Cell Contacts and Airway Epithelial Damage in Asthma

SHAHIDA SHAHANA
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Abstract


Airway epithelial damage is commonly found in asthma patients. Epithelial damage was investigated with special reference to contacts between epithelial cells.

Eosinophils, common in allergic asthma, secrete cationic proteins, particularly major basic protein (MBP). The effect of poly-L-arginine, an analogue of MBP, on airway epithelial cells was investigated. Poly-L-arginine induced membrane damage, resulting in increased permeability, loss of cell-cell contacts (tight junctions and desmosomes) and generalized cell damage.

Adhesion molecules on airway epithelial cells may be important in recruiting leukocytes. Interferon (IFN)-γ increased intracellular adhesion molecule-1 expression in airway epithelial cell lines. A combination of interleukin-4 and IFN-γ opened the tight junctions.

Epithelial damage in asthma was studied at the ultrastructural level in bronchial biopsies from patients with atopic or non-atopic asthma, and healthy controls. Epithelial damage was extensive in both asthma groups. In basal and columnar cells, relative desmosome length was reduced by 30-40%. In columnar cells, half-desmosomes were noticed. Changes tended to be more extensive in atopic asthma, but there was no significant difference between the two groups. Reduced desmosomal contact may be important in the epithelial shedding observed in asthma. The contact area between columnar cells and basal lamina is relatively small in the human airway. Attachment of columnar cells to the basal lamina occurs indirectly, via desmosomal attachment to basal cells. Direct attachment of columnar cells to the basal lamina is weakened in asthmatics.

Nasal polyposis is a chronic inflammatory disease often associated with asthma. An ultrastructural study showed that epithelial damage of columnar cells is more pronounced in allergic patients. The length of columnar cell desmosomes was significantly reduced in asthmatics vs. non-asthmatics, and in allergics vs. non-allergics.

Cell contacts in airway epithelium in asthmatics are weakened, which may be an intrinsic feature or due to the presence of eosinophils producing toxic proteins.

Keywords: Desmosomes, tight junctions, major basic protein, eosinophil, cytokines, epithelial damage.

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urn:nbn:se:uu:diva-4775 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-4775)
To

Mahdi
and my parents

If you have knowledge, let others light their candles at it

By Margaret Fuller
This thesis is based on the following papers:


V. S. Shahana, Z. Jaunmuktane, M. Stenkvist Asplund, G.M. Roomans. Ultrastuctural investigation of epithelial damage in asthmatic and non-asthmatic nasal polyps. (Submitted)
### CONTENTS

1. **Introduction** .............................................. 9  
   - Atopy and asthma ........................................ 9  
   - Structure of the airway wall ............................ 10  
   - Cell-cell contacts in epithelia .......................... 11  
   - Adhesion molecules ...................................... 15  
   - The inflammatory response in asthma ............... 16  
   - Inflammatory cells ....................................... 18  
   - Cytokines ................................................... 21  
   - Polyps and asthma ........................................ 22  
   - Rhinitis and asthma ....................................... 23  
   - Epithelial damage in asthma ............................. 23  
   - Treatment .................................................. 24  

2. **Aims** .......................................................... 26  

3. **Materials and Methods** ................................. 27  
   - Cell culture ................................................ 27  
   - Induction of cell cultures ................................ 27  
   - Bronchial biopsies ....................................... 28  
   - Rats ........................................................... 30  
   - Nasal polyps ................................................ 30  
   - Transmission electron microscopy ................... 31  
   - Scanning electron microscopy ........................... 32  
   - Immunocytochemical studies ............................ 33  
   - Light microscopy ......................................... 33  
   - Cell viability test ....................................... 33  
   - DNA labeling ............................................... 34  
   - Flow cytometry analysis of ICAM-1 .................... 34  
   - Western blot ............................................... 35  
   - X-ray microanalysis ...................................... 36  
   - Statistics .................................................... 36
4. Results ........................................................................................................... 37
   Paper I ........................................................................................................... 37
   Paper II ......................................................................................................... 38
   Paper III ....................................................................................................... 40
   Paper IV ........................................................................................................ 41
   Paper V .......................................................................................................... 42

5. Discussion ..................................................................................................... 44
   Studies on human tissues in situ ................................................................. 44
   Epithelial damage ......................................................................................... 44
   Desmosomes ................................................................................................. 45
   Eosinophils .................................................................................................... 46
   Neutrophils ..................................................................................................... 46
   Mast cells ....................................................................................................... 47
   Intercellular space ......................................................................................... 47
   Basal lamina ................................................................................................. 48

   Experimental studies ................................................................................... 48
   Effects of poly-L-arginine on airway epithelial cells .................................. 48
   Effects of different cytokines on bronchial epithelial cells ....................... 49

6. Conclusions and Future Perspectives ......................................................... 52

7. Acknowledgements ..................................................................................... 54

8. References .................................................................................................... 55
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AHR</td>
<td>Airway hyperreactivity</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BHR</td>
<td>Bronchial hyperresponsiveness</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BEGM</td>
<td>Bronchial epithelial growth medium</td>
</tr>
<tr>
<td>CCR-3</td>
<td>CC chemokine receptor 3</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>ECP</td>
<td>Eosinophilic cationic protein</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle's minimal essential medium</td>
</tr>
<tr>
<td>EPO</td>
<td>Eosinophil peroxidase</td>
</tr>
<tr>
<td>FEV1</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>FDA</td>
<td>Fluorescein di-acetate</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>16HBE14o-</td>
<td>Human bronchial epithelial cell line (transformed)</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocytic antigen</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula occludens-1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>MBP</td>
<td>Major basic protein</td>
</tr>
<tr>
<td>MCP-3</td>
<td>Membrane cofactor protein-3</td>
</tr>
<tr>
<td>NHBE</td>
<td>Normal human bronchial epithelial</td>
</tr>
<tr>
<td>PC20</td>
<td>Provocation concentration that reduces FEV1 by 20%</td>
</tr>
<tr>
<td>PEF</td>
<td>Peak expiratory flow</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on Activation, Normal T Expressed and Secreted</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope (microscopy)</td>
</tr>
<tr>
<td>STEM</td>
<td>Scanning transmission electron microscope (microscopy)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope (microscopy)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
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Introduction

Atopy and asthma

An Egyptian manuscript called Ebers Papyrus first described asthma-like symptoms 3500 years ago. The famous Greek physician Hippocrates 500 years later first use the word asthma to describe an illness[www.mercksharpdohme.com/disease/asthma/asthma_timeline/ancient.html]. In Greek asthma means “laboured breathing”. The Romans use to treat asthma by giving sufferers owl’s blood in wine. During the 17th and 18th centuries physicians realised that asthma was due to constriction of the bronchi. In the 1960’s physicians discovered that asthma is an inflammatory disease. At present, although profound insights have been obtained into the pathophysiology of asthma, the exact mechanisms inducing and regulating the disease are still not very clearly understood [Hamelmann & Gelfand, 2001]. The prevalence of asthma now is estimated at 300 million people worldwide [Masoli et al., 2004].

The scientific description of asthma has been continuously revised during the past 10 years. Now asthma is recognised as a complex disorder where genetic, environmental and allergenic factors interact, resulting in a significant airway inflammation [Schieken, 2002]. There is no age limit for this disease. Symptoms of asthma are recurrent episodes of wheezing, breathlessness, chest tightness, and cough, particularly at night and/or in the early morning. Asthma is characterised by airway hyperresponsiveness, reversible airflow obstruction and airway inflammation [Noguchi & Arinami, 2001]. Airway hyperresponsiveness is defined as exaggerated bronchoconstriction in response to a wide range of non-specific stimuli. Airflow limitation can be caused by several factors like bronchoconstriction, airway edema, mucous plug formation, smooth muscle hypertrophy and hyperplasia and this is partly reversible either spontaneously or with treatment. Airway inflammation is characterized by significant cellular infiltration that is mostly rich in eosinophils, mast cells and T-lymphocytes. The eosinophil granulocyte is now widely considered to be a major culprit in asthma. Asthma is often classified as extrinsic or atopic asthma and intrinsic or non-atopic asthma on the basis of the presence or absence of
immediate skin sensitivity reaction to common allergen [American Thoracic Society, 1987]. A more recent classification of asthma uses three types of criteria: (1) symptom-based, in which asthma is divided into chronic, acute severe, brittle, nocturnal and exercise-induced, (2) aetiological, in which asthma is divided into extrinsic (atopic), intrinsic (non-atopic), virally induced, occupational, and high-performance athletics, and (3) pathological, in which asthma is divided into eosinophilic, neutrophilic and fixed airflow obstruction [Wardlaw et al., 2002].

The Greek word ἀτοπία (strangeness) has given rise to the term atopy. The term was first used by Coca and Cooke [Coca & Cooke, 1923] to describe a tendency to develop an immediate-type hypersensitivity reaction. Allergy is associated with atopy and the hereditary predisposition to produce IgE to environmental allergens. An international consensus report proposed the following definition of atopy: "a skin reaction to one or more of the allergens with a mean diameter of ≥ 3 mm and no dermatographism" [European Academy of Allergology and Clinical Immunology, 1993; Anderson, 2001].

Atopy is one of the most important identifying risk factors for development of asthma [Burrows & Martinez, 1989] although not all asthmatics are atopic. Atopic asthma commonly begins in childhood. A positive family history and other allergic disorders (like allergic rhinitis and eczema) are often present. Non-atopic asthma is idiopathic and can begin at any age. This variety commonly occurs in adults, and the patient is not demonstrably allergic. In the majority of the cases the disease is chronic.

**Structure of the airway wall**

The conducting part of the airways is lined by epithelium and the airway wall contains epithelium, connective tissue, smooth muscle, cartilage and mucus-producing glands [McKay & Hogg, 2002]. The epithelium rests on a basement membrane that consists of a linear basal lamina together with a closely packed collagen fibres. Next to this is a lamina propria consisting of collagen, elastin, nerves, lymphatics, and blood vessels. Smooth muscle lies in between basement membrane and cartilage and wraps the bronchus spirally. The outer layer of the bronchus contains a thin layer of connective tissue that contains blood vessels and nerve fibres. Cartilage is absent in the bronchiole.
The airway epithelium is regarded as a pseudostratified columnar epithelium composed of three main cell types (ciliated columnar cells, secretory cells, and basal cells). In the lower airway the predominating ciliated cells maintain a constant upward flow of respiratory secretion with their cilia. They also transport water and ions, and release macromolecules [Rennard et al., 1994]. Ciliated cells are found down to the terminal bronchiole. Usually they reach the basal lamina by extending a slender process [Rennard et al., 1994] but they do not form hemidesmosomes to attach to the basal lamina [Hasleton, 1996]. Pyramidal-shaped basal cells play an important role in holding the columnar epithelium to the basal lamina by forming desmosome contacts with the columnar cells, whereas the basal cells themselves are connected to the basal lamina with hemidesmosomes [Davies & Devalia, 1992; Evans et al., 1990]. Basal cells together with goblet cells of the trachea, the Clara cells of the bronchiole, the type II pneumocytes of the alveoli, duct cells of submucosal mucous-glands in the trachea and bronchus, and maybe also neuroepithelial body cells have been considered to have the potential to repopulate the injured lung [Otto, 2002].

