over 5 years (Manninen *et al* 1997) and in the United States a prevalence of 47% (Jacobs *et al* 1998) has been observed. Overall in 15 countries 17% of isolates have been identified as beta-lactamase positive (Bandak *et al* 2001).

### 1.5 Prevention

There exists no method with complete success in prevention of AOM. Several methods are used including antimicrobial prophylaxis (Williams *et al* 1993, Rosenfeld & Bluestone 2003) and tympanostomy tube insertion with or without adenoidectomy (Gonzalez *et al* 1986, Casselbrant *et al* 1992, Rosenfeld & Bluestone 2003). Tympanostomy tube insertions may reduce the incidence of new AOM by 50% – 60% (Rosenfeld & Bluestone 2003). Modification of risk factors may also decrease AOM (Niemelä *et al* 2000).

Opportunities exist for immunoprophylaxis by targeting viral respiratory pathogens and bacterial otopathogens. Passive immunisation with different immunoglobulins has not had any proven effect on recurrent AOM (Kalm *et al* 1986, Jörgensen *et al* 1990, Shurin *et al* 1993). Vaccines for preventing disease caused by *S. pneumoniae* have focused on the capsule even though each of the described 90 serotypes has a unique polysaccharide composition with limited cross-reaction among serotypes. The earlier available vaccines against *S. pneumoniae* have not shown any clinically efficient prevention of AOM (Mäkelä *et al* 1980). Now new conjugated pneumococcal vaccines are available and have been tested in clinical studies. The reduction was 6% on overall AOM, 34% of the episodes that were caused by pneumococci, and 57% reduction on AOM caused by the pneumococcal serotypes included in the vaccine (Eskola *et al* 2001). Findings in these studies support the principle that high serum antibody concentrations result in better protection for AOM.

However, intervention strategies that target only a selected population of bacterial otopathogens are likely to have a limited effect as replacement of bacterial strains is observed that will potentially reduce the benefit of immunization (Veenhoven *et al* 2003).

### 1.6 Pathogenesis

The correlation between the pathogens found in the nasopharynx and in the MEE (Loos *et al* 1989) indicates that the bacteria in AOM originate in the nasopharynx. The middle ear cavity is normally a sterile compartment which is maintained by the mucociliary system together with the enzymes and antibodies secreted by the epithelial cells of the Eustachian tube (ET) and the middle ear (Lim *et al* 2000).
Abnormal anatomy and dysfunction of the tube are involved in the development of AOM (Bylander-Groth & Stenström 1998). An infection in the nasopharynx and ET lead to increased mucous secretion that can obstruct the ET resulting in a negative middle ear pressure (Bluestone 1996), and the bacteria can then enter the middle ear.

The bacteria then stimulate the host cellular responses leading to cytokine synthesis and a modulation on inflammatory cells (Henderson et al 1996). Cytokines are bioactive proteins that regulate proliferation, chemotaxis, and the activation of inflammatory cells (Nicod 1993). The pro-inflammatory cytokines are TNF-α, IL-1 and IL-6, while others are anti-inflammatory like IL-10 or TGF-β. IL-1β is the earliest cytokine detected in MEE (Sato et al 1999) and the concentration of IL-1 decreases during antibiotic treatment on days 4-5 (Barzilai et al 1999).

The inflammatory process has now started which is critical for the destruction of bacteria but it may also lead to mucosal damage in the host with scarring and polyp formation (Patel et al 1993). Vasodilatation occur (Robbins & Cotran 1979) and within 30 minutes the neutrophils transmigrate from the vessels. The cytokines also causes structural changes of the endothelial cell layer and later the tissues are infiltrated by monocytes and T-cells (Pober & Cotran 1990) and B-cells are recruited to the middle ear.

TGF-β is a potent multifunctional cytokine which regulates the proliferation of cells, embryonic development, wound healing, and angiogenesis (Dhainaut et al 2003, Fjellbirkeland et al 2003). TGF-β has well known anti-inflammatory and immune-suppressive properties. It is also capable of promoting inflammation whose sustained production underlies the development of tissue fibrosis (Dhainaut et al 2003). TGF-β can influence the process of T-cell migration and activation in local inflammation (Ludviksson & Gunnlaugsdottir 2003). In a murine model TGF-β was shown to regulate airway responses via its effects on T-cells (Schramm et al 2003). TGF-β has an important role in bone modelling and the development of bone quality (Geiser et al 1998).

1.7 Host responses to AOM

There are systemic and local immune reactions involved in the human immune response. In the middle ear mucosa, which is a part of the respiratory system, the healthy mucosa only has a few lymphocytes and no lymphoid tissue. The circulating antibodies that are secreted into the middle ear therefore play an important role in the defence (Ryan et al 1990).

Mucosal responses

The first line of defence is the mucosa of the upper respiratory tract which acts as a physical barrier. Multiple defence systems cooperate in controlling bacterial attachment. On the surface of these membranes there is a mucous layer that can trap pathogens, ciliary activity resulting in transport and epithelial cells that sheds outer epithelial layers (Wood & Davis 1980). Nasopharyngeal colonization with bacterial pathogens stimulates the production of mucosal as well as serum antibodies to the pathogens (Faden 2001). Decoys are presented, i.e glycoproteins, which may prevent bacterial adhesion (Zopf & Roth 1996). Secreted immunoglobulins and complement ligands may also trap or coat the bacteria and prevent bacterial adherence. S-IgA, IgM and IgG take part in this process (Brandtzæg 1992). Specific
IgA mucosal antibodies limit the duration and the frequency of colonization. When the specific immune response is not mature, between 6 to 18 months, the mannose-binding lectin is also an important component responsible for this function (Turner 1996).

**Systemic responses**

The specific immune system is divided into the B-cell system, in which immunoglobulins are secreted from plasma cells (mature B cells), and the T cell system, in which mature cytotoxic T lymphocytes kill infected cells. These systems are not fully matured before the age of about 18 months. Secretory IgA antibody prevents bacteria from attaching the epithelium and does not activate complement. IgG antibodies can opsonize the bacteria for phagocytosis and can activate a complement cascade (Stenfors 1999). It has been observed that humoral antibodies can prevent AOM caused by *S. pneumoniae* or NTHi (Barenkamp 1986, Rapola et al 2001). After unilateral middle ear infection and recovery, both ears are protected at rechallenge with the homologous isolate because of a humoral immune response (Melhus et al 1995). In a rat model protection against ipsilateral and contralateral rechallenge following middle ear infection with *S. pneumoniae* has also been reported (Svinhufvud et al 1991). Local immune responses following initial middle ear infection could protect the contralateral ear, but the role of local vs. humoral immunity could not be distinguished. Serum IgG antibodies can protect children against the development of AOM, but does not affect colonization (Faden 2001).

2. **Animal models for studies of middle ear pathology**

2.1 **Different models**

The common pathogens of AOM in humans have been extensively studied in animal models. The use of animal models has had a large impact on the knowledge we now have given the advantage of control over the animal and the microenvironment in the disease process which enables sequential and repeated observations of histological, immunologic, cytological and biochemical variables.

