EVIDENCE LINKING ALLERGIC OTITIS MEDIA WITH EFFUSION TO THE UNITED AIRWAYS CONCEPT

Dr. Ha-Nam Phan Nguyen

Department of Otolaryngology
McGill University, Montreal

Meakins-Christie Laboratories
McGill University, Montreal

October, 2003

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science.

© Ha-Nam Phan Nguyen, M.D., 2003
NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.
ACKNOWLEDGEMENTS

I extend my deepest gratitude to Dr. Q Hamid, my supervisor, for introducing me to the world of basic science research, and for his guidance and support throughout this entire process.

I am indebted to Dr. JJ Manoukian, my co-supervisor, for his wise advice and unfailing encouragement in all my endeavors.

My thanks to Dr. T Tewfik, Dr. M Schloss, Dr. Shapiro and Dr. J Rothstein for their assistance in the recruitment of patients.

I would like to acknowledge Dr. R Taha, E Schottman, May Kafilmout, P Fiset and Dr P Joubert for their invaluable technical assistance.

I would like to acknowledge Dr. T. Tewfik, Dr. B Mazer, Dr. M Tulic and Dr. P Christodoulopoulos for their assistance in editing the manuscript.

Dr. S Sobol, as a collaborator, for his guidance in conceptualizing the project at its earliest stages.

My appreciation goes to the Department of Otolaryngology, McGill University, for their support in arranging and carrying out this project, and to the Canadian Institute of Health and Research and the McGill University Head and Neck Research Fund for their financial support.

Finally, to my loving husband and caring family, thank you for your unconditional love and your unyielding support in the pursuit of my dreams.
ABSTRACT:

Otitis media with effusion (OME) is a chronic inflammatory disease of the middle ear space characterized by the accumulation of fluid. Previous investigations have found the composition of the inflammatory substrate in effusions of allergy-associated otitis media to be is similar to the late-phase allergic response seen elsewhere in the respiratory tract, such as in asthma and in allergic rhinitis. In addition, there is evidence suggesting that diseases of the upper and lower airways may represent different clinical manifestations of a single inflammatory airway syndrome, or the United Airway Concept. The objective of this research is to determine if the middle ear compartment may be a component of the United Airways in allergic disease. Middle ear fluid, torus tubaris (Eustachian tube mucosa at the nasopharyngeal orifice) and adenoidal tissue biopsies were obtained from 45 patients undergoing simultaneous tympanostomy tube placement for OME and adenoidectomy for adenoid hypertrophy. The cellular and cytokine profiles of each site were investigated using immunocytochemistry (elastase, CD3, MBP) and in-situ hybridization (IL-4, IL-5, IFN-γ mRNA). Atopic status was determined for each patient using skin-prick testing. Eleven of the 45 patients with OME (24%) were atopic. The MEE of atotics had significantly higher levels of eosinophils, T lymphocytes, IL-4 and IL-5 mRNA +cells (p < 0.01), and significantly lower levels of neutrophils and IFN-γ mRNA +cells (p < 0.01) when compared to non-atotics. The nasopharyngeal tissue biopsies also revealed similar cellular and cytokine profiles. Therefore, the allergic inflammation in atopic patients with OME occurs on both sides of the Eustachian tube,
both in the middle ear and in the nasopharynx. The results of this study support the concept that the middle ear may be part of the United Airway in atopic individuals.
SUMMAIRE

L'otite moyenne avec épanchement constitue une inflammation chronique de l'oreille moyenne. Des études portant sur la composition de l'épanchement ont démontrées que cette dernière est semblable à celle de la phase tardive allergiques des voies respiratoires supérieures et inférieures tel qu'observé dans l'asthme et la rhinite allergique. Il a également été suggéré que les maladies des voies respiratoires supérieures et inférieures peuvent représenter un seul syndrome inflammatoire avec deux tableaux cliniques différents, donc un "concept aérien uni." L'objectif de l'étude est de déterminer si l'oreille moyenne peut faire partie du "concept aérien uni" en cas d'otite allergique. Du liquide provenant de l'oreille moyenne, ainsi que des biopsies du torus tubaire (muqueuse de la trompe d'Eustache à l'orifice rhinopharyngé), et du tissu adénoidien ont été obtenus chez 45 patients ayant subi une insertion de tubes tympaniques pour OME et une adénoidectomie pour hypertrophie des adénoides. Le profil des cytokine et cytologique de chaque site a été examiné par immunocytochimie (elastase, CD3, MBP) et hybridation in situ (IL-4, IL-5, IFN-γ mRNA). L'état atopique de chaque patient a été déterminé par des épreuves cutanées. Onze des 45 patients avec OME (24%) étaient atopiques. Le liquide de l'oreille moyenne chez les patients atopiques contienait des niveaux élevés d'œsinophiles, de lymphocytes, de IL-4 et IL-5 mRNA + cellules (p<0.01), et des niveaux significativement bas des neutrophiles et IFN-γ mRNA + cellules en comparaison avec les patients non-atopiques. Les biopsies du nasopharynx ont révélé des résultats semblables. L'inflammation allergique chez les patients atopiques se manifeste des deux cotés de la trompe d'Eustache, soit dans l'oreille moyenne et le rhinopharynx. Les
résultats de cette étude confirment que l’oreille moyenne peut faire partie du “concept aérien uni” chez les individues atopiques.
ABBREVIATIONS

AP    alkaline phosphatase
APAAP alkaline phosphatase anti-alkaline phosphatase
ECP   eosinophilic cationic protein
ET    Eustachian tube
ICC   immunocytochemistry
IFN   interferon
Ig    immunoglobulin
IL    interleukin
ISH   in-situ hybridization
MBP   major basic protein
MEE   middle ear effusion
OCT   optimal cutting temperature
OME   otitis media with effusion
PBS   phosphate-buffered saline
RNA   ribonucleic acid
SEM   standard error of the mean
TBS   Tris buffered saline
TH    T helper cell
TNF   tumor necrosis factor
VCAM  vascular cell adhesion molecule
# LIST OF TABLES AND FIGURES

<table>
<thead>
<tr>
<th>FIGURES</th>
<th>PAGE NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1: Otitis Media with Effusion</td>
<td>13</td>
</tr>
<tr>
<td>Figure 2: T(<em>{H}1) and T(</em>{H}2) Immune Responses</td>
<td>15</td>
</tr>
<tr>
<td>Figure 3: Main Actions of IL-5</td>
<td>21</td>
</tr>
<tr>
<td>Figure 4: Main Actions of IL-4</td>
<td>22</td>
</tr>
<tr>
<td>Figure 5: The United Airways Concept</td>
<td>28</td>
</tr>
<tr>
<td>Figure 6: The Middle Ear and the United Airways Concept</td>
<td>31</td>
</tr>
<tr>
<td>Figure 7: The Torus Tubaris and the Adenoid Pad</td>
<td>34</td>
</tr>
<tr>
<td>Figure 8: APAAP technique of Immunocytochemistry</td>
<td>38</td>
</tr>
<tr>
<td>Figure 9A: T lymphocytes in Middle Ear Fluid</td>
<td>39</td>
</tr>
<tr>
<td>Figure 9B: Eosinophils in Adenoid Tissue</td>
<td>39</td>
</tr>
<tr>
<td>Figure 10A: IL-5 mRNA in Middle Ear Fluid</td>
<td>42</td>
</tr>
<tr>
<td>Figure 10B: IL-4 mRNA in Torus Tubaris Tissue</td>
<td>42</td>
</tr>
<tr>
<td>Figure 11: Eosinophil Levels</td>
<td>46</td>
</tr>
<tr>
<td>Figure 12: Neutrophil Levels</td>
<td>47</td>
</tr>
<tr>
<td>Figure 13: IL-4 mRNA Levels</td>
<td>48</td>
</tr>
</tbody>
</table>
Figure 14: IL-5 mRNA Levels 49

