BRONCHIAL EPITHELIAL CELLS AND PEPTIDASES

Modulation by cytokines and glucocorticoids *in vitro* and in asthma

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BRONCHIAL EPITHELIAL CELLS AND PEPTIDASES

Modulation by cytokines and glucocorticoids *in vitro* and in asthma

BRONCHUSEPITHEELCELLEN EN PEPTIDASEN

Beïnvloeding door cytokinen en glucocorticoïden *in vitro* en bij astmapatiënten

PROEFSCHRIFT

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Als je naar de zon kijkt
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1.1. STRUCTURE OF THE AIRWAYS

The airways can be divided in the upper respiratory tract, including the nose, the pharynx, and the larynx, and the lower respiratory tract, consisting of the trachea, bronchi, bronchioles, and alveoli (Fig. 1). This structure provides an enormous surface area where the exchange of oxygen and carbondioxide, the function of the lungs, can take place. Respiratory diseases may affect one or more of the different parts of the airways. For example, emphysema is characterized by a decreased number of alveoli which also have a reduced elasticity [1]. On the other hand, asthma, the main focus of this thesis, is considered to be a disease affecting predominantly the bronchi and bronchioli [2].

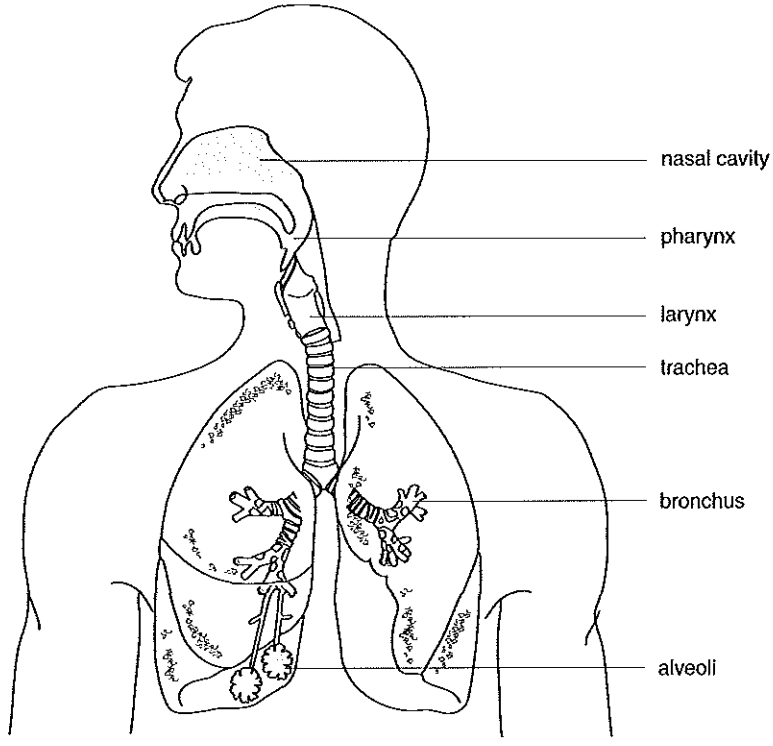

Figure 1. Structure of the human airways.

The bronchus can histologically be divided into a mucosa and a submucosa (Fig. 2) [3]. The bronchial mucosa consists of a lining layer of ciliated and non-ciliated epithelial cells, the basement membrane, and the lamina propria. In the lamina propria, a dense network of arterioles, capillaries, and postcapillary venules is embedded in collagenous, elastic, and reticular fibers [4, 5]. The submucosa of the airways contains cartilage, glands, and smooth muscle (Fig. 2). Nerves can be found both in the epithelium, lamina propria, and submucosa [6-8].

In asthmatic patients, several changes occur in the bronchi and this results in their clinical symptoms [9, 10]. But what is asthma and what exactly is going on in this disease?

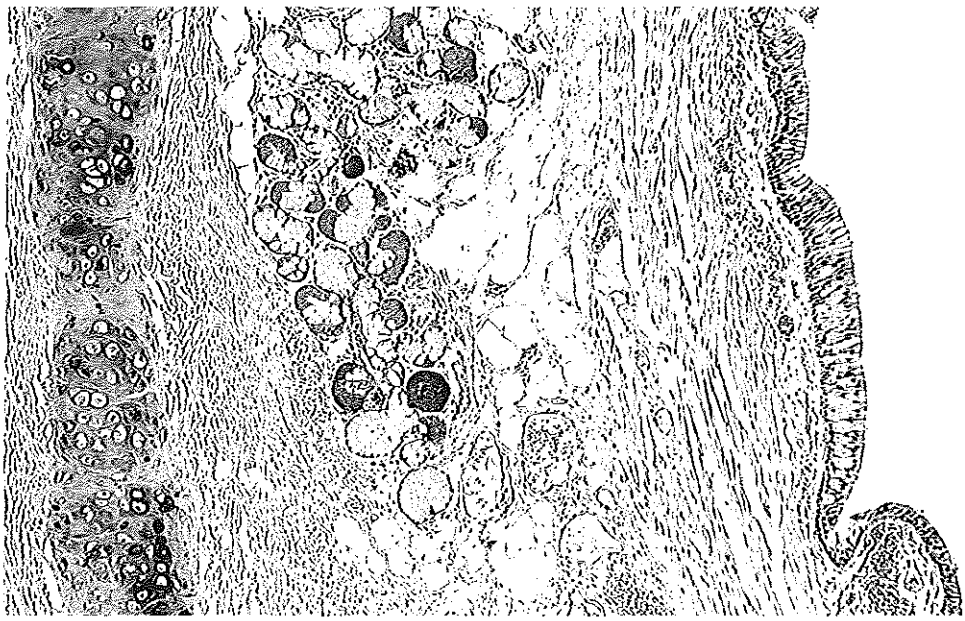

Figure 2. Light-microscopic photomicrograph of the human bronchus (magnification: 64x).

1.2. ASTHMA: AN INFLAMMATORY DISEASE OF THE AIRWAYS

The term asthma was first used by Hippocrates (460-357 B.C.), who gave this name to people suffering from "difficult breathing" ('ασθμα'). Nowadays, asthma is one of the most common chronic disorders in the Western World and affects almost 10% of the population [11]. Its prevalence, morbidity and mortality appear to be rising [2, 12-14]. From a simplified point of view, asthmatic patients may be categorized in two groups. The vast majority of the asthmatic patients develops an allergic reaction after exposure to specific stimuli, so called allergens (*e.g.* house dust mite, pollen, animal dander). These patients are called allergic (extrinsic or atopic) asthmatics and have enhanced serum levels of allergen-specific immunoglobulin E (IgE). Allergic asthma often develops during childhood, and the symptoms may show seasonal variation (due to the seasonal variation of allergens, like grass pollen, in the environment) [11]. Allergic asthma shows a strong genetic predisposition and several

studies have reported linkage of atopy genes to chromosome 5 (q23-31) or 11 (q13) [15-17]. In a much smaller percentage of asthmatic patients (10-20%), serum IgE levels are not enhanced and therefore these patients are categorized as non-allergic (intrinsic or non-atopic) asthmatics.

Because of the heterogeneity in asthmatic patients, it is difficult to define asthma. Nevertheless, based on the common characteristics, the following current working definition of asthma has been formed:

'Asthma is a *chronic inflammatory disorder* of the airways in which many cells play a role, including mast cells and eosinophils. In susceptible individuals this inflammation causes symptoms which are usually associated with widespread but variable *airflow obstruction* that is often *reversible* either spontaneously or with treatment, and causes an associated *increase in airway responsiveness* to a variety of stimuli' [2].

The keywords in this definition, which are in italic, will be discussed in the next paragraphs.

1.3. CLINICAL ASPECTS OF ASTHMA

Asthma is clinically defined by a reversible airway obstruction and a hyperreactivity of the airways [18]. The airway obstruction is mainly the result of the contraction of smooth muscle cells, the secretion of mucus, and enhanced vascular permeability with mucosal edema. In contrast to some other airway disorders like chronic bronchitis and emphysema, the airway obstruction in asthma is usually completely reversible, and between exacerbations the patient may have no airflow obstruction. The airway obstruction is considered to be reversible if the patients forced expiratory volume in one second (FEV_1) increases by at least 15% after inhalation of smooth muscle relaxing drugs, the β_2-agonists.

Hyperreactivity is defined as a decreased threshold of airway narrowing in response to a variety of non-specific stimuli, which under healthy conditions do not evoke an airway obstruction [2]. These non-specific stimuli include fog, tobacco smoke, ozone, viral infections, chemical irritants, inhaled pharmacological agents (such as histamine or methacholine), and physical stimuli (such as exposure to cold air and exercise) [19].

Asthmatic reactions can clinically be divided in an early asthmatic reaction (EAR) and a late phase asthmatic reaction (LAR) [20, 21]. The EAR develops immediately after the inhalation of allergens and is characterized by bronchoconstriction. This reaction, which is maximal at 15-30 min and resolves within 1-2 h, is due to the release of broncho-active substances, such as histamine and leukotrienes (LT), resulting in the contraction of smooth muscle, the secretion of mucus and vascular leakage [20]. In approximately half of the asthmatic patients, this EAR is followed by a LAR which begins at 3-4 h, is maximal at 6-12 h and generally resolves within 24 h. The LAR is accompanied by an infiltration of leukocytes in the airways [20].

1.4. IMMUNOLOGICAL ASPECTS OF ASTHMA

Nowadays it has widely been accepted that a chronic inflammation of the airways underlies the clinical features of asthma [17]. Indeed, bronchial tissue of asthmatic patients shows intense infiltration of leukocytes, especially eosinophils and T lymphocytes, damage and detachment of the bronchial epithelium, thickening of the epithelial basement membrane, edema of the submucosa, mucus gland hyperplasia, and smooth muscle hypertrophy [9, 10, 22-28].

The inflammatory response is the result of a complex interaction between the allergen(s), cells of the immune system, and their mediators (Fig. 3).

