BRONCHIAL MUCOSAL INFLAMMATION IN ASTHMA

Modulation by glucocorticoids

ONTSTEKING IN DE BRONCHIALE MUCOSA BIJ ASTMA

Beïnvloeding door glucocorticoïden

Cover: Electronmicroscopic photograph of an immunogold staining with anti-human IL-4 mAb of a secondary granule of an eosinophil from a patient with allergic asthma. Gold particles are seen in the crystalloid core of the secondary granule, indicating the presence of IL-4. Magnification x 30,000.

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"The patient is the centre of the medical universe around which all our work revolves and towards which all our efforts tend"
J.B. Murphy (1857-1916)

Aan mijn ouders
Aan Paul

BRONCHIAL MUCOSAL INFLAMMATION IN ASTHMA

Modulation by glucocorticoids

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 Asthma

In this thesis studies are presented concerning airway inflammation in the bronchial mucosa of allergic asthmatics and its modulation by glucocorticoids. Asthma is one of the most common chronic disorders in the Western world and affects about 10% of the population. Its prevalence, morbidity and mortality appear to be rising (1,2). Initially, the Greek term "αστμα" recognized by Hippocrates (460-357 B.C.) was the name given to the disorder occurring in people with "difficult breathing". At present, "asthma" is still difficult to define. The current working definition of asthma recommended by the National Asthma Education Program Expert Panel Report (3) is as follows:

'asthma is a lung disease with the following characteristics: (1) airway obstruction that is reversible (but not completely so in some patients) either spontaneously or with treatment; (2) airway inflammation; and (3) increased airway responsiveness to a variety of stimuli'.

Asthma is defined as a syndrome characterized by "variable airway obstruction". Pathophysiological changes that contribute to the airway narrowing are increased bronchial smooth muscle contraction, enhanced mucus production, and enhanced vascular permeability with mucosal edema. Histologic evaluation of the airways of asthmatics shows a chronic inflammation in the airway wall (4). The airflow obstruction in asthmatics is traditionally considered to be reversible if the patient's forced expiratory volume in one second (FEV_1) increases by at least 15% after inhalation of $ß_2$-agonists (5). Asthma is characterized by increased airway hyperresponsiveness, defined as a decreased threshold of airway narrowing in response to a variety of non-specific stimuli (6). These non-specific stimuli include tobacco smoke (7), fog, nitrogen dioxide (8), ozone (9), viral infections (10), and inhaled pharmacologic agents (histamine (11), methacholine (12)), as well as physical stimuli (exposure to cold air (13), exercise (14)). Exposure to specific stimuli such as allergens (house dust mite, pollen, animal dander) can lead to an increase in airway hyperresponsiveness (15), which often precedes asthma (16). Atopy, the genetic predisposition for directing an IgE response to common environmental allergens such as house dust mite and pollen, is another risk factor for the development of asthma (17) and may affect 40% of the population of western European countries (18). A recent study demonstrates that a trait for elevated level of serum total immunoglobulin E (IgE) is coinherited with a trait for bronchial hyperresponsiveness and that a gene governing bronchial hyperresponsiveness is located near a major locus on chromosome 5q that regulates IgE levels (19). IgE can bind with high

affinity to specific receptors (FcεRI) expressed on the surface of mast cells, basophils, monocytes and eosinophils, and with somewhat lower affinity to macrophages and eosinophils (FcεRII, CD23). Cross-linking of receptor-bound IgE with specific allergen results in the release of inflammatory mediators, including histamine, prostaglandin D_2 (PGD_2) and leukotriene C_4 (LTC_4) which, through their direct effects on airway smooth muscle and microvasculature, are responsible for the allergen-induced bronchial narrowing and wheezing (20).

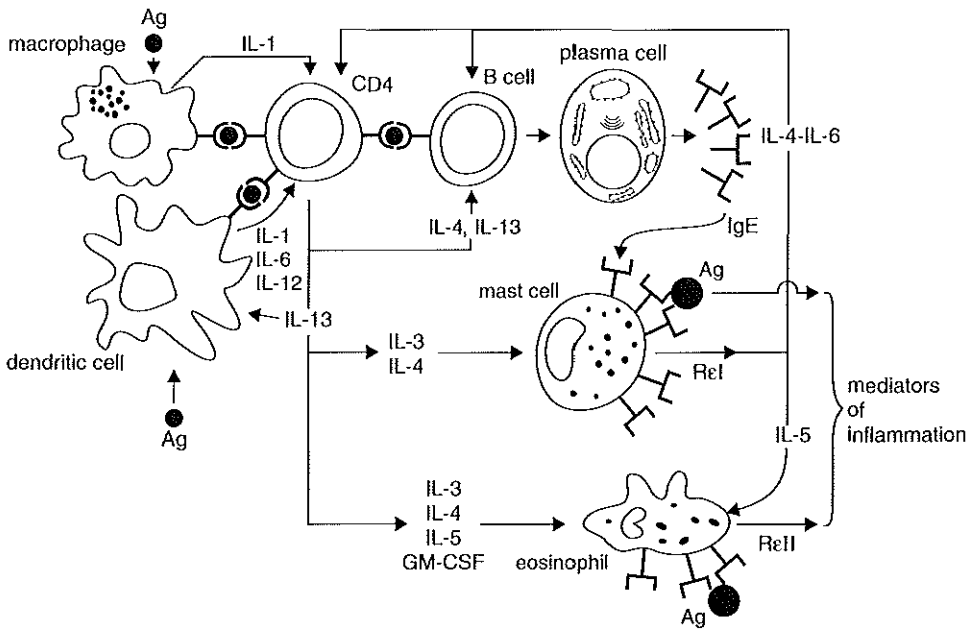

Figure 1. Schematic presentation of the allergic immune response.

1.2 Immunopathogenesis of allergic asthma

The initial event in the allergic immune reponse is thought to be the presentation of antigen to T lymphocytes by antigen-presenting cells (APC). APC comprise monocytes, macrophages, B lymphocytes and in particular dendritic cells (DC). Recognition of the presented antigen by the T lymphocyte and the generation of costimulatory signals by the APC result in proliferation of T lymphocytes and production of inflammatory mediators by both APC and T lymphocytes (Figure 1). T lymphocytes will develop into $CD4^+$ helper T lymphocytes, termed Th2 lymphocytes, which produce predominantly IL-4, IL-5, IL-6, IL-10 and IL-13. The development of Th2

lymphocytes is influenced by a balance between different cytokines. IL-4, IL-10 and prostaglandin E_2 (PGE_2), produced by APC promote the generation of Th2 cells, while IFN-γ and IL-12 strongly promote Th1 lymphocyte development (21,22). IL-4 stimulates the development and maturation of naive T lymphocytes towards the Th2 lymphocyte phenotype (23), and supports mast cell growth. In addition, IL-4 and IL-13 are obligatory cytokines required for isotype switching of B lymphocytes to IgE production (24,25). Although T lymphocytes have been proposed as the main origin of Th2-type cytokines, recent studies also identified the mast cell as an important source of IL-4 (26). In addition, IL-5 together with IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF) are involved in growth, differentiation, recruitment and increasing the effector capacity of eosinophils (27,28). Once recruited into the airway, activated eosinophils secrete a variety of lipid mediators such as platelet activating factor (PAF), LTC_4, and four cationic proteins with cytotoxic properties. These include major basic protein (MBP) localized to the crystalloid core of the specific or secondary granule, eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase (EPO) (29,30). These products are highly toxic to the respiratory epithelium and cause epithelial damage (shedding) (31). This loss of epithelial cells may further contribute to bronchial hyperresponsiveness, possibly by a reduced production of epithelium-derived relaxing factors (32,33). Respiratory viral infections are also suspected to be important in the pathogenesis of asthma (34). Many respiratory viruses, particularly respiratory syncytial virus (RSV) and parainfluenza virus, directly damage airway epithelium (35) and increase airway hyperresponsiveness (36).

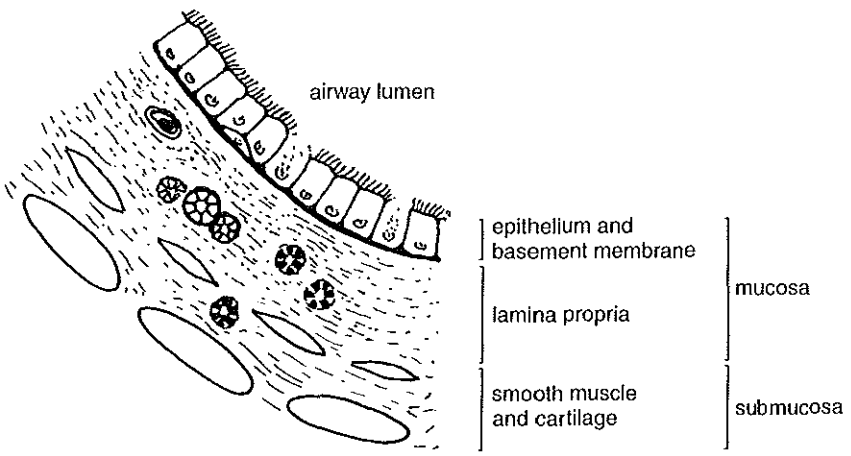

Figure 2. Schematic diagram of a bronchus.

1.3 Pathophysiology of the bronchial mucosa in asthma

The bronchial mucosa consists of epithelium, basement membrane and lamina propria. The submucosa contains smooth muscle, glands and cartilage (Figure 2) (37). More specific knowledge has become available since the introduction of the fibreoptic bronchoscope into asthma research, providing an opportunity to take bronchial biopsies (Figures 3 and 4) and to obtain cells from the epithelial lining fluid by bronchoalveolar lavage (BAL) (5). From several biopsy studies, it became apparent that inflammatory changes are already present in mild or newly diagnosed asthmatics (38,39).

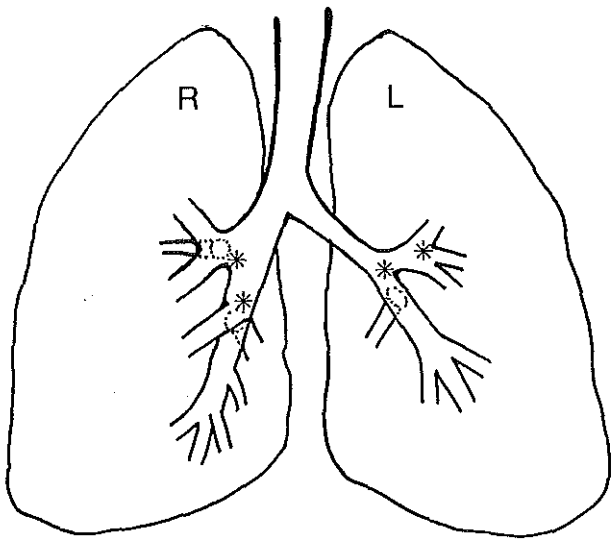

Figure 3. Schematic overview of the human lungs, indicating that biopsies were taken from the carinae of the lingula or the right upper, middle or lower lobes (*).

Bronchial epithelium

The mucosal lining of the airways consist of a pseudo-stratified columnar ciliated epithelium attached to the basement membrane (BM). Histologically, extensive damage to the epithelium and shedding are prominent in asthma, both in fatal asthma and in biopsy specimens from even mild asthmatics (32,39). The greater the epithelial damage in biopsy specimens, the greater appears to be the degree of airway hyperresponsiveness (40). Several hypotheses have been proposed to explain how epithelial damage may result in airway hyperresponsiveness.

These include increased permeability of the airway epithelium (41), loss of inhibitory mediators generated by airway epithelium such as epithelium derived relaxing factor (EpDRF) (42), exposure of sensory nerve endings to inflammatory stimuli, thereby facilitating axon reflexes with local release of tachykinins (43), and loss of neutral endopeptidase (NEP) (44).

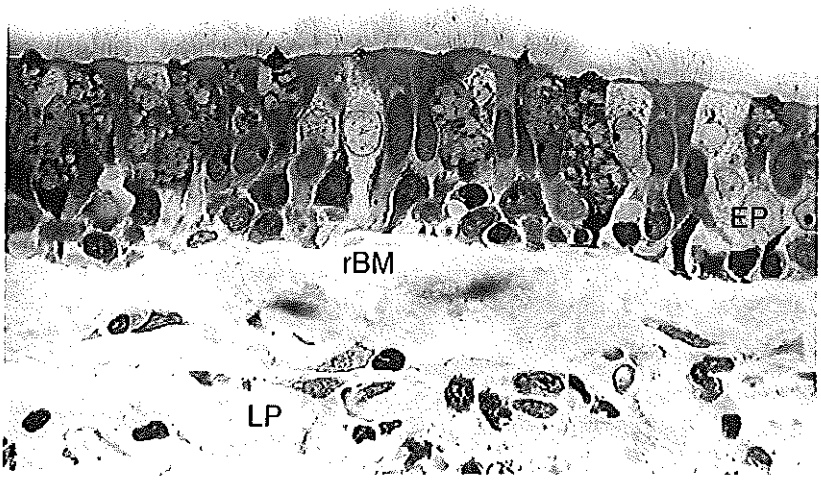

Figure 4. Bronchial biopsy specimen from an asthmatic patient of our study. Magnification x 63. EP = epithelium, rBM = reticular basement membrane, LP = lamina propria.

The bronchial epithelium is also an important source of inflammatory mediators including arachidonic acid metabolites, fibronectin, endothelin, nitric oxide (NO), substance P, and various cytokines including IL-1, IL-6, IL-8, GM-CSF, monocyte chemoattractant protein 1 (MCP-1), and RANTES (regulated upon activation, normal T expressed, and presumably secreted) (45-48). These chemotaxins can recruit neutrophils, eosinophils, monocytes and probably also DC to the lung (48,49). Furthermore, epithelial cells are capable of expressing receptors, which may play a role in the trafficking of inflammatory cells to the epithelium. In this regard, intercellular adhesion molecule 1 (ICAM-1), which is expressed on bronchial epithelium cells from asthmatics, has been of particular interest in the recruitment and migration of neutrophils and eosinophils (50,51). Immunohistochemical studies have shown that bronchial epithelial cells constitutively express MHC class II (52). Furthermore, it has been demonstrated that airway epithelial cells are capable of inducing T cell proliferation (53).

Reticular basement membrane

The "true" BM consists of a lamina lucida and a lamina densa, which is not different from that of normal subjects (54) (Figure 5). The main components are type IV collagen, proteoglycans, laminin, and fibronectin. There is a characteristic reticular component beneath the lamina densa, the so-called lamina reticularis, which becomes thickened in asthma and has been previously considered as "BM thickening" (55). In asthmatics this subepithelial collagen deposition consists of dense fibrillar collagen types III and V (Figure 6), indicating their fibroblast origin rather than epithelial origin (56). The number of myofibroblasts beneath the epithelium correlates well with the thickness of the subepithelial collagen deposition (57). Peptide growth modulating factors that may be important in initiating this response include transforming growth factor-ß (TGF-ß) released by eosinophils, platelets and fibroblasts, platelet-derived growth factor (PDGF) localized to the bronchial epithelium, and endothelin, a peptide found in endothelial and epithelial cells, the expression of which is increased in the epithelium of asthmatics (58).

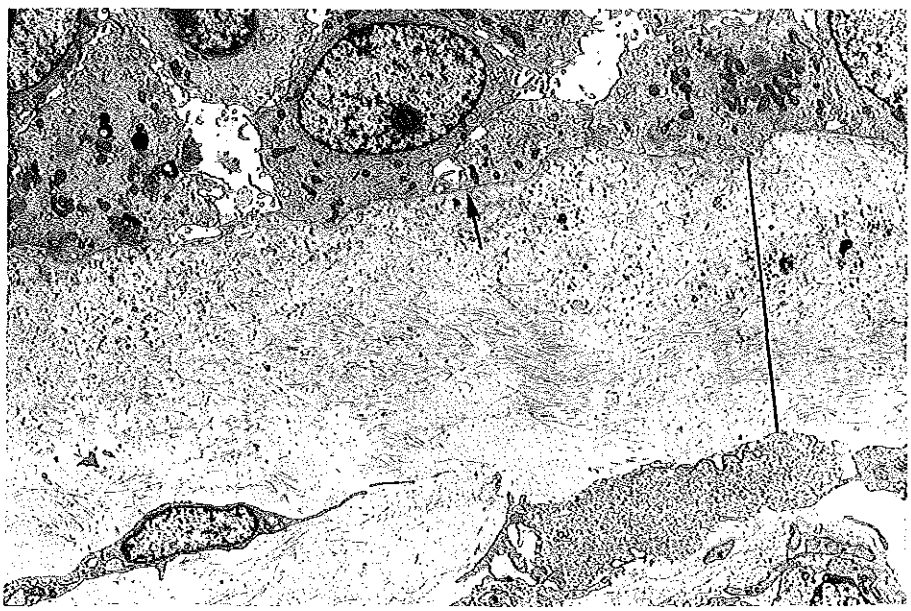

Figure 5. Electronmicroscopic photograph of a bronchial biopsy from an asthmatic patient of our study showing the 'true' basement membrane (*arrow*) and the thickened lamina reticularis (*bar*). Magnification x 1,100.

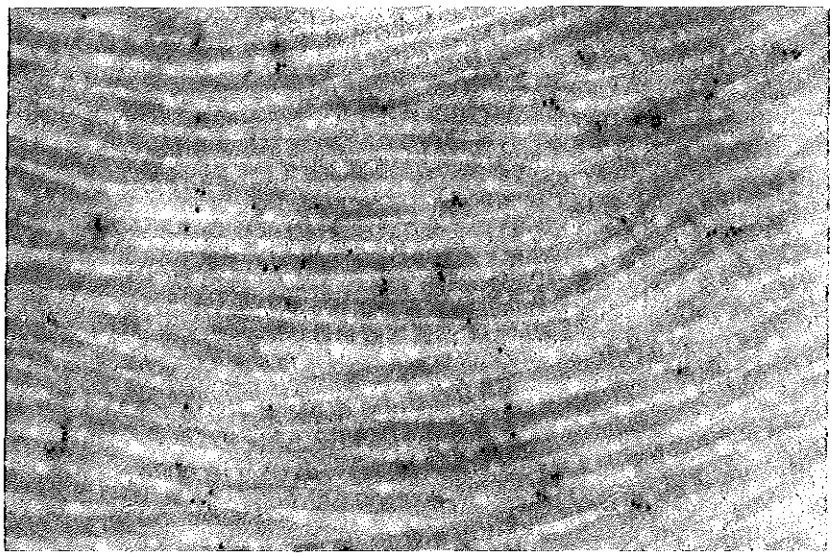

Figure 6. Immunoelectronmicroscopic photograph of dense fibrilar collagen type V in the lamina reticularis. Magnification x 20,000.

Dendritic cells

Pulmonary DC may be critical in the regulation of the allergic immune response in asthma. They are the most potent APC and serve a unique role in their capacity to stimulate naive T cells (59,60).

In the lung, DC are predominantly observed in epithelial and subepithelial tissue of the bronch(iol)us and in the bronchus-associated lymphoid tissue (61). Holt *et al.* described the intra-epithelial DC in the human bronchus as a tightly meshed network (62). Typical immunohistological features of DC are their long cytoplasmic extensions and a strong expression of MHC class II (Human leucocyte antigen (HLA-DR)), and expression of accessory molecules (ICAM-1 (CD54), B7-1 (CD80), B7-2 (CD86)) (63-65). Ultrastructural features of DC are the presence of intracytoplasmic structures termed Birbeck granules (Bg). A combination of dendritic morphology and the expression of CD1a and L25 are characteristic for DC (66,67).

Concerning the role of DC in asthma, only a few studies have been performed. One study has shown increased numbers of $RFD1^+$ cells (a marker for DC) in the bronchial mucosa of asthmatics (68). Another study reported increased numbers of intra-epithelial and mucosal $CD1a^+$ DC in atopic asthmatics as compared to nonasthmatic atopic controls sensitized to

the same allergen (69). However, the pathogenetic role of DC in allergic airway inflammation in asthma has not been completely established.

Macrophages and monocytes

Alveolar macrophages (AM) represent the largest cell population, accounting for 80% to 90% of the airway cells in BAL fluid in asthmatics and normal subjects (70). Increased numbers of macrophages were found in biopsies of atopic asthmatics (71). AM and monocytes express FcεRII (CD23) (72). Via these receptors, AM can be activated by antigen and release a variety of mediators, including LTB_4, LTC_4, PAF and PGD_2 (73,74). Moreover, macrophages and monocytes are also activated by antigen in an IgE-dependent manner to release IL-1ß, tumor necrosis factor-α (TNF-α), GM-CSF, and superoxide anion (O_2^-) (75-77).

In bronchial biopsies of asthmatics a reduced proportion of immunosuppressive macrophages was found, compared with normal lung tissue (78), supporting the hypothesis that T cell-mediated inflammation in asthma may partly result from a reduced capacity of a macrophage subpopulation to suppress T cell activity. Changes in subpopulations of AM, which favor proinflammatory functions, or lack downregulating capabilities, could be a mechanism by which airway inflammation in asthma is controlled (73). The contribution of AM to the pathogenesis of asthma has not yet been fully elucidated and requires additional study.

Lymphocytes

APC present antigenic peptides to T lymphocytes, and recognition of the peptide-MHC molecule complex by the T cell receptor is a crucial step in T cell stimulation, resulting in proliferation and cytokine production. Three distinct subsets, Th0, Th1 and Th2, can be distinguished, based on their different cytokine profiles (79). Th1 cells predominantly secrete IL-2 and IFN-γ, whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13, and Th0 cells produce all of these cytokines. Asthma is generally considered to be a Th2-mediated disorder. Recent studies have shown elevated percentages of cells that express mRNA encoding IL-3, IL-4, IL-5, and GM-CSF in BAL fluid from mild atopic asthmatics, consistent with a Th2-type pattern of cytokine synthesis (80,81).

IL-4 stimulates the development and maturation of naive T cells to the Th2 lymphocyte phenotype (82), and supports mast cell growth. IL-4 and IL-13 play a critical role in isotype switching of B lymphocytes to IgE production (25,83). For the induction of IgE synthesis by IL-4, a physical T-B cell interaction is required (84). After recognition of the peptide-MHC class II complex on B cells by the T cell receptor, T cells become activated, express

the CD40 ligand (CD40L, gp39), and stimulate B cell proliferation and IgE secretion (85). A second required costimulatory signal is mediated by the T cell antigen CD28. The antigen B7 expressed on DC, activated B cells and monocytes, has been shown to be the ligand for CD28 (86). CTLA-4 can also act as a ligand for B7 and is expressed on activated T lymphocytes (87). Furthermore, IL-4 increases expression of vascular cellular adhesion molecule (VCAM-1) on endothelial cells, which may be involved in eosinophil adhesion in the pulmonary circulation (88). IL-5 promotes the differentiation (27), recruitment (89), activation and survival of eosinophils (28). In several studies of bronchial biopsies taken from asthmatics, the expression of cell surface activation molecules, such as the IL-2 receptor (CD25), MHC class II, and very late activation antigen 1 (VLA-1), is increased on T-lymphocytes (90,91). Interestingly, the total numbers of both helper (CD4) and suppressor/cytotoxic (CD8) T lymphocytes in bronchial mucosa of asthmatics are comparable to those in controls (92); CD4 cells predominate over CD8 cells. The $CD4^+$ subset can be further subdivided by the expression of the leucocyte common antigen (CD45) isoforms by stage of maturation; CD45RA is expressed on naive cells, while CD45RO is expressed on "memory" cells, which have been previously activated by exposure to specific antigen (93). Immunohistochemical studies have shown that the numbers of activated T lymphocytes can be correlated with the degree of asthma severity and with the numbers of total and activated eosinophils (90,93).

There are virtually no B lymphocytes within histologic sections of the normal airway mucosa. However, distinct B cell areas were detected in the bronchus-associated lymphoid tissue (BALT) (64). In the bronchi of asthmatics a very few B lymphocytes are present (71).

Eosinophils

Eosinophils (Figure 7) in the bronchial mucosa are a characteristic feature of the inflammatory process in allergic and non-allergic asthma (94,95) (Figure 7). Increased numbers of eosinophils are also found in BAL fluid of asthmatics (94,96). In asthmatics, eosinophils are often degranulated and hypodense, suggesting that they are in an activated state (97). In association with the late-phase response to allergen, increased numbers of eosinophils are observed in biopsies and BAL fluid 3-24 hours after allergen provocation (98,99).

Eosinophils possess a wide array of biological properties, e.g. the release of toxic granule cationic proteins (described earlier in this chapter), oxygen radicals, lipid mediators such as PAF, LTC_4, and prostaglandins E, F and D_2, neuropeptides (substance P, vasoactive intestinal peptide (VIP) or somatostatin), and cytokines (100).

Eosinophils are a newly recognised source of several cytokines including IL-1α (101), IL-3 (102), IL-4 (103), IL-5 (104), IL-6 (105), IL-8 (106), GM-CSF (107), TNF-α (108), macrophage inflammatory protein-1α (MIP-1α) (109), transforming growth factor-α (TGF-α) (110) and TGF-ß (111). Important for eosinophil survival, activation and recruitment are IL-3, IL-5, GM-CSF, TNF-α, and MIP-1α, indicating an autocrine regulation which may prolong the eosinophilic inflammation in the airways.

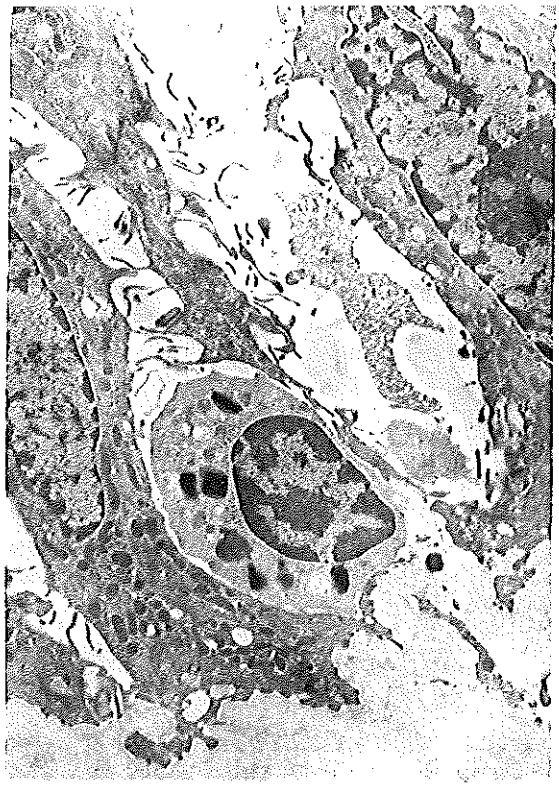

Figure 7. Electronmicroscopic photograph of an eosinophil in the bronchial epithelium of an asthmatic patient of our study. Magnification x 3,000.

Mast cells

There are two types of mast cells (Figure 8) that can be distinguished by their granule neutral protease content: the tryptase-containing (MC_T), and tryptase- and chymase-containing mast cells (MC_{TC}). Most of the mast cells in human airways are of the MC_T subtype (112). The epithelium of asthmatics contains more mast cells than the epithelium of controls.

