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Th2 cytokine expression in atopic children with otitis media with effusion

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Abstract

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.

Objectives: We hypothesize that atopic children with OME have a different inflammatory cell and cytokine profile than non-atopic children with the disease.

Methods: The atopic status of 26 children with OME was determined. Using immunocytochemistry, fluid specimens were assessed for T lymphocytes, eosinophils, neutrophils, mast cells, and basophils. The expression of IL-4, IL-5, and IFN-γ mRNA was assessed using in-situ hybridization.

Results: There is a higher percentage of eosinophils, T lymphocytes and cells expressing IL-4 and IL-5 mRNA in atopic children (n=8) compared to non-atopic controls (n=18) ($p<0.01$).

Conclusion: The predominance of eosinophils, T lymphocytes and Th2 mediators in the middle ear effusions of atopic children provides strong evidence that atopy plays a role in the pathogenesis of this condition.

Abregé

L'Otite moyenne avec épanchement (OMÉ) est plus courante chez les enfants atopiques. Peu d'études ont porté sur la présence de médiateurs chimiques d'allergie dans le cas d'OMÉ de cette population.

Nous proposons que les patients atopiques avec OMÉ auront un profil de cytokines et cellules inflammatoires différent des patients non-atopiques avec la maladie.

L'état atopique de 26 enfants avec OME a été déterminé. En utilisant l'immunocytochimie, des échantillons d'épanchement ont été évaluées pour la présence de neutrophiles, cellules T, éosinophiles, mastocytes, et basophiles. L'expression de l'ARNm de IL-4, IL-5, et IFN-γ a été considérée par l'hybridation in-situ.

Il y a un plus grand nombre d'éosinophiles, cellules T et de cellules qui exprime IL-4 and IL-5 chez les patients atopique (n=8) que chez les patients non-atopiques (n=18) ($p<0.01$).

La prédominance d'éosinophiles, cellules T et les médiateurs Th2 dans l'épanchements de l'oreille moyenne des patients atopiques supporte fortement le rôle joué par l'allergie dans la pathogénie de OMÉ.

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Contributions of authors

The work for this thesis was performed at the Meakins-Christie Laboratories, McGill University, from July, 2001-August, 2002.

My role in this study includes the following:

- Development of the research hypothesis and objectives.
- Development of the research design including the establishment of the middle ear effusion collection protocol (with help from Dr. Rame Taha).
- Obtainment of approval from the investigational review board of the Montreal Children's Hospital.
- Recruitment of all patients participating in this study.
- Skin prick testing of all patients participating in this study (under the guidance of Dr. Bruce D. Mazer).
- Performance of myringotomies and collection of middle ear fluid from all patients participating in this study (with help form Drs. Melvin D. Schloss, John J. Manoukian and Ted L. Tewfik).
- Performance of immunocytochemistry and in-situ hybridization (with help from Ms. Elsa Schotman)
- Counting of cells for each marker from every patient (with Dr. Qutayba Hamid).
- All data processing, statistical analysis, and writing of manuscripts was performed by myself with input from all of my coauthors.
- Presentation at the national meeting of the Canadian Society of Otolaryngology-Head and Neck Surgery, Vancouver, BC, May 26, 2001.

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Publications arising from this work

Papers

1. Sobol SE, Taha R, Schloss MD, Mazer BD, Manoukian JJ, Tewfik TL, Hamid Q: Th2 cytokine expression in atopic children with otitis media with effusion. J Allergy Clin Immunol *Submitted for publication.*
2. Sobol SE, Taha R, Schloss MD, Mazer BD, Manoukian JJ, Tewfik TL, Hamid Q: Increased expression of major basic protein, CD3 and BB1 in atopic children with otitis media with effusion. J Otolaryngol *Submitted for publication.*
3. Sobol SE, Schloss MD, Hamid Q: The role of atopy in the pathogenesis of otitis media with effusion. Laryngoscope *Submitted for publication.*

Abstracts

1. Sobol SE, Taha R, Schloss MD, Mazer BD, Hamid Q: Inflammatory cell and cytokine profiles in middle ear effusions of atopic and non-atopic children. J Otolaryngol 2001:30(suppl. 1):18.

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Abbreviations used in this text

BB1	basophil granule-specific monoclonal antibody
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
ECP	eosinophil cationic protein
ELISA	enzyme-linked immunosorbent assay
EN	eosinophil neurotoxin
EPO	eosinophil peroxidase
GM-CSF	granulocyte/macrophage colony stimulating factor
GM-CSFR	granulocyte/macrophage colony stimulating factor receptor
ICC	immunocytochemistry
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
ISH	in-situ hybridization
LT	leukotriene
MBP	major basic protein
MEE	middle ear effusion
mRNA	messenger ribonucleic acid
OME	otitis media with effusion
PAF	platelet activating factor
PG	prostaglandin
SEM	standard error of the mean

Th1	T helper lymphocyte type 1
Th2	T helper lymphocyte type 2
TNF-α	tumor necrosis factor alpha
VCAM-1	vascular cell adhesion molecule-1

Chapter 1. General introduction

1.1 Otitis media with effusion

Otitis media with effusion (OME) is a major pediatric health care issue, with an estimated prevalence of 15-20 % [1], and annual costs in the billions of dollars [2]. OME is the most common indication for surgery in children, and can have significant sequelae, including hearing loss, maldevelopment of communication skills, as well as permanent middle ear mucosal damage [3]. Although the exact etiology of OME is not known, it is thought to occur after a treated acute infection, in the setting of Eustachian tube dysfunction. However, 57-82 % of effusions do not have bacterial growth [4], and antibiotic therapy is no better than placebo in the resolution of OME [5-7]. A viral etiology has been proposed by some authors, but viral isolates are only present in 16-19 % of cases [8].

The inflammatory cell and cytokine profile of asthma [9], allergic rhinitis [10], and chronic sinusitis [11] 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-helper cells, and the Th2 cytokines (IL-4, IL-5, IL-13) are responsible for the late phase allergic response. Given that the middle ear mucosa is contiguous with that of the nasal cavity and sinuses via the Eustachian tube, it has been hypothesized that similar inflammatory responses may occur in patients with otitis media with effusion.

Whereas the role of allergy mediators in the pathogenesis of chronic sinusitis and allergic rhinitis has been well defined, the role of such mediators in the pathogenesis of OME is unclear. This introduction reviews both clinical and molecular biological studies

that have both supported and refuted the hypothesis that atopy plays a role in the pathogenesis of OME.

1.2 Clinical evidence of atopy in otitis media with effusion

1.2.1 Results of skin testing in patients with otitis media with effusion

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 0-88 % of patients [12-18]. While some investigators have concluded that atopy is a significant risk factor for recurrent otitis media [19-23], others have found no relationship between the two conditions [24,25]. The major problem with most of these studies is the lack of uniform diagnostic criteria for atopy. Moreover, in some studies the diagnosis of atopy was based on history rather than objective skin tests, provocation tests or serum IgE analysis.

The prevalence of positive skin tests in OME patients varies from 9-88 % between different studies [26-30]. A study by Lecks et al found that 88 % of OME patients had positive skin scratch tests [30]. Using the skin prick technique, Caffarelli et al found no difference in the prevalence of allergen reactivity between OME patients (27 %) and controls (31 %) [26]. The relatively high number of OME patients who were found to be positive for atopy in earlier compared to later studies, may be partly explained by the use of the skin scratch test, which has a high false positive rate compared to the prick test.