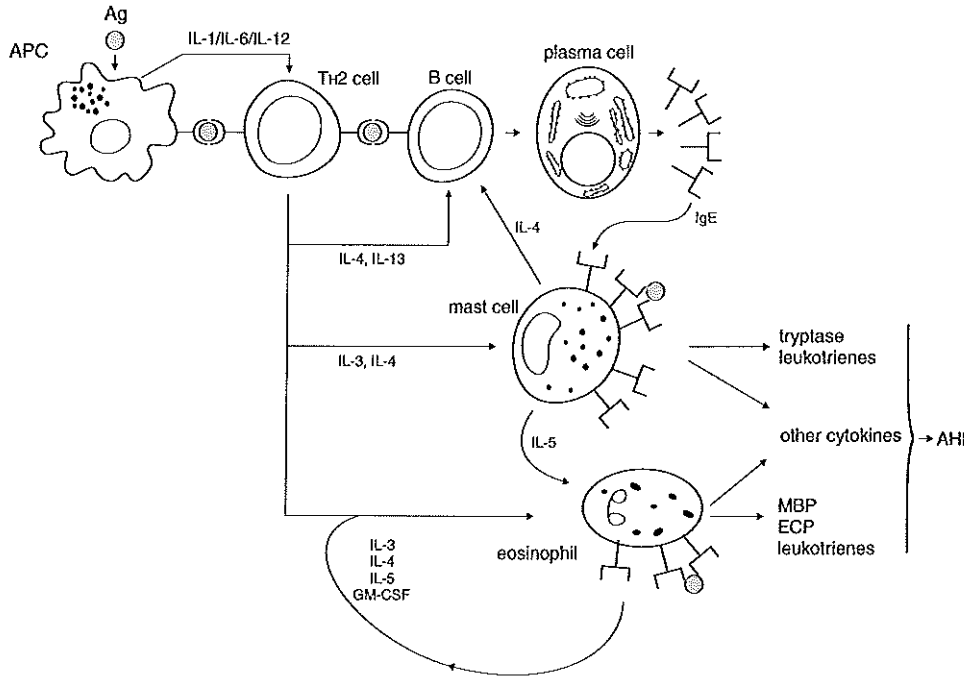

Figure 3. Schematic representation of the inflammatory process in airways of asthmatic patients. The initial event in the allergic immune response is thought to be presentation of antigen by APC. Subsequent recognition of the MHC class II-bound antigen by the CD4-positive T lymphocytes and simultaneous signaling via co-stimulatory molecules results in the release of cytokines and the development of Th2 cells, which are characterized by production of cytokines like IL-4, IL-5, and IL-13 [29-32]. These cytokines play an important role in the maturation of naive T lymphocytes towards the Th2 lymphocyte phenotype, in the recruitment and survival of eosinophils (together with GM-CSF and IL-3), support mast cell growth, and are required for isotype switching of B lymphocytes to IgE production [33-40]. The IgE present in the asthmatic airways can bind with high affinity to specific receptors (FcεRI) expressed on the surface of mast cells, basophils, monocytes, APC, and eosinophils [41, 42]. Binding of an inhaled allergen to receptor-bound IgE and subsequent cross-linking of these receptors results in the activation of the cell and thereby in the release of inflammatory mediators, including histamine, leukotrienes, and prostaglandins. These mediators can directly act on airway smooth muscle and vasculature, and are responsible for the airway obstruction seen during the EAR [43].

Eosinophils

Airways of asthmatic patients show an increased number of eosinophils, both in bronchial biopsies, bronchoalveolar lavage (BAL) fluid, and sputum [44-47]. Furthermore, the eosinophils in the asthmatic airways are often degranulated and hypodense, suggesting that they are in an activated state [48]. The number of eosinophils and their products have been shown to correlate significantly with the severity of the disease [45, 49-53]. Besides the elevated number of eosinophils under stable conditions, eosinophil numbers even further increase in the LAR after allergen provocation [54].

Several observations indicate that eosinophils play an important role in the epithelial damage seen in asthmatics. First, several *in vitro* studies have shown that activated eosinophils can alter the epithelial integrity by disruption of epithelial cells [55-57]. Second, it was shown that the cationic granule proteins of eosinophils are highly toxic to the respiratory epithelium [55-59], and increased levels of these proteins have been found in the BAL fluid of asthmatics compared to healthy subjects [60, 61]. Finally, bronchial biopsies of asthmatics show increased numbers of activated eosinophils and an association between eosinophils present near the epithelial layer and epithelial disruption has been found [10, 23, 61].

In addition to the cytotoxic proteins derived from the eosinophil granule, membrane phospholipid-derived mediators may also play a role in the pathogenesis of asthma. Upon activation, eosinophils produce considerable quantities of LTC_4 and platelet-activating factor (PAF), factors that are able to contract smooth muscle, increase vascular permeability, evoke vasodilatation, enhance mucus secretion and increase bronchial hyperresponsiveness [62, 63]. Eosinophils express FcεRI on their surface and inhalation of an allergen may therefore result in the activation of the eosinophils. The release of LTC_4 and PAF, together with other mediators, may subsequently be responsible for the airway obstruction seen during the EAR [43].

Eosinophils are also a newly recognized source of several cytokines, including interleukin (IL)-1α, IL-3, IL-4, IL-5, IL-6, IL-8, granulocyte/macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α, and macrophage inflammatory protein-1α (MIP-1α) [39, 64-73]. Since many of these cytokines are required for recruitment, activation and survival of eosinophils, an autocrine regulation may prolong the eosinophilic inflammation in the asthmatic airways, thereby contributing to the chronicity of the inflammatory reaction.

The recruitment of eosinophils into the airways requires the presence of chemokines and adhesion molecules. Several chemokines, including eotaxin, IL-5, monocyte chemoattractant protein (MCP)-3, MCP-4, MIP-1α and RANTES (Regulated upon Activation, Normal T cell Expressed, and presumably Secreted) are involved in the recruitment of eosinophils into the airways, predominantly via activation of the CCR-3 receptor [74-79]. Interaction between very late activation antigen (VLA)-4 on eosinophils and its ligand vascular cellular adhesion molecule (VCAM)-1 on endothelial cells seems to play a role in the selective recruitment of eosinophils into the airways [80-83]. Expression of VCAM-1 is up-regulated by IL-4, and immunohistochemical studies have shown an increased expression of VCAM-1 in asthmatic airways [81, 83].

Lymphocytes

T lymphocytes probably play a role in all inflammatory responses that are antigen driven, in the sense that they are the only cells that can recognize and respond directly to such antigens. T lymphocytes can be divided into two major functional subgroups: CD4-positive helper T cells and CD8-positive cytotoxic T cells (Fig. 4).

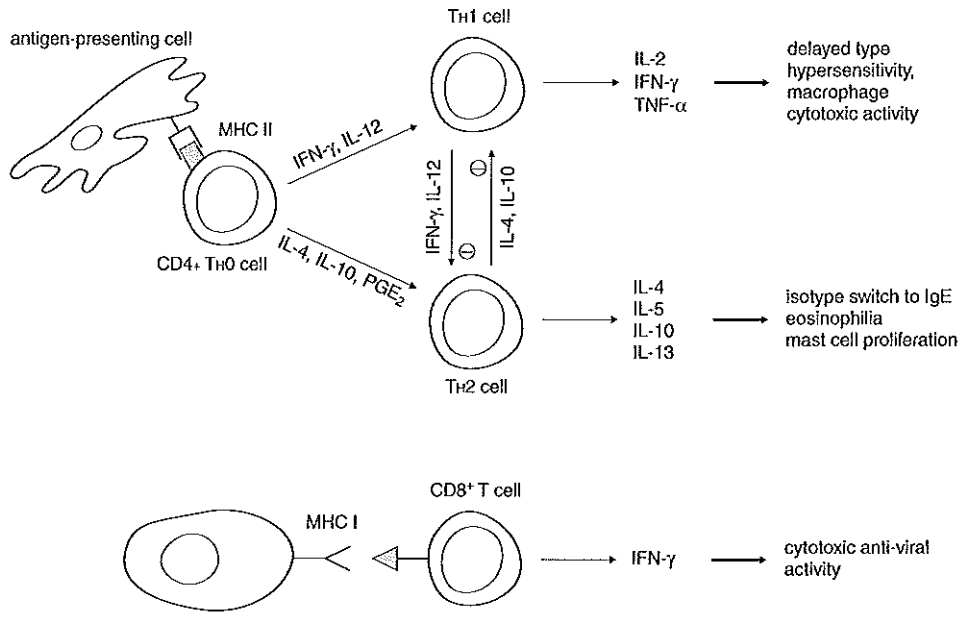

Figure 4. T cell subsets and their functions.

Helper T cells (CD4-positive) recognize antigen in the context of MHC class II molecules and this results in their activation, proliferation, and release of cytokines. Based on their cytokine profile produced, at least three distinct subsets can be distinguished: Th0, Th1, and Th2 [29, 84]. Th1 cells predominantly secrete IL-2 and interferon-γ (IFN-γ), whereas Th2 cells produce IL-4, IL-5, IL-9, IL-10 and IL-13 (Fig. 4). Other cytokines, like IL-3 and GM-CSF, can be secreted by both subtypes. Th0 cells are able to produce all of these cytokines. The development of Th1 cells is strongly promoted by IFN-γ and IL-12, whereas IL-4, IL-10, and prostaglandin (PG) E_2 stimulate the development of Th2 cells [85, 86]. IFN-γ inhibits the development of Th2 cells, whereas Th1 cell development is inhibited by IL-10 [86]. Thus, products of Th1 cells have the capacity to inhibit the growth of Th2 cells, and vice versa (Fig. 4). Recently, another CD4-positive T cell subset has been described [87]. This subset, designated T regulatory cells (Tr1), was shown to produce high levels of IL-10, to suppress antigen-specific immune responses and to down-regulate pathological immune responses *in vivo* [87].

Th2 cells, through their release of IL-3, IL-4, IL-5, and IL-9, favor the isotype switch of B lymphocytes to IgE [37], support mast cell growth, and promote the recruitment, activation and survival of eosinophils [39, 88, 89], and are therefore strongly implicated in the pathogenesis of asthma. Moreover, high levels of IL-9 may contribute to bronchial hyperresponsiveness [90]. The apparent predominance of Th2 cells in asthma is supported by the observation that asthmatic airways show increased numbers of cells expressing Th2-like cytokines [30, 91-93]. In addition, increased levels of Th2-like cytokines can be detected in BAL fluid from asthmatics compared to healthy individuals [94]. However, Th2 cells comprise a minor population in the airways of asthmatic patients and other T lymphocytes present within the airways may release IFN-γ upon activation [92, 95]. A preliminary

study showed that, after allergen challenge, not only IL-4 but also IFN-γ was increased in BAL fluid [96]. In a murine model of asthma, it was shown that the development of airway hyperresponsiveness was dependent on IFN-γ and independent of eosinophil infiltration [97]. Furthermore, IL-4 knockout mice sensitized and challenged with ovalbumin do not develop airway hyperresponsiveness [98, 99]. Therefore, it has been suggested that IL-4 is essential in the development and initial phase of the allergic reaction, but its function in the effector phase remains uncertain [97-99].

The total numbers of both CD4-positive and CD8-positive T lymphocytes in the bronchial mucosa of asthmatics do not differ significantly from healthy subjects and in both groups CD4-positive cells predominate over CD8-positive cells [100-102]. Only cells in the biopsies of asthmatics showed evidence of activation, as determined by the expression of the IL-2 receptor (CD25) and MHC class II and VLA-1 molecules [31, 101, 103-105]. Interestingly, in a study using asthmatic patients known to develop a LAR, a selective increase in CD4-positive T cells in BAL fluid 48 h after allergen challenge was reported [106]. This finding complements those of decreased CD4-positive T cells in peripheral blood after allergen challenge in atopic asthmatics and suggest that selective recruitment of CD4-positive T cells to the lungs may occur in association with the LAR to allergen challenge [107, 108]. The recent observation that the CCR-3 receptor is, in addition to basophils and eosinophils, selectively expressed on Th2 cells, and not on Th1 cells or CD8-positive T cells, clearly indicated that this receptor is of importance in allergic reactions [109].

