

T_H2 cytokine expression in atopic children with otitis media with effusion

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Background: Otitis media with effusion (OME) is more common in atopic children. Few studies have looked for the presence of inflammatory mediators in the middle-ear effusions of this population.

Objective: We hypothesize that atopic children with OME have a different inflammatory cell and cytokine profile than nonatopic patients with the disease.

Methods: Twenty-six patients with OME undergoing myringotomy and ventilation tube placement were recruited at the McGill University Hospital Center. The atopic status was determined for each patient by using standard skin testing. By means of immunocytochemistry, fluid specimens were assessed for T lymphocytes (CD3), eosinophils (major basic protein), neutrophils (elastase), mast cells (tryptase), and basophils (BB1). By using in situ hybridization, the expression of IL-4, IL-5, and IFN- γ was assessed.

Results: There is a higher percentage of eosinophils and T lymphocytes in atopic patients with OME ($n = 8$) compared with that seen in nonatopic patients ($n = 18$, $P < .01$). There is a higher percentage of neutrophils in nonatopic patients with OME compared with that seen in atopic patients ($P < .01$). In examining cytokine profiles, there is a higher percentage of cells expressing IL-4 and IL-5 in atopic patients with OME compared with that seen in nonatopic patients ($P < .01$).

Conclusion: The predominance of eosinophils, T lymphocytes, and T_H2 mediators in the middle-ear effusions of atopic children provides evidence that allergy might play a role in the pathogenesis of OME. (*J Allergy Clin Immunol* 2002;110:125-30.)

Key words: Otitis media, cytokines, allergies, atopy, children, effusion

Otitis media with effusion (OME) is a major pediatric health care issue, with an estimated prevalence of 15% to 20%¹ and high annual costs.² OME is the most common indication for surgery in children and can have significant sequelae, including hearing loss and permanent middle-ear mucosa damage.³ Longstanding OME might result in the

Abbreviations used

MEE: Middle-ear effusion

OME: Otitis media with effusion

maldevelopment of communication skills, although this has recently been questioned by Paradise et al.⁴ Although the exact cause of OME is not known, it is thought to occur after a treated acute infection in the setting of eustachian tube dysfunction. However, 57% to 82% of effusions do not have bacterial growth,⁵ and antibiotic therapy is no better than placebo in the resolution of OME.⁶⁻⁸ Some authors have proposed a viral cause, but viral isolates are only present in 16% to 19% of cases.⁹

The inflammatory cell and cytokine profile of asthma,¹⁰ allergic rhinitis,¹¹ and chronic sinusitis¹² is well documented in the literature. Mast cell degranulation, occurring in response to allergen binding of cross-linked IgE, is the predominant effector of early allergic responses. Eosinophils, basophils, T_H cells, and the T_H2-like cytokines (IL-4 and IL-5) are responsible for the late-phase allergic response. Given that the middle ear is contiguous with the upper airway, it is reasonable to hypothesize that allergy mediators play a role in the development of chronic middle-ear effusions (MEEs) in atopic patients.

We hypothesize that there is a higher percentage of eosinophils, basophils, mast cells, and T lymphocytes and increased expression of T_H2 cytokines, such as IL-4 and IL-5, in the middle-ear fluid specimens of atopic patients with OME compared with those of nonatopic control subjects.

METHODS

Patient selection

Patients were recruited from the Department of Otolaryngology at the McGill University Hospital Center over a 6-month period. Twenty-six patients with OME undergoing myringotomy and ventilation tube placement were included in the study. The criteria for myringotomy and ventilation tube placement in these patients was made on the basis of a history of persistent MEEs for greater than 3 months. Exclusion criteria included the presence of a known immunodeficiency disorder, craniofacial malformation, current middle-ear infection, or use of topical or systemic steroids within 6 weeks before surgery.

A history and physical examination were performed, assessing for general medical problems and risk factors for allergy. Eligible patients reviewed a detailed consent form approved by the inves-

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tigational review board of the Montreal Children's Hospital. At the time of preoperative testing or during general anesthesia, skin prick testing for perennial and seasonal allergens was done with a standard panel, including *Alternaria* species, *Aspergillus* species, *Cladosporium* species, grass, *Penicillium* species, ragweed, trees, cat, cockroach, dog, and the dust mites *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*. Histamine (1 mg/mL) was used as a positive control, and saline was used as a negative control. A positive skin reaction was judged as a wheal 3 mm or greater in diameter compared with that elicited by the negative control.