1.2.2 Association between otitis media with effusion and other atopic diseases

The presence of atopic conditions including allergic rhinitis, eczema, and asthma has been evaluated in OME patients [12-15,18,31]. Several authors have noted an increased prevalence of OME in allergic children compared to non-allergic controls [20,

[32-34]. Draper reported the presence of OME in 52 % of allergic rhinitis patients, compared to 24 % of non-allergic controls [34]. Other studies have found an increased prevalence of atopic conditions in OME patients compared to non-OME controls. For example, Caffarelli et al found an increased prevalence of eczema and allergic rhinitis in OME patients compared to controls [26], which is consistent with data found in other studies [29,35-40]. In an epidemiological study looking at the role of allergies in the development of acute otitis media, Pukander et al concluded that infants with a history of allergy had significantly longer persistence of MEE compared to non-allergic infants [41].

1.2.3 Allergen-induced Eustachian tube dysfunction

The nasal mucosa is continuous with that of the middle ear by way of the Eustachian tube. In theory, it is possible that allergic inflammation of the nasal mucosa could also affect the Eustachian tube resulting in edema and obstruction, with the subsequent development of OME.

Eustachian tube obstruction has been demonstrated in allergic patients after exposure to both natural and induced allergen exposure [42-49]. Clinical studies have shown that in atopic patients, exposure to allergen challenge results in a dose dependent decrease in Eustachian tube patency [42,48]. This response is consistently found regardless of the whether seasonal (ragweed) [42] or perennial (dust mite) allergen [48] was used in the challenge. What is not clear from these studies, however, is the role of allergen-induced Eustachian tube obstruction on the subsequent development of OME.

Animal models have been used to evaluate the histological response of the Eustachian tube to allergen exposure in sensitized subjects [50,51]. As early as one hour

after exposure, increased numbers of mast cells and eosinophils were found in the distal Eustachian tube mucosa [50]. Using a dog model of OME and allergic rhinitis, Mogi et al concluded that allergen challenge contributes to the chronicity but not the actual development of OME in sensitized animals [51].

1.3 Molecular evidence of atopy in otitis media with effusion (figure 1)

1.3.1 Role of the early allergic response in otitis media with effusion

Allergy mediators play an important role in the pathogenesis of chronic upper airway disease such as allergic rhinitis and chronic sinusitis (see below). Although there are few immunocompetent cells in the normal middle ear mucosa [52], mast cells have been found in the tympanic cavity of guinea pigs [53] and humans [54], leading to speculation that the middle ear may be a target of the early allergic response.

The mast cell plays an important role in mediating the early phase allergic response. Mast cells store a number of pro-inflammatory mediators, including tryptase, histamine, TNF-α and IL-4 [55,56]. Allergen inspiration and crosslinkage of IgE result in the degranulation and release of these stores, as evidenced by the detection of elevated tryptase and histamine levels in nasal lavage fluid of allergic rhinitis patients [57]. Allergen activation also induces mast cell synthesis of leukotrienes, prostaglandins, PAF and bradykinin [59,60].

Several investigators have looked at the levels of total and specific IgE in MEE and serum of children with OME. Whereas some authors have concluded that OME is an IgE mediated disease [61,62], others have not found increased IgE in MEE compared to serum samples [41,63-67]. A recent study by Labadie et al demonstrated the development of OME in sensitized rats when exposed to lipopolysaccharide [68]. Middle ear effusions in these rats could be prevented by the co-administration of the antihistamine diphenhydramine, leading the authors to conclude that OME may be an antigen-IgE mediated response. Bernstein et al found that levels of IgE are higher in MEE compared

to serum samples in 46 % of allergic children with OME [61]. They concluded that a local IgE response may be responsible for the generation of MEE in susceptible patients.

On the other hand, Mogi et al looked at total and specific IgE in the serum and MEE of children with OME [41,63]. They concluded that the presence of IgE in MEE was the result of systemic allergic disease rather than the cause of the OME. When exposed to allergen challenge, the middle ear mucosa of sensitized primates did not demonstrate any IgE response. Since IgE plays a major role in the early phase allergic response, its role in OME, which is a chronic condition, is questionable.

1.3.2 Role of the late allergic response in otitis media with effusion

1.3.2.1 Eosinophils

Eosinophils play a particularly important role in the late phase allergic response through the release of mediators including ECP, EN, EPO, and MBP [69,70], which cause extracellular matrix deposition, epithelial denudation and basement membrane disruption [71-77]. Eosinophils also secrete LTC_4 [78], which is known to cause mucosal edema and mucus secretion, and PAF [69,70] which stimulates eosinophil and neutrophil chemotaxis, increases vascular permeability, promotes mucosal edema and inhibits apoptosis of B cells [79]. Eosinophils appear to be an important source of cytokines such as IL-3, IL-5 and GM-CSF, which have been implicated in hematopoesis and may act in an autocrine fashion to promote eosinophil differentiation and survival [80].

There have been a number of studies that have attempted to identify the role of allergic inflammatory cells in OME [81-88]. Early studies demonstrated that eosinophils were not common in the effusions and middle ear mucosa of OME patients [81,89,90].

Most of these studies, however, were done before the advent of modern molecular biology techniques. Hurst et al recently demonstrated that 87.5 % of MEE and 80 % of middle ear mucosal biopsy specimens were positive for ECP [87]. He concluded that eosinophils play a major role in the pathogenesis of this condition. These studies, however, did not compare the cellular profiles of atopic and non-atopic OME patients. In order to overcome this deficiency, Hurst et al recently reported increased levels of ECP and tryptase in the supernatant of atopic OME patients compared to non-atopic controls [91]. Wright et al recently demonstrated increased expression of IL-5 and MBP in the middle ear mucosa of patients with OME compared to normal controls [82].

1.3.2.2 T lymphocytes

T lymphocytes ($CD3^+$ cells) are thought to play a central role in the initiation and regulation of the inflammatory response through the release of cytokines. The combination of cytokines secreted by the T lymphocyte determines what type of inflammatory reaction results. Initial studies of mouse T-helper cells ($CD4^+$ T lymphocyte clones) revealed that they could be differentiated from Th0 cells into two basic functional subsets termed Th1 and Th2 [92]. Th1 cells secrete IL-2, IFN-γ, and TNF-β, whereas Th2 cells secrete IL-4, IL-5, IL-6, and IL-10. Other cytokines including TNF-α, IL-3 and GM-CSF are secreted by both types of $CD4^+$ T lymphocytes. In general, the Th1 cytokines are crucial for the onset of delayed type hypersensitivity reactions, cell mediated immunity, and clearance of intracellular pathogens, whereas Th2 cytokines are thought to play a major role in the late phase allergic response [93,94].

The role of Th2 cells in allergic diseases including, allergic rhinitis, chronic sinusitis, and asthma has been well established in the literature [95]. There have been few studies that have looked for the presence of this cell in the middle ear of patients with OME. Wright et al demonstrated a significantly higher number of CD3 positive cells in the middle ear mucosa of atopic OME patients compared to controls [82]. Given the pivotal role of T lymphocytes in the development of allergic disease, further studies are needed to evaluate the role of this cell in OME.

1.3.2.3 Basophils

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 via IgE cross linkage and release histamine upon activation [96]. Since 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 [96-98]. The role of the basophil in OME has yet to be evaluated.

