High cytokine levels in perforated acute otitis media exudates containing live bacteria

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Abstract

Acute otitis media (AOM) is an inflammatory response to microbes in the middle ear, sometimes associated with rupture of the tympanic membrane. Human leukocytes produce different patterns of inflammatory mediators *in vitro* when stimulated with Gram-positive and Gram-negative bacteria, respectively. Here, we investigated the cytokine and prostaglandin E_2 (PGE_2) responses in middle ear fluids (MEFs) from children with spontaneously perforated AOM, and related the mediator levels to the presence of pathogens detected by culture (live) or PCR (live or dead). Furthermore, the *in vivo* cytokine pattern was compared with that induced in leukocytes stimulated by dead bacteria *in vitro*. MEFs with culturable pathogenic bacteria contained more interleukin (IL)-1β (median: 110 μg/L vs. <7.5 μg/L), tumour necrosis factor (TNF) (6.3 μg/L vs. <2.5 μg/L), IL-8 (410 μg/L vs. 38 μg/L) and IL-10 (0.48 μg/L vs. <0.30 μg/L) than culture-negative fluids, irrespective of PCR findings. IL-6 and PGE_2 were equally abundant (69–110 μg/L) in effusions with live, dead or undetectable bacteria. Cytokine levels were unrelated to bacterial species and to the presence or absence of virus. Similar levels of TNF and IL-6 as found in the MEFs were obtained by *in vitro* stimulation of leukocytes, whereas 11 times more IL-1β and 3.5 times more IL-8 were produced *in vivo*, and 22 times more IL-10 was produced *in vitro*. Vigorous production of proinflammatory cytokines accompanies AOM with membrane rupture, regardless of the causative agent, but the production seems to cease rapidly once the bacteria are killed and fragmented. IL-6 and PGE_2, however, remain after bacterial disintegration, and may play a role in the resolution phase.

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Introduction

Acute otitis media (AOM) is the most common bacterial infection in childhood. Bacteria ascend into the middle ear through the Eustachian tube, commonly after a viral infection. An inflammatory exudate is formed and trapped in the middle ear. In some cases, the tympanic membrane ruptures, by as yet unknown mechanisms.

The major causative agents of AOM are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*; *Streptococcus pyogenes* is uncommon today [1]. Respiratory viruses are detected in approximately 20% of AOM middle ear fluids (MEFs) by culture and/or search for antigens, and even more often by PCR [2]. Their role in pathogenesis is unclear.

A host of inflammatory mediators are produced by tissue macrophages in response to microbes and their products, including prostaglandin E_2 (PGE_2), which causes vasodilatation [3], and tumour necrosis factor (TNF), interleukin (IL)-1β and IL-8, which attract and guide neutrophils into the middle ear cavity [4,5]. Some macrophage mediators dampen the inflammatory response. IL-6 downregulates production of TNF and IL-1β, and appears to be important in the resolution phase [6]. IL-10 counteracts macrophage activation [7], and PGE_2 stimulates synthesis of IL-10 but suppresses production of TNF [8].

Human leukocytes produce different inflammatory mediators when stimulated with Gram-positive or Gram-negative bacteria *in vitro*. Intact Gram-positive bacteria induce more TNF than do Gram-negative bacteria, which instead induce more IL-6, IL-8, IL-10 and PGE_2 [9–11]. Dead and live bacte-

ria generally induce the same types and amounts of cytokines in human monocytes [10], but fragmented Gram-positive bacteria and isolated cell wall components induce smaller amounts of cytokines [10–12] and may even be inhibitory for cytokine production [13].

In the present study, we investigated the inflammatory mediator response in the middle ear in AOM causing rupture of the tympanic membrane, and related the levels to the presence of live bacteria, bacterial and viral DNA, cytokines and PGE_2. The mediator profile in the exudates was compared with that induced by *in vitro* stimulation of human leukocytes with AOM-causing microbes.

