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# Expression of Cytokine Genes during Pneumococcal and Nontypeable *Haemophilus influenzae* Acute Otitis Media in the Rat

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**Acute otitis media (AOM) elicits potent inflammatory responses from the cells of the middle ear mucosa as well as from infiltrating leukocytes. To explore host responses during experimental AOM induced by *Streptococcus pneumoniae* type 3 and nontypeable *Haemophilus influenzae* (NTHi), otomicroscopy findings and expression of cytokine genes in the middle ear were monitored up to 1 month postinoculation. The mucosa and infiltrating cells responded rapidly to the bacterial challenge. Otomicroscopically, AOM appeared 1 day after NTHi inoculation and 3 days after pneumococcus inoculation. Pneumococcal AOM was more severe than NTHi otitis, but in general, lower transcript levels were detected in pneumococcus-infected than in NTHi-infected animals. Interleukin-6 (IL-6) mRNA levels peaked at 3 to 6 h for both pneumococcus-infected and NTHi-infected animals. IL-1α, tumor necrosis factor alpha, and IL-10 mRNA levels peaked at 6 h for NTHi otitis and 1 to 3 days for pneumococcal otitis. Comparing otomicroscopy with expression profiles, it would appear that the majority of cytokine mRNAs had passed their peak before the AOM diagnosis could be made clinically. Only transforming growth factor β mRNA followed a slower time course, peaking very late and continuing expression even after the AOM was otomicroscopically resolved. IL-2 and IL-4 mRNAs were not detected in any animal at any time. Most of the investigated cytokines are very early markers for AOM and may be involved in initiation of inflammation, but they would be poor targets for pharmacological manipulation since their levels decline before clinical signs appear.**

Acute otitis media (AOM) is one of the most common illnesses diagnosed during early childhood. By 3 years of age, about 50 to 70% of all children will have experienced at least one episode of AOM (19, 36). Although the infection is often regarded as relatively benign, its complications and sequelae can be severe. AOM has been associated with mastoiditis, brain abscesses, and meningitis, and it can lead to rapid or insidious hearing loss or impairment in all age groups (4, 10, 29, 37).

The most frequently isolated bacteria in AOM are *Streptococcus pneumoniae* (20 to 55%), *Haemophilus influenzae* (15 to 40%), and *Moraxella catarrhalis* (10 to 25%) (17, 19). Data on how these organisms invade the middle ear cavity and how they interact with the host are limited. Like other parts of the respiratory tract, the middle ear is equipped with first- and second-line defense mechanisms, but it is unique in that it is endowed with only a few lymphocytes and no lymphoid tissue (5). When challenged, it must therefore develop immunity de novo and rely on nonspecific reactions or circulating antibodies that exude into the cavity until enough sensitized B cells are recruited to the middle ear (5, 33). Although AOM is usually treated as a single entity, both human and experimental-animal studies suggest that there are differences in host responses depending on the organism involved (14, 19, 26). There are indications that *H. influenzae* antigens evoke a greater local inflammatory response than pneumococcal antigens do (25). Pneumococcal infection, on the other hand, is clinically more severe and involves a higher risk of serious disease and intracranial complications (4, 29, 32). Pneumococcal infections also appear to induce better protection systemically against reinfections than do nontypeable *H. influenzae* (NTHi) and *M. catarrhalis* (6, 19, 26).

The treatment of middle ear infections is controversial. The spontaneous recovery rate of AOM varies between 20 and 80% depending on the organism (17, 19). It has been estimated that only approximately 30% of all AOM patients need antibiotic treatment (19), and pneumococci are the causative agents in the majority of these patients. Despite the risk of overconsumption of antibiotics, routine use of antibiotics in treatment is standard practice in most countries. With increasing problems of antibiotic resistance, questions have arisen about the efficacy and consequences of the current treatment strategy (9, 22). To better understand this infectious disease and its optimal treatment, the roles of different bacterial components and host responses initiating or sustaining the disease process must be defined.

Cytokines form a complex network of local mediators orchestrating both nonspecific responses and specific immunity to bacteria, and their presence has been reported in cases of otitis media with effusion (7, 8, 40). The present study was designed to delineate the expression of cytokine genes involved in both early and late inflammatory responses and to relate their production to the etiologic agent and the otomicroscopic and pathological findings during pneumococcal type 3 and NTHi AOM in two well-established animal models.

## MATERIALS AND METHODS

**Bacteria and media.** The two bacterial strains included in this study were a pneumococcal type 3 strain isolated from a patient at the Department of Otorhinolaryngology, Lund University Hospital, Lund, Sweden, and an NTHi (biotype II) strain kindly provided by Robert S. Munson, Jr., The Ohio State University, Columbus. The organisms were stored at −70°C, and all cultures were initially inoculated from these frozen stocks onto blood or chocolate agar.

Apart from a mouse passage of the pneumococcal strain prior to inoculation to enhance pathogenicity (23), the inocula for middle ear challenge were prepared as described previously (24). Only freshly prepared early-stationary-phase

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TABLE 1. Primer sequences used for RT-PCR and quantitative P

| Gene | Accession no. | Primer sequence | Size of PCR product (bp) | |
|---|---|---|---|---|
| | | | Target | Competitor |
| β-Actin | V01217, J00691 | 5′-TGG AGA AGA GCT ATG AGC TGC-3′<br>5′-TCC ACA CAG AGT ACT TGC GC-3′ | 315 | 510 |
| IL-1α | D00403 | 5′-GAG AAG ACA AGC CTG TGT TGC-3′<br>5′-CAT GCG AGT GAC TTA GGA CG-3′ | 311 | 514 |
| IL-2 | M22899 | 5′-AGC TGT TGC TGG ACT TAC AGG-3′<br>5′-AAT TCC ACC ACA GTT GCT GG-3′ | 307 | |
| IL-4 | X16058 | 5′-CGG TAT CCA CGG ATG TAA CG-3′ | 219 | |
| | | 5′-AAG CAC GGA GGT ACA TCA CG-3′ | 285 | |
| | | 5′-GCT ATT GAT GGG TCT CAG CC-3′ | 332 | |
| | | 5′-TTT CAG TGT TGT GAG CGT GG-3′ | 398 | |
| IL-6 | E02522 | 5′-TCT CTC CGC AAG AGA CTT CC-3′<br>5′-TCT TGG TCC TTA GCC ACT CC-3′ | 557 | 434 |
| IL-10 | L02926 | 5′-GCT CAG CAC TGC TAT GTT GC-3′<br>5′-TTC ATG GCC TTG TAG ACA CC-3′ | 469 | 355 |
| TNF-α | L00981 | 5′-AGT CTT CCA GCT GGA GAA GG-3′<br>5′-GCC ACT ACT TCA GCA TCT CG-3′ | 318 | 474 |
| TGF-β | X52498 | 5′-AGC TCC ACA GAG AAG AAC TGC-3′<br>5′-TCA TGT TGG ACA ACT GCT CC-3′ | 289 | 394 |