Figure 15: IFN-γ mRNA Levels 50

TABLES

Table 1: Allergy Data for Atopic Patients with OME 44

Table 2: Demographic and Clinical Characteristics 45
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CONTENTS</th>
<th>PAGE NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>02</td>
</tr>
<tr>
<td>Abstract</td>
<td></td>
</tr>
<tr>
<td>English</td>
<td>03</td>
</tr>
<tr>
<td>French</td>
<td>05</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>07</td>
</tr>
<tr>
<td>List of Tables and Figures</td>
<td>08</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>10</td>
</tr>
<tr>
<td><strong>Chapter I: Introduction</strong></td>
<td></td>
</tr>
<tr>
<td>1.1 Otitis Media with Effusion</td>
<td>13</td>
</tr>
<tr>
<td>1.2 Inflammation</td>
<td>15</td>
</tr>
<tr>
<td>1.3 Inflammation in Allergic OME</td>
<td>16</td>
</tr>
<tr>
<td>1.3.1 Cellular Profile</td>
<td></td>
</tr>
<tr>
<td>1.3.1.1 Early Phase Allergic Response</td>
<td>16</td>
</tr>
<tr>
<td>1.3.1.2 Late Phase Allergic Response</td>
<td>17</td>
</tr>
<tr>
<td>1.3.2 Cytokine Profile</td>
<td></td>
</tr>
<tr>
<td>1.3.2.1 Early Phase Allergic Response</td>
<td>18</td>
</tr>
<tr>
<td>1.3.2.2 Late Phase Allergic Response</td>
<td>19</td>
</tr>
<tr>
<td>1.3.3 Chemokine Profile</td>
<td>20</td>
</tr>
<tr>
<td>1.4 Inflammation in Non-Allergic OME</td>
<td>23</td>
</tr>
<tr>
<td>1.5 OME and Other Atopic Diseases</td>
<td>24</td>
</tr>
<tr>
<td>1.6 United Airways</td>
<td>24</td>
</tr>
<tr>
<td>1.6.1 Epidemiological Evidence</td>
<td>25</td>
</tr>
<tr>
<td>1.6.2 Immunopathophysiologic Evidence</td>
<td>26</td>
</tr>
</tbody>
</table>
1.6.3 Therapeutic Evidence

1.7 Objectives

Chapter II: Materials and Methods

2.1 Study Design

2.2 Diagnosis of Atopy

2.3 Sample Collection Method

2.4 Preparation for ICC and ISH
   2.4.1 Fluid Preparation
   2.4.2 Tissue Preparation

2.5 Immunocytochemistry
   2.5.1 Basic Principles
   2.5.2 Immunocytochemistry Protocols

2.6 In-situ Hybridization
   2.6.1 Basic Principles
   2.6.2 In-situ Hybridization Protocols

2.7 Quantification

2.8 Statistical Analysis

Chapter III: Results

3.1 Atopy in OME Patients

3.2 Cellular Profiles

3.3 Cytokine Profiles

Chapter IV: Discussion

4.1 Discussion of Results

4.2 Limitations
4.3 Clinical Implications and Future Directions 54
4.4 Conclusions 55

Chapter V: References 57

Chapter VI: Appendices

6.1 Consent Form (English) 73
6.2 Consent Form (French) 76
6.3 Data Collection Form 80
6.4 Ethics Certificate 82
CHAPTER I: INTRODUCTION

1.1 Otitis Media with Effusion

Otitis media with effusion (OME) is a chronic inflammatory disease of the middle ear mucosa characterized by the retention of fluid within the middle ear space (Figure 1). At a prevalence of 15-20%, OME represents both a major pediatric health care issue and a substantial economic burden estimated in the billions of dollars annually. OME is the single most important cause of hearing loss and the most common indication for surgery in children. Patients with OME can have significant sequelae including hearing loss, subsequent delayed speech development, as well as permanent middle ear mucosal damage.

Figure 1: Otitis Media with Effusion. Transcanal view of the tympanic membrane, demonstrating the presence of fluid in the middle ear space.
The development of OME results primarily from a complex interaction of numerous risk factors. Preceding bacterial or viral infection, as well as Eustachian tube (ET) dysfunction, are acknowledged contributors. Studies have indicated an additional effect related to craniofacial abnormalities, genetic predisposition, parental cigarette smoking and socioeconomic status. The controversial role of allergy in the pathogenesis of OME has been evaluated in recent years.

Many clinical studies have been designed to evaluate the relationship between allergies and OME. While some investigators have concluded that atopy is a significant risk factor for recurrent otitis media\textsuperscript{4-8}, others refute this hypothesis having found no relationship between the two conditions\textsuperscript{9,10}. The major problems with many of these studies is that there is a lack of uniform diagnostic criteria for atopy and that they are not based on present day standardized methods of allergy testing, such as objective skin tests, provocation tests or serum immunoglobulin (Ig) E analysis.

The prevalence of positive skin tests in OME patients varies widely, from 9 to 88\% between studies\textsuperscript{11-15}. Tomonaga et al found that 87\% of patients with OME were allergic by skin testing\textsuperscript{16}. He determined that the incidence of allergies among patients with OME to be 4 to 5 times higher that expected in a similar age-adjusted population of normal controls, and suggested that the relationship may be more than coincidential. On the other hand, Caffarelli et al used the skin prick technique and found no difference in the prevalence of allergen reactivity between OME patients (27\%) and controls (31\%)\textsuperscript{11}.
1.2 Inflammation

Adaptive immune response to antigens can be simplistically divided into two groups, depending on which T helper cell subtype, and its associated cytokines, predominates. T$_H$1 cells participate in cell-mediated immunity, predominate in bacterial/viral infections, and express cytokines IFN-$\gamma$ and IL-2. They are characteristically involved in delayed type hypersensitive responses and enhanced macrophage function. The inflammation in allergic diseases has been characterized by an abundance of T$_H$2 cytokines (particularly IL-4 and IL-5). It has been suggested that the immunopathologic mechanisms underlying the development of OME in allergic patients is largely attributed the effects of T$_H$2 cytokines and activation of their receptors. See Figure 2.

Figure 2: T$_H$1 and T$_H$2 Immune Responses. Adaptive humoral immune response to antigens can be divided into T$_H$1 and T$_H$2 responses. T$_H$1 response is typically seen in infectious diseases, while the T$_H$2 response is characteristically found in allergic diseases.
1.3 Inflammation in Allergic OME

Whereas the role of allergic 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. Preliminary studies have shown that the composition of the inflammatory substrate in allergic otitis media is similar to that seen in allergic rhinitis, allergy-associated chronic sinusitis and in the late-phase response to antigen challenge.

1.3.1 Cellular Profile

1.3.1.1 Early Phase Allergic Response

The healthy middle ear mucosa is composed of low cuboidal epithelium. Normally, it is devoid of lymphoid tissue and reacts weakly to antigen challenge. However, a sensitized ear is capable of mounting a vigorous immune response when an antigen is presented17. Indeed, immunocompetent cells are present in both the middle ear effusion and in the inflammed middle ear mucosa of atopic patients with OME.

Mast cell degranulation is the critical initiating event of early phase allergic response. Allergen exposure and crosslinkage of IgE result in the release of pro-inflammatory mediators, including tryptase, tissue necrosis factor-alpha (TNF-α), interleukin- (IL) 4 and histamine18,19. Elevated tryptase and histamine levels have been detected in nasal lavage fluid of allergic rhinitis patients20. Mast cells themselves have been found in the tympanic cavity of guinea pigs21 and humans22, leading to speculation that the middle ear may be a target of the early allergic response.
1.3.1.2 Late Phase Allergic Response

Many investigators have attempted to gain insight into the role of allergy mediators in OME. Sobol et al recently demonstrated significantly higher numbers of T lymphocytes and eosinophils in atopic middle ear effusions compared to non-atopic controls. Although not statistically significant, the number of mast cells and basophils were also higher in the atopic effusions compared to controls. The results of this study are consistent with the late phase allergic response seen in other areas of the respiratory tract, such as in asthma, allergic rhinitis, and chronic sinusitis.

Among the immune regulatory cells, eosinophils play a particularly important role in the late phase allergic response through the release of various mediators including eosinophilic cationic protein, major basic protein, and leukotriene C4. The presence of activated eosinophils is associated with extracellular matrix deposition, epithelial denudation and basement membrane disruption. Hurst et al recently reported increased levels of eosinophilic cationic protein (ECP) in the supernatant of atopic OME patients compared to non-atopic controls. Within each patient, the levels of ECP in serum and in middle ear effusion did not correlate with each other, suggesting that effusions result from localized middle ear eosinophil activation and do not represent transudates from a systemic response.

Although their exact role has yet to be clearly elucidated, basophils are also to contribute to the late phase allergic response. Similar to the mast cell, basophils bind allergen via IgE cross linkage and release histamine upon activation. In addition to being a major source of histamine, basophils secrete other proinflammatory cytokines,
such as IL-4 (and IL-13), that play an important role in the ongoing allergic response. Thus basophil-derived cytokines could potentially influence the involvement of eosinophils and lymphocytes by upregulating adhesion molecules, such as vascular cell adhesion molecule, and by promoting the development of T helper- (Th2) 2 type cells.

Interestingly, neutrophil activity has been found to be both increased and decreased in atopic patients. Hurst found that marked elevations of myeloperoxidase in nonpurulent middle ear effusion (MEE) in atopic patients when compared to nonatopics. They suggested that atopic patients respond differently to the microbial or viral products of acute inflammation owing to the presence of primed inflammatory cells, and that neutrophils were an integral part of the inflammatory process in OME.

1.3.2 Cytokine Profile

1.3.2.1 Early Phase Allergic Response

There have been conflicting evidence concerning the role of total and specific IgE in the etiology of OME. Whereas some investigators have concluded that OME is an IgE mediated disease, others have not found increased IgE in MEE compared to serum samples. Nonetheless, since IgE plays a major role in the early phase allergic response, its role in OME, which is a chronic condition, is questionable.