Sample-collection methods

At the time of myringotomy, middle-ear fluid was collected in a Juhn Tym-Taps (Xomed Treace Products, Jacksonville, Fla). One milliliter of sterile PBS was added to the sample. After transportation to the laboratory, the sample was resuspended and transferred to a siliconized centrifuge tube and centrifuged at 1200g for 10 minutes. The supernatant was stored at -80°C until assayed. One milliliter of mucollex (Baxter, McGraw Park, Ill) or erythrocyte lysing buffer (Sigma, St Louis, Mo) was added to the pellet for viscous or bloody samples, respectively. The pellet was resuspended and centrifuged, washed 3 times with sterile PBS, and fixed. The pellet was cytocentrifuged at 750g for 2 minutes onto frosted slides, and differential cell counts were made by means of Quick Diff (Baxter) staining and microscopic examination. Slides were also prepared for immunocytochemistry and in situ hybridization. For immunocytochemistry, the cytospin preparations were briefly fixed in a solution of acetone/methanol (40:60), air-dried, and stored at -80°C until further use. For in situ hybridization, the cytospin preparations were air-dried and fixed in 4% paraformaldehyde for 30 minutes, washed twice for 5 minutes with PBS, kept at 37°C overnight, and stored at -80°C until further use.

Immunocytochemistry

Immunocytochemistry was performed by using the alkaline phosphatase-antialkaline phosphatase method, as previously described.¹³ Monoclonal antibodies, including anti-CD3, major basic protein (a gift from Dr R.M. Moqbel, University of Alberta, Canada), BB1 (a gift from Dr A. Walls, Southampton University, United Kingdom), tryptase, and elastase, were used to detect T lymphocytes, eosinophils, basophils, mast cells, and neutrophils, respectively. Slides were developed with Fast Red substrate for alkaline phosphatase. A negative control slide was included in each immunocytochemistry experiment.

In situ hybridization

Radiolabeled complementary riboprobes (cRNA antisense) for IL-4, IL-5, and IFN- γ were used to identify the presence of mRNA, as previously reported.¹⁴ Cytospin preparations were permeabilized and treated with acetic anhydride and triethanolamine. Sulfur 35-labeled antisense probes were used (10^6 cpm/cytospin preparation), followed by high-stringency posthybridization washings. Unhybridized single-stranded RNA was removed by treating the preparation with a solution of RNase. Cytospin preparations were hybridized with the sense probe or pretreated with an RNase solution to ensure in situ hybridization specificity.

Quantification

Specimens were coded, and the percentage of positive cells for protein and mRNA were counted blind by using an Olympus microscope with an eyepiece graticule at $200\times$ magnification. Results were expressed as the mean percentage of positive cells per cytospin preparation $\pm$ SEM.

Statistical analysis

Cell counts were compared between atopic and nonatopic patients by using a Mann-Whitney *U* test, with a *P* value of less than .01 regarded as statistically significant.

RESULTS

Clinical data

In atopic patients with OME ($n = 8$), the mean age was 4.75 years (2-7 years) compared with 3.56 years (1-8 years) in the nonatopic group ($n = 18$). Two (25%) patients in the atopic group and 4 (22%) nonatopic control subjects had a history of asthma. Two (25%) patients in the atopic group and 5 (28%) nonatopic control subjects had a history of rhinitis. One (13%) patient in the atopic group and 1 (6%) nonatopic control subject had a history of eczema. One (13%) patient in the atopic group and 5 (29%) nonatopic control subjects had a family history of allergy.

Atopic patients were most commonly reactive to dog dander (62.5%). Fifty percent of atopic patients were reactive to dust mite, 25% to cat dander, and 13% to tree mix and ragweed. No patients were reactive to cockroach, grass, or molds.

Immunocytochemistry

Immunocytochemistry data are shown in Figures 1 and 2. The percentages of eosinophils and T lymphocytes are significantly higher in the MEEs of atopic patients ($7.7\% \pm 1.4\%$ and $20.0\% \pm 3.2\%$, respectively) compared with those in MEEs of nonatopic patients ($0.9\% \pm 0.2\%$ and $5.5\% \pm 0.6\%$, respectively; $P < .01$). On the other hand, a significantly higher percentage of neutrophils is found in the MEEs of nonatopic patients ($43.0\% \pm 2.4\%$) compared with that found in the MEEs of atopic patients ($15.3\% \pm 3.1$, $P < .01$). There is no significant difference in the percentage of mast cells found in the MEEs of atopic patients ($7.6\% \pm 2.2\%$) compared with that found in the MMEs nonatopic patients ($3.4\% \pm 0.6\%$, $P = .029$). There is no significant difference in the percentage of basophils found in the MEEs of atopic patients ($6.0\% \pm 2.0\%$) compared with that found in the MEEs of nonatopic patients ($1.8\% \pm 0.4$, $P = .015$).

