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Cytokines in nasopharyngeal secretions; evidence for defective IL-1β production in children with recurrent episodes of acute otitis media

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SUMMARY

The host–parasite relationship in the nasopharynx of young children with bacterial colonization and antigen uptake in the mucosa and lymphatic tissue provides an opportunity to investigate infectious/inflammatory processes and responses. IL-1β, IL-6 and tumour necrosis factor-alpha (TNF-α) were analysed in nasopharyngeal secretions and serum from children with or without recurrent episodes of acute otitis media, from healthy adults and adults with hypogammaglobulinaemia or selective deficiency of IgG3. Nasopharyngeal secretions generally contained substantial amounts of IL-1β, IL-6 and TNF-α. In contrast, IL-1β, IL-6 and TNF-α were not detectable in sera on the same occasion. Children were found to have higher levels of IL-1β, IL-6 and TNF-α than healthy adults and than adults with immunodeficiency. High levels of IL-1β were associated with low or undetectable levels of IL-6 and TNF-α, whereas the opposite pattern was seen in association with low levels of IL-1β. This was especially true for children with recurrent episodes of acute otitis media (RAOM). In children with nasopharyngeal colonization with *Haemophilus influenzae*, significantly higher levels of IL-1β, IL-6 and TNF-α ($P = 0{\cdot}0001$, respectively) were found compared with non-colonized children. Notably, the RAOM children exhibited significantly lower levels of IL-1β, IL-6, and TNF-α in nasopharyngeal secretions ($P = 0{\cdot}0001$, 0.01 and 0.0001, respectively) than healthy children. These results demonstrate local production of inflammatory cytokines in nasopharynx, related to bacterial colonization, and suggest that children with RAOM are poor nasopharyngeal cytokine producers.

Keywords IL-1β IL-6 tumour necrosis factor-alpha nasopharyngeal secretions children

INTRODUCTION

The human nasopharynx plays a crucial role for development of immune responses to foreign microorganisms [1], and is a natural reservoir for several bacterial species, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, the majority of which are non-typable [2], and *Moraxella (Branhamella) catarrhalis*.

Bacterial adherence to the mucosa is a well known phenomenon in children. Carriership of pneumococci has been monitored in several epidemiological studies, particularly in the age group 1–2 years [3], where the colonization rate is about 90%. There is a subsequent decline in preschool children, and the colonization rate in adults is 10%. The epidemiology of non-typable *H. influenzae* is less well known, because of earlier difficulties in classifying this species. In one recent study the colonization rate was 35% in preschool children [4]. A decline with increasing age has also been demonstrated. Carriership is a potential mechanism for pathogenicity, since bacteria might invade the eustachian tube and the middle ear and cause disease. In episodes of acute otitis media, the same bacterial strains are found in the nasopharynx and in the middle ear cavity, as demonstrated with genetic fingerprinting [5]. Acute otitis media (AOM) is a common problem in children. Thus, in an epidemiological investigation 5% of children under the age of 4 years were reported to have contracted between six and 11 episodes [6]. An early onset of episodes is associated with development of the otitis-prone condition. AOM before 6 months of age was recently shown to be highly predictive of recurrent acute otitis media (RAOM) [7].

The pharyngeal tonsil or adenoid is a part of the mucosa-associated lymphatic tissue (MALT), responsible for local immune functions in the nasopharynx. The adenoid is highly organized into T and B cell areas, and contains epithelial cells, macrophages and dendritic cells. Thus, it possesses the cellular prerequisites for antigen uptake, processing, presentation and T–B cell cooperation [1].

Cytokines form a complex network of local mediators orchestrating specific immunity as well as non-specific

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responses to infectious agents [8]. The cytokines IL-1, IL-6 and tumour necrosis factor-alpha (TNF-α) are glycoproteins produced by a wide variety of cell types when exposed to bacteria and viruses [9]. These polypeptide hormones mediate a broad spectrum of biological activities, such as triggering of the acute phase response, T and B cell proliferation and differentiation [10]. Increased serum cytokine levels have been reported in various bacterial infections with septicaemia.