Secretory cells include goblet cells, serous cells, Clara cells and neuroendocrine cells. Goblet cells are interspersed between the ciliated cells. In the normal airway there are about five times as many ciliated cells as goblet cells. The goblet cells, together with other mucous glands of the airway wall secrete mucus that lines the airway epithelium. Underlying the mucus layer there is a periciliary fluid layer that is less viscous. Together, the mucus and periciliary layer form the airway surface liquid (ASL).

Clara cells are found in the terminal bronchioles, which have a simpler epithelial lining consisting of a ciliated columnar or cuboidal epithelium and Clara cells, which are non-ciliated secretory cells that secrete a proteinaceous fluid. According to a recent theory, the Clara cell protein has an important role protecting the respiratory tract against oxidative stress and the inflammatory response [Singh & Katyal, 2000; Broeckaert et al., 2000].

**Cell-cell contacts in epithelia**

The plasma membrane of the epithelial cell forms specialized adhesion complexes for cell-cell contacts that are important for the organisation and behaviour of the cell. These contacts make it possible for the epithelium to fulfil its barrier function, provide anchoring sites
both for intermediate filaments and for actin filaments and are also thought to be important in the generation of different intracellular signals [Troyanovsky, 1999]. Cell-cell contacts can be divided structurally into four different types: tight junction or zonula occludens, intermediate junction or zonula adherens, desmosome or macula adherens, and gap junction or nexus. In addition, a cell-matrix contact, the hemidesmosome, connects epithelial cells to the connective tissue layer. Adhesion molecules on the other hand form adhesions between two cells or between a cell and extracellular matrix for only the time that is necessary for the indicated function to be carried out.

**Tight junctions**
The tight junction is located at the most apical part of the basolateral surface. The outer leaflet membrane of two adjacent cells creates a series of apparent fusions with completely obliterated intercellular spaces. In contrast, in gap junctions and desmosomes there is a 15-20 nm gap between the apposing membranes. Tight junctions form the barrier that regulates the paracellular movement of water and solutes across the epithelium. This transport is passive and driven by the gradients caused by transcellular transport. This process occurs by diffusion or electrodiffusion or osmosis, and the barrier appears to have a pore size up to 2 nm [Anderson, 2001]. Tight junctions show slight cation selectivity at physiological pH.

Tight junctions are formed by the transmembrane adhesion proteins claudin and occludin and a number of associated intracellular proteins such as ZO-1, ZO-2, and ZO-3 that organise the transmembrane proteins and connect them to other cytoplasmic proteins and to the actin filaments [Anderson, 2001]. Maintenance of this intercellular seal is important for the maintenance of the integrity of the epithelial barrier. Disruption of this barrier can lead to human diseases. The first described inherited disease of tight junctions is a magnesium wasting syndrome called renal hypomagnesemia with hypercalciuria and nephrocalcinosis, which is caused by a defect in claudin-16. Also, claudin-11 null mice showed sterility and neurological defects [Mitic et al., 2000]. Recent studies have focused on the importance of the interaction of peptidase allergens from e.g., dust mites with tight junction proteins, a process that might induce the development of a hypersensitivity reaction to a wide range of antigens [Robinson et al., 2001].
**Intermediate junctions**
The adhering junction or intermediate junction is located next to the tight junction in the junctional complex in the apical part of the basolateral membrane and, like the tight junction, it encircles the cell like a belt. Its primary function is to stabilize the tight junctions mechanically and to prevent cell separation during various cell activities.

**Desmosomes**
Desmosomes are highly organised adhesion complexes that express cell-specific transmembrane cadherins and plaque-associated proteins.

![Figure 1. Molecular model of the desmosome. This simplified model shows representative protein–protein interactions in which principal desmosomal components participate. [Green & Gaudry, 2000]](image)

Transmembrane cadherins can be subdivided into desmocilins and desmogleins. Plaque-associated proteins include the armadillo (plakoglobin and plakophilin) and the plakin (desmoplakin) protein family [Ishii & Green, 2001]. The plaque is linked with the intermediate filament cytoskeleton by desmoplakin. Cadherins are connected to desmoplakin by means of armadillo proteins [Jamora & Fuchs, 2002]. The assembly of adhesion junctional complex is a highly regulated process. Calcium is thought be one of the factors that stimulate the assembly of desmosomal cadherins [Jamora & Fuchs, 2002]. Desmosomes are an adherent structure in the epithelium and
they play an important role in maintaining the structural and functional interaction of neighbouring cells [Elias et al., 2001]. By anchoring the intermediate filaments, desmosomes form a highly resilient, intercellular network, which is required for tissue integrity. Recent research has considered an expanded role of desmosomes [Elias et al., 2001]. So far three main types of diseases involving desmosomal components have been found: autosomal diseases involving desmosomal components (e.g., pemphigus vulgaris, pemphigus foliaceus), congenital diseases affecting the calcium channels (e.g., Hailey-Hailey disease and Darier disease) and congenital diseases that directly affect the structural components of desmosomes [McMillan & Shimizu, 2001]. In addition, defective plakoglobin cause a disease called Naxos disease, which results in cardiomyopathy and growth of abnormal hair [McMillan & Shimizu, 2001]. Pemphigus is an autoimmune disease, where autoantibodies against desmoglein 1 and 3 develop and blisters arise in the epidermis and/or oral cavity. Patients with a congenital disease with a defect in the gene for plakophilin 1 develop skin fragility (ectodermal dysplasia syndrome) with skin, hair and nail defects [McMillan & Shimizu, 2001]. Recent studies have shown that desmosomes are not only adhesive intercellular glue but also possess signalling capability which can lead to integration of morphological changes and expression of genes during tissue and organ development [Jamora & Fuchs, 2002].

Previous studies in our laboratory [Kampf et al., 1999] have shown that TNF-α, IFN-γ, IL-1-β, and HOCl decrease expression of desmosomal proteins in airway epithelium in vitro. In asthma, epithelial desquamation is a common finding, but the pathological mechanism behind this observation is still not clear. One could speculate whether desmosomal abnormalities play a role in asthma pathophysiology.

**Gap junctions**

A nexus or gap junction is the direct path of communication between neighbouring cells and plays an important role in the intercellular communication by allowing a rapid exchange of ions and metabolites up to ~1kD in size [Shibata et al., 2001]. Gap junctions consist of two channels, connexons, which in turn each consist of six proteins, connexins, with limited tissue specificity. Gap junctions are not specific for epithelial cells, but are found in most cell types.
Hemidesmosomes

Hemidesmosomes are specialized junctional complexes that connect the epithelium to the underlying basement membrane. They also connect keratin filaments to the extracellular matrix. Although hemidesmosomes and desmosomes have similarities regarding connection with the cytoskeleton system, they consist of different structural proteins [Nievers et al., 1999]. They play an important role in maintaining tissue integrity. It is also thought that they are involved in signal transduction [Nievers et al., 1999]. Abnormalities of any of their components lead to tissue fragility and blistering disorders of the skin [Nievers et al., 1999].

Adhesion molecules

The adhesion molecules are membrane glycoproteins that mediate adhesion between two cells or between cell and extracellular matrix [Alvaro, 2000]. These contacts are formed only for the time that is necessary for the indicated function to be carried out. The most important function of the adhesion molecules is the transendothelial migration of the circulating leukocytes from the blood vessel to the inflammatory site. The adhesion molecules can act as a receptor or as a specific ligand for these receptors. They are expressed on leukocytes, endothelial cells and also on epithelial cells [Alvaro, 2000]. Adhesion molecules are divided into several superfamilies depending on their interaction, e.g. selectins, and integrins. Selectin and their ligands are involved in the rolling and tethering of leukocytes on the vascular wall. Intracellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAM-1), and some integrins, induce firm adhesion of inflammatory cells to the vascular wall, and platelet endothelial cell adhesion molecules (PECAM-1) are involved in extravasation of cells from the blood compartment [Blankenberg et al., 2003]. The inflammatory reaction of bronchial asthma has three sequential steps: the recognition-activation phase, the inflammation phase, and the solution phase. Adhesion molecules are important in the second phase [Alvaro, 2000]. Active bronchial asthma is associated with the presence of increased levels of soluble ICAM-1 in serum, and these levels are reduced following corticosteroid therapy [Shiota et al., 1996]. Serum concentrations of soluble ICAM-1 were similar in patients with either atopic or nonatopic asthma, which is an indication of active pulmonary
inflammation in both atopic and nonatopic asthma [Shiota et al., 1996].

Figure 2. Pathophysiological mechanism of allergic asthma

The inflammatory response in asthma

Asthma is a disease of chronic airway inflammation and it is not yet clear which aspects of inflammation are responsible for its chronic character. It is known that inflammation is associated with the recruitment of inflammatory cells, predominantly eosinophils, mast cells, lymphocytes and neutrophils and subsequent release of their mediators causing the symptoms of chronic asthma. After priming by T cells and antigen presenting cells, B cells are directed to produce allergen specific IgE. This IgE is released into the blood and attaches to high-affinity IgE receptors on the surface of the mast cells and peripheral blood basophils, as well as to the low-affinity IgE receptor or to the surface of lymphocytes, eosinophils, platelets and macrophages. Future exposure to the antigen causes mast cell activation and with release of many preformed mediators constitutes the "early phase reaction" [reviewed in Maddox & Schwartz, 2002]. Release of various leukotrienes as well as other inflammatory cytokines leads to the "late phase reaction" which primarily involves recruitment and activation of eosinophils, Th-2 lymphocytes, macrophages and neutrophils. Marked changes in the structure of the
airway are observed in this disease. The airway epithelium is damaged. Other changes include hyperplasia of goblet cells, thickening of the basement membrane, smooth muscle hypertrophy and hyperplasia, and airway wall oedema [McKay & Hogg, 2002]. A recent study suggests that airway smooth muscle myositis caused by mast cells could be an important factor in asthma pathology [Wardlaw et al., 2002]. Another recent study has shown that subjects with acute exacerbation of asthma have high serum ECP (eosinophilic cationic protein) levels and that bronchodilators were more effective in those patients who had lower serum ECP levels [Di Lorenzo et al., 2002].

This would implicate the eosinophil, which is the characteristic predominant cell type in atopic asthma, in the pathophysiology of this disease. The mechanism still needs to be elucidated. However, in non-atopic asthma neutrophils are predominant [Amin et al., 2000], and therefore this cell type also needs attention, because at present treatment and prevention strategies are almost exclusively focused on allergic or eosinophilic inflammation [reviewed in Douwes et al., 2002]. An association between increased neutrophils and IL-8 suggests that non-allergic neutrophil mediated airway inflammation could be the underlying mechanism of non-eosinophilic asthma [Douwes et al., 2002]. Another difference between atopic and non-atopic asthma appears to be that in non-atopic asthma, there were fewer IL-10 and IL-12 mRNA positive sputum macrophages, compared to atopic asthma. It has been suggested that since IL-12 is an anti-inflammatory cytokine, absence of this cytokine could be responsible for the inflammatory process in non-atopic asthma [Zeibecoglou et al., 2000].

