Effects of amoxicillin on the expression of cytokines during experimental acute otitis media caused by non-typeable Haemophilus influenzae

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Antibiotics are frequently prescribed when a diagnosis of acute otitis media (AOM) is made in childhood, but the effects of antibiotics on host–parasite interactions in the middle ear are not well defined. A rat model and PCR techniques were used to explore host responses during amoxicillin treatment of AOM caused by non-typeable Haemophilus influenzae (NTHi). The 5 day course of amoxicillin initiated at the otomicroscopic peak of the infection eradicated the bacteria and induced significant changes in the expression of cytokines. Interleukin (IL)-6, tumour necrosis factor-α and IL-10 were upregulated by the treatment, and the downregulation was slower than during the natural course. Amoxicillin inhibited the upregulation of transforming growth factor-β, whereas IL-1α expression remained unaffected by the treatment. By comparing inflammatory host responses during treated and untreated NTHi AOM, new targets for modification of the course, or more specified and individualized treatments, may evolve.

Introduction

Acute otitis media (AOM) is one of the most common infections diagnosed in early childhood. By 4 years of age, about 80% of all children will have experienced at least one episode of AOM.1 The three species of bacteria isolated most frequently in middle ear infections are Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis,2,3 three organisms with different potentials for causing severe disease.

Antimicrobial treatment of AOM is standard practice in most countries, independent of causative agent, and middle ear infections are currently the most common reason for outpatient antimicrobial therapy in the USA.4 The basis for this practice is the attribution of the rapid decline in incidence of mastoiditis and other intracranial complications of AOM seen in the late 1940s to the use of antibiotics.5

About 80–85% of all AOM episodes are cured with antimicrobial therapy.6 This should be compared with spontaneous clearance rates of 20% for pneumococci, 50% for H. influenzae, and 80% for M. catarrhalis within 2–7 days.6 With increasing problems of antibiotic resistance worldwide, questions have arisen about the consequences and the efficacy of the routine use of antimicrobial agents for a relatively benign infection.7,8 Furthermore, six out of seven children with AOM do not need or will not respond to antibiotic treatment,9 and effusion might persist in the middle ear for weeks after an AOM, irrespective of antimicrobial therapy.6,10

Despite the extensive use of antimicrobial agents, the data on the effects of these agents on host–parasite interactions in the middle ear cavity are limited. To optimize the treatment of AOM, basic knowledge of the events that take place in the middle ear during treated and untreated NTHi AOM is necessary. It was demonstrated recently in a rat otitis model that the majority of cytokine mRNAs had passed their peaks before the diagnosis of AOM could be made clinically,11 indicating that the antibiotic treatment may be introduced too late to have a major impact on the inflammation or the presence of effusion. However, amoxicillin treatment started at the clinical peak of the infection in the same model has been shown to reduce the inflammation and the histological changes induced by bacteria.12

To investigate the effects of amoxicillin in the middle ear cavity, the expression of mRNA for cytokines involved in inflammatory responses was measured during the natural course and during amoxicillin treatment of non-typeable H. influenzae (NTHi) AOM in the rat. The cytokine transcript profiles were thereafter related to the otomicroscopic, bacteriological and pathological findings.

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Materials and methods

Bacteria and media

The bacterial strain used in the study was NTHi (biotype II) strain 3655 (kindly provided by Robert S. Munson, Jr, The Ohio State University, Columbus, OH, USA). It was fully susceptible to amoxicillin according to Etest (MIC of amoxicillin 0.5 mg/L; AB Biodisk, Solna, Sweden) and a diagnostic disc with nitrocefin (AB Biodisk). The bacteria were grown on chocolate agar or in brain–heart infusion broth (BHI; Difco Laboratories, Detroit, MI, USA) supplemented with NAD and haemin (Sigma, St Louis, MO, USA), each at 10 mg/L.

The inoculum for middle ear challenge was prepared as described previously.13 The bacterial concentration was determined to be 10^7 cfu/mL after dose finding experiments, and only freshly prepared early S-phase bacteria were used. Viable count of the bacterial suspension was performed at the time of the challenge. Bacterial samples from the middle ears were obtained by inserting a swab into the middle ear cavity after opening up the bulla, the rat structure corresponding to the human middle ear. The swab was streaked directly onto chocolate agar. Growth was classified semi-quantitatively as sparse (1–10 cfu), moderate (11–100 cfu) or abundant (>100 cfu) after 24–48 h. All cultures were grown at 37°C in an atmosphere with 5% CO₂.

Animals and surgical procedures

Healthy male Sprague–Dawley rats weighing 350–450 g were used. The study was conducted according to standard practices concerning animal procedures, and the study protocol was approved by the ethics committee of Lund University. Whenever operated on or inspected under an otomicroscope, the animals were anaesthetized with chloralhydrate (Apoteksbolaget, Malmö, Sweden) administered ip. At challenge the bulla was reached bilaterally through a ventral midline incision, and c. 0.05 mL of the bacterial suspension was injected through the bony wall directly into each middle ear cavity of each animal.