1.3.2.4 Th2 cytokines

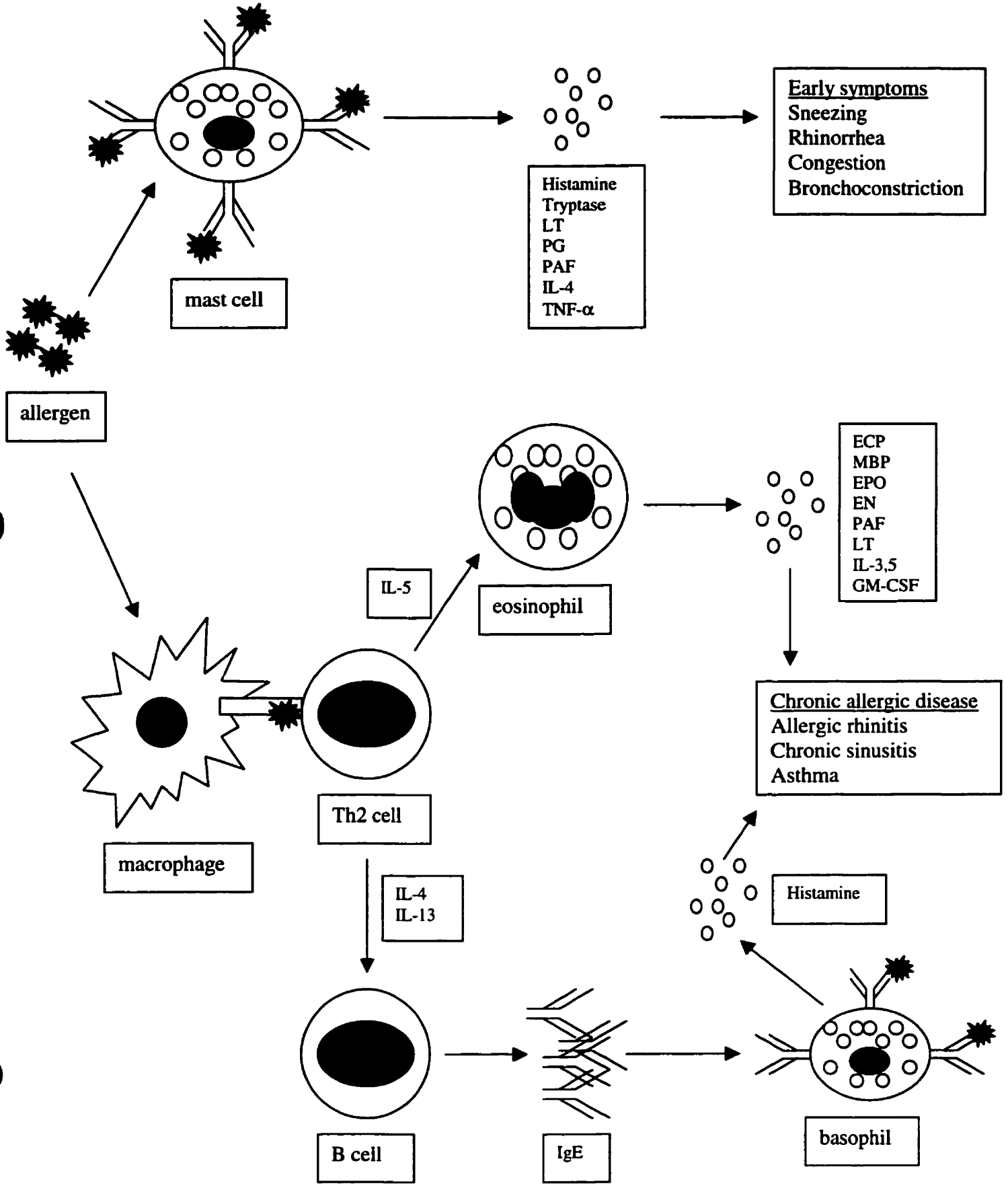
As mentioned above, the Th2 cytokines are thought to play a major role in the initiation and regulation of the late phase allergic inflammatory response. IL-5 is important for eosinophil differentiation from myeloid progenitors in the bone marrow, and is important for the recruitment, activation, and survival of mature eosinophils at the site of inflammation [99].

IL-4 is important for the differentiation of naïve T-helper cells (Th0) towards the Th2 phenotype [100], and plays an important role in the local production of IgE in tissues of atopic patients [101]. Through induction of endothelial VCAM-1, IL-4 is thought to promote eosinophil infiltration to the site of inflammation [102].

The recent literature has looked at the role of cytokines in MEE, both in animals and humans [103-106]. Most studies have assessed for the presence of non-specific inflammatory mediators such as IL-1, IL-6, IL-8 and TNF-α. Others have looked for the presence of antibodies in the systemic circulation and in MEE specimens [41].

Wright et al recently demonstrated an increased number of cells expressing IL-5 mRNA in the middle ear mucosa of atopic OME patients compared to controls [82]. The presence of IL-4 and IL-5 in the MEE of atopic and non-atopic OME patients has yet to be determined, and may play a critical role in the pathogenesis of this condition.

Figure 1


mast cell
Histamine
Tryptase
LT
PG
PAF
IL-4
TNF-α
Early symptoms
Sneezing
Rhinorrhea
Congestion
Bronchoconstriction
allergen
IL-5
eosinophil
ECP
MBP
EPO
EN
PAF
LT
IL-3,5
GM-CSF
Chronic allergic disease
Allergic rhinitis
Chronic sinusitis
Asthma
macrophage
Th2 cell
IL-4
IL-13
Histamine
B cell
IgE
basophil

1.4 The role of atopy in upper airway disease

1.4.1 Allergic rhinitis

Allergic rhinitis is a chronic inflammatory disorder of the nasal mucosa, which occurs after exposure to perennial or seasonal allergens, including dust mite, animal dander, mold spores and pollen. Encounter with the allergen results in the classical manifestations of allergic rhinitis such as sneezing, nasal pruritis, rhinorrhea and congestion. This response is thought to occur secondary to glandular hypersecretion, increased vascular permeability, afferent nerve stimulation, and the infiltration of inflammatory cells at the site of the nasal mucosa [107-110].

Allergen stimulation of the nasal mucosa results in an early phase reaction, which develops rapidly and lasts for 60 minutes. Mast cells, which are found in higher numbers within the nasal epithelium during allergy season, play an important role in mediating the immediate response to allergen by increasing their expression of the IgE receptor gene, indicating their increased ability to bind IgE [111-113]. Once activated, mast cells release histamine over a time course that correlates clinically with the early irritative and secretory manifestations of allergic rhinitis [114-116]. Other mediators of the early phase response include the cysLT and PGD_2, which irritate afferent nerve ending, increase vascular permeability and glandular secretion [116-119].

The early phase response is followed by a late phase reaction, which begins 3-6 hours after allergen exposure, and subsides within 12-24 hours. Dual responses are observed in 40-50% of patients [120]. Therefore, a single allergen challenge can precipitate chronic inflammatory alterations in the nasal mucosa and can enhance subsequent mediator release on rechallenge [114].

The late phase allergic response manifests as a re-occurrence of sneezing, rhinorrhea and congestion. This response is thought to arise from the expression of cytokines and chemokines, which lead to the recruitment and activation of inflammatory cells, including T lymphocytes, eosinophils and basophils [97,121].

Eosinophils play a particularly important role in the late phase reaction, through the release of mediators such as MBP and ECP, which have been shown to cause degranulation of other inflammatory cells as well as epithelial cell damage [72,73,75-77].

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 via IgE cross linkage and release histamine upon activation [96]. Since 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 [96-98].