In situ hybridization

In situ hybridization data are shown in Figure 3. The percentages of IL-4 and IL-5 mRNA-positive cells are significantly higher in the MEEs of atopic patients ($6.1\% \pm 0.5\%$ and $7.5\% \pm 0.6\%$, respectively) compared with those found in the MEEs of nonatopic patients ($1.7\% \pm 0.3\%$ and $2.6\% \pm 0.4\%$, respectively; $P < .01$). On the other hand, a significantly higher percentage of IFN- γ -positive cells is found in the MEEs of nonatopic patients ($6.0\% \pm 0.4\%$) compared with that found in the MEEs of atopic patients ($2.0\% \pm 0.5\%$, $P < .01$).

DISCUSSION

In our study population, we found a 30% incidence of atopy by means of routine skin testing. Seven of the 8

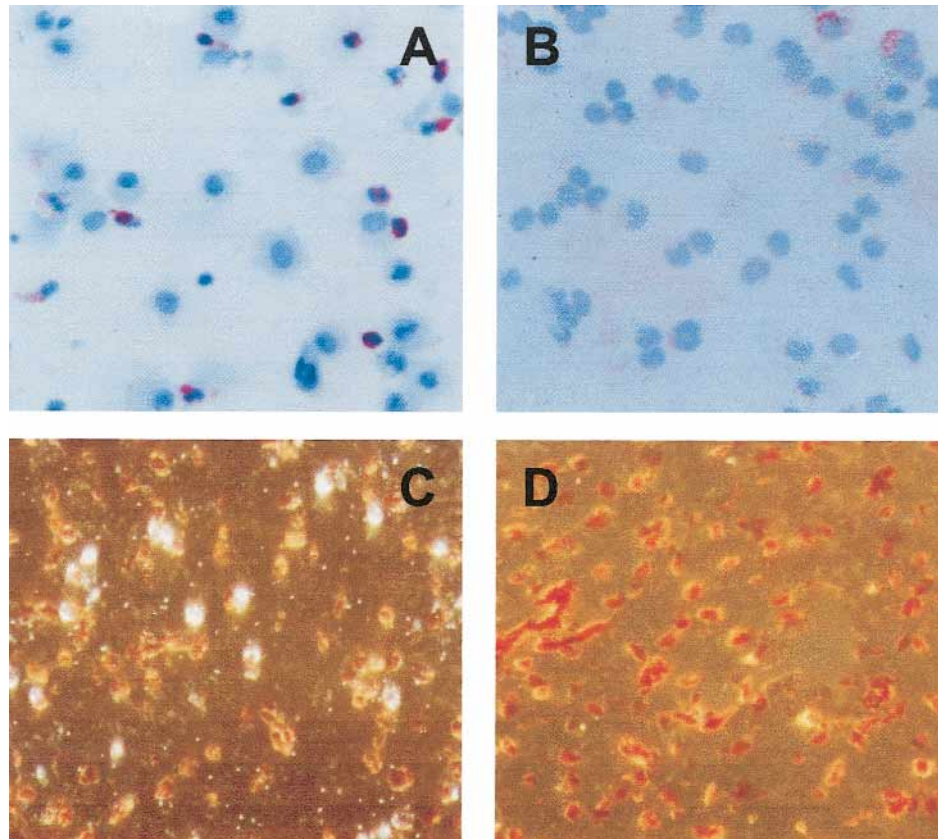


FIG 1. Immunocytochemistry and in situ hybridization of cytospin preparations from atopic and nonatopic patients with OME: **A**, major basic protein (MBP) immunostaining in atopic patients with OME; **B**, major basic protein immunostaining in nonatopic patients with OME; **C**, radioactive in situ hybridization for IL-5 in atopic patients with OME; **D**, IL-5 mRNA in nonatopic patients with OME (original magnification 200 $\times$).

atopic patients were reactive to perennial allergens. The role of allergy in the pathogenesis of recurrent otitis media and OME has been evaluated and has been reported to be a risk factor in 0% to 88% of patients.¹⁵⁻¹⁷ In theory, allergic inflammation can cause recurrent otitis media or OME indirectly by causing a transient blockage of the eustachian tube^{18,19} or directly by means of middle-ear mucosa inflammation. Although some investigators have concluded that atopy is a significant risk factor for recurrent otitis media,²⁰⁻²³ others have found no relationship between the 2 conditions.^{24,25} The major problem with most of these studies is the lack of uniform diagnostic criteria for atopy.