Virtually nothing is known concerning cytokine production in the nasopharynx. In this compartment, the heavy load of bacteria could imply a stimulus for cytokine production, leading to local cytokine levels at a detectable level. The aim of the present study was to elucidate the nasopharyngeal production of the inflammatory cytokines IL-1β, IL-6, and TNF-α in healthy children and otitis-prone children colonized with bacteria in the nasopharynx. All individuals studied, except the adults with immunodeficiency, lacked clinical signs of infection.

PATIENTS AND METHODS

Patient groups

The study groups consisted of 46 children, 18 healthy (2–89 months old, eight of which were < 18 months, median age 24 months), with 0–1 episodes of acute otitis media, and 28 children (8–38 months old, of which 13 were < 18 months, median age 20 months) at risk for development of the otitis-prone condition, with more than three episodes before 1 year of age or six episodes before 18 months of age, designated RAOM children. Of the 46 children, 41 were 3 years old or younger. Also, 21 healthy adults and 19 adults with hypogammaglobulinaemia or selective deficiency of IgG3 were included. The healthy adults and the healthy children did not receive prophylactic antibiotics, but 10 of the 28 RAOM children were treated. From three RAOM children, no information about antibiotics was available. From 23/28 RAOM and 12/18 healthy children information about other medical treatment was available, none of them received antihistamins, intranasal corticosteroids or decongestants. In 4/28 RAOM and 1/18 healthy children allergy was reported (two asthma, three to antibiotics and one to food).

Collection and preparation of secretions and sera

Specimens from the nasopharyngeal space were obtained more than 2 h after a meal with a suction device, the Juhn-Tym-Tap via the oral route, and care was taken not to damage the mucosa or to contaminate the sampled material with saliva or blood. In children, the collection had to be done under general anaesthesia (in healthy children due to non-otological surgery, and in RAOM children due to insertion of myringostomy tubes), all given the same type of premedication.

Because of our current regime with transmyringeal ventilating tube insertion in young patients at risk for recurrent episodes of middle ear disease, it was possible to collect nasopharyngeal secretions during the operating procedure. Due to small amounts (30–100 μl) and high viscosity of the nasopharyngeal specimens, the quantity from some individuals was not sufficient for all analyses planned.

The secretions were weighed and diluted 1:10 in PBS. For solubilization of the secretions, glass beads (2 mm in diameter) were added. The secretions were then centrifuged for 30 min at 4°C at 10 000 rev/min. The soluble fractions were immediately frozen at −70°C and stored until analysed. Sera were obtained from 39 children and from all healthy adults, and were centrifuged and stored as above.

Nasopharyngeal cultures

Specimens were obtained via the nasal route on the same occasion as the secretions, by using calcium alginate-tipped metal swabs (Calgiswab, Inolex, IL). For transport, Stewart's medium was used. The samples were submitted as ordinary routine specimens to the laboratory and inoculated within 3 h on sheep blood agar plates and haematin agar plates. All plates were incubated under aerobic conditions (haematin plates with addition of 5% CO_2) overnight at 37°C. *Streptococcus pneumoniae* was identified by typical colony morphology and optochin sensitivity. Growth requirements for X and V factor were determined for *H. influenzae*. *Moraxella catarrhalis* was diagnosed according to typical colony morphology, Gram staining and oxidase positivity.