Bernstein et al found that levels of IgE are higher in MEE compared to serum samples in 46% of allergic children with OME. They concluded that a local IgE response might be responsible for the generation of MEE in susceptible patients. On the
other hand, Boedts's\textsuperscript{36} results failed to support the concept of type 1 allergy as a major causative factor in OME. In 58 children with OME, IgE content was determined in middle ear effusions and matched sera. Eight cases (14\%) showed elevated serum IgE levels, indicating that an atopic disorder was very likely. However, in nearly all cases, higher IgE levels were found in the serum than in the effusion. Mogi et al also looked at total and specific IgE in the serum and MEE of children with OME\textsuperscript{31,32}. They determined 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.

1.3.2.2 Late Phase Allergic Response

The recent literature has looked at the role of cytokines in the orchestration of cellular recruitment and activation of the inflammatory infiltrate in allergic OME, both in animals and humans\textsuperscript{37-40}. Most studies have assessed for the presence of non-specific inflammatory mediators such as IL-1, IL-6, IL-8 and TNF-\(\alpha\). Others have looked for the presence of antibodies in the systemic circulation and in middle ear fluid specimens\textsuperscript{31}.

Like allergic rhinitis, it seems that the immunopathologic mechanisms underlying the development of OME in allergic patients has been largely attributed the effects of T\(_{H2}\) cytokines (namely IL-4, IL-5) and their receptors. 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\textsuperscript{41}. The eosinophil-associated cytokines IL-5 is especially up-regulated in allergic OME. Sobol et al demonstrated
significantly higher numbers of IL-4 and IL-5 messenger ribonucleic acid (mRNA) positive cells in atopic middle ear effusions compared to non-atopic controls. IL-5 plays a critical role in the recruitment, activation, differentiation and survival of eosinophils. See Figure 3.

There are significantly higher numbers of IL-4 mRNA within the middle ear effusion of allergic patients compared with nonatopic control subjects. The various functions of IL-4, an exclusive Th2 cytokine, include: a) influencing activated naïve T-cells to express predominately Th2 cytokines, b) promoting local IgE production by inducing isotype switching of B-cells in favor of the IgE, and c) facilitating eosinophil infiltration by enhancing endothelial expression of vascular cell adhesion molecules (VCAM-1). See Figure 4.

1.3.3 Adhesion Molecule Profile

Research on adhesion molecule activity in allergic OME is still in its infancy. Activation of adhesion molecules (i.e. VCAM) facilitates the selective transendothelial migration of inflammatory cells, particularly eosinophils, into the middle ear mucosa, and eventually into middle ear effusion, following allergen challenge. VCAM-1 is specific for eosinophils, lymphocytes and basophils (not neutrophils). Ohasi et al demonstrated that levels of soluble VCAM-1 in effusions from atopic patients were significantly higher than those from non-atopic patients.
Figure 3: Main Actions of IL-5. IL-5 plays a critical role in the preferential differentiation of eosinophils in the bone marrow, and its recruitment from the vasculature by adhering to the endothelium through the binding of integrins. IL-5 also prolongs the survival of eosinophils, and promotes the activation of eosinophils.
Figure 4: Main Actions of IL-4. The various functions of IL-4, an exclusive $\text{T}_{h2}$ cytokine, include: influencing naïve T-cells to differentiate into $\text{T}_{h2}$ cells, promoting local IgE production by inducing isotype switching of B-cells in favor of the IgE, facilitating eosinophil infiltration by enhancing endothelial expression of vascular cell adhesion molecules (VCAM-1), and inducing smooth muscle hyperplasia in asthma.
1.4 Inflammation in Non-Allergic OME

Non-allergic OME mostly results from the inflammation induced by viral or bacterial infection. Non-atopic patients reveal different cellular and cytokine profiles in their MEE, especially if the effusion is purulent. Macrophages and neutrophils are the predominant cell types in middle ear effusions, constituting a significant percentage of the total number of cells\textsuperscript{45,46}. These two cell types are critical in their ability to phagocytize bacteria. In addition, T-cells constituted the majority of lymphocytes found in the MEE\textsuperscript{47,48}.

In contrast to allergic OME, Th1 cytokines and their receptors seem to orchestrate the inflammatory response in nonallergic OME, reflecting the role of bacterial products in the stimulation of middle ear effusion production. Previous investigations have identified and characterized the presence of TNF-\alpha, TNF soluble receptor, interferon-\((\text{IFN})\) \(\gamma\) and IL-2, while certain cytokines such as IL-4 and GM-CSF were not present in sufficient quantities for detection.

IL-8 is a potent chemotactic factor and specifically recruits neutrophils to the site of inflammation. Numerous studies have shown that IL-8 is highly present in the MEE of children and adults\textsuperscript{49-51}. Nassif et al showed that IL-8 concentrations correlated positively with the total number of neutrophils in the effusion\textsuperscript{38}. In addition, purulent effusions had greater IL-8 and neutrophil concentrations than in other types of effusions (mucoid or serous).
1.5 **OME and Other Atopic Diseases**

Both allergy and otitis media are common entities in the pediatric population, and therefore a significant number of children are expected to have both conditions. Most, but not all, epidemiological studies have supported the association between OME and the presence of atopic conditions including allergic rhinitis, eczema, and asthma\(^{52-57}\). However, the nature of this association is debatable. It is possible that these conditions simply co-exist and do not affect each other. Other studies have presented evidence supporting causality.

Studies\(^{11,14,58-63}\) have shown that patients with OME have an increased prevalence of atopic conditions when compared to non-OME controls. Alles et al found in patients with OME an 89% incidence of allergic rhinitis, 36% asthma and 24% eczema\(^{64}\). Since the worldwide incidence of allergic rhinitis in children is estimated at 20%, the authors contend that the high prevalence of allergic rhinitis may suggest a causal relationship. However, the high frequency of allergy found in these patients may be explained by possible referral bias since the patients were recruited from a ‘glue ear/allergy’ clinic. Conversely, patients with known atopic conditions were also more likely to have OME\(^{5,16,65,66}\). In a study of 540 children, Draper reported the presence of OME in 52% of allergic rhinitis patients, compared to 24% of non-allergic controls\(^{66}\).

1.6 **United Airway Concept**

Historically, inflammatory diseases of the upper and lower airways, such as allergic rhinitis and asthma respectively, were diagnosed as distinct entities and thereby
managed separately. However, the respiratory mucosa, from the nasal cavity extending to the smaller bronchi, shares many common histopathological features. For example, it is covered by contiguous ciliated columnar epithelium possessing abundant mucinous glands and vascularity, and has similar innervation. Therefore, a link between the upper and lower airways has been proposed. In the past decade, substantial advances towards understanding this association have provided evidence supporting the premise of "one airway, one disease", or the United Airway Concept. It appears that asthma and allergic rhinitis may represent different clinical manifestations of a single inflammatory airway syndrome. The allergic inflammation is not confined to a specific target organ, but rather is present in continuum of the common airway.

1.6.1 Epidemiological Evidence

Collectively, epidemiological studies provide the main source of data concerning the association between the upper and lower airways. The frequent coexistence of allergic rhinitis and asthma has been documented in numerous cross-sectional studies: between 60% to 78% of asthmatic patients also have allergic rhinitis. Similarly, between 19% and 38% of patients with allergic rhinitis have coexisting asthma, a prevalence rate much higher than in the general population. A family history of atopic disease is recognized as a major risk factor for asthma and rhinitis.

There appears to be a temporal relationship between the onset of upper and lower airway diseases. The onset of nasal symptoms frequently precedes the onset asthma. In addition, the presence of allergic rhinitis may serve as a positive predictive factor in the
eventual development of lower airway dysfunction. In a prospective, 23-year follow-up study of 690 patients without evidence of asthma, 10.5% of patients who initially reported nasal symptoms developed asthma, compared to 3.6% of subjects without rhinitis\(^71\).

1.6.2 Immunopathophysiologic Evidence

The inflammatory cellular infiltrates and the cytokine profiles found in nasal mucosa of subjects with allergic rhinitis and in the bronchial mucosa of atopic asthmatics are very similar. To date, no consistent differences have been identified. In both upper and lower airways, allergic inflammation is characterized by an increased number of eosinophils, mast cells and T-helper lymphocytes expressing T\(_H2\)-type cytokines. These data clearly demonstrate that allergic rhinitis and atopic asthma share common immunological features.

These inflammatory profiles may occur even in the absence of symptoms. Chakir et al\(^72\) demonstrated an increased recruitment of inflammatory cells, such as lymphocytes and eosinophils, and an increased IL-5 immunoreactivity in bronchial mucosa biopsies of non-asthmatic subjects with allergic rhinitis during periods of natural allergen (pollen) exposure. On the other hand, Gaga et al\(^73\) demonstrated that the nasal mucosa of asthmatics, with or without rhinitis symptoms, had increased levels of activated eosinophils when compared to controls. The study demonstrates that the condition of the upper airways definitively influences the lower airway and vice versa, suggesting that the pathophysiologic interactions between the upper and lower airways are rooted in a
common underlying immunological pathway. The different clinical manifestations vary depending principally on the local environment.