bacteria grown in either Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) or brain heart infusion broth (Difco) supplemented with NAD and hemin (Sigma, St. Louis, Mo.), each at 10 μg/ml, and at a predetermined concentration of $10^7$ CFU/ml were used. Viable counts of the bacterial suspensions were performed at the time of the challenge. Bacterial samples from the middle ears and blood were cultured as previously described (24). Growth was classified semiquantitatively as sparse (1 to 10 CFU), moderate (11 to 100 CFU), and abundant (>100 CFU).

**Animals and surgical procedures.** Healthy male Sprague-Dawley rats weighing 250 to 350 g were used. The animals were kept under standard laboratory conditions and given water and food ad libitum. Whenever operated on or inspected under an otomicroscope, the animals were anesthetized with a rodent cocktail (13.3 mg of ketamine HCl per ml, 1.3 mg of xylazine per ml, 0.25 mg of acepromazine maleate per ml) administered intramuscularly. Inoculation was performed through the bony wall of the bulla, which was reached through a ventral midline incision and blunt dissection (16). Approximately 0.05 ml of the bacterial suspension was injected directly into to the middle ear cavities of the animals.

**Experimental design.** A total of 84 animals were challenged, 42 bilaterally and 42 unilaterally [$n = (2 \times 21) + (2 \times 21)$]. Three individuals from each bilaterally inoculated animal group were randomly selected for reverse transcription-PCR (RT-PCR) at 3 and 6 h and on days 1, 3, 6, 14, and 28 after the middle ear challenge. Prior to being sacrificed, all animals were inspected under an otomicroscope and blood was collected after 3 and 24 h from three pneumococcus-infected animals. The status of the tympanic membrane and the quantity and quality of the effusion behind the membrane were evaluated. After opening the bulla, pathological findings, including clot formations, hemorrhages, edema, adherences, and polyp-like formations, were registered and bacterial samples were collected. The middle ear tissues were removed bilaterally from each animal and pooled (two ears per sample). The samples were immediately frozen in dry ice and stored at −70°C until analyzed. The remaining unilaterally challenged animals were monitored otomicroscopically to allow at least nine challenged ears or six individuals to be examined at each time point. As day 0 controls, six unchallenged animals were sacrificed. The middle ear tissues of three control rats were individually frozen, while the tissues from the remaining six control ears were pooled.

**RT-PCR.** mRNA was extracted from the frozen middle ear samples using Dynabeads oligo$(dT)_{25}$ (Dynal A.S, Oslo, Norway) as specified by the manufacturer. After the mRNAs were eluted from the beads, they were reverse transcribed with the Superscript preamplification system for first-strand cDNA synthesis (Gibco BRL).

To determine when different cytokines were present in the middle ear cavity during and after AOM, seven specific primer sets were designed based on sequences reported in the GenBank database (Table 1). The RT mixture (2% of the total amount) was amplified in a conventional PCR using the *Taq* PCR core kit (Qiagen, Valencia, Calif.). The PCR mixture contained 10 μl of 10× PCR buffer, 2 μl of 25 mM $MgCl_2$, 2 μl of 10 mM deoxynucleoside triphosphate mix, 2.5 U of *Taq* DNA polymerase, and 100 pmol of each 5′ and 3′ primer (Oligos Etc. Inc., Wilsonville, Oreg.) in a total reaction volume of 100 μl. The mixtures were processed for 30 cycles (35 cycles for tumor necrosis factor alpha [TNF-α] and interleukin-10 [IL-10]) in a Perkin-Elmer Cetus DNA thermal cycler, with the cycling parameters consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Following the final cycle, an extension step of 10 min at 72°C was performed. A 10-μl volume of each PCR mixture was analyzed on a 2% agarose gel (Gibco BRL) with ethidium bromide. The sizes of the PCR products were compared with a DNA molecular size marker (100-bp DNA ladder; Gibco BRL), and the specificities of the products were verified by sequencing in an ABI model 373A DNA sequencing system (Applied Biosystems Inc., Foster City, Calif.). Appropriate positive and negative controls were included in each experiment. All PCR-positive samples were further analyzed in a competitive PCR assay.

**Construction, amplification efficiency, and titration of competitors.** Two competitors were constructed for the competitive PCR, one for quantification of the constitutively expressed product β-actin and one for the cytokines detected in the initial PCR.

The backbone of the β-actin competitor consisted of the 469-bp PCR product of the IL-10 primers (Table 1). β-Actin primers (Table 1) were added to the 5′ and 3′ ends of the backbone in a sequential PCR using partially overlapping 40-bp oligonucleotides. For the skeleton of the cytokine competitor, the 315-bp PCR product of the β-actin primers was used. Primers of IL-10, transforming growth factor β (TGF-β), IL-6, IL-1α, and TNF-α (Table 1) were thereafter successively added in a manner similar to that used when constructing the β-actin competitor. IL-2 and IL-4 primers were excluded due to the lack of PCR products. Both competitors were ligated into the pGEM-T vector (Promega, Madison, Wis.) and amplified, and the nucleotide sequences were determined.

The competitors had the same sets of primers as the target cDNA samples. Compared with the native PCR products, the lengths of the competitor products differed by 105 to 203 bp (Table 1). To control the amplification efficiency for both the native cDNA samples and the two different competitors, the ratio of target to competitor products was determined over a range of cycles. The experiments were performed twice and in duplicate, with different samples of target cDNA and different concentrations of the competitors.

To find the optimized working concentrations of competitor for each separate gene, serial 10-fold dilutions were made ($10^{-8}$ to $10^{-17}$ g/μl). The concentration of competitor yielding a band of equal or slightly higher density with respect to the target band was defined, and this concentration was used for threefold serial dilutions over a more restricted range. The same dilutional series ($2.7 \times 10^{-12}$ to $5 \times 10^{-17}$ g/μl) was used for all quantitations of cytokine cDNA to make a comparison between the different cytokines possible.

