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Cytokines in otitis media with effusion, Il-8 as the pivotal mediator of ongoing inflammation

Otitis media (OM) is one of the most common diseases affecting children (7, 9). It has been estimated that approximately 5% to 10% of children suffering from acute otitis media (AOM) have progression to a chronic form of this disease, known as chronic otitis media with effusion (COME). One of the most significant complications resulting from COME is hearing loss that adversely affects speech development in children, and permanent middle ear damage (4, 5, 7, 10).

Otitis media with effusion (OME) is a chronic inflammatory condition characterized by the collection of viscous, mucin-rich effusion in the middle ear cavity that cannot be cleared by the normal mucociliary transport mechanisms. Signs and symptoms of acute infection, such as otalgia and fever are absent and the tympanic membrane is intact (3, 11, 14).

Formation of the effusion in the middle ear cavity is associated with the differentiation of basal cells into goblet cells accompanied by subsequent proliferation in a modified respiratory epithelium (11).

Despite intensive research, the etiology and pathogenesis of OME still remains unclear. Nevertheless, inflammation in the middle ear mucosa is taken into account as the crucial event in the middle ear predisposing to OME development (11, 12). Both bacteria and viruses have been implicated as initial stimuli that evoke a local inflammatory reaction along with predisposing factors such as Eustachian tube and ciliary dysfunction, cleft palate, and obstructive adenoids (3, 12, 13, 14).

The most commonly cultured bacteria from the middle ear are *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* but in chronic OME cultures of middle ear effusions (MEEs) yield positive results for only 20–30% of patients (10).

Although the role of bacteria in OME has not been fully elucidated, it has been suggested that bacterial products are able to produce a mucosal and immunological response, which continues even after viable microorganisms are cleared. Therefore, retained bacterial antigens may contribute to the persistence of middle ear inflammatory process and effusion (3, 5, 12, 14).

Cytokines in the middle ear cavity are suggested to play a pivotal role in the regulation of ongoing inflammation in OME by participating in the inflammatory cascade in MEEs (9). Although

the control of inflammation mediated by cytokines represents a beneficial response to infection and injury, these substances are also responsible for pathologic changes, including mucosal hyperplasia, bone erosion, fibrosis and hearing loss (16).

A variety of cytokines, especially interleukin 1-β (IL-1β), tumor necrosis factor-α (TNF-α) and IL-8 have been considered to play a key role in the initiation and maintenance of the inflammatory response seen in OME (8).

Since studies of MEEs enable to gain insights into the local inflammatory process, the primary goal of our study was to measure levels of the pro-inflammatory cytokines such as IL-8, TNF-α and IFN-γ in MEEs obtained from children suffering from COME. Peripheral concentration of these cytokines in patients' serum samples was also determined.

MATERIAL AND METHODS

23 children suffering from COME were enrolled in this study. The children ranged in age between 2 and 9 years with a mean of 5.09 years.

MEEs were aspirated from consecutive patients. When the disease was bilateral (12 cases) the samples were taken from both ears. Serum samples for measurement of IL-8, TNF-α and IFN-γ concentrations also were collected from patients included in our study. Concentrations of IL-8, TNF-α and IFN-γ in the MEE and serum samples were measured using commercially available enzyme linked-immunosorbent assay (ELISA) – PharMingen USA (OptEIA™Set: Human IL-8, TNF-α and IFN-γ). Each kit consists of a murine monoclonal antibody against the relevant human cytokine (*Capture Antibody*), biotinylated anti-human cytokine monoclonal antibody (*Detection Antibody*), avidin-horseradish peroxidase conjugate (*Enzyme Reagent*), and tetramethylbenzidine and hydrogen peroxide (*Substrate Solution*). Tests were performed according to the manufacturer's instructions.

Briefly, microwells were first coated with diluted *Capture Antibody* and incubated overnight at 4°C. After washing and blocking plates with *Assay Diluent*, each standard, sample, and control were pippeted into appropriate wells. The plate was incubated for 2 hours at the room temperature and subsequently washed. After washing *Working Detector* (*Detection Antibody* + *Enzyme Reagent*) was added to each well. The plate was incubated for 1 h at the room temperature and subsequently washed. Next *Substrate Solution* was added to each well and plate was incubated for 30 min in the room temperature in the dark. In the final stage *Stop Solution* was added to each well. Absorbance was read at 450 nm within 30 min of stopping reaction. Standard curves were generated from known concentrations of cytokine provided by the manufacturer. Standard curves were subsequently used to predict the quantity of cytokine based on the level of spectrophotometric absorbance of each sample. Sensitivity of the used tests is 1 pg/ml, 2 pg/ml and 4 pg/ml for IL-8, TNF-α and IFN-γ, respectively.