CD8-positive T cells recognize antigens in the context of MHC class I molecules. These cells are involved in the elimination of cells expressing new antigens as a result of bacterial or viral infection or malignant transformation (Fig. 4). The role of CD8-positive cells in asthma is not completely clear yet. However, in a Th2-like environment virus-specific CD8-positive cells may decrease their IFN-γ production and increase their production of IL-5, which may cause eosinophilia after viral challenge [110, 111]. Such results may explain the link between viral infections and bronchial asthma, as an IL-4-dependent switch to CD8-positive cells secreting IL-5 may not only exacerbate asthma by recruiting eosinophils in to the airways, but impaired IFN-γ production may also lead to delayed viral clearance [110-112]. In addition, there is growing body of evidence suggesting that CD8-positive T cells play an important role in regulating IgE responses. The natural immune response to inhaled protein antigens includes a MHC class I-restricted CD8-positive T cell component, the appearance of which is associted with active suppression of IgE antibody production [113]. Furthermore, it has been shown that antigen-specific CD8-positive T cells inhibited IgE responses and IL-4 production by CD4-positive T cells in rats [114].

B lymphocytes and plasma cells are the producers of antibodies. Initially the B cell produces intracellular antigen-specific IgM, which then becomes bound to the surface of the cell and act as the antigen receptor for that cell. On exposure to that antigen and factors released by helper T cells, B cells become activated, may switch their isotype production, start to divide and differentiate in memory cells or plasma cells. Isotype-switching to IgE, as occurs in the development of allergy, requires the presence of IL-4 produces by Th2 cells.

B lymphocytes are rare in the bronchial mucosa and BAL fluid, both in asthmatics and in healthy subjects [115]. In contrast, distinct B cell areas can be detected in BALT [116]. In peripheral blood of allergic asthmatics, increased numbers of B cells bearing the low-affinity IgE receptor (CD23) can be found, indicating B cell activation [104].

Mast cells

The human respiratory tract is richly endowed with mast cells, particularly beneath the bronchial epithelium and in the alveolar walls. There are two types of mast cells that can be distinguished by their granule content of neutral proteases. Mast cells located at mucosal surfaces contain predominantly tryptase (MC_T), while at connective tissue sites mast cells are enriched with chymase and carboxypeptidase A in addition to tryptase (MC_{TC}) [117]. Increased numbers of mast cells (predominantly from the MC_T subset) have been found in patients with asthma, and this is accompanied by an increase in both cell-associated and cell-free histamine and tryptase in the BAL fluid [21, 118, 119]. Mast cells recovered from the airways of asthmatics by BAL exhibit spontaneous release of histamine and PGD_2 and also exhibit increased responsiveness to allergen [120]. This suggests that mast cells in asthma are primed for mediator release, possibly by cytokines like IL-4 and IL-10.

In human asthma, the EAR is largely caused by the IgE-dependent release of bronchoconstrictor mediators from activated airway mast cells. Indeed, after allergen challenge, increased levels of histamine, tryptase, PGD_2, and LTE_4 (the terminal metabolite of LTC_4) have been detected in BAL fluid [21, 119, 121].

Mast cells may also play a role in maintaining the chronicity of the inflammatory response by producing a variety of cytokines, including IL-4, IL-5, IL-6, IL-8, GM-CSF, and TNF-α [122]. Since IL-4 selectively stimulates the development of Th2 lymphocytes [29] and IL-5 promotes recruitment and survival of eosinophils [39, 88], it has been suggested that mast cells may also be important in the initial stage of the disease [17].

Mast cells can often be found in close proximity with sensory nerves and therefore an interaction between these two cells has been suggested [123, 124]. Indeed, tachykinins released from sensory nerves have been shown to activate human lung mast cells to release histamine [125].

Dendritic cells

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) of the body and are unique in their capacity to stimulate naive T cells [126, 127]. In the human lung, DC are predominantly located in epithelial and subepithelial tissue of the bronch(iol)us and the bronchus-associated lymphoid tissue (BALT) [128]. Although DC are a heterogeneous population of cells, typical immunocytological features are their long cytoplasmic extensions and a strong expression of major histocompatibility complex (MHC) class I and II molecules and co-stimulatory molecules (intercellular adhesion molecule-1 (ICAM-1; CD54), B7-1 (CD80), and B7-2 (CD86)) [116, 129, 130]. Ultrastructural features of DC are the presence of intracytoplasmic structures, the so-called Birbeck granules. Other features often used to characterize DC are the expression of CD1a, L25 or S100, but these markers are not necessarily present on each DC subtype [128, 131].

Increased numbers of DC have been described in the epithelium and lamina propria of asthmatic patients compared to healthy controls [132-134]. However, although it is clear that DC play an important role in the presentation of antigens to lymphocytes, less is known about their precise role in asthma.

Macrophages and monocytes

The airway macrophage is the most numerous cell within the airway lumen, accounting for 80-90% of the airway cells in BAL fluid in both healthy and asthmatic subjects [28]. Macrophages and monocytes can be activated in an IgE-dependent manner via FcεRII (CD23)

to release a variety of mediators. These mediators include lipid mediators like LTB_4, LTC_4, PGD_2 and platelet-activating factor, cytokines like IL-1β, TNF-α, and GM-CSF, reactive oxygen species (such as O_2^-), and hydrolytic enzymes [135-138]. Bronchial biopsies of asthmatic patients were found to have increased numbers of total macrophages [115], but a reduced number of immunosuppressive macrophages compared to healthy individuals [139]. The contribution of macrophages to the pathogenesis of asthma, however, still remains to be determined.

Neutrophils

The evidence that neutrophils by themselves play an important role in the pathogenesis of asthma is controversial. Comparison of the number of neutrophils in the bronchial mucosa or BAL fluid of stable asthmatics compared to healthy controls did not reveal significant differences [54, 115, 140]. However, some recent studies suggest that neutrophilia may be an early event preceding eosinophilia. Montefort *et al.* demonstrated an increased number of neutrophils in the submucosa of asthmatics 6 h after allergen challenge, and at this time point the magnitude of the neutrophil response was more pronounced than observed for eosinophils [141]. Finsnes *et al.* showed, in a rat model for asthma, an early but transient increase in neutrophils in BAL fluid, which preceded the influx of eosinophils [142]. Clearly, the role of the neutrophil in asthma needs further study.

Other cells and mediators

In addition to leukocytes, it is now generally accepted that also structural cells of the bronchus are involved in the initiation and perpetuation of inflammatory reactions within the airways. In this regard, bronchial epithelial cells are of particular importance. These cells form the interspace between the internal milieu of the lung and the inhaled air, and thus will be exposed to an array of stimuli present within the air. It has been shown that bronchial epithelial cells produce a variety of mediators that may contribute to the pathogenesis of asthma (reviewed in [143]). The structure and function of the bronchial epithelium as well as the pathophysiologic changes observed in asthma will be discussed in chapter 4.

Loss of epithelial integrity or epithelial damage may expose intra-epithelial, nonmyelinated, sensory nerves, which contain neuropeptides such as substance P and neurokinin A [144, 145]. Excitation of sensory nerves by inflammatory mediators might produce a retrograde conduction with local release of neuropeptides, a mechanism called the 'local axon reflex' [146]. Release of neuropeptides may subsequently result in contraction of smooth muscle cells, microvascular leakage, vasodilation, secretion of mucus, and cough, a process known as neurogenic inflammation [144, 145, 147]. The effects of neuropeptides are limited by rapid degradation by peptidases like neutral endopeptidase (NEP) [148]. Inhibition of NEP, as has been shown to occur by viruses [149], cigarette smoke [150], and chemical irritants [151], may therefore contribute to enhanced neurogenic inflammation. The innervation of the human airways and its possible role in asthma will be discussed in chapter 2. Peptidases will be discussed in detail in chapter 3.

Glucocorticoid therapy: suppression of the immune response

Asthmatic airways are chronically inflamed, even when patients are asymptomatic, and therefore anti-inflammatory drugs such as glucocorticoids should be used early in the course of the disease. Several studies have shown that glucocorticoids improve the clinical signs of asthma, and decrease the level of bronchial responsiveness, the requirements for other drugs

like β_2-agonists and oral steroids, and the inflammatory process [152-156]. Glucocorticoids and their beneficial effects in asthma will be discussed in more detail in chapter 5.

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Chapter 2

Autonomic innervation of human airways
Structure, function, and pathophysiology in asthma

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Autonomic innervation of human airways
Structure, function, and pathophysiology in asthma

The human airways are innervated via efferent and afferent autonomic nerves, which regulate many aspects of airway function, including airway smooth muscle tone, airway secretion, bronchial circulation, microvascular permeability, and the recruitment and subsequent activation of inflammatory cells [1, 2]. In addition to the classic cholinergic and adrenergic innervation of the airways, neural mechanisms that are not blocked by cholinergic or adrenergic antagonists are present [1-4]. Originally, it was thought that this non-adrenergic non-cholinergic (NANC) system was an anatomically separate nervous system, but at present it is clear that at least certain NANC neural effects are mediated by the release of neurotransmitters from classic parasympathetic (cholinergic) or sympathetic (adrenergic) nerves. The inhibitory NANC (i-NANC) system, which is the only neural bronchodilator pathway in the human airways, is co-localized with acetylcholine in the parasympathetic nerves [5, 6]. NANC vasoconstrictor responses are mediated by the release of neuropeptide Y from adrenergic nerves [7]. Finally, the excitatory NANC (e-NANC) system (which activation results in bronchoconstriction) is located in a subpopulation of non-myelinated sensory C-fibers [8].

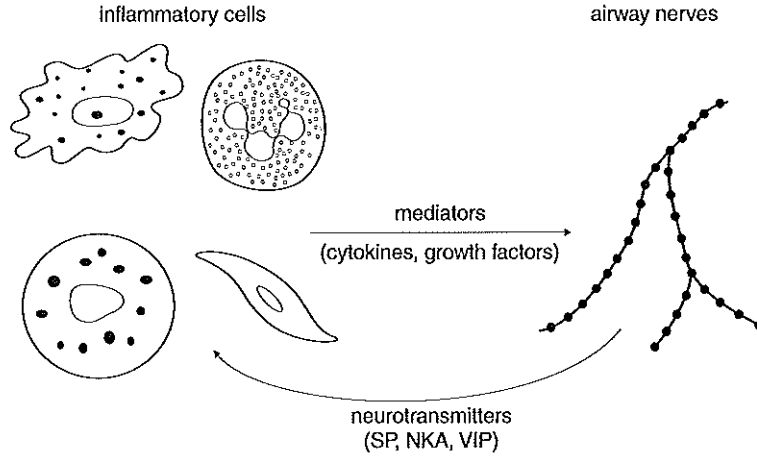

Figure 1. Interaction between airway nerves and inflammatory cells.

