



Presence of otitis media with effusion and its risk factors affect serum cytokine profile in children

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KEYWORDS

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Cigarette smoke

Summary Otitis media with effusion (OME) is a condition that has significant impact on the quality of life of children. Although the etiology is multi-factorial, certain risk factors such as an allergic predisposition, daycare, and cigarette smoke exposure contribute to its pathogenesis.

Objective: (1) To determine whether there is a tendency for children with chronic or recurrent OME (cases) to have higher serum levels of the T-helper 2 cell (Th-2) allergenic-type cytokines, interleukin-4 (IL-4), and IL-5, or the T-helper 1(Th-1) infectious-type cytokines, interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α), compared to children without a history of recurrent OME (controls) and (2) to determine any possible correlations between the cytokine levels and risk factors associated with OME.

Methods: We analyzed serum levels of these four cytokines by enzyme-linked immunosorbent assays of 19 cases and 17 controls.

Results: Cases, independent of age, had increased levels of serum IL-5 compared to controls ($p = 0.014$). While a significant difference in serum IL-4 levels did not exist between cases and controls, children exposed to cigarette smoke had significantly higher levels of serum IL-4 ($p = 0.003$). While serum levels of IFN- γ were statistically significantly higher in cases than controls with univariate analysis ($p = 0.011$), when controlling for age and smoke exposure with multivariate analyses, the difference did not reach significance ($p = 0.086$).

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Conclusion: These results suggest that patients with chronic or recurrent OME and those exposed to cigarette smoke mount a Th-2 allergic-like response, as demonstrated by their serum cytokines.

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1. Introduction

Otitis media (OM) is a ubiquitous condition of early childhood accounting for 16 million physician office visits a year [1] at a national cost of \$3–6 billion [2]. Otitis media with effusion (OME) is thought to result as a response to chronic inflammation in the middle ear, and is characterized by persistence of middle ear effusion [3]. Tympanostomy tube placement to treat OME is the most common pediatric surgical procedure requiring anesthesia in the United States [4]. OME with effusion is associated with hearing loss, delayed speech development, and the potential for permanent middle ear damage [5,6]. Although the etiology is multi-factorial, certain risk factors such as an allergic predisposition, daycare, and cigarette smoke exposure contribute to its pathogenesis [7].

Recently, allergic causes have been suspected in the etiology. The 'hygiene hypothesis' proposes that overuse of antibiotics, vaccination, and an increased general sterility sense have decreased infection and non-allergic immune responses via T-helper 1 in early childhood. At the same time, however, due to a lack of early antigen exposure, there has been an associated increase in allergic responses via T-helper 2 cells contributing to disease [8,9].

While many cell mediators produced by the T-helper 1 and 2 cells are the same, the two pathways of immune response are characterized by their differing mediator production. Allergic T-helper 2 cells produce interleukin-4 (IL-4), IL-5, IL-9, and IL-13. Non-allergic T-helper 1 cells are characterized by their interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) production [10,11].

The primary aim of this study was to investigate the hypothesis that children with chronic OME (cases) have higher systemic levels of either of the allergic T-helper 2 cell mediators IL-4 or IL-5, than of either of the non-allergic T-helper 1 cell mediators IFN- γ or TNF- α in comparison with controls. The secondary aim was to investigate whether serum levels of these cytokines are affected by risk factors for OME, including smoke exposure, age, hayfever, asthma, allergy, and daycare.

2. Methods

Cases and controls were selected from eligible children who participated in the Genetic Epidemiology of Chronic/Recurrent Otitis Media Study, which enrolled families with two or more affected siblings [12]. Cases had a history of chronic or recurrent otitis media (OM) treated with tubes. Exclusion criteria were history of Down's syndrome, immune deficiency, cleft palate or other craniofacial anomaly, or any other condition predisposing to OM. Controls were unaffected children who participated in study visits in the above referenced study with their families. They had two or fewer OM episodes per year and did not have a history of tubes. Controls were excluded for the same conditions as cases. The research was approved by the University of Minnesota Institutional Review Board.

Cases were enrolled in the pre-op area at the University of Minnesota Medical Center, Fairview. The exception was one child with recent tube surgery enrolled at the time of the study visit. Parents provided written consent and HIPAA authorization, a short history of OM and risk factors for the child, and signed medical record releases for the child. Blood samples were drawn for DNA analysis, antibody and cytokine assay in the operating room or the General Clinical Research Center (GCRC). Serum samples were processed in the GCRC laboratory, frozen at -20°C , and transferred to the Pneumococcal Antibody Laboratory for inventory and storage at -70°C .

Enzyme-linked immunosorbent assays (ELISA) for human serum IL-4, IL-5, IFN- γ , and TNF- α were performed for each of the 17 control and 19 case subjects to quantify systemic cytokine levels as follows. Unaliquoted samples were thawed and used immediately for IL-4 ELISAs. The remainder of each sample was aliquoted into three smaller containers and stored at -70°C until IL-5, IFN- γ , and TNF- α assays were run.