Numbers of mast cells in the lamina propria are not increased (113), but the elevated concentrations of tryptase in BAL fluid suggest that these mast cells are in a more activated state.

Mast cells probably play a key role in maintaining the chronicity of the inflammatory response. Human mast cells are an important source of IL-4, IL-5, IL-6, IL-8 and TNF-α (114). By immunohistochemistry and immunoelectronmicroscopy, it was shown that human mast cells contain granule-associated IL-4, IL-5, IL-6 and TNF-α (115). These cytokines are differentially expressed between the two mast cell subtypes with a predominance of IL-4 immunoreactive mast cells of the MC_{TC} phenotype in the bronchial mucosa of asthmatics (115). IL-4 is involved in IgE switching by B cells, and activated mast cells express the ligand for CD40 (CD40L) thereby providing the cell contact signal required for IgE synthesis by human B cells. Since IL-4 stimulates the commitment of T cells to the Th2 phenotype, it has been suggested that mast cells may be important in the initial phase of the allergic immune response (116,117).

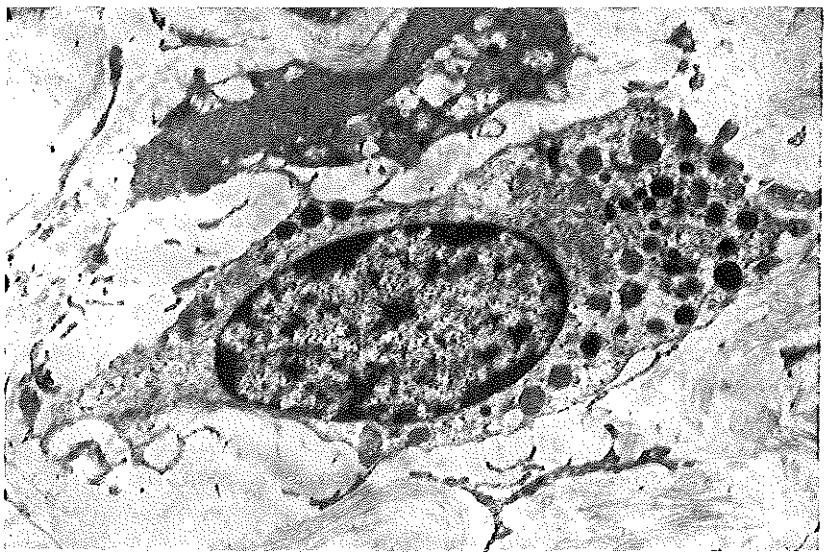

Figure 8. Electronmicroscopic photograph of a mast cell in the lamina propria of an asthmatic patient of our study. Magnification x 3,000.

Basophils

Basophils are bilobed metachromatic cells, expressing FcεRI. The blood basophil count increases during the pollen season (118) and may be influenced by allergens. A role for basophils has been suggested by the

finding that during the late-phase response to allergen, the numbers of basophils are increased in the BAL fluid (119). Clearly, the role of basophils in asthma requires further study.

Neutrophils

The role of neutrophils in asthma is not yet clear. The numbers of neutrophils seen in bronchial biopsies of asthmatics are extremely low (120). No differences in the numbers of neutrophils have been found in bronchial biopsies and lavages between asthmatics and healthy controls (5,71,98), suggesting that these cells do not play an important role in the mechanisms of the inflammatory process in asthma.

Adhesion molecules

Cellular adhesion mechanisms are necessary for the migration of inflammatory cells from the circulation into the surrounding tissue and the airway lumen. Several families of adhesion molecules have been described so far, but in asthma the main attention has been focused on ICAM-1 (CD54), a member of the immunoglobulin superfamily, ligand for the lymphocyte function-associated antigen (LFA-1 (CD11a/CD18) integrin on human T cells) (121) and the major receptor for rhinovirus (122). Another important adhesion molecule is endothelial leucocyte adhesion molecule (ELAM-1), which belongs to the selectin family and whose complementary ligand is Sialyl-Le^{x} (123). Vascular cellular adhesion molecule-1 (VCAM-1), a ligand for very late activation antigen (VLA-4), is expressed on eosinophils and is an important molecule in selective eosinophil recruitment (88). Expression of VCAM-1 appears to be upregulated by IL-4 on vascular endothelium (88). Immunohistochemical studies have demonstrated enhanced expression of ICAM-1, ELAM-1, and VCAM-1 in allergic asthma (124). In a monkey model of experimentally induced allergic asthma, antibodies to ICAM-1 were reported to reduce symptoms of hyperresponsiveness and to prevent eosinophil influx into the airways (51).

1.4 Glucocorticoids

In humans glucocorticoids, such as hydrocortisone (cortisol) and cortisone, are hormones naturally produced by the adrenal cortex. Synthetic glucocorticoids are also available, such as prednisolone and dexamethasone. Inhaled glucocorticoids are by far the most effective anti-inflammatory agents used in asthma. Inhaled glucocorticoids suppress inflammation in the airways of asthmatics, although their precise molecular mechanism of action

is not yet clear (125). There have recently been important advances in understanding the molecular mechanisms of glucocorticoids (126).

Glucocorticoids exert their regulatory effects after passive diffusion through the cell membrane by specific binding to the glucocorticoid receptor (GR) in the cytosol of the target cell. As a result of the binding of glucocorticoid to the GR, two types of heat shock proteins (hsp 90 and hsp 56) are lost and the activated GR-glucocorticoid complex migrates to the nucleus and binds to specific DNA sequences, glucocorticoid responsive elements (GRE), thereby either increasing or decreasing gene transcription. This regulation finally results in stimulation or inhibition of translation of mRNA to protein (Figure 9) (127).

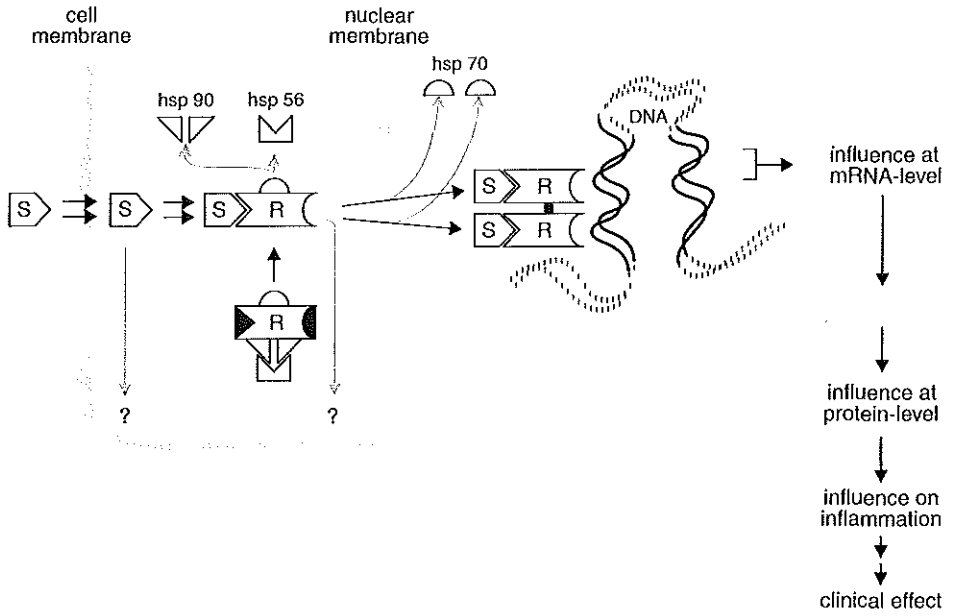

Figure 9. Hypothetical cellular events after glucocorticoid administration. Glucocorticoids (S) pass the cell membrane and bind to their receptors (R). Upon binding of glucocorticoids to their receptors, some heat shock proteins (hsp 56 and hsp 90) dissociate from the receptor in the cytoplasm. Others (hsp 70) probably dissociate from the receptor in the nucleus. Furthermore, in the nucleus liganded glucocorticoid receptors form homodimers and interact with specific DNA sequences. Eventually, this interaction results in modulation of transcription.

Beclomethasone dipropionate (BDP) (Figure 10) has been introduced in 1972 as a topical aerosol glucocorticoid in the treatment of asthma (128). Fluticasone propionate (FP) (Figure 10) is currently the most potent of the new inhaled glucocorticoids and has a high affinity for the GR, approximately 14-18 times greater than dexamethasone (129). FP has almost a 100% first-pass metabolism in the liver (130), resulting in fewer systemic side effects. FP shows almost twice the topical anti-inflammatory potency of BDP, with

less effect on adrenocortical function, and adequate control of severe asthma (131).

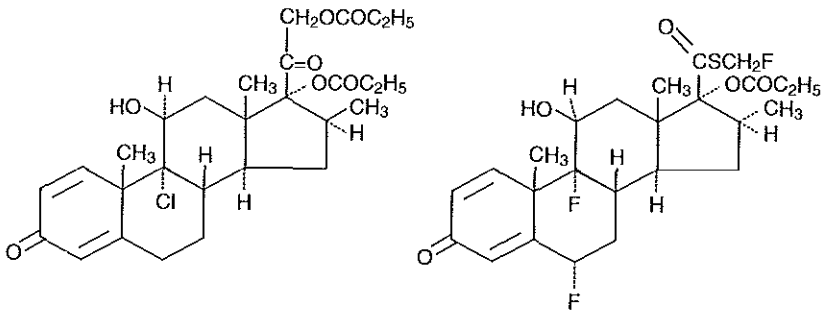

Figure 10. Structural formulae of beclomethasone dipropionate (BDP) and fluticasone propionate (FP).

Cellular effects of glucocorticoids

Glucocorticoids may have direct inhibitory effects on almost all cells involved in airway inflammation in asthma, including lymphocytes, eosinophils, macrophages, monocytes, mast cells and airway epithelial cells (132).

Biopsy studies in patients with asthma have confirmed that inhaled glucocorticoids reduce the number and activation of several inflammatory cell types in the airways (Table 1).

There are not many data on the effects of glucocorticoids on DC. Studies in the rat by Holt *et al.* have demonstrated that exposure to inhaled and systemic glucocorticoids leads to a rapid decrease in the number of airway intraepithelial DC, and in their MHC-class II expression (141).

An important target cell in asthma may be the T lymphocyte, since glucocorticoids are very effective in inhibition of activation of T lymphocytes and in blocking the release of cytokines. Glucocorticoids achieve their effects by inhibition of IL-2 production and inhibition of IL-2 receptor expression on T-lymphocytes (142). Systemic glucocorticoids also suppress antigen-stimulated production of IL-4 by lymphocytes (143).

Table 1. Cellular effects of inhaled glucocorticoids in bronchial biopsy studies

GC	Dose	Duration	Effects	Reference
BUD	1200 μg	3 months	increase in ciliated epithelial cells, reduction in eosinophils	133
BUD	400 μg	4 weeks	reduction mast cells, eosinophils, and macrophages; no reduction in lamina reticularis	134
BUD	400 μg	3.7 years	no reduction in lamina reticularis	134
BUD	800 μg	3 months	reduction in T cells, RFD1$^+$ DC, and expression of HLA-DR	68
BDP	1000 μg	4 months	reduction in eosinophils and mast cells; reduction in lamina reticularis	135
BDP	2000 μg + 1000 μg	2 weeks 4 weeks	reduction in eosinophils, mast cells, and T cells	136
BDP	1000 μg	8 weeks	reduction in GM-CSF staining (in epithelium)	45
BDP	400 μg	10 years	reduction in inflammatory cells; no reduction in lamina reticularis	137
FP	500 μg	6 weeks	reduction in eosinophils and mast cells	138
FP	1000 μg	6 weeks	reduction in IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, TNF-α, GM-CSF	139
FP	1000 μg	6 weeks	reduction in mast cells, LFA-1$^+$ cells, VLA-4$^+$ cells, adhesion molecules P-selectin and E-selectin	140

Glucocorticoids are effective in inhibiting release of certain cytokines from AM, such as IL-1, TNF-α, IFN-γ and GM-CSF (144). Oral prednisone inhibits the increased gene expression of IL-1ß in AM in BAL fluid from asthmatic patients (145). Glucocorticoids also reduce the number of circulating monocytes (146). After inhaled administration of glucocorticoids, it has been demonstrated that numbers of epithelial and mucosal eosinophils are reduced, correlating with improvement of symptoms (134-136).

Glucocorticoids have a direct inhibitory effect on the mediator release from eosinophils, although they are only weakly effective in inhibiting secretion of eosinophil basic proteins (147). Glucocorticoids also inhibit IL-3, IL-5 and GM-CSF-mediated eosinophil survival (148), presumably by activation of an endonuclease that leads to programmed cell death.

Although glucocorticoids do not have a direct inhibitory effect on mediator release from mast cells (149), recent studies in asthmatics have demonstrated significant reduction of mast cells in the epithelium and mucosa after inhaled glucocorticoids (135,137,138). Many of the effects of glucocorticoids on mast cells are likely to be due to their effect on the release of mast cell growth factors (IL-3, IL-4, GM-CSF).

The bronchial epithelium is another important target for inhaled glucocorticoids (150). Glucocorticoids inhibit the increased expression of GM-CSF and RANTES in the epithelium of asthmatic patients (45,47,135).

1.5 Aims of the studies

The aim of the studies presented in this thesis was to investigate the cellular aspects of chronic airway inflammation in the bronchial mucosa of allergic asthmatic patients by using (immuno)electronmicroscopy and immuno-histochemistry. Furthermore, the effects of glucocorticoids on the bronchial mucosal inflammation were studied, with a special emphasis on dendritic cells, eosinophils and cytokines, such as IL-4 and IL-5.

Chapter 2 describes the effects of long-term (2.5 years) and short-term (3 months) treatment with glucocorticoids, *i.e.* BDP and FP, respectively, on pulmonary function data, the number of several inflammatory cell types and the thickness of the lamina reticularis of the BM in the bronchial mucosa in allergic asthma.

In **chapter 3** the distribution of DC in the bronchial mucosa of asthmatics compared to controls is investigated. Furthermore, the influence of inhaled glucocorticoids on the presence of DC in the bronchial mucosa is investigated. The implications of these findings with regard to the role of pulmonary DC in the initiation and propagation of the allergic immune response are discussed.

Chapter 4 describes the localization of IL-4 immunoreactivity in the bronchial mucosa of allergic asthmatics and the ultrastructural immunogold localization of IL-5 in the crystalloid core compartment of the secondary granules of eosinophils.

In **chapter 5** the relationship between eosinophils in the bronchial mucosa and indices from the methacholine log-dose response curves in allergic asthmatics is studied.

In **chapter 6** the presented data are discussed in the context of the literature. Also a summary of the thesis is given.

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CHAPTER 2

EFFECTS OF GLUCOCORTICOID THERAPY ON AIRWAY INFLAMMATION

2.1 Effects of long-term treatment with beclomethasone on airway inflammation in the bronchial mucosa of atopic asthmatics
Submitted

2.2 Influence of fluticasone propionate on airway inflammation in atopic asthmatics
Submitted

CHAPTER 2.1

EFFECTS OF LONG-TERM TREATMENT WITH BECLOMETHASONE ON AIRWAY INFLAMMATION IN THE BRONCHIAL MUCOSA OF ATOPIC ASTHMATICS*

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ABSTRACT

Glucocorticoids are known to be the most effective drugs in improving asthma symptoms and airway hyperresponsiveness, but their precise mechanism of action is not yet clear.
We have investigated the effects of 2.5 years of inhaled beclomethasone dipropionate (BDP) on the interleukin (IL)-4 immunoreactivity, eosinophils, (activated) T lymphocytes, and mast cells and on the thickness of the lamina reticularis in sixteen mild to severe atopic asthmatics. These patients were randomly sampled from a double-blind, multicentre study in chronic non-specific lung disease (CNSLD) (1). Biopsies were obtained by fiberoptic bronchoscopy after 2.5 years of treatment with terbutaline, 2000 μg daily plus either inhaled BDP, 800 μg daily (BA+CS), ipratropium bromide, 160 μg daily (BA+AC) or placebo (BA+PL).
Following treatment, we found a significant increase in FEV_1 ($p=0.01$) and a significant decrease in hyperresponsiveness (PC_{20} histamine) ($p=0.01$) in the glucocorticoid group ($n=5$) compared to the bronchodilator (BA+AC/PL) group ($n=11$). In the glucocorticoid group the numbers of IL-4 expressing cells in the lamina propria were marginally significantly lower ($p=0.05$), as were the numbers of total eosinophils ($p=0.02$) compared to the bronchodilator group. The number of activated eosinophils and mast cells in the glucocorticoid group were generally lower, but this did not reach statistical significance. No difference in the number of T lymphocyte subsets and the thickness of the lamina reticularis were seen between both groups. These data support the hypothesis that the beneficial effects of long-term inhaled BDP treatment in asthma may result from a decrease in IL-4 immunoreactivity and from an inhibition of local bronchial eosinophilic inflammation.

INTRODUCTION

Inhaled glucocorticoids are the most effective drugs for asthma currently available. Numerous studies have documented their long-term clinical efficacy in adults and children (1-5) though the precise mode of action of these anti-inflammatory drugs is yet unknown. Several studies have demonstrated increased numbers of inflammatory cells, increased levels of cytokines in bronchial biopsies, bronchoalveolar lavage (BAL) fluid and peripheral blood (PB) even in mild asthmatics (6-12). Thickening of the lamina reticularis of the basement membrane is often reported in asthmatic subjects and is mainly attributed to increased depositions of collagen III and V and fibronectin but not laminin (13,14).

$CD4^+$ T helper lymphocytes can be classified according to their cytokine production pattern into a T helper 1 (Th1) subset, which produce IL-2 and IFN-γ, a T helper 2 (Th2) subset which produce IL-4, IL-5 and IL-10 and a naive Tho subset, producing all of these cytokines (15). The levels of mRNA for interleukin-4 (IL-4) and interleukin-5 (IL-5) are increased in BAL and PB (9) of asthmatics and have been shown to correlate positively with disease severity (10,12). IL-4 is involved in the isotype switching of B-cells to IgG4 and IgE (16,17), and is a growth factor for B cells (18,19). Though the initial source of IL-4 required for the Th2 commitment is not fully clear, mast cells do secrete IL-4 after crosslinking of the high affinity receptor for IgE (20). IL-5 promotes the differentiation (21), vascular adhesion, recruitment and activation of eosinophils (22,23). In addition to T lymphocytes, IL-4 and IL-5 are also produced by mast cells and eosinophils (24,25). Thus, IL-4 and IL-5 are considered to play a key role in the pathogenesis of asthma (26,27). Glucocorticoids have been shown to suppress cytokine production by inhibiting transcription factors which regulate gene expression (28-31).

We hypothesize that the beneficial anti-inflammatory actions of glucocorticoids in asthma may partly result from a reduction of cytokine production *in vivo*, concomitant with an inhibition of local bronchial eosinophilic inflammation. Therefore we have examined the effects of long-term beclomethasone dipropionate (BDP) on FEV_1, airway hyperresponsiveness (PC_{20} histamine), IL-4 immunoreactivity and the inflammatory cell infiltration in bronchial biopsies from 16 asthmatic patients randomly sampled from a previous double-blind, multicentre study in chronic non-specific lung disease (CNSLD) (1).

MATERIAL AND METHODS

Patients and non-asthmatic controls

Sixteen non-smoking atopic asthmatic patients (seven women, nine men, median age 43 yr, range 24 - 61 yr) were randomly sampled in two participating centres, Groningen and Rotterdam from the Dutch CNSLD study group (1,32).

The diagnosis of asthma was based on a history of attacks of breathlessness and wheezing without chronic cough or sputum production (i.e. for more than 3 months per year), according to the criteria of the American Thoracic Society (33). Atopy was defined as a positive skin prick test to house-dust mite or at least two of twelve common aeroallergens (mean wheal size > 0.7 times the histamine wheal size (32)). All patients showed airway hyperreactivity to histamine with a provocative concentration

of histamine causing a 20% decrease in FEV_1 (PC_{20}) of ≤ 8 mg/ml (32,34). The patients had a baseline reversibility ≥ 9% of predicted. Patients were treated double-blind with an inhaled β_2-agonist (terbutaline, two 250 μg puffs) plus either inhaled corticosteroid (BDP, two 100 μg puffs) (BA+CS) (n=5), an anticholinergic bronchodilator (ipratropium bromide, two 20 μg puffs) (BA+AC) (n=5) or placebo (BA+PL) (n=6). All medication was taken 4 times daily. As no significant differences were found between the BA+AC group and the BA+PL group with regard to FEV_1, PC_{20} and the profiles of inflammatory cells the data were subsequently pooled for analysis in one bronchodilator group (n=11). Fiberoptic bronchoscopy was performed at the end of the 2.5 year study in the same period (between August and December) in both centres, before breaking the code. A control group was composed of eight non-smoking non-asthmatic subjects (three women and five men with a median age of 23 years, range 19 to 52 years). All controls had a PC_{20} histamine of more than 8 mg/ml and a median FEV_1 of 103 (88-110)% of the predicted value. Patient and control characteristics are shown in Table 1. Further details of the study methods have been described previously (32). The study protocol was approved by the Medical Ethics Committee; all patients gave written informed consent.

Bronchoscopy

Fiberoptic bronchoscopy (Olympus model BF IT 10 Tokyo, Japan) was performed with atropine 0.5 mg intramuscularly as premedication. Terbutaline, 2 puffs of 250 μg per Nebuhaler, was given 30 min before the procedure. The nose, throat and vocal cords were anaesthetized with topical lidocaine spray. An Olympus alligator forceps model FB15C and the fenestrated forceps model FB19C were used to take two biopsies from segmental or subsegmental divisions of the main bronchi.

Bronchial biopsies

Each biopsy sample for immunohistochemistry was immediately placed in ice-cooled isotonic saline and snap-frozen within 20 min in Tissue-Tek II OCT embedding medium (Miles, Naperville, Illinois, USA). Samples were stored at -80°C until use. One biopsy sample for electron microscopic analysis was placed immediately in glutaraldehyde for future studies.
Frozen sections (6 μm) were cut on a Reichert-Jung 2800 Frigocut cryostat. From each biopsy two sections were placed on poly-L-lysine-coated (Sigma Diagnostics, St. Louis, MO, USA) microscopic slides. Sections were air dried for 30 min and stored at -80°C until use.

Table 1. Patient and control subject characteristics

Patient no.	Sex	Age (year)	Baseline FEV_1 % predicted	After 2.5 yrs FEV_1 % predicted	Baseline PC_{20} mg/ml	After 2.5 yrs PC_{20} mg/ml
			Glucocorticoid group			
1	F	32	51.8	56.4	0.17	0.10
2	M	61	84.0	98.7	0.94	5.38
3	F	44	75.0	88.5	0.06	0.45
4	F	60	59.7	90.3	0.06	0.96
5	F	45	49.2	69.6	0.05	0.21
Median		45	59.7	88.5	0.06	0.45
			Bronchodilator group			
1	M	32	70.4	51.7	0.79	0.13
2	M	38	48.6	31.2	0.03	0.01
3	F	43	54.3	53.4	0.03	0.04
4	M	44	63.5	66.3	0.02	0.05
5	M	24	61.5	54.7	0.19	0.58
6	M	50	65.1	62.7	0.28	0.18
7	M	57	81.6	42.3	0.14	0.04
8	F	30	100.0	96.4	4.72	0.42
9	M	26	38.3	60.0	0.24	0.40
10	M	42	48.7	56.5	0.24	0.87
11	F	38	57.0	43.6	0.13	0.06
Median		38	61.5	54.7	0.19	0.13
			Control subjects			
1	M	19	102			
2	M	23	109			
3	M	23	109			
4	F	24	103			
5	F	23	96			
6	M	23	88			
7	M	52	88			
8	F	35	110			
Median		23	103			

Immunohistochemistry

The following monoclonal antibodies (MoAb) were used: Anti-CD3 (Leu-4), anti-CD4 (Leu-3), anti-CD8 (Leu-2) and anti-CD25 (IL-2 receptor)

from Becton Dickinson, San Jose, CA, USA; anti-CD45RO (UCHL-1) from Dakopatts, Glostrup, Denmark; anti-IL-4 from Genzyme, Cambridge, MA, USA; EG1 recognizing eosinophil catonic protein (ECP) in resting and activated eosinophils, and EG2 recognizing the cleaved form of ECP in activated eosinophils from Pharmacia, Uppsala, Sweden; anti-tryptase recognizing mast cells from Chemicon Brunschwig Chemie, Temecula, CA, USA.

The MoAb staining was detected by the immuno-alkaline phosphatase anti-alkaline phosphatase (APAAP) method. The sections were fixed in acetone for 10 min at 20°C, rinsed in phosphate-buffered saline (PBS, pH 7.2) and placed in a half-automatic stainer (Shandon, Pittsburgh, PA, USA). In this stainer the slides were sequentially incubated with bovine serum albumin (BSA) 2% in PBS for 10 min, incubated with normal rabbit serum (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) for 10 min and incubated with the MoAb in the optimal dilution for 30 min at 20°C. The sections were subsequently rinsed in PBS for 5 min and incubated for 30 min with a rabbit anti-mouse (RaM) (1:20) immunoglobulin antiserum, rinsed in PBS, incubated with APAAP (1:40) (Dakopatts) for 30 min at 20°C, rinsed in PBS and TRIS buffer (pH 8.0), and incubated for 30 min with New Fuchsin substrate (Chroma, Stuttgart, Germany), which stained positive cells red. Finally, the sections were rinsed with distilled water, counterstained with Mayer's haematoxylin, and mounted in glycerin gelatin. Control staining was performed by substitution with PBS and incubation with an irrelevant MoAb of the same isotype and protein concentration.

For staining with anti-tryptase and anti-IL-4 a supersensitive immunodetection system (Biotin-Streptavidin Amplified Detection System AZ000UM, Biogenex, San Ramon, CA, USA) was used. This protocol followed the APAAP protocol up to the incubation with normal rabbit serum for 10 min. The sections were then incubated for 1 h with the MoAb. The sections were rinsed with PBS for 5 min and successively linked with biotinylated rabbit anti-mouse (1:50) for 30 min, rinsed with PBS for 5 min and labelled with streptavidine alkaline phosphatase (1:30) for 30 min, rinsed in PBS for 5 min and TRIS buffer (pH 8.0) for 5 min, and incubated with New Fuchsin. The rest of the protocol was identical to the APAAP protocol.

Quantification of the bronchial biopsies

Biopsies were coded and two sections 120 μm apart were counted in a blinded fashion for each antibody and each biopsy at a magnification of 10x40 by one person (G.M.M.) and the mean value was calculated. With an eye piece graticule the numbers of positively stained cells were counted in a zone 100 μm deep in the bronchial mucosa along the length of the epithelial

basement membrane (BM), which had to be covered with epithelium over at least 500 μm.

Cells were counted if they stained red and contained a nucleus. The cell counts were expressed as the number per unit length (1 mm) of basement membrane.

Quantification of the lamina reticularis

Biopsies were coded prior to analysis. Subepithelial reticular collagen thickness was measured by using a 10x100 oil objective and a digital image-processing system (IBAS 2000 system, Kontron, München, Germany). The thickness of the lamina reticularis was quantitated by measuring only the BM covered by at least a basal epithelial cell layer and where the lamina propria under the BM was at least 100 μm deep. The thickness of the BM was measured in five at random fields in two sections of each patient and control subject.