Values of the analyzed cytokines are given in the text as mean (pg/ml) ± standard error (SE).

Statistical analysis Depending on the results of the Normality test *Student's* t-test or Mann-Whitney's *U*-test was used to analyse significance of differences between various groups. Spearman's correlation test was performed and correlation coefficients were calculated to determine the correlation between peripheral and local concentrations of the pro-inflammatory cytokines.

RESULTS

The mean values for the cytokines found in the MEEs and in serum samples are shown in Table 1. We found that the mean concentrations of IL-8 in the MEEs were the highest among the measured pro-inflammatory cytokines and were significantly higher than its mean level in patients' serum ($p<0,001$). The mean values for TNF-α and IFN-γ in the MEEs were comparable to their mean concentrations in serum. Statistically significant correlation was found between the mean concentration of IFN-γ in the MEEs and its mean level in serum ($p=0.02$; $R=0.52$).

Table 1. The mean concentrations of IFN-γ, IL-8 and TNF-α in the MEEs and in patients' serum

Cytokine	Mean concentration (pg/ml) ± standard error (SE)
IFN-γ -MEEs	18.23 ± 4.29
IFN-γ -serum	17.82 ± 4.51
IL-8 -MEEs	381.71 ± 51.70 *
IL-8 -serum	6.9 ± 2.96
TNF-α -MEEs	36.15 ± 10.33
TNF-α -serum	47 ± 12.17

*Significantly higher ($p<0.001$) mean level of cytokine in the MEEs compared to its mean concentration in serum

IL-8 was detected in the highest percentage of the examined effusions. The detectable levels of this cytokine were found in 91.1% of the MEEs. 47.8% of the serum samples demonstrated the presence of IL-8. TNF-α was present in 61.7% of the analysed effusions versus 78.2% of the serum samples. The percentages of the MEEs and serum samples with detectable IFN-γ were 54.5% and 69.5%, respectively.

We also observed an interesting association between hearing loss degree and the mean concentrations of the cytokines in the MEEs (Table 2). A tendency toward elevated levels of the analyzed pro-inflammatory mediators both in the MEEs and in serum of patients suffering from more significant hearing loss degree (>20 dB) was demonstrated (Table 2). However, no significant differences were found in the mean concentrations of the cytokines between groups of patients suffering from more significant hearing loss (>20 dB) and subjects with less severe hearing loss degree (20 dB).

Table 2. The mean concentrations of IFN-γ, IL-8 and TNF-α in MEEs and in serum depending on the degree of hearing loss

	The mean cytokine concentration (pg/ml) ± standard error (SE)	
Cytokine	hearing loss 20 dB	hearing loss >20 dB
IFN-γ -serum	9.12 ± 4.11	19.07 ± 6.02
IFN-γ -MEEs	8.12 ± 5.55	17.29 ± 4.95
IL-8 -serum	1.32 ± 1.09	9.30 ± 4.63
IL-8 -MEEs	298.06 ± 75.70	395.49 ± 68.72
TNF-α -serum	28.84 ± 17.66	48.35 ± 13.82
TNF-α -MEEs	13.15 ± 8.12	45.25 ± 15.15

Additionally, we estimated concentrations of the pro-inflammatory cytokines in bilateral effusions and found differences for their levels in both ears in some patients (Table 3). The mean concentrations of IL-8 and TNF-α tended to be higher in the left ear effusions compared to their mean levels in the right ear effusions. The mean concentration of IFN-γ in the right ear effusions was elevated in comparison with its mean concentration in the left ear effusions. However, the results did not achieve statistical significance.