In each 96-well plate, 16 wells were used for the standardization curve and the 36 serum samples were assayed in duplicate. Quantikine[®] human IL-4, IL-5, IFN- γ , and TNF- α ELISA kits were purchased from R&D Systems (Minneapolis, MN) and run per manufacturer's protocol as follows. Standard dilution series were made from stock solutions. Assay diluent was added to each well, then standard,

sample, or control was added to each well within 15 min. After a 2.5-h room temperature incubation, wells were aspirated and washed multiple times. Conjugate was added to each well for 2.5 h. Wells were again aspirated and washed multiple times. Substrate solution was added to each well for 30 min while protected from light. Stop solution was added to each well. Plates were read at 450 nm within 30 min with a 560 nm correction. Sensitivity of the cytokine assays (pg/ml) was <10, <3, <8, and <1.6 for IL-4, IL-5, IFN- γ , and TNF- α , respectively. Readings below the standard control zero values were assumed to equal no cytokine level.

3. Statistical analysis

Fisher's exact test and Chi-square analysis were used to determine significant differences between case and control history of hayfever, asthma, allergies, infant feeding type, cigarette smoke exposure, and daycare attendance. Cytokine levels in case and control groups were compared using Wilcoxon Rank Sum tests. Univariate analyses of predictor for cytokine levels were performed. Predictors were affected status (case/control), smoke exposure (yes/no), and age group (<5 or $\geq$ 5). Affected status, smoke exposure, and age group were each analyzed as independent predictors in the multivariate model, controlling for the other two factors, regardless of significance in the univariate analysis. Wilcoxon Rank Sum *p*-values are reported for univariate comparisons of cytokine levels between groups. ANOVA models were fit with the three factors that were significant by univariate analyses.

4. Results

Nineteen cases and 17 controls were group matched on age (<5 years, $\geq$ 5 years); the age range was 8 months–15 years. Twenty were male and 16 were female.

Case and control groups were comparable for most risk factors, but cases were younger than controls (Table 1). A trend existed for cases being more likely exposed to cigarette smoke than controls (42.1% versus 11.8%, *p* = 0.065). Those with a history of allergies comprised 15.8% of OME patients, while none of the controls reported such history. Likewise, while 10.5% of OME patients had a history of asthma, none of the controls did. However, due to the small number of subjects, excluding age, statistical significance between these groups was not demonstrated.

The mean age for cases was 4.3 years with a standard deviation of 3.9 years, a median of 2.5 years, and a 25–75 percentile range of 1.3–5.1 years. The mean age of the controls was 7.8 years with a standard deviation of 3.8 years, a median of 7 years, and a 25–75 percentile range of 4.7–10.6 years. This discrepancy in age is explained by the fact that in the genetic study from which the cases and controls were drawn, cases were younger, because of the fact that recurrent and chronic OM and tympanostomy tube treatment are more common in younger age groups. Controls were older so they would be less likely to have active OM, and old enough to have passed through the high risk ages for OM, so we would know they represented true controls. Given that age <5 has been shown to be an independent factor for otitis media [13,14] we analyzed it as a predictive category for each cytokine level both with univariate and multivariate analysis as described below.

On univariate analysis, cases had increased levels of serum IL-5 (mean = 4.75 pg/ml) and IFN- γ (mean = 2.39 pg/ml) compared to controls (IL-5 mean = 2.00 pg/ml and IFN- γ mean = 1.24 pg/ml) (*p* = 0.014 and *p* = 0.018, respectively, Fig. 1). Smoke exposure (yes/no), and age group (<5 or $\geq$ 5) were also examined as predictors for cytokine levels with univariate analysis (Table 2). Other predictors (hayfever, asthma, allergies, infant feeding type, daycare attendance, and gender) were not included in the univariate analyses because they were not significantly different between cases

Table 1 Demographic characteristics and otitis media with effusion (OME) predisposing factors in children with OME and controls

Factor	Cases, <i>N</i> = 19	Controls, <i>N</i> = 17	<i>p</i>
Age (<5 years)	12 (70.5%)	5 (29.4%)	0.043
Gender (male)	12 (70.5%)	8 (47.1%)	0.332
Cigarette smoke exposure	8 (47.1%)	2 (11.8%)	0.065
Breastfed	5 (26.3%)	4 (23.5%)	0.847
Daycare	13 (68.4%)	12 (70.6%)	0.888
Allergies	3 (15.8%)	0 (0%)	0.107
Hayfever	1 (5.2%)	1 (5.9%)	0.935
Asthma	2 (10.5%)	0 (0%)	0.169