Assays

Assays for IL-1β, IL-6 and TNF-α were performed with commercially available EASIA kits (Medgenix Diagnostics, Brussels, Belgium). The kits are based on the oligoclonal system, in which several MoAbs directed against distinct epitopes of IL-1β, IL-6 and TNF-α, respectively, were used. The optical density of the wells was measured at 490 nm by a Microplate autoreader (BioTekinstruments). A standard curve was plotted and concentrations of the cytokines in the samples

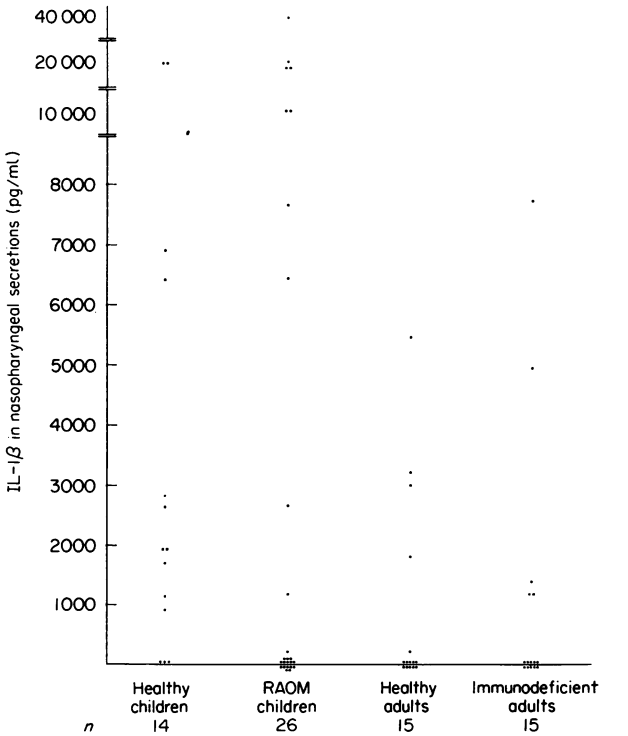

Fig. 1. The levels of IL-1β in nasopharyngeal secretions from healthy children, children with recurrent episodes of acute otitis media (RAOM), healthy adults and adults with immunodeficiency syndrome.

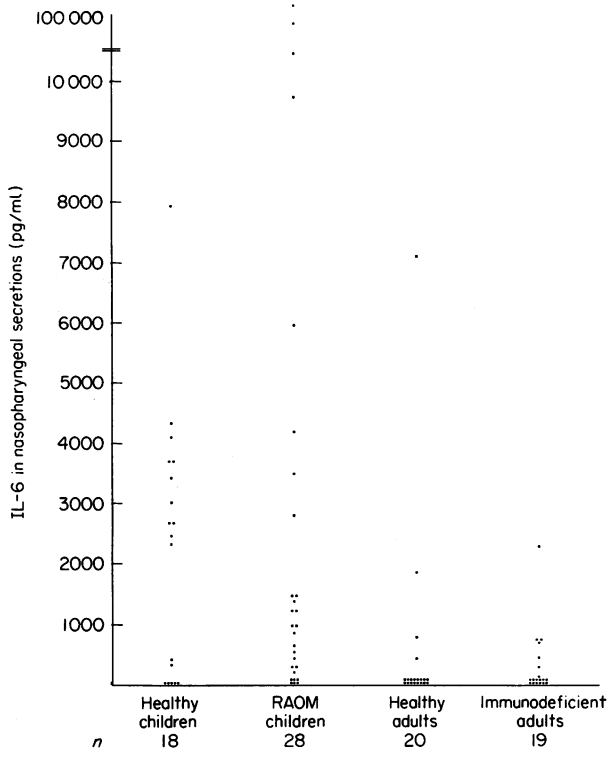

Fig. 2. IL-6 levels in nasopharyngeal secretions from healthy children, children with recurrent episodes of acute otitis media (RAOM), healthy adults and adults with immunodeficiency syndrome.

were determined by interpolation from the standard curve.The separate EASIA assays have been studied with respect to specificity for IL-1β, IL-6 and TNF-α, respectively. Thus, the IL-1β EASIA does not detect IL-6 and TNF-α, the IL-6 EASIA does not detect IL-1β and TNF-α, and the TNF-α EASIA does not detect IL-1β and IL-6. Minimum detectable concentrations of IL-1β were estimated to be 2 pg/ml, and for IL-6 and TNF-α, 3 pg/ml.