There is evidence that neural control of the airways may be abnormal in asthmatic patients, and that neurogenic mechanisms may contribute to the pathogenesis and pathophysiology of asthma. A complex interaction between cells and mediators of the immune system and the

nervous system is present within the airways (Fig. 1). Inflammatory mediators may modulate or facilitate the release of neurotransmitters from airway nerves, whereas neural mechanisms may contribute to the inflammatory process in the airways by causing neurogenic inflammation.

2.1. PARASYMPATHETIC NERVOUS SYSTEM

Parasympathetic nerves are the dominant neural pathway in the control of airway smooth muscle tone and secretion in human airways. Their major neurotransmitter is acetylcholine (Ach). It acts via binding to the muscarinic receptors, of which at least 3 subtypes can pharmacologically be recognized in the human lung [9]. Excitatory M_1 receptors are present in airway parasympathetic ganglia and may facilitate neurotransmission, which is mediated via nicotinic receptors (Fig. 2) [10]. In contrast, M_2 receptors on postganglionic nerve terminals in human central airways and subsegmental and terminal bronchi inhibit the release of Ach, thus reducing the stimulation of postjunctional M_3 receptors which constrict airway smooth muscle (Fig. 2) [11-14].

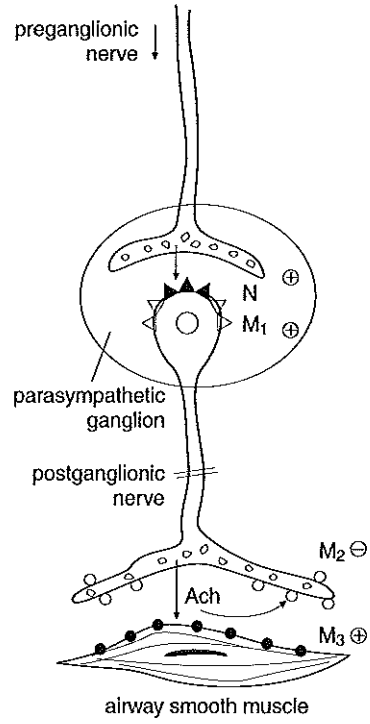

Figure 2. Muscarinic receptor subtypes in the human airways. M_1 receptors in parasympathetic ganglia may facilitate neurotransmission. In contrast, M_2 receptors inhibit the release of Ach, thereby reducing the stimulation of postjunctional M_3 receptors on airway smooth muscle.

Several mechanisms may contribute to cholinergic bronchoconstriction in asthma. First, asthmatic patients may have an increased cholinergic reflex bronchoconstriction due to stimulation of sensory receptors in the airways by inflammatory mediators like histamine, bradykinin, and prostaglandins (PG) [13, 15, 16]. Second, the release of Ach may be increased in asthmatics. Several mediators known to be increased in asthmatics (such as tachykinins, thromboxane, and PGD_2) have been shown to facilitate Ach release from postganglionic nerves in the airways [17, 18]. Third, evidence for a dysfunction of M_2 receptors has been found [19, 20]. Such a defect may then result in exaggerated reflexes in asthma, since the normal feedback inhibition of Ach release may be lost (Fig. 2). Some recent studies suggest that major basic protein, released by eosinophils, may contribute to the dysfunction of the M_2 receptors [21-23]. Interestingly, in asthmatic patients many eosinophils and their granule proteins are seen in association with airway nerves [23]. It has also been shown that viral infection may result in a loss of M_2 receptor function, due to the action of viral neuraminidase on the sialic acid residues of M_2 receptors, which are necessary for their function [24, 25]. Recently, it has been shown that immunoglobulin E (IgE) may facilitate Ach release from cholinergic nerves, an effect that also appears to be related to M_2 receptor dysfunction [26]. Finally, inflammatory mediators may directly increase the sensitivity of human airway smooth muscle cells to cholinergic stimulation resulting in an enhanced bronchoconstriction [27].

2.2. SYMPATHETIC NERVOUS SYSTEM

The sympathetic or adrenergic nervous system is less prominent than the parasympathetic nervous system within the human airways. Its main neurotransmitters are noradrenaline and neuropeptide Y [1, 28]. Noradrenalin is able to activate α- and β-adrenergic receptors on target cells in the airways. There is a sparse adrenergic innervation of the human airways, with adrenergic fibers especially present in close association with submucosal glands and bronchial arteries. Airway smooth muscle does not seem to be innervated by the adrenergic nerve system, but it is possible that adrenergic nerves may influence bronchomotor tone indirectly via pre-junctional α- and β-adrenergic receptors [29-33].

The α_1-adrenergic receptor, which mediates the contraction of smooth muscle, is relatively sparse and may only be demonstrated under certain conditions [34-38]. Prejunctional α_2-adrenergic receptors (autoreceptors) may inhibit the release of both norepinephrine and of neuropeptide Y (NPY) from adrenergic nerves and the release of tachykinins from sensory nerves [39-41]. Cholinergic neurotransmission may also be inhibited via prejunctional α_2-adrenergic receptors [39].

β-adrenergic receptors, which mediate bronchorelaxation, are widely distributed in the human lung [36, 42-45]. At least three β-adrenergic receptors can be distinguished: β_1-, β_2, and β_3-adrenergic receptors [46-49]. In the human lung, β-adrenergic receptors on smooth muscle cells are entirely of the β_2-subtype and their number increases towards the peripheral airways [36, 37, 50]. The epithelial and mast cell β-adrenergic receptors are also of the β_2-subtype, whereas in human submucosal glands and alveolar walls, receptors of the β_1-subtype have also been found [36]. Thus far, no β_3-adrenergic receptors have been detected in the human lung [47-49].

A reduced respiratory β-adrenergic receptor function in asthma has been postulated [34]. Such a defect would lead to impaired relaxation of airway smooth muscle and could increase

cholinergic tone and mediator release from mast cells. Investigations into the function of β-adrenergic receptors in asthmatics, however, have shown conflicting results. Several investigators reported decreased β-adrenergic receptor function in isolated airways of asthmatic patients [51-53], whereas others found normal relaxations of airways smooth muscle from asthmatics [54, 55]. Studies on polymorphisms in the β_2-adrenergic receptor gene have shown that the frequency of most polymorphisms (Gly16, resulting in enhanced agonist-promoted down-regulation; Glu27, resulting in resistance to down-regulation; and Ile164, resulting in altered coupling to adenyl cyclase) is not different between asthmatic patients and healthy controls [56-58]. However, some recent studies indicate that polymorphic forms may promote asthmatic phenotypes or influence the response to β-agonist therapy [59-61]. Expression of mRNA encoding the β_2-adrenergic receptor has been reported to be increased in patients with asthma [62]. However, the density of the receptor expression in asthmatic patients is not different from those observed in healthy subjects [63, 64]. Lack of β_2-adrenergic receptor dysfunction may be demonstrated most convincingly by the fact that β-agonists have excellent bronchodilatory effects in asthmatic patients.

In addition to norepinephrine, adrenergic nerves contain NPY, a 36 amino acid peptide which is a cotransmitter with norepinephrine and usually amplifies its effects [4]. NPY, which is part of the e-NANC nervous system, has no direct effect on airway smooth muscle but may cause bronchoconstriction via release of prostaglandins [65]. In addition, NPY is a potent vasoconstrictor in some vascular beds [4, 66]. NPY may also modulate immune cell functions, such as T cell adhesion to fibronectin [67, 68].

In asthmatic patients, no difference in the number of NPY-immunoreactive nerves in the airways has been found compared to healthy controls [69]. In contrast, serum levels of NPY have been shown to be increased during exacerbations of asthma [70].

2.3. INHIBITORY-NANC NERVOUS SYSTEM

The i-NANC nervous system is the only neural bronchodilator pathway in the human airways. Anatomically, it is co-localized with acetylcholine in the parasympathetic nerves [5, 6]. Neurotransmitters of the i-NANC system include neuropeptides such as vasoactive intestinal peptide (VIP), peptide histidine methionine (PHM), and pituitary adenylate cyclase activating peptide (PACAP) [71, 72], and nitric oxide (NO) [73-76].

2.3.1. Vasoactive intestinal peptide and peptide histidine methionine

VIP is a 28 amino acid peptide, which acts through binding to the VIP receptors [3]. Receptors for VIP are localized in pulmonary vascular smooth muscle, airway smooth muscle of large, but not small airways, airway epithelium, and submucosal glands [77-80]. At present, at least two VIP receptors can be distinguished [81-84]. Binding of VIP to its receptors (which is dependent on its C-terminal part [85]) activates adenyl cyclase, resulting in elevated cyclic AMP levels [86]. The effects of VIP are, therefore, often similar to the effects of β-adrenergic receptor agonists. VIP is one of the most potent relaxants of airway smooth muscle. It may be co-released from cholinergic nerves (together with NO) and act as functional

antagonist of cholinergic bronchoconstriction. In addition, VIP and NO may act prejunctionally to inhibit Ach release (Fig. 3).

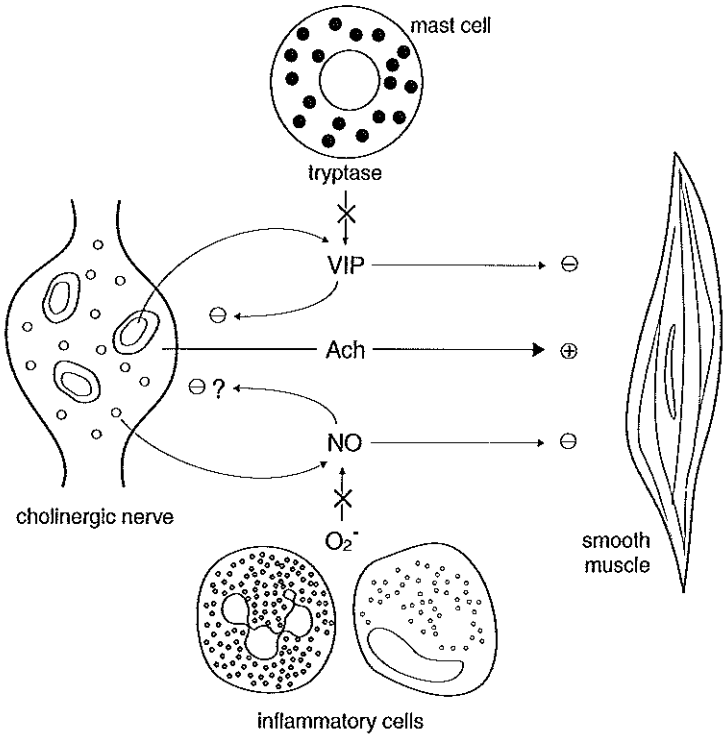

Figure 3. Functional antagonism of cholinergic neurotransmission. VIP and NO, coreleased from cholinergic nerves, may act as functional antagonists of cholinergic bronchoconstriction and may act prejunctionally to inhibit Ach release [87]. In asthmatic airways, VIP and NO may more rapidly be inactivated, thereby leading to exaggerated bronchoconstriction.