Another study found increased hyperresponsiveness in atopic asthma after adenosine 5'-monophosphate (AMP) challenge compared to non-atopic asthma. However, no difference between these two groups was observed after metacholine and cold air challenge. The same study also found that in atopic asthma, the slope of the dose-response curve for AMP was correlated with serum levels of eosinophil peroxidase (EPO) and eosinophil cationic protein (ECP) while no correlation between BHR and airway inflammation markers was observed in non-atopic asthma [Zeibecoglou et al., 2000].

In conclusion, differences in the pattern of inflammatory cells between atopic and non-atopic asthma have been noted. However, no definitive HLA association could be established with atopic or non-atopic asthma [Zeibecoglou et al., 2000]. Therefore, differences between atopic and non-atopic asthma need to be investigated further.
Inflammatory cells

Eosinophils

Once the inflammatory reaction starts in asthma, eosinophils become the prime mediators of chronic inflammation [Maddox & Schwartz, 2002]. Recent reviews have described that eosinophils with their secreted mediators are heavily implicated as effector cells in the pathophysiological changes in the airways in asthma [Walsh et al., 2003]. The Th2 cytokines IL-3, IL-5 and GM-CSF are primarily involved in the selective differentiation and activation of eosinophils and are thought to regulate eosinophil priming, activation, and survival [Lopez et al., 1988; Rothenberg et al., 1988].

![Diagram of eosinophil products in asthma](image)

Figure 3. Eosinophil products that may be acting in asthma [Woolf, 1998]

Eosinophils are attracted to the lung by the chemoattractants leukotriene B4, IL-16, and eotaxin released by mast cells, T cells and also by epithelial cells. By releasing highly charged cationic proteins, like major basic protein (MBP), eosinophilic cationic protein (ECP), pro-inflammatory mediators, oxygen free radicals, and cytokines, eosinophils cause direct damage to the airway mucosa and associated nerves, hypersecretion of mucus, smooth muscle contraction, and
increased vascular permeability [Kay et al., 2004]. This latter effect recruits more eosinophils and Th-2 type lymphocytes to the airway [Maddox & Schwartz, 2002]. The number of eosinophils is broadly correlated with disease severity [Corrigan & Kay, 1992]. Shedding and epithelial damage caused by highly basic granule proteins such as MBP, eosinophil peroxidase, and ECP are a major event in asthma pathophysiology [Gleich, 2000]. Among all mediators produced by eosinophils, MBP is the most cytotoxic to airway epithelium. Once started, this cycle of tissue damage and cellular recruitment is repetitive and thereafter becomes chronic. Even in the absence of sustained allergens the inflammatory process persists. Eosinophils are considered as a prime effector cell in the inflammatory mechanism of asthma and other allergic disease [Wardlaw et al., 2000]. They have been shown to synthesise up to 28 cytokines, chemokines and growth factors [Lacy & Moqbel, 2001]. Recently it has been found that eosinophils also play a role in antigen presentation to activated CD4+ T cells [MacKenzie et al., 2001]. Eosinophils might also be involved in tissue repair and airway remodelling because they contain preformed pro-fibrogenic cytokines [Puxeddu & Levi-Schaffer, 2004]. However, it is increasingly becoming clear that eosinophil-epithelium interactions are an important part of asthmatic disease in relation to both the initiation and resolution of inflammation [Walsh et al., 2003].

Neutrophils

Neutrophils represent 60 to 70% of the total circulating leukocytes. Within minutes of tissue damage or pathologic invasion (bacteria, fungi, protozoa etc.), neutrophils migrate into involved tissues as a “first line of defense” to engulf and eliminate foreign material. Neutrophils contain different types of granules, which contain different kinds of proteins and proteinases such as myeloperoxidase (MPO), defensins, lysozyme, elastase, cathepsinG and collagenase. Lipid mediators, reactive oxygen intermediates (ROI), nitric oxide and small amounts of some cytokines including IL-1β, IL-6, IL-8 and TNF-α [Sampson, 2000] are also produced by neutrophils. Exocytosis of granules and secretory vesicles plays an important role in most neutrophil functions from early activation to the destruction of phagocytosed microorganisms. Although the role of the neutrophil in asthma remains obscure, high numbers of neutrophils have been found in the airways of patients with chronic severe asthma [Caramori et al., 2005]. Neutrophil products such as lipid mediators, reactive oxygen intermediates (ROI) and proteases such as elastase, may contribute to
airflow obstruction, epithelial damage and remodeling [Sampson, 2000].

*Mast Cells*

Mast cells originate from CD34⁺ progenitor cells in the bone marrow [Kirshenbaum et al., 1991]. The progenitor cells circulate as undifferentiated mononuclear cells in the peripheral circulation and express c-kit (CD117), the receptor for stem cell factor. After migration into the tissue they mature under the influence of locally derived growth factors and cytokines including stem cell factor. How mast cells reach the tissue is poorly understood. Mast cells are widely distributed through the body in both connective tissue and mucosal surfaces. There are two subtypes of mast cell known as the mucosal type and the tissue type. The mucosal type predominantly contains tryptase and is designated as MC₁ and the tissue type contains both tryptase and chymase and is designated as MC₁C [Schwartz et al., 1987]. The mast cell is well known as an important mediator-secreting cell in asthma. Present on the surface of and within the airway, they are well positioned to respond to a provocative stimulus. They interact with allergens by way of IgE bound to the high affinity receptor FcεRI and within minutes release both preformed mediators such as histamine and tryptase and newly synthesized mediators such as PDG₂ and LTC₄ [Wenzel et al., 1988, 1990], which are potent bronchoconstrictors and cause changes in vascular permeability. By releasing different kinds of cytokines such as IL-4, IL-5 and IL-13 mast cells may further contribute to the inflammatory changes in asthma [Puxeddu & Levi-Schaffer, 2004; Bradding et al., 1992; Jaffe et al., 1995; Jaffe et al., 1996].

*Lymphocytes*

Lymphocytes are mononuclear white blood cells. They are divided into two major types, B lymphocytes and T lymphocytes. B lymphocytes produce specific antibodies that help to destroy foreign substances. T cells are formed in the bone marrow, mature in the thymus and consist of several distinct subpopulations such as helper T cells or CD4 cells, cytotoxic T cells or CD8 cells and suppressor T cells, most of which are also CD8 cells. T helper cells are divided into two functionally distinct subsets, named Th1 and Th2 lymphocytes. Th1 cells produce IL-2 and IFN-γ and enhance cell mediated immune response and inhibit Th2 cell activity. Th2 cells produce IL-4, IL-5,
IL-9, and IL-13 [Prescott, 2003]. Th2 cells are thought to play an important role in humoral immunity and downregulate the Th1 cells in their neighborhood. It has been found that the asthmatic airway is dominated by activated CD4+ T helper cells after an allergen challenge [Azzawi et al., 1990]. Eosinophils within the lumina of airways could process inhaled antigen and function in vitro and in vivo as antigen-presenting cells to promote expansion of Th2 cells in the lungs [Xie et al., 2005]. The previous hypothesis was, that a deficient Th1 activity would play a key role in the development of asthma. However, recent studies found no conclusive evidence of impaired Th1 function either in asthma or in atopy [Ng et al., 2002; Smart & Kemp, 2002; Kimura et al., 2002]. On the other hand, Th2 cytokines (IL-4, IL-5, IL-9, IL-13) appear to have a critical role in the expression and development of airway inflammation and hyperactivity (AHR) [Prescott, 2003].

**Macrophages/ Monocytes**

Both in asthmatic and non-asthmatic persons, macrophages are the predominant cells observed in BAL. Monocytes, one of the mononuclear leukocytes, migrate into tissues and develop into macrophages. The functions of these cells are phagocytosis and antigen presentation to T cells. Although the role of macrophages in asthma pathophysiology is being debated [Hamid et al., 2003], these cells may participate in airway inflammation. Macrophages also produce pro-inflammatory cytokines such as IL-1, TNF-α, IL-6 and GM-CSF that may participate in endothelial activation, cellular recruitment, and prolonged eosinophil survival. Macrophages may also play a role in asthma by mast cell activation and late phase responses independently of repeated allergen exposure [Hamid et al., 2003].

**Cytokines**

Atopic asthma is associated with local Th2 cytokine expression, observed initially in the early 1990s. Cytokines such as IL-4, IL-5, IL-9, IL-10, IL-13 and GM-CSF were upregulated in asthmatic patients compared to healthy controls and associated with pathological changes in asthma [Chung & Barnes, 1999; Wilson & Li, 1997]. IL-5 plays an important role in proliferation, differentiation, activation, and survival of eosinophils [Lampinen et al., 2004]. Recent research is
focused towards interaction between cytokines and local airway epithelial cells, and the role of cytokines in the prediction, assessment, and treatment of the disease [reviewed in Prescott, 2003]. Cytokine research is bringing new interesting concepts regarding asthma pathophysiology. There is a growing interest in how local immune networks interact with resident airway cell populations such as epithelial cells. These epithelial cells are also a key producer of cytokines, chemokines and growth factors. Studies on Calu-3 human lung epithelial cells have shown that IL-4 and IL-13 directly affect these cells by impairing epithelial barrier function and wound healing [Ahdieh et al., 2001]. These effects may be mediated by direct effects on airway epithelial cells [Kuperman et al., 2002; Venkayya et al., 2002] without the need for an associated inflammatory infiltrate [Prescott, 2003]. A recent study on animal models has shown that IL-10 and TGF-β can reduce airway inflammation mediated by mycobacteria administration [Prescott, 2003].

Polyps and asthma

Nasal polyposis is a chronic inflammatory condition of the nose and sinuses often associated with rhinitis, asthma and aspirin intolerance [Bachert et al., 2002; Holmberg & Karlsson, 1996]. The aetiology of nasal polyposis is unknown. Symptoms are primarily nasal obstruction and disturbance of the sense of smell. Nasal polyps are found in 36% of patients with aspirin intolerance, 7% of those with asthma, 0.1% in children, and in about 20% of patients with cystic fibrosis [Settipane, 1996]. Nasal polyps have long been associated with rhinitis and asthma. However, the role of allergy in the etiology and pathogenesis of nasal polyps is a controversial issue. High levels of mast cells have been found in nasal polyps [Otsuka et al., 1993], but these were not related to allergy [Ruhno et al., 1990]. The cytokine profile of nasal polyps resembles neither Th1 nor Th2. IL-4, IL-5, IL-12 and IFN-γ have been shown to be upregulated in nasal polyposis tissue [Bachert et al., 2002; Holmberg & Karlsson, 1996]. Nasal polyps are histologically associated with dense infiltration with eosinophils [Stammberger, 1999]. Recent studies have observed a strong upregulation of IgE synthesis with specific IgE to Staphylococcus aureus enterotoxin, suggesting a possible role of superantigens in the pathologic process [Bachert et al., 2002; Holmberg & Karlsson, 1996]. It has also been suggested that a fungal etiology may underlie severe nasal polyposis, although this notion is
still controversial [Ponikau et al., 1999; Pitzurra et al., 2004]. As a possible mechanism by which fungi act on the respiratory epithelium, it has been suggested that fungi produce proteases that bind to protease-activated receptors; this binding would activate intracellular signalling pathways that would give rise to multiple responses, including cytokine production and disruption of tight junctions [Reed & Kita, 2004].