Absorption experiments overnight with selected IL-6-containing nasopharyngeal fluids using immobilized anti-human IL-6 antibodies (Genzyme, code 1618–01) were performed. Albumin was analysed according to standard laboratory methods (Department of Clinical Chemistry, Huddinge Hospital, Sweden).

Statistical analysis

The non-parametric, Mann–Whitney *U*-test was used when the concentrations of albumin were compared. χ^2 analysis was used when comparing levels of cytokines.

RESULTS

IL-1β, IL-6, and TNF-α in nasopharyngeal secretions

IL-1β. Seventy samples of nasopharyngeal secretions were analysed (Fig. 1). In 22 of 40 children (healthy 11/14 and RAOM 11/26), in five of 15 healthy adults, and in five of 15 adults with immunodeficiency, detectable levels of IL-1β were found. Healthy children showed significantly ($P = 0{\cdot}0001$) higher levels of IL-1β in nasopharyngeal secretions than RAOM children. The levels of IL-1β in healthy children were significantly ($P = 0{\cdot}0001$) higher than in healthy adults.

IL-6. Eighty-five samples from nasopharyngeal secretions were analysed (Fig. 2). Detectable levels of IL-6 were found in 35 of 46 children (healthy 13/18 and RAOM 22/28), in four of 20 healthy adults, and in six of 19 adults with hypogammaglobulinaemia. Healthy children exhibited significantly ($P = 0{\cdot}01$) higher levels of IL-6 than RAOM children. A significant difference was also found when healthy children were compared with healthy adults ($P = 0{\cdot}0001$).

TNF-α. Eighty-one samples of nasopharyngeal secretions were analysed (Fig. 3). In 14 of 42 children (healthy 7/15 and RAOM 7/27), in two of 20 healthy adults, and in one of the 19 adults with immunodeficiency, detectable levels of TNF-α were seen. The levels of TNF-α in nasopharyngeal secretions were significantly higher in healthy children ($P = 0{\cdot}0001$) than in RAOM children. Healthy children showed significantly ($P = 0{\cdot}01$) higher levels than healthy adults. In all but three children with detectable TNF-α, IL-6 was also demonstrated.

No differences were found in the levels of IL-1β, IL-6 and TNF-α in nasopharyngeal secretions from healthy adults and adults with hypogammaglobulinaemia, respectively.

IL-1β, IL-6 and TNF-α in children with H. influenzae *colonization in nasopharynx*

In total, nine of the 40 children investigated were culture positive for *H. influenzae* in the nasopharynx. These children showed significantly higher nasopharyngeal levels of IL-1β ($P = 0{\cdot}0001$), IL-6 ($P = 0{\cdot}0001$) and TNF-α ($P = 0{\cdot}0001$) than the ones not colonized with *H. influenzae* (Fig. 4).

Possible effects of antibiotics on cytokine production

Ten of the 26 RAOM children were treated with prophylactic antibiotics. Cytokine levels were compared in 14 non-treated healthy and 16 non-treated RAOM children (Fig. 5). The 16