Materials and Methods

Patients

We recruited 47 children presenting at an outpatient ear, nose and throat clinic (Lundby Hospital, Gothenburg, Sweden) between January 2004 and May 2005 within 24 h of spontaneous rupture of the tympanic membrane due to AOM. Patients with chronic otitis media or tympanostomy tubes were excluded. The fluid in the ear canal was sucked up into a container, and the tube was flushed with 0.5 mL of sterile NaCl solution, presumed to result in a 1 : 5 dilution. After culture (see below), the fluid was stored at −20°C.

Culture of bacteria from exudate

Semiquantitative culture of the middle ear exudate was performed by streaking, at the bedside, a calibrated loop (10 μL) on suitable agars, prepared in-house at the Bacteriological Laboratory, Sahlgrenska University Hospital, Gothenburg, Sweden.

The plates were transported within 4 h to the laboratory, where they were incubated anaerobically (*Streptococcus* plates) or in air with 5% CO_2 at 37°C. After 24 h and 48 h of culture, AOM pathogens were enumerated and identified using standard methods.

PCR for bacterial pathogens

S. pneumoniae, *H. influenzae* and *Moraxella* spp. were detected in MEF by specific PCR. Bacterial DNA was extracted from 100 μL of exudate, using the Puregene Yeast & Gram-positive Bacteria kit (Gentra Systems, Minneapolis, MN, USA) [14]. A duplex PCR assay was used for simultaneous detection of *S. pneumoniae* and *M. catarrhalis*, and another PCR assay for detection of *H. influenzae*, as described previously [15,16]. The common lower primer was 5′-CTA CGC ATT TCA CCG CTA CAC-3′, and the specific upper primers were as follows: for *S. pneumoniae*, 5′-AAG GTG CAC TTG CAT CAC TAC C-3′; for *H. influenzae*, 5′-CGT ATT ATC GGA AGA TGA AAG TGC-3′; and for *Moraxella* spp., 5′-CCC ATA AGC CCT GAC GTT AC-3′ [15]. The PCRs were considered to be specific for *S. pneumoniae* and *H. influenzae*, whereas moraxellae other than *M. catarrhalis* could give positive results (data not shown).

PCR for viral pathogens

Rhinovirus, coronavirus, influenza A virus, influenza B virus, respiratory syncytial virus, adenovirus, metapneumovirus and enterovirus were detected by PCR as previously described [17]. Nucleic acid from 200 μL of diluted specimen was extracted (Magnapure LC robot; (Roche Molecular Systems, Mannheim, Germany), using the Total Nucleic Acid protocol) and amplified (ABI 7500 genetic analyzer; Applied Biosystems, Foster City, CA, USA) using published primers and conditions [17].

In vitro stimulation of blood mononuclear cells

MEF isolates of *S. pneumoniae* (n = 3), *H. influenzae* (n = 3) and *M. catarrhalis* (n = 1) were cultured overnight, harvested in phosphate-buffered saline, washed, suspended at 10^9/mL (optical density at 580 nm of 1.19), inactivated by exposure to UV light for 18 min (confirmed by a negative viable count) and stored at −80°C.

Peripheral blood mononuclear cells were prepared from blood donor buffy coats (Blood Bank, Sahlgrenska University Hospital) by density gradient centrifugation (Lymphoprep; Axis-Shield, Oslo, Norway) for 20 min at 820 ***g***, washed in endotoxin-free RPMI-1640 with 2 mM L-glutamine (PAA Laboratories GmbH, Linz, Austria), and suspended in triplicate at 2×10^6/mL in RPMI-1640 with 5% inactivated fetal bovine serum (Invitrogen, San Diego, CA, USA) and 50 mg/L gentamicin (Sigma-Aldrich, St Louis, MO, USA) in 96-well flat-bottomed plates (Nunc, Roskilde, Denmark). Bacteria were added to achieve final concentrations of 5×10^5/mL, 5×10^6/mL or 5×10^7/mL. After 24 h of incubation in a 37°C humidified atmosphere with 5% CO_2, supernatants were harvested and stored at −20°C until being analysed.