**Quantification of PCR products.** Equal amounts of competitor and target i.e., 1 μl of each preparation, were used in the competitive PCR. The coamplified

PCR products were separated on a 2% agarose gel and visualized as described above. Appropriate bands were scanned and photographed by the computerized AlphaImager system (version 3.3; Alpha Innotech Corp., San Leandro, Calif.), which also analyzed the band densities. Each gel was scanned and analyzed two or three times. To assess the reproducibility, approximately 20% of the samples were run twice or in duplicate.

**Statistical analysis.** To control how the statistical method affected the significance, both Student's *t* test and the Mann-Whitney U test were used for all comparisons of the mRNA levels between the pneumococcus-infected and NTHi-infected animals. Analysis was performed on each separate observation and over the whole observation period. There were only minor differences between the methods. To avoid choosing the method with the highest significance for each analysis, the *P* values given in the figures were analyzed by Student's *t* test whereas the asterisks are based on the Mann-Whitney U test. A difference was considered statistically significant at $P < 0.05$.

## RESULTS

**Clinical findings and otomicroscopy.** Apart from the induced middle ear infection, all animals challenged with NTHi appeared clinically healthy throughout the study. The pneumococcus-infected animals exhibited signs of a systemic reaction (ruffled fur and lethargy) over a postoperative period of about 24 h, and for another 5 days their susceptibility to anesthesia was increased. These reactions were independent of the number of ears inoculated per animal. Although a bilateral inoculation entailed a 100% increase in bacterial load per individual, the course and time needed for eradication of pneumococci or NTHi appeared the same in both uni- and bilaterally challenged animals. The otomicroscopy findings in the right and left ears were not always identical, but animals with a slow recovery rate were without exception slow to recover in both ears. No animal developed a bilateral infection unless it was bilaterally inoculated. All blood cultures were negative by the method used.

The otomicroscopy findings and culture results differed between the two bacterial groups. In general, the pneumococcal infection had a slower onset but was more severe and had a longer duration. The typical course of pneumococcal infection was as follows. After 3 h, a dilation of the manubrial vessels and a clear effusion filling most of the cavity could be observed. Compared with the injected bacterial suspension, the effusion had an increased viscosity. The mucosa was generally edematous. After 6 h, the effusion had turned turbid and the viscosity had increased further. A hemorrhagic fibrin clot had been formed, and there were signs of hemolysis. On day 1, the clot was massive and purulent, although the otomicroscopy findings still only suggested a turbid effusion behind the tympanic membrane. Spotted bleeding could be found in both the pars tensa and pars flaccida, and the mucosa had thickened notably. Two days later, vessel dilatation reached its maximum and the effusion was opaque. The mucosal lining seemed to be disintegrating. All the bleeding had disappeared, and the first cases of perforation and myringosclerosis were registered. From 3 to 72 h, all cultures showed abundant growth. On day 6, the infection had started to resolve, and for the last time all middle ear cultures were positive (sparse growth in two of three cultures and moderate in the third). Instead of the former pale, almost white tone, the mucosa had turned red and was less edematous. On day 14, the otomicroscopic status was normalized in all but one animal, which was still culture positive (sparse growth) and had small amounts of turbid effusion behind the pars flaccida. After a month, all infections were resolved. A thickening of the bullular bone was evident, and the mucosa remained thick and rich with vessels. A polyp-like formation was observed at this point. Adherences were never recorded.

A typical NTHi-induced infection had the following course. After 3 h, the middle ear cavity was filled with clear effusion without any vessel reaction. A slightly hemorrhagic fibrin clot was formed, and the mucosa was edematous and pale. After 6 h, the clot had turned purulent and the manubrial vessels were visible. Small white spots were diffusely distributed over the mucosal lining. On day 1, the purulent clot extended through the entire cavity and could be observed as an opaque effusion behind the tympanic membrane. Petechiae covered the promontorium, and the mucosa was generally thickened and edematous. On day 3, the vessel reaction reached its peak. All the petechiae had disappeared. The mucosa remained edematous, but the effusion was less purulent and more turbid. For the first time the growth of bacteria was reduced (abundant growth in two of three cultures and moderate in the third). On day 6, the cultures were positive for the last time (sparse growth in two cultures and moderate in the third), and only small amounts of pus could be observed behind the lower part of the pars flaccida. A polyp-like formation was recorded for the first time. Eight days later, the middle ear infections were all clinically resolved. The middle ears had a yellowish protruding tissue formation in the atticus region, and the mucosa was red and thick. The first cases of myringosclerosis were observed, and one animal exhibited a protruding bone formation and adherences inside the middle ear cavity. After a month, the mucosa was still thickened and had an abundance of dilated vessels. The hole in the bulla caused by the surgical procedure had healed, but otherwise there were no obvious signs of new bone formation. Perforations of the tympanic membrane were never recorded in the NTHi-infected group.

**Construct controls and β-actin levels.** For the results of the competitive PCR to be reliable, the efficiency of the amplification for both target and competitor should be independent of the number of amplification cycles. In the range from 25 to 40 cycles, the amplification efficiencies were essentially identical for the native cDNA and the two constructs, indicating that the targets and competitors were amplified with equal efficiency (Fig. 1a). Furthermore, the factor of the amplification throughout the PCR should be the same for both target and competitor, i.e., the curve relating the log of the product of target and competitor to the initial log of competitor should be linear. As shown in Fig. 1b, the curves fitted closely to a line and the correlation coefficients approximated −1 or 1. The reproducibility of the competitive PCR was high. Samples run twice or in duplicate yielded exactly the same concentrations or the same concentrations ± 1 dilution step. Representative gels stained for coamplified PCR products are shown in Fig. 2. No cytokines could be detected in the individually frozen or pooled unchallenged ears.

The β-actin serves as an internal control for total cell mass and RNA recovery, and expressed levels of investigated genes are usually corrected to the β-actin levels. In the rat middle ear, there was an elevation of the β-actin concentrations during and after the AOM, and this elevation was dependent on the organism (Fig. 3). For animals challenged with NTHi, the β-actin levels increased early and reached a plateau on days 1 to 3. In contrast, the β-actin levels in the pneumococcus-infected animals remained similar to those in the unchallenged animals until day 14, when they increased to be equivalent to the levels in the NTHi-infected animals on day 28. The two patterns suggest two different backgrounds to the β-actin elevation. To better describe the actual events in the middle ear cavity and to avoid problems with cellular influx versus proliferation when comparing the host reactions to pneumococci versus NTHi, the total and not the relative levels of the cytokine transcripts are shown in the following figures. Major differences between the figures and the results observed after normalization of the β-actin levels are indicated in the text.