Several different species have been used including chinchilla (Bakaletz et al 1999), rat (Hermansson et al 1988), mouse (Sabirov et al 2001), gerbil (Parra et al 2002) and guinea pig (Sato 1997). AOM models of both *S. pneumoniae* and *H. influenzae* have been established in several species, but it has been difficult to develop an animal model for *M. catarrhalis* (Doyle 1989). The animals may develop AOM, but the bacteria are rapidly eliminated from the middle ear (Chung et al 1994, Doyle 1989, Fulghum & Marrow 1996).

It has been difficult to find an ideal animal model for studies of AOM but the various models have made it possible to study several aspects of AOM. One of the most frequently used models has been the chinchilla (Giebink 1999). The middle ear cavity and Eustachian tube structures of chinchillas and gerbils are similar but differ from those of the rat. The middle ear of the chinchilla also differs from that of humans mainly in that it has a multilobular tympanic cavity and patulous or semipatulous ET (Doyle 1985). The rat has been increasingly used since the introduction of an AOM model in the 1980s (Hermansson et al 1988) due to the advantage of inbred strains, commercially available antibodies, and near-complete genetic data. One animal that now is being increasingly studied in otitis media research is the
mouse, mainly because of the vast possibilities with knock-out strains for the studies of pathogenesis (Melhus & Ryan 2003).

2.2 Anatomy and histopathology of rat middle ear

The rat middle ear has small bullae and almost horizontal ETs that are usually closed. The ET mucosa contain large concentrations of goblet cells but relatively few mucous glands (Daniels et al. 1982). The opening pressure of the rat ET corresponds to that in humans (Hellström & Stenfors 1983). The ET is connected to the epi tympanon via two distinct tracts of ciliated and secretory cells. Rat and human mucosa show similarities in the distribution of the mucociliary transport system (Albiin et al. 1986). The rat middle ear is located in the temporal bone and well protected but its TM is easily accessible for inspection with an ordinary otomicroscope. The three-dimensional structure of the rat tympanic cavity has similarities to that of man but lacks mastoid air cells and has tympanic bullae that protrude from the floor of the cavity (Hebel & Stromberg 1978, Hellström et al. 1982). The TM, with the pars tensa and the relatively large pars flaccida, forms the major portion of the lateral wall. In the medial wall the promontory, the round and oval windows with stapes, and the tympanal opening of the ET are located. Except the two tracts of ciliated and secretory cells the tympanic cavity is lined with a simple, squamous-cuboidal, non-ciliated epithelium. During pathological conditions this simple epithelium will change and the ciliates and secretory cells appear in large numbers outside the tracts. The laboratory rat can develop AOM spontaneously but has rarely done so in our hands.
AIMS

The aims of the present study were to explore various aspects of the Scandinavian treatment policy in a rat model.

The specific aims were:

- To evaluate the response of AOM to amoxicillin treatment with special reference to the otomicroscopic appearance of the TM, morphological changes in the middle ear and serum-antibody response.

- To study the influence of amoxicillin treatment on recurrent AOM caused by NTHi.

- To study the effects of amoxicillin treatment on AOM induced by penicillin-resistant non-typeable *H. influenzae*, with both beta-lactamase-mediated and non-beta-lactamase-mediated resistance.

- To evaluate if it is possible to eradicate a penicillin-susceptible strain of *S. pneumoniae* with amoxicillin when the bacteria co exist with a beta-lactamase-producing *H. influenzae* in the middle ear.

- To establish an animal model of AOM caused by *M. catarrhalis* and to compare differences in clinical courses between AOM pathogens.
MATERIAL AND METHODS

1. Animals and surgical procedures

Healthy male Sprague-Dawley rats were used in all studies. When starting the experiments the animals weighed 200-450g. The animals were kept under standard laboratory conditions and given food and water ad libitum. For otomicroscopic examinations or during operations, the animals were anesthetised intravenously with methohexital or intraperitoneally with chloral hydrate. All experiments were approved by the Animal Ethics Committee in Lund/Malmö.

To induce AOM, bacterial inoculations were made directly into the middle ear bulla. The bulla was exposed through a ventral midline incision and blunt dissection of the soft tissue. With a fine needle approximately 0.05 ml of the bacterial suspension was instilled through the bony wall. The animals were then sutured. The procedure was performed in exactly the same manner during rechallenge.

2. Otomicroscopy

In all studies animals were inspected under an otomicroscope at various intervals. The inspections were carried out by a team member unaware of the identity of each animal. The status of the TM and the quality and quantity of the effusion behind the TM were evaluated. Direct visualisation of opaque fluid behind the TM was required for diagnosis of AOM.

3. Experimental design

Paper I

To study the effects of a 5-day course of amoxicillin for recurrent AOM caused by NTHi, 43 animals were challenged with NTHi on day 0. On day 3, when AOM was established, antibiotic therapy with amoxicillin was introduced in 20 animals via drinking water. The treatment continued for 5 days, until day 8 after inoculation.

One month after the initial inoculation a rechallenge was performed. Five unchallenged animals were then added as controls to ensure established infection.

Table 1. Experimental design paper I

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment group (number of animals)</th>
<th>Controls (number of controls)</th>
<th>Evaluated (number of animals)</th>
<th>Excluded due to interventions (number of animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>23</td>
<td>Otomicroscopy 43, serology 10</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>23</td>
<td>Otomicroscopy 43, middle ear culture 3</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>20</td>
<td>Otomicroscopy 40, middle ear culture 6, serology 20</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>17</td>
<td>17</td>
<td>Otomicroscopy 34, middle ear culture 6, serology 20, histology 6</td>
<td>12</td>
</tr>
<tr>
<td>28 (re-challenge)</td>
<td>11</td>
<td>11 + 5 (not challenged earlier)</td>
<td>Otomicroscopy 27, serology 20</td>
<td>4</td>
</tr>
<tr>
<td>32</td>
<td>8</td>
<td>10 + 5</td>
<td>Otomicroscopy 23, middle ear culture 3</td>
<td>3</td>
</tr>
<tr>
<td>36</td>
<td>8</td>
<td>9 + 2</td>
<td>Otomicroscopy 19, middle ear culture 8, serology 17</td>
<td>8</td>
</tr>
<tr>
<td>56</td>
<td>5</td>
<td>6</td>
<td>Otomicroscopy 11, histology 5</td>
<td>11</td>
</tr>
</tbody>
</table>
Paper II

To study the effects of amoxicillin treatment on the outcome of AOM after challenge with non-typeable *H. influenzae* strains with and without chromosomal changes mediating reduced susceptibility to beta-lactams, 70 rats were used.

On day 0 the animals were challenged with wild-type strain 3655 (n = 35) or transformant strain 3655/4700 (n = 35). Antibiotic therapy with amoxicillin was introduced on day 3 in 25 animals in each challenge group. Middle ear fluid samples for culture were obtained from all animals on day 8 except those that were randomly selected from the treatment groups for morphological examination.