$CD4^+$ T lymphocytes elevated in the nasal mucosa of allergic patients and are thought to play an important role in the pathogenesis of allergic rhinitis by the release of Th2 type cytokines including, IL-3, IL-4, IL-5, IL-13 and GM-CSF [10,122,123]. The role of the Th2 cytokines has already been described in the previous section. Co-localization studies have demonstrated that approximately 70-80% of IL-4, IL-5 and IL-13 mRNA positive cells were T lymphocyte-associated [122,124,125].

1.4.2 Allergic Chronic Sinusitis

Allergic chronic sinusitis is an inflammatory condition of the paranasal sinuses, which is thought to result, in the majority of cases, from allergic stimulation of the nasal

and paranasal mucosa. Like allergic rhinitis, the inflammatory infiltrate in allergic chronic sinusitis is consistent with the late phase response [10,71]. The allergen-induced inflammation leads to hypertrophy of the sinus mucosa and polypoid changes, which leads to a narrowing of the sinus ostia and obstruction of mucous drainage.

The sinus mucosa of patients with allergic chronic sinusitis is characterized by a higher percentage of eosinophils, T lymphocytes and B cells, compared to normal controls [11,126]. Like allergic rhinitis, the pathophysiology of chronic sinusitis has been largely attributed to the effects of Th2 cytokines, including IL-3, IL-4, IL-5 and IL-13 and GM-CSF, which are found in a higher percentage of cells within the sinus mucosa allergic chronic sinusitis patients compared to normal controls [126,127]. Receptors for IL-4 and IL-5 are upregulated in the mucosa of patients with allergic chronic sinusitis, while the GM-CSF receptor was more predominantly expressed in non-allergic chronic sinusitis patients [128].

1.5 Management of atopic diseases of the upper airway

There have been very few studies that have looked at the response of OME to anti-allergy therapy. Kawauchi et al found that azelastine hydrochloride was more effective than placebo in clearing MEE in children with OME and allergic rhinitis [41]. They found a similar response in a Guinea pig model, where accelerated MEE clearance was noted when azelastine hydrochloride was used [129]. Hurst reported promising results in the management of specific IgE positive OME patients with anti-allergy therapy [130].

The use of corticosteroids for the treatment of chronic inflammatory diseases such as allergic rhinitis and chronic sinusitis has been well established. Steroids reduce the irritative and hypersecretory manifestations of allergic rhinitis and restore the normal architecture of the nasal mucosa [131-133].

Steroid administration is also seen to coincide with a reduction in the number of inflammatory cells and Th2 type cytokines within the nasal mucosa of patients with allergic rhinitis. Corticosteroids treatments have been found to reduce the number of basophils, eosinophils and the level of ECP release of patients with allergic rhinitis [97,112]. Although they do not appear to reduce mast cell or T lymphocyte number within the nasal mucosa, steroids are thought to reduce the activity of these inflammatory cells [97,134-136].

Corticosteroids are thought to play a role in limiting cytokine and chemokine synthesis by inhibiting allergen-induced expression of mRNA encoding IL-4, IL-13, and eotaxin, a potent eosinophil chemotactic factor [112,122,124,135,137,138]. In allergic

chronic sinusitis, steroids have been seen to reduce the production of IL-4 and IL-13 [127] as well as the number of cells expressing receptors for IL-4, IL-5 and GM-CSF [139].

Immunotherapy is recommended for the small group of patients with atopic disease who fail to respond to standard pharmacotherpy. Durham et al. have recently shown that grass pollen immunotherapy is associated with a markedly reduced response to allergen challenge, including decreased infiltration of T lymphocytes and reduced expression of IL-4 mRNA in response to allergen challenge [140]. The beneficial effect of immunotherapy is thought to result from an inhibition of the late phase response by decreasing the infiltration of CD4+ T lymphocytes and eosinophils, and decreasing the release of histamine from basophils [141-143]. Although the exact mechanism of this effect is unclear, it appears to be mediated by the induction of Th1 cytokine expression, including IL-12 and IFN-γ, which leads to the subsequent downregulation of the Th2 response [141,144-146].

1.6 Rationale for this study

The role of atopy in the pathogenesis of upper airway inflammatory diseases such as allergic rhinitis and chronic sinusitis has been clearly established in the literature. The role of atopy in the development of middle ear disease, however, remains unclear with evidence both supporting and refuting this hypothesis.

Recent studies have demonstrated that cells implicated in the allergic response are present in the middle ear effusions and mucosal specimens of atopic children with OME. There is a paucity of information, however, concerning the role of the Th2 cytokines in the pathogenesis of OME in atopic and non-atopic children.

Given that the middle ear mucosa is contiguous with that of the nasal cavity and sinuses via the Eustachian tube, it is reasonable to hypothesize that allergic inflammatory responses may play a role in the pathogenesis of OME.

1.7 General hypothesis

We hypothesize that the MEE of atopic children with OME is characterized by cellular and cytokine profiles consistent with the late-phase allergic response.

1.8 Aims of this study

1. To determine the atopic status of children with OME by skin testing for common perennial and seasonal allergens.
2. To investigate the cellular profile of MEE from atopic and non-atopic children using immunocytochemistry with markers for eosinophils (MBP), T lymphocytes (CD3), basophils (BB1), mast cells (tryptase) and neutrophils (elastase).
3. To investigate the expression of mRNA for IL-4, IL-5, and IFN-γ in MEE of atopic and non-atopic children using in-situ hybridization.

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Chapter 2. Th2 cytokine expression in atopic children with otitis media with effusion

2.1 Abstract

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.

Objectives: We hypothesize that atopic children with OME have a different inflammatory cell and cytokine profile than non-atopic children 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 using standard skin testing. Using immunocytochemistry, fluid specimens were assessed for T lymphocytes (CD3), eosinophils (MBP), neutrophils (elastase), mast cells (tryptase) and basophils (BB1). Using in-situ hybridization the expression of IL-4. IL-5, and IFN-γ mRNA was assessed.

Results: There is a higher percentage of eosinophils and T lymphocytes in atopic OME patients (n=8) compared to the non-atopic group (n=18) ($p<0.01$). There is a higher percentage of neutrophils in non-atopic OME patients compared to the atopic group ($p<0.01$). In examining cytokine profiles, there is a higher percentage of cells expressing IL-4 and IL-5 mRNA in atopic OME patients compared to the non-atopic group ($p<0.01$).

Conclusions: The predominance of eosinophils, T lymphocytes and Th2 mediators in the middle ear effusions of atopic patients provides strong evidence that allergy plays a role in the pathogenesis of this condition and emphasizes the need to introduce anti-allergy therapy into the treatment algorithm of OME.

2.2 Introduction

Otitis media with effusion (OME) is a major pediatric health care issue, with an estimated prevalence of 15-20 % [1], and annual costs in the billions of dollars [2]. OME is the most common indication for surgery in children, and can have significant sequelae, including hearing loss, maldevelopment of communication skills, as well as permanent middle ear mucosal damage [3]. Although the exact etiology of OME is not known, it is thought to occur after a treated acute infection, in the setting of Eustachian tube dysfunction. However, 57-82 % of effusions do not have bacterial growth [4], and antibiotic therapy is no better than placebo in the resolution of OME [5-7]. Some authors have proposed a viral etiology, but viral isolates are only present in 16-19 % of cases [8].

The inflammatory cell and cytokine profile of asthma [9], allergic rhinitis [10], and chronic sinusitis [11] 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-helper cells, and the Th2 cytokines (IL-4, 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 in atopic children.

We hypothesize that there is a higher number of eosinophils, basophils, mast cells and T lymphocytes and increased expression of Th2 cytokines such as IL-4, and IL-5 in the middle ear fluid specimens of atopic children with OME compared non-atopic controls.