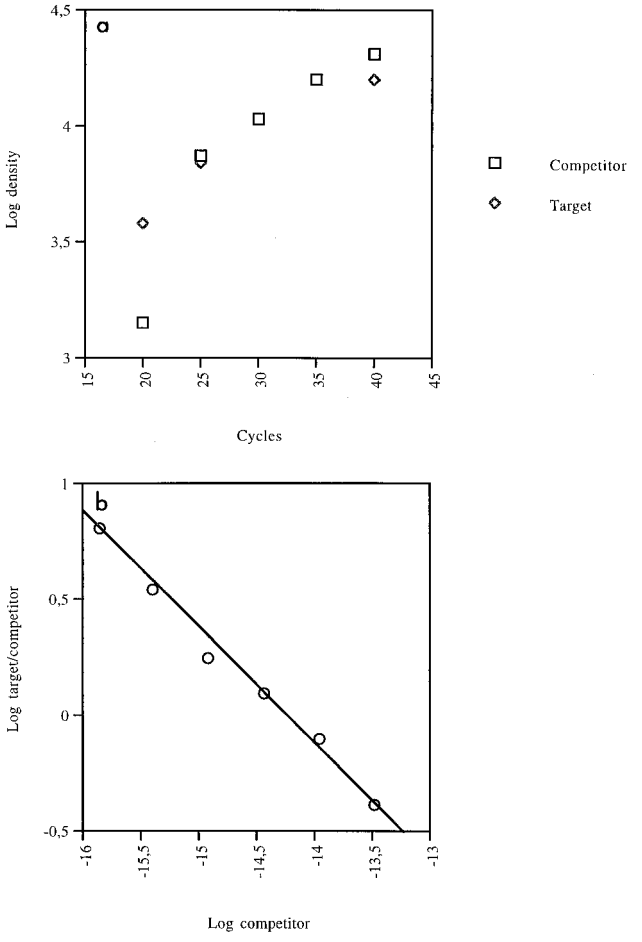


FIG. 1. (a) Kinetics of amplification of competitor and IL-1α target cDNA. Equal amounts of competitor and target were run in a PCR. Aliquots were removed after each five cycles from 20 to 40 cycles. The densitometric values of amplified competitor and target were plotted as a function of number of PCR cycles. (b) Graph relating the logarithm of the ratio of the final PCR products of target and competitor to the logarithm of the initial amount of competitor added to the PCR mixtures. The graph corresponds to the IL-6 gel in Fig. 2.

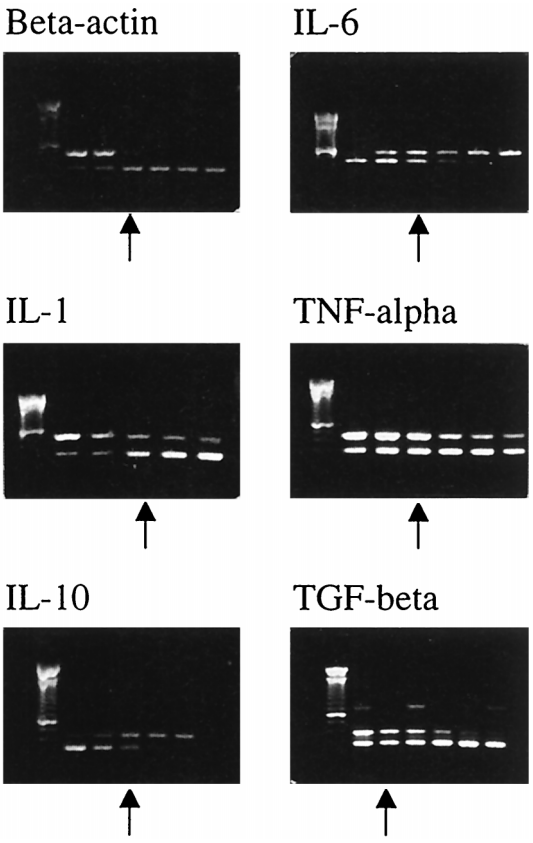


FIG. 2. Representative gels stained for coamplified PCR products of β-actin, IL-6, IL-1α, TNF-α, IL-10, and TGF-β. Lane 1 in each gel contains a molecular weight marker. The arrows below the gels indicate the lanes in which the concentrations of the competitor and the target were approximately equal.

**Profiles of cytokine mRNAs.** The cytokine mRNA levels are reported in Fig. 4. The first cytokine transcript to be recovered was IL-6, which could be measured in all pneumococcus- and NTHi-infected animals after 3 h. Its appearance was transient, and its level dropped to almost 0 after 24 h, with a peak at 6 h (or at 3 h when corrected to the β-actin levels). It was followed by IL-1, TNF-α, IL-10, and TGF-β mRNAs, which could all be detected within 24 h. For these four cytokine transcripts, the time point for appearance and maximum concentration depended on the bacterial species. Compared with NTHi, pneumococci induced a slower upregulation of the cytokine genes, the peaks were delayed with at least one observation point, and, in general, lower mRNA levels were expressed. For IL-1, the dominating cytokine for both bacterially challenged groups in terms of mRNA concentration, the pneumococcus-infected animals showed a peak at 3 days postinoculation, compared with 6 h for the animals challenged with NTHi. Moreover, the peak concentration of IL-1 mRNA was approximately 4 times higher in the latter group. Just one cytokine mRNA reached a higher level in pneumococcus-infected animals than in NTHi-infected animals, and that was TGF-β. The difference in levels was statistically significant ($P = 0.04$), but only when the β-actin was normalized. TGF-β was the only cytokine for which sustained expression was recorded. Although it appeared early in the course of AOM, it reached its maximum level late. The highest mRNA levels for this cytokine were recorded on days 6 and on 28 for the NTHi- and pneumococcus-infected ani-

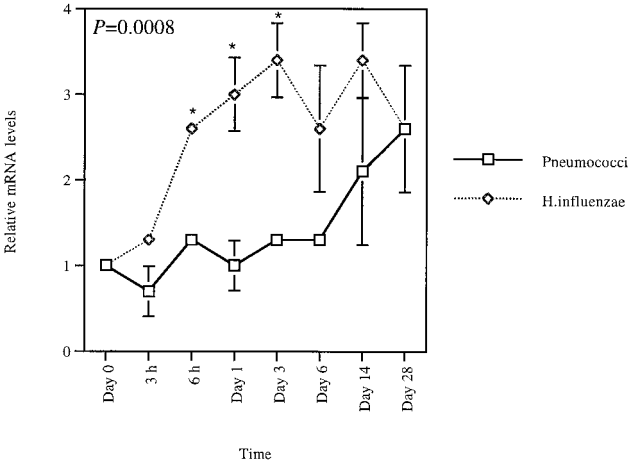


FIG. 3. Relative levels of β-actin mRNA in the middle ear mucosa of animals challenged with pneumococci and NTHi. The concentrations given are the mean and standard error of the mean for three individuals at each time point. *, $P < 0.05$. The $P$ value over the entire period is indicated in the upper left corner.