Table 2. Experimental design paper II

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment groups (strains 3655+3655/4700) (number of animals)</th>
<th>Controls (strains 3655+3655/4700) (number of animals)</th>
<th>Evaluated (number of animals)</th>
<th>Excluded due to interventions (number of animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25 + 25</td>
<td>10 + 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>25 + 25</td>
<td>10 + 10</td>
<td>Otomicroscopy 35 + 35</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>25 + 25</td>
<td>10 + 10</td>
<td>Otomicroscopy 35 + 35, histology 5 + 5</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>20 + 20</td>
<td>10 + 10</td>
<td>Otomicroscopy 30 + 30</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>20 + 20</td>
<td>10 + 10</td>
<td>Otomicroscopy 30 + 30</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>20 + 20</td>
<td>10 + 10</td>
<td>Otomicroscopy 30 + 30</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>20 + 20</td>
<td>10 + 10</td>
<td>Otomicroscopy 30 + 30, middle ear culture 15 + 15, histology 5 + 5</td>
<td>50</td>
</tr>
<tr>
<td>56</td>
<td>5 + 5</td>
<td>0</td>
<td>Histology 5 + 5</td>
<td>10</td>
</tr>
</tbody>
</table>

Paper III

To investigate if amoxicillin treatment could eradicate pneumococci even if a beta-lactamase-producing NTHi co existed in the middle ear, a total of 78 animals were challenged with *S. pneumoniae* (groups A and D), NTHi (group E) or a combination of both bacteria (groups B and C). Forty-four animals were treated with amoxicillin while the remaining 34 animals served as controls.

A rechallenge was performed 1 month after the initial inoculation in which a total of 20 animals from groups A-C were challenged in the left contralateral middle ear with the pneumococcal strain. At this rechallenge four unchallenged animals were added as controls.

From day 0 to day 56 after challenge, the animals were repeatedly inspected under an otomicroscope. Bacterial samples from the middle ears were collected either from the ear canal of animals with spontaneously perforated TM or bilateral infections (day 3, n = 25) or by inserting a swab directly into the middle ear cavity after opening up the bulla (day 8, n = 40). For histological studies animals were sacrificed on days 3 (groups A-B, n = 3 + 3), 8 (groups A-C, n = 3 + 3 + 3) and 56 (groups A-C, n = 6 + 4 + 5). Animals were also sacrificed on day 56 (groups B and C, n = 8 + 8) to study the gene expression of TGF-β.
**Paper IV**

To study AOM caused by beta-lactamase-producing *M. catarrhalis*, 55 animals were challenged in the right middle ear with strain BC 1, 30 animals with viable bacteria and 25 with heat-killed bacteria. Six animals died during or after anesthesia.

<table>
<thead>
<tr>
<th>Day</th>
<th>Viable M. catarrhalis (number of animals)</th>
<th>Heat-killed M. catarrhalis (number of animals)</th>
<th>Evaluated (number of animals)</th>
<th>Excluded due to interventions (number of animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30</td>
<td>25</td>
<td>otomicroscopy 30 + 25</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>25</td>
<td>otomicroscopy 30 + 25</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>25</td>
<td>Otomicroscopy 30 + 25, histology 5 + 5, middle ear cultures 5</td>
<td>10 + 5</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>20</td>
<td>Otomicroscopy 20 + 20, histology 4 + 5</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>10</td>
<td>15</td>
<td>Otomicroscopy 10 + 15, histology 5 + 5</td>
<td></td>
</tr>
<tr>
<td>3m</td>
<td>5</td>
<td>10</td>
<td>Otomicroscopy 5 + 10, histology 2 + 5</td>
<td></td>
</tr>
<tr>
<td>6m</td>
<td>3</td>
<td>5</td>
<td>Otomicroscopy 3 + 5, histology 3 + 5</td>
<td></td>
</tr>
</tbody>
</table>

To further study the local and systemic effects occurring after middle ear challenge with *M. catarrhalis*, and the rate of protection after a resolved infection or subcutaneous immunisation, animals were immunised and challenged. Twenty-eight animals were inoculated with the bacterial suspensions in the middle ears for intrabullar immunisation with strains 12075, 101018 or heat-killed 101018 (\(n=18+5+5\)). Six animals were subcutaneously immunised and injected in the right hip area with \(10^8\) formalin-fixed whole bacteria emulsified in Freund's adjuvant at the first immunisation, and in incomplete adjuvant at the booster immunisation 3 weeks later.

All animals were challenged 5 weeks after the initial immunisation with either the homologous or a heterologous strain. To each challenge group, untreated control animals were added (\(n=5+5+6+6\)). An additional 5 animals were challenged with heat-killed 101018. Otomicroscopy was performed prior to immunisation (day 0), before challenge (pre-operatively), and on days 4 and 8. Animals immunised intrabullarly were, in addition, examined under the otomicroscope on days 2, 4 and 8 after the initial inoculation to ensure that an infection had been induced. Blood samples were collected for serological studies on day 0, preoperatively, and on days 4 and 8 after challenge.

MEE for culture was obtained on days 4 and 8. Twenty-one additional animals were included for cultures at day 4.

4. **Bacterial strains and preparations**

**Strains**

In papers I and II, beta-lactamase negative, non-typeable *H. influenzae* strain 3655 was used to induce AOM. Another three wild-type strains and two transformant non-typeable *H. influenzae* strains were used in paper II: the wild-type strains 1161 (control strain for lipopolysaccharide [LPS] analysis), 4700 and 4089.
A beta-lactamase-producing non-typeable *H. influenzae* strain 3144 was used in paper III together with a penicillin-susceptible strain of *S. pneumoniae* type 3.

In paper IV four different strains of *M. catarrhalis* were used: strains BC 1, 101018, 12075 and 101224.

All bacteria that were not donated from other laboratories were identified with conventional methods.

**Inoculum preparations for challenge**

The bacteria were stored at -70°C, and all cultures were initially inoculated from these frozen stocks. The media used were chocolate agar, chocolate agar supplemented with 1% enrichment (an in-house mixture corresponding to IsoVitaleX [BBL]), and 1% hemin or BHI broth or agar supplemented with NAD and hemin, each at 10µg/ml, and if indicated, ampicillin at 50µg/ml.

The inocula for challenge or rechallenge of the middle ear were prepared by growing the bacteria at 37°C in an atmosphere with 5% CO₂. The bacteria were harvested by centrifugation and resuspended in fresh culture medium to an optical density of 1 at 620 nm. The bacterial suspensions were thereafter diluted with supplemented fresh medium to the varying inoculum concentrations between 10⁴ - 10⁸ cfu/ml.

For paper IV whole cell formalin-fixed *M. catarrhalis* strain 12075 for subcutaneous immunisation was prepared according to a modified version of the method described by Green *et al* (1993). Three 1-ml portions of strains BC1 and 101018 were heat-killed by boiling in water for 2 minutes.