Various pathophysiologic mechanisms have been proposed to explain the interaction between the nose and the lung, and they include pulmonary aspiration, neural reflex mechanisms and systemic induction of inflammatory mediators and cells. In recent years, there is growing data to suggest that the mechanism for the upper-lower airway interaction occurs through shared systemic inflammatory processes. Although the local response to an allergen is usually most severe at the primary site of contact, there follows a more generalized inflammatory reaction in the remaining airway.

In a study by Braunstahl et al\textsuperscript{74}, subjects with allergic rhinitis underwent nasal allergen provocation testing. They showed an induced increase in eosinophil and vascular adhesion molecule levels in both nasal and, interestingly, bronchial biopsies. Conversely, in another study by Braunstahl\textsuperscript{75}, segmental bronchial provocation induced nasal inflammation in nonasthmatic allergic rhinitis patients. The nasal inflammation was characterized by an enhanced expression of IL-5 and an increase in eosinophils. They also demonstrated that, after bronchial provocation, there was an increase in total blood eosinophil counts in allergic patients as compared to controls. Therefore, the observed inflammatory process was not limited to the provoked bronchial segment, but was detected simultaneously in other part of the lung, in the nasal mucosa, and in peripheral blood. Saito et al\textsuperscript{76} analyzed the inflammatory changes in the nasal mucosa and bone marrow in experimental rhinitis. They showed that allergen deposition in the
nose resulted in an increase in eosinophil progenitors in the bone marrow. The results of these studies suggest there exists a stimulation of bone marrow eosinopoiesis. The migration of these activated eosinophils through the systemic circulation is thought to be involved in the generation of the airway allergic inflammation in both the upper and lower respiratory mucosa. See Figure 5.

Figure 5: United Airways Concept. The interaction between the upper and lower airways in the United Airways Concept likely involves the systemic induction of inflammatory mediators and cells and bone marrow eosinopoiesis.
1.6.3 Therapeutic Evidence

Therapeutic outcomes studies provide indirect evidence of a common pathologic mechanism where local treatment at one site leads to an improvement at the other site. Particularly, successful treatment of allergic rhinitis results in reduced bronchial hyperresponsiveness in patients suffering from concomitant asthma. The effects of intranasal corticosteroid treatment on nasal and pulmonary responses in patients exposed to cat allergens were examined by Wood and Eggleson. When compared to placebo, treated patients had improved nasal and respiratory symptoms, as well as improvement in their FEV$_1$ values.

A variety of other modalities in the treatment of allergic rhinitis have also lead to the improvement of concomitant asthma through modulation of numerous aspects of the inflammatory cascade. For example, combinations of antihistamine/decongestant and antihistamine/antileukotrienes have been evaluated with positive effects.

1.7 Objectives

Extensive research has supported the concept of a united airway where an intimate interconnection exists between the upper and lower airways in allergic disease. For example, the treatment of allergic rhinitis may bring benefit to the patient’s asthma. Given that the middle ear space is an anatomical extension of the airway by way of the Eustachian tube, and given that the middle ear is capable of mounting an allergic inflammation, we propose that the middle ear may be a component of this United Airways Concept. To study this hypothesis, we compared the cellular and cytokine
profiles of middle ear effusion to those of the upper airway in both atopic and non-atopic children with OME.

The objectives of this study are twofold: 1) to confirm findings that the inflammatory cellular and cytokine profiles in middle ear effusion of atopic children with OME are different than non-atopic patients, and 2) to determine if the middle ear is part of the United Airway Concept by comparing the inflammatory profiles of the middle ear effusion to the upper airway, namely the torus tubaris (mucosa at the Eustachian tube opening) and adenoid tissue located in the nasopharynx. See Figure 6.
Figure 6: The Middle Ear and the United Airways Concept. The objective of this thesis is to determine if the middle ear is part of the United Airway Concept by comparing the inflammatory profiles of the middle ear effusion to the upper airway.
CHAPTER II: MATERIALS AND METHODS

2.1 Study Design

Forty-five children (ages 2 to 18 years) undergoing myringotomy, tympanostomy tube placement and adenoidectomy were prospectively and consecutively recruited for the study undertaken at the Department of Otolaryngology, Montreal Children's Hospital (McGill University). Participation was by parental informed consent and the study obtained full scientific and ethical approval from the institutional review board.

All patients had documented conductive hearing loss, flat tympanograms, MEE persisting for > 3 months unresponsive to antibiotics, and symptomatic nasal obstruction due to adenoid hypertrophy. Patients were excluded if they used medications containing antihistamines in the preceding week or immunosuppressive agents in the preceding six weeks, if there was a history of acute otitis media in the preceding three weeks, or if frank pus was found in the nasopharynx at the time of surgery. Other exclusion criteria consisted of the presence of congenital malformations (i.e. cleft palate), known immunodeficiency disorder or ciliary dyskinesia.

2.2 Diagnosis of Atopy

Children underwent skin-prick tests for twelve common perennial and seasonal allergens, consisting of Alternaria, Aspergillus, Cladosporium, Penicillium, ragweed, grass mix, trees mix, cockroach, dust mites Dermatophagoides farinae and pteronyssinus,
cat and dog epithelium. Histamine (1 mg/ml) and saline served as positive and negative controls. All testing was performed intra-operatively by one investigator.

The results were evaluated after 10 minutes. Wheals ≥ 3mm in diameter than wheals at the site of the negative control were considered positive. Patients were defined as having atopy if they reacted positively to at least one allergen. Children <2 years old were not included since diagnosis of atopy by skin testing is less reliable.

2.3 Sample Collection Methods

Middle ear fluid was collected in a Juhn Tym-Taps (Xomed Treace Products, Jacksonville, FL) at the time of myringotomy and tympanostomy tube placement. During adenoidectomy, a cupped forcep was used to take a 3mm biopsy specimen of both the adenoid pad and the ET mucosa at the nasopharyngeal orifice, namely the mucosal surface of the torus tubaris. The torus tubaris specimen was taken on the ipsilateral side of the middle ear fluid sampled. See Figure 7.

For ethical reasons, the ET mucosa was not biopsied. Instead, we sampled the torus tubaris which we felt was the most easily accessible and representative tissue. In addition, biopsies of adenoid tissue were taken only in patients with adenoidal hypertrophy, again for ethical reasons.
Figure 7: The Torus Tubaris and the Adenoid Pad. Transoral view of the nasopharynx, demonstrating the adenoids, the torus tubaris and the Eustachian tube opening.
2.4 Preparation for ICC and ISH

2.4.1 Fluid Preparation

One milliliter (ml) of sterile phosphate-buffered saline (PBS) was added to the sample. At the laboratory, the sample was resuspended and transferred to a 15ml falcon tube, and centrifuged at 200g (1500 rpm) for five minutes. The supernatant was stored at –80°C until assayed. One ml erythrocyte lysing buffer (Pharmalyse, St. Louis, MO) was added to the pellet for bloody samples, respectively. The pellet was resuspended and centrifuged, washed three times with sterile PBS and fixed. The pellet was cytopun 300rpm for ten minutes onto frosted slides, and differential cell counts were made by Quick Diff (Baxter) staining and microscopic examination. Slides were also prepared for immunocytochemistry (ICC) and in-situ hybridization (ISH). For ICC, the cytopins were briefly fixed in a solution of acetone:methanol (60:40), air dried, and stored at -20°C until further use. For ISH, the cytopins 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.

2.4.2 Tissue Preparation

For ICC, adenoid and torus tubaris tissue was immersed in 15% PBS on ice for 15 minutes. After tissue was blocked in OCT (optimal cutting temperature) medium, cryostat sections (thickness 5 μm) were mounted on microscope slides and air-dried for one hour. The sections were then fixed by immersion in an acetone-methanol (60:40) mixture for seven minutes at room temperature, air-dried for one hour and stored at -20°C until further use.
For ISH, fresh tissue specimens were immediately placed into freshly prepared 4% paraformaldehyde for two hours. Three washes were performed with 15% sucrose in DEPC treated 0.1 M PBS, pH 7.4 (first two washes for one hour at room temperature and then overnight at 4°C). After biopsies were blocked in OCT medium, snap frozen in isopentane precooled in liquid nitrogen, and stored at -80°C until further use. Cryostat sections of 10 µm in thickness were mounted on microscope slides, air dried for 1 hour, and left to incubate at 37°C overnight.

2.5 Immunocytochemistry:

2.5.1 Basic Principles

Immunocytochemistry (ICC) is commonly used method of both detecting and localizing cellular- or tissue-associated antigens through the use of antigen-antibody affinity. Since the 1940’s, ICC has proven to be an invaluable tool in biological research. It allows for precise identification of the various cell types, through phenotypic markers, as well as their many products. Ultimately, by being able to examine the different aspects of cell function, our understanding of the disease processes has greatly been enhanced.