Statistical analysis

The numbers of positive cells for most of the MoAb used showed a positive skewed distribution and therefore were analyzed using non-parametric statistics. Median cell counts and thickness of the lamina reticularis from biopsies from asthmatics who received inhaled glucocorticoids were compared with those who received bronchodilators only using the Mann-Whitney U test. The thickness of the lamina reticularis of asthmatics was compared with the thickness of the control subjects using the Mann-Whitney U test. A value of $p<0.05$ was considered significant.

RESULTS

Pulmonary function data

Details of patient and pulmonary function data have been given before (1). In short: Two and a half years of inhaled BDP resulted in a significant improvement in FEV_1 of 14.7% of predicted as compared to the bronchodilator group (-3.6% of predicted, $p=0.01$) (Table 2). Log_2PC_{20} in the glucocorticoid group improved 2.5 dose steps, compared to an deterioration of -0.63 dose steps in the bronchodilator group ($p=0.01$) (Table 2).

Table 2. Pulmonary function data of the patients included in the study

	Glucocorticoid group	Bronchodilator group	P value
ΔFEV_1	14.7 (4.6-30.6)	-3.6 (-39.3-21.7)	0.01
Δlog_2PC_{20}	2.5 (-0.8-4.0)	-0.6 (-3.5-1.9)	0.01

ΔFEV_1 in % predicted: difference in median change (ranges) of FEV_1 % predicted from baseline to 2.5 yrs.
Δlog_2PC_{20} in mg/ml: difference in median change (ranges) of log_2PC_{20} mg/ml to histamine from baseline to 2.5 yrs.

Bronchial epithelium

Some sections could not quantitatively be evaluated for bronchial epithelial infiltration because they did not show enough intact epithelium. In the bronchial epithelium we did not see any differences on the number of eosinophils, IL-4$^+$ cells, mast cells or T lymphocyte subsets between the glucocorticoid and the bronchodilator group.

Table 3. Median cell counts (ranges) after 2.5 yrs treatment per mm of basement membrane in the bronchial mucosa

Marker	Glucocorticoid group	Bronchodilator group	P value
CD3	10.8 (0-90.0) n=4	40.0 (10.3-123.0) n=7	NS
CD4	15.5 (3.8-81.1) n=4	5.5 (0-44.2) n=10	NS
CD8	7.6 (0-45.5) n=4	8.3 (0-37.8) n=9	NS
CD45RO	6.9 (4.0-26.3) n=4	4.1 (0-21.7) n=10	NS
CD25	0 (0-0.6) n=4	0.4 (0-13.3) n=10	NS
EG1	0 (0-1.0) n=5	8.2 (0-19.2) n=7	0.02
EG2	0.6 (0-1.3) n=5	1.3 (0-15.1) n=11	NS
IL-4	1.1 (0-6.5) n=5	10.8 (0-15.4) n=6	0.05
Tryptase	1.1 (0-4.0) n=4	2.5 (0-12.4) n=8	NS

Lamina propria

Counting of cells stained immunohistochemically was possible in the lamina propria of all 16 patients. However, some sections were excluded, because they were too small or the epithelial layer was not well preserved or did not meet the criteria of at least 500 µm BM as a result of biopsy trauma (Table 3). In the glucocorticoid-treated group borderline significantly lower median numbers of IL-4$^+$ cells ($p=0.05$) (Figure 1A) and total eosinophils (EG1$^+$, $p=0.02$) (Figure 1B) were observed compared to the bronchodilator group (Table 3). Lower number of mast cells and activated eosinophils (EG2$^+$) were observed in the BDP group, although these differences did not reach significance. We did not find significant differences in the numbers of CD3$^+$, CD4$^+$, CD8$^+$, CD25$^+$ and (memory) CD45RO$^+$ lymphocytes between the glucocorticoid group and the bronchodilator group (Table 3).

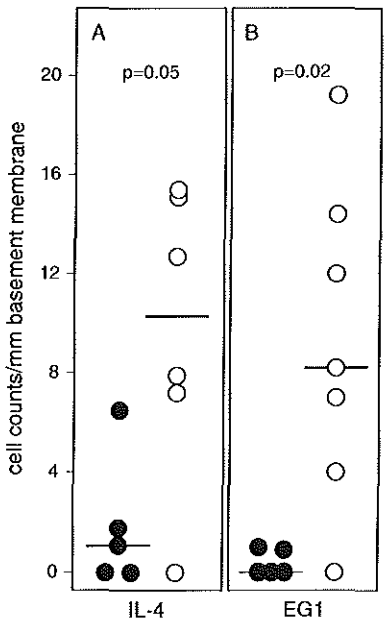

Figure 1A. Individual counts for cells expressing IL-4 in the bronchial mucosa, expressed as the number of positive cells per millimeter basement membrane. Median values are represented by the horizontal bars.

● = glucocorticoid; O = bronchodilator.

Figure 1B. Individual counts for cells expressing EG1 (pan eo-marker) in the bronchial mucosa, expressed as the number of positive cells per millimeter basement membrane. Median values are represented by the horizontal bars.

● = glucocorticoid; O = bronchodilator.

Lamina reticularis

The thickness of the lamina reticularis of the asthmatics and the normal controls is shown in Table 4. There was a significant thickening of the lamina reticularis in the asthmatics both in the glucococorticoid (median 12.2 (8.1-14.9) µm) and in the bronchodilator group (median 10.3 (6.6-15.5)

μm) as compared with control subjects (median 6.5 (4.9-8.8) μm). However, long-term treatment with BDP did not reduce the thickness of the lamina reticularis significantly (Table 4).

Table 4. Median thickness of basement membrane (lamina reticularis) in μm in bronchial biopsies of atopic asthmatics and control subjects

glucocorticoid group (n=5)	bronchodilator group (n=11)	control subjects (n=8)
12.2 (8.1-14.9)	10.3 (6.6-15.5)	6.5 (4.9-8.8)

p = 0.280 : glucocorticoid group compared with bronchodilator group
p = 0.001 : glucococorticoid group compared with controls
p = 0.003 : bronchodilator group compared with controls

DISCUSSION

Inhaled corticosteroids are clinically the most effective drugs for asthma, but their precise mechanism of action is still unknown. In atopic mild to severe asthmatic patients from the Dutch CNSLD study (1), BDP administered over a period of 2.5 years significantly improved FEV_1 and reduced airway hyperresponsiveness. Our results show that this clinical improvement is accompanied by borderline significantly lower numbers of IL-4^+ cells and significantly lower numbers of total eosinophils in the mucosa of the glucocorticoid group as compared to the group treated with bronchodilators only.

Increased levels of IL-4 and IL-5 in BAL as well as in supernatants of peripheral blood T cells from allergic asthmatics were observed by Walker (9). A higher number of cells expressing mRNA for a Th2-type pattern of cytokines has been reported in the BAL of atopic asthmatics compared with healthy volunteers (11). Hamid and colleagues observed increased mRNA for IL-5 in mucosal biopsies from asthmatics, which was related to an increased degree of infiltration by activated eosinophils (10). The glucocorticoid induced decrease of the number of IL-4^+ cells we observed in our biopsies suggests that the anti-inflammatory actions of topical glucocorticoids may depend on downregulation of cytokine synthesis *in vivo*. This is consistent with the *in vitro* finding that hydrocortisone inhibits the production of IL-4 and also decreases the transcription of IL-4 mRNA in PBMC cultures (31). Recent studies demonstrated that 2 weeks of treatment with prednisolone, 0.6 mg/kg/day in asthma resulted in a reduction of BAL T cells expressing mRNA for IL-4 and IL-5 and an increase in mRNA for IFN-γ (35). One can therefore speculate that inhaled glucocorticoids may exert their anti-

inflammatory actions by suppressing IL-4 production in the bronchial mucosa. The importance of IL-4 as a mediator of allergic airway inflammation regulating antigen-induced eosinophil recruitment is supported by studies of Brusselle *et al.* (36). Unfortunately, because this study was set up later as a part from the multicentre study (1), we do not have biopsies at the beginning of the study prior to treatment. While it is extremely likely that the reduced number of IL-4$^+$ cells and eosinophils present in the glucocorticoid group is a direct effect of beclomethasone, it can not be excluded with this design of the study that there has not been an adverse effect in the group given bronchodilators alone without BDP.

Eosinophils are known to be effector cells in the pathophysiology of asthma (37,38). Bronchial biopsies from patients treated with long-term (3.7 yr) inhaled BDP showed reduced numbers of mast cells and eosinophils. These changes were not seen in subjects treated with terbutaline (14). An uncontrolled study by Djukanovic and colleagues on 6 weeks of treatment with inhaled BDP has shown a significant decrease in airway hyperresponsiveness accompanied by a reduction in eosinophils, mast cells and T lymphocytes in bronchial mucosal biopsies (39). Our data, showing a reduction in the number of total eosinophils in the bronchial mucosa after long-term treatment with BDP compared with the bronchodilator group are in agreement with these observations. The reduction in eosinophils numbers we found, could possibly be a result of an inhibition of chemoattractants for eosinophils, like IL-3, IL-5 and granulocyt-macrophage colony stimulating factor (GM-CSF), since oral glucocorticoids reduce the concentrations of circulating IL-3, IL-5 and GM-CSF in asthmatics (40). In culture, glucocorticoids decrease cytokine-mediated survival of eosinophils, presumably by stimulating programmed cell death (apoptosis) (41). This process may also explain the reduction in the number of eosinophils in the bronchial mucosa after glucocorticoid treatment.

Interestingly, we detected a significant reduction in total eosinophils, as identified by EG1, but not in EG2$^+$ eosinophils in the glucocorticoid group. EG2 identifies the secreted form of ECP and hence only stains eosinophils that are undergoing activation and/or secretion. Thus, our results suggest that glucocorticoids have no effect on eosinophil degranulation. Recently, it has been reported that eosinophil degranulation is not an important direct target of glucocorticoid action in humans (42).

Topical glucocorticoids have been shown to reduce the numbers of mast cells in bronchial biopsies, in parallel with clinical improvement (43,44). In our study we also observed a reduction in the median numbers of mast cells in the bronchial mucosa of the glucocorticoid group, although it did not reach statistical significance in this limited number of patients. Finally, in agreement with Wilson and colleagues (44), treatment with BDP did not differ from bronchodilator therapy with regard to the total number of T

lymphocytes, or their $CD4^+$, $CD8^+$ or $CD45RO^+$ subsets in the bronchial mucosa.

We did not observe a difference in the thickening of the lamina reticularis between the glucocorticoid and the bronchodilator group as measured by staining with hematoxylin and eosin and the use of a digital image-processing system. These findings are in agreement with another study, which showed that neither short-term (four weeks) nor long-term (3.7 years) corticosteroid treatment reduced the thickening of the reticular basement membrane (14). These results indicate that even long-term treatment with beclomethasone does not reduce the deposition of collagen fibrils and suggest that this deposition may be associated with an irreversible subepithelial fibrosis and airflow limitation.

In conclusion, our data support the hypothesis that the beneficial effects of long-term inhaled BDP therapy may result at least in part from an inhibition of IL-4 production in the bronchial mucosa and in a downregulation of the local eosinophilic inflammation. Recent studies suggest that mast cells as well as eosinophils can store and release IL-4 (24,25). Our findings suggest that inhaled glucocorticoids are very important in suppressing airway inflammation in asthma possibly through their ability to downregulate cytokine production.

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CHAPTER 2.2

INFLUENCE OF FLUTICASONE PROPIONATE ON AIRWAY INFLAMMATION IN ATOPIC ASTHMATICS*

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ABSTRACT

Fluticasone propionate is a new, topically active inhaled glucocorticoid with a high affinity for the glucocorticoid receptor. We investigated the effects of 3 months inhaled fluticasone propionate therapy on bronchial mucosal inflammation and the thickness of the lamina reticularis in subjects with atopic asthma.

Bronchial biopsies were obtained from 24 asthmatics before and after 3 months of treatment with fluticasone propionate 1000 μg daily, or matched placebo in a randomized, double-blind parallel group study. Sections (6 μm) were stained for T lymphocyte subsets, eosinophils, mast cells and adhesion molecules.

When the treated ($n=11$) and placebo ($n=13$) groups were compared, an improvement in FEV_1 ($p=0.009$) and in methacholine PC_{20} (an increase of 3.7 doubling doses, $p=0.001$) were observed after treatment with fluticasone propionate. This was accompanied by a significant reduction in eosinophils ($p=0.005$) and a trend toward a decrease in mast cells ($p=0.087$) and expression of intercellular adhesion molecule-1 (ICAM-I) ($p=0.091$). In the treated group there was a significant correlation between the reduction in the number of total eosinophils and the improvement in airway hyperresponsiveness ($r = 0.96$, $p=0.011$). No change in total helper or activated helper T cells was seen. The thickness of the bronchial lamina reticularis was not reduced after 3 months treatment with fluticasone propionate.

In conclusion, 3 months fluticasone propionate treatment reduces the bronchial mucosal infiltration of eosinophils and mast cells, and downregulates ICAM-1 expression.

INTRODUCTION

Fluticasone propionate (FP) is a new, topically active, inhaled glucocorticoid with a high affinity for the human glucocorticoid receptor and a negligible oral bioavailability (12). Glucocorticoids are the most effective therapy in asthma and suppress the chronic airway inflammation largely by inhibiting transcription factors that regulate abnormal gene expression (1,19).

Bronchial biopsies obtained from asthmatics have demonstrated local infiltration by inflammatory cells in the bronchial mucosa even in mild asthmatics (2). In addition, thickening of the lamina reticularis of the basement membrane is often reported as a characteristic feature of the bronchial mucosa in asthma and is mainly composed of Type III and Type V collagen (11,20). Cell adhesion molecules play a key role in the recruitment of leucocytes from the circulation to the site of the inflammation (14).

Vascular cell adhesion molecule-1 (VCAM-I) is important for eosinophil migration, because very late activation antigen-4 (VLA-4), a ligand for VCAM-I, is expressed on the surface of eosinophils (21). In primates with experimentally induced allergic asthma, antibodies to intercellular adhesion molecule-1 (ICAM-I, CD54) were reported to reduce symptoms of hyperresponsiveness and airway eosinophilia (27). E-selectin is also involved in eosinophil accumulation in the bronchial mucosa (7). To date, increased expression of these molecules has been demonstrated in bronchial biopsies of asthmatics (17). However, the influence of FP therapy on the expression of these adhesion molecules has not been elucidated.

At the time of design of this study, placebo-controlled studies on the effects of 3 months inhaled FP on inflammatory cells in the bronchial mucosa were not available. Treatment with another inhaled glucocorticoid, beclomethasone dipropionate (BDP), 1000 μg daily dose for 4 months in a placebo controlled study showed a significant reduction in mast cells, eosinophils and no change in T lymphocyte subsets (22). Six weeks BDP (daily dose 2000 μg for 2 wks and 1000 μg for 4 wks) did not alter the total numbers of T cells or the $CD4^+$ and $CD8^+$ subsets when analyzed in peripheral blood or bronchoalveolar lavage (BAL) fluid. However, activated T cells recovered by BAL were reduced (28). Intranasal FP treatment of rhinitics for 3 months did reduce Langerhans' cells, but did not reduce the number of T lymphocytes in nasal mucosa (9).

The aim of this study was to examine the effect of FP, 500 μg twice a day, on the number of bronchial mucosal inflammatory cells, the expression of adhesion molecules and the thickness of the lamina reticularis after a period of 3 months in comparison with a placebo group receiving inhaled $ß_2$-agonists alone.

MATERIALS AND METHODS

Patients and non-asthmatic controls

Bronchial mucosal biopsy specimens were obtained from 24 non-smoking atopic asthmatic subjects (six women and eighteen men with a median age of 26 years, range 21 to 56 years). Asthma was defined as a history of episodic wheezing and reversible airway obstruction characterized by an increase in forced expiratory volume in one second (FEV_1) of ≥ 9 % after inhalation of 1000 μg terbutaline. The asthmatic subjects had a mean FEV_1 of 84.0 ± 16.1 % of the predicted value; the mean of the ^{2}logs of the provocative concentrations of inhaled methacholine required to reduce their FEV_1 by 20% (PC_{20}) was 0.32 ± 2.94 mg/ml. Individual PC_{20} data were obtained by linear interpolation of the logs of adjacent concentration values

(8). Atopy was defined by one or more positive skin-prick tests to extracts of 16 common aeroallergens. All patients were receiving inhaled $ß_2$-agonists, and none had taken oral or inhaled glucocorticoids in the month prior to the study. After a run-in period of 2 weeks, during which time they took inhaled $ß_2$-agonists only, spirometry was performed and methacholine PC_{20} was measured according to the standardized 2 min tidal breathing technique (8), followed by the first fiberoptic bronchoscopy. Patients were randomized in a double-blind fashion to receive 3 months FP, 500 µg or matched placebo twice daily, in addition to their inhaled $ß_2$-agonists. After 3 months of therapy, patients reattended the hospital for repeat measurement of spirometry and bronchial responsiveness, followed by a second fiberoptic bronchoscopy one week later. All measurements of PC_{20} and both fiberoptic bronchoscopies were performed at the same time of day. There were no significant differences at baseline between the FP and the placebo group with regard to FEV_1 and PC_{20}.

A control group was composed of eight non-smoking non-asthmatic subjects (three women and five men with a median age of 23 years, range 19 to 52 years). All controls had a PC_{20} histamine of more than 8 mg/ml and a median FEV_1 of 103 (88-110)% of the predicted value. Patient and control characteristics are shown in Table 1. The study was approved by the local Ethics Committee and all participants gave their written informed consent.

Bronchial biopsy and tissue processing

Bronchial biopsy specimens were taken from the carinae of the lingula or the right upper, middle or lower lobes via an Olympus BF IT 10 fiberoptic bronchoscope (Tokyo, Japan) using alligator forceps, Olympus FB 15C by the same operator (S.E.O.). For light microscopic examination, each biopsy specimen was immediately placed in isotonic saline and frozen within 20 min in Tissue-Tek II OCT embedding medium (Miles, Naperville, IL, USA). Samples were stored at -80°C until use. Frozen sections (6µm) were cut on a Reichert-Jung 2800 Frigocut cryostat. Two sections from each biopsy were placed on poly-L-lysine-coated (Sigma, Diagnostics, St Louis, MO, USA) slides, air dried and stored at -80°C until use.

Immunohistochemistry

The following monoclonal antibodies (MoAb) were used: anti-CD3 (Leu 4) for identification of total T lymphocytes, anti-CD4 (Leu 3) and anti-CD8 (Leu 2) for identification of T helper and T suppressor/cytotoxic lymphocyte subpopulations, anti-CD25 (IL-2R) for identification of cells expressing the activation marker interleukin-2 receptor (IL-2R), all from Becton Dickinson,

Table 1. Patient and control subject characteristics

Patient no.	Sex	Age (years)	Baseline FEV_1 % predicted	After 3 months FEV_1 % predicted	Baseline $^2log\ PC_{20}$ mg/ml	After 3 months $^2log\ PC_{20}$ mg/ml
			Fluticasone group			
1	F	26	90.2	115.0	0.77	5.50
2	M	51	84.3	96.0	4.12	4.99
3	M	21	93.2	97.8	2.40	8.0
4	M	24	90.3	93.2	-0.91	3.40
5	M	23	77.7	84.0	0.12	2.06
6	M	23	104.4	107.6	2.85	6.72
7	M	21	99.2	103.2	-5.18	0.15
8	M	42	55.9	63.7	0.25	-0.18
9	F	20	90.5	90.8	-0.06	2.08
10	M	43	67.3	62.5	3.43	8.00
11	M	47	81.4	87.3	-1.05	6.85
Median		24 (21-51)	90.2 (55.9-99.2)	93.2 (62.5-115)	0.25 (-5.18-4.12)	4.99 (-0.18-6.72)
			Placebo group			
1	F	44	80.0	81.0	0.58	5.50
2	M	52	62.8	58.9	1.47	4.99
3	M	41	87.4	83.2	-1.83	8.00
4	M	55	60.4	61.8	-2.34	3.40
5	M	55	72.1	72.3	3.57	1.06
6	M	22	92.1	86.8	-0.52	-1.53
7	M	56	84.2	79.0	3.40	1.93
8	F	21	104.6	106.1	-3.42	-1.80
9	F	26	96.7	95.1	-2.56	-0.58
10	M	24	46.8	21.3	0.04	1.19
11	M	32	91.4	101.9	0.64	1.41
12	F	26	94.6	92.4	7.98	2.86
13	M	23	108.3	85.3	-3.89	-1.10
Median		32 (21-56)	87.4 (46.8-108.3)	83.2 (21.3-106.1)	-0.52 (-3.89-7.89)	0.58 (-2.34-2.86)
			Control subjects			
1	M	19	102			
2	M	23	109			
3	M	23	109			
4	F	24	103			
5	F	23	96			
6	M	23	88			
7	M	52	88			
8	F	35	110			
Median		23 (19-52)	103 (88-110)			

San Jose, CA, USA; EG1 recognizing eosinophil catonic protein (ECP) in resting and activated eosinophils, and EG2 recognizing the cleaved form of ECP in activated eosinophils from Pharmacia, Uppsala, Sweden; anti-tryptase recognizing mast cells from Chemicon Brunschwig Chemie, Temecula, CA, USA; anti-intercellular leucocyte adhesion molecule-1 (ICAM-I) (BBA3; British Bio-technology, Abingdon, Oxon, UK), anti-vascular cellular adhesion molecule-1 (VCAM-I) (BBA5; British Bio-technology) and anti-E-selectin (BBA1; British Biotechnology) for identification of vessels expressing these adhesion molecules.

The MoAb staining was detected by the immuno-alkaline phosphatase anti-alkaline phosphatase (APAAP) method. The sections were fixed in acetone for 10 min at 20°C, rinsed in phosphate-buffered saline (PBS, pH 7.2) and placed in a half-automatic stainer (Shandon, Pittsburgh, PA, USA). In this stainer the slides were sequentially incubated with bovine serum albumin (BSA) 2% in PBS for 10 min, incubated with normal rabbit serum (CLB, Amsterdam, the Netherlands) for 10 min and incubated with the MoAb in the optimal dilution for 30 min at 20°C. The sections were subsequently rinsed in PBS for 5 min and incubated for 30 min with a rabbit anti-mouse (RaM) (1:20) immunoglobulin antiserum, rinsed in PBS, incubated with APAAP (1:40) (Dakopatts, Glostrup, Denmark) for 30 min at 20°C, rinsed in PBS and TRIS buffer (pH 8.0), and incubated for 30 min with New Fuchsin substrate (Chroma, Stuttgart, Germany) as a chromagen. The sections were counterstained with Mayers hematoxylin.

Control slides were treated similarly, but in one case excluding the primary antibody and in the other using an unrelated antibody of the same isotype and protein concentration.

Quantification

Biopsies were coded and two sections were counted in a blinded fashion for each antibody and each biopsy at a magnification of 10x40 by one person (G.M.M.) and the mean value was calculated. With an eye piece graticule the numbers of positively stained cells were counted in the epithelium and in a zone 100 µm deep in the lamina propria along the length of the epithelial basement membrane (BM), which had to be covered with epithelium over at least 500 µm.

Cells were counted if they stained red and contained a nucleus. The cell counts were expressed as the number per unit (1 mm) of basement membrane. The adhesion molecules were scored semiquantitatively on a 0-3 scale (0 = negative; 1 = weak; 2 = strong; 3 = very strong). There were no significant differences at baseline between the FP and the placebo group with regard to the profiles of the inflammatory cell types and the expression of the adhesion molecules.

Quantification of the lamina reticularis

Biopsies were coded prior to analysis. Subepithelial reticular collagen thickness was measured by using a 10x100 oil objective and a digital image-processing system (IBAS 2000 system, Kontron, München, Germany). The thickness of the BM was quantitated by measuring only the BM covered by at least a basal epithelial cell layer and where the lamina propria under the BM was at least 100 μm deep. The thickness of the BM was measured in five at random fields in two sections of each patient and control subject.

Statistical analysis

The median cell numbers showed a positive skewed distribution and therefore were analyzed using non-parametric statistics. Comparison of treatment effects between FP and placebo was performed by Mann-Whitney U test on percentual changes (after treatment minus before treatment divided by before treatment, multiplied by 100%). Correlation coefficients were obtained by Pearson's rank method.

Median thickness of the lamina reticularis in biopsies from asthmatics taken before treatment was compared with median thickness of biopsies from control subjects using the Mann-Whitney U test. A value of $p<0.05$ was considered significant.

RESULTS

Pulmonary function data

Three months of FP resulted in a significant improvement in FEV_1 of 6.06% of predicted value as compared with placebo group (-4.33% of predicted, $p=0.009$) (Table 2). $^2LogPC_{20}$ in the FP group improved 3.71 dose steps, compared with 0.04 dose steps in the placebo group ($p=0.001$) (Table 2). After FP treatment, the reduction in the number of total eosinophils correlated significantly with the improvement in aiway hyperresponsiveness ($r = 0.96$, $p=0.011$). In the placebo group no correlations were found.

Table 2. Pulmonary function data of the patients included in the study

	Fluticasone group	Placebo group	P value
ΔFEV_1	6.06 ± 7.50	-4.33 ± 9.77	0.009
$\Delta^2 log\ PC_{20}$	3.71 ± 2.38	0.039 ± 2.18	0.001

ΔFEV_1 in % predicted: difference in mean change ± SD of FEV_1 % predicted from baseline to 3 months.
$\Delta^2 log\ PC_{20}$ in mg/ml: difference in mean change ± SD of $^2log\ PC_{20}$ mg/ml to methacholine from baseline to 3 months.

Bronchial epithelium

Some sections could not be evaluated quantitatively for bronchial epithelial infiltration because they did not show enough intact epithelium. In the asthmatics the loss of epithelium from the BM (shedding) was 30% of the total length of epithelium and significantly higher as compared to 10% shedding in the controls ($p=0.003$). In the sections of the asthmatic patients the epithelium consisted for approximately 50% of a mono-layer and for 20% of a multi-layer of partially stratified cuboidal epithelium; the epithelium of the controls consisted for approximately 30% of a mono-layer and for 60% of a multi-layer of cuboidal epithelium. Three months of FP treatment did not reverse the shedding of the epithelium.

In the bronchial epithelium we did not see any treatment effects on the numbers of $CD4^+$ and $CD8^+$ T cells, mast cells, eosinophils, and on ICAM-I expression.

Lamina propria

The number of $CD4^+$, $CD8^+$ and $CD25^+$ T lymphocytes had not changed after 3 months of glucocorticoid therapy. FP treatment was accompanied by a significant reduction in the number of total eosinophils ($p=0.005$) (Table 3). Small reductions in numbers of activated eosinophils, mast cells, and in expression of ICAM-1^+ vessels were observed after FP therapy, although these differences were not significant compared to placebo (Table 3). No significant changes were observed in expression of E-selectin and VCAM-1.