**Genetic transformation**

Genetic transformation of *H. influenzae* was performed in paper II. DNA from non-typeable strains *H. influenzae* 4700 and 4089 was prepared by a modification (Poulsen *et al* 1988) of the method described by Moxon *et al* (1984). Non-typeable *H. influenzae* 3655 cells were made competent by a modified (Barnhart *et al* 1963) aerobic-anaerobic incubation procedure (Goodgal & Herriot 1961) and were transformed with approximately 1µg of sheared chromosomal DNA. The transformation mixtures were plated, and colonies that grew on the selective agar were purified.

**Growth rate**

In paper II the growth rate was assessed by measuring the turbidity of bacterial cultures in a spectrophotometer.

**Determination of MIC and MBC**

The determinations of MICs in paper II of penicillin V, amoxicillin, cefaclor, cefuroxime and cefotaxime were made by an agar dilution method. Antibiotics were added to the agar at twofold dilutions in freshly prepared solutions. Inocula were applied to the surface of the agar and the plates were incubated overnight *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* 29212 and *H. influenzae* NCTC 8468 were included as quality control
strains. The lowest antibiotic concentration which completely inhibited bacterial growth was recorded as the MIC.

The MICs of bensylpenicillin, ampicillin, cefuroxime and cefotaxime were determined by Etest (Biodisk AB, Solna, Sweden) as an extra control. The MICs and MBCs in paper III were determined by Etest.

Purification of LPS

LPS for paper II were purified by a small scale modification (Fomsgaard et al 1993) of the hot phenol-water extraction method (Westphal et al 1965) from wild type strain 1161, 3655, 4700 and 4089 and transformed strains 3655/4700 and 3655/4089 grown overnight on chocolate agar. Electrophoretic separation was then done (Schägger & von Jagow 1987), and the LPS were visualised with the Instaview silver staining kit (BDH Laboratory Supplies, Poole, England).

5. Pharmacokinetics and pharmacodynamics of amoxicillin

The antibiotic treatment in papers I, II and III was administered via drinking water. Amoxicillin (Imacillin; AstraZeneca, Södertälje, Sweden) was used, which is a first-line therapeutic drug in the treatment of AOM in many countries (Dowell & Schwartz 1997, Froom et al 1997). A dose of 250mg/500ml was administered, the recommended dose for rats by veterinary standards. Water consumption was measured in all studies with amoxicillin treatment on a daily basis during the treatment period.

The water consumption was followed more closely in these studies in several animals in whom the serum concentrations of amoxicillin were measured during two periods of 12 hours in each animal.

6. Morphological examination and tissue preparation

In papers I-IV middle ear tissues were examined for structural changes. In paper I the whole bullae were removed and fixation was performed in 4% paraformaldehyde for 24 hours. The middle ears were then decalcified and after dehydration embedded in paraffin, sectioned and stained. Serial sections, in which both the pars tensa and the cochlea were present, were examined under light microscopy. The epithelial lining of the middle ear cavity was studied. The degree of inflammation was registered with emphasis on the presence of inflammatory cells and vascular changes. Metaplastic changes, representing a transformation of normally flat epithelium into a cuboidal or cylindrical epithelium with or without cilia, were registered. The changes were graded as minor, moderate or extensive. After sacrifice the tympanic bullae was opened (papers II, III, IV) and the middle ear cavity filled with a fixative solution containing 3% glutaraldehyde. The pieces of tissue were collected from well-defined areas of the middle ear (Hermansson et al 1990) as seen in the schematic drawings of the medial upper and lateral lower walls of the rat middle ear. (fig 1)
Fig 1. Schematic drawing of rat middle ear. The areas represent (a) tympanal orifice of Eustachian tube, (b) posterior portion of sulcus promontorialis occipitalis, (c) fossa nasalis, (d) attic space, (e) pars flaccida and (f) pars tensa.

In papers II and IV, pieces of tissue were collected from all of these areas. Pieces were collected from areas c and e in paper III and from areas b, c and e in paper I.

The tissue samples were collected under an otomicroscope and processed for microscopy. The changes were graded semi-quantitatively or the degree of inflammation was registered with emphasis on inflammatory cells, vascular reaction, metaplasia and epithelial proliferation.

Specimens from the sulcus promontorialis occipitalis in paper IV were also evaluated by a point-counting technique (Weibel 1989), and the relative volumes occupied by goblet cells were determined. In addition these specimens were sectioned for transmission electron microscopy (TEM) and the goblet cells further scrutinised.

7. **Immunoassays**

The IgG response (paper I, III) was analysed with an enzyme-linked immunosorbent assay (ELISA). Blood samples were collected and stored at -20°C until analysed. In paper I the serum antibodies were measured with a previously described ELISA (Melhus et al., 1995) with modifications. Microtiter plates were coated overnight with whole strain 3655 cells with antigen concentrations predetermined to yield optimal readings. The plates were blocked and after each step washed. Diluted test sera were allowed to react. Anti-rat IgG was added and the plates incubated. The process was then halted and the optical densities were measured in a spectrophotometer.

Experiments were performed in triplicate and two internal controls were included on each plate. The titres were averages of the three optical density readings. The blood samples from paper IV were also analysed with the above-described ELISA technique except that the plates were coated with whole strain 101018 bacteria.

8. **RT-PCR**

To detect the gene expression of TGF-β in paper IV, mRNA was extracted from frozen middle ear samples using Dynabeads Oligo (dT)25 (Dynal A.S., Oslo, Norway). After eluting the mRNA from the beads, it was reverse transcribed and amplified as described previously (Melhus & Ryan 2000) and all samples were further analysed in a competitive PCR assay (Melhus & Ryan 2000).
9. Statistical analysis

Fisher’s exact test was used for statistical analyses of the otomicroscopy results. Mann-Whitney’s test was used for statistical analysis of the quantitated pathological and serological findings in paper I. Student’s t-test was used for the serological findings in paper III and in paper IV for comparison of mRNA-levels. A difference was considered statistically significant if $P \leq 0.05$. 
RESULTS

1. Amoxicillin treatment in recurrent AOM caused by NTHi (paper I)

Clinical observations and otomicroscopy

Apart from the middle ear infections the animals appeared clinically healthy throughout the study. The amoxicillin was well tolerated. All animals developed AOM after the first challenge and once the amoxicillin had been introduced there was no progress of the AOM otomicroscopically.

Table 4. Otomicroscopic appearance after challenge. At day 28 rechallenge was performed. No: no effusion. Clear, turbid or opaque describes the appearance of the MEE behind the TM.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 8</th>
<th>Day 28</th>
<th>Day 32</th>
<th>Day 36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>Challenge</td>
<td>10 opaque</td>
<td>8 no</td>
<td>1 turbid</td>
<td>Rechallenge</td>
<td>6 no</td>
</tr>
<tr>
<td>No treatment</td>
<td>Challenge</td>
<td>10 opaque</td>
<td>1 turbid</td>
<td>3 clear</td>
<td>1 turbid</td>
<td>No</td>
</tr>
</tbody>
</table>

After 8 days, 8 out of 10 animals in the treatment group had recovered fully while none had in the untreated control group ($P = 0.03$)

After rechallenge the presence of white plaques in the TMs was observed in 4 animals in the treatment group.