The antibodies, which are raised in the laboratory, are used to link the antigens to a specific stain that can be more readily detected under the microscope. Both monoclonal and polyclonal antibodies can be employed in ICC investigations. There exists three principle methods of ICC. The direct method simply involves the application of a labeled
antibody targeted to a specific tissue antigen. The indirect method utilizes a secondary unlabelled antibody raised to the Ig of the species providing the primary antibody. The final unlabelled antibody-enzyme method is a more sensitive variant of the indirect method and allows for the detection of extremely low levels of antigens. Here, an unconjugated bridging antibody between the primary antibody and the label detection reagent is used.

2.5.2 Immunocytochemistry Protocols

For this thesis, ICC was performed using the unlabelled antibody-enzyme method, specifically the alkaline phosphatase anti-alkaline phosphatase (APAAP) method, as previously described86. See Figure 8. Sections were hydrated in Tris buffered saline (TBS) solution and incubated with a mixture of commercially available, non-specific blocking antibodies for 10 minutes. Monoclonal mouse antibodies directed against MBP and elastase were used to detect eosinophils and neutrophils, respectively. Sections were incubated with 45-60 µl of primary antibody solution in a humid chamber overnight at 4°C. The following day, slides were washed for 6 minutes (2 washes for 3 minutes each) in TBS and incubated with a secondary layer of rabbit anti-mouse polyclonal IgG at room temperature for 30 minutes. Slides were then washed in TBS for 6 minutes and a third layer of alkaline-phosphatase (AP)-conjugated rat anti-rabbit polyclonal IgG was applied for 30 minutes. Slides were incubated with a mixture of AP substrate and Fast Red TR chromogen (0.5mg/ml). Positive cells stained red. See Figure 9. Negative control experiments were performed by replacing the primary antibody with an isotype-matched control.
Figure 8: Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) technique for Immunocytochemistry. A primary unlabelled antibody is applied directly to the tissue preparation for antigen binding. An unconjugated secondary antibody serves as a bridge between the primary antibody and the enzyme-anti-enzyme antibody complex. A label detection reagent is then used to visualize antigen positivity.
Figure 9A:  **T lymphocytes in Middle Ear Fluid.** Using the APAAP technique of ICC, T lymphocytes (stained red) are identified in the middle ear fluid of an atopic child.

Figure 9B:  **Eosinophils in Adenoid Tissue.** Using the APAAP technique of ICC, eosinophils (stained red) are identified in the adenoid biopsy of an atopic subject.
**In-situ Hybridization**

2.6.1 Basic Principles

*In-situ* hybridization was first introduced in 1969 by Pardue and Gall for the cellular localization of specific DNA sequences through the use a labeled complementary stranded probe. ISH can also localize messenger RNA (mRNA), which is the intermediate molecule between genomic DNA and the final functional polypeptide. By detecting mRNA, valuable information concerning both gene expression and protein synthesis is provided. The advantage of ISH over techniques of complementary sequence hybridization (i.e. Northern blot or polymerase chain reaction) is that the signal is localized to a particular cell type within the tissue.

Various probes are available to the ISH technique of detecting mRNA. Single-stranded RNA probes (riboprobes) have gained popularity in recent years due to its high sensitivity and specificity. Once a probe has been constructed, it is labeled with either radioactive (i.e. $^3$H, $^{32}$P, $^{35}$S) or non-radioactive agents (i.e. biotin, digoxigenin-11 UTP).

2.6.2 In-situ Hybridization Protocol

Slides were defrosted. Digoxigenin(Dig)-labelled ISH was conducted using digoxigenin-11-UTP labeled IL-4, IL-5 and IFN-$\gamma$ mRNA riboprobes. The permeabilization, prehybridization and the hybridization protocols were carried out as previously discussed. The hybridization mixture consisted of hybridization buffer (50% deionized formamide, 5X Denhardt’s solution, 10% dextran sulphate, 0.5% sodium pyrophosphate, 0.5% SDS and 100mM dithiothreitol (DDT). To this, 10mM of DDT
and 0.75x10^6 cpm/section of radiolabelled riboprobe (IL-4, IL5, IFN-γ) was added. Each tissue section was incubated in 15μl of this mixture (37°C), covered with dimethyldichorosilane-coated coverslips, transferred to a humid chamber and incubated overnight at 42°C.

Color development in Dig-labeled *in-situ* hybridization is achieved by adding a freshly prepared substrate solution consisting of 0.175 mg X-phosphate-5-bromo-4-chloro-3-indoly phosphate (BCIP) and 0.37 mg nitroblue tetrazolium (NBT) salt per milliliter of equalization buffer to the slides (for 20 to 40 minutes, at room temperature). Slides were transferred to TBS and washed in tap water, and counter-stained with hematoxylin for 5 seconds. Hematoxylin, a basic blue dye that combines with acid substances, creates a contrast for histological analysis with light field microscopy. Positive signals were identified by a purple stain on the cells under light microscopy. See Figure 10. Negative control experiments using sense probes and RNase treatment (100μg/ml of RNase A at 37°C) before antisense probe application were performed to confirm probe specificity.
**Figure 10A: IL-5 mRNA in Middle Ear Fluid.** The digoxigenin(Dig)-labelled ISH technique was used to identify IL-5 mRNA in middle ear fluid in an atopic subject.

**Figure 10B: IL-4 mRNA in Torus Tubaris Tissue.** The digoxigenin(Dig)-labelled ISH technique was used to identify IL-4 mRNA in torus tubaris biopsy of an atopic child.
2.7 Quantification:

For the middle ear fluid, specimens were coded and the percentage of positive cells for protein or mRNA transcript of interest was counted using an Olympus microscope with an eyepiece graticule at 200x magnification. Results were expressed as the mean percentage of positive cells per cytospin ± the standard error of the mean (SEM). For the tissue specimens, slides were analyzed for positive signal in a blinded fashion by an investigator using an Olympus light microscope at 200x magnification with an eye piece graticule of 0.202 mm². Number of positive cells were counted and expressed as the mean per square millimeter. Counting was done in a blinded fashion by two independent examiners.

2.8 Statistical Analysis

Cell counts were compared between atopic and non-atopic patients using an ANOVA t-test, with values of $p < 0.01$ considered as statistically significant.
CHAPTER III: RESULTS

3.1 Atopy of OME Patients

Out of the 45 children included in the final analysis, 11 (24.4%) had at least one positive skin-prick test to any antigen and were classified as atopic. See Table 1. The remaining 34 (75.6%) children served as non-atopic controls. Table 2 summarizes the selected demographic and clinical characteristics for patients in each subgroup.

Table 1: Allergy data for Atopic Patients with OME

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Positive skin-prick test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>6.5</td>
<td>Dust mite, mold</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>13</td>
<td>Dust mite</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>4.5</td>
<td>Dust mite, tree mix</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>5.4</td>
<td>Cat</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>5.5</td>
<td>Tree mix</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>5.9</td>
<td>Dust mite, ragweed, tree mix, dog</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>5.1</td>
<td>Dust mite, dog, cockroach</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>5.5</td>
<td>Dust mite</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>4.9</td>
<td>Ragweed, cat</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>8.8</td>
<td>Dust mite, cockroach</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>6.5</td>
<td>Dog, mold</td>
</tr>
</tbody>
</table>

(M = male, F = female)
Table 2: Demographic and Clinical Characteristics:

<table>
<thead>
<tr>
<th></th>
<th>Atopic Group</th>
<th>Non-Atopic Group (Controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>34</td>
</tr>
<tr>
<td>Mean Age (SEM)</td>
<td>6.5 years (0.86)</td>
<td>5.3 years (0.45)</td>
</tr>
<tr>
<td>Gender (male : female)</td>
<td>2.7 : 1</td>
<td>1 : 1</td>
</tr>
</tbody>
</table>

(SEM = standard error of the mean)
3.2 Cellular Profiles

The percentage of MBP positive cells (indicating eosinophils) was significantly higher in the MEE of atopic patients (7.2 ± SEM 0.4) compared to non-atopic patients (1.2 ± SEM 0.2) (p<0.01). In addition, eosinophil levels were significantly higher in both the torus tubaris tissue and the adenoid tissue of atopic patients (6.1 ± SEM 0.6; 6.4 ± SEM 0.9) compared to non-atopic patients (3.1 ± SEM 0.2; 2.5 ± SEM 0.3) (p<0.01). See Figure 11.