Lamina reticularis

The thickness of the lamina reticularis of the asthmatics before and after treatment and controls is shown in Table 4. There was a significant thickening of the lamina reticularis in the asthmatics, both in the FP (median 11.9 (7.3-14.5) μm) and in the placebo group (median 10.0 (6.1-14.3) μm)

as compared with controls (median 6.5 (4.9-8.8) μm). Three months treatment with FP did not reduce the thickness of the lamina reticularis (Table 4).

Table 3. Median cell counts (ranges) in lamina propria per mm basement membrane before and after Fluticasone or Placebo treatment*

	Fluticasone group			Placebo group			P value
	Before	After	Median difference	Before	After	Median difference	
EG1+ eos	11.2	0	-10.5 (-17.6, -1.6) *(n=5)*	12.3	7.5	-6.2 (-55.9, 11.1) *(n=8)*	0.005
EG2+ eos	3.2	0	-3.2 (-3.8, -0.4) *(n=3)*	7.9	5.6	-2.3 (-19.7, 8.3) *(n=8)*	0.149
Mast cells	9.7	0.5	-6.4 (-11.9, -1.2) *(n=4)*	6.8	3.8	-1.8 (-4.8, -0.4) *(n=6)*	0.087
ICAM-1†	1.5	0	-1.5 (-1.5, 0.5) *(n=3)*	1	1	0 (-0.7, 0.5) *(n=5)*	0.091

* Comparison of treatment effects between Fluticasone and Placebo was performed by the Mann-Whitney U test on percentual changes ($\frac{\text{after treatment minus before treatment}}{\text{before treatment}} \times 100\%$)

† Median score (ranges)

Table 4. Median thickness of the lamina reticularis in μm in bronchial biopsies from asthmatics, before and after Fluticasone or Placebo treatment and controls

Fluticasone group		Placebo group		Controls
Before	After	Before	After	
11.9 (7.3 - 14.5) *(n=9)*	10.6 (6.2 - 16.7) *(n=8)*	10.0 (6.1 - 14.3) *(n=11)*	10.6 (8.3 - 12.2) *(n=10)*	6.5 (4.9 - 8.8) *(n=8)*

Comparison of treatment effects between Fluticasone and Placebo : $p=0.659$
Asthmatics (before) compared with controls : $p < 0.0005$

DISCUSSION

This double-blind placebo-controlled study has shown the antiinflammatory benefit of inhaled FP, 1000 μg daily, in asthma. After 3 months of treatment there was a significant improvement in FEV_1 and airway hyperresponsiveness (PC_{20} methacholine) accompanied by a significant reduction in numbers of total eosinophils in bronchial mucosal biopsies in the FP group. The decrease in eosinophils after FP therapy was significantly correlated with improvement in hyperresponsiveness. There was a concomitant trend toward a reduction in mast cells and ICAM-I expression.

The effect on eosinophils and mast cells was in line with preliminary findings of others in short- and long-term studies with FP (6,15,10) and BDP (5,22). In a study with FP therapy, 500 μg for six weeks significantly reduced eosinophil counts in both BAL and peripheral blood (15). Moreover, six weeks treatment with FP, 500 μg daily dose was effective in reducing the numbers of eosinophils and mast cells in the lamina propria (6). In an uncontrolled study, after 6 weeks treatment with inhaled BDP there was a reduction in eosinophils, mast cells and T lymphocytes in bronchial mucosal biopsies (5). Glucocorticoids appear to inhibit the cytokine-mediated survival of eosinophils, presumably by stimulating programmed cell death (apoptosis) (25). This process may partly explain the reduction in the number of eosinophils in the bronchial mucosa after glucocorticoid treatment. IL-5 is an important selective eosinophil chemoattractant (16) and IL-3 and GM-CSF can induce chemotactic activity for eosinophils (26). Glucocorticoids reduce the concentrations of circulating IL-3, IL-5 and GM-CSF in asthmatics (3) and inhibit the increased expression of GM-CSF in the epithelium of asthmatics (24). This may be another important mechanism for the eosinopenic effect of glucocorticoids.

Interestingly, we detected a significant reduction in total eosinophils, as identified by EG1, but not in $EG2^+$ eosinophils in the FP group. EG2 identifies the secreted form of ECP and hence only stains eosinophils that are undergoing activation and secretion. Thus, our results suggest that glucocorticoids are only weakly effective in inhibiting secretion of eosinophil basic proteins. This observation has been confirmed by others in the literature (13).

Modification of cytokines by glucocorticoids, which we did not investigate, has been reported (3,10,18). In a double-blind study of mild atopic asthmatics, prednisolone therapy was associated with clinical improvement and a reduction in the percentages of BAL fluid cells expressing mRNA encoding IL-4 and IL-5 (18). In biopsies from asthmatic patients receiving 6 weeks FP, 500 μg twice daily, there was a significant reduction in immunostaining for IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, TNF-α, and GM-CSF but not IFN-γ (10). Oral glucocorticoid therapy of severe asthmatics is

associated with a reduction in the percentages of peripheral blood $CD4^+$ T lymphocytes expressing IL-3, IL-5, and GM-CSF mRNA and corresponding proteins, which are responsible for eosinophil survival (3).

There were no significant changes in the number of other inflammatory cells, including total T lymphocytes or their $CD4^+$ or $CD8^+$ subsets. These data are in agreement with findings of others (22,28). However, activated T cells recovered by BAL were reduced by steroids (28). FP treatment does not appear to affect the number of T cells or their subpopulations, although the function of T lymphocytes and their cytokine production profile may be influenced.

The downregulation of the numbers of eosinophils may be only one of the mechanisms whereby glucocorticoids modulate inflammation in asthma. The anti-inflammatory effects of FP may be related to the inhibition of the expression of adhesion molecules on endothelial cells. We did find a trend towards a reduction in the expression of ICAM-1 on vessels after FP treatment. It did not reach significance, probably because of the low number of biopsies. Recently, it was shown that dexamethasone was able to downregulate ICAM-1 expression in both a human monocytic and a bronchial epithelial cell line (23). Another study showed that dexamethasone inhibited the expression of adhesion molecules ELAM-1 and ICAM-1 by endothelial cells (4).

In this study, we found a significant thickening of the lamina reticularis of asthmatics compared to controls and we did not observe a reduction in thickening of the lamina reticularis by 3 months FP therapy. These findings are in agreement with earlier data on 2.5 years treatment with beclomethasone which did not show a reduction of the subepithelial collagen deposition (data not shown). Jeffery *et al.* showed that neither short-term (four weeks) nor long-term (3.7 years) glucocorticoid treatment reduced the thickening of the lamina reticularis (11). These results may implicate that the subepithelial collagen deposition in asthmatics may be associated with irreversible fibrosis and airflow limitation.

Lung function and airway hyperresponsiveness improved after 3 months FP therapy. The PC_{20} methacholine improved 3.7 doubling doses and the FEV_1 6.1 % predicted in the FP group. Our observation that the reduction in eosinophils after FP treatment correlated significantly with the improvement in airway hyperresponsiveness provides strong evidence that there exists a relationship between airway responsiveness and eosinophils in the lamina propria.

Other investigators have also reported improvement in hyperresponsiveness. Donno *et al.* (6) and Li *et al.* (15) found improvement in PC_{20} methacholine after 6 weeks of FP (500 µg daily), whereas Montefort *et al.* (18) showed improvement in PC_{20} histamine after 6 weeks of FP, 1000 µg daily. These placebo-controlled studies showed clearly that FP

modifies airway inflammation in association with clinical benefit.

In conclusion, this study shows that three months treatment with 1000 μg FP reduces the number of eosinophils and mast cells, and downregulates the expression of ICAM-I on vessels in the bronchial mucosa of asthmatic patients in parallel with improved FEV_1 and decreased hyperresponsiveness.

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CHAPTER 3

DENDRITIC CELLS IN ASTHMA AND MODULATION BY GLUCOCORTICOIDS

3.1 Increased numbers of dendritic cells in the bronchial mucosa of atopic asthmatics: downregulation by inhaled corticosteroids
Clin Exp Allergy 1996 (in press)

3.2 Fluticasone propionate therapy is associated with modulation of dendritic cells in the bronchial mucosa of atopic asthmatics
Submitted

CHAPTER 3.1

INCREASED NUMBERS OF DENDRITIC CELLS IN THE BRONCHIAL MUCOSA OF ATOPIC ASTHMATICS: DOWNREGULATION BY INHALED CORTICOSTEROIDS*

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SUMMARY

Background: Dendritic cells (DC) are the most potent antigen-presenting cells (APC) and stimulators of T cells. Dendritic cells are also likely to be essential for the initiation of allergic immune responses in the lung. However, there are not many data on the presence of dendritic cells in the airways of patients with atopic asthma and on the effects of corticosteroid-treatment on such dendritic cells.
Objective: We investigated the distribution of dendritic cells in the bronchial epithelium and mucosa of 16 non-smoking atopic asthmatic patients and 8 healthy control subjects using detailed immunohistochemistry (CD1a, HLA-DR, L25 as markers for dendritic cells).
Methods: Eleven asthmatics were treated for 2.5 years with bronchodilators only and five with bronchodilators and inhaled beclomethasone dipropionate (BDP), 800 µg daily. The patients were randomly sampled from a double-blind multicentre study.
Results: There were higher numbers of $CD1a^+$ DC ($p=0.003$), $L25^+$ DC ($p=0.002$) and HLA-DR expression ($p=0.042$) in the bronchial mucosa of asthmatic patients compared with healthy controls. After 2.5 years of treatment, we found a significant increase in flow expiratory volume in 1 second (FEV_1) ($p=0.009$) and a significant decrease in hyperresponsiveness (PC_{20} histamine) ($p=0.013$) in the corticosteroid group ($n=5$) compared with the bronchodilator group ($n=11$). This clinical improvement in the corticosteroid-treated group was accompanied by significantly lower numbers of $CD1a^+$ DC ($p=0.008$), and HLA-DR expression ($p=0.028$) in the bronchial mucosa than in the bronchodilator-treated group.
Conclusion: Our data suggest that dendritic cells are involved in asthmatic inflammation and that corticosteroids may downregulate the number of dendritic cells.

INTRODUCTION

Atopic asthma is associated with a chronic T cell-mediated inflammatory process in the airways (1-3). Antigen-presenting cells (APC) play an essential role in asthma since T lymphocytes cannot respond to antigens without the help of APC (4). Dendritic cells (DC) are the most potent APC and in contrast to other APC unique in their capacity to stimulate naive T cells (5,6). Therefore, they may play a key role in the initiation of immune responses. In the lung, dendritic cells are predominantly observed in epithelial and subepithelial tissue of the bronch(iol)us and in the bronchus-associated lymphoid tissue (7). Typical immunohistological features of DC are a strong major histocompatibility complex (MHC) class II(HLA-DR)

expression (8-10), long cytoplasmic extensions, and the absence or a juxtanuclear spot of acid phosphatase. A combination of dendritic morphology and the expression of CD1a is characteristic for DC (11,12). Increased numbers of $CD1a^+$ DC and increased expression of MHC class II have been described in bronchial biopsies of asthmatic patients (13).
Corticosteroids are the most effective therapy for asthma, but their precise mechanism of action is still unclear (14). Corticosteroids inhibit cytokine gene transcription and cytokine effects, thereby reducing chronic inflammation in asthma (14-17). To date, the effects of inhaled corticosteroids on DC in asthma are unknown. We hypothesized that the beneficial effects of corticosteroid therapy in atopic asthmatics may in part result from a downregulation of the numbers of DC, resulting in a decreased local T cell stimulation. Therefore we examined the distribution of the DC in the bronchial epithelium and mucosa of atopic asthmatics by immunohistochemistry. To this aim the effects of 2.5 years of double-blind treatment with bronchodilators alone or with bronchodilators plus beclomethasone dipropionate (BDP) were evaluated. The patients were randomly sampled from the Dutch Chronic Non-Specific Lung Disease (CNSLD)-study (18).

MATERIALS AND METHODS

Patients and Control subjects

Sixteen non-smoking atopic asthmatic patients (seven women, nine men, median age 43 years, range 24 - 61 years) were randomly sampled from the Dutch CNSLD study group (18,19) in two participating centres, Groningen and Rotterdam.

The diagnosis of asthma was based on a history of attacks of breathlessness and wheezing without chronic cough or sputum production (i.e. for more than 3 months per year), according to the criteria of the American Thoracic Society (20). Atopy was defined as a positive skin-prick test to house-dust mite or at least two of 12 common aeroallergens (mean weal size > 0.7 times the histamine weal size (21)). All patients showed airway hyperreactivity to histamine with a provocative concentration of histamine causing a 20% decrease in FEV_1 (PC_{20}) of $\leq$ 8 mg/mL (19,21). Patients were treated in a double-blind fashion with an inhaled β_2-agonist (terbutaline, 250 µg two puffs) plus either inhaled corticosteroid (BDP, 100 µg two puffs) (BA+CS) ($n=5$), an anticholinergic bronchodilator (ipratropium bromide, 20 µg two puffs) (BA+AC) ($n=5$) or placebo (BA+PL) ($n=6$). All medication was taken four times daily. Because no significant differences were found between the BA+AC group and the BA+PL group with regard to

FEV_1 and PC_{20} the data were subsequently pooled for analysis in one bronchodilator group ($n = 11$). The fibreoptic bronchoscopy was performed at the end of the 2.5 years study in the same period (between August and December) in both centres, before breaking the code. Eight healthy non-smoking subjects (three women, five men, median age 23 years, range 19 - 52 years) without medication were studied as controls. All controls had a PC_{20} of more than 8 mg/mL and a median FEV_1 of 103 (88-110) % predicted. Patient and control characteristics are shown in Table 1. Further details of the study methods have been described previously (19). The study protocol was approved by the Medical Ethics Committee; all patients and controls gave written informed consent.

Bronchoscopy

Fibreoptic bronchoscopy (Olympus model BF IT 10, Tokyo, Japan) was performed with atropine 0.5 mg intramuscularly as premedication. Terbutaline, two puffs of 250 µg per Nebuhaler, was given 30 min before the procedure. The nose, throat and vocal cords were anaesthetized with topical lidocaine spray. An Olympus alligator forceps model FB15C and the fenestrated forceps model FB19C were used to take two biopsies from segmental and subsegmental divisions of the main bronchi.

Bronchial biopsies

Each biopsy was immediately placed in isotonic saline and frozen within 20 min in Tissue-Tek II OCT embedding medium (Miles, Naperville, Illinois, USA). Samples were stored at -80°C until use. Frozen sections (6 µm) were cut on a Reichert-Jung 2800 Frigocut cryostat. Two sections from each biopsy were placed on poly-L-lysine-coated (Sigma Diagnostics, St Louis, MO, USA) microscopic slides. Sections were air dried for 30 min and stored at -80°C until use.

Immunohistochemistry

The following monoclonal antibodies (MoAb) were used: OKT6 (CD1a) (American Type Culture Collection, Rockville, Maryland, USA), directed against the CD1a antigen of DC (11); L25, a MoAb directed against B cells and DC (12) was kindly provided by Dr T. Takami (Gifu, Japan); HLA-DR (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service

Table 1. Patient and control subject characteristics

Patient no.	Sex	Age (year)	Baseline FEV_1 % predicted	After 2.5 years FEV_1 % predicted	Baseline PC_{20} mg/mL	After 2.5 years PC_{20} mg/mL
			Corticosteroid group			
1	F	32	51.8	56.4	0.17	0.10
2	M	61	84.0	98.7	0.94	5.38
3	F	44	75.0	88.5	0.06	0.45
4	F	60	59.7	90.3	0.06	0.96
5	F	45	49.2	69.6	0.05	0.21
Median		45	59.7	88.5	0.06	0.45
			Bronchodilator group			
1	M	32	70.4	51.7	0.79	0.13
2	M	38	48.6	31.2	0.03	0.01
3	F	43	54.3	53.4	0.03	0.04
4	M	44	63.5	66.3	0.02	0.05
5	M	24	61.5	54.7	0.19	0.58
6	M	50	65.1	62.7	0.28	0.18
7	M	57	81.6	42.3	0.14	0.04
8	F	30	100.0	96.4	4.72	0.42
9	M	26	38.3	60.0	0.24	0.40
10	M	42	48.7	56.5	0.24	0.87
11	F	38	57.0	43.6	0.13	0.06
Median		38	61.5	54.7	0.19	0.13
			Control subjects			
1	M	19	102			
2	M	23	109			
3	M	23	109			
4	F	24	103			
5	F	23	96			
6	M	23	88			
7	M	52	88			
8	F	35	110			
Median		23	103			

(CLB), Amsterdam, the Netherlands), and biotin-conjugated HLA-DR MoAb (Becton Dickinson, San Jose, CA, USA). The MoAb staining was detected by the immuno-alkaline phosphatase anti-alkaline phosphatase (APAAP) method.

The sections were fixed in acetone for 10 min at 20°C, rinsed in phosphate-buffered saline (PBS, pH 7.2) and placed in a half-automatic stainer (Shandon, Pittsburgh, PA, USA). In this stainer the slides were sequentially incubated with bovine serum albumin (BSA) 2% in PBS for 10 min, incubated with normal rabbit serum (CLB, Amsterdam, the Netherlands) for 10 min and incubated with the MoAb in the optimal dilution for 30 min at 20°C. The optimal dilutions were determined by previous titration studies of the specific MoAb (OKT6 10 μg/mL; L25 1 μg/mL and HLA-DR 0.1 μg/mL). The sections were subsequently rinsed in PBS for 5 min and incubated for 30 min with a rabbit anti-mouse (RaM) (1:20) immunoglobulin antiserum, rinsed in PBS, incubated with APAAP (1:40) (Dakopatts, Glostrup, Denmark) for 30 min at 20°C, rinsed in PBS and TRIS buffer (pH 8.0), and incubated for 30 min with New Fuchsin substrate (Chroma, Stuttgart, Germany), which stained positive cells red. Finally, the sections were rinsed with distilled water, counterstained with Mayer's haematoxylin, and mounted in glycerin gelatin. Control staining was performed by substitution with PBS and incubation with an irrelevant MoAb of the same isotype and concentration.
To establish that $L25^+$ DC were also expressing HLA-DR we performed a double-labelling with L25 and biotinylated HLA-DR. The procedure followed the APAAP protocol up to the incubation with APAAP for 30 min. After rinsing in PBS and blocking with normal mouse serum (1:10) for 10 min, the slides were incubated for 45 min with the second MoAb biotinylated HLA-DR. Slides were rinsed in PBS and incubated with streptavidine - β galactosidase (Biogenex, San Ramon, CA, USA) for 45 min, and rinsed again with PBS. Slides were sequentially incubated with β galactoside (Serva, Heidelberg, Germany) which stained positive cells green, rinsed in TRIS buffer (pH 8.0) and stained with New Fuchsin, which stained $L25^+$ cells red. Finally, the sections were rinsed in distilled water and mounted in glycerin gelatin.

The immuno-double staining with L25 and HLA-DR was performed on three sections of three different patients, because of scarcity of biopsy material.

Quantification

Biopsies were coded and two sections 120 μm apart were counted in a blinded fashion for each antibody and each biopsy at a magnification of 10x40 by one person (G.M.M.) and the mean value was calculated. With an eye piece graticule the numbers of positively stained cells were counted in a zone 100 μm deep in the bronchial mucosa along the length of the epithelial basement membrane (BM), which had to be covered with epithelium over at least 500 μm. Cells were counted if they stained red and contained a nucleus. The cell counts were expressed as the number per unit (1 mm) of

basement membrane. The (biotinylated) HLA-DR expression was scored semiquantitatively on a 0-3 scale (0 = negative; 1 = weak; 2 = strong; 3 = very strong). Double-stained cells $L25^+$ and biotinylated $HLA\text{-}DR^+$ were counted if they stained violet and contained a nucleus.

Statistical analysis

The numbers of positive cells for most of the MoAb used showed a positive skewed distribution and therefore were analyzed using non-parametric statistics. Median cell counts from biopsies from asthmatics who received inhaled corticosteroids were compared with those who received only bronchodilators using the Mann-Whitney *U*-test. Median cell counts from biopsies from atopic asthmatics of the bronchodilator group were compared with median cell counts of the control subjects using the Mann-Whitney *U*-test. A value of $p<0.05$ was considered significant.

Table 2. Pulmonary function data of the patients included in the study

	Corticosteroid group	Bronchodilator group	P value
ΔFEV_1	14.7 (4.6-30.6)	-3.6 (-39.3-21.7)	0.009
Δlog_2PC_{20}	2.5 (-0.8-4.0)	-0.63 (-3.5-1.9)	0.013

ΔFEV_1 in % predicted: difference in median change (ranges) of FEV_1 % predicted from baseline to 2.5 years.
Δlog_2PC_{20} in mg/mL: difference in median change (ranges) of log_2PC_{20} mg/mL to histamine from baseline to 2.5 years.

RESULTS

Pulmonary function data

Details of patient and pulmonary function data have been described before (18). In short:
2.5 years of inhaled BDP resulted in a significant improvement in FEV_1 of 14.7% of predicted as compared with bronchodilator group (-3.6% of predicted, $p=0.009$) (Table 2). Log_2PC_{20} in the corticosteroid group improved 2.5 dose steps, compared with - 0.63 dose steps in the bronchodilator group ($p=0.013$) (Table 2).

Bronchial epithelium

Some sections could not quantitatively be evaluated for bronchial epithelial infiltration because they did not show enough intact epithelium (Table 3). In the sections of the asthmatic patients who received

bronchodilators only (n=7) the epithelium consisted for approximately 60% of a monolayer and for 10% of a multilayer of partially stratified cuboidal epithelium; the epithelium of the group of corticosteroid-treated patients consisted for approximately 45% of a monolayer and for 40% of a multilayer of cuboidal epithelium. The loss of epithelium from the BM (shedding) was 30% of the total length of epithelium in the bronchodilator group whereas it was 15% in the corticosteroid group.

Though there were clearly dendritic-shaped CD1a$^+$ cells expressing MHC class II in the epithelium of all the groups studied, differences between groups did not reach statistical significance (Table 3).

Table 3. Median cell counts (ranges) in bronchial epithelium and mucosa in atopic asthmatics after 2.5 yrs treatment and in control subjects per mm of basement membrane

	Corticosteroid group	Bronchodilator group	Control subjects	*P value	**P value
			Bronchial epithelium		
CD1a$^+$DC	0 (0) n=4	0.4 (0-5.3) n=10	0 (0-3.6) n=7	0.106	0.601
L25$^+$cells	2.5 (0-10.4) n=4	0.5 (0-9.6) n=8	1.0 (0-3.2) n=5	0.461	1
HLA-DR†	0(0-3.0) n=5	1 (0-2.0) n=10	1 (0-2.0) n=6	0.594	0.635
			Bronchial mucosa		
CD1a$^+$DC	0.7 (0-1.4) n=4	2.5 (1.1-6.7) n=10	0 (0-4.0) n=7	0.008	0.003
L25$^+$cells	5.7 (1.6-16.3) n=4	10.3 (6.7-13.6) n=8	0.5 (0-1.7) n=5	0.282	0.002
HLA-DR†	1.0(0-2.0) n=5	2.0 (0-3.0) n=10	1.5 (1.0-2.0) n=6	0.028	0.042

† Median score (ranges).
* P Corticosteroid group compared with bronchodilator group.
** P Bronchodilator group compared with controls.

Bronchial mucosa

The bronchial mucosa of the asthmatic patients consisted of a subepithelial cell-rich layer and a deeper cell-poor layer. Higher numbers of CD1a$^+$ DC were found in the bronchial mucosa of patients of the bronchodilator group (p=0.008) (Figure 1). In addition, the numbers of L25$^+$ cells (Figures 1 and 2) and the expression of MHC class II (Figure 1) in the bronchodilator group were higher than in the healthy controls (Table 3). The double-staining of HLA-DR and L25 showed that virtually all L25$^+$ DC

expressed HLA-DR. The HLA-DR expression was not only confined to cells in the mucosa showing an irregular outline with marked cytoplasmic extensions (DC), but there were also other cells like lymphocytes and macrophages positive for HLA-DR.

The numbers of $CD1a^+$ DC were significantly lower in the corticosteroid-treated group than in the bronchodilator group ($p=0.008$) (Figure 1). The number of $L25^+$ cells in the corticosteroid group was also lower (median 5.7/mmBM) than in the bronchodilator group (median 10.3/mmBM), but this difference did not reach significance ($p=0.282$) (Table 3). However, a significantly lower MHC class II expression was found in the corticosteroid group compared with the bronchodilator group (Figure 1) ($p=0.028$).

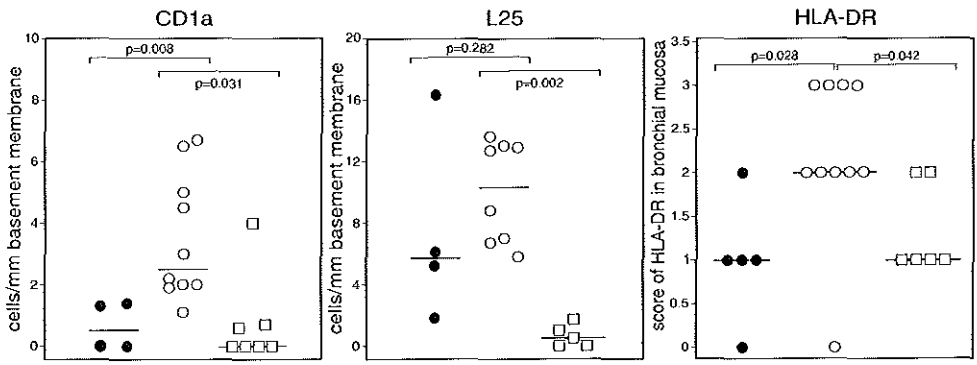

Figure 1. Individual counts for cells expressing (a) $CD1a^+$ DC and (b) $L25^+$ DC in the bronchial mucosa, expressed as the number of positive cells/mm basement membrane and individual score of (c) HLA-DR expression in the bronchial mucosa. Median values are represented by the horizontal bars.

● = Corticosteroid. ○ = Bronchodilator. □ = Control subjects.

DISCUSSION

In this study we have observed that the numbers of DC in the bronchial mucosa of atopic asthmatic patients are significantly higher than in healthy control subjects and that 2.5 years of inhaled corticosteroid therapy downregulate the number of DC to normal levels, accompanied by a significantly improved FEV_1 and reduced hyperresponsiveness.

Our data of increased numbers of $CD1a^+$DC in atopic asthma are in agreement with data of a study of Bellini *et al.* (22) who also showed increased numbers of intraepithelial and mucosal $CD1a^+$ DC in atopic asthmatics as compared to nonasthmatic atopic controls sensitized to the same allergen. Furthermore, an increased frequency of DC has been reported in the nasal mucosa of patients with atopic rhinitis during the pollen season

(23).