Atopic patients in our study have significantly higher percentages of T lymphocytes, eosinophils, and IL-4- and IL-5-positive cells compared with nonatopic patients. Although not statistically significant, the percentages of mast cells and basophils were also higher in the atopic effusions compared with those seen in effusions of control subjects. These results are consistent with the late-phase allergic response seen in other areas of the respiratory tract, which is implicated in the pathogenesis of asthma, allergic rhinitis, and chronic sinusitis. Eosinophils play a particularly important role in the late-phase allergic response through the release of mediators,

including eosinophilic cationic protein, major basic protein, and leukotriene C4, which causes extracellular matrix deposition, epithelial denudation, and basement membrane disruption.^{13,26-30} Basophils are thought to play an important role in the late-phase allergic response, although their exact role has yet to be clearly elucidated. Similar to the mast cell, basophils bind allergen through IgE cross-linkage and release histamine on activation.³¹ Because the level of histamine, but not tryptase, is found to be elevated during the late-phase response, basophils are thought to be the major source of this mediator as opposed to secondary mast cell degranulation.³¹⁻³³

IL-5 is important for eosinophil production, recruitment, activation, and survival.³⁴ IL-4 is important for the differentiation of naive T_H0 cells toward the T_H2 phenotype and plays an important role in the local production of IgE in tissues of atopic patients.³⁵ Through induction of endothelial vascular cell adhesion molecule 1, IL-4 is thought to promote eosinophil infiltration to the site of inflammation.

Neutrophils and IFN- γ -positive cells were present in significantly higher percentages in nonatopic children in our study. These findings are similar to those found in a subset of asthmatic patients. Wenzel et al³⁶ found a 2-fold higher number of neutrophils in bronchoalveolar

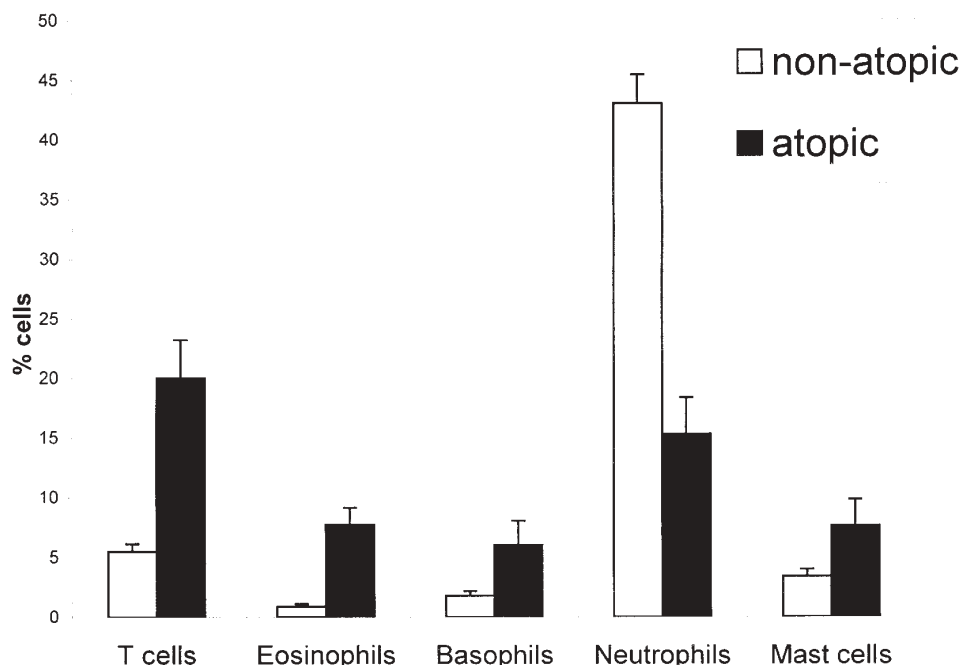


FIG 2. Inflammatory cells in the MEEs of atopic and nonatopic children with OME.