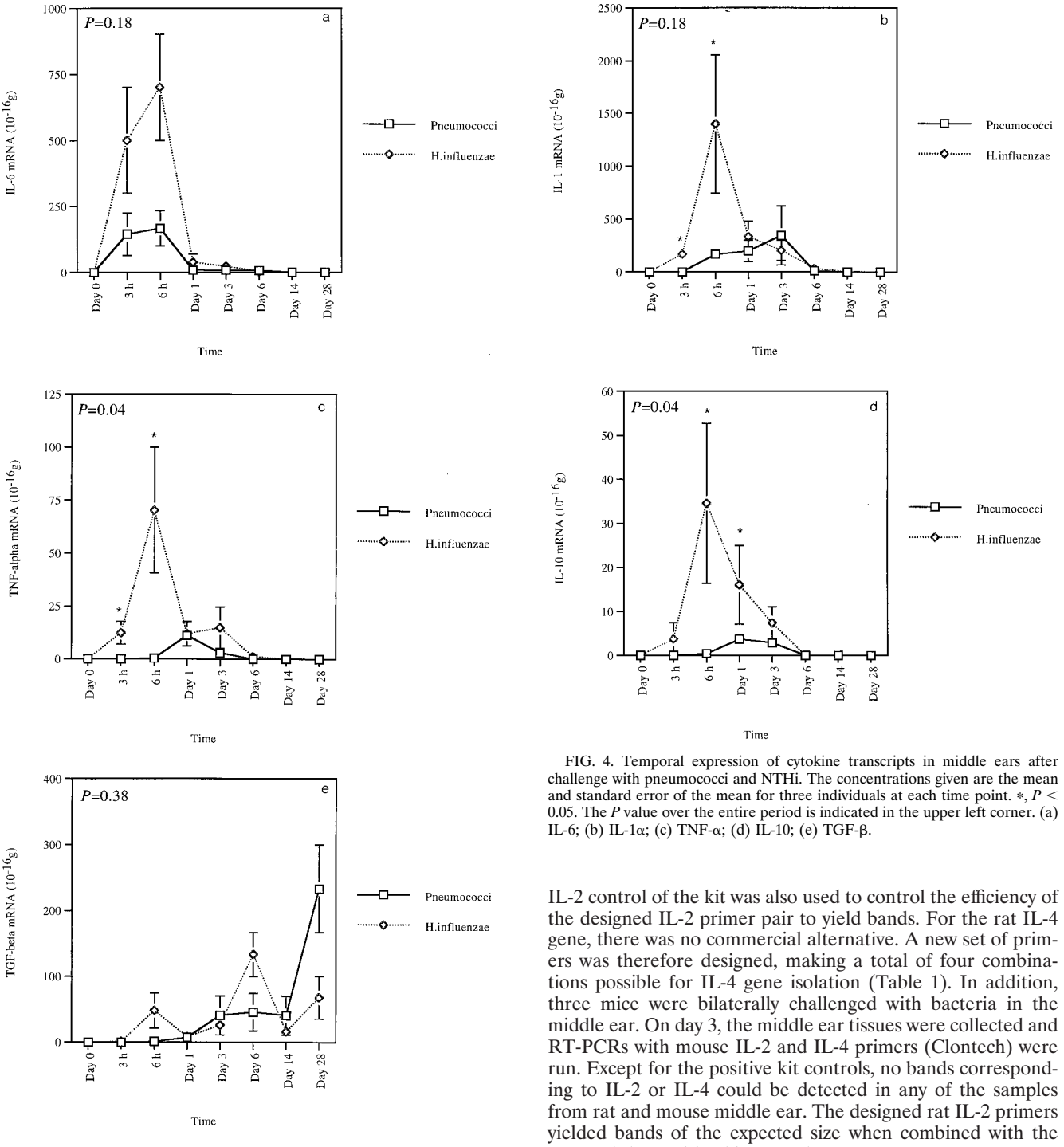


FIG. 4. Temporal expression of cytokine transcripts in middle ears after challenge with pneumococci and NTHi. The concentrations given are the mean and standard error of the mean for three individuals at each time point. *, $P < 0.05$. The $P$ value over the entire period is indicated in the upper left corner. (a) IL-6; (b) IL-1α; (c) TNF-α; (d) IL-10; (e) TGF-β.

mals, respectively. A downregulation of TGF-β was never observed in the latter group. Compared with IL-6, IL-1, and TGF-β, TNF-α and IL-10 mRNAs were expressed at much lower levels. These two transcripts had very similar profiles, and both appeared and peaked at the same time.

Two cytokine mRNAs could never be detected in any of the samples at any time, IL-2 and IL-4. Due to these negative results, commercially available IL-2 primers for rats (Clontech Laboratories, Inc., Palo Alto, Calif.) were tested. The positive IL-2 control of the kit was also used to control the efficiency of the designed IL-2 primer pair to yield bands. For the rat IL-4 gene, there was no commercial alternative. A new set of primers was therefore designed, making a total of four combinations possible for IL-4 gene isolation (Table 1). In addition, three mice were bilaterally challenged with bacteria in the middle ear. On day 3, the middle ear tissues were collected and RT-PCRs with mouse IL-2 and IL-4 primers (Clontech) were run. Except for the positive kit controls, no bands corresponding to IL-2 or IL-4 could be detected in any of the samples from rat and mouse middle ear. The designed rat IL-2 primers yielded bands of the expected size when combined with the DNA of the positive kit control.

Summaries of the otomicroscopic findings in the two infection groups are shown in Fig. 5. The findings are also related to the cytokine transcript profiles in this figure. Apart from the early appearance of IL-6 mRNA, the delay of the otomicroscopic findings in the pneumococcus-challenged animals corresponded to their slower cytokine transcript pattern. Independent of the speed of the host reaction, none of the infections reached their clinical maximum until the TNF-α and IL-10 transcript levels had peaked, and for both bacterial groups the appearance of polyp-like formations coincided with the peak expression of TGF-β.