Table 3. Concentrations of the pro-inflammatory cytokines in bilateral effusions

Patient number	Left ear IL-8	Right ear IL-8	Left ear TNF-α	Right ear TNF-α	Left ear INF-γ	Right ear INF-γ
1	219.4	553.6	8.2	0.0	0.0	0.0
2	1073.6	29.9	26.2	88.2	6.7	59.6
3	251.5	708.4	0.0	43.5	6.7	65.9
4	904.7	170.4	0.0	47.8	79.3	65.9
5	334.2	349.9	250.9	66.7	0.0	0.0
6	329.9	402.1	1.3	2.2	0.0	0.0
7	511.5	639.9	0.0	75.3	0.0	0.0
8	0.0	323.6	75.3	3.0	0.0	0.0
9	370.5	188.4	231.1	21.1	41.7	58.7
10	111.5	0.0	0.0	ND	26.5	ND
11	473.6	89.9	0.0	0.0	3.1	0.0
12	178.9	ND	0.0	0.0	26.5	ND
The mean level (pg/ml)±standard error (SE)	396.6 ± 90.58	314.19 ±73.49	49.41 ±26.61	31.61 ±10.24	15.87 ±7.02	25.01 ±10.23

DISCUSSION

An ongoing, chronic inflammatory state, which is one of the salient features of COME, results from retention of inflammatory products and cells in the middle ear cavity in the course of infection and eustachian tube dysfunction with poor drainage. The presence of neutrophils, lymphocytes, macrophages, and cellular remnants in the effusion fluid and subepithelial space of the middle ear cavity has been shown (7, 17).

Cytokines are potent mediators of middle ear inflammation and regulators of the immune response. These substances also play a significant role in the stimulation of the molecular-pathological processes in middle ear tissues, leading to histopathological changes in the middle ear cavity and the pathogenesis of OME (9, 12, 14, 16, 17).

The presence of a variety of potent inflammatory cytokines including TNF-α, IL-1β, IL-2, IL-6, IL-8 and IFN-γ has been demonstrated in MEEs (1–9, 13, 16, 17). IL-8 is related to chemokine family of cytokines and is produced mainly by monocytes, macrophages, neutrophils, endothelial cells, epithelial cells and fibroblasts. A wide range of stimuli encompassing pro-inflammatory cytokines such as IL-1 and TNF-α as well as bacterial, viral products, and cellular stress rapidly induces IL-8 production. IL-8 participates in the initiation and maintenance of the inflammatory process in a variety of tissues; high levels of this chemokine are observed in association with both acute and chronic inflammatory conditions. IL-8 is responsible for the accumulation of leukocytes in the middle ear cavity, which constitutes a hallmark of COME. This cytokine acts as chemotactic

factor for different types of leukocyte cells such as neutrophils, T lymphocyte subsets, and basophils. In addition to its potent chemoatractant function, IL-8 activates polymorphonuclear leukocytes to release intracellular enzymes, undergo a respiratory burst, and degranulate. It also regulates the adherence of neutrophils to endothelial cells and mediates the transmigration of neutrophils across endothelium (2, 3, 4, 6, 7, 9, 11, 12, 13).

The toxic effects of neutrophils can be attributed to the degranulative release of various proteolytic enzymes but also to the production of reactive oxygen intermediates during the respiratory burst. Hence, primarily due to its potent and selective chemotactic and activating properties toward polymorphonuclear leukocytes, IL-8 may play a key role in causing tissue damage, which leads to the prolongation of middle ear inflammation and the chronicity of the disease (2, 15).

Smirnova et al. demonstrated that goblet cells, whose population is increased in the course of OME, were able to secrete IL-8 upon stimulation with TNF-α and IL-1β. These authors also observed that this chemokine stimulated the prolonged secretion of mucin from goblet cells; this activity of IL-8 could be associated with converting inflammation of the middle ear to the chronic stage and with the maintenance of the chronic disease (11).

The results of our study are in keeping with other previous reports indicating that the level of IL-8 and the percentage of effusions demonstrating the presence of this cytokine are the highest among pro-inflammatory mediators detected in MEEs (3, 7).

Our study demonstrated the presence of IL-8 in 91.1% of the examined MEEs. The mean values for this cytokine in the MEEs were the highest among the other measured pro-inflammatory cytokines such as TNF-α and IFN-γ. The mean concentrations of IL-8 for the analyzed effusions were significantly higher ($p<0.001$) than its mean level in serum. These findings reflect a strong inflammatory response confined to the middle ear cavity and suggest a central role played by IL-8 in producing inflammation.