Besides effects on smooth muscle, VIP potently stimulates mucus secretion [88] and is a potent vasodilator [89, 90]. Interestingly, an increased number of VIP-positive nerves can be found around glands of patients with chronic bronchitis or hypertrophic rhinitis, suggesting a role for VIP in sputum production and hypersecretive changes [91, 92]. VIP also has several immunomodulatory functions (reviewed in [93]). These effects include inhibition of mediator release from mast cells [94], inhibition of T lymphocyte proliferation, IL-2, IL-4, and IL-10 production [95-97], regulation of isotype-switching in B lymphocytes [98], and stimulation of IL-6 and IL-8 release from human bronchial epithelial cells [99].

PHM is produced by alternative splicing of the gene encoding VIP [3, 100]. PHM stimulates adenyl cyclase and appears to activate the same receptor as VIP [78]. Therefore, the effects are similar to the effects of VIP, although some differences in potencies have been described [1, 101, 102].

2.3.2. Nitric oxide

NO appears to be the major neurotransmitter of i-NANC nerves in human airways [73-76]. NO is formed during the conversion of L-arginine and oxygen to L-citrulline by the enzyme nitric oxide synthase (NOS). After production, NO is released by simple diffusion. NOS-containing nerves can be found in tracheal and bronchial smooth muscle, around submucosal glands and around blood vessels [71, 103]. As mentioned above, NO may be coreleased with Ach and VIP, and has potent smooth muscle relaxing properties. Furthermore, increased NO production in the airways may result in hyperemia, plasma exudation, and mucus secretion [104, 105]. NO also has been implicated in skewing T lymphocytes towards a Th2 phenotype, through inhibition of Th1 cells and their production of IFN-γ [106].

Whether i-NANC responses are impaired in asthma is not clear. In patients with severe asthma, immunohistochemistry showed a striking depletion of VIP-positive nerves, but this may be due to rapid degradation of VIP during the processing of the biopsies [107, 108]. Indeed, no differences in VIP-immunoreactivity in tracheal or parenchymal tissue was observed between healthy subjects and asthmatic patients [109]. Also, no difference in VIP-receptor expression was observed between healthy subjects and asthmatic patients [80]. NANC bronchodilation has been demonstrated in human airways *in vivo* but does not appear to be defective in patients with mild asthma [51, 110]. Nevertheless, decreased plasma levels of VIP have been described during exacerbations of asthma [70]. Studies using guinea pigs have shown that antigen exposure results in impairment of neural NO-mediated relaxation [111, 112]. This effect was not due to reduced expression of NO synthase, but probably reflects rapid inactivation of NO by free radicals from inflammatory cells [113]. Similar, rapid degradation of VIP by mast cell tryptase [114-116] or other peptidases may result in exaggerated cholinergic neural bronchoconstriction (Fig. 3).

2.4. EXCITATORY-NANC NERVOUS SYSTEM

Excitatory NANC bronchoconstrictor responses are believed to be mediated via the release of neuropeptides from a subpopulation of non-myelinated sensory C-fibers in the airways. C-fibers of this nociceptive sensory nervous system transmit the sensations of itch and pain and are associated with tissue injury.

C-fibers are stimulated both by exogenous substances, such as cigarette smoke, capsaicin (the pungent principle of red pepper), or inhaled irritants, and by endogenous substances, such as histamine, bradykinin, and prostaglandins [117]. Upon stimulation, C-fibers transmit information to the central nervous system, where reflex responses may be evoked. In addition, neuropeptides are released from the peripheral ends of these afferents into the airway microenvironment, where they can bind to specific receptors and exert their effects (the so called 'local axon reflex') [118]. Among the best-studied neuropeptides of sensory nerves are the tachykinins (TK) substance P (SP) and neurokinin A (NKA), and calcitonin gene-related peptide (CGRP). Recently, secretoneurin has also been found to be a neurotransmitter of sensory nerves [119].

2.4.1. Tachykinins

2.4.1.1. Structure, localization and receptors

TK are a family of peptides with the common C-terminal sequence Phe-X-Gly-Leu-Met-NH_2 [3] At present, five tachykinin peptides have been identified: SP, NKA, neurokinin B (NKB), neuropeptide K (NPK), and neuropeptide-γ (NP-γ). Two distinct preprotachykinin (PPT) genes control the synthesis of TK peptides [3]. The PPT-A gene produces three mRNA by alternative splicing: αPPT-A encoding only SP; βPPT-A encoding SP, NKA, and NPK; and γPPT-A encoding SP, NKA, and NP-γ. The PPT-B gene encodes NKB.

Nerve fibers containing TK have been detected in the human airways by immunohistochemistry [8, 120-126]. SP-immunoreactive nerves can be found beneath and within the airway epithelium, around mucosal arterioles and submucosal glands, within bronchial smooth muscle and around local parasympathetic ganglia. A similar pattern is found for NKA-immunoreactivity, indicating co-localization of both TK. However, SP- or NKA-immunoreactive nerves are relatively sparse compared to VIP-immunoreactive nerves [69, 123].

Three mammalian neurokinin (NK) receptors have been cloned and characterized thus far [127, 128]. NK_1 receptors are activated preferentially by SP (>NKA>NKB), NK_2 receptors by NKA (>SP>NKB), and NK_3 receptors by NKB (>NKA>SP). All NK receptors are members of the superfamily of guanine nucleotide binding protein-coupled receptors. They are glycoproteins with seven putative alpha-helical transmembrane segments, an extracellular amino-terminus and an intracellular carboxy tail. The amino acid sequence of each receptor type is well conserved among species, but species-dependent pharmacological heterogeneity is evident [127-129].

In human airways, only SP and NKA are known to be present and the expression of NK_1 and NK_2 receptors predominate in various target cells of the airways. In general, NK_1 receptors are primarily responsible for mediating the inflammatory effects of TK, such as stimulation of mucus secretion and microvascular leak [130, 131], whereas NK_2 receptors mediate bronchoconstriction [132-134].

The localization of NK_1 receptors in the airways, as determined by autoradiography, shows a distribution that parallels the known actions of TK. NK_1 receptors are present on smooth muscle, pulmonary vessels, airway epithelium and submucosal glands [135, 136]. NK_2 receptor expression in the human lung has not yet been carefully characterized. However, *in vitro* studies have indicated that the contractile effects of TK on smooth muscle are mediated mainly, but not exclusively, by NK_2 receptors [132-134].

2.4.1.2. Effects of tachykinins

Upon release by sensory nerves and subsequent activation of specific NK receptors, TK are able to exert a wide variety of effects. Among the possible target cells are smooth muscle cells, submucosal glands, epithelial cells, blood vessels, nerves, and cells of the immune system.

Effects on airway smooth muscle

Studies using isolated human airways have shown that both SP and NKA, but not NKB, are able to contract human bronchi and bronchioli [124, 137-140]. NKA is a more potent

constrictor than SP and was reported to be, on a molar base, 2-3 orders of magnitude more potent than histamine or Ach [137]. The contractile response to NKA is significantly greater in smaller bronchi than in more proximal airways, indicating that TK may have a more important constrictor effect on more peripheral airways [139]. Using selective NK-receptor agonists and antagonists, it has been demonstrated that the constrictor effect is mediated mainly via NK_2 receptors [132-134]. However, NK_1 receptors may also be involved in SP-induced contraction of human small bronchi [141].

Interestingly, the contractile effects of SP and NKA on human bronchi *in vitro* can be modulated by passive sensitization. Human bronchi incubated overnight with serum from asthmatic patients atopic to *Dermatophagoides pteronyssinus* showed an enhanced sensitivity and an enhanced maximal contractile response to SP and NKA [142]. These enhanced effects were independent of changes in peptidase activities [142].

Several studies have determined the bronchoconstrictor effect of SP and NKA *in vivo*, both in healthy subjects and in asthmatics [143-151]. In accordance with the *in vitro* studies, it was found that both NKA and SP are bronchoconstrictors, NKA being more potent than SP. Furthermore, asthmatic patients were found to be hyperresponsive to SP and NKA. Some reports have suggested a role for the mast cell in the bronchoconstrictor effect of TK, but although SP has been shown to stimulate the release of histamine from human lung mast cells *in vitro* [152], it is not clear whether such a mechanism occurs *in vivo*.

In addition to the contractile effects of TK on airway smooth muscle cells, TK also increase the proliferation of these cells, an effect that is mediated via NK_1 receptors [153].

Effects on submucosal glands

SP and NKA stimulate mucus secretion from submucosal glands in human airways, both *in vitro* and *in vivo* [130, 154]. SP is more potent than NKA, indicating the involvement of the NK_1 receptor. As mentioned above, NK_1 receptors indeed have been identified on submucosal glands in human bronchi [135]. In addition, SP has been shown to be a potent stimulator of goblet cell secretion [155]. Because goblet cells are the only source of mucus in peripheral airways, SP may play a role in mucus secretion in peripheral airways.

Effects on blood vessels

In rodents, stimulation of sensory nerves or administration of TK causes microvascular leakage through the opening of endothelial gaps at postcapillary venules [156-159]. Among the TK, SP is the most potent and NK_1 receptors have been identified on postcapillary venules [160, 161]. Whether TK cause microvascular leakage in humans is not certain, since no direct measurements have been made. Nevertheless, SP and NKA increased the nasal protein output in patients with allergic rhinitis, suggesting the occurrence of microvascular leakage [131]. More definitive evidence was recently provided by the demonstration that capsaicin induced plasma extravasation in the human nose via a neuronally mediated pathway [162] and by the observation that SP is generated *in vivo* following nasal challenge of allergic individuals with BK [163].

TK also have potent effects on airway blood flow [66, 164, 165], presumably via NK_1 receptors [166, 167]. Both SP and NKA cause vasodilation which, together with the SP- or NKA-mediated increase in microvascular permeability, may contribute to the formation of oedema.

SP has been shown to promote endothelial-leukocyte interaction via increased expression of adhesion molecules [168]. However, this effect may be indirect via activation of mast cells

and subsequent release of TNF-α [169]. A recent study indicates that SP stimulates endothelial cell differentiation into capillary-like structures [170]. This may indicate that increased levels of SP, found in chronic inflammatory conditions, may play a role in tissue repair by promoting the development of new vessels.