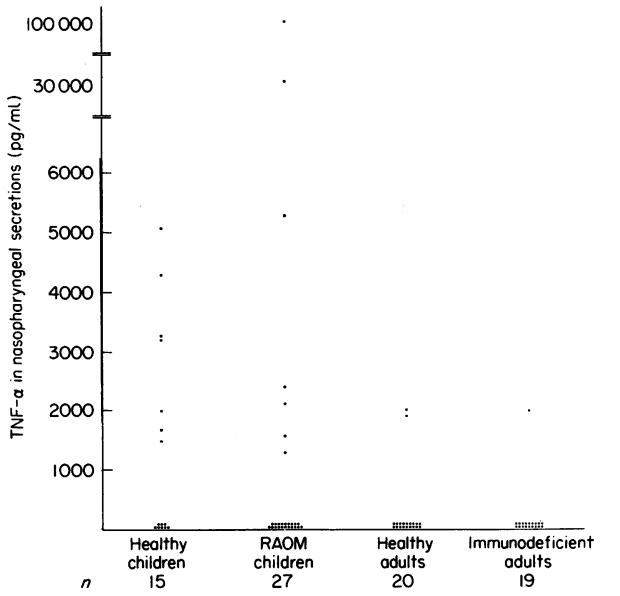

Fig. 3. The levels of tumour necrosis factor-alpha (TNF-α) in nasopharyngeal secretions from healthy children, children with recurrent episodes of acute otitis media (RAOM), healthy adults and adults with immunodeficiency syndrome.

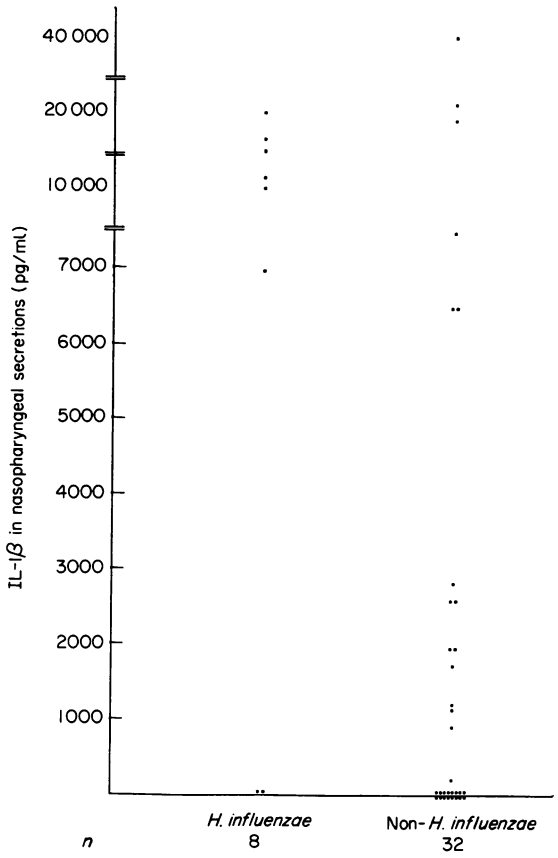

Fig. 4. IL-1β levels in nasopharyngeal secretions in children, culture-positive or -negative for *Haemophilus influenzae*.

non-treated RAOM children exhibited significantly lower levels than the 14 healthy children. This difference was significant ($P = 0{\cdot}001$). Higher levels of both IL-6 and TNF-α were also seen in the healthy children, but the difference was not significant.

Comparison of IL-1β, IL-6 and TNF-α in individual nasopharyngeal secretions

In all 14 healthy children and in 22 of the 26 RAOM children, one or more of the three cytokines were elevated. Two different patterns were found. Sometimes, high levels of IL-1β were associated with low or undetectable levels of IL-6 and TNF-α, whereas low levels of IL-1β were associated with high levels of IL-6 and TNF-α, especially in RAOM children.