Experimental design

A total of 30 animals were challenged with bacteria. Antibiotic therapy with amoxicillin (AstraZeneca, Sodertalje, Sweden) was introduced on day 3 via the drinking water as described previously.12 Fifteen animals were treated and the remaining 15 animals served as controls. Water consumption was measured on a daily basis until day 8, when the remaining 15 animals served as controls. Water consumption was measured on a daily basis until day 8, when the remaining 15 animals served as controls.

RT–PCR

To detect mRNAs for interleukin (IL)-1α, IL-6, IL-10, tumour necrosis factor (TNF)-α and transforming growth factor (TGF)-β, a previously described RT–PCR protocol was used.11 Briefly, mRNAs extracted with Dynabeads Oligo(dT)₂₅ (Dynal A.S., Oslo, Norway) were reverse transcribed and then amplified. The primer sequences were as follows:

- IL-1α, 5'-GAGAAGACACGCTGTAGTGCG-3' (sense) and 5'-CATGCGGAGTACTGAGACG-3' (antisense);
- IL-6, 5'-TCTCTCAGAAGAGACTTCC-3' (sense) and 5'-TCTTGTCTAGGACACTCC-3' (antisense);
- IL-10, 5'-GCTAGCAGCTGCTGTTGC-3' (sense) and 5'-TTATGGCCTTGTAGACACC-3' (antisense);
- TNF-α, 5'-AGTCTTCCAGCTGGAGAAGG-3' (sense) and 5'-GCCACTAATTCAGCATCTCG-3' (antisense);
- TGF-β, 5'-AGCTCCACAGGAAAGACTGC-3' (sense) and 5'-TCATGTTTGGACAACTGCTCC-3' (antisense).

The PCR products were subjected to electrophoresis. All PCR-positive samples were further analysed in a competitive PCR assay.

Quantification of PCR products

The competitive PCR was performed as described previously.11 Two different competitors were used, one for the house-keeping β-actin gene, primer sequences 5'-TGAGAAGAGACTTGGCTGC-3' (sense) and 5'-TCTCACGAGATCTGGCGC-3' (antisense), and one for the five cytokine genes. The β-actin was used for detecting the initial amplifiable mRNA levels. The expressed levels of the investigated genes were corrected to the β-actin transcript levels in each sample. The co-amplified PCR products were separated on a 2% agarose gel. Appropriate bands were scanned and photographed by the computerized Molecular Analyst system (version 1.5; Bio-Rad, Hercules, CA, USA), which also analysed the band densities. Each gel was scanned and analysed twice. Approximately 20% of the samples were run twice or in duplicate to assess the reproductibility.
Amoxicillin affects cytokine mRNA profiles

Statistical analysis

Student’s t-test was used for comparison of the mRNA levels between the two treatment groups. Analysis was performed on each separate observation and over the entire observation period. A difference was considered statistically significant when $P \leq 0.05$.

Results

Clinical findings and otomicroscopy

The animals appeared clinically healthy throughout the study apart from the induced middle ear infection, which could be diagnosed in all animals on day 3. The amoxicillin was well tolerated, and the only observed side effect was slight diarrhoea. The daily dose of amoxicillin was $51 \pm 9$ mg/kg body weight per day, deduced from the water consumption, yielding peak concentrations in serum of $3.9 \pm 3.1$ mg/L. There was no difference in water consumption between treated and untreated animals.

The otomicroscopy findings between treated and untreated animals differed. The natural AOM was more severe and lasted approximately 2 days longer than the treated AOM. The typical course of an AOM with or without antibiotic therapy is summarized in the Table, together with the culture results.

Profiles of cytokine transcripts

The reproducibility of the competitive PCR was high. Samples run twice or in duplicate yielded exactly the same concentrations or the same concentrations within one dilution step.

The mRNA levels of the cytokines during untreated and treated NTHi AOM are shown in the Figure. There were several differences in host reaction between the two animal groups. Of the five cytokine genes investigated, only IL-1α, the dominant gene product in terms of transcript concentration, remained unaffected by the amoxicillin therapy. For IL-6, TNF-α and IL-10, the maximum mRNA concentrations occurred at the same time point in both animal groups, but the peak levels were two to three times higher in the treated animals. Furthermore, the transcripts of these three cytokines were detected for a longer time period and at higher concentrations in the treatment group than in the non-treatment group. On day 6, the mRNA concentrations of IL-6, TNF-α and IL-10 had dropped to zero in all but one of the untreated animals. The levels detected in this animal were two to four times lower than those found in the treated animals.

In contrast to the enhanced upregulation of IL-6, TNF-α and IL-10, the naturally occurring upregulation of TGF-β was inhibited by the amoxicillin therapy. On day 6, i.e. the day of the natural peak, the TGF-β transcript levels in treated animals were about one-twelfth of those found in untreated animals. When the antibiotic treatment had ceased, there was a slight increase in the TGF-β mRNA levels, but the levels never reached the peak levels found during the natural course. Although TGF-β transcripts remained at a relatively low level, a downregulation was never recorded during the observation period. TGF-β was the only cytokine for which a sustained gene expression was observed.