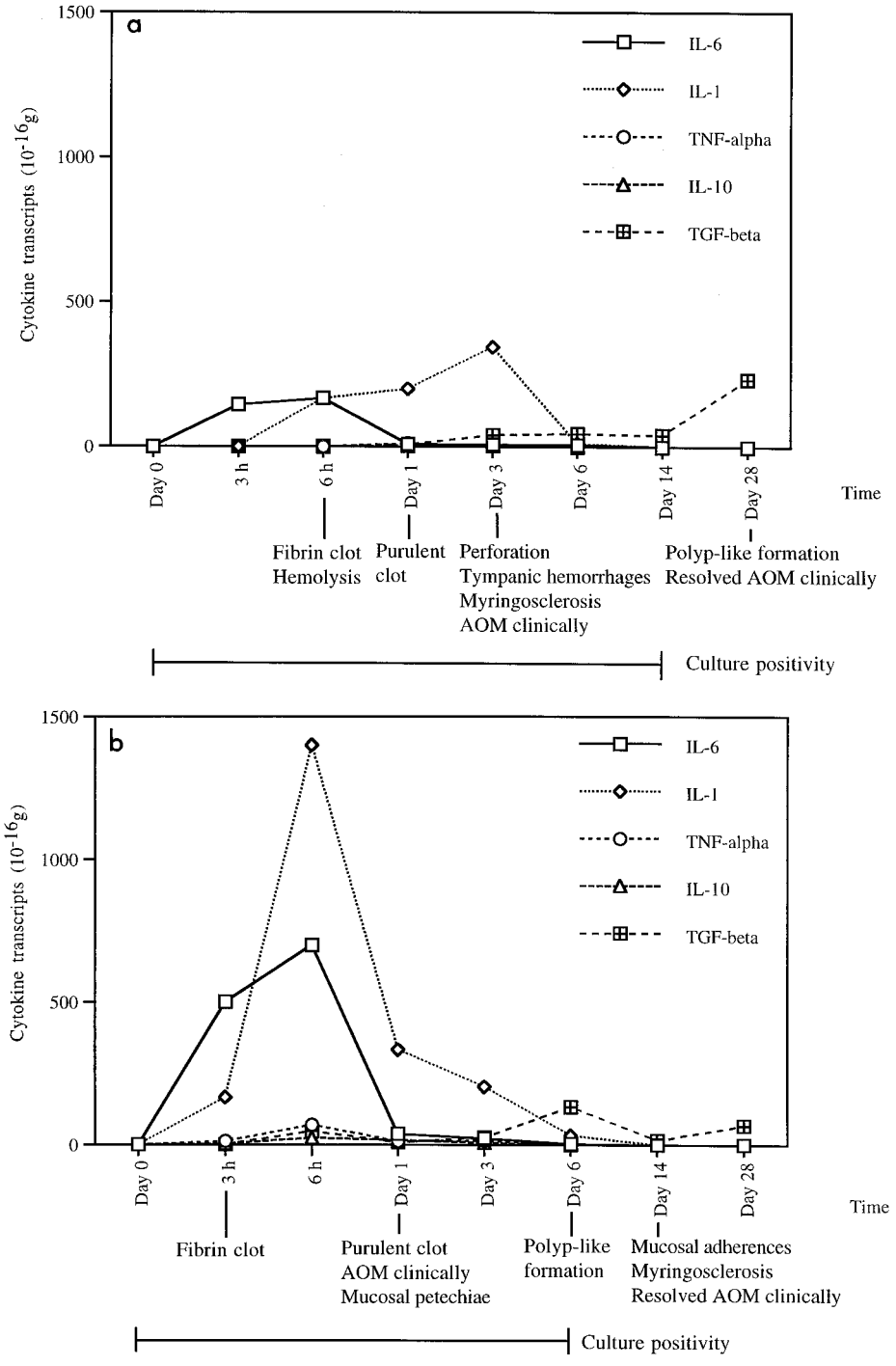


FIG. 5. Summary of the clinical findings in pneumococcus-infected (a) and NTHi-infected (b) animals in relation to their cytokine mRNA profiles. The concentrations given are the mean for three individuals at each time point.

## DISCUSSION

The present study compared the temporal expression of different cytokines during AOM in two well-established animal models. The study clearly demonstrated that the host responses to a bacterial middle ear challenge depended on the organism involved and that cytokine mRNA profiles were related to the evolution of AOM and its course. These profiles by themselves could not, however, explain all of the differences in clinical findings between middle ear infections induced by pneumococci and NTHi. These differences included signs of systemic reaction to the challenge, severity and duration of AOM, hemolysis, localization of mucosal bleeding, adherences, and time point for the appearance of myringosclerosis.

Cytokines are crucial mediators of cell-to-cell signals in immune and inflammatory responses, and monocytes/macrophages are one of the central determinants of which effector

mechanism will be primarily induced. In vitro and in vivo studies have shown that the profile of the cytokine production in an infection is dependent on the causative agent and can even vary with the strain (2, 3, 41). This is in accordance with the results of the present study. In general, NTHi mediated a more rapid influx of cells, as inferred by the increase in β-actin mRNA levels, and a more rapid transcription of cytokine genes at a higher level than did pneumococci. Even when the cytokine transcripts were corrected for β-actin mRNA levels, these differences remained significant in most cases.

An explanation for the more rapid response to NTHi could be the surface-exposed lipopolysaccharides (LPS) of the gram-negative cell wall. The LPS are well-documented stimulators of an acute, early release of cytokines such as IL-1 and TNF from macrophages (15). LPS strongly stimulate phosphorylation of the stress-activated kinase, p38 (12). In turn, p38 is involved in the production of inflammatory cytokines, including IL-1 and TNF (21). The upregulation of cytokines by pneumococci depends on other pathways (28). In contrast to NTHi, the upregulation of cytokines in the ears challenged with pneumococci was remarkably slow. The pneumococcal AOM was equally slow to develop, and the animals showed systemic signs of infection long before the AOM was clinically diagnosed. It has been suggested that the pneumococcal inflammation process is not fully developed unless the capsule, which is less immunogenic than the lipoteichoic acid of the cell wall, is shed or the bacteria have lysed (1, 30). This process requires time (38), and before the host reacts locally to a challenge, the pneumococcal infection may develop into an invasive disease with intracranial complications.