**Rhinitis and asthma**

Rhinitis is an inflammation of the nasal mucosa associated with sneezing, itching, rhinorrhoea, and nasal congestion. Rhinitis is classified as allergic or non-allergic rhinitis. Allergic rhinitis (AR) is again subdivided into seasonal allergic rhinitis and perennial rhinitis. As the disease asthma is commonly present with allergic rhinitis it is thought that there is a relationship between these diseases. Asthma and rhinitis are connected epidemiologically, anatomically, physiologically, immunopathologically, and therapeutically [reviewed in Ciprandi et al., 2004]). Both diseases are characterized by infiltration of T cells, mast cells, and eosinophils [Christodoulopoulos et al., 2000], have similar mediators such as histamine, leukotrienes [Foresi et al., 1997], and their cytokine pattern is typically of the Th2 type [Hamid & Minshall, 2000]. Vascular tissue and smooth muscle cause nasal congestion and bronchospasm, and the same drugs, such as antihistamines, antileukotrienes, and topical corticosteroids can be used with different efficacy for both diseases [Ciprandi et al., 2004].

**Epithelial damage in asthma**

Epithelial damage is a prominent characteristic feature of asthma, and can be observed even in patients with mild asthma [Jeffery et al., 1989]. The epithelium is the first line of defence, which protects the airway from environmental stress, allergens and microbes. Disruption of this barrier can lead to an imbalance of homeostasis of the airway. Therefore, loss of airway epithelial cells can increase airway hyperresponsiveness by allowing easy entrance of allergenic and noxious agents into the airway wall. Asthmatics have a highly abnormal bronchial epithelium, which includes both structural (separation of columnar cells from basal cells) and functional (increased expression and production of pro-inflammatory cytokines, growth factors and mediator-generating enzymes) abnormalities.
In asthma there may be a primary defect in the epithelium, which leads it to respond abnormally to various stimuli and also prevents it from proceeding towards a normal repair process [reviewed in Holgate, 2000]. In addition, the airway epithelium is more than just a barrier: it can generate different mediators which may play an important role in the inflammatory and remodelling procedure in asthma [Holgate, 2000].

The epithelium is the prime source of granulocyte macrophage colony-stimulating factor (GM-CSF) and also of IL-5, which are cytokines prolonging the survival of eosinophils. The bronchial epithelium also synthesizes eotaxin, which is a potent chemokine of eosinophils and which is elevated in allergic asthma [Lilly et al., 1997]. The eosinophils in turn cause an inflammatory reaction and may damage the airway epithelium by their toxic cationic granule proteins [Gleich et al., 1988].

Epidermal growth factor (EGF) is a key stimulator of bronchial epithelial proliferation and migration [Holgate, 2000] produced by the epithelium [Holgate et al., 2000] and found to be increased in asthma. However, there is no increase in proliferation, response and restitution of normal airway epithelium in asthma. Transforming growth factor β (TGF-β), which is produced by the epithelium and also by the inflammatory cells, is responsible for collagen synthesis. It is thought that there is an abnormal communication between the myofibroblasts that deposit collagen in the sub-epithelial basement membrane, and the epithelium [Holgate et al., 2000].

The abnormal epithelium in asthma may be a primary defect, so that children are born with it. There are some studies that support this thought. For instance, Devalia and colleagues [Bayram et al., 1998; Devalia et al., 1999] have shown that bronchial epithelium grown from biopsies of asthma patients generated more cytokines than epithelium grown from healthy subjects. Epithelial damage is therefore a very important aspect of asthma. Indeed, within the epithelium there may be one of the secrets that underlie the chronic character of asthma [Holgate, 2000].

**Treatment**

Asthma is now considered a major health care burden. At present there is no curative drug for asthma because the exact defect is not yet known. The treatment strategy to control asthma is unchanged from what it was 30 years ago, using β-2 adrenoreceptor agonists and
glucocorticosteroids [Spina & Page, 2002; Holgate, 2004]. Many
drugs have been developed during this period, for example,
thromboxane receptor antagonists [Stenton et al., 1992], thromboxane
synthase inhibitors [Gardiner et al., 1993], platelet activating factor
receptor antagonists [Kuitert & Barnes, 1995], recombinant IL-12
[Bryan et al., 2000] and a monoclonal antibody against IL-5 [Bryan et
al., 2000]. However, all these treatments have failed. A soluble IL-4
receptor, which neutralizes the effects of IL-4 alone, provided modest
improvements in moderate asthma according to one study [Chung,
2003] but was disappointing in another study [Stokes & Casale, 2004].
A recent development is anti IgE-therapy available as Omalizumab,
which is effective in allergic asthma in children and adults [Schulman,
2001; Stokes & Casale, 2004].
Therefore, we need to understand more about the inflammatory
mechanism to find the right therapeutic target. As asthma is a
multifactorial disease, a combination therapy would possibly be more
effective to cure or prevent the disease. The genetics of asthma also
need to be evaluated more extensively. In the present study, we
attempt to evaluate the mechanism of epithelial damage and structural
changes of the asthmatic airway.
Aims

The aims of this study were:

1. To evaluate the mechanism of damage to airway epithelial cells caused by poly-L-arginine (an analogue of eosinophil-derived major basic protein) against the background formed by the fact that the eosinophil is considered to be a crucial factor in the inflammatory process in atopic asthma.

2. To study the effects of IL-8, IL-4, IL-13 and IFN-γ on cell viability and ICAM-1 and ZO-1 expression on human airway epithelial cells since these cytokines may play a role in recruiting leukocytes to the epithelium from where they pass into the airway lumen.

3. To carry out an ultrastructural study comparing epithelial damage in atopic and non-atopic asthma in order to determine whether there is a difference between these two forms of asthma in this respect.

4. To carry out a quantitative study of cell attachment in the human airway epithelium to confirm that the attachment of columnar epithelial cells to the basal lamina mainly occurs indirectly, via desmosomal attachment to basal cells, and to compare attachment in asthmatics versus healthy controls.

5. To study epithelial damage and cell contacts in nasal polyposis and compare these with changes in the lower airways found in asthma.
Materials and Methods

Cell culture
16HBE14o- cells, a columnar cell line (a kind gift from Dr D.C Gruenert, University of California, San Francisco, CA, USA) was cultured in Eagle's minimal essential medium (EMEM)(National Veterinary Institute, Uppsala, Sweden) supplemented with 10% fetal bovine serum (Gibco BRL/Life Technologies, Paisley, UK), 100 U/ml penicillin and 100μg/ml streptomycin sulfate. The culture flasks were coated with fibronectin-coating solution containing 0.01 mg/ml fibronectin, 0.029 mg/ml collagen and 0.1 mg/ml BSA (bovine serum albumin), at least one hour before culturing the cells.
As a basal cell line, normal human bronchial epithelial (NHBE) cells (Clonetics, San Diego, CA, USA) were used. Culture of these cells was established at the Clonetics cell culture facility from normal human tissue taken from a 16-yr-old female. The cells were cultured in plastic culture flasks (Corning Costar Corporation, Cambridge, MA, USA) in bronchial epithelial growth medium (BEGM) (Clonetics) supplemented according to the manufacturer's instruction.
Both cell lines were cultured in a humidified atmosphere of 5% CO2/95% air at 37°C and the culture medium was changed every 48 hours. Desmosome formation in NHBE cells required a different medium, namely Dulbecco’s modified Eagle’s medium (DMEM):F12 (1:1) (Gibco) supplemented with 5% fetal bovine serum, penicillin (100 U/ml) streptomycin (100 μg/ml) and non-essential amino acids (Sigma, St. Louis, MO, USA). The cells were cultured on Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY, USA) or in Petri dishes (Becton Dickinson, Plymouth, UK). (Paper I, Paper II)

Induction of cell cultures
Poly-L-arginine induction
Cells were cultured to about 90% confluence and then incubated with poly-L-arginine. In initial experiments, cell viability was assessed using poly-L-arginine in the concentration range of 2.5 μM to 20 μM. Based on the data obtained in those experiments, a concentration of 15 μM for 16HBE14o- and 2.5 μM for NHBE cells was chosen.
Unexposed cells served as controls. The cells were incubated for 24 h with poly-L-arginine, except for the desmosome experiments, where an incubation period of 48 h was used, since this was the time required for desmosome formation. (Paper I)

Cytokine induction
Cells were cultured to 90% confluence and then incubated with either IL-8 (25 ng/ml), IL-4 (25 ng/ml), IL-13 (25 ng/ml) or IFN-γ (1000 U/ml) or a combination of either IL-8, IL-4 or IL-13 with IFN-γ (PeproTech, Rockey Hill, NJ, USA) or cycloheximide (CHX) (Sigma) (25 µg/ml) for 48 hrs, unless otherwise stated. Unstimulated cells served as controls. (Paper II)

Bronchial biopsies
Bronchial biopsy specimens were taken from 23 adults; 11 of these were atopic asthmatics, 7 non-atopic asthmatics and the remaining 5 were healthy controls. The study was conducted in accordance with the declaration of Helsinki and was approved by the ethics committee at the Faculty of Medicine at the University of Uppsala.

All asthma patients had a clinical diagnosis, current asthma symptoms and increased responsiveness to inhaled methacholine, defined by a provocative dose of methacholine causing a ≥ 20% fall in FEV₁ (PC20) ≤ 32 mg. Clinically diagnosed asthma patients were being followed as out-patients at the Department of Respiratory Medicine and Allergology at the hospital. The participants had gone through a questionnaire about airway symptoms during the last 12 months before they were included in the study. The questionnaire was based on the European Community Respiratory Health Survey Questionnaire [Burney et al., 1994]. None of the patients were smokers. They had been free from respiratory infections for at least six weeks before taking biopsy and none had a history of cardiovascular disease.