Discussion

The present study delineated the temporal expression of five different cytokines in relation to clinical findings during treated and untreated NTHi AOM in a rat otitis model. The study demonstrated that amoxicillin treatment
initiated at the clinical peak of the infection changed the severity of the infection and its natural course. The amoxicillin exhibited an efficient bactericidal activity, but it also appeared to induce modifications of the inflammatory responses in the middle ear. These modifications included a significant upregulation of TNF-α and IL-10, and an absent upregulation of TGF-β.

Bacterial killing is the main attribute of antibiotics. In recent years, however, reports have appeared describing abilities of antimicrobial drugs to modify host cell responses. Cytokines are probable mediators for the majority of these effects. So far, few studies have been performed in vivo, but the rat otitis model is well-suited for these investigations, especially since the infection occurs in a confined space in which different parameters and the clinical course can be monitored with relative ease.

Penicillins are frequently used to treat AOM. Like all β-lactams, they induce a liberation of endotoxin from Gram-negative bacteria by interfering with the bacterial cell wall synthesis. A strong correlation between endotoxin levels and TNF-α production has been demonstrated during ampicillin killing of H. influenzae type b. In the present study, there was an increase of TNF-α transcripts 24 h after the introduction of amoxicillin, probably secondary to an endotoxin release. This increase coincided with increased expression of IL-10, the anti-inflammatory activity of which may have subdued or counteracted the inflammatory effects of TNF-α.

![Figure](image_url)
Amoxicillin affects cytokine mRNA profiles

TNF-α and IL-10 concentrations have been demonstrated previously, both in vitro and in vivo.

Although antibiotics have different potentials for inducing endotoxin release, there is at present no conclusive evidence that the induced release is associated with an adverse clinical outcome. In the middle ear, the background for this could be the demonstrated and immediate anti-inflammatory response mediated by IL-10. In children less than 18 months of age, clinical signs of AOM often persist even though the bacteria are eradicated. It is therefore possible that the IL-10 response or some detoxifying or other anti-inflammatory function is not fully developed in this age group. Thus, endotoxin and other bacterial products from non-viable bacteria may sustain the inflammatory process. However, this speculation needs to be verified.

The most striking effect of the amoxicillin treatment was the inhibition of the naturally occurring upregulation of TGF-β during the resolution phase of the middle ear infection. This effect has not previously been described during treatment with a β-lactam. TGF-β is an important wound-healing agent, but it also has a crucial role in the pathogenesis of tissue fibrosis. By keeping the TGF-β transcription at a low level, the amoxicillin probably minimized the otherwise pronounced histological changes that develop during an untreated rat AOM. Since the peak concentrations of TGF-β transcripts were recorded comparatively late during the natural AOM, i.e. on day 6, an introduction of amoxicillin on day 3 could still have an impact on the development of the structural changes in the rat. A factor often disregarded when discussing clinical treatment failures and non-responsive patients is the time factor. For an antibiotic treatment to be effective and disrupt events in progress associated with morphological alterations, fibrosis and sequelae, treatment should probably be initiated as early as possible. Noteworthy in this context is that there was no difference in the frequency of myringosclerosis in the two animal groups, indicating that the initial tissue injury and the healing process took place early during the AOM course and long before the amoxicillin was introduced, and that this form of tissue healing does not involve TGF-β to any greater degree. This suggestion is further supported by the fact that myringosclerosis may appear within 24 h in pneumococcal AOM, but the peak expression of TGF-β is not reached until day 28 after a challenge with pneumococci.

Of the IL-1, IL-6 and TNF-α patterns demonstrated in vitro for different antibiotics after endotoxin stimulation, none have exhibited the same cytokine pattern as amoxicillin did during the NTHI AOM in the rat. β-Lactams, and penicillins in particular, are usually considered to exhibit only modest immunomodulating actions. However, a more complex cellular system than that which an in vitro system can offer might be necessary to demonstrate the activity of most of these compounds in vivo. Owing to the time consuming techniques used for inducing the middle ear infection and collecting the mucosa, a more extensive study including additional controls, i.e. animals challenged with other NTHI strains with β-lactam resistance or animals treated with drugs with well-documented immunomodulatory effects, was not possible. New studies are therefore in progress, in which the effects of other antibiotics as well as anti-inflammatory substances are explored in the same model.

In conclusion, the amoxicillin treatment significantly modified cytokine transcript profiles in the rat middle ear after challenge with NTHI. It is possible that some of these effects, the increased expression of IL-10 in particular, prevented antibiotic-associated toxicity or counteracted effects secondary to bacterial killing, whereas others, i.e. the inhibited upregulation of TGF-β, reduced secondary structural changes. By studying host responses in the middle ear during different types of treatment, new targets for modification of the course, or more specified and individualized treatments, may evolve.

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References


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