Quantification of inflammatory mediators

TNF, IL-1β, IL-6, IL-8 and IL-10 were quantified by ELISA as previously described [18], using monoclonal antibodies and recombinant cytokines from BD Pharmingen (San Diego, CA, USA) or R&D Systems (Minneapolis, MN, USA) (anti-IL-1β antibodies). PGE_2 was measured using the Biotrak Enzyme Immunoassay System (Amersham Biosciences UK Limited, Little Chalfont, UK).

Statistics

Differences between groups were tested for significance with the Mann–Whitney test. Spearman rank correlation was used

to evaluate relationships between bacterial numbers and cytokine levels.

Results

Bacterial pathogens detected by culture and PCR

Forty-seven patients (median age, 26 months; range, 4 months to 14 years) with ruptured tympanic membranes were included. Cultures were positive for a bacterial pathogen in 22 cases (47%), mainly *S. pneumoniae* and *H. influenzae* (Table 1). Among the 25 pathogen-negative samples, 19 yielded skin commensals such as coagulase-negative staphylococci, *Staphylococcus aureus*, or diptheroid rods, considered to be contaminants from the external ear canal.

All culture-positive samples were also positive by PCR, except for one sample yielding *S. pneumoniae* in culture but not by PCR (Table 1). Another sample yielded *S. pyogenes* in culture, but the PCR was not designed to detect this pathogen. In seven culture-positive samples, the PCR revealed not only the bacteria found by culture in each sample, but also an additional one or two bacterial pathogens.

Among the 25 culture-negative samples, 21 were also negative by PCR. Thus, only a few additional pathogens were detected with PCR in the samples negative by culture. Dilution (1 : 5 and 1 : 25) to minimize the effect of possible inhibitors did not increase the detection rate, and admixed pneumococcal DNA was readily detected by PCR, ruling out the possibility that negative results were caused by the presence of inhibitory substances in the MEFs.

Presence of viral pathogens

Twenty-five MEFs were analysed by PCR for the presence of rhinovirus, coronavirus, influenza A virus, influenza B virus, respiratory syncytial virus, adenovirus, metapneumovirus and enterovirus. Five samples (20%) yielded viruses: rhinovirus (n = 4) and coronavirus (n = 1). These were all culture-positive for bacterial pathogens: *H. influenzae* (n = 4) and/or *S. pneumoniae* (n = 2).

Cytokine levels in relation to microbiological findings

Cytokines and PGE_2 were measured in the MEFs and related to culture and PCR findings. The results are shown in Fig. 1.

MEFs from which bacterial pathogens were cultured contained high levels of IL-1β, IL-8, TNF and IL-10, whereas culture-negative, but PCR-positive, samples had similar levels of these cytokines as MEFs that were negative by both methods. Thus, culture-positive fluids contained 11 times more IL-1β (median 110 μg/L vs. <7.5 μg/L, p <0.0001), 5.5 times more IL-8 (410 μg/L vs. 38 μg/L, p <0.0001), at least 2.5 times more TNF (6.3 μg/L vs. <2.5 μg/L, p <0.0001) and at least 1.5 times more IL-10 (0.48 μg/L vs. <0.30 μg/L, p 0.0001) than culture-negative specimens. In contrast, IL-6 and PGE_2 were found at equally high levels in both culture-positive and culture-negative samples (110 μg/L vs. 100 μg/L, and 83 μg/L vs. 69 μg/L, respectively). Samples in which viruses were detected did not contain higher cytokine levels than virus-negative samples (data not shown).

Positive MEF samples contained 100–100 000 bacteria/mL. Among culture-positive samples, there was no correlation between population numbers and cytokine levels (data not shown).

Inflammatory mediators in relation to bacterial pathogens

We compared inflammatory mediators in effusions that contained the Gram-positive *S. pneumoniae* or the Gram-negative *H. influenzae*, as detected by culture or PCR. No significant difference was seen for any of the investigated mediators (Table 2 and data not shown).