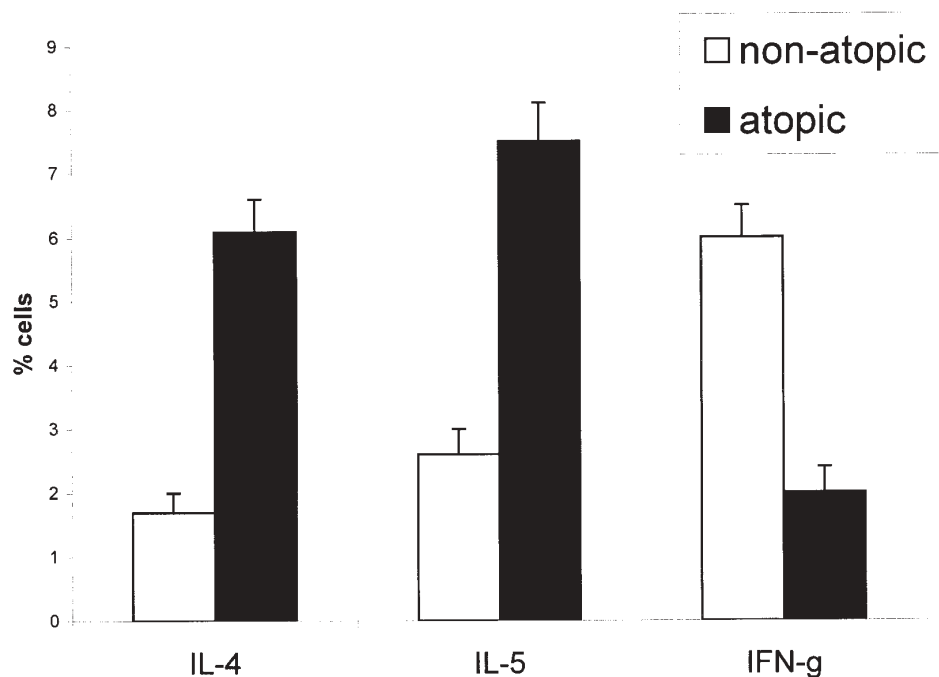


FIG 3. T_H1 and T_H2 cytokine mRNA expression in the MEEs of atopic and nonatopic children with OME.

lavage fluid of patients with severe asthma compared with that seen in patients with mild-to-moderate asthma and control subjects. Alternatively, the high number of neutrophils in the MEEs of these patients might represent the persistence of an infectious process in the presence of eustachian tube dysfunction.

The recent literature has looked at the role of cytokines in MEEs, both in animals and in human subjects.^{37,38} Most studies have assessed for the presence of nonspecific inflammatory mediators, such as IL-1, IL-6, IL-8, and TNF- α . Others have looked for the presence of IgE antibodies in the systemic circulation and in middle-ear fluid specimens.³⁹

There have been a number of studies that have attempted to identify the role of allergic mediators in OME. Bikhazi and Ryan⁴⁰ demonstrated high numbers of IL-5-positive, but not IL-4-positive, cells in a mouse model of chronic otitis media. Hurst et al^{41,42} demonstrated that eosinophils and mast cells were present in the fluid of atopic patients with OME. Wright et al⁴³ recently demonstrated increased expression of IL-5 and major basic protein in the middle-ear mucosa of patients with OME compared with that seen in normal control subjects. Although these studies provide preliminary evidence for the role of allergy mediators in OME, they do not examine nonatopic patients with OME and are limited by the necessity of using middle-ear mucosa biopsies to measure appreciable levels of cytokines. To overcome these deficiencies, Hurst and Venge⁴⁴ recently demonstrated elevated levels of eosinophil cationic protein and tryptase in atopic, compared with nonatopic, MEEs.

In summary, we have found that 30% of children with OME are atopic, as determined by means of skin testing. Seven of the 8 atopic patients were reactive to perennial allergens. MEEs of atopic patients with OME are characterized by higher percentages of eosinophils, T lymphocytes, and T_H2-positive cells compared with that seen in nonatopic control subjects. This inflammatory profile is consistent with that seen in other upper airway diseases, the pathogenesis of which is related to the late-phase allergic response. It is likely that in atopic patients MEEs develop after exposure to allergen, resulting in a sustained late-phase allergic response in the middle-ear mucosa.

Because of the small sample size, this study does not aim to make any conclusions about the management of patients with OME. The results of this study, however, emphasize the need to further investigate the role of antiallergy therapy in selected patients with OME.

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