High concentration of IL-8 in the analyzed MEEs obtained from children with COME is also suggestive of involvement of this powerful leukocyte chemotactic factor in the prolongation of the inflammatory process in the middle ear and the maintenance of OM in the chronic stage.

Since IL-8 expression and secretion during middle ear inflammation is controlled by the primary pro-inflammatory cytokines, IL-1β and TNF-α, this chemokine can be considered as the secondary mediator of the ongoing inflammatory process in the middle ear cavity (12).

Considerable MEE levels of TNF-α and IFN-γ were also detected in our study. TNF-α is considered to be the primary cytokine in OME since it is produced in the early stage of inflammatory process by the middle ear mucosa and in the late stage by accumulating inflammatory cells (12).

TNF-α is mainly produced by activated macrophages and involved in various pathologic processes associated with OM such as generation of mucoid effusion, fibrosis, bone resorption, and tissue damage, given its effects in stimulating production of prostaglandin E_2 and collagenase, enhancing proliferation of fibroblasts and activity of this cytokine as factor activating osteoclasts and polymorphonuclear leukocytes (5, 7, 12, 17). The high level of TNF-α production by macrophages present in the middle ear seems to correlate with the more protracted clinical courses of COME. Therefore, this cytokine appears to be a marker for OME chronicity and related to the persistence of this disease process (1, 2, 3, 12, 16, 17).

IFN-γ is produced by T-lymphocytes in response to viral or bacterial challenge. This cytokine activates macrophages, stimulates cytotoxic cell activity and increases B-cell differentiation and antibody production. Similarly to other cytokines, IFN-γ has the potential to help clear infection from the middle ear but it can also contribute to tissue damage (17).

Additionally, we found an interesting association between hearing loss degree and the mean concentrations of the analyzed cytokines. A tendency toward elevated levels of the analyzed pro-inflammatory cytokines both in the MEEs and in serum was demonstrated in children with hearing loss >20 dB. We conclude that significant hearing loss observed in some subjects with COME could be associated with higher levels of inflammatory mediators such as TNF-α, IL-8 and IFN-γ in MEEs. Since these cytokines have the potential to promote adverse changes in the middle ear such as stimulation of cartilage and bone resorption, fibrosis, leukocyte recruitment and activation leading to tissue damage, mucosal changes and stimulation of mucin production, they may contribute to hearing loss.

Measurement of concentrations of pro-inflammatory cytokines in bilateral effusions revealed that pathological changes might occur independently in each ear. Johnson et al. demonstrated significant differences for reduced specific viscosity, mucin content, protein content and levels of IL-8 in paired ears and postulated that a magnitude of inflammatory processes or a phase of inflammatory response (such as chronicity) could differ between the two ears (3).

Although differences for the mean concentrations of IL-8, TNF-α and IFN-γ between patients' ears did not achieve statistical significance in our study, we observed differences for their mean levels in bilateral effusions. Our findings corroborate the hypothesis that inflammatory processes might occur independently when the disease affects both ears.

CONCLUSIONS

In the light of the present study, pro-inflammatory cytokines such as IL-8, TNF-α and IFN-γ appear to be important factors involved in the local immune response in the middle ear cavity. The results of our study emphasize the role of IL-8 as the pivotal mediator of the chronic middle ear inflammation as its mean concentration and the percentage of the effusions demonstrating the presence of this cytokine were the highest among the measured pro-inflammatory cytokines.

The increased levels of the inflammatory mediators in the MEEs seem to be responsible for pathologic changes such as hearing loss observed in some subjects suffering from COME; differences for their mean concentrations in bilateral effusions might indicate the possibility of independent inflammatory processes in each ear.

Hence, detection and persistent presence of the pro-inflammatory cytokines in MEEs would be helpful in illuminating and elucidating the problem of OM pathogenesis and chronicity.