TABLE 1. Presence of bacterial pathogens in middle ear fluids (MEFs) from spontaneously ruptured acute otitis media as detected by culture and PCR

		A single pathogen				
	n	*Streptococcus pneumoniae*	*Haemophilus influenzae*	*Moraxella catarrhalis*	*Streptococcus pyogenes*	More than pathogen
Assayed MEF	47					
Culture-positive	22	10	9	1	1	1[a]
PCR-positive	20	6	6	0	–[b]	8[c]
PCR-negative	2	1	0	0	1[b]	0
Culture-negative	25					
PCR-positive	4	0	0	2	–	2[d]
PCR-negative	21					

[a] *S. pneumoniae* + *H. influenzae*.
[b] *S. pyogenes* was not assessed in the PCR assay.
[c] *S. pneumoniae* + *H. influenzae* (n = 1); *S. pneumoniae* + *M. catarrhalis* (n = 2); *H. influenzae* + *M. catarrhalis* (n = 2); *S. pneumoniae* + *H. influenzae* + *M. catarrhalis* (n = 3).
[d] *S. pneumoniae* + *H. influenzae* (n = 1); *S. pneumoniae* + *M. catarrhalis* (n = 1).

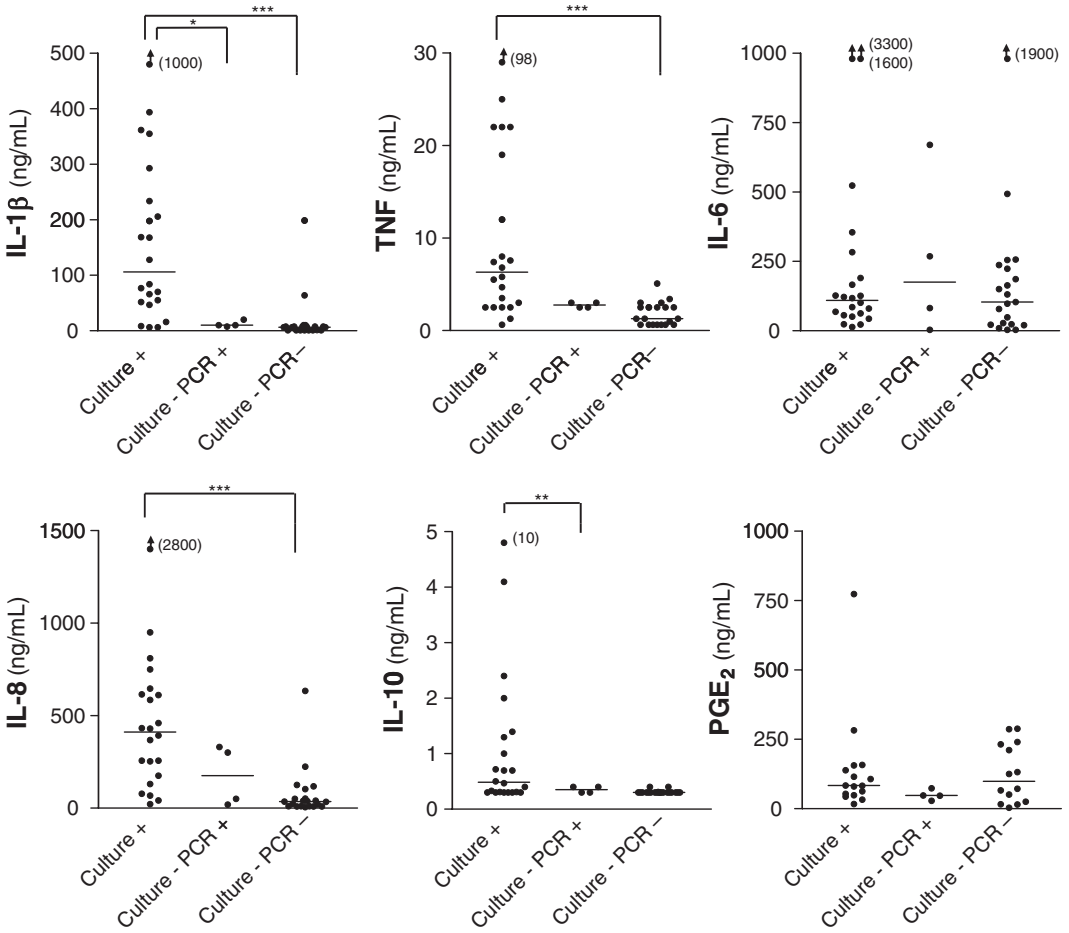

FIG. 1. Inflammatory mediators in middle ear effusions with positive bacterial culture, negative culture with positive PCR and negative culture with negative PCR, measured using ELISA. The levels of interleukin (IL)-1β, tumour necrosis factor (TNF), IL-8 and IL-10 were higher in culture-positive middle ear fluids (MEFs) than in culture-negative specimens, irrespective of PCR findings. In contrast, the levels of IL-6 and prostaglandin E_2 (PGE_2) were equally elevated in effusions with live, dead or undetectable bacteria. *p <0.05, **p <0.01, ***p ≤0.0001.