Dendritic cells in the epithelium of the airways form a network (24,25). For primary responses they are strategically positioned to pick up inhaled antigens, migrate to regional lymph nodes and present the inhaled antigens to lymphnode T cells. For secondary responses in the mucosa local antigen presentation by DC to T cells and local activation of T cells are thought to occur as well (26). In asthma it has been suggested that DC play a prime role in inducing cytokine production by the local activation of T cells (27,28). In this way they are thought to act as important pro-inflammatory cells. It has indeed been demonstrated both *in vivo* (29) as well as *in vitro* (22,30) that DC are able to activate Th2 cells. The mechanisms by which DC induce the expansion of Th2 cells are still unclear, but some consider - despite the low production of cytokines by DC - the secretion of interleukin-1, a co-stimulator for Th2 cells, as important.
DC might, however, also induce Th1 expansion (31). *In vitro* IL-12 production by DC favours such Th1 expansion, as observed by secretion of interferon-gamma (IFN-γ) and immunoglobulin G2a (IgG2a) humoral responses (32).

Biopsy studies in patients with asthma have confirmed that inhaled corticosteroids reduce the number and activation of several inflammatory cells (33-36). Although this study was carried out on a limited number of patients, our data show that clinical improvement induced by local BDP administration is accompanied by significantly lower numbers of $CD1a^+$ DC and $HLA\text{-}DR^+$ cells in the bronchial mucosa. The here described inhibitory effects of 2.5 years of corticosteroids on DC numbers and HLA-DR expression, are in agreement with data of Burke *et al.* who described a reduction in $RFD1^+$ DC and HLA-DR expression after 3 months of budesonide in asthma (13). Thus, long-term treatment with inhaled corticosteroids also reduces the number of DC in the bronchial mucosa. Studies in the nasal mucosa of patients with atopic rhinitis showed that 3 months of fluticasone therapy decreases the $CD1a^+$ and $HLA\text{-}DR^+$ cells (37).

It may be that chemoattractive factors such as leukotriene B_4 (38), monocyte chemoattractant protein 1 (39), and substance P play a role in attracting DC into the epithelium in allergic inflammation. Substance P, secreted by C-fibres was found to be a highly effective chemoattractant for DC (40) and in this way neurogenic inflammation might contribute to the DC migration. Interestingly, corticosteroids have been reported to suppress the release of chemotactic factors from epithelial cells, indicating a possible important role of the epithelium in DC kinetics (38) under such circumstances.

In conclusion, our data show that DC numbers are elevated in atopic asthma, and can be normalized by local corticosteroid therapy. We suggest

that DC may play an important role in the initiation and propagation of the asthmatic inflammation because DC are situated strategically in bronchial mucosa and DC have been described as the most potent APC population for T cell stimulation. In this way, reduction of DC numbers after 2.5 years of corticosteroid therapy may result in a downregulation of T cell activation and hence affect allergic airway inflammation.

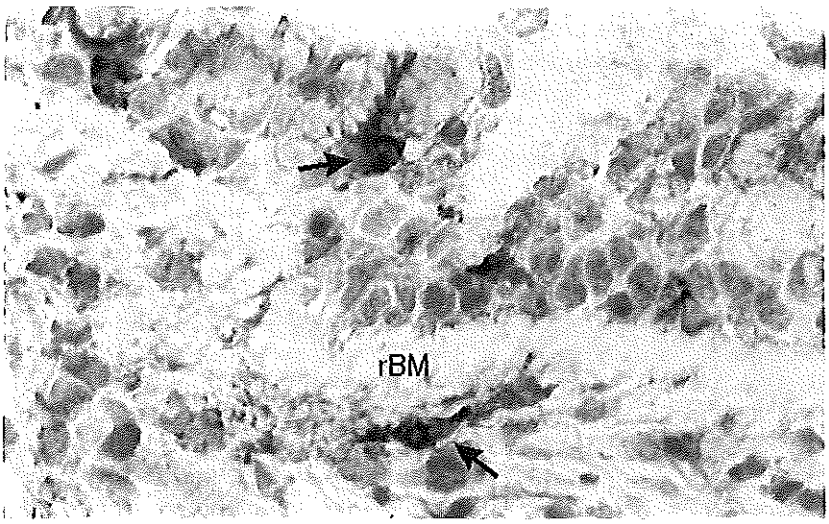

Figure 2. Cryostat section of a bronchial mucosal biopsy of an asthmatic patient. $L25^+$ dendritic-shaped cells in the bronchial epithelium and mucosa, situated just under the rBM (arrows).

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CHAPTER 3.2

FLUTICASONE PROPIONATE THERAPY IS ASSOCIATED WITH MODULATION OF DENDRITIC CELLS IN THE BRONCHIAL MUCOSA OF ATOPIC ASTHMATICS*

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* *Submitted for publication*

ABSTRACT

Background: Dendritic cells (DC) are professional antigen-presenting cells (APC) and potent stimulators of resting T cells. Moreover DC express the high affinity receptor for IgE (FcεRI) and membrane bound IgE, instruments for IgE mediated antigen presentation. DC are therefore considered to play an important role in the initiation and propagation of allergic immune responses in the lung.
Objective: We examined (1) the distribution of DC in the bronchial epithelium and lamina propria of 24 atopic asthmatics and 8 non-asthmatic controls using immunohistochemistry and (2) the effects of 3 months of an inhaled glucocorticoid (fluticasone propionate (FP)) on the numbers of DC.
Methods: Bronchial biopsy specimens were taken from 8 control subjects and 24 subjects with atopic asthma before and after 3 months of treatment with FP, 1000 μg daily, or matched placebo in a randomized double-blind parallel group study. Sections (6μm) were stained for CD1a, L25, HLA-DR, IgE, and the high affinity receptor for IgE (FcεRI).
Results: There were higher numbers of $CD1a^+$ DC ($p = 0.025$), $L25^+$ DC ($p = 0.008$), IgE^+ cells ($p = 0.0003$) and a higher HLA-DR expression ($p = 0.057$) in the lamina propria of asthmatics compared with non-asthmatic controls. When the treated ($n = 11$) and placebo ($n = 13$) groups were compared, there was a decrease in hyperresponsiveness (PC_{20} methacholine) ($p = 0.001$) after FP. This was accompanied by a decrease in bronchial mucosal $CD1a^+$ DC ($p = 0.018$), a reduction in HLA-DR expression ($p = 0.008$), numbers of IgE^+ cells ($p = 0.010$), but not by a change in number of cells expressing FcεRI. Electron microscopic studies showed that electronlucent DC in the epithelium contained the characteristic intracytoplasmic Birbeck granules.
Conclusion: This study shows that there are significantly more DC in the bronchial mucosa of asthmatics than in non-asthmatic controls and that fluticasone propionate therapy of asthmatics is associated with a reduction in the numbers of DC.

INTRODUCTION

Dendritic cells (DC) are professional antigen-presenting cells (APC) in the pulmonary tissue (1) and in contrast to other APC unique in their capacity to activate resting naive T cells (2-4). In the lung, DC are predominantly situated in epithelial and subepithelial tissue of the bronch(iol)us and in the bronchus-associated lymphoid tissue (5).

DC are characterized by their high constitutive expression of major histocompatibility complex (MHC) class II (HLA-DR) antigens (6-8), accessory

molecules (intercellular adhesion molecule 1(CD54), B7-1(CD80), B7-2(CD86)), long cytoplasmic extensions, the absence or a juxtanuclear spot of acid phosphatase, and the presence of intracytoplasmic structures termed Birbeck granules (Bg). A combination of dendritic morphology and expression of CD1a and L25 are characteristic for DC (9,10).

It is now known that a varying proportion of Langerhans' cells (LC), the representatives of the DC in the skin, carries surface immunoglobulin E (IgE) (11,12). There is evidence that, this membrane IgE not only can capture antigen, but also that DC use the IgE for antigen presentation (13). It is also clear that the IgE-binding capacity of these cells is in fact due to the membrane bound expressing of a high affinity receptor for IgE (FcεRI) (14,15). FcεRI has been traditionally described on basophils and mast cells (16). More recently, the FcεRI has also been found on monocytes of atopic dermatitis patients (17) and on eosinophils in parasitic infections (18).

Atopic asthma is associated with a chronic T cell-mediated inflammatory process in the airways (19,20). Since T lymphocytes cannot respond to antigens without the help of APC (21) and DC are superb APC, strategically positioned in the bronchial mucosa and carrying FcεRI and IgE on their membrane, it is likely that pulmonary DC play a key role in the initiation and propagation of asthma. In an earlier study we found increased numbers of $CD1a^+$ DC and increased expression of MHC class II in bronchial mucosal biopsies from asthmatics (22).

Glucocorticoids are by far the most effective therapy for asthma, but their precise mechanism of action is still not certain (23). Glucocorticoids inhibit cytokine gene transcription and cytokine effects, thereby reducing chronic inflammation in asthma (23-26). Fluticasone propionate (FP) is a new topically-active glucocorticoid with a high affinity for the human glucocorticoid receptor and a negligible oral bioavailibility (27). To date, the effects of inhaled FP on DC in asthma are unknown. We hypothesized that one of the beneficial effects of glucocorticoid therapy of asthmatics might be associated with a reduction in the numbers of DC and other IgE-bearing cells, resulting in a decreased local T cell stimulation.

Our aim, therefore, was to evaluate the number of DC, the expression of MHC class II, the number of membrane bound IgE^+ cells, and of FcεRI expression in the bronchial epithelium and the lamina propria in biopsy specimens from a group of asthmatics before and after 3 months FP therapy and from a group of non-asthmatic controls.

MATERIALS AND METHODS

Patients and non-asthmatic controls

Bronchial mucosal biopsy specimens were obtained from 24 non-smoking atopic asthmatic subjects (six women and eighteen men with a median age of 26 years, range 21 to 56 years). Asthma was defined as a history of episodic wheezing and reversible airway obstruction characterized by an increase in forced expiratory volume in one second (FEV_1) of ≥ 9 % after inhalation of 1000 µg terbutaline. The asthmatic subjects had a mean FEV_1 of 84.0 ± 16.1 % of the predicted value; the mean of the ^{2}logs of the provocative concentrations of inhaled methacholine required to reduce their FEV_1 by 20% (PC_{20}) was 0.32 ± 2.94 mg/ml. Atopy was defined by one or more positive skin-prick tests to extracts of 16 common aeroallergens. All patients were receiving inhaled ß-agonists, and none had taken oral or inhaled corticosteroids in the month prior to the study. After a run-in period of 2 weeks, during which time they took inhaled ß-agonists only, spirometry was performed and methacholine PC_{20} was measured according to the standardized 2 min tidal breathing technique (28), followed by the first fiberoptic bronchoscopy. Patients were randomized in a double-blind fashion to receive 3 months FP, 500 µg or matched placebo twice daily, besides their inhaled ß-agonists. After 3 months patients reattended the hospital for repeat measurement of spirometry and bronchial responsiveness, followed by a second fiberoptic bronchoscopy one week later. All measurements of PC_{20} and both fiberoptic bronchoscopies were performed at the same time of day. There were no significant differences at baseline between the FP and the placebo group with regard to FEV_1 and PC_{20}.

A control group was composed of eight non-smoking non-asthmatic subjects (three women and five men with a median age of 23 years, range 19 to 52 years). All controls had a PC_{20} histamine of more than 8 mg/ml and a median FEV_1 of 103 (88-110)% of the predicted value. Patient and control characteristics are shown in Table 1. The study was approved by the local Ethics Committee and all participants gave their written informed consent.

Bronchial biopsy and tissue processing

Bronchial biopsy specimens were taken from the carinae of the lingula or the right upper, middle or lower lobes via an Olympus BF IT 10 fiberoptic bronchoscope (Tokyo, Japan) using alligator forceps, Olympus FB 15C by the same operator (S.E.O.). For light microscopic examination, each biopsy specimen was immediately placed in isotonic saline and frozen within 20 min in Tissue-Tek II OCT embedding medium (Miles, Naperville, IL, USA).

Table 1. Patient and control subject characteristics

Patient no.	Sex	Age (years)	Baseline FEV_1 % predicted	After 3 months FEV_1 % predicted	Baseline $^2log\ PC_{20}$ mg/ml	After 3 months $^2log\ PC_{20}$ mg/ml
			Fluticasone group			
1	F	26	90.2	115.0	0.77	5.50
2	M	51	84.3	96.0	4.12	4.99
3	M	21	93.2	97.8	2.40	8.0
4	M	24	90.3	93.2	-0.91	3.40
5	M	23	77.7	84.0	0.12	2.06
6	M	23	104.4	107.6	2.85	6.72
7	M	21	99.2	103.2	-5.18	0.15
8	M	42	55.9	63.7	0.25	-0.18
9	F	20	90.5	90.8	-0.06	2.08
10	M	43	67.3	62.5	3.43	8.00
11	M	47	81.4	87.3	-1.05	6.85
Median		24 (21-51)	90.2 (55.9-99.2)	93.2 (62.5-115)	0.25 (-5.18-4.12)	4.99 (-0.18-6.72)
			Placebo group			
1	F	44	80.0	81.0	0.58	5.50
2	M	52	62.8	58.9	1.47	4.99
3	M	41	87.4	83.2	-1.83	8.00
4	M	55	60.4	61.8	-2.34	3.40
5	M	55	72.1	72.3	3.57	1.06
6	M	22	92.1	86.8	-0.52	-1.53
7	M	56	84.2	79.0	3.40	1.93
8	F	21	104.6	106.1	-3.42	-1.80
9	F	26	96.7	95.1	-2.56	-0.58
10	M	24	46.8	21.3	0.04	1.19
11	M	32	91.4	101.9	0.64	1.41
12	F	26	94.6	92.4	7.98	2.86
13	M	23	108.3	85.3	-3.89	-1.10
Median		32 (21-56)	87.4 (46.8-108.3)	83.2 (21.3-106.1)	-0.52 (-3.89-7.89)	0.58 (-2.34-2.86)
			Control subjects			
1	M	19	102			
2	M	23	109			
3	M	23	109			
4	F	24	103			
5	F	23	96			
6	M	23	88			
7	M	52	88			
8	F	35	110			
Median		23 (19-52)	103 (88-110)			

Samples were stored at -80° C until use. Frozen sections (6μm) were cut on a Reichert-Jung 2800 Frigocut cryostat. Two sections from each biopsy were placed on poly-L-lysine -coated (Sigma, Diagnostics, St Louis, MO, USA) slides, air dried and stored at -80°C until use.

For electron microscopic evaluation, tissue was fixed in 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 0-4°C for 24 hr, postfixed in 1% OsO_4 in the same buffer, dehydrated, and embedded in LX 112 resin (Zeiss, Weesp, The Netherlands). Ultrathin (40-60 nm) sections were examined using a Zeiss electron microscope 902 operating at 80 kV.

Immunohistochemistry

The following mouse IgG1 monoclonal antibodies (mAb) were applied at previously titrated optimal dilutions: OKT6 (10 μg/ml) (American Type Culture Collection, Rockville, MA, USA) directed against the CD1a antigen of DC[9]; L25 (0.1 μg/ml), directed against B cells and DC[10], kindly provided by Dr. T. Takami, Gifu, Japan; anti-CD83 (2 μg/ml), an IgG3 mAb reacting with HB 15, expressed by DC subsets (29) from Dr. T. F. Tedder, Dana-Farber Cancer Institute, Boston, MA, USA; S100 (21.6 μg/ml), a rabbit anti cow polyclonal antibody from Dakopatts, Glostrup, Denmark, which stains LC; anti-HLA-DR (0.01 μg/ml) from Becton Dickinson, San Jose, CA, USA; IgG2b anti-IgE (10 μg/ml) from Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands; and mAb 15-1 (anti-FcεRI), kindly provided by Dr. J.-P. Kinet (National Institutes of Health, Bethesda, MA, USA).

The mAb staining was detected by the immuno-alkaline phosphatase anti-alkaline phosphatase (APAAP) method. The sections were fixed in acetone for 10 min at 20°C, rinsed in phosphate-buffered saline (PBS, pH 7.2) and placed in a half-automatic stainer (Shandon, Pittsburgh, PA, USA). In this stainer the slides were sequentially incubated with bovine serum albumin (BSA) 2% in PBS for 10 min, incubated with normal rabbit serum (CLB) for 10 min and incubated with the mAb in the optimal dilution for 30 min at 20°C. The sections were subsequently rinsed in PBS for 5 min and incubated for 30 min with a rabbit anti-mouse (RaM) (1:20) immunoglobulin antiserum, rinsed in PBS, incubated with APAAP (1:40) (Dakopatts) for 30 min at 20°C, rinsed in PBS and TRIS buffer (pH 8.0), and incubated for 30 min with New Fuchsin substrate (Chroma, Stuttgart, Germany) as a chromogen. The sections were counterstained with Mayers hematoxylin. For staining with S100, the sections were incubated with normal goat serum (1:10) (CLB) for 10 min before incubation with the primary antibody, subsequently incubated for 30 min with biotinylated goat-anti-rabbit immunoglobulin antiserum (1:2) (Biogenex, San Ramon, CA, USA), rinsed in

PBS and incubated for 30 min with streptavidin-alkaline phoshatase (1:50) (Biogenex). The sections were rinsed in PBS, incubated with APAAP (1:40) and further processed as above.

Control slides were treated similarly, but in one case excluding the primary antibody and in the other using an unrelated antibody of the same isotype and protein concentration.

Quantification

Biopsies were coded and two sections were counted in a blinded fashion for each antibody and each biopsy at a magnification of 10x40 by one person (G.M.M.) and the mean value was calculated. With an eye piece graticule the numbers of positively stained cells were counted in the epithelium and in a zone 100 μm deep in the lamina propria along the length of the epithelial basement membrane (BM), which had to be covered with epithelium over at least 500 μm.

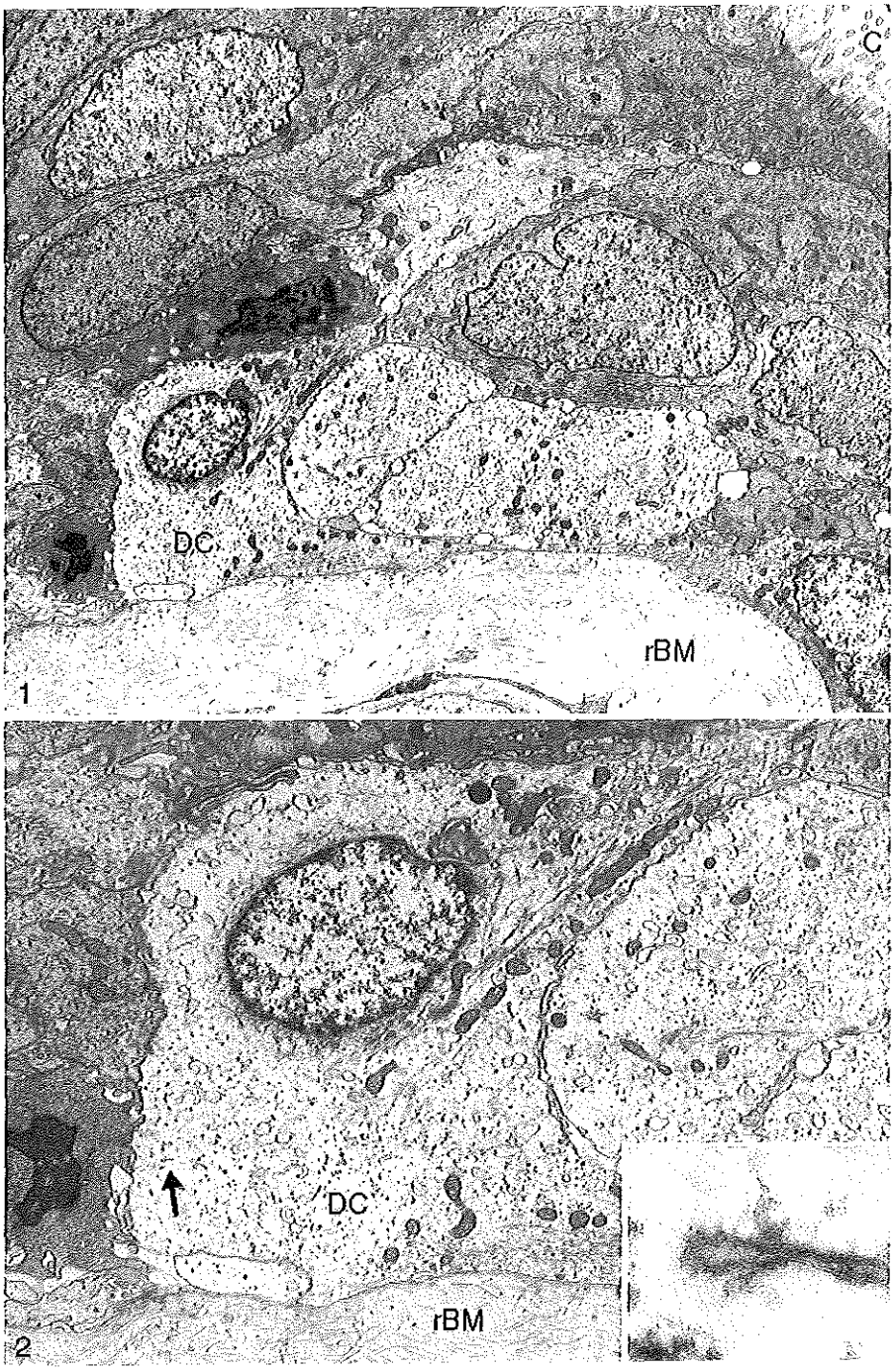
Cells were counted if they stained red and contained a nucleus. The cell counts were expressed as the number per unit (1 mm) of basement membrane. Since the HLA-DR expression was not only confined to inflammatory cells, HLA-DR expression was scored semiquantitatively on a 0-3 scale (0 = negative; 1 = weak; 2 = strong; 3 = very strong).

Statistical analysis

The median cell numbers showed a positively skewed distribution and therefore were analyzed using non-parametric statistics. There were no significant differences at baseline between the FP and the placebo group with regard to the profiles of the inflammatory cell types and the expression of HLA-DR. Comparison of treatment effects between Fluticasone and placebo was performed by Mann-Whitney U test on percentual changes (after treatment minus before treatment divided by before treatment, multiplied by 100%). Median cell counts from the biopsy specimens from the asthmatic subjects taken before treatment were compared with median cell counts of the control subjects using the Mann-Whitney U test. A value of $p<0.05$ was considered significant. The staining with two DC-markers (S100 and CD83) was performed on a few sections, because of scarcity of biopsy material; statistical analysis of these data was not attempted.

Figure 1. Electron microscopical ultrathin section of the bronchial mucosa showing dendritic cells (DC), close to the basement membrane (BM) in the basal layers of the epithelium. Cilia (C). x 1,100.

Figure 2. Dendritic cell in the bronchial epithelium, which contains a Birbeck granule (*arrow*) (see inset, x 50,000). x 3,000.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RESULTS

Pulmonary function data

Three months of FP treatment resulted in a significant improvement in FEV_1 of 6.06% of predicted as compared with placebo group (-4.33% of predicted, p=0.009) (Table 2). $^2LogPC_{20}$ in the FP group improved 3.71 dose steps, compared with 0.04 dose steps in the placebo group (p=0.001) (Table 2).

Table 2. Pulmonary function data of the patients included in the study

	Fluticasone group	Placebo group	P value
ΔFEV_1	6.06 ± 7.50	-4.33 ± 9.77	0.009
$\Delta^2 log\ PC_{20}$	3.71 ± 2.38	0.039 ± 2.18	0.001

ΔFEV_1 in % predicted: difference in mean change ± SD of FEV_1 % predicted from baseline to 3 months.
$\Delta^2 log\ PC_{20}$ in mg/ml: difference in mean change ± SD of $^2log\ PC_{20}$ mg/ml to methacholine from baseline to 3 months.

Bronchial epithelium

Some of the sections could not quantitatively be evaluated for bronchial epithelial distribution of DC because they did not show (enough) intact epithelium, this has been indicated in Table 3. Nevertheless in the cryostat sections a significantly raised number of $CD1a^+$ DC was present in the basal and middle layers of the epithelium of asthmatics compared to non-asthmatic controls (p= 0.047) (Table 3). There were also higher numbers of $L25^+$ DC in asthmatics, but this difference did not reach statistical significance (p=0.065) (Table 3). Many of these DC were also positive for CD83 and S100.

Electron microscopically, DC could easily be recognized in the epithelium of asthmatics, by their electron-lucent cytoplasm and marked cytoplasmic extensions between adjoining epithelial cells (Figure 1). Birbeck granules (Bg) were observed in almost all of these epithelial DC (Figure 2).

Though there were clearly dendritic-shaped $CD1a^+$ and $L25^+$ DC in the epithelium of both groups studied, differences of treatment effects between FP and placebo did not reach statistical significance (Table 4).

Table 3. Median cell counts (ranges) in bronchial epithelium and lamina propria in atopic asthmatics and in non-asthmatic controls per mm of basement membrane

	Asthmatics	Non-asthmatics	P value
Bronchial epithelium			
$CD1a^+$ DC	1.7 (0, 9.1) *n=19*	0 (0, 3.6) *n=8*	0.047
$L25^+$ DC	5.1 (0, 8.0) *n=6*	1 (0, 3.2) *n=5*	0.065
HLA-DR†	0.9 (0, 2.0) *n=20*	1.25 (0, 2.0) *n=6*	0.107
IgE^+ cells	0.8 (0, 6.4) *n=17*	0 (0, 1.5) *n=6*	0.159
$Fc\varepsilon RI^+$ cells	0 (0, 1.2) *n=20*	3.2 (0, 5.7) *n=5*	0.132
Lamina propria			
$CD1a^+$ DC	1.7 (0, 7.0) *n=19*	0 (0, 4.0) *n=8*	0.025
$L25^+$ DC	5.0 (1.7, 8.0) *n=6*	0.5 (0, 1.7) *n=5*	0.008
HLA-DR†	2.0 (0, 3.0) *n=20*	1.5 (1.0, 2.0) *n=6*	0.057
IgE^+ cells	7.1 (2.1, 12.7) *n=17*	0 (0, 2.0) *n=6*	0.0003
$Fc\varepsilon RI^+$ cells	3.2 (0, 11.7) *n=20*	2.0 (1.2-2.8) *n=5*	0.102

† Median score (ranges)

Lamina propria

The numbers of $CD1a^+$ DC, $L25^+$ DC and membrane bound IgE^+ cells, found in the lamina propria of patients with asthma, were significantly higher than in controls (Table 3). These IgE^+ cells included dendritic cells as

well as mast cells and an occasional plasma cell, as observed in double-staining experiments (data not shown).

Staining with anti-HLA-DR was not confined to cells showing an irregular outline with cytoplasmic extensions (DC), as other cells likebronchial epithelial cells, activated lymphocytes and endothelial cells were also positive for HLA-DR. HLA-DR expression in the lamina propria of asthmatics was also increased as compared to controls, but only with a marginal significance ($p = 0.057$).

Although the number of cells expressing FcεRI in asthmatics also tended to be higher as compared to controls, this difference did not reach appreciable significance.