2.3 Methods

2.3.1 Patient selection

Patients were recruited from the Department of Otolaryngology at the McGill University Hospital Center over a six-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 based on a history of persistent MEE for greater than three 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 six weeks prior to surgery.

2.3.2 Determination of atopy

A history and physical examination was performed, assessing for general medical problems and risk factors for allergy. Eligible patients reviewed a detailed consent form approved by the investigational review board of the Montreal Children's Hospital. At the time of preoperative testing or during general anesthesia, skin testing for perennial and seasonal allergens was done, using a standard panel including alternaria, aspergillus, cladosporium, grass, penicillium, ragweed, trees, cat, cockroach, dog, and dust mites D. Farinae, and D. Pteronyssinus. Histamine (1 mg/ml) was used as a positive control and saline as a negative control. A positive skin reaction was judged as a wheal great or equal to 3mm greater than the negative control. Children were considered atopic if they had a positive skin test, and non-atopic if all skin tests were negative, regardless of clinical history.

2.3.3 Middle ear effusion collection methods

At the time of myringotomy, middle ear fluid was collected in a Juhn Tym-Taps (Xomed Treace Products, Jacksonville, FL). One milliliter (ml) of sterile phosphate-buffered saline (PBS) was added to the sample. After transportation to the Meakins-Christie Laboratories, the sample was resuspended and transferred to a siliconized centrifuge tube, and centrifuged at 1200 g for 10 minutes. The supernatant was stored at – 80 °c until assayed. One ml of mucolexx (Baxter, McGraw Park, IL) 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 three times with sterile PBS and fixed. The pellet was cytocentrifuged at 750 g for 2 minutes onto frosted slides, and differential cell counts were made by Quick Diff (Baxter) staining and microscopic examination. Slides were also prepared for immunocytochemistry and in-situ hybridization. For ICC, the cytospins were briefly fixed in a solution of acetone:methanol (40:60), air dried, and stored at – 80 °c until further use. For ISH the cytospins were air-dried and fixed in 4 % paraformaldehyde for 30 minutes, washed twice for five minutes with PBS, kept at 37 c overnight, and stored at – 80 °c until further use.

2.3.4 Immunocytochemistry

Immunocytochemistry was performed using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method, as previously described [12]. Monoclonal antibodies including anti-CD3, MBP, BB1, tryptase and elastase were used to detect T lymphocytes, eosinophils, basophils, mast cells and neutrophils, respectively. Slides were developed

using Fast Red substrate for alkaline phosphatase. A negative control slide was included in each ICC experiment.

2.3.5 In-situ hybridization

Radiolabelled complementary riboprobes for IL-4, IL-5, and IFN-γ were used to identify the presence of mRNA. The cDNA of each of these individual cytokines was inserted into pGEM vectors, grown in E.Coli, and linearized with the appropriate enzymes. Before ISH, in vitro transcription to generate sense and antisense probes was performed in the presence of ^{35}S-uridine triphosphate and the appropriate polymerases as previously reported [13]. Cytospins were permeabilized, treated with acetic anhydride and triethanolamine to reduce non-specific binding, and pre-hybridized in formamide at 42 °c. ^{35}S-labelled antisense probes were used (10^6cpm/section), followed by high-stringency post-hybridization washings. Unhybridized single-stranded RNA was removed by treating the preparation with a solution of RNAse. After hybridization, the slides were immersed in emulsion fluid and exposed for 10-14 days. The autoradiographs were developed, fixed and counterstained with hematoxylin. To ensure ISH specificity, sections and cytospins were hybridized with the sense probe or pre-treated with an RNAse solution.

2.3.6 Quantification

Specimens were coded and the number of positive cells for protein and mRNA were counted 'blind' using an Olympus microscope with an eyepiece graticule at 200x

magnification. At least 6 fields were counted for each patient at each time point. Results were expressed as the mean percentage of positive cells ± the SEM.

2.3.7 Statistical analysis

Cell counts were compared between atopic and non-atopic patients using a Mann Whitney U test, with $p<0.01$ regarded as statistically significant. At least two high power fields were counted for each marker, and the mean value of these slides was reported. To avoid observer bias, slides was coded before analysis and read in a blinded fashion.

2.4 Results

2.4.1 Clinical data

Tables 1 and 2 demonstrate the clinical data of atopic and non-atopic children with OME. In atopic OME patients (n=8), the mean age was 4.75 (2-7) years compared to 3.56 (1-8) years in the non-atopic group (n=18). Forty-six percent of patients greater than 3 years of age had at least one positive skin test, compared to 17 % of children less than 3 years.

Two patients (25 %) in the atopic group and 4 (22 %) non-atopic controls had a history of asthma. Two patients (25 %) in the atopic group and 5 (28 %) non-atopic controls had a history of rhinitis. One patient (13 %) in the atopic group and 1 (6 %) of the non-atopic controls had a history of eczema. One patient (13 %) in the atopic group and 5 (29 %) non-atopic controls 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.

2.4.2 Cellular composition of middle ear effusion specimens (figures 1,2)

The percentage of eosinophils and T lymphocytes is significantly higher in the MEE of atopic patients (7.7 ± 1.4; 20.0 ± 3.2) compared to non-atopic patients (0.9 ± 0.2; 5.5 ± 0.6) ($p<0.01$). On the other hand, a significantly higher percentage of neutrophils are found in the MEE of non-atopic patients (43.0 ± 2.4), compared to atopic patients (15.3 ± 3.1) ($p<0.01$). There is no significant difference in the percentage of mast cells found in MEE of atopic patients (7.6 ± 2.2) compared to non-atopic patients (3.4 ± 0.6)

(p=0.029). There is no significant difference in the percentage of basophils found in MEE of atopic patients (6.0 ± 2.0) compared to non-atopic patients (1.8 ± 0.4) (p=0.015).

2.4.3 Cytokine profile of middle ear effusion specimens (figures 3,4)

The percentage of IL-4 and IL-5 mRNA positive cells is significantly higher in the MEE of atopic patients (6.1 ± 0.5; 7.5 ± 0.6), compared to non-atopic patients (1.7 ± 0.3; 2.6 ± 0.4) ($p<0.01$). On the other hand, a significantly higher percentage of IFN-γ positive cells are found in the MEE of non-atopic patients (6.0 ± 0.4), compared to atopic patients (2.0 ± 0.5) ($p<0.01$).

2.5 Discussion

In our study population, we found a 30 % incidence of atopy by routine skin testing. Seven of the eight atopic children were reactive to perennial allergens. The role of atopy in the pathogenesis of recurrent otitis media and OME has been evaluated, and has been reported to be a risk factor in 0-88 % of patients [14-20]. In theory, allergic inflammation can cause recurrent otitis media or OME indirectly by causing a transient blockage of the Eustachian tube [21, 22], or directly by middle ear mucosal inflammation. While some investigators have concluded that atopy is a significant risk factor for recurrent otitis media [23-28], others have found no relationship between the two conditions [29,30]. The major problem with most of these studies is the lack of uniform diagnostic criteria for atopy.

Atopic children in our study have significantly higher percentages of T lymphocytes, eosinophils, IL-4 and IL-5 mRNA positive cells compared non-atopic children. Although not statistically significant, the percentage of mast cells and basophils was also higher in the atopic effusions compared to controls. 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, ECP, MBP, and LTC_4, which causes extracellular matrix deposition, epithelial denudation and basement membrane disruption [12,31-36]. 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 via IgE cross linkage and release histamine upon activation [37].

Since 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 [37-39].