TABLE 2. Inflammatory mediators in middle ear fluids (MEFs) culture-positive for *Streptococcus pneumoniae* or *Haemophilus influenzae*

	Inflammatory mediators in MEF (median, range)		
	***S. pneumoniae* (*n* = 10)**	***H. influenzae* (*n* = 9)**	**p-value**
IL-1β (μg/L)	110 (6.3–390)	170 (8.6–1000)	0.97
TNF (μg/L)	7 (<1.3–98)	2.5 (<2.5–25)	0.44
IL-6 (μg/L)	160 (24–1600)	85 (14–170)	0.22
IL-8 (μg/L)	430 (22–810)	590 (180–2800)	0.48
IL-10 (μg/L)	0.48 (<0.30–2.0)	0.33 (<0.30–4.1)	0.68
PGE_2 (μg/L)	83[a] (17–280)	130[b] (33–770)	0.78

IL, interleukin; PGE_2, prostaglandin E_2; TNF, tumour necrosis factor.
[a]*n* = 8.
[b]*n* = 6.

Inflammatory mediator response after *in vitro* stimulation of leukocytes with bacterial pathogens

We investigated whether the cytokine pattern seen in MEFs could be replicated by stimulation of human leukocytes with AOM pathogens *in vitro*. Mononuclear cells from three blood donors were stimulated with three strains each of *S. pneumoniae* and *H. influenzae* and one of *M. catarrhalis*. UV-inactivated bacteria were used at 5×10^5/mL, 5×10^6/mL and 5×10^7/mL, and cytokines were measured in the supernatant after 24 h, previously found to be optimal [10,11]. MEF levels of IL-1β and IL-8 were, on average, 11 and 3.5 times higher than could be induced *in vitro* with an optimal dose of bacteria (5×10^7/mL) (p <0.0001 and p 0.004, respectively) (Fig. 2). In contrast, IL-10 levels were 22 times lower in exudates than those obtained