Detectability of cytokines in nasopharyngeal secretions

In order to study the specificity of the detection of cytokines in nasopharyngeal fluid, a number of experiments were undertaken. Table 1 shows the capacity of the EASIA assays to detect different concentrations of IL-1β, IL-6 and TNF-α when added to negative samples of nasopharyngeal secretions. Separate nasopharyngeal secretions did not influence the detectability of the added cytokines, and there was negligible variation in degree of recovery among the samples in each of the three cytokine-specific immunoassays. Because the EASIA assays routinely are applied to cytokine determinations in serum, it was considered valuable to elucidate whether nasopharyngeal cytokines were detectable in a serum environment. Therefore, three IL-1β-positive and three IL-1β-negative nasopharyngeal secretions were dialysed, lyophilized and subsequently diluted in normal human serum (IL-1β-negative). When analysed with respect to IL-1β the three IL-1β-positive serum-diluted nasopharyngeal samples were shown to contain 1036, 162, and 118 pg/ml, respectively, whereas the three negative contained 0, 0, and 0 pg/ml. It follows that the detectability of cytokines in nasopharyngeal fluid does not seem different from the detectability in serum. In order to elucidate further the specificity of the cytokine EASIA measurements in nasopharyngeal fluid, two IL-6-positive samples were absorbed on an anti-IL-6-affinity matrix. This absorption decreased the IL-6 concentration of one sample from > 1000 pg/ml to 163 pg/ml, and of the second sample from 377 pg/ml to 176 pg/ml. Control absorption caused negligible reduction of the IL-6 concentration of the samples. Measurements of cytokine IL-1β, IL-6 and TNF-α concentration after dilution of samples yielded similar results as measurements of undiluted ones, showing that there was no interference by factors in the undiluted samples.

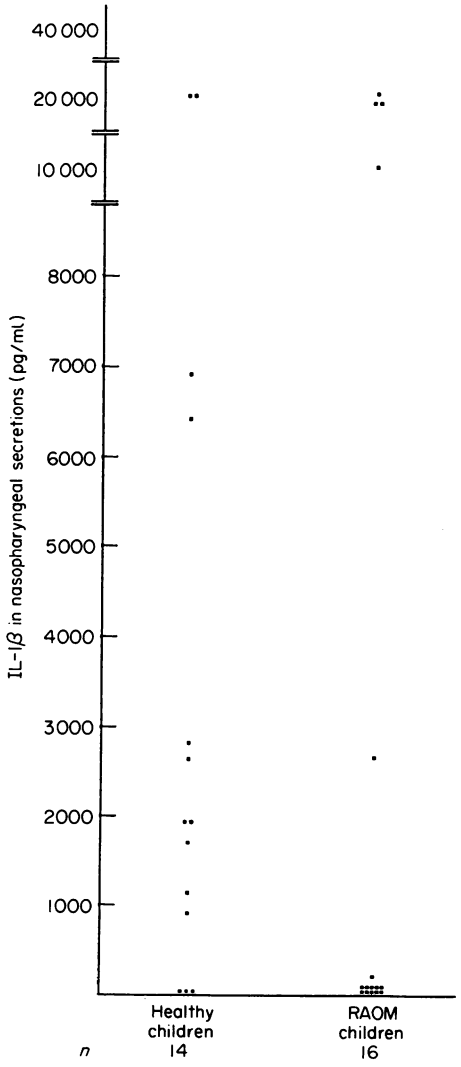

Fig. 5. The levels of IL-1β in nasopharyngeal secretions in children not treated with antibiotics.

Table 1. Detectability of cytokines in nasopharyngeal secretions. Known concentrations of IL-1β, IL-6 and TNF-α added to negative samples

	Added (pg)	Detected (pg)	Added (pg)	Detected (pg)	Added (pg)	Detected (pg)
IL-1β	25	42 (38–47)	250	338 (311–366)	500	538 (495–582)
IL-6	10	30 (21–39)	75	91 (88–94)	500	414 (371–457)
TNF-α	6	2 (0–5)	65	64 (55–72)	190	195 (151–240)

Cytokine levels in sera

Selected serum samples, 13 of which were analysed with respect to IL-1β, and 17 with respect to TNF-α, showed low or undetectable levels of these cytokines (IL-1β < 112 pg/ml, TNF-α < 43 pg/ml). Forty-two sera from the children and healthy adults with IL-6 exceeding 20 pg/ml in nasopharyngeal secretions generally showed negligible levels of IL-6.