IL-5 is important for eosinophil production, recruitment, activation, and survival [40]. IL-4 is important for the differentiation of naïve T-helper cells (TH0) towards the TH2 phenotype, and plays an important role in the local production of IgE in tissues of atopic patients [41]. Through induction of endothelial VCAM-1, IL-4 is thought to promote eosinophil infiltration to the site of inflammation.

The recent literature has looked at the role of cytokines in middle ear effusions, both in animals and humans [42-45]. Most studies have assessed for the presence of non-specific 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 [46].

There have been a number of studies that have attempted to identify the role of allergic mediators in OME. Hurst et al showed that eosinophils were present in the fluid of atopic patients with OME [47-52]. Wright et al recently demonstrated increased expression of IL-5 and major basic protein (eosinophils) in the middle ear mucosa of patients with OME compared to normal controls [53]. Although these studies provide preliminary evidence for the role of allergy mediators in OME, they do not look at non-atopic patients with OME, and are limited by the necessity to do middle ear mucosal biopsies in order to measure appreciable levels of cytokines.

In summary, we have found that 30 % of children with OME in our study were atopic by skin testing. Seven of the eight atopic children were reactive to perennial

allergens. Middle ear effusions of atopic children with OME are characterized by higher percentages of eosinophils, T lymphocytes and Th2 positive cells compared to non-atopic controls. This inflammatory profile is consistent with that seen in other upper airway diseases whose pathogenesis is related to the late phase allergic response. It is likely that in atopic patients MEE develops after exposure to allergen, resulting in a sustained late phase allergic response.

Neutrophils and IFN-γ positive cells were present in significantly higher percentages in non-atopic patients, providing evidence that MEE in these patients are likely the result of an infectious process. It is likely that MEE in these patients develop after an acute infection in the presence of Eustachian tube dysfunction.

Demonstration of allergy mediators provides evidence for the need to introduce anti-allergy therapy in to the treatment algorithm of OME. This may include avoidance therapy and the use of specific pharmacotherapy for certain patients in order to prevent the complications of OME. These results would also provide the rationale for doing studies to further elucidate the role of allergy in the pathogenesis of OME.

2.6 Acknowledgments

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2.8 Tables

2.8.1 Table 1: Clinical presentation of atopic and non-atopic children with otitis media with effusion

	Atopic OME patients	Non-atopic OME patients
Age	4.75 (2-7 yrs)	3.56 (1-8 yrs)
Sex (M:F)	7:1	12:6
Asthma	2 (25%)	4 (22%)
Rhinitis	2 (25%)	5 (28%)
Eczema	1 (13%)	1 (6%)
Family history of allergy	1 (13%)	5 (29%)

2.8.2 *Table 2:* Results of skin prick testing in atopic children with otitis media with effusion

Dog	5
Dust Mite	4
Cat	2
Tree mix	1
Ragweed	1
Cockroach	0
Grass	0
Molds	0

2.9 Figure legend

Figure 1. *Inflammatory cells in the middle ear effusions of atopic and non-atopic children with otitis media with effusion*

This figure demonstrates the mean percentage of positive cells ± the standard error of the mean present in each patient group. The percentage of eosinophils and T lymphocytes is significantly higher in the MEE of atopic patients compared to non-atopic patients ($p<0.01$). On the other hand, a significantly higher percentage of neutrophils are found in the MEE of non-atopic patients compared to atopic patients ($p<0.01$). There is no significant difference in the percentage of basophils and mast cells found in MEE of atopic patients compared to non-atopic patients.

Figure 2. *Results of immunocytochemistry: MBP, CD3, and BB1 positive cells in the middle ear effusions of atopic children with otitis media with effusion*

Development of the slide using Fast Red substrate for alkaline phosphatase allows for the identification of (A) T lymphocytes (CD3), (B) eosinophils (MBP) and (C) basophils (BB1) in atopic patients with OME.

Figure 3. *Th1 and Th2 cytokine mRNA expression in the middle ear effusions of atopic and non-atopic children with otitis media with effusion*

This figure demonstrates mean percentage of cells expressing cytokine mRNA ± the standard error of the mean in each patient group. The percentage of IL-4 and IL-5 mRNA positive cells is significantly higher in the MEE of atopic patients compared to non-atopic patients ($p<0.01$). On the other hand, a significantly higher percentage of IFN-γ

mRNA positive cells are found in the MEE of non-atopic patients compared to atopic patients ($p<0.01$).

Figure 4. ***Results of in-situ hybridization: IL-5, IL-4, and IFN-γ mRNA expression in the middle ear effusions of atopic children with otitis media with effusion***

Development of the slide by autradiography allows for the demonstration of cells containing (A) IL-5, (B) IL-4, and (C) IFN-γ mRNA positive cells in the middle ear effusions of atopic OME patients.