Figure 11: Eosinophil Levels. A comparison of eosinophil levels between atopic and non-atopic subjects. Middle ear fluid levels measured in percentage of total cells. Torus tubaris and adenoid tissue levels measured in number of cells per mm².
On the other hand, a significantly lower percentage of elastase positive cells (indicating neutrophils) are found in the MEE of atopic patients (18.4 ± SEM 0.9), compared to non-atopic patients (44.4 ± SEM 1.3) (p<0.01). In addition, neutrophil levels were significantly lower in both the torus tubaris tissue and the adenoid tissue of atopic patients (26.6 ± SEM 1.9; 22.9 ± SEM 1.2) compared to non-atopic patients (37.2 ± SEM 1.2; 34.1 ± SEM 1.0) (p<0.01). See Figure 12.

**Figure 12: Neutrophil Levels.** A comparison of neutrophil levels between atopic and non-atopic subjects. Middle ear fluid levels measured in percentage of total cells. Torus tubaris and adenoid tissue levels measured in number of cells per mm².
3.3 Cytokine Profiles

The percentage of IL-4 mRNA positive cells is significantly higher in the MEE of atopic patients (8.4 ± SEM 0.7) compared to non-atopic patients (1.7 ± 0.3) (p<0.01). In addition, IL-4 levels was significantly higher in both the torus tubaris tissue and the adenoid tissue of atopic patients (8.0 ± SEM 1.1; 10.3 ± SEM 0.9) compared to non-atopic patients (1.3 ± SEM 0.2; 1.5 ± SEM 0.2) (p<0.01). See Figure 13.

**Figure 13:** IL-4 mRNA Levels. A comparison of IL-4 mRNA levels between atopic and non-atopic subjects. Middle ear fluid levels measured in percentage of total cells. Torus tubaris and adenoid tissue levels measured in number of cells per mm².
The percentage of IL-5 mRNA positive cells is significantly higher in the MEE of atopic patients (10.5 ± SEM 0.9) compared to non-atopic patients (1.5 ± SEM 0.15) (p<0.01). In addition, IL-5 levels was significantly higher in both the torus tubaris tissue and the adenoid tissue of atopic patients (8.4 ± SEM 1.6; 11.1 ± SEM 1.0) compared to non-atopic patients (1.45 ± SEM 0.2; 1.55 ± SEM 0.15) (p<0.01). See Figure 14.

**Figure 14: IL-5 mRNA Levels.** A comparison of IL-5 mRNA levels between atopic and non-atopic subjects. Middle ear fluid levels measured in percentage of total cells. Torus tubaris and adenoid tissue levels measured in number of cells per mm$^2$. 

* p < 0.01
On the other hand, levels of IFN-γ positive cells were found to be significantly lower in the MEE of atopic patients (5.3 ± SEM 1.1), compared to non-atopic patients (9.3 ± SEM 0.7) (p<0.01). In addition, IFN-γ positive cells levels was significantly lower in the adenoid tissue of atopic patients (3.3 ± SEM 0.6) compared to non-atopic patients (6.7 ± SEM 0.5) (p<0.01). However, the levels of IFN-γ positive cells in the torus tubaris tissue was not significantly lower in atopics than in non-atopics, although there was a trend (p = 0.09). See Figure 15.

**Figure 15: IFN-γ mRNA Levels.** A comparison of IFN-γ mRNA levels between atopic and non-atopic subjects. Middle ear fluid levels measured in percentage of total cells. Torus tubaris and adenoid tissue levels measured in number of cells per mm².
CHAPTER IV: DISCUSSION

4.1 Discussion of Results

In this study, we found that the MEE of atopics had significantly higher levels of eosinophils, T lymphocytes, IL-4 and IL-5 mRNA positive cells \( (p < 0.01) \), and significantly lower levels of neutrophils and IFN-\( \gamma \) mRNA +cells \( (p < 0.01) \) when compared to non-atopics. The nasopharyngeal biopsies, consisting of torus tubaris and adenoid tissue, also revealed very similar cellular and cytokine profiles. The incidence of atopy among our study population was 24%.

Our findings suggest that the middle ear is capable of participating in and sustaining late-phase, allergen-specific inflammation. The data is consistent with the T\(_{H2}\) model of late-phase allergen response characterized by the increased expression of T\(_{H2}\)-type cytokines (i.e. IL-4, IL-5) and in infiltration of eosinophil and T-cells. IL-4 is essential for the promotion of eosinophil adhesion and infiltration by enhancing endothelial expression of vascular cell adhesion molecules (VCAM-1)\(^{88}\). IL-4 also induces isotype switching of antibody production by B-cells in favor of the IgE\(^{89}\) and influences activated naïve T-cells to express predominately T\(_{H2}\) cytokines. IL-5 plays a critical role in the recruitment, activation, differentiation and survival of eosinophils\(^{90}\). Among the immune regulatory cells, eosinophils play a particularly important role in the late phase allergic response through the release of various mediators including eosinophilic cationic protein, major basic protein, and leukotriene C4. The presence of activated eosinophils is associated with extracellular matrix deposition, epithelial
denudation and basement membrane disruption\textsuperscript{91-95}. These effector cells are rapidly recruited and activated after allergen stimulation\textsuperscript{93,94}. The increase in eosinophils in atopic patients (compared to nonatopic patients) is much greater in the MEE than that seen in the torus tubarius or adenoid tissue. These results suggest that, in addition to participating in a generalized allergic inflammation of the airway, the middle ear is capable of a more intense local inflammation. These may be due to superimposed local factors in the middle ear, such as chemokines, which can be produced by structural cells.

Much of the recent studies have concentrated on improving our understanding of the differences in the inflammatory and structural changes between allergic and non-allergic OME. Wright\textsuperscript{41} et al demonstrated increased expression of IL-5 and major basic protein (eosinophils) in the middle ear mucosa of patients with OME compared to normal controls. Hurst\textsuperscript{24} et al recently reported increased levels of eosinophilic cationic protein (ECP) in the supernatant of atopic OME patients compared to non-atopic controls. Thus, our study confirms the findings of previous studies that support allergy as a contributing factor in the persistence of OME in allergic patients.

In addition, our study, for the first time, correlates inflammatory profiles found in the MEE to those found in the nasopharynx, or the upper airway. We have shown that the allergic inflammation in atopic children with OME is not isolated to the middle ear. In fact, it occurs uniformly on both sides of the Eustachian tube, in both the middle ear and the nasopharynx. These observed cellular and cytokine profiles resemble the inflammatory substrates in late phase-allergic response previously demonstrated in other
areas of the respiratory tract and implicated in the pathogenesis of asthma, allergic rhinitis, and allergy-associated chronic sinusitis. Our data supports the concept that the middle ear may be part of the United Airway Concept, and may behave in a similar fashion to the lungs under allergic inflammatory insults.

In contrast to atopic patients, non-atopic patients revealed different cellular and cytokine profiles in the MEE and nasopharynx, with a predominantly TH1-mediated inflammatory response. Neutrophils and IFN-γ positive cells were the predominant cell types, reflecting the possible role of bacterial and/or viral products in the stimulation of middle ear effusion production. Although at significantly lower levels, the presence of neutrophils and IFN-γ in atopic patients may represent the residual effects of a previous infectious process, despite the fact that patients with purulent effusions, pus in the nasopharynx or a history of a recent ear infection were excluded. As well, within the atopic patients, another possible explanation for the low levels of IFN-γ levels seen is that there is suppression of the basal levels of IFN-γ by the increased levels of IL-4, thereby maintaining a balance of TH1/TH2 cytokines. Alternatively, since the atopic group was younger than the control group, repeated antigen exposure may be needed in these young children to alter inflammatory reactivity by strengthening their eosinophil participation in the late-phase reaction and diminishing neutrophil involvement.

4.2 Limitations

There are limitations to the study due to ethical reasons. Firstly, mucosa biopsies within the ET mucosa were not taken. Therefore, we sampled the torus tubaris which we
felt was the most easily accessible and representative tissue. Secondly, patients with adenoidal hypertrophy were enrolled in the study. Ideally, tissues would be sampled from patients undergoing only tympanostomy tube insertion, regardless of their adenoid size. That being said, Bernstein et al examined the cytokine profiles in adenoids and found no difference between patients who underwent adenoidectomy for otitis media or for nasal obstruction.  

4.3 Clinical Implications and Future Directions

The relation between the upper and lower airway is subject to ongoing investigations and is slowly being understood. Epidemiological surveys and clinical trials have established a link between allergic rhinitis and asthma, where the condition of the upper airways can influence that of the lower airways and vice versa. Nonetheless, the underlying immunopathophysiological mechanisms remain elusive.

This study has introduced the notion that the middle ear may be included in the United Airways Concept. The immunological link between allergic otitis media with effusion and allergic diseases of the airway requires extensive future research. Further confirmation of these findings, followed by epidemiological studies and eventual therapeutic clinical trials, are required to elucidate the proposed interaction between the middle ear and the airway. Examination for allergic inflammation of middle ear fluid or mucosa during provocation studies, where the nasal or bronchial mucosa are exposed to allergens, would provide more direct evidence to this interaction.
The possible integration of the middle ear in the United Airway Concept will have major clinical implications on the diagnosis and management of allergic airway diseases. Patients presenting with allergic OME may require routine evaluation of the lower airway in an effort toward early detection of bronchial involvement. Conversely, nasal and otologic assessments in asthmatic patients may become standard clinical practice.