Bacterial cultures

Table 5. Growth of NTHi in bacterial cultures

<table>
<thead>
<tr>
<th>Growth</th>
<th>Day 4</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No treatment</td>
<td>Treatment</td>
</tr>
<tr>
<td>Abundant</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Sparse</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>No growth</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Serum antibodies

Fig 2. Mean ELISA values (± S.D) of serum IgG antibodies to whole NTHi cells for untreated (□) and amoxicillin-treated (○) animals on day 0 to day 36 (day 8 after rechallenge)
Low levels of IgG antibodies to NTHi cells were detected in the pre-challenge sera. During the treatment period there was no significant difference between the ELISA values in either the treatment or no treatment groups. All animals developed IgG antibodies after the challenge and after 28 days the mean ELISA values were similar for the two groups. After rechallenge, on day 36, the ELISA values were higher in the treatment group ($P = 0.003$) which had received amoxicillin during the first middle ear infection.

**Protection**

After rechallenge only 2 animals developed AOM otomicroscopically, both from the previously untreated group (2/10). The difference in protection between the groups was not significant and none were culture-positive.

**Structural changes**

The tissue samples were divided into three categories: (1) no changes, into which an unchallenged animal and 1 treated animal from day 28 were assigned; (2) minor changes, into which treated animals from day 28 and 1 from day 56 were assigned; and (3) major changes, into which untreated animals from day 28 and treated animals on day 56 were assigned. The category with major changes was characterised by the presence of inflammatory cells, dilated vessels, metaplasia of the epithelium and increased numbers of goblet cells and ciliated cells.

**Summary:** The protection of recurrent AOM was not improved by abstaining from antibiotic therapy.

2. **Amoxicillin treatment of NTHi with non-beta-lactamase-mediated resistance (paper II)**

**Characterisation of strains and growth rates**

The activities of five antibiotics were tested against the various nontypeable H. influenzae strains. The degree of resistance to beta-lactams was similar amongst the donor and the transformant strains, except in the cephalosporins.

To induce AOM the donor strains required a dose of at least 100-fold greater than required for the recipient and the transformant strains. Despite the high concentrations used the course of the AOM was shorter than that caused by any other strain. Recipient strain 3655 demonstrated the highest growth rate followed by transformant strain 3655/4700. Lowest growth rate was observed with donor strain 4700.

The LPSs of the transformant strains were characterised both physiochemically and antigenically. The amounts of LPS expressed, the electrophoretic mobilities and the immunoreactive patterns of the LPSs for the transformant strains were identical to those for the recipient strain. The transformant strain had the same biotype as strain 3655.
Clinical observations and otomicroscopy

Apart from the middle ear infections the animals appeared clinically healthy throughout the study. The amoxicillin was well tolerated. With antibiotics the duration of a middle ear infection caused by the fully susceptible strain was shortened by at least 2 days compared with the course in the control animals. For the animals challenged with the strain with reduced susceptibility, recovery was not quite as rapid, especially not during the first days of treatment. The gain in recovery time for animals treated compared with the control animals was at least 1 day.

Table 6. Number of animals with no effusion/normal appearance during the treatment period as seen otomicroscopically.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Treatment</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type strain 3655</td>
<td>Amoxicillin (n=10)</td>
<td></td>
<td>1</td>
<td>6</td>
<td>10</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>No treatment (n=10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transformant strain 3655/4700</td>
<td>Amoxicillin (n=10)</td>
<td></td>
<td>3</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>No treatment (n=10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bacterial cultures

All cultures of specimens from middle ears challenged with donor strains were negative on day 8. Cultures from animals challenged with the fully susceptible strain were negative in 9/10 after amoxicillin treatment compared with 5/10 in the controls. The corresponding figures for animals challenged with the strain with reduced susceptibility were 8/10 after amoxicillin treatment and 5/10 in the no treatment group.

Structural changes

The structural changes observed in animals challenged with 3655/4700 were comparable to those seen after strain 3655 with only minor and no definite differences in either the quantity or the quality of the changes observed at any observation time. The histological changes observed in the middle ear of animals infected with the susceptible strain that were amoxicillin-treated were less severe compared with the changes observed after no treatment or treatment of rats infected with bacteria with reduced susceptibilities to beta-lactams. The most substantial difference was that the pars flaccida of the TM exhibited a normal appearance in most animals after 2 months (4/5 vs. 0/5 animals; \( P = 0.02 \)).

Summary: Chromosomal changes mediating a relatively low level of resistance to beta-lactams seem to be advantageous for *H. influenzae* during amoxicillin treatment of AOM in the rat.
3. The ability of beta-lactamase-producing NTHi to shelter a penicillin-susceptible S. pneumoniae in mixed AOM during amoxicillin treatment (paper III)

Clinical observations and otomicroscopy

All animals developed AOM after first challenge. In 8 (10%) animals the infection progressed into a bilateral middle ear infection on day 4. Of these animals, 4 belonged to the mixed group with no treatment, 2 belonged to the mixed group with amoxicillin treatment, and 2 belonged to the group challenged with S. pneumoniae with no treatment. Seven (9%) animals developed a severe systemic infection and succumbed. Four (57%) of these animals had a bilateral infection. Of the 7 animals that succumbed, 2 were from the pneumococcal group and the deaths occurred early (days 3 and 4), 2 were from the group with mixed infection that were treated (they succumbed on days 4 and 5), and 3 were from the group with mixed infection and succumbed on days 4 (n=1) and 6 (n=2).

The treatment significantly accelerated the resolution of the pneumococcal AOM in contrast to mixed infection. On day 8, 94% of the animals had cleared otomicroscopically, with no or clear effusion, whereas 60% of the amoxicillin-treated animals with mixed infections had cleared otomicroscopically and 45% of the non-treated animals. Opaque effusion could only be observed in the two groups with mixed infection after day 7. On day 56 the presence of white plaques in the TM was substantial in the right ear of all animals with a resolved mixed infection, independent of antibiotic therapy.