2.10 Figures

Figure 1

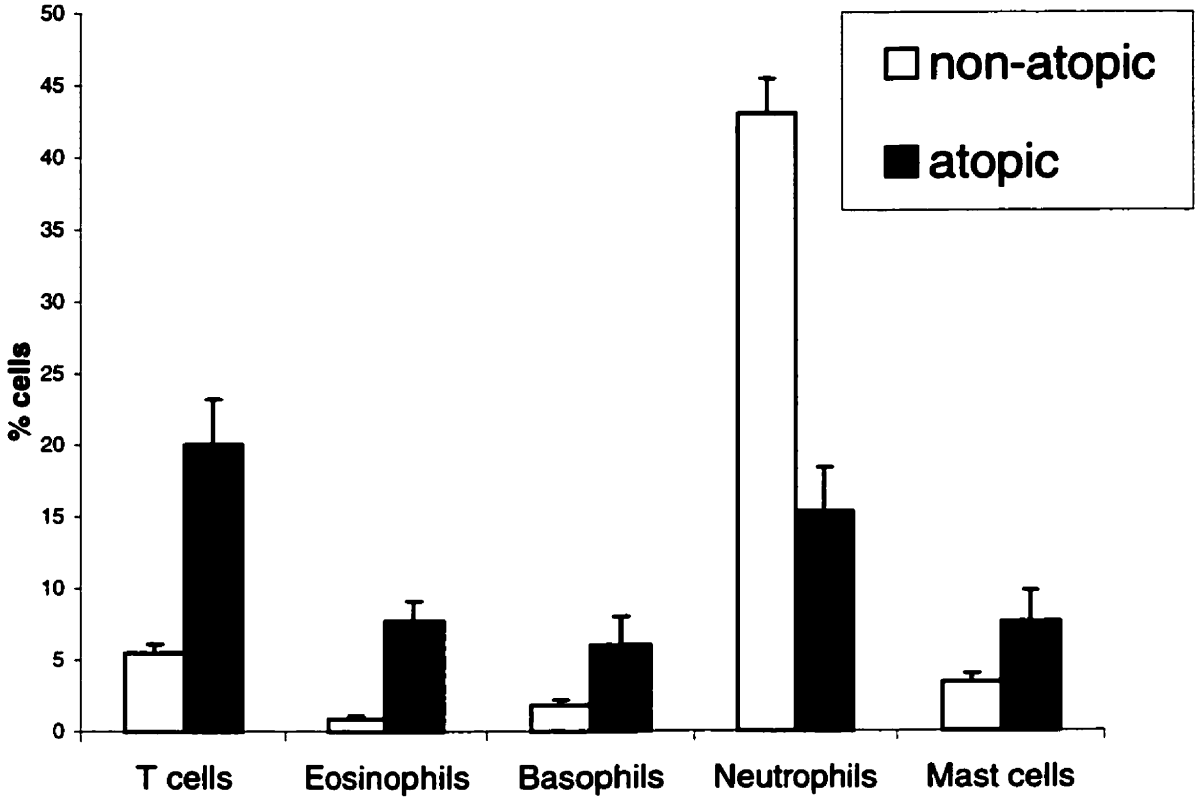


Figure 2

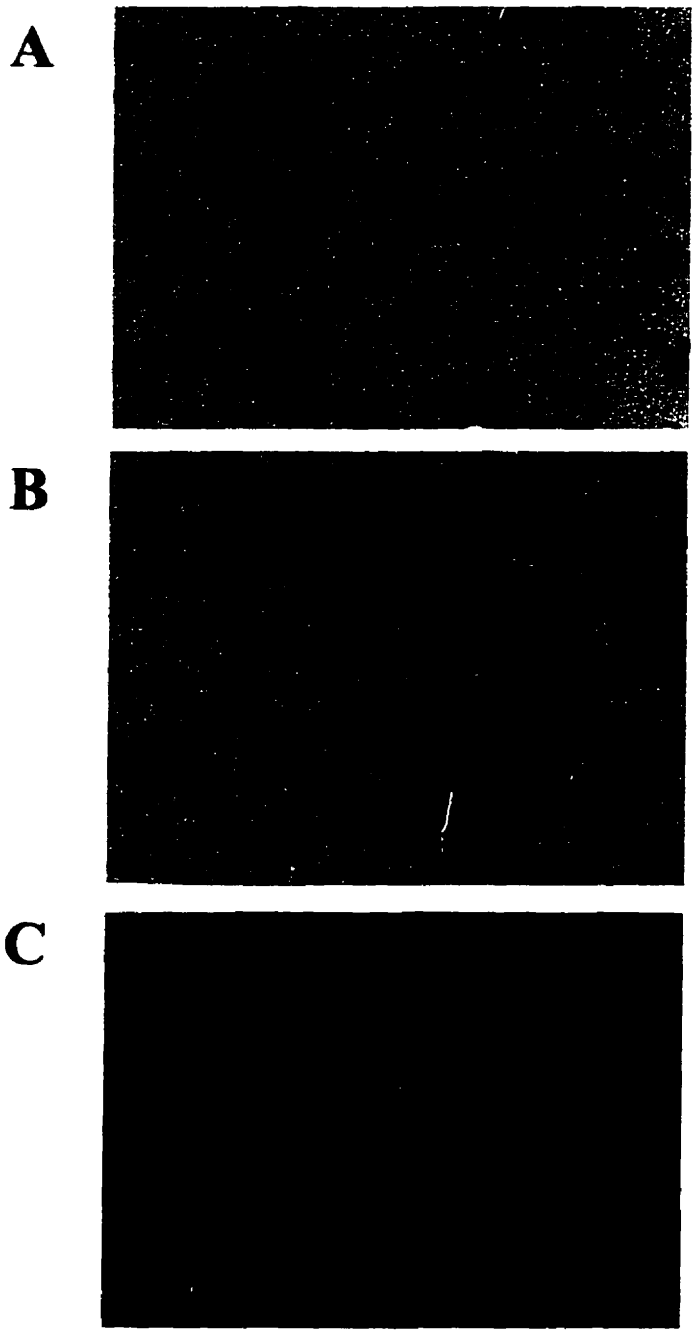
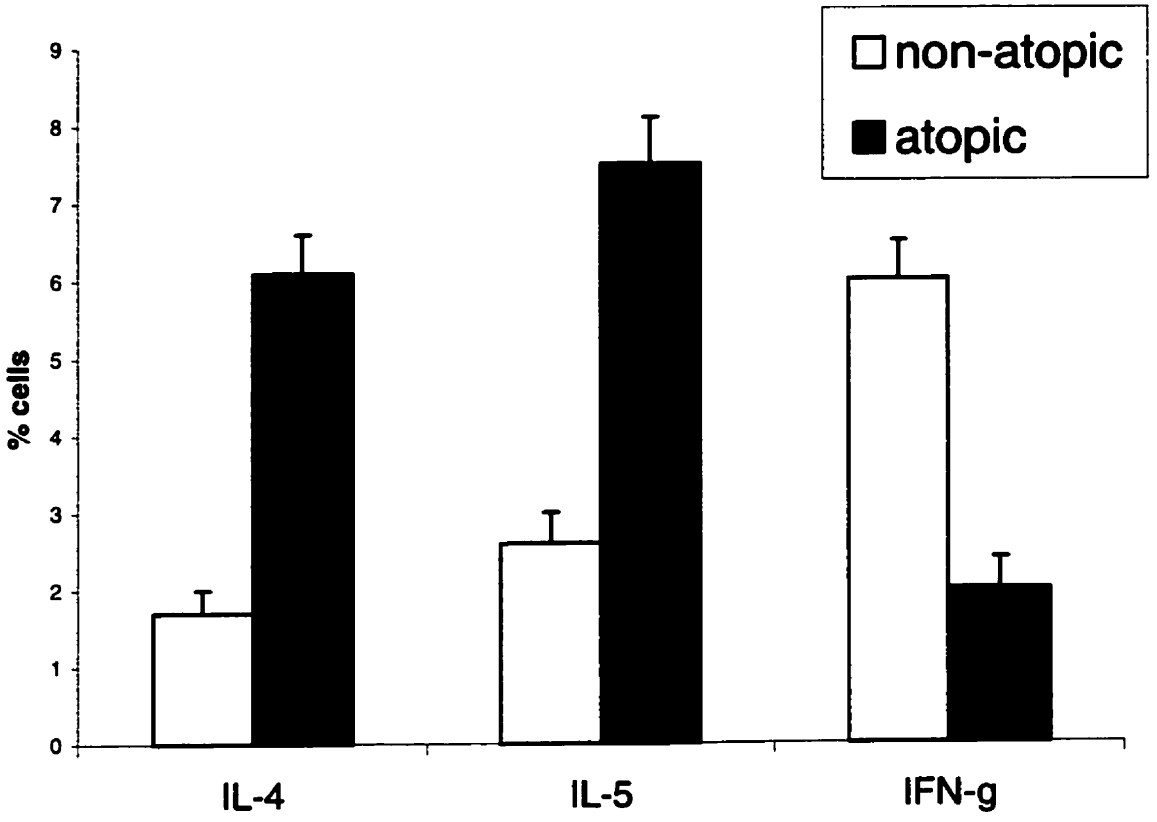
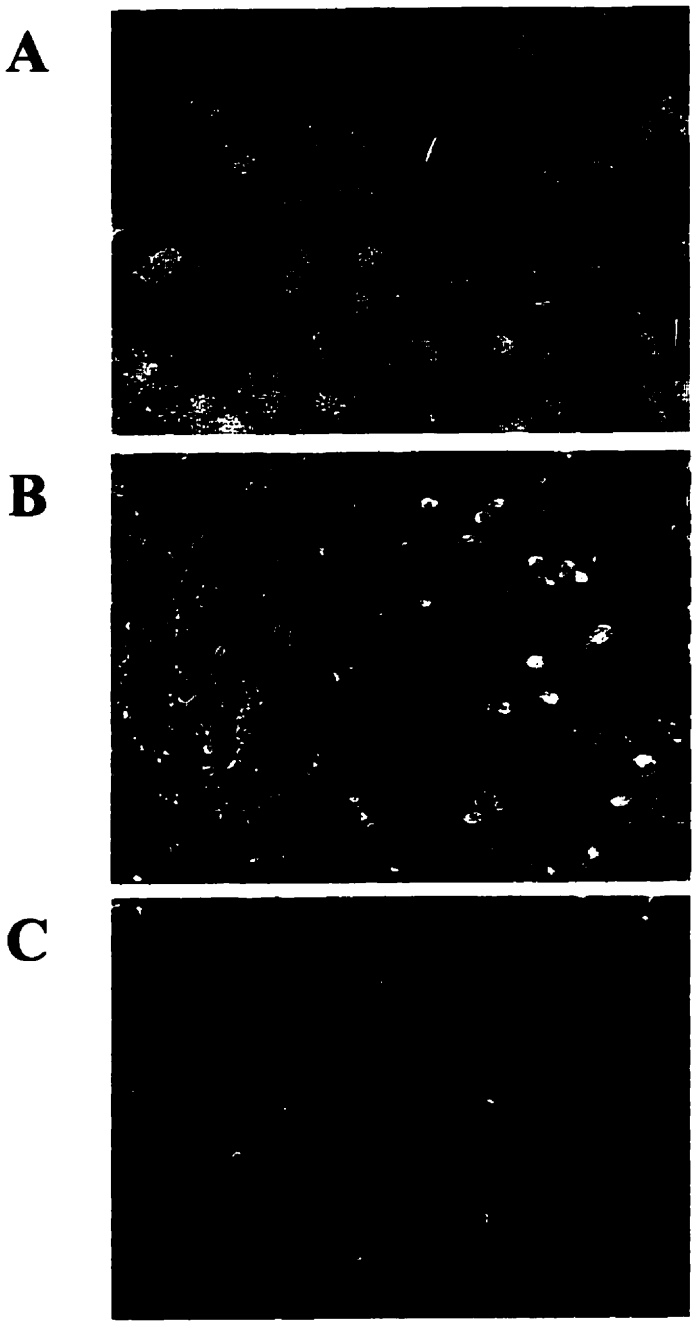