The current medical management of OME is often unsuccessful, and a significant number of refractory cases require surgical management consisting of myringotomy and insertion of tympanostomy (ventilation) tubes. Identification of the subgroup of atopic patients with OME may allow for a different treatment approach. In addition, if the middle ear proves to play a role in this chronic allergic respiratory syndrome, then treatment of OME should take into consideration the common underlying systemic inflammation and the unity of airways. An integrated management approach would thus possibly include avoidance of offending allergen and the concomitant treatment of nasal or bronchial inflammation. In addition to topical intranasal or inhaled corticosteroids, it may also include systemic steroids, antihistamines and allergy immunotherapy (hyposensitization).

4.4 Conclusions

Our findings confirm that the MEE in atopic children with OME is characterized by a \(T_{H2}\)-mediated inflammation. Our study supports the hypothesis that allergic inflammation may contribute to the etiology of OME in atopic children. In addition, we have shown for the first time that the allergic inflammation seen in atopics with OME is
not isolated to the middle ear but occurs on both sides of the Eustachian tube, in both the middle ear and the nasopharynx. This inflammation is typically seen in the rest of the respiratory tract in allergic diseases. Therefore, our data supports the concept that the ME is part of the United Airways.

Identification of the middle ear as part of the United Airways implies that the middle ear is not an isolated organ system. Therefore the diagnosis of allergic OME must be done in conjunction with the rest of the respiratory tract, and the treatment of allergic OME must address the underlying inflammatory process. Alternative treatment options may be available to atopic patients with OME. For example, allergen avoidance, topical or systemic anti-inflammatory medications, or immunotherapy may prove to be beneficial to this subgroup of patients. More studies that stratify specifically for atopy status are needed to further elucidate the role of allergy in OME. As the incidence of allergy in children continues to rise, these issues will play an increasingly important role.
CHAPTER IV: REFERENCES


41. Wright ED, Miotto D, Giguere C, Hamid Q. Increased expression of major basic protein (MBP) and interleukin-5 (IL-5) in middle ear biopsy specimens from atopic patients with persistent otitis media with effusion. Otolaryngol Head Neck Surg 2000;123:533-8

42. Kotsimbos AT, Hamid Q. IL-5 and IL-5 receptor in asthma. Mem Inst Oswaldo Cruz 1997;92 Suppl 2:75-91


47. Palva T, Tolvanen E, Konttinen YT, Reitamo S. Inflammatory cells in the middle ear mucosa in cases of chronic otitis media. Arch Otolaryngol 1981;107(9):528-31


68. Pederson PA, Weeke ER. Asthma and allergic rhinitis in the same patients. Allergy 1983;38:25-9.


90. Kotsimbos AT, Hamid Q. IL-5 and IL-5 receptor in asthma. Mem Inst Oswaldo Cruz 1997; 92 Suppl 2:75-91


CHAPTER VI: APPENDICES

6.1 Informed Consent (English)

THE ROLE OF ALLERGY, ADENOIDS AND EUSTACHIAN TUBE IN THE PATHOPHYSIOLOGY OF OTITIS MEDIA WITH EFFUSION IN ATOPIC CHILDREN

Lily HP Nguyen, M.D., John J Manoukian, M.D., Steve Sobol, M.D., Melvin D Schloss, M.D., Ted L Tewfik, M.D., Bruce D. Mazer, M.D., Qutayba A. Hamid, MD, Ph.D.

Introduction

Otitis media with effusion (fluid in the middle ear) is one of the most common problems seen by the pediatrician and ear, nose, and throat specialist. Our current belief is that this condition is caused by a blockage of the Eustachian tube, which normally allows drainage of middle ear fluid into the back of the nose. As the child grows, this tube opens up and the middle ear condition resolves.

Potential complications of long-term middle ear fluid accumulation include recurrent ear infections, scarring in the middle ear, and decreased hearing, which may result in delayed speech development. The treatments of unresolved middle ear fluid are 1) placement of ear tubes, which serves to remove the fluid and prevent its re-accumulation, or 2) placement ear tubes, and adenoidectomy, which either removes the focus of infection or relieves the obstruction of the Eustachian tube.

Allergies are common in children, and can be the cause of many problems including asthma, nasal congestion, and sinusitis. Studies have also shown that allergies may cause fluid to accumulate in the ear. Enlarged adenoids, which are tissue found in the back of the nose, may also cause fluid to collect in the ear by blocking the Eustachian tube. Our goal is to see how allergy, adenoids and Eustachian tube contributes to the accumulation of ear fluid in children with allergies.

Description of the Study

Your child is scheduled for ear tube placement and adenoidectomy as recommended by his/her physician. At the time of your child’s routine preoperative evaluation, the doctor
will take a brief history and do a physical examination to determine if your child is eligible for the study. If eligible, you will be asked to read this consent form to determine whether you are willing to participate in the study. If you agree to participate, these are the steps that will be undertaken:

1) At the time of general anesthesia, standard skin testing will be done to assess whether your child is susceptible to having allergies. The skin test involves putting small drops of fluid that contains different sorts of allergens onto the forearm of your child. Following that, a sterile needle will be used to make multiple light scratches to the skin to see if there is a skin reaction.

2) If this skin test does show that your child is susceptible to having allergies, a consultation with an allergy specialist will be arranged at your convenience.

3) While your child is under anesthesia, the operation will be carried out as usual by your specialist. The following specimens will be brought to a laboratory for analysis: 1) the fluid that is removed from the ear, 2) the adenoid tissue removed during the surgery and 3) a very small biopsy of the torus tubaris, which is found right next to the adenoids. The torus tubaris is a small shelf that separates the adenoids from the Eustachian tube.

Potential Benefits

One potential benefit is that we will be determining if your child is susceptible to having allergies. If this is found on the skin test, your child will be evaluated by an allergy specialist who will counsel you on how to prevent allergies, and possibly help to prevent the development of asthma, skin, and sinus disease.

This study will analyze the fluid from your child’s ears and the biopsies from your child’s adenoids and torus tubaris. The fluid/biopsies will be analyzed only for this study, and will then be discarded. Although the results of these analyses will not change the management of your child directly, it will help determine if allergy is one of the causes of ear fluid accumulation, and if the Eustachian tube and adenoids play a role this accumulation. These findings would have future implications on how we treat children with this condition.

Potential Risks

Since participation in this study does not change how your child is being managed, the risks are the same as those discussed with your specialist concerning the operation.

1) The removal of the middle ear fluid is standard step in the insertion of a tympanostomy tube. There are no additional risks other than the risks inherent to the operation itself.
2) The biopsy of the torus tubaris is very small, only 2 mm wide. The biopsy is taken from a site that should not affect the function of the Eustachian tube. To our knowledge, this procedure does not add any additional risks or morbidity to the patient other than those inherent to a simple adenoidectomy (i.e. minimal bleeding and infection).

3) The allergy skin test is sometimes associated with mild discomfort (like a mosquito bite) which lasts for approximately 10-15 minutes. This reaction may likely be gone by the time your child wakes up from the general anesthesia.

Important information

You understand that participation in this research study is voluntary and that you are free not to participate in the study without penalty or loss of benefits or treatment to which your child is entitled.

If you have any further questions about this study, you may contact the physician at 514-412-4303.

Confidentiality

Information gathered during this study will be identified only by patient initials and a number. In the event of publication of the study, patient identity will not be disclosed. You will be given a copy of this consent form for your own records.

Contact person: Lily HP Nguyen, MD Tel: 514-412-4303
Ombudsman: Elizabeth Gibbon Tel: 514-412-4400, ext. 22223

By signing this letter you hereby give consent to your child’s participation in this study.

You have had an opportunity to ask the doctor questions about the study and you understand the conditions and procedures of the research study, as written above, and you give your voluntary informed consent to have your child participate.

_________________________________  _______________________________________
Child’s name                                      Parent or guardian’s signature

_________________________________
Witness’ signature                          Principle investigator’s signature
6.2 Informed Consent (French)

Consentement éclairé

RÔLE DE L’ALLERGIE, DES VÉGÉTATIONS ADÉNOÏDES ET DE LA TROMPE D’EUSTACHE DANS LA PATHOPHYSIOLOGIE DES OTITES MOYENNES AVEC ÉPANCHEMENT CHEZ LES ENFANTS ALLERGIQUES

Lily HP Nguyen, M.D., John J Manoukian, M.D., Steve Sobol, M.D., Melvin D Schloss, M.D., Ted L Tewfik, M.D., Bruce D. Mazer, M.D., Qutayba A. Hamid, M.D., Ph.D.