Modern therapeutic approaches have focused on the modulation of host responses and on cytokine responses in particular, but the results have not always been convincing. A suggested explanation for this has been the time factor; the treatment is introduced too late to interrupt events already in progress (39). The results of the present study reinforce this conclusion. Most cytokine responses peaked well before clinical symptoms of AOM could be detected. Intervention directed at early cytokines might therefore have little influence on the clinical course.

Cytokine expression may play a role in the detection and diagnosis of AOM. To both increase the efficacy of antibiotic treatment and identify patients at risk for intracranial complications, an early marker of AOM and especially pneumococcal infection is needed. The cytokine transcript profiles of rats suggest that IL-6 is a potential candidate. After NTHi inoculation, the levels of mRNAs encoding most cytokines tested, including IL-6, were briskly upregulated. However, following pneumococcal inoculation, the IL-6 level did not follow the slower pattern seen in most cytokines but was immediately upregulated in all animals.

It should be noted that rapid expression of IL-6, but at a much lower level, has also been observed after middle ear surgery without bacterial challenge (reference 13 and unpublished data). This suggests that IL-6 upregulation may be a general host response to stress, with the level depending on the cause. In an earlier study by Heikkinen et al. (14), serum IL-6 levels were monitored in neonatal bacterial AOM. These workers found that pneumococci induced significantly higher IL-6 levels in serum than did *H. influenzae* or *M. catarrhalis* and that the specificity and sensitivity for IL-6 detection were relatively high (91 and 61%, respectively). The value of IL-6 as an early marker in serum or in middle ear effusion for pneumococcal disease or severity of disease deserves further evaluation.

Apart from IL-6, TGF-β was the only other cytokine transcript whose kinetics varied from the typical pattern. TGF-β is widely recognized as an anti-inflammatory cytokine. It deactivates the release of $H_2O_2$ and thereby suppresses destructive aspects of the inflammatory host response while facilitating the anabolic effects of growth factors on tissue repair (31). As indicated by the hemolysis, the tympanic hemorrhages, and the early appearance of myringosclerosis, pneumococci and their products may more directly contribute to the tissue destruction. TGF-β expression was significantly higher in pneumococcal animals, and the clinical severity and the duration of the pneumococcal infection could be indicators of an increased need for a reduced host reaction. Due to the prolonged expression of this cytokine, it might act as a possible therapeutic target. Enhancement of TGF-β levels could contribute to the downregulation of inflammation in AOM. However, the relation of this cytokine to chronic central tympanic membrane perforations (35), otitis media with effusion (8), new bone formation (34), polyp formation, and other negative and positive effects in the middle ear must be better defined.

Of the cytokine mRNAs investigated, only IL-2 and IL-4 were never detected. This phenomenon has been observed in other animal models of infection (11, 18) and also in human middle ears and tissues (20, 27). Whether this reflects a lesser role for IL-2 and IL-4 in protective immunity of the middle ear or an inability to detect their expression is not clear.

In conclusion, this study demonstrated that cytokine mRNA profiles are relevant for the evolution of AOM and that NTHi induces a more rapid and prominent host response to middle ear challenge than does the pneumococcus. However, only a single strain of each bacterial species was used. Future studies with other animal models and other strains are needed to verify if these results might be generally applicable. Based on the expression of cytokines, early markers for bacterial AOM and more specific and individualized treatments may evolve.

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## REFERENCES

1. **AlonsoDeVelasco, E., A. F. M. Verheul, J. Verhoef, and H. Snippe.** 1995. *Streptococcus pneumoniae*: virulence factors, pathogenesis, and vaccines. Microbiol. Rev. **59:**591–603.
2. **Arvå, E., and B. Andersson.** 1999. Induction of phagocyte-stimulating cytokines by *in vitro* stimulation of human peripheral blood mononuclear cells with *Haemophilus influenzae*. Scand. J. Immunol. **49:**411–416.
3. **Arvå, E., and B. Andersson.** 1999. Induction of phagocyte-stimulating and Th1-promoting cytokines by *in vitro* stimulation of human peripheral blood mononuclear cells with *Streptococcus pneumoniae*. Scand. J. Immunol. **49:** 417–423.
4. **Barry, B., J. Delattre, F. Vie, J. P. Bedos, and P. Gehanno.** 1999. Otogenic intracranial infections in adults. Laryngoscope **109:**483–487.
5. **Bernstein, J. M., and P. L. Ogra.** 1980. Mucosal immune system: implications in otitis media with effusion. Ann. Otol. Rhinol. Laryngol. **89**(Suppl.)**:** 326–332.
6. **Branefors-Helander, P., T. Dahlberg, and O. Nylén.** 1975. Acute otitis media. A clinical, bacteriological and serological study of children with frequent episodes of acute otitis media. Acta Otolaryngol. (Stockholm) **80:**399–409.
7. **Chonmaitree, T., J. A. Patel, R. Garofalo, T. Uchida, T. Sim, M. J. Owen, and V. M. Howie.** 1996. Role of leukotriene B4 and interleukin-8 in acute bacteria and viral otitis media. Ann. Otol. Rhinol. Laryngol. **105:**968–974.
8. **Cooter, M. S., R. J. Eisma, J. A. Burleson, G. Leonard, D. Lafreniere, and D. L. Kreutzer.** 1998. Transforming growth factor-beta expression in otitis media with effusion. Laryngoscope **108:**1066–1070.
9. **Culpepper, L., and J. Froom.** 1997. Routine antimicrobial treatment of acute otitis media. Is it necessary? JAMA **278:**1643–1645.
10. **Goldstein, N. A., M. L. Casselbrant, C. D. Bluestone, and M. Kurs-Lasky.** 1998. Intratemporal complications of acute otitis media in infants and children. Otolaryngol. Head Neck Surg. **119:**444–454.
11. **Haak-Frendscho, M., J. F. Brown, Y. Iizawa, R. D. Wagner, and C. J. Czuprynski.** 1992. Administration of anti-IL-4 monoclonal antibody 11B11