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SUMMARY

Otitis media with effusion (OME) is a chronic inflammatory disease characterized by the collection of viscous, mucin-rich effusion in the middle ear cavity that cannot be cleared by the normal mucociliary transport mechanisms. Signs and symptoms of acute infection, such as otalgia and fever are absent and the tympanic membrane is intact. Cytokines in the middle ear cavity are suggested to play a pivotal role in the regulation of ongoing inflammation in OME by participating in the inflammatory cascade in middle ear effusions (MEEs). The primary goal of our study was to measure levels of the pro-inflammatory cytokines such as IL-8, TNF-α and IFN-γ in MEEs obtained from children with chronic OME. Peripheral concentration of these cytokines in serum samples also was determined. 23 children with chronic OME were enrolled in this study. Concentrations of IL-8, TNF-α and IFN-γ in patients' sera and in the MEE samples were measured using enzyme linked-immunosorbent assay (ELISA). The mean MEE concentration and the percentage of the effusions demonstrating the detectable levels of IL-8 were the highest among the measured pro-inflammatory cytokines. The mean value for this cytokine in the MEEs was 381.7 ± 51.7 pg/ml and was significantly higher ($p<0.001$) than its mean concentration in serum, which was 6.9 ± 2.96 pg/ml. Additionally, a tendency toward elevated levels of the analyzed pro-inflammatory cytokines both in the MEEs and in serum was demonstrated in children with more significant hearing loss degree (>20 dB). Differences

were also observed for the mean concentrations of the cytokines in bilateral effusions. The results of our study emphasize the role of IL-8 as the pivotal mediator of the chronic middle ear inflammation. Significant hearing loss observed in some subjects with chronic OME could be associated with higher levels of the inflammatory mediators such as TNF-α, IL-8 and IFN-γ in MEEs. Differences for the mean concentrations of the analysed cytokines in bilateral effusions might indicate the possibility of independent inflammatory processes in each ear.

Cytokiny w wysiękowym zapaleniu ucha: IL-8 jako zasadniczy mediator procesu zapalnego

Wysiękowe zapalenie ucha środkowego (*otitis media secretoria,* OMS) jest przewlekłą chorobą o charakterze zapalnym, przejawiającą się nagromadzeniem bogatego w mucynę, kleistego wysięku w jamie ucha środkowego, który nie ulega usunięciu przez mechanizm śluzowo-rzęskowy. W przebiegu OMS nie występują objawy ostrej infekcji, jak ból ucha i gorączka; błona bębenkowa pozostaje nienaruszona. Obecność cytokin w jamie ucha środkowego wydaje się odgrywać kluczową rolę w regulacji toczącego się procesu zapalnego w przebiegu WZUŚ poprzez ich udział w kaskadzie reakcji zapalnych. Celem badania było określenie stężenia prozapalnych cytokin, takich jak IL-8, TNF-α oraz IFN-γ w wysiękach z ucha środkowego pobranych od dzieci, u których zdiagnozowano przewlekłe OMS. Określono także stężenie tych cytokin we krwi obwodowej. Grupę badaną stanowiło 23 dzieci z przewlekłym OMS. Stężenie IL-8, TNF-α oraz IFN-γ w surowicy pacjentów oraz w wysiękach z ucha środkowego oznaczono z wykorzystaniem metody immunoenzymatycznej (ELISA). Średnie stężenie oraz procent wysięków wykazujących wykrywalne ilości IL-8 były najwyższe wśród cytokin, które poddano analizie. Średnie stężenie IL-8 w wysiękach wynosiło 381,7 ± 51,7 pg/ml i było znacząco wyższe ($p<0,001$) niż jej stężenie w surowicy, które wynosiło 6,9 ± 2,96 pg/ml. Dodatkowo wykazano tendencję w kierunku podwyższonego poziomu analizowanych cytokin prozapalnych zarówno w wysiękach, jak i w surowicy u dzieci z wyższym stopniem niedosłuchu (>20dB). Zaobserwowano także różnice w średnim stężeniu cytokin w przypadku wysięków obustronnych. Wyniki badania wskazują na zasadniczą rolę IL-8 jako mediatora przewlekłego procesu zapalnego toczącego się w obrębie ucha środkowego. Znaczący stopień utraty słuchu, obserwowany niekiedy w przypadku przewlekłego OMS, może być związany z podwyższonym poziomem mediatorów zapalnych, włączając IL-8, TNF-α oraz IFN-γ, w wysiękach z ucha środkowego. Różnice w obrębie średnich stężeń analizowanych cytokin, obserwowane w przypadku wysięków obustronnych, mogą wskazywać na możliwość niezależnie przebiegającego procesu zapalnego w obu uszach.