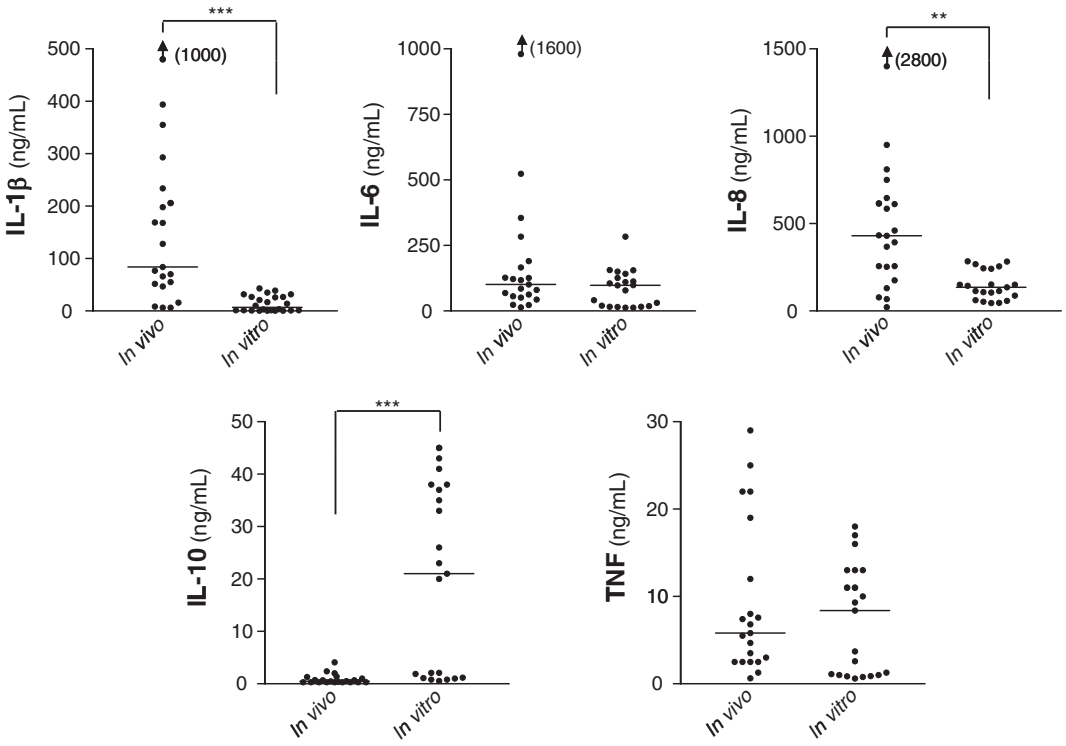

FIG. 2. Mononuclear cells from three blood donors were stimulated with clinical acute otitis media (AOM) isolates of UV-inactivated *Streptococcus pneumoniae* (n = 3), *Haemophilus influenzae* (n = 3) or *Moraxella catarrhalis* (n = 1) at optimal concentrations (5×10^7 bacteria/mL), and the levels of inflammatory mediators were compared with the levels measured in middle ear fluids (MEFs) containing these pathogens. Whereas the levels of interleukin (IL)-6 and tumour necrosis factor (TNF) in the MEF samples were similar to those in the supernatants from stimulated leukocytes, the AOM exudates contained more IL-1β and IL-8, but less IL-10, than was obtained *in vitro*. **p <0.001, ***p <0.0001.

in vitro (p <0.0001) (Fig. 2), mainly owing to high IL-10 levels induced *in vitro* by *H. influenzae* and *M. catarrhalis*. IL-6 and TNF were produced at approximately the same levels *in vitro* as those found in culture-positive MEFs (Fig. 2).

Discussion

In this study, middle ear effusions obtained after spontaneous rupture of the tympanic membrane were examined for live and dead bacteria, viruses and inflammatory mediators.

A major finding was that of strikingly higher levels of IL-1β, TNF, IL-8 and IL-10 in culture-positive than in culture-negative MEFs. Accordingly, in MEFs obtained by tympanocentesis, the levels of IL-1β, TNF and IL-8 were two to ten times higher in samples containing live bacteria than in sterile samples [19–21], and in our studies of secretory otitis media, a condition with long-standing fluid in the middle ear, the levels of not only IL-1β and IL-8, but also IL-6, were higher in culture-positive than culture-negative MEFs [16]. Interestingly, the presence of dead bacteria, as revealed by positive PCR but negative culture, was not associated with enhanced cytokine levels. Furthermore, only a few samples that were negative by culture were positive by PCR, indicating that killing of the bacteria is followed quite soon by elimination of bacterial DNA.