Nasopharyngeal cultures

In total, 19 of the 42 children (healthy children 8/16 and RAOM children 11/26) and two of the 21 healthy adults were considered as culture-positive. *Streptococcus pneumoniae* was found in four of the healthy children, in one of the RAOM children and in two of the healthy adults. *Haemophilus influenzae* was found in two of the healthy children and in six of the ROAM children, whereas *M. catarrhalis* were found in two of the RAOM children. In one of the healthy and in five of the RAOM children a mixture of bacteria was found. In total, nine of the children were colonized with *H. influenzae*, either alone or in combination with *Strep. pneumoniae* and/or *M. catarrhalis*.

Albumin

The albumin concentration was measured both in secretions (mg/*l*) and in serum (g/*l*). In secretions, no difference was found between healthy children and RAOM children. The quotient of albumin in secretions (mg/*l*) and in sera (g/*l*) was 0·16 in the adult group and 0·38 in the two groups of children ($P = 0{\cdot}0002$). The higher levels in children indicate a higher degree of leakage from serum.

DISCUSSION

Although cytokines have been implicated in inflammatory reactions in many diseases, little is known about their role in immune responses to bacteria colonizing mucosal membranes in individuals without clinical signs of infection. The present study provides information about cytokines in nasopharyngeal secretions, and about cytokine production in the nasopharyngeal mucosa.

Thus, nasopharyngeal secretions of children, mainly 1–3 years old, were found to contain substantial quantities of IL-1β (22 positive of 40), IL-6 (35 positive of 46), and TNF-α (14 positive of 42). In a few cases extremely high levels of IL-1β, IL-6 and TNF-α were detected in the absence of upper respiratory tract infection. In contrast, children with recurrent episodes of otitis media exhibited significantly lower nasopharyngeal IL-1β, IL-6 and TNF-α levels than healthy children. Healthy adults had IL-1β, IL-6 and TNF-α levels which were significantly lower than in healthy children. TNF-α was detected less frequently than IL-1β and IL-6, but was correlated to positive IL-6 determinations. The very high IL-1β levels in nasopharyngeal fluid was associated with low levels of IL-6 and TNF-α, whereas low levels of IL-1β were found in children with high levels of IL-6 and TNF-α. This pattern was most pronounced in RAOM children. Of healthy adults and adults with immunodeficiency approximately 30% were found to have detectable levels of IL-1β. It is possible that serum leakage into nasopharynx, a phenomenon known to be more prominent in children than adults [11], caused dilution of the nasopharyngeal fluid. Thus, were it not for this dilution it is likely that the levels of cytokines in nasopharyngeal secretions of children, as measured in this study, would have been even more elevated.

The present results contain several findings which merit consideration and discussion. Accordingly, the high and even extremely high levels of IL-1β, IL-6 and TNF-α in nasopharyngeal secretions in healthy children, and in a minority of adults, clearly indicate an activation of the local synthesis in epithelium and/or lymphatic tissue. The high cytokine levels in nasopharyngeal secretions probably depend primarily on the presence of inducing factors such as lipopolysaccharide (LPS) from Gram-negative bacteria. However, it is also possible that the nasopharynx of children contain a relatively large number of potent producer cells in the epithelium, the subepithelial region or the organized lymphatic tissue. Epithelial cells in the urinary tract have been found to produce IL-6 [12] and the cryptepithelium in the respiratory tract may also play a role in cytokine production besides intra- or subepithelial macrophages and T cells. The relatively weak production of cytokines in patients with immunodeficiency diseases, in spite of heavy *H. influenzae* colonization, may reflect that the mucosa of these patients contain few producer cells. Furthermore, the poor cytokine production may constitute a primary reason for defective local immunity.

The LPS component of Gram-negative bacteria has been shown to induce production of IL-1β, IL-6, TNF-α and possibly other mediators of inflammation [9,13]. In several models of experimental urinary tract infections in mice and humans, *Escherichia coli* has been found to be a potent inducer of IL-6 [12,14]. *Haemophilus influenzae* is considered the most pathogenic organism associated with chronic secretory otitis media [15], which is an inflammatory process in the middle ear

cleft. Endotoxin is present in a high percentage of middle ear effusions [16], and significant levels of IL-1β, IL-6 and TNF-α have been found in the fluid [17]. Also, teichoic acid from Gram-positive bacteria can induce cytokine production, and high cytokine levels were demonstrated in serum of sepsis patients with both Gram-negative and Gram-positive infections [18,19].