Effects on nerves

In human airways the interaction between TK and airway nerves is not certain. However, studies using rodents suggest that TK may amplify or facilitate cholinergic neurotransmission [17, 171-174] and may modulate i-NANC mediated bronchodilation [175, 176], thereby contributing to exaggerated bronchoconstriction.

Effects on epithelial cells and fibroblasts

TK stimulate ciliary beat frequency through activation of NK_1 receptors on bronchial epithelial cells, and thereby contribute to the clearance of mucus, bacteria and inhaled particles [177, 178]. TK also stimulate ion transport in airway epithelium, and exert a protective effect on bronchial epithelial barrier function under conditions of challenge [179].

Stimulation of bronchial epithelial cells with TK results in the release of PGE_2 and possibly the epithelium-derived relaxing factor (EpDRF) [180, 181]. In addition, TK are involved in the migration and proliferation of bronchial epithelial cells [182].

SP has been shown to increase the expression of adhesion molecules on bronchial epithelial cells and to stimulate the release of neutrophil chemoattractant mediators by bronchial epithelial cells [183, 184]. Therefore, SP may be involved in the recruitment of neutrophils into the airways.

TK may also stimulate chemotaxis and proliferation of human lung fibroblasts, an effect that is mediated via release of PGE_2 and prostacyclin [185-187]. Activation of fibroblasts by TK may therefore contribute to the structural abnormalities observed in the asthmatic airways.

Effects on inflammatory cells

TK have effects on a number of inflammatory cells, including neutrophils, eosinophils, T lymphocytes, mast cells, monocytes and macrophages, lymphocytes, and dendritic cells (Table 1) [188]. Several of these effects may be explained by the ability of TK to activate transcription factors like nuclear factor (NF)-κB [189], activating protein (AP)-1, and cAMP-responsive element-binding protein (CREB) [190].

Table1. Main effects of TK on inflammatory cells.

Cell	Effect of TK*
Neutrophils	Chemotaxis ↑, adherence ↑, O_2^- production ↑
Eosinophils	Migration ↑, degranulation
Mast cells	Histamine release
T lymphocytes	Proliferation ↑, cytokine production ↑, chemotaxis ↑
B lymphocytes	Differentiation, immunoglobulin isotype switch
Monocytes/macrophages	Release of inflammatory cytokines
Dendritic cells	Chemotaxis ↑, antigen presentation ↓

* see text for details.

TK stimulate a number of *neutrophil* functions, including chemotaxis, aggregation, superoxide production, and adherence to epithelium and endothelium [191-199]. The expression of an endothelial leukocyte adhesion molecule (ICAM-1) by the microvascular endothelium following application of SP could be of relevance for the latter effect [192]. However, relatively high concentrations of SP may be required for activation of neutrophils. This may be explained by the high levels of the SP-degrading enzyme neutral endopeptidase (NEP) on the surface of these cells [199]. At low concentrations, SP may have a priming effect on neutrophils, i.e., SP can enhance the neutrophil response to other stimuli at concentrations that may otherwise be ineffective [200, 201].

SP has a degranulating effect on *eosinophils* and induces human eosinophil migration *in vitro* [202, 203]. Priming of human eosinophils with SP (via the NK_1 receptor) has been shown to enhance platelet-activating factor (PAF)- or IL-5-stimulated migration [204]. In an *in vivo* study with allergic rhinitis patients, it was shown that SP given after repeated allergen challenge enhanced the recruitment of eosinophils [205]. Eosinophils also may produce SP themselves [206, 207] and may activate ganglion neurons to release SP [208].

Mast cells can be found in close proximity with sensory nerves [209, 210]. It has been demonstrated that SP can cause histamine release from human lung mast cells [152, 211], and that SP-induced histamine release from BAL mast cells from asthmatic patients is significantly higher than in healthy subjects [212]. Although NK_1 receptors have been located on mast cells, some data suggest that the effect of SP on mast cells is mediated via a non-receptor mediated pathway, since the effect is dependent on the N-terminal sequence of SP [129, 213, 214]. SP in low concentrations can act as a mast cell primer to other agents (like allergens) when released from sensory nerves [215].

SP activates *monocytes* to release inflammatory cytokines, including IL-1, TNF-α, IL-10, and IL-6 [216-218]. Again, this effect does not seem to be mediated via classic NK receptors [219, 220]. The effect of TK on human *macrophages* is less clear. Although SP may increase the production of oxygen radicals by guinea pig macrophages (via both NK_1 and NK_2 receptors), no effect was observed on human alveolar macrophages [221-223]. A recent preliminary report did demonstrate the presence of NK_1 receptors on human alveolar macrophages and showed that SP may be involved in cytokine production by these cells [224]. Rat alveolar macrophages have been shown to express PPT-A mRNA and to display SP-like immunoreactivity, indicating that macrophages may also be a source for SP in the airways [225].

NK receptors are present on certain subsets of *T and B lymphocytes*, and TK-containing nerves have been demonstrated in lymphoid tissue [226, 227]. These findings suggest a role for TK in regulating lymphocyte functions. Indeed, several effects of TK on T and B cells have been described yet. SP may be a late-acting B lymphocyte differentiation cofactor regulating immunoglobulin production and secretion [228-231]. TK can also activate proliferation of T lymphocytes and stimulate their cytokine production [232-234]. Furthermore, SP has recently been shown to be a lymphocyte chemoattractant [235]. In addition to NK receptors, other receptors or non-receptor-mediated pathways may be involved in the effects of TK on lymphocytes [236-238].

Little data is available on the effects of TK on *dendritic cells* (DC). In rats, pulmonary DC were shown to bind SP and to display increased motility in response to graded concentrations of SP, suggesting a role for SP in the recruitment of DC into the airways [239]. SP receptors have recently been identified on Langerhans cells and it was shown that high concentrations of SP inhibit antigen presentation by these cells [240].

2.4.2. Calcitonin gene-related peptide

CGRP is a 37 amino acid peptide formed by the alternative splicing of the precursor mRNA coded by the calcitonin gene [241]. It occurs in two forms, A and B, which differ by three amino acids. Both forms are expressed in sensory nerves, often colocalized with TK, and act via binding to the CGRP type I receptor [125, 242, 243]. CGRP is a potent vasodilator, especially of arterial and arteriolar vessels [244]. Its effect, which is long-lasting, is mediated via direct action on receptors on vascular smooth muscle. Indeed, receptors for CGRP are most dense on arterial vessels, with little expression on smooth muscle or epithelial cells in the human airways [117, 245]. CGRP itself has no direct effect on airway microvascular leak, but amplifies the plasma protein extravasation induced by SP [244]. This is likely due to a synergistic combination of the potent arteriolar vasodilator effect of CGRP, which increases mucosal blood flow, and the SP-induced venular vasodilation and increased vascular permeability, which increases the extravasation of plasma fluid. CGRP has also been reported to cause constriction of human bronchi *in vitro* [246]. However, since airway smooth muscle cells in humans possess few receptors for CGRP [117], this bronchoconstrictor effect may be mediated indirectly.

CGRP may also affect immune functions. It has been shown that nebulized CGRP causes eosinophilia in the rat lung [247]. CGRP inhibits SP-induced superoxide production in human neutrophils [248] and stimulates the chemotaxis and adhesion of lymphocytes [68, 235]. In addition, CGRP may be involved in epithelial repair by stimulating bronchial epithelial cell migration [249] and modulates B lymphocyte differentiation [250, 251].

2.5. NEUROGENIC INFLAMMATION

The set of responses produced through the release of TK from the peripheral endings of sensory nerves via an axon reflex is now widely known as 'neurogenic inflammation'. The basis for this term is twofold: the biological effects produced by TK in the airways in general are pro-inflammatory and provide a neurogenic contribution to the overall inflammatory process; and the stimuli that elicit TK release in the airways include mediators of inflammation, such as bradykinin (BK), histamine, and prostanoids. Since neurogenic inflammation mimics many of the pathophysiological features of asthma, a role for neuropeptides in the pathogenesis of asthma has been suggested. Several mechanisms may underlie the apparent upregulation of the sensory neuropeptide effects (Fig. 4).

First, sensory nerves in asthmatic airways may be hyperreactive. On one hand, this may be due to epithelial shedding, thereby exposing sensory nerve endings. On the other hand, sensory nerves in the asthmatic airways may be hyperalgesic, making them more responsive to activation by several mediators, including bradykinin. Certain inflammatory mediators such as PG (particularly PGE_2) and cytokines, like IL-1β and TNF-α, may sensitize sensory nerve endings, causing a hyperalgesic state [252, 253]. BK is a potent activator of sensory nerves and causes asthma-like symptoms in asthmatics, but its effects in healthy subjects are much less prominent [254-256].

Second, the sensory innervation of the asthmatic airways may be different from that in healthy controls. In airways of patients with fatal asthma, both the length and the number of SP-immunoreactive nerves has been shown to be increased when compared to airways of

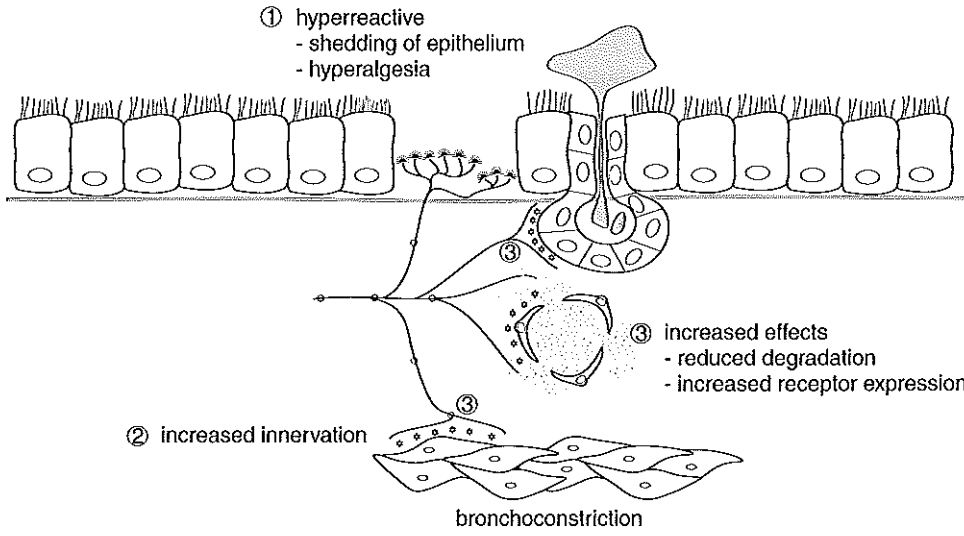

Figure 4. Possible mechanisms underlying the apparent upregulation of sensory neuropeptide effects in the asthmatic airways.

healthy subjects [126]. However, this finding could not be reproduced in another study using bronchial biopsies of mild asthmatics, which may suggest that increased innervation is a feature of either prolonged or severe asthma [109]. SP-immunoreactivity has also been detected in induced sputum of asthmatics, but not in sputum of healthy subjects [257]. In addition, increased amounts of SP were observed in BAL fluid of allergic asthmatics compared to healthy controls [258], and after segmental allergen challenge of allergic asthmatics an additional increase in SP levels in BAL fluid was observed. Furthermore, increased levels of SP have been detected in serum during exacerbations of asthma [70]. These findings suggest an increased e-NANC response in asthmatic patients. Interestingly, recent findings suggest that inflammatory mediators may have neuropoietic effects. For instance, IL-11 may induce the production of SP by sympathetic neurons [259].