Atopic asthmatics
All patients had a positive skin prick test (>3mm) for at least one common allergen tested: birch, timothy grass (Phleum pratense), mugwort (Artemisia vulgaris), cat, dog, horse, house dust mite (Dermatophagoides pteronyssinus), Cladosporium, and Alternaria. All but two of the patients were on regular treatment with inhaled
glucocorticosteroids (budesonide 200-800µg/day) and inhaled beta agonists as needed. (Paper III, IV)

Non-atopic asthmatics
All had a negative skin prick test and all but two non-atopic patients were on regular treatment with inhaled glucocorticoids (budsonide or beclomethasone 200-800µg/day) and inhaled β₂-agonists as required. The average use of inhaled glucocorticosteroids was similar in both asthma groups. (Paper III)

Healthy controls
Controls were healthy persons who responded to a request for volunteers. None had asthmatic symptoms and all of the subjects showed a negative skin prick test. (Paper III, IV)

Symptom score and peak flow variability
Records of symptoms and peak flow variabilities were kept during a 17 day period starting on the day of the metacholine challenge. The peak expiratory flow rate (the best of three measurements) was recorded four times daily with a Mini-Write Peak Flow Meter (Clement Clare, London, UK). Minimum requirements were at least two recordings a day, one of which was in the morning. Peak flow variability was calculated by dividing the difference between the highest and lowest daily PEF reading by the daily mean PEF value. The results were expressed as the daily percentage of variability (vPEF%). In the symptom score, the subjects stated on awakening whether they had had: breathing difficulties during the previous night, wheezing in the chest, attacks of breathlessness or attacks of coughing during the previous 24 hours. Each affirmative answer was given a score of one and a symptom score was calculated [Ludviksdottir et al., 1999]. (Paper III, IV)

Bronchoscopy
The patients were given 10 mg of diazepam (Stesolid®, Dumex, Copenhagen, Denmark) orally and 0.5 mg of atropin (Atropin, NM Pharma, Stockholm, Sweden) subcutaneously 30 min before the investigation. The upper airways were anaesthetised with lidocaine hydrochloride (Xylocain, Astra, Södertälje, Sweden). Using a flexible fibre bronchoscope (Olympus p 20D) with an FB 15C 2.0 mm forceps
(Olympus), one or two biopsies for transmission electron microscopy were taken in the right lung in the upper lobe bronchus immediately after the division from the main bronchus. In the first series of biopsies (5 controls, 6 patients with allergic asthma, and 3 patients with non-allergic asthma) one biopsy was taken, in the second series (5 patients with allergic asthma and 4 patients with non-allergic asthma) two biopsies were taken from each patient. The specimens were examined immediately by light microscopy to ensure the presence of a complete mucosa and fixed as described below. The patients were instructed to take their regular asthma sprays on the morning of the bronchoscopy. (Paper III, IV)

Rats

Three adult male Sprague Dawley rats (weight about 300 grams) were purchased from B&K Universal (Sollentuna, Sweden) and housed in a conventional animal care facility at the Biomedical Center, Uppsala University one week before experimentation. The Regional Committee on Animal Experimentation, Uppsala, approved the protocol. All rats were anesthetized intraperitoneally by sodium pentobarbital injection, and were dissected when reflexes could no longer be elicited. The tracheas were removed from the rats, and tissue taken from the middle part was fixed for electron microscopy. (Paper IV)

Nasal polyps

Nasal polyps were taken from 15 adults, 6 of them asthmatics, of which 3 were allergic and 3 non-allergic; 2 adults were suffering from allergy but not from asthma, and the remaining 7 had neither allergy nor asthma. Allergy had previously been documented by either skin test or blood sample (Phadiatop, Pharmacia Diagnostics, Uppsala, Sweden). Clinically diagnosed patients with nasal polyps were being followed up at the Department of Ear Nose and Throat Diseases at Uppsala Academic Hospital. All patients had chronic nasal congestion, chronic rhinorrhea and all except one variable degrees of anosmia. One of the patients in the no allergy/no asthma group was a smoker. None of the subjects suffered from respiratory infection during surgery. Out of 15 patients 2 were female and 13 male. Seven of the patients had had no previous surgery. All patients except one were using nasal steroids and all patients except two were periodically
using oral cortisone tablets; the exceptions were due to advanced age and stomach problems, respectively. Treatment with corticosteroids had not been suspended prior to surgery. Polyps were removed during functional endoscopic sinus surgery (FESS) from ethmoidal cells and meatus medius bilaterally, immediately examined macroscopically and fixed in glutaraldehyde for electron microscopy. (Paper V)

Transmission electron microscopy (TEM)

16HBE14o- and NHBE cells grown in Petri dishes, as well as biopsy tissues and polyps were fixed in 2.5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (Agar Scientific, Stansted, UK) for 1 day. After being washed in cacodylate buffer, the specimens were post-fixed in 1% OsO₄ in cacodylate buffer for 60 min. A second wash in buffer was followed by dehydration in graded series of ethanol, before the specimens were finally embedded in Agar 100 Resin (Agar Scientific).

The paracellular permeability barrier function of both the 16HBE14o- and the NHBE cells was investigated by visualizing the tight junctions by means of lanthanum tracer. Lanthanum nitrate (1%) was added to the fixative.

Sections were cut and stained with uranyl acetate/lead citrate, and examined in a Hitachi H7100 transmission electron microscope (Hitachi, Tokyo, Japan) at 75 kV. (Paper I, II, III, IV, V)

Microscopic evaluation of biopsy sections

All specimens were coded and examined without any knowledge of the diagnosis. The degree of damage was judged by two independent observers, using a scale of 1-5, where 1 indicates no damage, 2 the presence of intercellular spaces between the cells, 3 detachment of a few columnar cells, 4 detachment of many, but not all columnar cells, and 5 a total loss of columnar epithelium.

To quantify the relative length of the desmosomes 5-10 columnar and 5-10 basal cells were randomly selected for each patient for whom these parameters were quantified. The TEM image was transferred via a digital camera to a Synopsis (Cambridge, UK) Synapse frame capture system. The length of all desmosomes of a columnar cell, or a basal cell, respectively, was determined and divided by the perimeter of the cell. The thickness of the basal lamina was determined at 5-10 randomly selected positions along the basal lamina. The presence of
eosinophils, mast cells, lymphocytes and neutrophils as well as that of goblet cells was scored qualitatively for each specimen. Degranulation of mast cells was defined as granules surrounding the cell or crossing the cell membrane. (Paper III, V)

To determine the contact area between columnar cells and basal cells and the basal lamina, in each human specimen measurements were taken from two non-adjacent areas randomly chosen from areas with an intact epithelium. For the rat specimens measurements were taken from three separate areas in each rat. An average total length of 81 µm of basal lamina was studied in each human control specimen, 99 µm was studied for each asthma patient, and 119 µm was studied for each rat. The number of nucleated basal cells were counted for every area and expressed per 100 µm of basal lamina. The height of the columnar cells was determined by measuring the height of a randomly chosen cell in the center of each area. The height of the epithelium (i.e., columnar plus basal cells) was also determined in the center of each area. Cilia were not included in these measurements. Columnar and basal cell surfaces in contact with the basal lamina were determined by tracing the length of their attachment to the basal lamina. The data are presented as columnar or basal cell contacts per 100 µm of basal lamina. The columnar cell surface in contact with basal cells was also determined by tracing, and the data were normalized with regard to the length of the basal lamina. (Paper IV)

**Scanning electron microscopy (SEM)**

Both cell lines were grown on cover slips and fixed with glutaraldehyde as described above for TEM. After washing in 0.1 M cacodylate buffer post fixation in 1% OsO₄ in the same buffer for about 1 hr was carried out. Then the cells were washed again in 0.1 M cacodylate buffer and dehydrated in a graded series of acetone. Subsequently, the cells were dried by means of the critical point drying method. Finally, the cover slips were mounted on the holders and sputter-coated with gold. The specimens were examined in a LEO 1530 field emission scanning electron microscope (LEO, Cambridge, UK) at an accelerating voltage of 1kV. (Paper I) Two biopsies in each group were, after fixation and dehydration, dried by critical point drying, and examined uncoated in a LEO (Cambridge UK) 1530 field emission scanning electron microscope at 1 kV. For this purpose, biopsy material left over from a previous study was used. (Paper III)
Immunocytochemical studies

Confluent 16HBE14o- and NHBE cells grown on glass slides were exposed to poly-L-arginine (Paper I) or to cytokines (Paper II) as described above. After incubation, the cells were fixed in methanol for 3 min at -20°C, rinsed with tris-hydroxymethyl-amino methane (Tris)-buffered saline (TBS) (0.05M Tris HCl, 0.15M NaCl, pH 7.6) for 5 min and then blocked with 10% normal human serum (NHS) (Sigma) in TBS for 15 min. Following primary incubation with monoclonal anti-plakoglobin (Sigma) at a dilution of 1:6000 for 16HBE14o- cells and 1:10,000 for NHBE cells and for both cell lines anti-ICAM-1 (R&D Systems) at a dilution of 1:100 or polyclonal anti-ZO-1 (Zymed Laboratories) at a dilution of 1:100 in TBS for 1 hr at 37°C, the cells were rinsed twice with TBS. The cells were again blocked with 10% NHS for 15 min and incubated with a FITC (Fluorescein isothiocyanate)-conjugated secondary antibody (Dako, Glostrup, Denmark) at a dilution of 1:40 for 1 hr. After rinsing with TBS the slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) mounting medium and examined with a Leica DMR fluorescent microscope (Leica, Wetzlar, Germany). (Paper I, II)

Light microscopy

Sections were cut to 2µm thickness, stained with toluidin blue and examined with a Motic (Wetzlar, Germany) 1B light microscope. Using Motic Image Plus Software, the length of different types of epithelium relative to the length of the basal lamina was determined. The epithelium was divided into the following categories: 1= normal epithelium, 2 = goblet cell hyperplasia, 3 = epithelium with several layers of basal and intermediate cells plus columnar cells, 4 = epithelium with one basal cell layer and damaged or lacking columnar cells, 5 = several layers of basal or intermediate cells, but no columnar cells, 6 = no epithelium, 7 = metaplastic epithelium. (Paper V)

Cell viability test

A fluorometric assay was carried out using fluorescein diacetate (FDA) (Sigma) at a final concentration of 10 µg/ml. Absorbance was taken at 485 nm with a reference wavelength of 538 nm using a 96-well multiscanner for fluorescence. Intact viable cells take up FDA,
which is cleaved by intracellular esterases to fluorescein showing green fluorescence. (Paper I, II)

A tetrazolium salt colorimetric assay based on the method previously described by Mosmann [Mosmann, 1983] was used. Absorbance was taken at 540 nm with a reference wavelength of 690 nm using a 96-well multiscanner autoreader. (Paper II)

**Analysis of apoptotic and necrotic cells**
Both cell types were cultured in 24-well plates and exposed to poly-L-arginine (PLA). The cells were then stained with bisbenzimide H 33342 (20 µg/ml) and propidium iodide (10µg/ml) (Sigma), and analyzed using a Leica DMR fluorescent microscope. (Paper I)

In addition, experiments with NHBE cells were carried out after 30 hrs instead of 48 hrs of exposure with cytokines, in some cases combined with treatment with CHX for 24 hrs starting 6 hrs after exposure to cytokines. Treated cells were stained with propidium iodide (Sigma) (20µg/ml) and bisbenzimide (Sigma) (5µg/ml) for 15 min at 37 °C. After careful washing with PBS, cells were placed on coverslips and examined by fluorescence microscopy with a UV-2B filter using the Openlab 3.0.4 (Improvision, Lexington, MA, USA) software. The frequencies of apoptosis and necrosis were added together and designated as cell death. (Paper II)

**DNA labeling**

NHBE cells were cultured, gathered, and resuspended in phosphate-buffered saline (PBS). They were permeabilised overnight with ethanol followed by RNA degradation (RNase, final concentration 0.4 mg/ml) (Sigma) and stained with propidium iodide (final concentration 50 µg/ml). The cells were incubated in the dark at room temperature for 15 min and kept on ice until analysis. Histograms of stained cells were formed using CellQuest (Becton Dickinson). (Paper II)

**Flow cytometry analysis of ICAM-1**

Cells were cultured in 24-well plates. They were gathered, pelleted and washed twice with 0.1% bovine serum albumin (Sigma)/PBS. Cells were resuspended, blocked with 10% human serum (Sigma) for 20 min and incubated with FITC-conjugated anti-human ICAM-1 (CD54) monoclonal antibody (R&D Systems, Abingdon, Oxon, UK)
for 45 min at +4°C. After two washings with 0.1% BSA/PBS, the cells were resuspended and maintained on ice in the dark until analyzed using CellQuest (Becton Dickinson, San Jose, CA, USA). (Paper II)

**Western blot**

**ICAM-1 and ZO-1**

NHBE cells were stimulated with IL-4, IL-8, IL-13, IFN-γ or a combination of cytokines for 6 hrs and then treated with CHX for 24 hrs. Stimulated cells were washed in cold PBS and directly lysed in sodium dodecyl sulfate (SDS)-b-mercaptoethanol sample buffer containing 1mM phenylmethylsulfonyl fluoride (PMSF). Samples were then run on 6% SDS-polyacrylamide gel electrophoresis (PAGE) gel and electroblotted to Immobilon-P filters. The filters were then incubated with monoclonal anti-human ICAM-1 (CD54) antibodies (R&D Systems) and polyclonal rabbit anti ZO-1 antibodies (Zymed Laboratories) diluted 1:1000 in tris-hydroxymethyl-amino methane (Tris) -buffered saline (TBS) (0.05M Tris HCl, 0.15M NaCl, pH 7.6) supplemented with 2.5% BSA. Horseradish peroxidase linked sheep anti-mouse immunoglobulin G (IgG) and horseradish peroxidase linked donkey anti-rabbit immunoglobulin (Ig) was used as a second layer. The immunodetection was performed as described for the ECL immunoblotting detection system (Amersham Pharmacia Biotech, Uppsala, Sweden).