Figure 3




A
B
C

Chapter 3. Overall conclusions

3.1 The role of atopy in otitis media with effusion

3.1.1 Clinical

3.1.2 Cellular and cytokine profiles

3.1.3 Recommendations

3.2 Limitations of this study

3.3 Future studies

3.1 The role of atopy in otitis media with effusion

3.1.1 Clinical

Thirty percent of patients with OME in our study were atopic by skin testing, having an average age of 4.75 years. Seven of the eight atopic patients were reactive to perennial allergens. Non-atopic patients represented 70 % of our patient group, having an average age of 3.56 years. These clinical findings are consistent with those found in other studies. Given that the sample size in this study is only 26, it is difficult to draw conclusions regarding the epidemiological significance of our data. It seems apparent, however, that older children with OME have a predisposition for allergies, given that nearly half of them are skin test positive. Moreover, the nearly uniform reactivity to perennial allergens in OME patients is consistent with that seen in asthmatic patients. Taken together, this clinical data supports the hypothesis that OME is an atopic disease, although further studies are necessary in order to properly resolve this question.

3.1.2 Cellular and cytokine profiles

Middle ear effusions of atopic patients with OME are characterized by higher percentages of eosinophils, T lymphocytes and Th2 mRNA positive cells compared to non-atopic controls. This inflammatory profile is consistent with that seen in other upper airway diseases whose pathogenesis is related to the late phase allergic response. It is likely that in atopic patients middle ear effusions develop after exposure to allergen, which may reach the middle ear cleft through the Eustachian tube. The allergen may then be processed by dendritic cells located in the middle ear mucosa, and presented to Th2 cells, which subsequently produce and secrete Th2 mediators, including IL-4 and IL-5.

IL-5 may then promote the production, recruitment, activation, and survival of eosinophils within the middle ear cleft. IL-4 may perpetuate the Th2 response within the middle ear by promoting the differentiation of naïve T-helper cells (TH0) towards the TH2 phenotype. Through induction of endothelial VCAM-1, IL-4 may also contribute to the eosinophilic infiltrates found in the middle ear of atopic OME patients.

Neutrophils and IFN-γ mRNA positive cells were present in significantly higher percentages in non-atopic patients, providing evidence that middle ear effusions in these patients are likely the result of an infectious process. It is likely that middle ear effusions in these patients develop after an acute infection in the presence of Eustachian tube dysfunction.

3.1.3 Recommendations

Demonstration of allergy mediators provides evidence for the need to introduce anti-allergy management in to the treatment algorithm of OME. Based on the results of this study we recommend the following.

1) Children with OME over three years of age should be referred to a pediatric allergist for an evaluation, which should include skin prick testing.
2) Children with OME less than three years of age should be referred to a pediatric allergist if they are at moderate to high risk of being atopic (strong family history, history of asthma, eczema, rhinitis), with consideration for skin prick testing in selected cases.
3) Therapy for atopic OME patients should include specific avoidance of the reactive allergen if possible.

4) The role of pharmacotherapy, including systemic or nasal corticosteroids, and antihistamines needs to be evaluated by randomized controlled trials.

5) The role of immunotherapy in older children with refractory OME needs to be evaluated by randomized controlled trials.

6) Myringotomy and ventilation tube placement is the treatment of choice in selected OME patients having middle ear effusions for greater than three months with hearing loss of greater than 15 decibels.

7) Observation of selected patients with OME may be considered, given that the long-term effects of middle ear effusions on speech and language development is controversial.

3.2 Limitations of this study

The limitations of this study lie in its inability to answer several questions related to the pathogenesis of OME. For example, one question that needs to be answered is whether or not the middle ear mucosa is the sole target of allergic inflammation in OME patients, or is the inflammatory process continuous along the Eustachian tube and nasopharyngeal mucosa ? Another question that needs to be addressed is the role of the adenoid in the pathogenesis of OME in atopic patients. The role of these two anatomical structures in the pathogenesis of OME needs to be established before any conclusions can be made about the role of allergies.

Another limitation of our study is the inability to determine whether or not the cells and cytokines present in atopic middle ear effusions represent inflammation within the middle ear mucosa, or if they emanate from the circulation (i.e. a systemic allergic response). Unfortunately, it is not possible to overcome this limitation without taking biopsy specimens of middle ear mucosa from OME patients. Even if middle ear effusions in atopic patients are the result of a systemic response, the goal of management should include treating the underlying allergies contributing to OME.

The control group in our study was comprised of non-atopic children with OME. It was not possible to have a control group of normal children without OME, given that the middle ear cleft does not normally have fluid. Therefore, another limitation of our study is that we cannot determine what cells and cytokines are present in the middle ear cleft of children without OME. Unfortunately, it is not possible to overcome this limitation without taking biopsy specimens of middle ear mucosa from both OME patients and normal children.

3.3 Future studies

Future studies are necessary in order to further elucidate the role of allergies in the pathogenesis of OME, and to assess the role of anti-allergy therapy in the treatment of OME patients.

We are currently in the process of designing a study, which will evaluate the cellular and cytokine profiles of the nasopharyngeal mucosa, adenoid, and middle ear effusions of atopic and non-atopic patients with OME. The goal of this study will be to answer questions concerning the role of the adenoid and Eustachian tube in the pathogenesis of OME in both atopic and non-atopic children.

The role of other inflammatory cells and cytokines, including B cells, macrophages, and IL-13 should be evaluated in future studies in order to determine their role in the pathogenesis of OME.

Randomized clinical trials are needed in order to assess the efficacy of anti-allergy therapies for the treatment of OME. In specific, the role of avoidance, immunotherapy and specific pharmacotherapy needs to be evaluated.