We have shown that fragments from decayed Gram-positive bacteria are rather inefficient in triggering cytokine production [10,11] and, in fact, may even potently downregulate the production of IL-12, TNF and interferon-γ [13]. Our results in this study confirm these findings, as the highest cytokine levels were only seen in the presence of intact, live bacteria, suggesting that fragmented bacteria are less stimulatory.

Interestingly, the levels of IL-6 and PGE_2 were strongly elevated even in exudates devoid of viable bacteria. We have observed that IL-6 is also produced in response to fragmented bacteria [12]. IL-6 is a cytokine that inhibits the production of IL-1β and TNF, favours the resolution of the neutrophil infiltrate and initiates an acquired immune response [6,22], thus playing a role in initiating the resolution phase of the infection. It is also possible that the production of IL-6 and PGE_2 could continue for a longer time after the initial stimulus than that of other cytokines, and/or that these mediators are not eliminated at the same rate as the other cytokines.

Thus, in a pneumococcal AOM rat model, IL-6 was observed in the middle ear tissue for at least 5 days after initiation of the infection, whereas its production ceased within 24 h [23], suggesting slow elimination of this cytokine.

When blood mononuclear cells were stimulated with optimal doses of inactivated bacterial pathogens, similar levels of TNF and IL-6 were obtained as those present in the AOM fluids. However, MEFs contained 11 times more IL-1β and three times more IL-8 than could be obtained *in vitro*, but, conversely, 22 times less IL-10 than was generated upon *in vitro* stimulation of leukocytes. Thus, the inflammatory reaction associated with rupture of the tympanic membrane is associated with exceptionally high levels of IL-1β, but little IL-10.

Secretion of biologically active IL-1β requires two signals: one signal inducing production of the inactive precursor, pro-IL-1β; and another signal for activation of caspase-1, leading to the cleavage and export of active IL-1β [24]. Caspase-1 is activated by formation of an inflammasome in response to cell stress [24]. Thus, the high levels of IL-1β seen in spontaneously perforated AOM may be the consequence of severe tissue stress and destruction. However, IL-1β may also contribute to destruction, as it induces production of metalloproteases, enzymes that digest connective tissue, a component of the tympanic membrane [25].

We could not detect any significant differences in the levels of inflammatory mediators between effusions yielding the Gram-positive *S. pneumoniae* or the Gram-negative *H. influenzae*, despite *in vitro* findings of different responses to these bacteria and to Gram-positives and Gram-negatives in general [10,11,26]. This shows that viable bacteria interacting with host tissue produce additional inflammatory stimuli to those provided by the bacterial structures themselves. Serum levels of IL-6 have actually been shown to be higher in AOM caused by Gram-positive pneumococci than in AOM caused by Gram-negative *H. influenzae* [27], although Gram-negatives *in vitro* elicit more IL-6 than do Gram-positives [11].

Respiratory viruses could be detected in 20% of the samples that were invariably culture-positive for bacteria. This low rate contrasts with those found in other studies [2], and might be explained by dilution of samples. However, AOM with spontaneous rupture of the tympanic membrane may have a mainly bacterial aetiology. Studies have shown higher levels of IL-8 in virus-containing exudates [28], but we saw no effect of the absence or presence of viruses on any of the inflammatory mediators.

In half of the MEFs, we found no recognized pathogen, either by culture or by PCR. Could novel species, perhaps difficult to culture, be responsible for infection in apparently pathogen-negative otitis media effusions?

The bacterium *Alloiococcus otitidis* has been detected by PCR in 25% of MEFs from children with AOM, but the clinical significance is doubtful [29]. However, an infection so powerful as to sever the tympanic membrane is probably caused by highly virulent bacteria such as pneumococci [30]. In conclusion, we assume that the pathogens of importance are *S. pneumoniae*, *H. influenzae* and, to a lesser extent, *M. catarrhalis* and *S. pyogenes*, and that the absence of detectable bacteria depends on their active elimination shortly after killing of the bacteria. Our results further suggest that once the bacteria are dead and fragmented, the production of pro-inflammatory cytokines ceases rapidly.

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Transparency Declaration

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