increases the resistance of mice to *Listeria monocytogenes* infection. J. Immunol. **148:**3978–3985.
12. **Han, J., J. D. Lee, L. Bibbs, and R. J. Ulevitch.** 1994. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science **265:**808–811.
13. **Hebda, P. A., C. M. Alper, W. J. Doyle, G. J. Burckart, W. F. Diven, and A. Zeevi.** 1998. Upregulation of messenger RNA for inflammatory cytokines in middle ear mucosa in a rat model of acute otitis media. Ann. Otol. Rhinol. Laryngol. **107:**501–507.
14. **Heikkinen, T., F. Ghaffar, A. O. Okorodudu, and T. Chonmaitree.** 1998. Serum interleukin-6 in bacterial and nonbacterial acute otitis media. Pediatrics **102:**296–299.
15. **Henderson, B., S. Poole, and M. Wilson.** 1996. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol. Rev. **60:**316–341.
16. **Hermansson, A., P. Emgård, K. Prellner, and S. Hellström.** 1988. A rat model for pneumococcal otitis media. Am. J. Otolaryngol. **9:**97–101.
17. **Howie, V. M., R. Dillard, and B. Lawrence.** 1985. In vivo sensitivity test in otitis media: efficacy of antibiotics. Pediatrics **75:**8–13.
18. **Iizawa, Y., J. F. Brown, and C. J. Czuprynski.** 1992. Early expression of cytokine mRNA in mice infected with *Listeria monocytogenes*. Infect. Immun. **60:**4068–4073.
19. **Klein, J. O.** 1994. Otitis media. Clin. Infect. Dis. **19:**823–833.
20. **Kotake, S., H. R. Schumacher, Jr., and R. L. Wilder.** 1996. A simple nested RT-PCR method for quantitation of the relative amounts of multiple cytokine mRNAs in small tissue samples. J. Immunol. Methods **199:**193–203.
21. **Lee, J. C., J. T. Laydon, P. C. McDonnell, T. F. Gallagher, S. Kumar, D. Green, D. McNulty, M. J. Blumenthal, J. R. Heys, S. W. Landvatter, J. E. Strickler, M. M. McLaughlin, I. Siemens, S. Fisher, G. P. Livi, J. R. White, J. L. Adams, and P. R. Young.** 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature **372:**739–746.
22. **McCaig, L. F., and J. M. Hughes.** 1995. Trends in antimicrobial drug prescribing among office-based physicians in the United States. JAMA **273:**214–219.
23. **Melhus, Å., A. Hermansson, A. Forsgren, and K. Prellner.** 1997. A resolved pneumococcal infection protects against nontypeable *Haemophilus influenzae*: an evaluation of different routes of whole cell immunization in protection against experimental acute otitis media. Int. J. Pediatr. Otorhinolaryngol. **39:**119–131.
24. **Melhus Å., A. Hermansson, and K. Prellner.** 1994. Nontypeable and encapsulated *Haemophilus influenzae* yield different clinical courses of experimental otitis media. Acta Otolaryngol. (Stockholm) **114:**289–294.
25. **Miller, M. B., P. J. Koltai, and S. V. Hetherington.** 1990. Bacterial antigens and neutrophil granule proteins in middle ear effusions. Arch. Otolaryngol. Head Neck Surg. **116:**335–337.
26. **Murphy, T. F.** 1996. *Branhamella catarrhalis*: epidemiology, surface antigenic structure, and immune response. Microbiol. Rev. **60:**267–279.
27. **Okamoto, Y., K. Kudo, K. Ishikawa, E. Ito, K. Togawa, I. Saito, I. Moro, J. A. Patel, and P. L. Ogra.** 1993. Presence of respiratory syncytial virus genomic sequences in middle ear fluid and its relationship to expression of cytokines and cell adhesion molecules. J. Infect. Dis. **168:**1277–1281.
28. **Paul, A., S. Wilson, C. M. Belham, C. J. Robinson, P. H. Scott, G. W. Gould, R. Plevin.** 1997. Stress-activated protein kinases: activation, regulation and function. Cell. Signal. **9:**403–410.
29. **Petersen, C. G., T. Ovesen, and C. B. Pedersen.** 1998. Acute mastoidectomy in a Danish county from 1977–1996 with focus on the bacteriology. Int. J. Pediatr. Otorhinolaryngol. **45:**21–29.
30. **Ripley-Petzoldt, M. L., G. S. Giebink, S. K. Juhn, D. Aeppli, A. Tomasz, and E. Tuomanen.** 1988. The contribution of pneumococcal cell wall to the pathogenesis of experimental otitis media. J. Infect. Dis. **157:**245–255.
31. **Roberts, A. B., and M. B. Sporn.** 1990. The transforming growth factor-βs, p. 419–472. *In* A. B. Roberts and M. B. Sporn (ed.), Peptide growth factors and their receptors. I. Springer-Verlag, New York, N.Y.
32. **Rudberg, R. D.** 1954. Acute otitis media: comparative therapeutic results of sulphonamide and penicillin administered in various forms. Acta Otolaryngol. (Stockholm) **113**(Suppl.)**:**9–79.
33. **Ryan, A. F., P. A. Sharp, and J. P. Harris.** 1990. Lymphocyte circulation to the middle ear. Acta Otolaryngol. (Stockholm) **109:**278–287.
34. **Solheim, E.** 1998. Growth factors in bone. Int. Orthop. **22:**410–416.
35. **Somers, T., G. Goovaerts, L. Schelfhout, S. Peeters, P. J. Govaerts, and E. Offeciers.** 1998. Growth factors in tympanic membrane perforations. Am. J. Otol. **19:**428–434.
36. **Stangerup, S.-E., and M. Tos.** 1986. Epidemiology of acute suppurative otitis media. Am. J. Otolaryngol. **7:**47–54.
37. **Tarlow, M.** 1998. Otitis media: pathogenesis and medical sequelae. Ear Nose Throat J. **77**(6 Suppl.)**:**3–6.
38. **Tomasz, A.** 1981. Surface components of *Streptococcus pneumoniae*. Rev. Infect. Dis. **3:**190–211.
39. **Wang, H., O. Bloom, M. Zhang, J. M. Vishnubhakat, M. Ombrellino, J. Che, A. Frazier, H. Yang, S. Ivanova, L. Borovikova, K. R. Manogue, E. Faist, E. Abraham, J. Andersson, U. Andersson, P. E. Molina, N. N. Abumrad, A. Sama, and K. J. Tracey.** 1999. HMG-1 as a late mediator of endotoxin lethality in mice. Science **285:**248–251.
40. **Willett, D. N., R. P. Rezaee, J. M. Billy, M. B. Tighe, and T. F. DeMaria.** 1998. Relationship of endotoxin to tumor necrosis factor-alpha and interleukin-1 beta in children with otitis media with effusion. Ann. Otol. Rhinol. Laryngol. **107:**28–33.
41. **Xiong, H., I. Kawamura, T. Nishibori, and M. Mitshuyama.** 1994. Cytokine gene expression in mice at an early stage of infection with various strains of *Listeria* spp. differing in virulence. Infect. Immun. **62:**3649–3654.

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