The high nasopharyngeal cytokine levels may seem reasonable in the light of the fact that the upper respiratory tract is a common site of Gram-negative bacterial colonization in children [4] and adults with antibody deficiency diseases [20]. IL-1β, IL-6 and TNF-α levels were significantly higher in children with demonstrable *H. influenzae* growth in nasopharyngeal cultures than in culture-negative children. The capacity of *M. catarrhalis* to induce cytokine production is less well known, but a contribution of LPS from this species to cytokine production cannot be excluded. Also, pneumococci may contribute to cytokine induction, but only few children were culture-positive in the present study.

Bacterial adherence to epithelial and pharyngeal cells has been extensively studied [21], but still the mechanisms of colonization are only partly understood. It is a clearly age-dependent phenomenon, and a role of the local immune response in the clearance of bacteria from the mucosa has been postulated [11]. In a recent study of bacterial load on homogenized adenoid lymphatic tissue, *H. influenzae* was found in only 50% of the nasopharyngeal cultures corresponding to a positive quantitative culture. This suggests that bacteria are harboured in crypts or in the lymphatic tissue [22]. In a recent study it has been possible to demonstrate *H. influenzae* intracellularly in macrophages by electron microscopy and by RNA hybridization [23]. *Haemophilus influenzae* LPS may thus exert a chronic stimulation of local immune reactivity.

The present observation that children at risk for development of the otitis-prone condition often lacked nasopharyngeal cytokine activity, points to the possibility that they have a local defect in cytokine production. Such a putative defect may be responsible for defective immune reactivity. A defective or immature immune system, mainly demonstrated as low levels of the IgG2 subclass and of low specific antibody activity against common pneumococcal serotypes in serum, in a significant number of highly otitis-prone children, has been reported in several prospective controlled studies [24–26], and may be consistent with the present findings.

The poor cytokine production in RAOM children is probably not a result of antibiotics. Prophylactic treatment at the time of operation was given to 10 otitis-prone children, and might have reduced bacterial load on mucosa and lymphatic tissue, resulting in lower cytokine levels. However, the fact that the 16 individuals not treated with antibiotics also showed statistically low cytokine production indicates that antibiotics did not suppress cytokine induction. In a prospective study of otitis-prone children, *H. influenzae* was isolated from the nasopharynx even after antibiotic treatment of AOM in about 70% of the episodes [25]. Despite the fact that it is justified to assume that children requiring antibiotics were more severely affected than those not given any treatment, RAOM children without antibiotic prophylaxis were found to have significantly lower levels of IL-1β.

One important implication of the present study is that measurements of cytokines in serum do not necessarily reflect a pronounced local synthesis. Thus, the high cytokine levels in nasopharynx were undetectable in serum. These results are consistent with a number of earlier studies demonstrating cytokine production in compartments such as cerebrospinal fluid [9] and urine [12], but not in serum. Most studies show low or undetectable levels of IL-1, IL-6 or TNF-α in sera of healthy individuals [12,18,27]. Elevated levels of serum cytokines have been found in relation to surgery and trauma [28], and in patients with sepsis [18]. Elevated levels of cytokines in other serious diseases in infants and children [29], or in myeloma in adults [30], have been found.

To our knowledge high cytokine levels have not been reported earlier in healthy individuals, and there is little or no information about defective production in certain individuals. Elucidation of nasopharyngeal cytokine function requires further studies, and the cells responsible for the nasopharyngeal cytokine response remain to be identified. One plausible function of cytokines in nasopharyngeal secretions may be to regulate the local inflammatory response to microorganisms.

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