Table 4. Median cell counts (ranges) in bronchial epithelium and lamina propria per mm basement membrane before and after Fluticasone or Placebo treatment*

	Fluticasone group			Placebo group			P value
	Before	After	Median difference	Before	After	Median difference	
Bronchial epithelium							
CD1a$^+$ DC	1.5	0	-1.2 (-2.6, -0.4) *n=3*	2.1	1.6	-1.2 (-1.5, 0.6) *n=4*	0.079
IgE$^+$ cells	0	0	0 (-2.1, 0) *n=6*	1.1	1.1	0 (-2.7, 0.4) *n=1*	-
HLA-DR†	0.3	0	-0.5 (-0.7, 0) *n=3*	0.5	1.0	0.7 (-0.5, 1.0) *n=6*	0.794
FcεRI$^+$ cells	0	0	0 (0, 0.6) *n=6*	0	0	0 (-1.1, 1.2) *n=6*	-
Lamina propria							
CD1a$^+$ DC	3.1	0	-2.3 (-7.0, -1.6) *n=4*	3.8	1.4	-1.8 (-3.0, 0) *n=4*	0.018
IgE$^+$ cells	2.9	1.9	-2.7 (-5.1, -0.5) *n=5*	7.3	6.5	1.0 (-2.1, 2.7) *n=6*	0.010
HLA-DR†	2.0	1.0	-1.0 (-2.5, -0.5) *n=5*	1.8	2.0	0 (-1.3, 1.0) *n=8*	0.008
FcεRI$^+$ cells	2.2	1.3	-1.2 (-11.7, 1.3) *n=4*	3.6	2.2	-2.5 (-3.6, -0.1) *n=7*	0.923

* Comparison of treatment effects between Fluticasone and Placebo was performed by the Mann-Whitney U test on percentual changes ($\frac{\text{after treatment minus before treatment}}{\text{before treatment}} \times 100\%$)

† Median score (ranges)

The numbers of $CD1a^+$ DC and IgE^+ cells were significantly decreased in the glucocorticoid-treated group compared to the placebo group. No significant change in expression of FcεRI was seen after treatment (Table 4). There was a significant reduction in the score of MHC class II expression in the glucocorticoid group as compared to the placebo group.

DISCUSSION

In this double-blind placebo controlled study we firstly have shown that in the bronchial mucosa of asthmatics there are raised numbers of $CD1a^+$ and $L25^+$ DC, and of IgE^+ cells. Secondly, we have shown that a decreased bronchial hyperresponsiveness after 3 months FP treatment was accompanied by a decrease in the numbers of $CD1a^+$ DC, a reduction in MHC class II expression, and a reduction in the number of IgE^+ cells in the bronchial mucosa of subjects with atopic asthma.

The finding of Bg in the putative DC in the bronchial epithelium definitely shows them to be DC.

The DC network within the epithelium of the airways is strategically positioned to pick up inhaled antigens/allergens (30). For primary sensitization of naive T cells, DC are able to migrate to regional lymph nodes and present the inhaled antigens to lymph node T cells. For secondary responses in the mucosa, local antigen presentation by DC to T cells and local activation of T cells are thought to occur as well (31). The data showing increased numbers of $CD1a^+$ and $L25^+$ DC in atopic asthma are in agreement with our earlier findings (22) and with data of others, in which higher numbers of intraepithelial and mucosal $CD1a^+$ DC in atopic asthmatics were described as compared to non-asthmatic atopic controls sensitized to the same allergen (32). In the nasal mucosa, DC and HLA-DR expression have been found to increase during symptomatic atopic rhinitis (33).

The increased number of cells bearing IgE in the lamina propria of asthmatics, of which the majority are DC and mast cells, likely contributes to an enhanced IgE-mediated antigen/allergen presentation. It has been proposed by Mudde et al (34) that IgE, bound to the FcεRI on the surface of APC or mast cells plays a role in antigen capturing before antigen processing and presentation. Although IgE is able to bind to mast cells, and to mediate antigen-induced degranulation, its role in maintaining chronic asthma is not yet fully understood. Increased expression of the high affinity receptor on monocytes of atopic dermatitis patients has been described (17). In our study the FcεRI expression in the lamina propria of asthmatics was upregulated as compared to controls, although not significant, probably due to the small number of control subjects.

Biopsy studies in patients with asthma have confirmed that

glucocorticoids reduce the number and activation of several inflammatory cells (35-37). Although this study was carried out on a limited number of patients, our hypothesis that inhaled FP is able to downregulate the numbers of airway DC, was clearly supported by our results.

The described inhibitory effects of 3 months of FP on DC numbers and HLA-DR expression, are consistent with our previous findings indicating downregulation of DC and MHC-class II expression by 2.5 years beclomethasone therapy (22) and are in agreement with a study, which showed a reduction in RFD1 $^{+}$ DC and HLA-DR expression after three months of budesonide in asthma (37). In the present study, we found a considerable decrease in the number of CD1a^{+} DC in the lamina propria during FP therapy (median numbers from 3.1 to 0) and this decrease was larger than that reported in the study with 400 µg budesonide treatment for three months (38). Similar studies in the rat by Holt *et al.* (39) have demonstrated that exposure to inhaled and systemic glucocorticoids leads to a rapid decrease in the number of airway intraepithelial DC, and in their MHC-class II expression. Studies in the nasal mucosa of patients with perennial allergic rhinitis showed a significant reduction in the number of CD1a^{+} cells and HLA-DR expression after 3 months of fluticasone therapy (40).

The precise mechanisms by which glucocorticoids downregulate DC, remain to be established. One possible mode of action is that glucocorticoids downmodulate the precursor recruitment from the circulation (39). For instance by suppressing the release of chemotaxins by bronchial epithelial cells, like monocyte chemoattractant protein 1 (41) and granulocyte macrophage colony stimulating factor (GM-CSF), which are involved in the recruitment and differentation of DC. The downregulation of DC by glucocorticoid therapy is probably associated with, and presumably dependent on, a decreased accessory function *in vivo*, since we showed previously that glucocorticoids are able to suppress the T cell stimulatory function of airway DC *in vitro* (42).

In conclusion, our data show that 3 months of topically-active glucocorticoid therapy downregulate DC and MHC class II expression in the bronchial mucosa of atopic asthmatics.
Since DC play an important role in the initiation and propagation of the immune response, the decrease in DC numbers and their functional activity by glucocorticoids likely results in a downregulation of T cell activation.

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CHAPTER 4

CYTOKINE EXPRESSION IN ASTHMA

4.1 Immunolocalization of interleukin 4 in eosinophils in the bronchial mucosa of atopic asthmatics
Am J Respir Cell Mol Biol 1996 (in press)

4.2 Ultrastructural immunogold localization of interleukin 5 to the crystalloid core compartment of eosinophil secondary granules in patients with atopic asthma
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CHAPTER 4.1

IMMUNOLOCALIZATION OF INTERLEUKIN 4 IN EOSINOPHILS IN THE BRONCHIAL MUCOSA OF ATOPIC ASTHMATICS*

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ABSTRACT

Several studies have shown that human eosinophils can synthesize and release a number of cytokines. The aim of this study was to investigate whether eosinophils contain interleukin (IL)-4 protein. We examined the ultrastructural localization of IL-4 in eosinophils in bronchial mucosal biopsies of twenty-nine non-smoking atopic asthmatics and seven controls. No eosinophils were detected in the bronchial mucosa of controls. In the eosinophils ($n=42$) of the asthmatics IL-4 was localized to the electron-dense crystalloid core compartment of 85% of the secondary or specific eosinophil granules ($n=468$). Other structures in the eosinophils were unlabeled. Control sections, incubated with an irrelevant primary antibody were negative. This study demonstrates that (pre-formed) IL-4 is stored in the secondary eosinophil granules. These results were extended by light microscopic immunodouble-staining for IL-4 protein and eosinophil cationic protein, which showed that a subpopulation of (activated) eosinophils express IL-4 immunoreactivity in bronchial mucosal biopsies of asthmatics as well as controls. These data indicate that eosinophils may be an important source of IL-4 in allergic inflammation.

INTRODUCTION

Asthma is characterized by chronic infiltration and activation of several types of inflammatory cells in the bronchial mucosa, particularly eosinophils and activated mast cells (1-3). Interleukin (IL)-4 and IL-5 are important immunoregulatory cytokines which appear to play a dominant role in the pathogenesis of asthma. IL-4 induces the isotype switching of B cells to IgE synthesis (4) and therefore contributes to the excessive IgE synthesis in atopic patients. Furthermore, IL-4 stimulates T cell proliferation, especially of the T helper 2 (Th2) phenotype (5) and increases the endothelial VCAM-I expression as well as the VLA-4 dependent selective accumulation of eosinophils in the lung (6). These cells are thought to play a critical role in epithelial cell damage and in mediating the airway inflammation. IL-5 promotes the differentiation (7), recruitment (8), activation and survival of the eosinophils (9).

Increased levels of IL-4 protein (10), and increased messenger RNA encoding IL-4 have been found in T cells recovered by bronchoalveolar lavage (BAL) of mild atopic asthmatics by *in situ* hybridization (11). IL-4 has been demonstrated in mast cells using immunoelectron microscopic analysis of mucosal samples taken from resected lung and nasal polyp tissues (12) and by immunohistochemistry studies with glycol methacrylate (GMA) embedded bronchial biopsies of asthmatics (13,14). Recently, IL-4 synthesis

by eosinophils in nasal polyp tissues and bronchial biopsies of asthmatics has been described (15). Taken together, the cellular sources of IL-4 include T cells, mast cells, basophils and as it seems eosinophils. However, the subcellular immunolocalization of IL-4 protein in eosinophils in the bronchial mucosa of atopic asthmatics and of controls has not been described so far.

Therefore, in the present study we examined the (ultrastructural) localization of IL-4 by immunoelectron microscopy and immunodouble-staining in bronchial mucosal biopsies of atopic asthmatics and controls.

MATERIALS AND METHODS

Bronchial biopsies

Bronchial biopsies were obtained by fiberoptic bronchoscopy (Olympus model BF IT Tokyo, Japan) by the same operator (S.E.O.) from the segmental divisions of the main bronchi of twenty-nine patients (9 women, 20 men median age 30 yrs, range 18-55) with mild atopic asthma who were on bronchodilators only for at least 3 weeks prior to the study and seven nonatopic controls (3 women, 4 men median age 28 years, range 23-52). All were nonsmokers. The diagnosis of asthma was based on a history of attacks of breathlessness and wheezing without chronic cough or sputum production, according to the criteria of the American Thoracic Society (16). Atopy was defined as a positive skin-prick test to at least one of 16 common aeroallergens. The mean FEV_1 was 82.0 (46.8-108.3) % predicted and the geometric mean provocative concentration of inhaled histamine required to reduce their FEV_1 by 20% (PC_{20}) was 0.87 mg/ml (0.03-4.72). The control subjects had a mean FEV_1 99.3 (88-110) % predicted and a PC_{20} histamine of more than 8 mg/ml. Due to the low frequency of eosinophils in the biopsies processed for immunoelectron microscopy, a selection was made on semithin sections of the twenty-nine patients. Five biopsies each of five different patients, containing eosinophils were further processed for immunoelectron microscopy. In the biopsies of the controls processed for immunoelectron microscopy, no eosinophils were seen. Because of scarcity of biopsy material, light microscopic immunodouble staining was carried out on only twelve patients and three controls. The study protocol was approved by the local Medical Ethics Committee and all participants gave their written informed consent.

Fixation and embedding for immunoelectron microscopy

Biopsies were immediately placed into ice-cooled isotonic saline and within 20 min fixed in 1 % acroleine plus 0.4% glutaraldehyde (in 0.1 M

phosphate buffer pH 7.2 at 0-4°C for 1-4 h). After fixation the biopsy samples were stored in a sucrose buffer (1 M sucrose in 1.1 M phosphate buffer pH 7.2 containing 1% paraformaldehyde) at 0-4°C. The samples were dehydrated and processed into Lowicryl K4M resin by the Progressive Lowering of Temperature technique. Polymerization took place under UV light at -40°C for 24h and at room temperature for an additional 48h. From the Lowicryl embedded material 1 µm sections were cut and stained with toluidine blue to select appropiate areas for ultrathin sectioning. The ultrathin sections (40-60 nm) were collected on carbon coated Formvar filmed mesh 200 copper grids.

Immunogold labelling to demonstrate IL-4

Grids were rinsed for 10 min in 0.14% glycine in 0.1 M PBS pH 7.2. The following sequence of steps was followed to stain the grids, by placing them into reagent drops: 10% normal goat serum in PBS, 30 min; mouse IgG1 anti-human IL-4 mAb (0.1µg/ml) (# 1842-01, Genzyme, Cambridge, MA, USA) and another mouse IgG1 anti-human IL-4 mAb (0.05 mg/ml) (# 1-41-1 Sandoz, Vienna, Austria) in PBS for 2 h; 10 min wash in 0.14% glycine in 0.1 M PBS pH 7.2; secondary gold-labelled antibody 1:20 dilution of 10-nm colloidal gold conjugated to goat anti-mouse immunoglobulins (GAM IgG/M) (Aurion, Wageningen, the Netherlands) in 0.1 M PBS, pH 7.2, for 2 h; 3 times wash for 3 min in 0.14% glycine in 0.1 M PBS; wash for 3 min in distilled water. Control sections were incubated with a) GAM IgG/M with omission of the primary antibody; b) with an irrelevant IgG1 monoclonal antibody PSA/ER-PR8 1µg/ml (Dakopatts, Glostrup, Denmark) of the same isotype and concentration. After drying, all grids were stained with uranyl acetate and lead citrate. Transmission micrographs were made on a Zeiss electron microscope 902 operating at 80 kV.

Immunohistochemistry

Biopsies were frozen in tissue-Tek (Miles, Naperville, IL, USA) and stored at -80°C until use. Two cryostat sections (6 µm) were cut from each biopsy and placed on poly-L-lysine-coated (Sigma Diagnostics, St. Louis, MO, USA) slides and air-dried. For immunodouble-staining the following mAb were used: anti-human IL-4 (# 1842-01 Genzyme), anti-CD3 biotinylated (Leu- 4), and anti-CD4 biotinylated (Leu 3) (Becton Dickinson, San Jose, CA, USA). EG1 recognizing eosinophil cationic protein (ECP) in resting and activated eosinophils, and EG2 recognizing the cleaved form of ECP in activated eosinophils (Pharmacia, Uppsala, Sweden) were both biotinylated according to standard procedures using the biotin succinimide ester (Sigma). Anti-tryptase mAb were alkaline phosphatase(AP)-conjugated (Brunschwig

chemie, Amsterdam, the Netherlands). For immunodouble-staining the following procedure was followed: the sections were fixed in paraformaldehyde (PF) 4°C for 15 min, washed in PBS (pH 7.4) for 5 min, washed for 30 min in PBS/0.45% hydrogenperoxide/0.1% natriumazide, 5 min in PBS, 3 times for 5 min in PBS/1% BSA, and 3 times for 5 min with PBS/BSA/0.1% saponin (17). After blocking with normal rabbit serum (Dakopatts) 10% in PBS for 10 min, the slides were incubated with the primary mAb for 1h. After 3 washes with PBS/BSA/0.1% saponin the slides were incubated for 30 min with rabbit anti-mouse antiserum conjugated with horseradish peroxidase (Dakopatts) diluted in PBS with 10% normal human serum and washed again 3 times for 5 min in PBS/BSA/0.1% saponin. After blocking with 10% normal mouse serum for 10 min, the slides were incubated for 30 min with a directly biotinylated second mAb or AP-conjugated second mAb. Slides were washed 3 times for 5 min in PBS/BSA/0.1% saponin and incubated with streptavidine-biotin complex conjugated with AP (Dakopatts) for 30 min. Slides were rinsed again in PBS/BSA/0.1% saponin and TRIS buffer (pH 8.5) and incubated with Fast Blue BB Base (Sigma), rinsed in distilled water and acetate buffer 1% and incubated with 3-amino-9-ethylcarbazole (Sigma). Finally, the slides were rinsed in distilled water and mounted in glycerin gelatin. Using this method, the IL-4 immunoreactivity stained red, whereas the cellular phenotypic markers were blue. Double-positive cells exhibited a red-blue color. Control slides were treated similarly but in five sections the anti IL-4 mAb was replaced by an unrelated IgG1 mAb (Becton Dickinson), in one the cellular marker was replaced by biotinylated IgG1 mAb, and in the seventh section both mAb were replaced by IgG1 mAb and biotinylated IgG1 mAb subsequently.

RESULTS

Identification and enumeration of IL-4$^+$ cells by immunoelectron microscopy

Immunogold labelling of IL-4 was detected within the crystalloid core compartment of 85% of the eosinophil secondary granules ($n=468$). We examined 42 eosinophils in the bronchial mucosa from 5 atopic asthmatic patients (Figures 1 and 2).

The surrounding matrix compartment of these eosinophil granules often was electron-lucent. When the matrix compartment was visible, it was not detectably labeled for IL-4. No labeling was detected in other organelles of the eosinophils, nucleus, or cytoplasm. Background labeling was negligible. No staining was observed in mast cells ($n=3$) nor in T lymphocytes ($n=32$). These results were corroborated by using another

mouse IgG1 anti-human IL-4 mAb (# 1-41-1 from Sandoz, Vienna, Austria). No eosinophils were found in biopsies of controls.

Omission of the primary antibody or substitution with an irrelevant IgG1 antibody (PSA/ER-PR8) in the same concentration resulted in absence of gold labeling of the eosinophil granules (Figure 3).

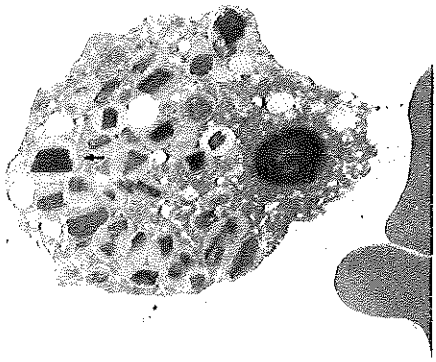
Identification of IL-4$^+$ cells by immunohistochemistry

The immunodouble-stained cells present in the bronchial mucosa were identified independently by two investigators (G. M. M. and A. F. W.-W.) at a magnification of 10x40. The localization of IL-4 immunoreactivity was examined in double-stained sections separately stained for IL-4 and EG1, EG2, tryptase, CD3 and CD4. Quantification was performed in a zone 100 µm deep in the lamina propria under the basement membrane (Table 1).

Table 1 Median percentages (ranges) of IL-4$^+$ cells coexpressing EG1, EG2, CD3, CD4, and tryptase immunoreactivity in biopsies from atopic asthmatics and control subjects

	Asthmatics (n = 12)	Control subjects (n = 3)
IL-4-positive cells that were EG1$^+$ eosinophils	9 (0-22)	0 (0-20)
IL-4-positive cells that were EG2$^+$ eosinophils	35 (0-100)	0 (0-20)
IL-4-positive cells that were mast cells	0 (0-24)	0
IL-4-positive cells that were CD3$^+$ T lymphocytes	0 (0-10)	0
IL-4-positive cells that were CD4$^+$ T lymphocytes	0	0

A few IL-4$^+$ cells stained with EG1 and EG2 in the bronchial mucosa from both asthmatics and control subjects, identifying them as eosinophils and activated eosinophils, respectively (Figure 4). Very sporadically IL-4 could be localized to mast cells and to CD3 or CD4 positive T lymphocytes in bronchial mucosa of asthmatics. No significant staining was seen in any of the control sections (data not shown).



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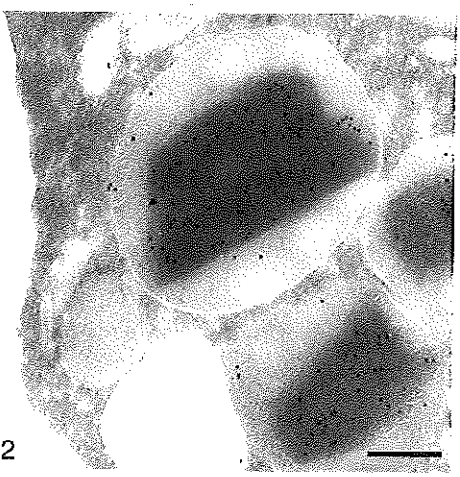

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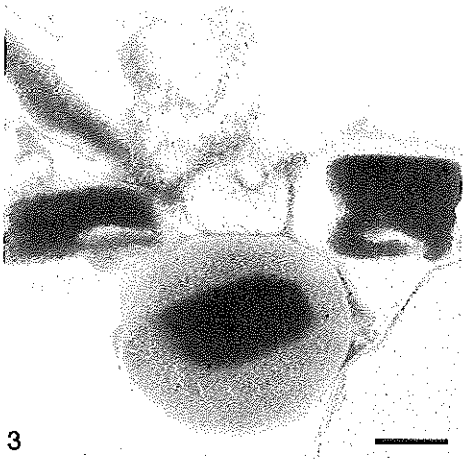

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Figure 1. Immunogold staining with anti-human IL-4 mAb of an eosinophil with secondary granules (*arrow*) from a patient with atopic asthma. Bar = 1.1 µm.

Figure 2. Gold particles are present in the crystalloid core of 85% of the secondary granules, indicating the presence of IL-4; the surrounding matrix compartment is not labeled.
Bar = 0.25 µm.

Figure 3. The eosinophil granules do not label with the irrelevant IgG1 antibody PSA/ER-PR8.
Bar = 0.25 µm.

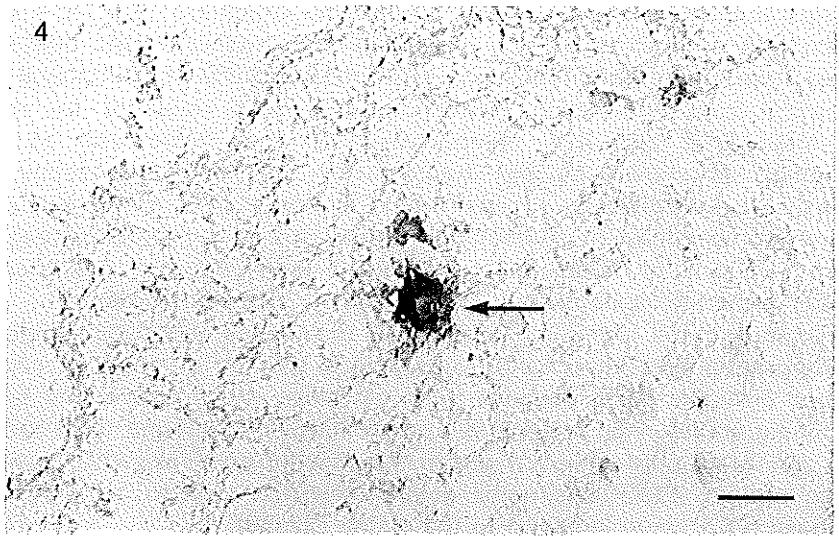

Figure 4. Cryostat section of a bronchial mucosal biopsy from a patient with atopic asthma. IL-4$^+$ cell (red) staining double with EG2 (blue) within the lamina propria (*arrow*).
Bar = 6 μm.

DISCUSSION

This study demonstrates that IL-4 protein was localized by immunogold labeling to the crystalloid core compartment of the major secondary or specific granule population of eosinophils in the bronchial mucosa of five atopic asthmatic patients. In contrast, the surrounding matrix compartment of these granules did not label for IL-4. The findings from light microscopic immunodouble-staining showed that most of the cells expressing IL-4 immunoreactivity appear to be (activated) eosinophils both in asthmatics and in controls.

Our ultrastructural localization of IL-4 suggests that either pre-formed IL-4 is stored in the secondary granules of eosinophils, before being released and may therefore provide a ready source of this cytokine in response to allergen stimulation or that IL-4 is directly secreted and the granule-bound IL-4 represents only a minor fraction of the total eosinophil-specific IL-4 production. However, we find that the frequency of IL-4 staining activated eosinophils is slightly higher compared to the EG1$^+$ eosinophils positive for IL-4. These findings could suggest that activated eosinophils might still constitute a pool of IL-4 that could be released immediately, having important biological implications. Other studies have also shown that human peripheral blood eosinophils are capable of releasing IL-4 after stimulation

with sIgA-immune complexes (15) or calcium ionophore (18). IL-4 mRNA expression by a small percentage of activated eosinophils in BAL fluid and in biopsies from atopic asthmatics has been observed (19). In peripheral blood eosinophils from asthmatics mRNA for IL-4 was detected by reverse transcriptase-PCR and IL-4 immunoreactivity was colocalized to blood eosinophils, as shown by cell fractionation and light microscopy (20). Obviously, IL-4 expression in eosinophils is not a chracteristic feature of pulmonary eosinophils. In combination these data on synthesis, storage and secretion of IL-4 by eosinophils, suggest a novel role for these cells in maintaining the IgE response and stimulating the Th2 commitment.

Ultrastructural immunohistochemical techniques with gold conjugated secondary antibodies have made it possible to identify a variety of enzymes and four different basic proteins with cytotoxic properties in the specific eosinophil granules (21). Major basic protein (MBP) was found to be localized to the crystalloid core compartment of the specific granules, while eosinophil cationic protein (ECP), eosinophil derived-neurotoxin (EDN), and eosinophil peroxidase (EPO) were localized to the matrix (22,23).

It is now well established that eosinophils are a source of several other cytokines in addition to IL-4, including IL-1α (24), IL-3 (25), IL-5 (26), IL-6 (27), IL-8 (28), GM-CSF (29), TNF-α (30), MIP-1α (31), TGF-α (32) and TGF-ß (33). However, the substructural localization of most of these cytokines remains to be determined.

Immunoelectron microscopic and immunohistochemical studies have demonstrated the presence of IL-4 in mast cells in bronchial and nasal mucosal biopsies from both patients with allergic asthma and rhinitis and controls (12,14). We could not confirm by immunogold labeling IL-4 immunoreactivity localized to mast cells granules. This could be due to the low number of mast cells examined in the ultrathin sections and to the variable distribution in the number of mast cells expressing cytokines (14). By immunodouble-staining we saw in only one patient a solitary mast cell positive for IL-4. We did not detect IL-4 immunoreactivity in CD3 or CD4 positive T lymphocytes. This does not exclude that these cells can produce and synthesize IL-4. It is likely, that once generated, T cells immediately secrete IL-4. In contrast to eosinophils and mast cells, they have little capacity for mediator storage.

In conclusion, our data indicate that eosinophils in the bronchial mucosa store IL-4 in the crystalloid core of secondary granules and may be an important source of IL-4 in chronic allergic airway inflammation.

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CHAPTER 4.2

ULTRASTRUCTURAL IMMUNOGOLD LOCALIZATION OF INTERLEUKIN 5 TO THE CRYSTALLOID CORE COMPARTMENT OF EOSINOPHIL SECONDARY GRANULES IN PATIENTS WITH ATOPIC ASTHMA*

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* *J Histochem Cytochem 1996; 44:67-69*

SUMMARY

Bronchial biopsies from two patients with atopic asthma were analyzed by immunogold labeling to detect the ultrastructural location of interleukin 5 (IL-5). In eosinophils, IL-5 was localized to the electron-dense crystalloid core compartment of the secondary or specific eosinophil granules. Other structures in the eosinophils were unlabeled. No IL-5 was detected in mast cells. Control sections incubated with an irrelevant primary antibody were negative. This study demonstrates that pre-formed IL-5 is stored within the major population of secondary granules in human eosinophils.