**IL-4 receptor**

Cells were washed, suspended in 100 μl of lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1.0 mM dithiothreitol, 0.2 U/ml aprotinin and leupeptin, 50 μg/ml phenylmethylsulfonyl fluoride), and incubated for 30 min on ice. After centrifugation, supernatants were collected and protein concentrations were determined using the Bradford assay. Samples with equal amounts of protein were loaded on an SDS-7.5% polyacrylamide gel. After PAGE, protein was transferred to a nitrocellulose membrane (pore size 0.2 μm). The membrane was blocked with 5% nonfat milk in TTBS (20mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.5% Tween 20). Monoclonal anti-human IL-4R antibody (R&D Systems) at a final concentration of 2 μg/ml was then added. After incubation for 1 hr at room temperature, the membrane was washed and incubated with horseradish peroxidase-linked immunoglobulin directed against the primary antibody. After
additional washes, bound secondary antibody was detected by using Super Signal CL-HRP Substrate System Kit (Pierce, Rockford, IL, USA). (Paper II)

**X-ray microanalysis**

Cells were cultured on 75-mesh titanium grids (Agar Scientific) covered with a carbon coated Formvar film (Merck, Darmstadt, Germany), sterilized by ultraviolet light and coated with fibronectin-coating solution for 16HBE14O cells. Both types of cells were then incubated in a 5% CO₂/95% air atmosphere at 37°C. About 48-72 hrs later, cells were exposed to poly-L-arginine. Unexposed cells served as controls. After 24 hour exposure cells were rinsed for a few seconds with cold distilled water and frozen in liquid propane cooled by liquid nitrogen (-180°C) and freeze dried overnight under vacuum at -130°C [Roomans, 2002]. Before analysis the specimens were coated with a conductive carbon layer. Analysis was performed in a Hitachi H7100 electron microscope in the scanning transmission electron microscopy (STEM) mode at 100 kV with an Oxford Instruments (Oxford, UK) ISIS energy-dispersive spectrometer system. Quantitative analysis was done based on the peak-to-continuum method after correction for extraneous background and by comparing the spectra from the cells with those from a standard [Roomans, 1988]. Spectra were acquired for 100s and each cell was analyzed only once. (Paper I)

**Statistics**

Data are presented as mean±standard error of the mean. When two groups were compared, statistical analysis was performed using an unpaired Student's t-test, or the Mann-Whitney test when the distribution of data was not normal or when the number of experiments was low (Paper I, II, IV, V). In paper III, differences between groups were determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test for determination of the significance of differences between controls and the two asthma groups, as well as Student's t-test for determination of the significance of differences between the two asthma groups. Unless otherwise indicated, significance was attributed to probability values <0.05.
Results

Paper I: Epithelial damage induced by poly-L-arginine

Cell viability
The viability of the cells was determined by the FDA dye exclusion test. 16HBE14o- cells exposed to 5-20 \( \mu \)M and NHBE cells exposed to 1-15 \( \mu \)M poly-L-arginine for 24 hrs showed a concentration-related increase in the number of dead cells. NHBE cells appeared more sensitive than 16HBE14o- cells. Heparin (100 \( \mu \)g/ml) inhibited the toxic effect of 15 \( \mu \)M poly-L-arginine completely.

Apoptotic and necrotic cells
In 16HBE14o- cells 15 \( \mu \)M poly-L-arginine increased the number of necrotic cells by 30% and the number of apoptotic cells by 5%, whereas in NHBE cells 2.5\( \mu \)M poly-L-arginine increased the number of necrotic cells by about 28% and the number of apoptotic cells by about 6%.

Transmission electron microscopy
Control 16HBE14o- cells had a central dome-shaped elevation containing the nucleus, and showed normal and compact mitochondria. In most of the poly-L-arginine exposed cells, the mitochondria had fewer cristae than normal. Control NHBE cells were flat with a dome-shaped elevation containing the nucleus. After 24 h treatment with poly-L-arginine, most of the cells showed an irregularly shaped nucleus, where the nuclear membrane started to invaginate and the nucleus appeared to be divided into small lobes. In both cell lines, no lanthanum was detected in the intercellular spaces in the controls. However, after 24 hr treatment lanthanum penetrated the tight junctions of most of the cells in both cell lines.

Scanning electron microscopy
Control 16HBE14o- cells grown in EMEM were flat and evenly covered with cell processes. Poly-L-arginine (15 \( \mu \)M, 24 hrs)-exposed cells showed shorter and fewer cell processes with an even distribution. Control NHBE cells grown in BEGM also had a flat shape and were evenly covered with cell processes. After exposure to poly-L-arginine
the shape of all the cells became rounded and cell processes with an uneven distribution were longer and present mainly on the top of the cell. The cell membrane appeared damaged with many pores.

**X-ray microanalysis**
Exposed 16HBE14o-cells showed a significant decrease in the cellular content of potassium, phosphorus, sulfur, magnesium and chloride, but the intracellular sodium content was not significantly altered compared to control. However, the total intracellular content of calcium was raised significantly in the treated cells. When NHBE cells were treated with poly-L-arginine there was a significant reduction in the intracellular content of potassium, phosphorus and magnesium. No significant difference was observed in the cellular content of sodium, chloride and sulfur between the control and treated cells. Like in 16HBE14o-cells, the total intracellular content of calcium was raised significantly in the NHBE cells.

**Immunocytochemistry**
In the control cells there was a continuous immunofluorescent band of desmosomes lining the cell membrane. Treatment of the cells with poly-L-arginine caused a marked reduction in the number of desmosomes. Treated NHBE cells also showed a reduced number of desmosomes compared to the controls.

**Paper II: Effect of cytokines on ICAM-1 and ZO-1 expression on human airway epithelial cells**

**Cytotoxicity**
As determined by the MTT assay none of the cytokines IL-8, IL-4, or IL-13 affected cell viability in the NHBE cells, and only IL-8 caused a small proliferative effect in 16HBE14o- cells after 48 hrs. In both cell types, IFN-γ caused significant cell death, and this effect was not significantly affected by any of the cytokines tested. Exposure of the cells to CHX caused a marked decrease in cell viability. NHBE cells were much more sensitive than 16HBE14o- cells. IL-4 and IL-13 inhibited the effect of CHX in NHBE cells. In 16HBE14o- cells, the effect of CHX was significantly inhibited by IL-8. Exposure of the cells to CHX caused a marked decrease in cell viability. The same results were found if the exposure time to the cytokines was shortened to 30 hrs and the exposure time to CHX to 24 hrs.
Adhesion molecule and IL-4 receptor expression
ICAM-1 is constitutively expressed in both cell lines. Flow cytometry analysis showed that ICAM-1 expression was increased by IFN-γ alone but not by IL-8, IL-4, or IL-13 alone. In 16HBE14o- cells IL-8 inhibited the effect of IFN-γ. For NHBE cells, the results of the flow cytometry experiments for ICAM-1 were confirmed by Western blot. CHX inhibited both basal ICAM-1 expression and the IFN-γ induced increase in ICAM-1 expression. Expression of ZO-1 as determined by Western blot was not affected by IFN-γ, IL-4, IL-8 or IL-13, but it was inhibited by CHX. The IL-4 receptor was detected only on NHBE cells and not to a significant amount on 16HBE14o- cells.

Immunocytochemistry and transmission electron microscopy
Immunocytochemistry confirmed that ICAM-1 is expressed in both cell lines. IL-4, IL-8, or IL-13 alone did not induce any increase in ICAM-1 expression, but IFN-γ alone increased ICAM-1 expression in both cell lines. None of the cytokines IL-4, IL-8, IL-13 or IFN-γ affected ZO-1 expression noticeably. When the cells were exposed to IFN-γ and IL-4 in combination, the cells did not appear to form proper cell contacts; although ZO-1 was expressed, there was a small gap in between the cells. Exposure of the cells to CHX caused a marked decrease in cell quantity and broken tight junctions.
Transmission electron microscopy confirmed that a gap had formed in the tight junctions of cells exposed to the combination of IFN-γ and IL-4, whereas cells were connected at the junctions in cultures exposed to IL-4 or IFN-γ only.

DNA labeling
IL-4, IL-8 and IL-13 alone had no effects on apoptosis levels in both cell lines. There was a significant increase in apoptosis after stimulation with CHX alone or in combination with either IL-4, IL-8 or IL-13 in NHBE cells whereas IFN-γ had no effect on these cells. In contrast, there was a significant increase in apoptosis after stimulation with either IFN-γ, CHX and IFN-γ or CHX in combination with either IL-4, IL-8 or IL-13 in 16HBE14o- cells. IL-4 or IL-13 in combination with CHX significantly increased apoptosis compared to cells stimulated with CHX alone in NHBE cells but not in 16HBE14o- cells.
**Paper III: Bronchial biopsies of patients with atopic and non-atopic asthma**

*Epithelial damage*
In the healthy controls, an intact epithelium was observed by both scanning and transmission electron microscopy. Both in non-atopic patients and in atopic patients, sites of damage were observed. In severe cases the columnar cells had been lost over larger areas and in extreme cases only a few basal cells were left covering the basement membrane. In all of the patients with non-atopic asthma, areas with flattened rather than columnar cells were observed, but such areas were not seen in patients with atopic asthma. Only in one biopsy (allergic asthma) also the basal cells were lost. A semi-quantitative scoring system confirmed that the degree of epithelial damage was significantly more extensive in atopic and non-atopic asthmatics than in controls, but that there was no significant difference between atopic and non-atopic patients.