Third, the effects of the sensory neuropeptides may be increased in asthmatics. Asthmatic airways show an increased expression of NK_1 and NK_2 receptors [260] and the reactivity to TK is greater in allergic subjects both regarding NKA-mediated bronchoconstriction and SP-mediated nasal congestion [145, 261, 262]. The effects of sensory neuropeptides may also be exaggerated due to impaired degradation. NEP seems to be the major enzyme involved in the metabolism of neuropeptides in the airways [263, 264]. Many of the agents that lead to exacerbations of asthma, including viruses, cigarette smoke and chemical irritants, appear to reduce the activity of NEP in the airways [265-272]. The role of NEP in the modulation of neurogenic inflammation will be discussed in more detail in chapter 3.

Although several studies suggest a contribution of neurogenic inflammatory processes in the pathophysiology of asthma, the exact contribution of TK remains to be determined. The availability of highly potent nonpeptide NK receptor antagonists definitively will contribute to a better understanding of the role of sensory neuropeptides in the pathogenesis of asthma.

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Chapter 3

Peptidases
Structure, function, and modulation of peptide-mediated effects in the human lung

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Chapter 3

Peptidases

Structure, function, and modulation of peptide-mediated effects in the human lung

Peptidases are enzymes capable of cleaving, and thereby often inactivating, small peptides. They are widely distributed on the surface of many different cell types, with the catalytic site exposed at the external surface. Peptidases are involved in a variety of processes, including peptide-mediated inflammatory responses, stromal cell-dependent B lymphopoiesis, and T cell activation. In addition, some peptidases may have functions that are not based on their enzymatical activity.

Peptidases are classified according to the location of the cleavage site in the putative substrate (Table 1) [1]. *Endo*peptidases recognize specific amino acids in the middle of the peptide, whereas *exo*peptidases recognize one or two terminal amino acids. Exopeptidases that attack peptides from the N-terminus (removing either single amino acids or a dipeptide) are termed (dipeptidyl) *amino*peptidases, whereas peptidases attacking the C-terminus are termed *carboxy*peptidases.

Table 1. Peptidases and their substrates.

Peptidase	Specificity*	Possible substrates
Aminopeptidases		
APN	Ala/Leu + X -	IL-8, fMLP, opioid peptides, enkephalins
APA	Glu/Asp + X -	angiotensins
APP	Pro + X -	BK, SP
DPP IV	X - Pro/Ala + X -	SP, BK
Carboxypeptidases		
CPN	- X + Arg/Lys	anaphylatoxins
Endopeptidases		
NEP	- X - Phe/Leu/Ile/Val/Tyr/Trp/Ala + X -	BK, SP, NKA, NPY, VIP, enkephalins, BLP, ET-1, ANF, angiotensins
ACE	relatively non-specific	angiotensins, enkephalins, SP
ECE	-Ile-Ile-Trp + X -	big-ET-1

* X = random amino acid. The cleaved bond is represented by a +.

3.1. NEUTRAL ENDOPEPTIDASE 24.11

3.1.1. Characteristics

Biochemical and molecular characterization

Neutral endopeptidase (NEP, neprilysin, EC 3.4.24.11) was first characterized from rabbit kidney brush border [2, 3]. It soon became apparent that NEP was similar to enkephalinase, originally discovered in the brain [4-7]. Furthermore, cloning of the NEP gene and subsequent cloning of the common acute lymphoblastic leukemia antigen (CALLA, CD10) showed that both sequences were similar [8-11].

NEP is a glycoprotein of 750 amino acids, with a single 24 amino acid hydrophobic segment that functions as both a transmembrane region and a signal peptide (Fig. 1). The C-terminal 700 amino acids compose the extracellular domain, whereas the 25 N-terminal amino acids form the cytoplasmic tail [8, 10, 11]. The extracellular domain contains six potential N-glycosylation sites. Tissue-specific glycosylation may result in different molecular masses, ranging from approximately 90 to 110 Kd [12-15]. The extracellular domain contains the pentapeptide consensus sequence (His-Glu-[Ile, Leu, Met]-X-His) of zinc binding metalloproteases, in which the two histidines coordinating zinc and the glutamic acid residue, together with an aspartic acid residue, are critically involved in the catalytic process [16, 17].

Gene structure

Characterization of the human NEP gene, which is located at chromosome 3 (q21-q27) [19], showed that it spans more than 80 kilobases (kb) and is composed of 25 exons [20]. Exons 1, 1bis, and 2 encode 5' untranslated sequences; exon 3 encodes the initiation codon and the transmembrane and cytoplasmic domain; 20 short exons (exons 4-23) encode most of the extracellular region; and exon 24 encodes the C-terminal 32 amino acids of the protein and contains the entire 3' untranslated region (UTR). Within exon 24 are five poly(A) addition signals. Alternative splicing of exon 1, exon 1bis, exon 2 (2a), or part of exon 2 (2b) to the common exon 3, resulting in four different transcripts, may be the origin of the tissue- or stage of development-specific expression of NEP [21, 22]. Indeed, two separate regulatory elements have been found in the NEP promoter region and these elements may be regulated by the transcription factor CBF/NF-Y in a tissue-specific manner [22, 23]. A cDNA clone lacking the complete exon 16 has been isolated from human lung tissue [24]. Deletion of this 27 amino acid segment was shown to reduce enzyme activity to barely detectable levels. However, the physiologic relevance of this truncated form remains to be determined. In the rat, an exon 5-18 deletion has been described, but no evidence was found to support the expression of this variant in the human lung [25].

Distribution

NEP is expressed by a variety of hematopoietic and non-hematopoietic cells [18, 26]. NEP is abundantly present in renal proximal tubular epithelial cells, small intestinal epithelium, and biliary canaliculae. In addition, NEP can be found in synaptic membranes of the central nervous system, bone marrow stromal cells, fibroblasts, placenta, lymphoid progenitors, and neutrophils [12-14, 27-29]. Given the expression of NEP on lymphoid progenitors,

expression of NEP is used as a diagnostic marker for several lymphoid malignancies, including Burkitt's lymphomas and certain myelomas [30-33].

In the human lung, NEP is expressed by bronchial epithelial cells, submucosal glands, bronchial smooth muscle, and endothelium [34]. In addition, NEP can be found on alveolar epithelial cells [35].

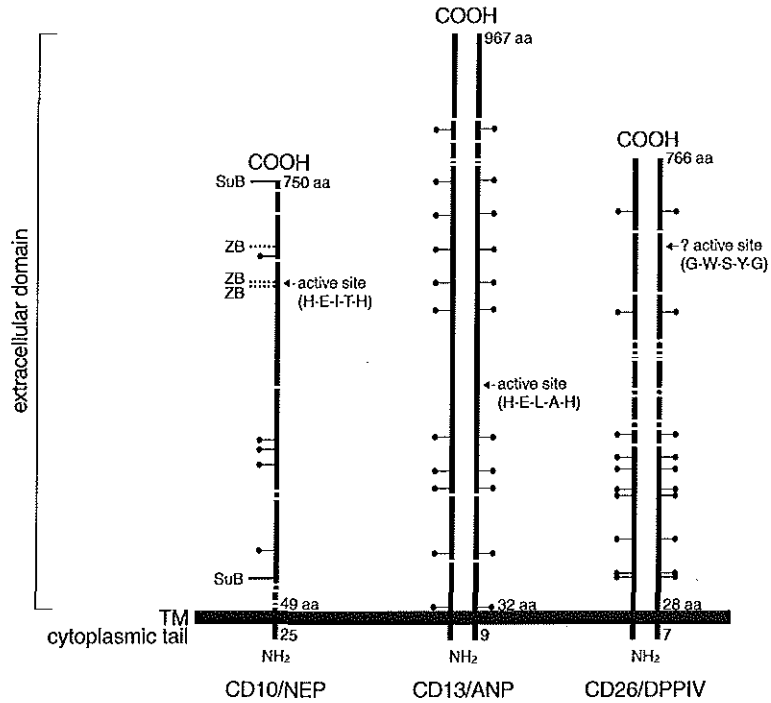

Figure 1. Molecular structure of NEP, APN and DPP IV. The size, monomeric or dimeric structure, and orientation of each protein with respect to the cytoplasm, transmembrane (TM) region, and extracellular domain are shown. Proven or potential active sites (zinc-binding (ZB) or substrate-binding (SuB)) are indicated. Glycosylation sites are indicated by a black dot (adapted from reference [18]).

3.1.2. Enzymatic activity and biological functions

NEP is able to hydrolyze peptide bonds on the N-terminal site of hydrophobic amino acids, like Phe, Leu, Ile, Val, Tyr, Ala, and Trp (Table 1) [2]. However, sub-site interactions and conformational factors greatly influence the efficiency of hydrolysis [36]. Among the possible substrates of NEP are substance P (SP), neurokinin A (NKA), formyl-metheonyl-leucyl-phenylalanine (fMLP), atrial natriuretic factor (ANF), endothelin-1 (ET-1), bombesin-like peptides (BLP), angiotensins, vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), bradykinin (BK), enkephalins, cholecystokinin, and neurotensin [37]. Although NEP predominantly cleaves simple peptides, it has been reported that NEP may also be able to hydrolyze certain larger substrates, including cytokines such as IL-1β and IL-6 [36].

The general biologic function of NEP is to reduce cellular responses to peptide hormones. Target cells express both NEP and the peptide-receptor; by degrading the peptide

substrate, NEP reduces the local concentration of the peptide available for binding to the receptor. For example, NEP reduces ANF-mediated hypotension [38], fMLP-mediated chemotaxis of neutrophils [39], and enkephalin-mediated analgesia [4]. Targeted disruption of the NEP locus in mice results in enhanced lethality to endotoxin, indicating an important protective role for NEP in septic shock [40]. A role for NEP in lymphoid development has been suggested by studies showing that inhibition of NEP resulted in increased proliferation and maturation of B cells, both *in vitro* and *in vivo* [41, 42]. Therefore, it has been suggested that NEP functions to regulate B cell development by inactivating a peptide that stimulates B cell proliferation and differentiation. Alternatively, NEP may activate a pro-peptide that inhibits proliferation and differentiation of B cells. The role of NEP in the regulation of cellular proliferation and differentiation will be discussed below in some more detail. The role of NEP in the modulation of neurogenic inflammation will be discussed in paragraph 3.6.