INTRODUCTION

Interleukin-5 (IL-5) was demonstrated to be localized in eosinophils and, to a lesser extent, in mast cells in a recent immunohistochemical study using glycol methacrylate (GMA)-embedded bronchial biopsy specimens from asthmatic patients (1). The clinical importance of IL-5 is demonstrated by the fact that IL-5 is a growth and differentation factor (2), activator (3), and chemoattractant (4) for eosinophils and, as a consequence, is considered a pivotal cytokine in allergen-mediated eosinophilic responses. In the lower airways, increased IL-5 mRNA has been reported in the mucosa of mild asthmatics (5). In addition, increased concentration of IL-5 protein (6) and increased transcription of IL-5 has been found in T-cells recovered by bronchoalveolar lavage (BAL) from allergic asthmatics (7). In the present investigation we wanted to localize the IL-5 immunoreactivity by immunoelectron microscopy. We detected IL-5 exclusively in the major eosinophil granule population in mucosal biopsy specimens taken from two atopic asthmatic patients. Specifically, IL-5 was present in the electron-dense crystalloid core compartment but not in the surrounding matrix of the secondary granules of eosinophils.

MATERIALS AND METHODS

Bronchial tissues

Bronchial biopsy specimens were obtained by fiberoptic bronchoscopy (Olympus model BF IT Tokyo, Japan) from the segmental divisions of the main bronchi of two non-smoking atopic asthmatic patients who were on bronchodilators only (terbutaline 500 µg four times a day). The diagnosis of asthma was based on a history of attacks of breathlessness and wheezing without chronic cough or sputum production, according to the criteria of the

American Thoracic Society. The two patients were randomly sampled from the Dutch CNSLD study group (8). The study protocol was approved by the Medical Ethics Committee.

Fixation and embedding for immunoelectron microscopy

Biopsies were immediately placed in ice-cooled isotonic saline and within 20 min were fixed in 1% acrolein plus 0.4% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.2, at 0-4°C for 1-4 hr). After fixation the biopsy samples were stored in a sucrose buffer (1 M sucrose in 1.1 M phosphate buffer, pH 7.2) with 1% paraformaldehyde at 0-4°C. The samples were dehydrated and processed into Lowicryl K4M resin by the progressive lowering of temperature technique. Polymerization took place under UV light at -40°C for 24 hr and at room temperature (RT) for an additional 48 hr. From the Lowicryl-embedded material, 1-µm sections were cut and stained with toluidine blue to select appropiate areas for ultra-thin sectioning. The ultra-thin sections (40-60 nm) were collected on carbon-coated Formvar-filmed mesh 200-mesh copper grids.

Immunogold labelling to demonstrate IL-5

Grids were rinsed for 10 min in 0.14% glycine in 0.1 M PBS, pH 7.2. The following sequence of steps was used to stain the grids, by placing them into reagent drops: (a) 10% normal goat serum (NGS) in PBS, 30 min; (b) 1:100 dilution of primary mouse anti-human IL-5 monoclonal antibody (Mab7) (9) (Dr. L. A. McNamee, Glaxo, Greenford, Middlesex, UK) in PBS for 2 hr at RT; (c) wash in 0.14% glycine in 0.1 M PBS, pH 7.2; (d) secondary gold-labeled antibody [1:20 dilution of 10-nm colloidal gold conjugated to goat anti-mouse immunoglobulins (GAM IgG/M) (Aurion, Wageningen, the Netherlands) in 0.1 M PBS, pH 7.2, for 2 hr]; (e) wash in 0.14% glycine in 0.1 M PBS; (f) wash for 3 min in distilled water. Control sections were incubated (a) with GAM IgG/M with omission of the primary antibody and (b) with an irrelevant IgG1 MAb PSA/ER-PR8 (Dakopatts, Glostrup, Denmark) of the same isotype and concentration. After drying, all grids were stained with uranyl acetate and lead citrate. Transmission micrographs were made on a Zeiss electron microscope 902 operating at 80 kV.

RESULTS

Bronchial mucosa

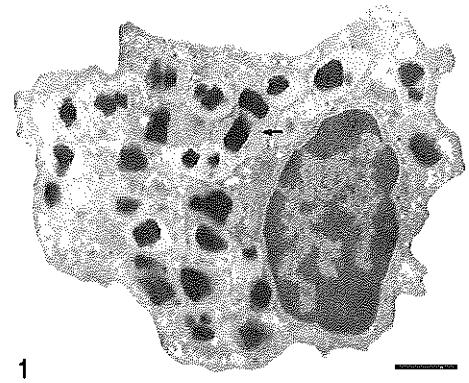
Immunogold labeling of IL-5 was detected within the crystalloid core compartment of more than 90% of the eosinophil secondary granules visualized in ultra-thin sections of several eosinophilic granulocytes in the bronchial mucosa of two atopic asthmatic patients (Figures 1 and 2). The surrounding matrix compartment of these eosinophil granules was often electron-lucent. When matrix compartment was visible, it was not detectably labeled for IL-5. Background labeling was negligible. Other eosinophil granule populations (i.e., primary and small granules), as well as lipid bodies, were not labeled for IL-5, nor was nuclear or cytoplasmic staining detected. No staining was observed in mast cells or in T-lymphocytes. These results were confirmed by two other anti-human IL-5 antibodies [monoclonal mouse IgG1 anti-human IL-5 (#2374-01) and polyclonal rabbit anti-human IL-5 (#1722-01)] from Genzyme (Cambridge, MA).

Specificity Controls

Omission of the primary antibody or substitution with an irrelevant IgG1 antibody PSA/ER-PR8 (Dakopatts, Glostrup, Denmark) resulted in absence of gold labeling of the eosinophil granules (Figure 3). The specificity of the anti-human IL-5 monoclonal antibody (MAb7) has been demonstrated by Bradding et al. (1) by immunostaining cultured Chinese hamster ovarian (CHO) cells transfected with cDNA for human IL-5.

DISCUSSION

This study demonstrates that pre-formed IL-5 was localized by immunogold labeling to the crystalloid core compartment of the major secondary or specific granule population of eosinophils in the bronchial mucosa of two atopic asthmatic patients. In contrast, the surrounding matrix compartment of these granules did not label for IL-5. No labeling was detected in other organelles of the eosinophils, nucleus, or cytoplasm.
IL-5 mRNA has been previously reported in eosinophils (10,11), and in an immunohistochemical study using GMA-embedded bronchial biopsies IL-5 protein has been detected in eosinophils (1). Our ultrastructural localization of IL-5 suggested that the presence of IL-5 in stored forms in the secondary granules of eosinophils would provide a ready source of this cytokine in response to allergen stimulation. No staining was observed in mast cells or in T-lymphocytes. However, this does not mean that these cells are not



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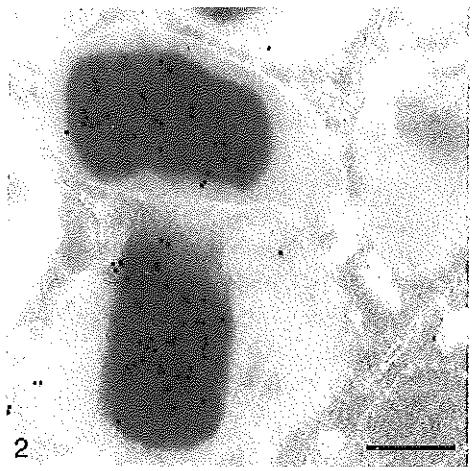

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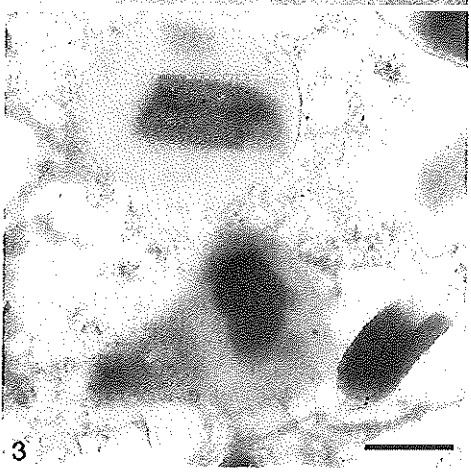

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Figure 1. Ultra-thin section of an eosinophil with secondary granules from a patient with atopic asthma. Original magnification x 7,000. Bar = 1.1 µm.

Figure 2. Gold particles are seen in the crystalloid core of the secondary granules, indicating the presence of IL-5; the surrounding matrix compartment is not labeled. Original magnification x 30,000. Bar = 0.25 µm.

Figure 3. The eosinophil granules do not label with an irrelevant IgG1 antibody, PSA/ER-PR8. Original magnification x 20,000. Bar = 0.4 µm.

producing and secreting IL-5. It is likely that once generated, T-cells immediately secrete IL-5. In contrast with eosinophils, lymphocytes have little capacity for mediator storage.

Eosinophils have recently been identified as a pivotal source of several other cytokines in addition to IL-5, including IL-1α (12), IL-3 (13), IL-6 (14), IL-8 (15), GM-CSF (16), TNF-α (17), MIP-1α (18), TGF-α (19), and TGF-ß (20). However, the substructural location of most of these cytokines remains to be determined.

Ultrastructural immunohistochemical techniques with gold conjugated secondary antibodies have made it possible to identify subcellular sites for a variety of eosinophil constituents (21). Four different basic proteins with cytotoxic properties and a number of enzymes are recognized in the specific eosinophil granules (22). The crystalline core compartment consists of major basic protein (MBP), and the surrounding matrix is composed of eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO).

Although the distribution of these eosinophil granule proteins has been known for some time, the ultrastructural localization of IL-5 in the crystalloid core of the secondary granules of eosinophils in bronchial mucosa of asthmatic patients has not previously been determined.

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CHAPTER 5

EOSINOPHILS IN THE BRONCHIAL MUCOSA IN RELATION TO INDICES FROM METHACHOLINE LOG-DOSE RESPONSE (MLDR) CURVES IN ATOPIC ASTHMATICS

5.1 Eosinophils in the bronchial mucosa in relation to indices from methacholine log-dose response (MLDR) curves in atopic asthmatics
Am J Respir Crit Care Med 1995; 151:4:A133 (modified)

CHAPTER 5.1

EOSINOPHILS IN THE BRONCHIAL MUCOSA IN RELATION TO INDICES FROM METHACHOLINE LOG-DOSE RESPONSE (MLDR) CURVES IN ATOPIC ASTHMATICS*

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INTRODUCTION

Atopic asthma is characterized by local infiltration of several activated inflammatory cells in the bronchial mucosa, even in mild asthmatics (1). Particularly eosinophils, through their release of different basic proteins with cytotoxic properties (major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN) and eosinophil peroxidase (EPO)) have been associated with epithelial shedding (2,3). The numbers of eosinophils in the bronchial mucosa and in bronchoalveolar lavage (BAL) fluid has been correlated with the severity of asthma and the mechanisms which underlie airway hyperresponsiveness (4-7), one of the most prominent functional characteristics of asthma (8). Airway hyperresponsiveness is defined as a decreased threshold of airway narrowing in response to a variety of non-specific stimuli (9). At present the airway hyperresponsiveness is usually measured in log dose response curves as the provocative concentration histamine or methacholine causing a fall of 20% in the forced expiratory volume in one second (FEV_1) and is called the sensitivity (S; log_2PC_{20}). Previous studies (10,11) have focussed on the importance of defining airway hyperresponsiveness by a more detailed characterization of the entire histamine or methacholine log-dose response (MLDR) curve, *i.e.* the sensitivity (S), the steepest part of the slope of the curve (reactivity; R; Δ FEV_1 (%)/ log_2 PC) and the maximal response value of airway narrowing (plateau; P, % FEV_1). It was found that the log dose response curves from asthmatics, show a shift to the left, had a steeper slope and a higher maximal response value as compared to normals (12).

For the interpretation of the entire log- dose response curve a model fit to the data was necessary, because reaching of a plateau can be associated with a large drop in FEV_1, and therefore extrapolation may be necessary. Moreover the model gives a smoothing of the individual data which often show marked random fluctuations. In our investigation indices were obtained as parameters of a Cumulative Gaussian Distribution (CGD) function fitted to the log-dose response curves (13).

Another pulmonary function variable, the specific (volumic) airway conductance (sG_{aw}) can be derived from bodyplethysmography and can be considered as indicator for the airway lumen (14).

Airway inflammation produces hyperemia in the lamina propria (15). The process of vasodilation and plasma leakage may increase airway wall thickness, which may directly contribute to airway narrowing (15,16). Therefore, we tested the hypothesis that in mild to moderate asthma the indices from the entire MLDR curves and the baseline specific airway conductance (sG_{aw}) are related to the presence and activation of eosinophils in the lamina propria.

MATERIALS AND METHODS

Twenty non-smoking atopic asthmatics (15M; 5F; median age 26 years (21-56)), who were on bronchodilators only, entered the study. Bronchial biopsies were obtained from the segmental divisions of the main bronchi by fiberoptic bronchoscopy. Cryostat sections (6μm) were stained with two monoclonal antibodies using the alkaline-phosphatase anti-alkaline phosphatase (APAAP) technique: EG1, recognizing both resting and activated eosinophils and EG2, recognizing the cleaved epitope of eosinophil cationic protein of activated eosinophils (Pharmacia, Uppsala, Sweden). Cell counts were expressed as the median number of positive cells (ranges) per unit length (1mm) of basement membrane.

Methacholine challenge test was performed using the standardized 2 min tidal breathing technique (17). Dose-response (Δ FEV_1%) curves were obtained after inhalation of doubling concentrations of acetyl-β-methyl choline bromide (0.03-256 mg/ml) in normal saline. Dose was expressed as $\log_2$ concentration. Sensitivity ($\log_2PC_{20}$) was obtained by linear interpolation between adjacent points of the log dose-response curve. A sigmoid Cumulative Gaussian Distribution (CGD) model fit (13) yielded the reactivity as steepest slope (R) and the plateau value (P). All reactivity and plateau values were derived as fit parameters. Smoothing and extrapolation was considered as an absolute prerequisite for the accuracy of the plateau value if the last three PC showed a variation coefficient of more than 5% of the mean value (13).

Besides the FEV_1-MLDR curve also baseline specific airway conductance (s*G*aw) was measured in a volume-constant bodyplethysmograph (Jaeger bodytest, Würzburg, Germany). Correlation coefficients were obtained by Spearman's Rank method (Table 1).

RESULTS

Airway hyperresponsiveness and s*G*aw

Median values and range of $\log_2PC_{20}$, reactivity and plateau values were 0.08 (-5.18-7.98) mg/ml, 9.06 (3.31-16.7) % FEV_1/doubling dose, and 52.95 (23.8-80.5) % FEV_1 respectively. In 12 patients the plateau value had to be obtained with the model fit, because experimental determination was less accurate. The median s*G*aw was 0.63 (0.25-2.28) $s^{-1}.kPa^{-1}$.

Correlation between (activated) eosinophils and airway hyperresponsivenes and s*G*aw

The median values and range for $EG1^+$ (total) eosinophils and $EG2^+$

(activated) eosinophils were 11.6 (1.6-68)/mm BM, and 3.5 (0-29.3)/mm BM respectively, indicated in Table 1, where also the relationship between the lung function variables and (activated) eosinophils was shown. There were no significant correlations between log_2PC_{20} and eosinophils, neither were the eosinophils significantly related to the reactivity. The plateau value was significantly related to the number of activated eosinophils (Table 1; $r = 0.62$, $p<0.05$) (Figure 1) and showed a trend of association with the total eosinophil number. The s*G*aw was inversely related to the number of activated eosinophils (Table 1; $r = -0.52$, $p<0.05$) and showed also a trend of association with the total eosinophil number.

Table 1. Correlation coefficients

	Median (ranges)	log_2PC_{20} (S)	reactivity (R)	plateau (P)	s*G*aw
EG1	11.6	- 0.10	0.28	0.37**	-0.40**
(n=20)	(1.6 - 68)				
EG2	3.5	- 0.32	0.12	0.62*	-0.52*
(n=20)	(0 - 29.3)				

(** $p \leq 0.1$; * $p < 0.05$)

CONCLUSION

In the present study we have demonstrated a positive relationship between the number of activated eosinophils in the lamina propria, an important cellular marker in bronchial mucosal inflammation, and the plateau value. Furthermore, the numbers of activated eosinophils were inversely correlated with the s*G*aw.

The total eosinophil number showed a trend of association with the plateau value and the s*G*aw. Although the sign of the correlation coefficients fitted into an increase of airway hyperresponsiveness and reactivity, with an increased total eosinophil count and activity, no significance of the correlations was present.

The aim of this study was to investigate whether indices from the MLDR curves were associated with the presence and activity of eosinophils in the bronchial mucosa in asthma. In previous studies with bronchial biopsies from asthmatic patients relationship between eosinophils and the degree of airway hyperresponsiveness has been investigated. An inverse correlation was found

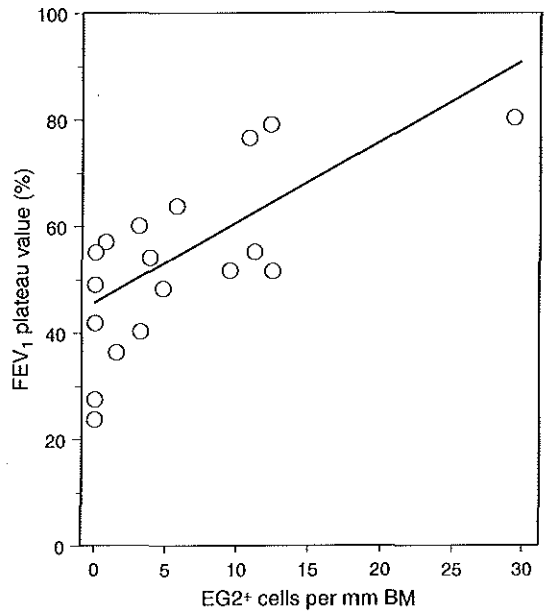

Figure 1. Correlation between the plateau value and the number of activated ($EG2^+$) eosinophils per mm basement membrane (BM)(r = 0.62, $p<0.05$).

between the number of activated eosinophils in the lamina propria and airway hyperresponsiveness (PC_{20} methacholine) (18). Another study showed a significant inverse correlation between the intraepithelial eosinophils and the PC_{20} methacholine values (19). In a recent study (20) total inflammatory cells and mast cells were significantly inversely correlated with the position of MLDR curve, so with the PC_{20} methacholine. Djukanovic *et al.* (21) were unable to demonstrate a correlation between $EG2^+$ cells and the PC_{20} methacholine, and neither were we. Apparently, the number of eosinophils in the lamina propria does not reflect all the inflammatory events in the airway wall, which determine airway hyperresponsiveness.

We did find a significant correlation between the number of activated eosinophils and the plateau value, one of the indices of the MLDR-curve. Interestingly, only the activated eosinophils were significantly correlated with the plateau value and the sG_{aw}. The maximal response plateau has been considered as determined by "postjunctional" mechanisms as smooth muscle contractility, viscous and elastic loads on airway smooth muscle shortening, swelling of the airway wall and intraluminal exudate (10). Like the sG_{aw}, the plateau value is linked to the airway resistance characteristics.

Our findings therefore support the hypothesis that the activated eosinophils and their mediators cause bronchoconstriction, enhance airway inflammation and

epithelial damage, and possibly cause an increase in airway hyperresponsiveness.

In conclusion, indices of MLDR curves and also baseline s*G*aw are partly significantly related to the number of activated eosinophils in the bronchial mucosa. These results suggest a direct relationship between bronchial mucosal inflammation, characterized by eosinophil activity, and decrease in airway lumen. Our results stress the importance of modelling the entire MLDR curve and not only the use of $\log_2 PC_{20}$ in follow up and characterization of airway hyperresponsiveness.

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CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

The objectives of the studies described in this thesis were:

1. To assess the modulatory effect of inhaled glucocorticoids on the bronchial mucosal inflammation in relation to pulmonary function data of allergic asthmatics.
2. To examine the occurrence of dendritic cells (DC) in the bronchial mucosa of allergic asthmatics and the modulation by inhaled glucocorticoids.
3. To investigate the immunolocalization of interleukin (IL)-4 and IL-5 in eosinophils in the bronchial mucosa of asthmatic patients.

Limitations of the study

The group of allergic asthmatic patients, who were randomly sampled from the Dutch chronic non-specific lung disease (CNSLD) study group, was relatively small. Furthermore, the patients from the CNSLD study group were biopsied only once at the end of the 2.5 year study period. This made it difficult to perform statistical analyses between the two treatment groups, *i.e.* the glucocorticoid group and the bronchodilator group. We tested for significance between these groups with the Mann-Whitney U test for small groups. Although the number of 24 allergic asthmatic patients studied in chapters 2.2 and 3.2 was reasonably large, the number of paired biopsies for immunohistochemical evaluation taken from these patients at the beginning and at the end of the study was unfortunately much lower. The biopsies were too small or not well preserved, due to biopsy trauma. In addition, one has to keep in mind that bronchial biopsies likely represent static "snapshots" of the inflammatory process in the bronchial mucosa, which is in fact a highly dynamic process (1). Several inflammatory cell types and the local cytokine production may vary with time and with the progress of the disease. Studies with more detailed comparisons, performed by taking biopsies at different time points, may be required in the future. However, it is difficult to take bronchial biopsies from patients with severe asthma. Taken together, it is clear that studying allergic asthma by taking bronchial biopsies has its limitations.

Effects of glucocorticoid therapy on airway inflammation and airway hyperresponsiveness

Glucocorticoids have been the most effective treatment for asthma since 1950 (2), but their precise mechanism of action is still unclear (3). In the studies described in chapter 2, the effects of two different types of

inhaled glucocorticoids on clinical parameters, several inflammatory cells and the thickness of the lamina reticularis of the basement membrane (BM) were investigated. Therapy with 2.5 years of beclomethasone dipropionate downregulated the number of eosinophils and IL-4$^+$ cells. Therapy with 3 months of fluticasone propionate reduced the eosinophilic inflammation in the bronchial mucosa of asthmatics and improved the airway hyperresponsiveness to methacholine. In addition, a significant relationship between the improvement in airway hyperresponsiveness and the reduction in eosinophils was found. This suggests that the reduction in eosinophil counts is one of the anti-inflammatory effects of glucocorticoids. IL-3, IL-5 and GM-CSF promote maturation, activation and adhesion, and prolong survival of eosinophils (4-6). An explanation for the reduction in eosinophils may be that glucocorticoids inhibit cytokine-mediated eosinophil survival (7). Furthermore, the accumulation of eosinophils may also be inhibited since prednisolone therapy reduces the secretion of IL-3, IL-5 and GM-CSF and the percentages of peripheral blood T lymphocytes expressing mRNA of these cytokines (8). We also found that the numbers of IL-4$^+$ cells were decreased after glucocorticoid therapy. Similar inhibitory effects of prednisolone therapy on bronchoalveolar lavage (BAL) fluid cells expressing IL-4 and IL-5 mRNA have been reported (9). The inhibition of cytokines may be an important mechanism whereby glucocorticoids suppress bronchial mucosal inflammation.

In both studies, a significant thickening was found of the reticular BM from asthmatics compared to controls. No effect was seen on the thickness of the lamina reticularis of the BM by inhaled glucocorticoids. Neither 3 months nor 2.5 years of treatment reduced the thickening of the reticular BM. Studies by others reported similar results (10,11). These data suggest that subepithelial collagen deposition may be associated with an irreversible fibrosis and airflow limitation. The increase in subepithelial collagen in asthmatics may be due to increased synthesis, decreased degradation of procollagens in the cytoplasm, or reduced proteolysis of these extracellular matrix proteins. This aspect of the cell biology of asthma is largely unexplored. Recent studies showed that the thickness of the lamina reticularis in asthma was significantly inversely correlated with airway hyperresponsiveness to methacholine (12,13). Thus, thickening of the reticular BM may be associated with increased airway responsiveness. However, the influence of excessive subepithelial collagen deposition on airway function remains to be further established.

There are some patients with asthma in whom glucocorticoids do not improve the clinical symptoms; these patients are so-called glucocorticoid resistant (14). This may be due to a defect in the cellular response to glucocorticoids (15,16). Glucocorticoid resistance cannot be explained by a defect in the binding of glucocorticoids to glucocorticoid receptors. In a

recent study, it was demonstrated that the ability of the activated glucocorticoid receptor to bind to glucocorticoid responsive elements is decreased because of a reduced number of glucocorticoid receptors available for binding to DNA (17). The underlying mechanism of this abnormality remains to be elucidated. Thus, to gain further insight into the working mechanisms of glucocorticoids, it would be interesting to also study patients who fail to respond to glucocorticoid therapy.

Dendritic cells in asthma and modulation by glucocorticoids

In the studies described in chapter 3, we investigated the numbers of DC in the bronchial mucosa of allergic asthmatics and the modulation by inhaled glucocorticoids. We found that the numbers of DC in the bronchial mucosa of asthmatics were increased compared to controls. A similar increase of mucosal DC has been reported in allergic asthmatics compared to controls sensitized to the same allergen (18). In the nasal mucosa, numbers of DC have been found to increase during symptomatic rhinitis (19). The discovery of increased numbers of DC in the bronchial wall of allergic asthmatics led to the hypothesis of an increased antigen presentation and T cell activation *in situ*. In future studies, it would be of interest to investigate the number of DC also in non-allergic 'intrinsic' asthmatic patients and in patients with inflammatory lung diseases.

In addition, we found that two types of inhaled glucocorticoids (beclomethasone dipropionate and fluticasone propionate) downregulate the numbers of DC in the bronchial mucosa of asthmatics. Similar studies in the rat by Holt *et al.* (20) have demonstrated that exposure to inhaled and systemic glucocorticoids leads to a rapid decrease in the number of airway epithelial DC. Studies in the nasal mucosa of patients with perennial allergic rhinitis showed a significant reduction in the number of $CD1a^+$ cells after 3 months of fluticasone therapy (21). Electronmicroscopic studies are necessary to determine whether DC disappear from the bronchial mucosa during glucocorticoid therapy or to exclude the possibility that these findings are the result of change in DC surface markers. As discussed in chapter 3, the precise mechanisms by which glucocorticoids downregulate DC remain to be established. A possible explanation may be that glucocorticoids suppress the release by epithelial cells of monocyte chemoattractant protein-1 (22) and GM-CSF, which are involved in the recruitment and differentiation of DC in the lung (23). Furthermore, glucocorticoids may decrease the accessory function of DC, since glucocorticoids are able to suppress T cell stimulatory function of pulmonary DC *in vitro* (24). We hypothesize, that the decrease in DC numbers and their functional activity by glucocorticoids downregulates T cell activation and hence reduces the allergic inflammation.

The results of the studies described in chapters 2 and 3 are incorporated in Figure 1, which shows a hypothetic scheme of the allergic immune response in asthma and its modulation by glucocorticoids.