Introduction

L’otite moyenne avec épanchement (présence de fluide dans l'oreille moyenne) est une des principales affections conduisant à une consultation par les pédiatres et les spécialistes du nez, de la gorge et de l’oreille. Notre hypothèse actuelle est que ce phénomène résulte de l’obstruction de la trompe d’Eustache, qui permet normalement le drainage du fluide de l'oreille moyenne vers l’arrière-fond des fosses nasales. Pendant la croissance de l’enfant, la trompe d’Eustache se développe et conduit alors à la disparition de l'épanchement au niveau de l'oreille moyenne.

Les complications potentielles à long terme de l'accumulation de liquide dans l'oreille moyenne concernent les infections répétées de l'oreille, les lésions de l'oreille moyenne, et la perte de l'acuité auditive, pouvant ainsi entraîner un retard dans le développement du langage.

Les traitements de l’otite moyenne avec épanchement sont les suivants: 1) l’installation d’un tube dans l’oreille afin d’éliminer le fluide et de prévenir son accumulation ultérieure, ou 2) l’installation d’un tube dans l’oreille associée à l’ablation des adénoïdes, ou adénoïdectomie, afin d’éliminer le centre infectieux et de dégager la trompe d’Eustache.

Les allergies sont communes chez les enfants, et peuvent être la cause de plusieurs problèmes de santé, comprenant l’asthme, la congestion nasale, et la sinusite. Certaines études ont démontré que les allergies peuvent, de plus, donner lieu à l’accumulation de fluide dans l'oreille moyenne. L’hypertrophie des végétations adénoïdes, situées dans l’arrière-fond des fosses nasales, peut également conduire à l’accumulation de fluide dans l'oreille moyenne bloquant ainsi la trompe d’Eustache.
Le but de notre étude est de déterminer comment l'allergie, les adénoides et la trompe d'Eustache contribuent à l'accumulation du fluide dans l'oreille moyenne chez les enfants présentant des allergies.

**Description de l'étude**

Votre médecin vous a recommandé de faire subir à votre enfant l'insertion d'un tube dans l'oreille ainsi qu'une adénoidectomie. Au moment de l'évaluation pré-opératoire, le docteur vous interrogera brièvement sur les antécédents de santé de votre enfant et lui fera un examen médical, afin de déterminer si votre enfant peut participer à cette étude.

Dans le cas où votre enfant présente les conditions requises, vous serez invité à lire ce formulaire de consentement. Si vous acceptez que votre enfant participe à cette étude, les étapes suivantes seront alors réalisées:

1) Au moment de l'anesthésie générale, un test cutané sera effectué afin d'évaluer si votre enfant est susceptible de présenter des allergies. Pour cela, de petites gouttes contenant différents types d'allergènes seront déposées sur l'avant-bras de votre enfant. Une aiguille stérile sera ensuite utilisée pour pratiquer de légères éraflures à la surface de la peau afin de mettre en évidence une éventuelle réaction allergique.

2) Dans le cas où le test cutané révèle que votre enfant est susceptible d'avoir des allergies, un rendez-vous pour une consultation avec un spécialiste allergologue sera alors programmé en fonction de votre disponibilité.

3) Lors de l'anesthésie de votre enfant, l'opération chirurgicale sera réalisée de façon habituelle par votre médecin spécialiste, et les échantillons suivants seront prélevés et apportés à un laboratoire à des fins d'analyse: 1) le fluide collecté au niveau de l'oreille moyenne, 2) le tissu adénoidé enlevé lors de la chirurgie et 3) une très petite biopsie du torus tubaris, tissu se situant au voisinage immédiat des adénoides. Le torus tubaris est une petite protubérance qui sépare les végétations adénoides de la trompe d'Eustache.

**Risques potentiels**

La participation de votre enfant à cette étude ne modifiant pas les soins qui lui sont dispensés, les risques associés sont les mêmes que les risques liés à l'opération qui vont ont été expliqués par votre médecin spécialiste.

- L'élimination du fluide de l'oreille interne est une étape habituelle lors de l'insertion du tube de tympanostomie. Elle ne présente aucun risque supplémentaire à ceux inhérents à l'opération elle-même.
La biopsie du torus tubaris ne mesure que 2 mm, et est donc très petite. Cette biopsie est réalisée à un endroit qui ne devrait pas affecter la fonction de la trompe d'Eustache. À notre connaissance, cette procédure n’augmente pas les risques ou la morbidité pour les patients par comparaison à ceux inhérents à une simple adénoïdectomie (c’est-à-dire saignement ou infection minimes).

Le test cutané réalisé pour diagnostiquer les allergies peut quelquefois être associé à une légère sensation inconfortable, semblable à une piqûre de moustique. Cette sensation ne dure alors que 10 à 15 minutes et devrait normalement avoir disparu lors du réveil de votre enfant de l’anesthésie générale.

**Bénéfices potentiels**

Un des bénéfices potentiels pour votre enfant est l’évaluation de sa susceptibilité à développer des allergies. Si le test cutané met en évidence la présence de réactions allergiques, votre enfant sera examiné par un spécialiste allergologue qui vous conseillera sur les moyens de prévenir l’apparition des allergies, ce qui pourrait éventuellement contribuer à limiter le développement de l’asthme et de certaines maladies de la peau et des sinus.

Au cours de cette étude, nous analyserons le fluide de l’oreille et les biopsies provenant des adénoïdes et du torus tubaris de votre enfant. Ces prélèvements ne seront utilisés que dans le cadre de cette étude, et seront ensuite détruits. Bien que les résultats de ces analyses n’influent pas de façon directe les soins dispensés à votre enfant, ils permettront de déterminer si l’allergie est une des causes de l’accumulation de fluide dans l’oreille, et si la trompe d’Eustache et les adénoïdes jouent un rôle dans ce phénomène. De telles découvertes devraient avoir des implications futures concernant le mode de traitement des enfants concernés.

**Information importante**

La participation à ce protocole de recherche est volontaire. Vous êtes libre de refuser que votre enfant participe à cette étude sans que cela n’affecte en aucune manière la qualité des soins médicaux qui lui sont dispensés. Si vous avez des questions supplémentaires concernant cette étude, vous pouvez contacter le médecin référent au 514-412-4303.

**Clause de confidentialité**

Les données collectées au cours de cette étude ne seront identifiées que par les initiales du patient associées à un numéro. Dans le cas où cette étude donne lieu à une publication scientifique, l’identité du patient ne sera pas communiquée.
Nous vous fournirons une copie de ce formulaire de consentement pour vos archives personnelles.

Personne à contacter: Lily HP Nguyen, M.D. Tel: 514-412-4303
Représentant des patients: Elizabeth Gibbon Tel: 514-412-4400, ext. 22223

En signant ce formulaire, vous acceptez la participation de votre enfant à cette étude. Vous reconnaissez avoir eu l’opportunité de questionner le médecin responsable à propos de cette étude, et vous avez compris les formalités et procédures associées à ce protocole expliquées ci-dessus. Vous donnez volontairement votre consentement éclairé à ce que votre enfant participe à cette étude.

_____________________________   ______________________________
Nom de l’enfant                       Signature du parent ou du tuteur

_____________________________   ______________________________
Signature du témoin                      Signature de l’investigateur
6.3 Data Collection Form

Date: ___________________________   Unit #: _______________________

Name: ________________________________________________________________

Date of Birth: _______________________   Age: __________________________

Gender:   Allergies:

_____ M   _____ No
_____ F   _____ Yes

Medications:

_____ Benadryl   _____ Atarax   _____ Antihistamines
_____ Gravol   _____ Cough Syrup   _____ Others

Other medical conditions:

Presence of Allergic Symptoms:

_____ Runny, Itchy Eyes   _____ Sneezing
_____ Runny, Itchy Nose   _____ Scratchy Throat

Indication for OR:

Procedure:

_____ PET & Adenoidectomy
_____ PET & T & A

Surgeon:

_____ Manoukian
_____ Tewfik
_____ Schloss
Fluid:

- R
- L
- Both
- Pus
- Mucoid
- Regular
- Bloody

Presence of Lymphoid Tissue on Torus Tubaris:
- Yes
- No

Adenoids:
- Degree of Hypertrophy _______
- Abutment _______
  - R
  - L
  - Both
  - None

Skin Test:

<table>
<thead>
<tr>
<th>Pollens / Molds</th>
<th>Erythema (mm)</th>
<th>Wheal (mm)</th>
<th>Perennial Allergens</th>
<th>Erythema (mm)</th>
<th>Wheal (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternia</td>
<td></td>
<td></td>
<td>Cat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus</td>
<td></td>
<td></td>
<td>Cockroach</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cladosporium</td>
<td></td>
<td></td>
<td>Dog</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grass Mix</td>
<td></td>
<td></td>
<td>Dust mite DF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillum</td>
<td></td>
<td></td>
<td>Dust Mite DP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ragweed</td>
<td></td>
<td></td>
<td>(+) Histamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tree Mix</td>
<td></td>
<td></td>
<td>(-) Saline</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>