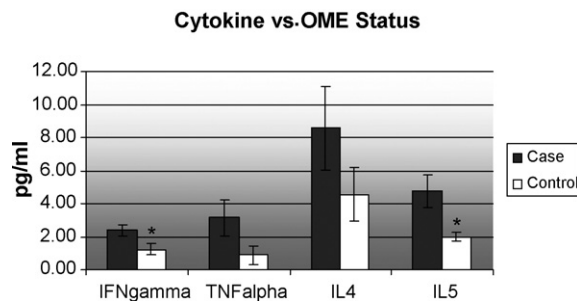


Fig. 1 Serum cytokine levels in chronic otitis media with effusion cases vs. controls. Values represent mean cytokine level. Error bars represent standard error of the mean. Statistical significance denoted with (*). IFNgamma = interferon-gamma, TNFalpha = tumor necrosis factor-alpha, IL4 = interleukin-4, IL5 = interleukin-5.

and controls (Table 1). In the multivariate analyses, all three predictors were included in the models to account for their contribution to cytokine levels (Table 3). While a significant difference in serum IL-4 levels did not exist between cases and controls, children exposed to cigarette smoke had significantly higher levels of serum IL-4 (mean = 13.73 pg/ml) than children not exposed to smoke (mean = 4.54 pg/ml, $p = 0.003$) (Table 3, Fig. 2). Although, as stated above, serum levels of IFN- γ were significantly higher in cases than controls with univariate analysis ($p = 0.011$, Fig. 1), these were not significant when controlling for age in multivariate analysis ($p = 0.065$). Using multivariate analyses, levels of IL-4, TNF- α , and IFN- γ were not found to be significantly different between cases and controls, although IFN- γ was borderline significant ($p = 0.496, 0.407, 0.082$, respectively).

5. Discussion

Otitis media with effusion (OME) is a common childhood condition posing a widespread impact in the

Table 2 p -Values from univariate analysis of serum cytokine levels vs. case/control status, age group (<5), and cigarette smoke exposure

Variable	Affected status	Age group	Smoke exposure
IFN gamma	0.018*	0.011*	0.846
IL4	0.200	0.965	0.003*
IL5	0.014*	0.231	0.133
TNF alpha	0.078	0.017*	0.099+

(*) Signifies statistically significant p -value < 0.05, (+) signifies borderline statistically significant risk factor, and therefore included in multivariate analysis. IFNgamma = interferon-gamma, TNFalpha = tumor necrosis factor-alpha, IL4 = interleukin-4, IL5 = interleukin-5.

Table 3 Multivariate models for cytokine level

Parameter	Estimate	p -Value
INF gamma		
Intercept (pg/ml)	0.41	
Affected (case)	0.83	0.08
Age group (<5 years)	0.95	0.05*
Smoke exposure (no.)	0.59	0.27
TNF alpha		
Intercept (pg/ml)	1.26	
Affected (case)	1.12	0.40
Age group (<5 years)	2.36	0.08
Smoke exposure	0	0.41
IL4		
Intercept (pg/ml)	14.42	
Affected (case)	2.11	0.49
Age group (<5 years)	0	<0.01*
Smoke exposure (no.)	0	<0.01*
IL5		
Intercept (pg/ml)	2.71	
Affected (case)	2.36	0.05*
Age group (<5 years)	0.35	0.76
Smoke exposure (no.)	0	0.48

(*) Signifies statistically significant p -value < 0.05.

quality of life of children [1,2,5,6]. Its etiology is multi-factorial. Some propose that allergic processes, mediated by humoral Th2-type cytokines, comprise an important mechanism in the occurrence of OME. A study by Sobol et al. found a predominance of eosinophils, T lymphocytes, and Th2 cell mediators in middle ear effusions of atopic children undergoing myringotomy with tube placement [15]. Several other studies have also examined local inflammatory mediators and cytokine expression in middle ear fluid samples, as summarized by Ryan et al. [16]. Only a few studies, however, have looked at systemic cytokines in this condition.

Harimaya et al. compared the cytokines secreted by adenoidal and peripheral blood lymphocytes cultured cells taken from children with otitis media.

Serum Cytokines in OME and Smoke Exposure

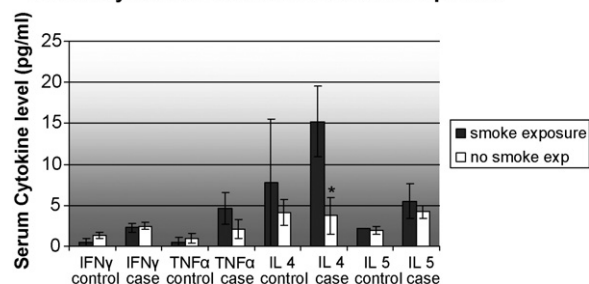


Fig. 2 Multivariate analysis of serum cytokine levels in cases and controls with and without cigarette smoke exposure. (*) Denotes statistical significance between smoke exposure and no smoke exposure. Error bars represent standard error of the mean.