Bacterial cultures

Table 7. Bacterial cultures from day 3, when amoxicillin-treatment started, and from day 8, at the end of the treatment period

<table>
<thead>
<tr>
<th>Inocula</th>
<th>Treatment</th>
<th>Growth on day 3</th>
<th>Growth on day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pnc</td>
<td>Amoxicillin</td>
<td>-</td>
<td>0/10</td>
</tr>
<tr>
<td>Pnc/NTHi</td>
<td>Amoxicillin</td>
<td>-</td>
<td>Pnc 0/11</td>
</tr>
<tr>
<td>Pnc/NTHi</td>
<td>No</td>
<td>NTHi 16/16</td>
<td>NTHi 5/11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pnc 7/16</td>
<td>Pnc 4/10</td>
</tr>
<tr>
<td>Pnc</td>
<td>No</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>NTHi</td>
<td>No</td>
<td>5/6</td>
<td>2/6</td>
</tr>
</tbody>
</table>

TGF-β

The expression of TGF-β differed between animal groups with mixed infections. In the amoxicillin-treated group the transcript levels were lower (mean 25.9 ± 11.1 fg) compared with the untreated group (mean 35.4 ± 27.5 fg), but the difference was not statistically significant (P = 0.34)
Protection

Table 8. Protective rate after rechallenge in the left ear. The results are similar in the different groups with no significant differences

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inocula</th>
<th>Protective rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>Pnc</td>
<td>2/4 (50%)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>Pnc/NTHi</td>
<td>5/8 (62%)</td>
</tr>
<tr>
<td>No treatment</td>
<td>Pnc/NTHi</td>
<td>4/8 (50%)</td>
</tr>
</tbody>
</table>

Structural changes

The specimens were categorised and assigned to 3 groups depending on the inflammatory reaction observed. The different groups were: no or minor changes, moderate changes and major changes.

On day 3 there were 2 groups, moderate and major changes. The group with major changes showed a massive inflammatory response with abundant inflammatory cells.

On day 8 the degree of inflammation had decreased in 3 animals with amoxicillin treatment. The specimens with mixed infections were all but one assigned to the category with major changes with numerous inflammatory cells still present. Ciliated cells and goblet cells were increased in number and also present in the inner epithelium of the pars flaccida.

After 56 days the middle ears of treated animals challenged with *S. pneumoniae* had no/minor or moderate changes whereas the specimens from the groups with mixed infections exhibited moderate or major changes. The major changes were characterised by extensive metaplastic changes in the epithelium in fossa nasalis with several newly formed layers of epithelial cells and connective tissue and increased numbers of ciliated and secretory cells. Islands of epithelial cells were observed in subepithelial tissue, and polyps extended into the middle ear cavity (fig 3). The pars flaccida was thickened (fig 4) and ciliated cells were present in the inner epithelium. Three out of 4 specimens from the amoxicillin-treated group B and 3/5 from the non-treated group C were assigned to this category.

Fig 3. Polyp formation observed in the middle ear mucosa in fossa nasalis after mixed infection on day 56.
Fig 4. Pars flaccida with substantial changes after mixed infection on day 56.

Summary: The beta-lactamase producing *H. influenzae* did not demonstrate ability to protect *S. pneumoniae* during amoxicillin treatment, but the mixed infection appeared to increase the mucosal changes.

4. AOM caused by *M. catarrhalis* (paper IV)

Clinical observations and otomicroscopy

Apart from the middle ear infections the animals appeared clinically healthy throughout the study. All strains of *M. catarrhalis* used induced AOM at a concentration of $10^8$ cfu/ml. The course of a *M. catarrhalis*-induced middle ear infection was generally as follows: on day 2 a clear and slightly yellow effusion could be observed otomicroscopically. Small amounts of pus-like effusion appeared in 50%. Two days later the animals presented an opaque effusion filling approximately 2/3 of the middle ear cavity. On day 8 a normalised status was observed or there were small amounts of clear or turbid fluid behind the pars flaccida. After 5 weeks no changes were noted except in animals inoculated with strain BC 1. In these animals a transparent effusion was present on day 16, and after 3 months. After 6 months all TMs appeared normal and no effusion was noted. Heat-killed bacteria generated no opaque effusion but a transparent effusion was observed in a majority of animals that lasted no longer than 16 days.

Bacterial cultures

Middle ear cultures, from non-immunised animals challenged with viable bacteria, on days 4 and 8 after inoculation were positive in 58% (15/26) and 12% (1/8), respectively.

Serum antibodies

Intrabullar challenge with viable strain 101018 induced the highest levels of IgG response. In contrast the antibody response elicited by heat-killed bacteria was weaker. The protection against AOM did not correlate with the induced antibody response.


Protection

Table 9. Frequency of AOM on day 4 in challenged animals as related to immunisation and bacterial strain.

<table>
<thead>
<tr>
<th>Strain used for immunisation</th>
<th>Immunisation route</th>
<th>Bacterial strain used at challenge</th>
<th>Ear challenged after immunisation</th>
<th>Frequency of AOM</th>
</tr>
</thead>
<tbody>
<tr>
<td>12075</td>
<td>Intrabullarly</td>
<td>12075</td>
<td>Ipsilateral</td>
<td>3/6 (P = 0.30)</td>
</tr>
<tr>
<td>12075</td>
<td>Intrabullarly</td>
<td>12075</td>
<td>Contralateral</td>
<td>4/6 (P = 0.45)</td>
</tr>
<tr>
<td>12075</td>
<td>Intrabullarly</td>
<td>101018</td>
<td>Ipsilateral</td>
<td>2/6 (P = 0.03)</td>
</tr>
<tr>
<td>12075</td>
<td>Subcutaneously</td>
<td>101018</td>
<td>Right ear</td>
<td>2/6 (P = 0.03)</td>
</tr>
<tr>
<td>Heat-killed</td>
<td>Intrabullarly</td>
<td>101018</td>
<td>Ipsilateral</td>
<td>5/5</td>
</tr>
<tr>
<td>101018</td>
<td>Intrabullarly</td>
<td>101224</td>
<td>Ipsilateral</td>
<td>1/5 (P = 0.02)</td>
</tr>
</tbody>
</table>

The protective rate after immunisation was about 50%. The exception was mainly after heat-killed bacteria that did not confer any protection to rechallenge. There was no immunised group in which all animals were protected. All observed differences between the routes were statistically invalid, but a cross-protection was observed which was statistically significant (P = 0.02-0.03) and independent of ribotype.

Structural changes

Mucosal changes were observed in all tissue sites in ears challenged with viable or heat-killed bacteria.

Viable bacteria: In the acute phase inflammatory cells, mainly PMNs, were abundant and the mucosa was thickened with increased numbers of ciliated and secretory cells. Dilated vessels were observed. These changes gradually diminished and at 6 months they were almost normalised.

Heat-killed: The changes were fewer after challenge with fewer PMNs and less thickened mucosa. After the acute phase and until the end of the observation period the changes observed did not differ from those caused by viable bacteria.

Summary: Inoculation of viable *M. catarrhalis* into the rat middle ear caused an AOM that was characterised by a mild acute reaction, and the infection was clinically and structurally different from that elicited by *S. pneumoniae* and *H. influenzae* in the rat. The AOM caused by *M. catarrhalis* was followed by a protection for reinfection which was not strain-specific.
DISCUSSION

In this study several questions related to the Scandinavian treatment policy were addressed.