*Desmosomes*
In asthmatic patients, the relative length of the desmosomes was reduced. The reduction appeared more pronounced in atopic patients than in non-atopic patients, although this difference was not statistically significant between the two asthma groups. In addition, in both atopic and non-atopic patients, many "half-desmosomes" were found, which are characterised by the presence of a single plaque in one of the neighbouring cells. In some cases, the cell lacking the corresponding plaque was damaged, but in other cases neither of the cells was damaged. Compared to the columnar cells, the desmosomes of the basal cells were larger and denser. In the basal cells, the relative desmosome length was significantly reduced both in atopic and non-atopic asthma, compared to control.

*Basal lamina*
Both atopic and non-atopic asthma patients had a thickened basal lamina compared to the controls. The thickness of the basal lamina was irregular in atopic asthma. Hemidesmosomes were observed attaching the basal cells to the basal lamina but no obvious differences in this structure between the groups were noted.
**Inflammatory cells**
A qualitative assessment of the occurrence and structure of white blood cells was carried out. Eosinophils were a common finding in atopic asthma, and these cells showed mostly piece-meal degranulation. They were most commonly observed in the subepithelial connective tissue, but some were seen penetrating the epithelium and others were seen inside the capillaries. Accumulations of eosinophils showed a spatial correlation with sites of epithelial damage.

In atopic asthma, about equal numbers of mast cells were degranulating and non-degranulating, whereas in non-atopic asthma most mast cells were non-degranulating, and in controls hardly any mast cells were found. A quantitative evaluation of the number of degranulating and non-degranulating mast cells confirmed this tendency of more degranulating mast cells in the patients with atopic asthma.

The number of lymphocytes was increased both in atopic asthma and non-atopic asthma, and they were found both in the submucosa and in the epithelium.

Neutrophils were more frequently found in non-atopic asthma compared to atopic asthma.

A few cases of atopic asthma showed goblet cell hyperplasia. Overactive fibroblasts with enlarged rough endoplasmic reticulum was another common finding in patients with atopic asthma.

**Paper IV: Attachment of columnar airway epithelial cells in asthma**

**Rat trachea**
The rat airway epithelium is less high than the human airway epithelium (21 µm vs. 40 µm), and the basal cells are fewer in number and smaller in size compared to the corresponding cells in humans.

The percentage of basal lamina covered by columnar cells was much higher in the rats (36% vs. 5%). At higher magnifications desmosome attachments between columnar and basal cells, as well as between basal cells were observed. The desmosomes in the rat tracheae seemed to be shorter than those found in the human tissue. Hemidesmosomal attachments were observed between basal cells and the basal lamina. The columnar cells send out basal extensions that are closely apposed to the basal lamina, but that do not have hemidesmosomes.
Human bronchial biopsies
Transmission electron microscopy of human respiratory epithelium showed several layers of basal cells. Tissue from patients with atopic asthma showed, in contrast to control tissue, damage ranging from minor changes up to shedding of the epithelium. In both the controls and the asthmatics, desmosomes were present between columnar cells, between basal cells, and between columnar and basal cells. Hemidesmosomes were observed attaching the basal cells to the basal lamina in both groups. As in the rat, no hemidesmosomes were observed attaching the columnar cells to the basal lamina, neither in the controls nor in the asthmatics. In the asthma patients the basal lamina was curved and thickened, with an irregular thickness. In asthma patients, widening of the interstitial space between the basal cells was observed. Goblet cell hyperplasia was a common finding in asthmatics. Intercellular spaces adjoining the basal lamina were larger in the human airway epithelium compared to the rat. The only significant difference between controls and asthmatics was a decrease in contact area between columnar cells and basal lamina.

Paper V: Ultrastructural investigation of epithelial damage in asthmatic and non-asthmatic nasal polyps
Epithelial damage was commonly observed. The data for asthmatic patients were compared with those for non-asthmatic patients (irrespective of the presence of allergy) and the data for allergic patients were compared to the data for non-allergic patients (irrespective of the presence of asthma). Statistical analysis of the data showed no significant difference between any of the groups, except that allergic patients had a larger percentage of type 4 epithelium (one layer of basal cells, lacking or with damaged columnar cells).

The presence of inflammatory cells was determined in a semi-quantitative manner. The allergic, but-non-asthmatic, patients had the lowest frequency of eosinophils and neutrophils. Some patients showed massive infiltration of leukocytes, in particular eosinophils, in the epithelium. All patients had dilated capillaries in the connective tissue, which was oedematous. In some patients, it was noted that leukocytes were migrating out of the capillaries. In a few samples, plasma cells were observed.

The relative length of the desmosomes of the columnar and basal cells was determined quantitatively. The relative length of columnar cell desmosomes was significantly reduced in asthmatics vs. non-
asthmatics, and in allergies vs. non-allergics. For the basal cells, however, no significant difference between the groups was noted. No significant difference in thickness of the basal lamina was found in any of the groups. The intercellular space between the epithelial cells was significantly larger in non-asthmatics vs. asthmatics, but there was no significant difference between allergic and non-allergic patients.
Discussion

Studies on human tissues in situ (Paper III, IV, V)

Epithelial damage, possibly related to a predominantly eosinophilic inflammation, is a characteristic feature both in asthma and in nasal polyps. Therefore, an ultrastuctural investigation of human bronchial biopsies and nasal polyp biopsies was carried out. Since cell-cell contacts play an important role maintaining epithelial integrity and epithelial barrier function, which is important for the protection of the airways from extraneous noxious substances, special attention was paid to cell contacts, in particular desmosomes and tight junctions. Our data allowed us to compare atopic and non-atopic patients as well as upper and lower airways.

Transmission electron microscopy of bronchial biopsies showed that in many respects changes in the airway epithelium were similar in atopic and non-atopic asthma patients: epithelial damage, thickened basement membrane, and weakened desmosomal connections. There was a tendency for these changes to be more extensive in patients with atopic asthma, but the differences were not significant. However, we also found differences between atopic and non-atopic asthma patients, namely more eosinophil accumulation and goblet cell hyperplasia in atopic asthma.

Epithelial damage

We observed that epithelial damage is a general characteristic of asthma and nasal polyposis. This feature mostly comprises the columnar cells of which many were damaged or completely lost, especially in the atopic asthmatics and allergic nasal polyposis. This agrees with findings by other groups [Laitinen et al., 1985; Ohashi et al., 1992; Wladislovsky-Waserman et al., 1984; Beju et al., 2004]. In non-atopic asthmatics, many flattened cells rather than columnar cells were seen, which could be part of the airway remodelling process. Epithelial damage is an important aspect of asthma, because it makes the submucosal tissue layer more accessible to irritants, which can
cause hyperresponsiveness [Laitinen et al., 1985; Laitinen & Laitinen, 1994a, 1994b]. It is likely that there is a relationship between eosinophils and epithelial damage [Brown et al., 1998; Jatakanon et al., 2000].

**Desmosomes**

The quantitative assay applied in this study provides important data on the desmosomal contacts between epithelial cells. To our knowledge, this is the first time that this type of cell contact is objectively quantified in the airway epithelium and in connection to asthma. The relative length of desmosomes was reduced in both types of asthma. Frequently, half-desmosomes were found in asthmatics. In the nasal polyps the desmosomal contacts in columnar cells were significantly reduced. Several explanations are possible for our findings. First, it could be speculated that asthma patients have an intrinsic or acquired deficiency in the synthesis of desmosomes. This would make the epithelium more prone to loss of columnar cells. Secondly, it could be speculated that inflammatory cells, in particular eosinophils, specifically accelerate the breakdown of desmosomes and/or inhibit the synthesis of desmosomes. Finally, the reduction in desmosomal contact could be a more or less unspecific effect of a decrease in metabolism in the epithelial cells, which would inhibit synthesis of desmosomes as well as other proteins. The present study does not allow a distinction between these different possibilities. It is, however, of interest that in cell pairs in which half-desmosomes were found, the cell lacking the desmosome plaque was not necessarily damaged. This would indicate that the decrease in desmosomal contacts is not per se a result of cell death. Half-desmosomes have been observed in epithelial cells cultured in a low calcium medium, where desmosomal proteins were synthesized but not assembled, or formed partially assembled unstable half desmosomes [Burdett, 1998]. It is therefore possible that the half-desmosomes observed in the present study were due to a disturbance of desmosome assembly.

A statistically significant difference was found in the percentage of basal lamina in direct contact with columnar cell surface between asthma patients and controls. Columnar cells contact the basal lamina by means of slender processes, but have no hemidesmosome connections. Therefore, the indirect contact by desmosome attachments with the basal cells, which in turn are connected to the basal lamina with hemidesmosomes, is very important. The small
contact area and weak direct contact between columnar cells and basal lamina, in combination with the decrease in size of the desmosomes between columnar cells and basal cells that we observed could be an important factor in the development of epithelial shedding in asthma.

**Eosinophils**

Many activated eosinophils were observed in the bronchial biopsies of nearly all atopic asthmatics and in many nasal polyp biopsies. Massive infiltration of eosinophils was observed in both epithelium and connective tissue in these biopsies. The most common degranulation mechanism *in vivo* appears to be the so-called "piece-meal degranulation" mechanism [Karawajczyk *et al.*, 2000; Erjefält *et al.*, 2001] but in some cases, lysis was observed [Erjefält & Persson, 2000]. The fact that piece-meal degranulation was observed more commonly than lysis agrees with the literature [Erjefält *et al.*, 1999]. Activated eosinophils play an important role in the pathogenesis of bronchial asthma and nasal polyposis. It is known that asthmatics as compared with controls, have significantly more serum ECP, and that serum ECP increases with an increase in severity of asthma [Badar *et al.*, 2004]. The eosinophilic infiltration and subsequent release of many proinflammatory cytokines and mediators such as the cytotoxic major basic protein could be a cause of the extensive epithelial damage in atopic asthma. In a previous light microscopical study on the same patients [Amin *et al.*, 2000] a statistically significant correlation between the number of eosinophils in a biopsy and the epithelial damage was observed. Also other groups have shown a relationship between eosinophils and epithelial damage [Brown *et al.*, 1998; Jatakanon *et al.*, 2000].

Another aspect worth noting was that eosinophils were often found in between the cells of the epithelium, both in the bronchial biopsies of allergic asthma patients and in nasal polyps. Rarely, eosinophils undergoing apoptosis were seen. This agrees with a recent study [Uller *et al.*, 2004] where it was proposed that tissue eosinophils were cleared by transepithelial transport to the lumen rather than by apoptosis.

**Neutrophils**

Neutrophils were more common in bronchial biopsies from non-atopic asthma patients compared to atopic asthma patients, and most of the
polyp biopsies showed a moderate infiltration of neutrophils. As neutrophils respond poorly to corticosteroids, it has been suggested that they could be in part responsible for the epithelial damage, the extensive mucus plugging, and the abnormalities of epithelial and endothelial permeability which are associated with severe acute asthma [Tillie-Leblond et al., 2005].

**Mast cells**

Degranulating mast cells were found in bronchial biopsies of both types of asthma. Cross-linking of surface IgE with antigen releasing preformed mediators, and synthesis of new mediators, are known to play an important role in the allergic reaction and this is the most extensively studied mechanism for mast cell degranulation. Furthermore, there is an IgE-independent mechanism in which eosinophils stimulate mast cells to degranulate by major basic protein (MBP) or its analogues [Furuta et al., 1998].