Levels of IFN- γ and IL-4 were significantly increased after *in vitro* stimulation with *S. aureus*, simulating more of an acute than chronic otitis media condition. Although exact levels of the cytokines were not displayed, graphs revealed that cells from recurrent otitis media patients secreted more IFN- γ than IL-4 at baseline, a finding of unknown significance [17].

Our data support the hypothesis that a Th2-type immune response plays a role in chronic OME, as demonstrated by the increased level of systemic IL-5 in cases versus controls. No relationship was demonstrated between IL-5 levels and age group or cigarette smoke exposure. This supports previous studies showing elevated Th2-type cytokine levels in middle ear effusions and that perhaps the etiology (or response) to recurrent OME is a systemic one. Whether systemic cytokines function to cause chronic or recurrent OME or are released in response to the condition is unknown. The increased level of systemic IL-5 may either be the etiologic mechanism or simply released in response to OME. We have confirmed an association between IL-5 and OME.

Several studies have evaluated the association between systemic cytokine levels and cigarette smoke exposure. Byron et al. found that peripheral blood mononuclear cells of smokers produce more IL-4 than those of non-smokers, and cells of heavy smokers produced higher levels than light smokers. They found no relationship between IFN- γ levels and smoking status [18]. Nordskog et al. showed that IL-4 and IL-8 secretion rapidly increased in human aortic endothelial cells but not in THP-1 monocyte macrophages upon *in vitro* exposure to cigarette smoke condensate [19]. They concluded that cigarette smoke condensate uniquely influences the expression of cytokines, depending on cell type. To further delineate the mechanism of cigarette smoke exposure on cytokine release, Vassallo et al. demonstrated that cigarette smoke extract inhibited dendritic cell-mediated priming of T cells. Specifically, secretion of IFN- γ was inhibited and secretion of IL-4 was increased. They concluded that soluble components extracted from cigarette smoke suppress dendritic cell function and favor the development of Th-2 immunity [20].

The association of elevated levels of IL-4 with OME has been established in middle ear effusions [21]. Although the serum level of IL-4 was not significantly elevated in cases in our study (which would reinforce the activity of Th2 cytokine etiology), it was significantly elevated in children exposed to cigarette smoke ($p = 0.008$). Diaz-Sanchez et al. have recently confirmed the increased Th2 cytokine levels in nasal lavage after a second-hand smoke exposure challenge, namely IL-4, IL-5, and IL-13. In contrast, IFN- γ levels (Th1-type cyto-

kine) decreased after the challenge [22]. In our study IFN- γ was significantly higher in cases versus controls ($p = 0.018$) with univariate analysis, but not with multivariate analysis when controlling for age and smoke exposure. Therefore our results support a systemic response consistent with the Th2-type response.

As mentioned in Section 4, cases (42.1%) were more likely to be exposed to cigarette smoke than controls (11.8%), although the number of subjects limited significance ($p = 0.065$). From this and the finding of elevated IL-4 in smoke-exposed subjects, one could conclude that smoke exposure elevates systemic IL-4 and therefore causes the Th-2 response in OME. However, cases did not have elevated IL-4 levels, so the Th-2 response of smoke exposure and recurrent OME may be assumed to be two distinct processes. Future studies that increase our sample size and power could perhaps better delineate a relationship between smoke exposure, IL-4, and OME status.

Our study was limited by the overall low levels of serum cytokines compared to the sensitivities of cytokine detection kits. This may be a result of repeat freeze–thaw cycles and could be addressed in future studies by running ELISAs on fresh serum or by aliquoting serum samples upon collection from subjects before freezing. Serum IL-4 levels were higher than the other three cytokines for both cases and controls. This was likely because the IL-4 assay was run before serum aliquots were made and therefore underwent one fewer freeze–thaw cycles than the other cytokine assay serum samples. More accurate comparison between the cytokines could be achieved in future by running ELISAs on fresh serum or by aliquoting serum samples upon collection from subjects before freezing.

In conclusion, the preliminary results presented herein although limited by a small sample size demonstrate that recurrent OME is associated with elevated Th-2 cytokines, implying an allergic basis to the condition. This is clinically significant because localized treatment of recurrent OME may not be sufficient if the condition is associated with a systemic response. In addition, cigarette smoke exposure is associated with systemically elevated IL-4, independent of case-control status. This finding supports previous studies. In order to achieve better statistical power and confirm statistical significance, future research could look at systemic cytokine levels in a larger OM patient cohort. Studies could also be done prospectively to compare pre- and post-OME cytokine levels in the same patients to help determine whether the systemic release of Th-2 cytokines is causing or released in response to recurrent OME.

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