**Question 1: Is an untreated middle ear infection better than a treated one in promoting protective host responses?**

It is often assumed that antibiotic treatment affects the host responses negatively. In paper I the production of IgG antibodies was slightly affected by the amoxicillin treatment during the initial AOM induced by NTHi. However, after rechallenge there was a significantly higher serum antibody production in the earlier amoxicillin-treated animals compared with the untreated animals (fig 2.). The background to this significantly increased IgG-production after rechallenge is not clear. Immune-responses are modulated by two different types of immunity. Type 1 immunity is characterized by intense phagocytic activity and type 2 immunity is characterized by high antibody titres. Severe systemic stress or overwhelming microbial load can cause an imbalance in the immune responses. Administration of antibiotics can restore the systemic balance, which allows successful host responses to clear the infection (Spellberg & Edwards 2001). Thus, the amoxicillin treatment may have initiated an earlier shift to type 2 immunity by reducing the bioburden.

High serum IgG levels do not necessarily entail a good protection. Only by challenging immunised animals, the protective function of mounted serum antibodies can be tested. The antibody-production after AOM and subcutaneous vaccination by *M. catarrhalis* was cross-protective, i.e. the protection observed was not only against the homologous strain, but also against heterologous strains of various ribotypes. In contrast, the protection after an NTHi infection has been shown to be strain-specific (Karasic et al 1985). NTHi is associated with recurrent AOM and older children, whereas *M. catarrhalis* mainly causes AOM in younger children (Kilpi et al 2001). It is possible that the host only needs to be exposed to a few *M. catarrhalis* strains to develop adequate immune responses to all strains.

The antibiotic treatment reduced the inflammatory reaction and prevented structural changes in the middle ear mucosa. A resolution without major structural changes appeared to be advantageous at renewed contact with bacteria, since the protection in treated animals was excellent, 100%. However, after rechallenge structural changes were seen in all animals independent of earlier treatment. Therefore there seemed to be no long-term beneficial effect of antibiotic treatment on the development of structural changes during subsequent AOM episodes.

An important participant in inflammatory processes is TGF-β, an anti-inflammatory cytokine that facilitates the anabolic effects of growth factors on tissue repair. In experimental AOM induced by NTHi, it has been demonstrated that the occurrence of polyps and adhesions in the middle ear coincide with higher levels of TGF-β transcripts. Furthermore, during antibiotic treatment the expression of the TGF-β gene is decreased (Melhus 2001). In paper III, the TGF-β expression levels were also reduced after antibiotic treatment. The reduction was not significant. A possible explanation to this could be the resistant NTHi cells, which maintained the inflammatory process and the demand for a continued expression of TGF-β.
Question 2: Are bacteria with acquired resistance less virulent?

Acquisition of resistance usually costs some loss in virulence for bacteria. However, in the presence of antibiotics carriage of genes encoding resistance can give a bacterial strain an advantage. Resistance in bacteria is not normally considered to be a virulence factor, but during selective antibiotic pressure these genes are important for the survival of a bacterium, especially when entering into competition with other bacteria.

In papers II and III, the NTHi strains with acquired resistance to amoxicillin exhibited a reduced growth rate and a relatively low ability to persist at the infectious site when not under a selective pressure. During amoxicillin treatment, the NTHi cells with non-beta-lactamase-mediated resistance to amoxicillin influenced the course of the AOM by delaying the resolution of the purulent effusion. Furthermore, the late histological changes in the middle ear corresponded to those found in untreated animals challenged with the susceptible strain. The acquisition of chromosomal resistance mediating increased MICs of several beta-lactams probably cost the micro-organisms more than the acquisition of beta-lactamase genes. The chromosomal resistance involves a structural alteration of the PBPs in the cell wall. This alteration may mediate possible defects in the metabolism or the replication of the bacterium.

Antibiotic use promotes bacterial resistance. If antibiotics are not present the resistant bacteria may be exchanged in the competition with other more susceptible bacteria (Seppälä et al 1997). Widely diverse patterns of antibiotic resistance can be found in adjacent countries in Europe, which suggests that national patterns in the practice of medicine and prescribing may be important factors in determining the frequency of resistance. Micro-organisms do not respect national boundaries. In Sweden, the resistance pattern among AOM pathogens and their isolation frequency are probably the result of the antibiotics prescribed. PcV is efficient in eradicating S. pneumoniae but it is less efficient in eradicating gram-negative bacteria, such as NTHi and M. catarrhalis. The native NTHi population is probably more resistant to PcV than M. catarrhalis but the beta-lactamase produced by M. catarrhalis is notably very efficient in neutralising penicillins. The high frequency of beta-lactamase production in M. catarrhalis is probably promoted by the ecological selection exerted by PcV. In Sweden, and also in Finland, the prevalence of M. catarrhalis in AOM is higher than in other countries where PcV is not prescribed (Kilpi et al 2001, Gehanno et al 2001).

Question 3: Can beta-lactamases always neutralize penicillins?

The widespread use of beta-lactams has led to prevalent beta-lactamase production among the gram-negative pathogens. Experimental evidence exists for a sheltering effect of beta-lactamase producing bacteria in several types of mixed infections (Brook & Gilmore 1993, Hol et al 1994).

In paper III, the beta-lactamase-producing H. influenzae did not demonstrate an ability to protect S. pneumoniae during amoxicillin treatment. Thus, the presence of beta-lactamase-producing bacteria in a polymicrobial middle ear infection does not automatically render penicillins ineffective against otherwise susceptible bacteria. Several studies have reported that there are differences in the ability of the beta-lactamases to protect concomitant bacteria (Renneberg & Walder 1989, Hol et al 1994). M. catarrhalis has been shown to produce a beta-lactamase...
with a better degrading effect than that NTHi may harbour. It is therefore possible that the results obtained in paper III would have been different with a *M. catarrhalis* strain instead of an NTHi strain.

With this as a background, should the spectrum of the prescribed antimicrobial drugs for AOM always cover beta-lactamase-producing bacteria? A consequence would be an increased use of broad-spectrum antibiotics and thereby a greater ecological impact on the gastrointestinal and nasopharyngeal flora (Brook & Gober 1998). The competition between the upper respiratory tract pathogens and commensals in the nasopharynx may be of greater importance for the development of AOM and recurrent disease than earlier assumed (Roos et al 2001, Joki-Erkkilä et al 2002). It is even possible that not all beta-lactamase-producing bacteria should be eradicated, as the presence of beta-lactamase-producing *M. catarrhalis* appears to prevent the development of penicillin resistance among pneumococcal isolates in the nasopharynx (Joki-Erkkilä et al 2002).

**Question 4: Are all AOM pathogens equally important?**

To answer this question it was necessary to establish an AOM model for *M. catarrhalis*. In paper IV inoculation of viable *M. catarrhalis* into the rat middle ear caused an AOM that was characterised by a mild acute reaction. The infection was clinically and structurally different from that elicited by *S. pneumoniae* and *H. influenzae* in the rat (Hermansson et al 1988, Melhus et al 1994, Magnusson et al 1997).

Various properties of *M. catarrhalis* have made it difficult to develop an animal model (Chung et al 1994, Doyle 1998, Fulghum & Marrow 1996, Melhus 2003). When developing the rat model it was obvious that this bacterium must be handled delicately. It had to be used shortly after its isolation from the infectious site, and it required high inoculation doses (Paper IV). Furthermore, the strain used should probably be isolated from the middle ear of an AOM patient and not from the nasopharynx of a healthy individual, since it is difficult to induce AOM in the rat in the latter case (unpublished data).