**Intercellular space**

Asthma patients and all groups of polyp patients showed widening of the intercellular spaces between the basal cells (paper IV, V). In nasal polyps the intercellular space was significantly larger in non-asthmatics than in asthmatics. In the nasal polyp study, all patients also had dilated capillaries in the connective tissue. This finding may be related to the results of a study by Cauna et al. [Cauna et al., 1972] who observed that the sensory nerves and the autonomic vasomotor and secretory nerves invariably found in normal and abnormal nasal mucosa could not be identified within the stroma of polyps. This led to the assumption that denervation of nasal polyps causes a decrease in secretory activity of the glands and induces an abnormal vascular permeability, leading to an irreversible tissue oedema. Nasal polyps develop in areas where the lining of the nasal cavity joins that of the sinuses, and these marginal zones contain thin nerve fascicles [Cauna et al., 1972] which may be more sensitive to damage from, for example, eosinophil derived proteins [Mygind, 1990; Mygind et al., 2000]. Diffusion of this oedema fluid to the epithelial layer could be an explanation of the increased intercellular space in asthmatics and nasal polyps. Disturbance of normal fluid characteristics of the airway epithelium is also likely to occur both as a result of the gross epithelial damage that is observed and as a result of damage to the tight
junctions. It has previously been shown that breaking the tight junctions causes changes in transepithelial fluid transport in the airway wall [Högman et al., 2002; Relova & Roomans, 2001].

**Basal lamina**

Thickening of the basal lamina was found in both groups of asthmatics, but more prominently in atopic asthma. There was a moderate increase in the thickness of the basal lamina in all types of polyps in comparison to the control bronchial biopsies, but there was no significant difference between any of the groups of nasal polyps. Also other studies have noticed thickening of the basement membrane in mild to moderate asthmatics [Cokugras et al., 2001; Laitinen et al., 1997]. This thickening may be caused by an increase in type III collagen synthesis resulting from fibroblast activation [Cokugras et al., 2001; Roche et al., 1989]. It is also thought that some mast cell mediators may topically affect fibroblasts [Vignola et al., 1998] and that this process is responsible for the thickening of the basement membrane. It is also thought that eosinophils co-operate with mast cells in this process, as eosinophils through the production of cytokines such as transforming growth factor β (TGF-β) have been shown to interfere with fibroblast growth and the production of matrix metalloproteins [Minshall et al., 1997].

**Experimental studies (Paper I, II)**

**Effects of poly-L-arginine on airway epithelial cells**

In previous experimental studies, it was found that one of the toxic eosinophilic proteins, or its analogue poly-L-arginine could damage airway epithelial cells in a dose- and time-dependent manner[Gleich et al., 1988; Robinson et al., 1992]. It was suggested that cationic proteins could be responsible for the hyperresponsiveness found in asthma patients [Coyle et al., 1994]. In in vitro studies from our laboratory, it had been shown that several factors produced by different types of leukocytes can result in the reduction of the number of desmosomes between the epithelial cells [Kampf et al., 1999]. Therefore, we studied the effect of poly-L-arginine on cell contacts in airway epithelial cells in vitro. We found indeed that treatment with poly-L-arginine gave rise to a reduced number of desmosomes. This suggests that eosinophils could be responsible for the reduction in desmosomal contacts, and possibly the exfoliation of epithelial cells in
the airway of asthmatic patients where MBP levels are increased [Gleich, 1990; Wardlaw et al., 1988].

In addition, poly-L-arginine disrupted tight junctions, which are important for the maintenance of the integrity of the epithelial barrier. Even if eosinophils did not cause total loss of columnar cells from the airway epithelium, opening of the tight junctions as such could already cause hyperreactiveness by allowing entrance of irritant substances. It should be noted that there are no tight junctions between basal cells in situ, so that the integrity of the epithelial barrier is compromised even if a layer of basal cells is left. Very recently, a study by Ohtake et al. showed that poly-L-arginine appears to increase predominantly the paracellular transport of hydrophilic macromolecules by disorganisation of tight junction and adherence junction proteins of the nasal epithelium [Ohtake et al., 2003a]. These authors believe that the regulatory mechanism of poly-L-arginine depends on energy-requiring cellular processes. The same group also showed that the PLA-enhanced paracellular permeability of macromolecules (FD-4) is associated with both phosphorylation and dephosphorylation of tight junction proteins [Ohtake et al., 2003b].

Furthermore, poly-L-arginine induces non-specific cell damage in several ways. One of the interesting findings is that of small pores in the membrane observed by high-resolution SEM. It has been claimed that MBP interacts with the negatively charged plasma membrane lipids due to hydrostatic interactions [Abu-Ghazaleh et al., 1992]. Eosinophilic cationic protein (ECP) was found to cause pores in the cell membrane [Young et al., 1986]. The membrane damage in its turn could be an explanation for the leakage of ions that was demonstrated by X-ray microanalysis. The X-ray microanalysis data show a loss of K, but at the same time an increase in Ca which is the hallmark of damaged cells [Trump et al., 1980]. Loss of K ions from the cell can induce cell death [Hughes et al., 1997]. The ultrastructural changes in the mitochondria are likely to be an unspecific effect of poly-L-arginine treatment. It has been found that increased cellular Ca concentrations result in damage to mitochondria [Duchen, 2000a, 2000b].

**Effects of different cytokines on bronchial epithelial cells**

As discussed above, infiltration of the epithelium by eosinophils is found both in the lower airways of allergic asthma patients and in nasal polyps. This may be a way for the tissue to remove the eosinophils, but at the same time, the invasion of the epithelium by
eosinophils can contribute to the destruction of the epithelial cell layer and sustain or aggravate inflammation. Trafficking and activation of inflammatory cells may be dependent on adhesion molecule expression on epithelial cells. This adhesion molecule expression is probably mediated by the effect of various cytokines produced by lymphocytes or bronchial epithelial cells themselves. Also, in order to pass into the airway lumen, the leukocytes need to pass through the tight junctions. Therefore, the effects of different cytokines (IL-8, IL-4, IL-13, and IFN-\(\gamma\)) on cell viability and ICAM-1 and ZO-1 expression on human airway epithelial cells were investigated. Our results imply that IFN-\(\gamma\) may contribute to leukocyte recruitment through increased ICAM-1 expression in epithelial cells. This confirms the role of the airway epithelium in sequestering leukocytes to the airways in patients suffering from airway inflammation. Although we found that the cytokines tested individually did not affect ZO-1 expression appreciably, the combination of IL-4 and IFN-\(\gamma\) appeared to cause a separation of the cells. It has previously been observed that cytokines can affect tight junctions in a large variety of cells. Tumour necrosis factor (TNF)-\(\alpha\) disrupts tight junctions in MDCK cells, without decreasing the amount of ZO-1 [Poritz et al., 2004], similar to the effect of IFN-\(\gamma\) and IL-4 observed in the present paper. IL-8 affects tight junctions between endothelial cells [Talavera et al., 2004]. IL-4, IL-13 and IFN-\(\gamma\) decrease the expression of ZO-1 and occludin in Calu-3 cells [Ahdieh et al., 2001], and IFN-\(\gamma\) downregulates claudin-1 in primary cultured thyrocytes [Tedelind et al., 2003]. A decrease of transepithelial resistance after treatment with IL-4 and IFN-\(\gamma\) was also found in podocytes [Coers et al., 1995]. IFN-\(\gamma\) but not IL-4 decreased ZO-1 expression in cultured mouse cholangiocytes [Hanada et al., 2003]. Since IFN-\(\gamma\) and IL-4 are increased in the airways of patients with asthma [Prescott, 2003], this could contribute to a decrease in barrier function of the epithelium. This could be responsible for increased penetration of irritant or noxious substances across the epithelium, and, as discussed above, for a disturbed fluid transport.

In summary, the experimental studies show that factors produced by inflammatory cells may (1) cause epithelial damage, and in particular affect desmosomal contacts and open tight junctions, and (2) increase the potential of epithelial cells to recruit inflammatory cells to the epithelium, which may both assist in the removal of the epithelial cells, but also cause increased damage, thus setting in motion the
vicious circle of epithelial damage and inflammation that is a hallmark of the airway epithelium in asthma and nasal polyposis. The experimental studies thus point to possible mechanisms underlying the observations in the in situ studies, with as main points: decrease of cell contacts resulting in epithelial shedding, and increased recruitment of inflammatory cells, in particular eosinophils, to the airway epithelium.
Conclusions and Future Perspectives

The results of this study may provide a direction for focusing future research which may result in an improved treatment strategy for a complex disease such as asthma.

- Eosinophil derived toxic proteins reduce the number of desmosomes and cause other generalized damage (such as membrane damage, leakage of ions, and damage to the mitochondria).
- Inflammatory mediators from leukocytes (cationic proteins, and also IFN-γ and IL-4 in combination) open up the tight junctions between airway epithelial cells and also play a crucial role in sequestering leukocytes to the epithelium.
- No significant difference in epithelial damage between atopic asthma and non-atopic asthma could be shown, but there is more eosinophil infiltration in atopic asthma.
- The columnar epithelium in asthmatics has less attachment to the basal lamina compared to that in healthy persons, which may contribute to epithelial shedding.
- In the bronchial biopsies from asthmatics and in nasal polyps from allergic or asthmatic patients the following common features were found: reduced desmosomal contacts between airway epithelial cells, epithelial damage, eosinophilic infiltration into the epithelium and piece-meal degranulation of eosinophils.

An important question to answer is whether the defect in the asthmatic epithelium is an intrinsic, inherited or congenital defect, or whether it is secondary to inflammatory mediators. Further studies are needed to provide more details of desmosomal structure and function in healthy and asthmatic airway epithelium.

To regulate eosinophil arrest and extravasation, blocking of cellular adhesion molecules may be a useful mechanism. The transport of eosinophils through the epithelium is another aspect that should be further investigated.

Another strategy may be to redirect the maladaptive, antigen-induced polarization of CD4+ T cells from a type-2 phenotype toward a less
injurious, type-1 phenotype. This might be accomplished by gene transfer to the bronchial epithelium [Factor, 2003].

The reasons for the failure of the anti-IL-5 therapy need to be analyzed. It has been suggested that, in addition to IL-5, activation of the CCR3 receptor on eosinophils by eotaxins, RANTES or MCP3 may be required to draw eosinophils into the airways and that antagonism of both IL-5 and CCR3 receptors is necessary to totally deplete the airways of eosinophils. CCR3 antagonists are in clinical development as are blocking antibodies against eotaxin [Holgate, 2004].

Asthma is a complex heterogeneous disease and therefore it cannot be expected to be associated with or depend on a single cell type such as the eosinophil, although eosinophils are certainly an important factor in epithelial damage and airway hyperreactivity. As complex diseases require complex therapeutic approaches, combination therapies may dominate future disease management in asthma and include anti-eosinophilic strategies. There are different subtypes of asthmatic patients with apparently different degrees of eosinophil activity. Therefore, a strategy that targets a functional pathway common to all cellular infiltrates involved in asthma may finally produce the best drug to reduce the burden of asthma in the future. Further studies are required to understand the common functions of these cellular infiltrates.
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