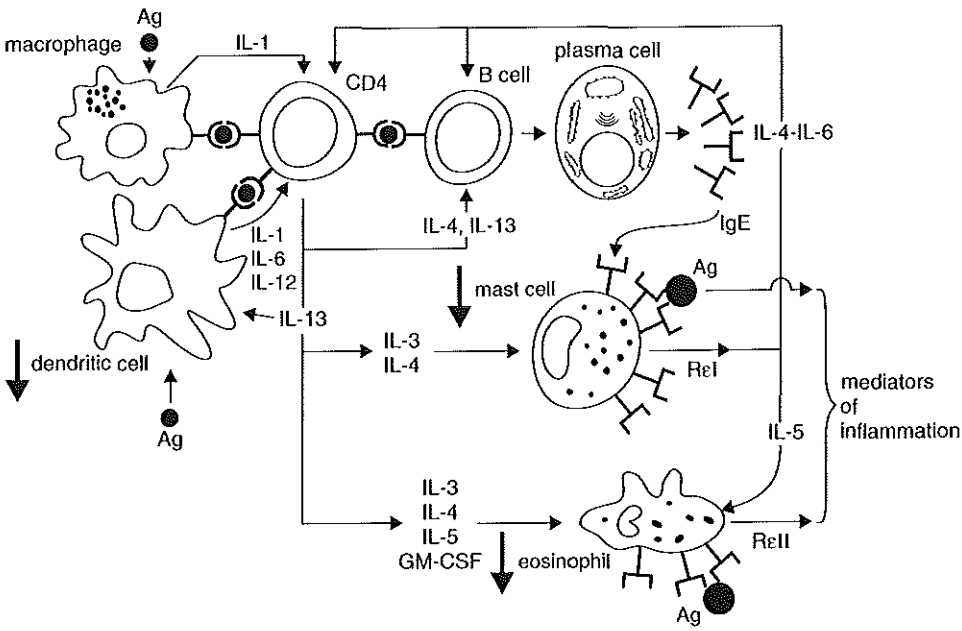

Figure 1. Hypothetic scheme of the allergic immune response in asthma and its modulation by glucocorticoids. This figure is in essential the same as Figure 1 presented in chapter 1, in which we incorporated the results from chapters 2 and 3. The effects of glucocorticoids on different inflammatory cell types are indicated by bold arrows.

Localization of IL-4 and IL-5 in eosinophils

In chapter 4.1 we describe that IL-4 protein is stored in eosinophils in the bronchial mucosa of asthmatics, as seen by the use of immunoelectron microscopy and light microscopic immunodouble-staining. IL-4 was predominantly localized to the crystalloid core compartment in the secondary granules of eosinophils. We suggest that this is preformed IL-4 which is stored in the eosinophil following *de novo* synthesis. Another possibility may be uptake by endocytosis via IL-4 receptors on eosinophils (25). This remains to be determined in future studies. In recent studies others have detected mRNA for IL-4 by reverse transcription-PCR in peripheral blood eosinophils from allergic asthmatics (26). Using sequential immunocytochemistry and *in situ* hybridization, mRNA for IL-4 was detected in eosinophils from the BAL fluid from asthmatics (26). These results are compatible with our hypothesis that the IL-4 protein detected in the granules of eosinophils is derived from

de novo synthesis. Furthermore, it was shown that human peripheral blood eosinophils release IL-4 *in vitro* after stimulation with secretory IgA immune complexes (27). The release of IL-4 by eosinophils is of particular interest since we know that IL-4 may induce selective eosinophil infiltration by upregulating the expression of vascular cellular adhesion molecule-1 on endothelial cells (28). Furthermore, IL-4 may be a chemoattractant for eosinophils (29). These findings suggest that eosinophils (by releasing IL-4) can affect and attract other eosinophils in a paracrine way. IL-4 protein and mRNA for IL-4 have initially been found in T lymphocytes (30-32). In addition to T lymphocytes and eosinophils, IL-4 is also a product of mast cells, which are capable of releasing this cytokine (33-35). However, we could not localize IL-4 to T lymphocytes or mast cells. This could be due to the low number of mast cells present for examination and to the proportion of mast cells expressing IL-4. T lymphocytes, on the other hand, immediately secrete IL-4 after its synthesis, because they have little capacity for mediator storage. Taken together, the storage of IL-4 in the secondary granules of eosinophils suggests that these cells may be an important source of IL-4 in allergic airway inflammation.

In chapter 4.2 we demonstrate IL-5 also to be localized to the crystalloid compartment of eosinophil secondary granules in the bronchial mucosa of allergic asthmatics. Recent studies using immunocytochemistry followed by *in situ* hybridization showed that increased mRNA for IL-5 colocalized with activated T lymphocytes, mast cells and eosinophils in the mucosa of mild asthmatics (1). Eosinophils could therefore translate IL-5 mRNA and store the IL-5 protein in their granules following de novo synthesis. Another study showed that blood eosinophils from patients with hypereosinophilic syndromes are capable of releasing IL-5 after stimulation with IgA-, IgE-, or IgG-immune complexes (36). Thus, eosinophils can synthesize, store and secrete IL-5.

It is known that IL-5 promotes the differentiation of eosinophil precursors, activates eosinophils and prolongs their survival (5,37,38). In addition, eosinophils were found to express IL-5 receptors (39). These data suggest that eosinophils, via the synthesis of IL-5, provide an autocrine and/or paracrine pathway that is involved in the eosinophil survival and activation.

In conclusion, in chapter 4 it is demonstrated that eosinophils in the bronchial mucosa of asthmatics store IL-4 and IL-5 in their secondary granules. Both cytokines play a central role in eosinophilic inflammation in asthma, and the genes encoding IL-4 and IL-5, as a part of a cluster of cytokines, are situated close together on chromosome 5q (40). It could be possible that their expression is partly under common genomic control. A major locus regulating serum IgE levels and a gene regulating bronchial hyperresponsiveness have recently also been mapped to this region of

chromosome 5q (41,42).

Eosinophils and indices from the methacholine log-dose response curves

The number of eosinophils in the bronchial mucosa of asthmatics has been correlated with the severity of asthma and the mechanisms which underly airway hyperresponsiveness (43,44). Airway hyperresponsiveness is defined as the provocative concentration of histamine or methacholine causing a fall of 20% in the forced expiratory volume in one second, PC_{20}, and is reflected by a leftward shift of the log-dose response curve. The log-dose response curves from asthmatics have a steeper slope (reactivity) and a higher level of maximum airway narrowing (plateau value) as compared to normals (45). Mucosal swelling, which may be induced by several inflammatory mediators, is one of the contributors to an increased plateau value (46).

The finding described in chapter 5 that activated eosinophils in the lamina propria of allergic asthmatics were significantly related to the plateau value, stresses the importance of modelling the entire methacholine log-dose response curve and not only the use of PC_{20} in the follow-up and characterization of airway hyperresponsiveness. The number of activated eosinophils was inversely correlated with the baseline specific airway conductance. These results suggest a direct relationship between bronchial mucosal inflammation, characterized by eosinophil activity, and decrease in airway lumen. However, it should be kept in mind that bronchial mucosal inflammation and airway hyperresponsiveness are not induced by the action of a single cell type, but by a variety of cells and mediators.

Concluding remarks

The studies described in this thesis show the following with regard to the bronchial mucosa of allergic asthmatics:

1. Glucocorticoids inhibit local eosinophilic inflammation and IL-4 immunoreactivity. In addition, glucocorticoids improve airway hyperresponsiveness. The reduction in numbers of eosinophils after glucocorticoid therapy correlates with the improvement in airway hyperresponsiveness. Therefore, eosinophils may be an important factor in determining airway hyperresponsiveness.
2. In asthma the lamina reticularis of the BM is thickened by subepithelial collagen deposition; neither short- nor long-term treatment with inhaled glucocorticoids reduce the thickness of the reticular BM.
3. DC numbers are increased in the bronchial mucosa of allergic

asthmatics compared to controls. Inhaled glucocorticoids downregulate the number of DC to normal levels. Since DC play an important role in the initiation and propagation of the allergic immune response, the decrease in DC numbers by glucocorticoids may downregulate T cell activation.

4. IL-4 and IL-5 are predominantly stored in the crystalloid core compartment of the secondary granules of eosinophils. Since eosinophils synthesize and release these cytokines after stimulation, these data suggest a novel role for these cells in the chronic mucosal inflammation in asthma.

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SUMMARY

The main subject of this thesis is the inflammatory process in the bronchial mucosa of allergic asthmatic patients. Bronchial biopsy studies were performed to gain more insight into the cellular aspects of the inflammation in the airways of asthmatic patients. Furthermore, the effects of inhaled glucocorticoids were studied on several inflammatory cell types in the bronchial mucosa, which may be useful in the treatment of asthmatic patients.

In *chapter 1*, asthma is defined as a chronic inflammatory disease of the airways with variable airway obstruction and airway hyperresponsiveness. Pathological changes that contribute to airway narrowing are increased bronchial smooth muscle contraction and enhanced mucus secretion in the airways. Asthma is characterized by increased airway hyperresponsiveness to a variety of non-specific stimuli, including tobacco smoke, fog, cold air and exercise. Atopy, the genetic predisposition for directing an IgE response to common allergens such as house dust mite and pollen, is a risk factor for the development of asthma. The clinical symptoms of asthma are recurrent episodes of cough, dyspnoea, wheezing and chest tightness.

Histological examination of the bronchial mucosa of asthmatics shows epithelial damage (shedding) which is associated with increased airway hyperresponsiveness. Also, the lamina reticularis of the basement membrane (BM) is thickened by a subepithelial collagen deposition, probably produced by myofibroblasts.

Allergic asthma is induced and propagated by the action of a variety of cells and mediators. In *chapter 1* the role and function of bronchial epithelial cells, dendritic cells (DC), monocytes/macrophages, eosinophils and mast cells in the process of allergic asthma are described. The mechanisms of action of glucocorticoids and a summary of the most important bronchial biopsy studies of the anti-inflammatory actions of inhaled glucocorticoids (budesonide, beclomethasone dipropionate and fluticasone propionate) are described. Finally, the aims of the studies presented in this thesis are described.

Chapter 2.1 describes the effects of 2.5 years of treatment with beclomethasone dipropionate and bronchodilators on eosinophils, IL-4 immunoreactivity, T lymphocytes, mast cells and the thickness of the reticular BM in bronchial mucosal biopsies from allergic asthmatics from the Dutch Chronic Non-Specific Lung Disease (CNSLD) study group. The numbers of eosinophils and cells expressing IL-4 immunoreactivity were lower in the lamina propria of patients treated with glucocorticoids than in patients treated with bronchodilators only. In *chapter 2.2* we show that 3 months of fluticasone propionate therapy is associated with a significant improvement in FEV_1 and PC_{20} methacholine (an increase of 3.7 doubling doses). This clinical improvement is associated with a reduction in eosinophils and mast

cells, and decreased expression of intercellular adhesion molecule-1 (ICAM-I) on blood vessels in the lamina propria. The reduction in eosinophils after fluticasone propionate treatment was significantly correlated with the improvement in airway hyperresponsiveness. In both studies in *chapter 2* we found a significant thickening of the reticular BM of asthmatics compared to controls and the thickness of the lamina reticularis was not reduced after treatment with glucocorticoids.

In *chapter 3.1* the distribution of DC in the bronchial mucosa of asthmatics from the CNSLD study group is investigated, as well as the effects of long-term beclomethasone dipropionate treatment on DC. Allergic asthma is associated with a chronic T cell-mediated inflammatory process in the airways. Since T lymphocytes cannot respond to antigens without the help of antigen-presenting cells (APC), and DC are the most potent APC, it is likely that pulmonary DC play a key role in the initiation and propagation of asthma. Increased numbers of $CD1a^+$ DC, $L25^+$ DC and a higher expression of HLA-DR were observed in the bronchial mucosa of asthmatics compared to controls. With double-staining we showed that virtually all DC expressed HLA-DR, but there were also other $HLA\text{-}DR^+$ cells such as activated T lymphocytes and macrophages. After 2.5 years of treatment with beclomethasone dipropionate, the numbers of DC and the MHC class II expression were downregulated to normal levels. *Chapter 3.2* describes the increased numbers of DC and IgE^+ cells in the lamina propria of asthmatics compared to controls, and the effects of 3 months of fluticasone propionate therapy of asthmatics on the numbers of DC, IgE^+ cells, and cells positive for the high affinity receptor for IgE ($Fc\varepsilon RI$) and on the expression of MHC class II. The IgE^+ cells, of which the majority are DC and mast cells, likely contribute to an enhanced IgE-mediated antigen/allergen presentation. Fluticasone propionate therapy resulted in a decrease in $CD1a^+$ DC and IgE^+ cells, and in a reduction in the expression of MHC class II. This was accompanied by a decrease in bronchial hyperresponsiveness. No effects were seen on the number of $Fc\varepsilon RI^+$ cells. The inhibitory effects of fluticasone propionate on DC are in agreement with the results of the study with beclomethasone dipropionate in *chapter 3.1*. The precise mechanisms by which glucocorticoids reduce the number of DC remain to be established. We conclude that DC numbers, IgE^+ cells, and MHC class II expression are elevated in the bronchial mucosa of atopic asthmatics and that glucocorticoid therapy reduces these DC, IgE^+ cells and MHC class II expression. This may downregulate T cell activation and hence reduce allergic airway inflammation.

The immunolocalization of IL-4 in the bronchial mucosa of asthmatics is studied in *chapter 4.1*. Biopsy specimens (containing eosinophils) were processed for immunoelectron microscopy. Immunogold labelling of IL-4 was detected within the crystalloid core compartment of 85% of the eosinophil secondary granules ($n = 468$). No staining was detected in mast cells nor in T

lymphocytes. These results were extended by light microscopic immunodouble-staining for IL-4 protein and eosinophil cationic protein, which showed that a subpopulation of (activated) eosinophils expressed IL-4 immunoreactivity in bronchial mucosal biopsies of allergic asthmatics as well as controls. We conclude that eosinophils in the bronchial mucosa store IL-4 in the crystalloid core of secondary granules. These results in combination with data from the literature on synthesis and secretion of IL-4 by eosinophils, suggest a novel role for these cells in the mucosal inflammatory response.

In *chapter 4.2* we describe the ultrastructural immunogold localization of IL-5 in the crystalloid core of the secondary granules of eosinophils in the bronchial mucosa of asthmatic patients. IL-5 is a growth and differentiation factor, activator and chemoattractant for eosinophils and considered a pivotal cytokine in allergen-mediated responses. Our ultrastructural localization of IL-5 suggests that IL-5 stored in the secondary granules of eosinophils would provide a ready source of this cytokine in response to allergen stimulation.

In *chapter 5*, the relationship between eosinophils and indices from the methacholine log-dose response curves in allergic asthmatics is studied. The number of $EG2^+$ (activated) eosinophils in the lamina propria was significantly related to the maximum response value (the plateau value). Furthermore, the number of activated eosinophils was inversely correlated with the specific (volumic) airway conductance (sG_{aw}), which can be considered as an indicator for the airway lumen. These results suggest a direct relationship between bronchial mucosal inflammation, characterized by eosinophil activity, and decrease in airway lumen.

In dit proefschrift staat het chronische ontstekingsproces in de bronchuswand van allergische astmapatiënten centraal. Bij astmapatiënten zijn perifere bronchusbiopten genomen om inzicht te krijgen in het onderliggende ontstekingsproces en om de effecten van therapie met inhalatie-glucocorticoïden te bestuderen. Het uiteindelijke doel van de in dit proefschrift beschreven studies was om bij astmapatiënten het anti-inflammatoire effect van glucocorticoïden en hun invloed op verschillende celtypen en mediatoren in de luchtwegen te onderzoeken. Inzicht hierin is van groot belang voor de behandeling van astmapatiënten.

In *hoofdstuk 1* wordt astma gedefinieerd als een ziekte, die wordt gekenmerkt door een chronisch ontstekingsproces in de luchtwegen en een reversibele luchtwegvernauwing. Deze luchtwegvernauwing ontstaat door contractie van glad spierweefsel in de bronchuswand en door ophoping van slijm in de luchtwegen. Tevens is er een toename van prikkelbaarheid (hyperreactiviteit) van de luchtwegen voor niet-allergische prikkels, zoals rook, mist, koude lucht en lichamelijke inspanning. Deze prikkels veroorzaken een luchtwegvernauwing met verschijnselen van hoesten, piepende ademhaling en kortademigheid. Bij atopische astmapatiënten bestaat een genetische predispositie tot sensibilisatie door allergenen zoals huisstof en pollen. Daardoor worden door zulke patiënten veelal specifieke IgE antistoffen tegen deze allergenen gevormd.

Bij microscopisch onderzoek van de luchtwegen van astmapatiënten wordt er schade aan het luchtwegepitheel gezien (shedding), die is geassocieerd met een toename in bronchiale hyperreactiviteit. Eveneens wordt er een verdikte lamina reticularis van de basaalmembraan (BM) gezien, waarschijnlijk veroorzaakt door subepitheliale collageen deposities door myofibroblasten.

In *hoofdstuk 1* worden tevens de rol en typische functie van het bronchusepitheel, dendritische cellen (DC), monocyten/macrofagen, lymfocyten, eosinofiele granulocyten en mestcellen in allergisch astma beschreven. Uit de immunopathogenese van astma blijkt wel dat astma wordt geïnduceerd en in stand gehouden door een heel netwerk van verschillende cellen en mediatoren. Tot slot wordt ingegaan op de werkingsmechanismen van glucocorticoïden en worden de doelstellingen van de studie beschreven.

Hoofdstuk 2.1 beschrijft de effecten van 2,5 jaar behandeling met beclomethason dipropionaat en van luchtwegverwijders op eosinofiele granulocyten, T lymfocyten, mestcellen, IL-4$^+$ cellen en de dikte van de lamina reticularis in bronchusbiopten van astmapatiënten, die deel uitmaken van het Stimuleringsprogramma Gezondheidonderzoek Chronische Aspecifieke Respiratoire Aandoeningen (SGO-CARA onderzoek). Het aantal eosinofiele granulocyten en IL-4$^+$ cellen was significant lager in de mucosa van patienten behandeld met glucocorticoïden, vergeleken met patiënten behandeld

met luchtwegverwijders. Er werd geen effect gezien op de dikte van de lamina reticularis.

Hoofdstuk 2.2 behandelt de effecten van 3 maanden therapie met het inhalatieglucocorticoïd fluticason propionaat op longfunctie parameters en het cellulaire ontstekingsproces in de lamina propria. Significante klinische verbeteringen in FEV_1 en in PC_{20} methacholine (3,7 verdubbelingsdoses) gingen gepaard met een afname in het aantal eosinofiele granulocyten en mestcellen, en verminderde expressie van het intercellulair adhesiemolecuul-1 (ICAM-I), dat aanwezig is op het endotheel in de lamina propria. De afname van het aantal eosinofielen onder invloed van fluticason propionaat was significant gecorreleerd met de verbetering in bronchiale hyperreactiviteit. De dikte van de lamina reticularis nam niet af onder invloed van glucocorticoiden. In beide studies werd er een significant dikkere reticulaire BM bij astmapatiënten gevonden, vergeleken met gezonde controles.

In *hoofdstuk 3* is het voorkomen van DC in de bronchiale mucosa van astmapatiënten bestudeerd. DC zijn gespecialiseerde cellen van het immuunsysteem, die antigenen kunnen opnemen en tot peptiden kunnen afbreken om deze vervolgens te presenteren aan T-lymfocyten. Indien T-lymfocyten het peptide als lichaamsvreemd herkennen, worden ze geactiveerd. In astma spelen DC een essentiële rol in het op gang brengen van de allergische reactie.

In *hoofdstuk 3.1* wordt beschreven dat het aantal $CD1a^+$ DC en $L25^+$ DC, en de expressie van HLA-DR in bronchusbiopten van astmapatiënten uit het SGO-CARA onderzoek, die geen inhalatie-glucocorticoïden gebruikten, was verhoogd in vergelijking met gezonde controles. Met dubbelkleuringen werd aangetoond dat bijna alle DC MHC klasse II tot expressie brengen. Bij patiënten die 2,5 jaar behandeld zijn met beclomethason dipropionaat werden aantallen DC gevonden, die vergelijkbaar waren met die van controles.

In *hoofdstuk 3.2* wordt het effect van 3 maanden inhalatie-therapie met fluticason propionaat op DC, IgE^+ cellen, de expressie van MHC klasse II en op cellen positief voor de hoog affiene IgE receptor $(Fc\varepsilon RI)^+$ onderzocht. De IgE^+ cellen, waarvan het merendeel DC en mestcellen zijn, dragen bij tot een verhoogde IgE gemedieerde antigeenpresentatie. Fluticason propionaat therapie resulteerde in een afname van het aantal DC en IgE^+ cellen en een reductie in MHC klasse II expressie. Er werd geen effect gevonden op het aantal $Fc\varepsilon RI^+$ cellen. Uit deze resultaten kan worden geconcludeerd dat het aantal DC in astma is toegenomen en dat glucocorticoïden het aantal DC doen verminderen, wat mogelijk resulteert in een verminderde activatie van T-lymfocyten. Het mechanisme van de afname van het aantal DC door glucocorticoïden is nog niet opgehelderd.

In *hoofdstuk 4.1* wordt de immunolocalisatie van IL-4 beschreven in de bronchiale mucosa van astmapatiënten. Immunogoud labelling van IL-4 werd aangetroffen in het kristalloïde internum in 85% van de secundaire of

specifieke granula ($n = 468$) van eosinofiele granulocyten. Mestcellen noch T-lymfocyten waren positief voor IL-4. Deze resultaten zijn uitgebreid met immunohistochemische dubbelkleuringen voor IL-4 en (geactiveerde) eosinofielen. Een subpopulatie van (geactiveerde) eosinofielen was IL-4 positief, zowel bij astmapatiënten als bij gezonde controles. Uit deze resultaten en gegevens uit de literatuur over synthese en uitscheiding van IL-4 door eosinofiele granulocyten kan worden geconcludeerd dat deze cellen een belangrijke rol spelen in allergisch astma.

In *hoofdstuk 4.2* wordt de ultrastructurele immunolocalisatie van IL-5 in het kristalloïde internum van de secundaire granula van eosinofiele granulocyten beschreven. IL-5 is als groei- en differentiatiefactor en als chemoattractant voor eosinofiele granulocyten betrokken bij de allergische reactie. De aanwezigheid van IL-5 in opgeslagen vorm in de eosinofiele granulocyt maakt een snelle reactie na allergeen stimulatie mogelijk.

In *hoofdstuk 5* wordt de relatie tussen indices van de methacholine log-dose respons curve van allergische astmapatiënten en de aanwezigheid van eosinofiele granulocyten in de bronchiale mucosa onderzocht. Het aantal geactiveerde eosinofiele granulocyten in de lamina propria bleek gecorreleerd met de maximale respons (plateau waarde). Tevens was het aantal geactiveerde eosinofiele granulocyten negatief gecorreleerd met de specifieke luchtwegconductantie (sG_{aw}), wat een maat is voor de luchtwegweerstand. Deze resultaten suggereren dat er een directe relatie bestaat tussen de mate van ontsteking in de bronchiale mucosa en de luchtwegvernauwing.

De belangrijkste *conclusies* uit de in dit proefschrift beschreven studies zijn:

1. Inhalatie-glucocorticoïden verminderen het aantal eosinofiele granulocyten en IL-4^+ cellen in het bronchiaal slijmvlies van allergische astmapatiënten. De afname van het aantal eosinofiele granulocyten na glucocorticoïd therapie is gecorreleerd met een vermindering in bronchiale hyperreactiviteit.
2. In allergisch astma is de lamina reticularis van de BM verdikt door subepitheliale collageendeposities; kort- noch langdurende behandeling met inhalatie-glucocorticoïden doet de dikte van de reticulaire BM afnemen.
3. Het aantal DC in het bronchiaal slijmvlies van astmapatiënten is verhoogd vergeleken met gezonde controles. Inhalatie-glucocorticoïden reduceren het aantal DC tot normale waarden, wat zou kunnen resulteren in een verminderde T-lymfocyt activatie.
4. IL-4 en IL-5 zijn voornamelijk gelocaliseerd in het kristalloïde internum van de secundaire granula van eosinofiele granulocyten in het bronchiaal slijmvlies.

ABBREVIATIONS

AC	:	anticholinergica
AM	:	alveolar macrophages
AP	:	alkaline phosphatase
APAAP	:	alkaline phosphatase anti-alkaline phosphatase
APC	:	antigen-presenting cells
BA	:	β_2 agonists
BAL	:	bronchoalveolar lavage
BALT	:	bronchus-associated lymphoid tissue
BDP	:	beclomethasone dipropionate
Bg	:	Birbeck granules
BM	:	basement membrane
BSA	:	bovine serum albumin
BUD	:	budesonide
CD	:	cluster of differentiation
CGD	:	Cumulative Gaussian Distribution
CHO	:	chinese hamster ovarian
CNSLD	:	chronic non-specific lung disease
CS	:	corticosteroids
DC	:	dendritic cells
DNA	:	deoxyribonucleic acid
e.g.	:	exempli gratia (for example)
ECP	:	eosinophil cationic protein
EDN	:	eosinophil derived neurotoxin
ELAM	:	endothelial leucocyte adhesion molecule
EpDRF	:	epithelium derived relaxing factor
EPO	:	eosinophil peroxidase
FcεR	:	receptor for constant part of heavy chain of IgE
FEV_1	:	forced expiratory volume in 1 second
FP	:	fluticasone propionate
GαM	:	goat anti-mouse
GC	:	glucocorticoid
GM-CSF	:	granulocyte macrophage colony-stimulating factor
GMA	:	glycol methacrylate
GR	:	glucocorticoid receptor
GRE	:	glucocorticoid responsive elements
HLA	:	human leucocyte antigen
hsp	:	heat shock protein
i.e.	:	id est
ICAM	:	intercellular adhesion molecule
IFN	:	interferon

Ig	:	immunoglobulin
IL	:	interleukin
LC	:	Langerhans cells
LFA	:	lymphocyte function-associated antigen
LT	:	leukotriene
mAb or MoAb	:	monoclonal antibodies
MBP	:	major basic protein
MCP	:	monocyte chemoattractant protein
MC_T	:	tryptase-containing mast cells
MC_{TC}	:	tryptase- and chymase-containing mast cells
MHC	:	major histocompatibility complex
MIP	:	macrophage inflammatory protein
MLDR	:	methacholine log-dose response
mRNA	:	messenger RNA
n	:	number in study or group
NEP	:	neutral endopeptidase
NGS	:	normal goat-serum
NO	:	nitric oxide
NS	:	not significant
P	:	plateau value
PAF	:	platelet activating factor
PB	:	peripheral blood
PBS	:	phosphate-buffered saline
PC_{20}	:	provocative concentration (of a non-specific agent, e.g. histamine) which causes a 20% decrease in FEV_1
PCR	:	polymerase chain reaction
PG	:	prostaglandin
PGDF	:	platelet-derived growth factor
PL	:	placebo
R	:	reactivity
RαM	:	rabbit anti-mouse
RANTES	:	regulated upon activation, normal T expressed, and presumably secreted
rBM	:	reticular basement membrane
RNA	:	ribonucleic acid
RSV	:	Respiratory Syncytial Virus
RT	:	room temperature
S	:	sensitivity
s*G*aw	:	specific airway conductance
TGF	:	transforming growth factor
Th	:	T helper
TNF	:	tumor necrosis factor

VCAM	:	vascular cellular adhesion molecule
VIP	:	vasoactive intestinal peptide
VLA	:	very late activation antigen

DANKWOORD

DANKWOORD

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Trudelie

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