Paper IV demonstrated that the AOM caused by *M. catarrhalis* is a self-limiting disease with few changes in the middle ear after resolution compared with the other major AOM pathogens. This suggests a low virulence and it is therefore not surprising that *M. catarrhalis* was not considered a pathogen until the mid 1980s (Kovatch et al 1983, Shurin & Van Hare 1986).

*S. pneumoniae* has the lowest spontaneous clearance rate and is the microorganism most often associated with severe and fatal complications in AOM. Thus the antimicrobial drug routinely used for AOM treatment must have a spectrum which covers this bacterium in order to be efficacious. The spontaneous clearance rates for the two other major AOM pathogens are higher and for *M. catarrhalis* it is approximately 80%. Differences between the courses of AOM in the rat caused by *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* include inoculation dose, duration of MEE, frequency of positive middle ear cultures, structural changes in the middle ears, frequency of severe systemic infections, frequency of myringosclerosis, and protection rate. These findings are similar to those found in humans.

The most pronounced structural changes observed in the rat model occurred after the mixed infection in paper III. It occurred both in untreated and antibiotic treated animals with polyp formation, metaplasia in the epithelium with several layers of cells, and presence of inflammatory cells, dilated vessels and
epithelial proliferation. Otherwise the most prominent inflammation on day 3, in the acute phase, was observed in middle ears challenged with *S. pneumoniae* (paper III), with massive inflammation and deranged middle ear epithelium with dilated vessels. After the acute inflammatory reaction had abated, the long-term inflammatory changes were most pronounced after the NTHi-induced AOM. Vascular changes and metaplasia occurred after 2 months in pars flaccida and in fossa nasalis in untreated animals challenged with the susceptible strain and in treated animals inoculated with the resistant strain (paper II). The morphological changes after a *M. catarrhalis* infection were the mildest, but the bacterium still induced a long-term inflammation.

Changes in the goblet cells after a *M. catarrhalis* AOM differed from those observed after a pneumococcal AOM, with an increased total volume of goblet cells and various alterations in the appearance of the goblet cell granules (paper IV). NTHi have been demonstrated to induce even more pronounced changes in the goblet cells (Magnusson et al. 1997). Cayé-Thomassen and co-workers have also shown that capsulated and non-typeable *H. influenzae* induce the most severe and protracted histopathologic changes, as evaluated by the increase of goblet-cell density and the formation of polyps and fibrous adhesions. According to their studies, *S. pneumoniae* induces almost the same changes, but has a tendency to promote new bone formation. *M. catarrhalis*, finally, induces the slightest changes (Cayé-Thomassen & Tos 2001).

The effect on the middle ear mucosa can be an aspect of how the bacterial cell wall is constructed, and what type of toxin the bacterium therefore mainly rely on to injure the host. *S. pneumoniae*, which is a gram-positive bacterium, causes a direct and intense acute reaction probably through the production of exotoxins. NTHi and *M. catarrhalis* are gram-negative bacteria, whose endotoxin in the cell membrane induces inflammatory host responses. Secondary to these host responses, the tissues are destroyed. Due to their endotoxin, gram-negative bacteria are capable of inducing inflammation even if they no longer multiply or are viable in the middle ear (DeMaria 1999). Thus, the inflammatory process is prolonged by the residual endotoxins. When gram-positive bacteria are mixed with gram-negative bacteria, exotoxins are combined with endotoxins, and the most pronounced structural changes are therefore observed.

The use of the penicillins pcV and amoxicillin as first-line drugs in AOM seems to be a wise choice due to their high ability to eradicate *S. pneumoniae*, the most virulent bacterium. In contrast to pcV, amoxicillin is effective in eradicating the majority of NTHi strains and even PNSP in higher doses (Lister et al. 1997, Canafax et al. 1998). Beta-lactamase producing bacteria, and *M. catarrhalis* in particular, are not eradicated with this treatment, but these microbes appear to be less virulent and are not a target for the initial therapy. If penicillins are used, a majority of the AOM pathogens are eradicated without the negative ecological risks of more broad-spectrum antibiotics.
CONCLUSIONS

Amoxicillin treatment:

• eradicated *S. pneumoniae* and NTHi from the middle ear.
• eradicated *S. pneumoniae* in the presence of beta-lactamase.
• shortened the duration of AOM caused by NTHi or *S. pneumoniae*.
• reduced the morphological changes normally observed after an untreated AOM but did not prevent further tissue damage in the long run.
• did not reduce the protection against reinfection with *S. pneumoniae* and NTHi and induced significantly higher IgG levels after rechallenge.
• seemed to be advantageous for NTHi with beta-lactamase production and chromosomal alterations mediating a relatively low level of resistance to beta-lactams.

Moraxella catarrhalis:

• induced an AOM in the rat model that was a self-limiting disease.
• induced a protection that was not strain-specific.

In conclusion: There is still a lot that supports the Scandinavian treatment policy. Penicillins are not sufficient to treat all causative agents, but the majority of pathogens including the most virulent bacteria are eradicated from the middle ear.


Syfte: De frågor vi försöker besvara är: Är en obehandlad öroninflammation bättre än en antibiotikabehandlad öroninflammation för utvecklingen av infektionsförsvaret? Har bakterier med förvärvad antibiotikaresistens mindre sjukdomsförmedlande förmåga? Kan beta-laktamas alltid neutralisera penicilliner? Är alla bakterier som orsakar öroninflammationer lika viktiga att behandla?

Metod: En djurmodell användes för att undersöka olika aspekter av behandling, skydd och histologiska förändringar vid bakteriell öroninflammation.


I artikel II, där öroninflammationen orsakades av *H. influenzae* med en kromosomal resistens mot beta-laktam antibiotika, observerades att den genetiska förändringen som leder till resistens hos bakterien kan ske utan alltför stor minskning i den sjukdomsframkallande förmågan hos bakterien. Resistensutvecklingen mot antibiotika kan också vara till fördel för bakterien då den under antibiotikabehandlingen överlevde längre.

I artikel III undersökte vi om en bakterie som producerar beta-laktamas möjligen skulle kunna skydda en penicillinkänslig bakterie mot antibiotikabehandling när de förekommer samtidigt i en infektion. Vi visade att amoxicillin kunde avdöda de *S. pneumoniae* som fanns i örat, trots närvaro av beta-laktamasproducerande *H. influenzae*. 

SUMMARY IN SWEDISH


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Konklusion: Penicilliner kan inte behandla alla bakterier som orsakar öroninflammation, men de kan sannolikt ta bort de bakterier som ger allvarliga infektioner med risk för komplikationer. Trots att den skandinaviska behandlingsmodellen funnits i många år och att känsligheten för penicilliner har minskat hos en del av de bakterier som orsakar öroninflammationer, finns det mycket som talar för att den fortfarande fungerar bra.
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