Role of NEP in cellular differentiation and proliferation in the lung

NEP plays an important role in the cellular differentiation and proliferation of bronchial epithelial cells by inactivating BLP [43]. BLP are potent growth factors for bronchial epithelial cells and are involved in lung development. The temporal and cellular patterns of NEP expression implicate the enzyme in the regulation of BLP-mediated fetal lung development [44]. Indeed, both *in vitro* and *in vivo* it was shown that inhibition of NEP resulted in increased maturation of the developing fetal lung [44, 45]. Reduced NEP activity may also promote BLP-mediated proliferation of bronchial epithelial cells. Indeed, the growth and proliferation of BLP-dependent carcinomas is inhibited by NEP and potentiated by NEP inhibition [43]. NEP expression by epithelial cells is inversely correlated with cellular proliferation [46]. Therefore, reduced NEP activity may promote BLP-mediated proliferation and facilitate the development of small-cell carcinomas of the lung [43, 47]. A role for NEP in the regulation of tumor cell proliferation is also supported by studies using a human T cell line (Jurkat). In these cells, NEP is required for phorbol ester-induced growth arrest [48].

3.2. AMINOPEPTIDASE N

3.2.1. Characteristics

Biochemical and molecular characterization

Aminopeptidase N (APN; EC 3.4.11.2) is a widely studied peptidase, which is known under a variety of names, including aminopeptidase M, alanine aminopeptidase, arylamidase, and microsomal α-aminoacyl-peptide hydrolase [49]. APN is a glycoprotein of 967 amino acids with 11 potential sites of asparagine-linked oligosaccharide addition (Fig. 1) [50, 51]. The unglycosylated protein has a molecular size of 110 Kd; posttranslational modification results in the 130 Kd precursor (gp130) and the 150 Kd mature protein (gp150) [52-55]. The 23 amino acid retained signal also functions as the membrane-spanning segment, orientating the APN N-terminus inside and the C-terminus outside the cell (thereby defining APN as a type II integral membrane protein) [50, 51]. The intracellular domain of APN is only nine amino acids long, whereas the extracellular domain contains 935 amino acids. Similar to NEP, the extracellular domain contains a pentapeptide consensus sequence characteristic of

members of the zinc-binding metalloprotease family. On the surface of cells, APN is expressed as a non-covalently bound homodimer [56-59]. Cloning of the APN cDNA revealed that its sequence was identical to the myeloid marker CD13 [50-52].

Gene structure

The APN gene is located on the long arm of chromosome 15 (q25-26) and exists of 20 exons [60-62]. Northern blot analysis of RNA extracted from several tissues revealed two distinct APN transcripts: a 3.7 kb transcript expressed by monocytes, myeloid leukemia cells, and fibroblasts, and a 3.4 kb transcript expressed by intestinal epithelium and kidney cells [63]. In epithelial cells, transcripts originate 47 base pairs upstream from the initiation codon and 22 base pairs downstream from a TATA box. In contrast, the longer transcripts found in myeloid cells and fibroblasts originated from several sites clustered in an upstream exon located 8 kb from the exon containing the initiation codon. Nevertheless, both transcripts encode the same protein, indicating that separate promoters control the tissue-specific expression of the APN gene [63]. In addition, a 300 base pair region with enhancer activity, located 2.7 kb upstream of the transcriptional start site which is used in epithelial cells, may also be important for the tissue-specific expression [64].

Distribution

The non-hematopoietic distribution of APN shows a pattern comparable to NEP. Thus, APN is expressed on renal proximal tubular epithelial cells, small intestinal epithelium, biliary canaliculae, synaptic membranes of the central nervous system, bone marrow stromal cells, fibroblasts, osteoclasts, placenta, and granulocytes [6, 56, 65, 66]. In contrast to NEP, APN is also expressed on monocytes and all myeloid progenitors [67-69]. Expression of APN may be used as a marker for myeloid leukemias [67, 68, 70-72]. Mast cells may also express APN [73], whereas peripheral blood lymphocytes do not express this enzyme. However, expression of APN on lymphocytes can be induced after mitogenic stimulation or after adhesion to fibroblast-like synoviocytes, endothelial cells, epithelial cells and monocytes/macrophages [74-76].

3.2.2. Enzymatic activity and biological function

APN is a peptidase which hydrolyses preferentially natural or synthetic substrates with an N-terminal alanine residue (Table 1) [1]. Other amino acids, especially neutral ones, may also be removed hydrolitically, with the exception of proline. Natural APN substrates appear to be small peptides rather than larger proteins, although the enzyme is more effective in removing residues from oligopeptides than dipeptides. Among the possible substrates for APN are enkephalins, tachykinins, bradykinin, fMLP, and possibly cytokines such as IL-1β, IL-6, and IL-8 [77-79]. However, in certain cases initial cleavage by endopeptidases (like NEP) may be required.

Several functions of APN have been described. First, APN expressed on the brush border of the intestine may be involved in the final stages of digestion of small peptides [56]. Second, comparable with NEP and often in collaboration with NEP, APN may function to reduce cellular responses to peptide hormones [65, 69, 80, 81]. Third, a recent report implicates APN in the processing of peptides bound to major histocompatibility (MHC) class II molecules [82]. Fourth, APN may be involved in tumor invasion and metastasis by degrada-

tion of collagen type IV [83, 84]. Finally, APN serves as a receptor for coronaviruses, which are RNA viruses that cause respiratory disease in humans [85].

3.3. DIPEPTIDYL PEPTIDASE IV

3.3.1. Characteristics

Biochemical and molecular characterization

Dipeptidyl (amino)peptidase IV (DPP IV; EC 3.4.14.5) is an atypical serine protease of 766 amino acids with type II membrane topology (Fig. 1) [86, 87]. It contains a short, highly conserved intracellular domain of six amino acids, a 22 amino acid hydrophobic transmembrane region (which also functions as signal peptide), and a 738 amino acid extracellular domain. The extracellular domain, which contains nine potential glycosylation sites, can be divided into three regions: an N-terminal glycosylated region containing seven glycosylation sites and starting with a 20 amino acid flexible 'stalk region'; a cysteine-rich region; and a 260 amino acid C-terminal domain containing the putative catalytic sequence. On the surface of cells, DPP IV probably is present as a homodimer comprising two identical subunits of approximately 110 Kd molecular mass [88-91]. Recent studies indicate that several isoforms of DPP IV can be found [92-94].

In contrast to NEP and APN, DPP IV does not contain zinc in its catalytic center. Based upon its structural homology with other nonclassic serine proteases, DPP IV is assigned to the prolyl oligopeptidase family. Members of this family share a catalytic site in which the essential residues are arranged in the unique sequence Ser-Asp-His [95]. Cloning of the DPP IV cDNA revealed that its sequence was identical to the T cell activation antigen CD26 [86, 87].

Gene structure

The human DPP IV gene, located on chromosome 2 (q24.3), spans approximately 70 kb and contains 26 exons [96]. The serine recognition site is split across two exons, the first half Gly-Trp is in exon 21 and the second half Ser-Tyr-Gly is in exon 22. The three residues comprising the catalytic site are each present in a distinct exon: Ser in exon 22, Asp in exon 24, and His in exon 26. This latter exon also contains the stop codon and the 3' untranslated region of the gene. The 5' flanking domain of the DPP IV gene contains neither a TATA box nor a CAAT box, but a 300 base pair region extremely rich in C and G contains potential binding sites for several transcription factors, including Sp-1 and activating protein (AP)-1 [97]. The human DPP IV gene encodes two RNA transcripts of approximately 4.2 and 2.8 kb, which differ in sequence only at the 3' untranslated region [96]. Probably, the two mRNA arise from the use of different polyadenylation sites in the last exon of the DPP IV gene.

Distribution

In many respects, the non-hematopoietic tissue distribution of DPP IV resembles that of NEP and APN. DPP IV is constitutively expressed on renal proximal tubular epithelial cells, epithelial cells in the small intestine, and biliary canaliculae, but can also be found on alveolar pneumocytes and endothelia [88, 98]. The expression of DPP IV on hematopoietic cells

is regulated stringently. DPP IV is absent from the majority of human resting peripheral blood T lymphocytes, but some subsets of resting peripheral blood T cells weakly express the molecule [89, 90, 99]. DPP IV expression on T lymphocytes is increased after T cell activation [90, 100-102]. Thus, DPP IV is a suitable marker for T cells activated *in vivo*. Recent data indicate that DPP IV expression on T cells may correlate with T helper (Th) subsets [103-105]. High DPP IV expression was found on Th1 and Th0 cells, whereas Th2 cells displayed lower expression of DPP IV. The amount of IL-4 secretion was responsible for this correlation [104]. Memory T cells have been reported to reside in the DPP IV-positive T cell fraction [106], although this was not found in another study [107]. DPP IV is also expressed by medullary thymocytes in humans [108] and can be induced on activated natural killer cells by cytokines [109].

3.3.2. Enzymatic activity and biological functions

DPP IV is a serine peptidase with a unique specificity: it cleaves dipeptides from the N-terminus of polypeptides if proline is at the penultimate position [88, 110, 111]. Peptides with alanine in the penultimate position may also be cleaved, although with a much lower efficiency. Since N-termini containing X-Pro are not easily cleaved by other peptidases, the action of DPP IV is a rate-limiting step in the degradation of such peptides. Several biologically active peptides have the X-Pro sequence at their N-terminus and therefore DPP IV may play an important role in modulating their action. These peptides include SP and bradykinin [112, 113]. Hydrolysis of SP by DPP IV yields two products (SP_{3-11} and SP_{5-11}) which both are more potent bronchoconstrictors than intact SP_{1-11} [114]. Both products can rapidly be inactivated by APN [115]. A proline residue is also present at the penultimate position of several cytokines and chemokines, like IL-1β, IL-2, tumor necrosis factor (TNF)-β, RANTES, and granulocyte-colony-stimulating factor (G-CSF) [78].

DPP IV may have several functions, dependent upon the tissue in which it is expressed. DPP IV plays an obligatory role in the renal transport and intestinal digestion of proline-containing polypeptides [116, 117]. However, most attention has been given to the function of DPP IV on T lymphocytes.

Role of DPP IV on T lymphocytes

Although the role of DPP IV on activated T cells is not completely understood yet, recent studies indicate that it may act as a costimulatory molecule that can up-regulate the signal transducing properties of the T cell receptor (TCR). Stimulation of DPP IV (using monoclonal antibodies) leads to the activation of all functional programs of the T cells, including cytotoxicity and production of IL-2. This activation requires the expression of the TCR and DPP IV enzymatic activity [118, 119]. Furthermore, antibody-induced cross-linking of DPP IV induced tyrosine phosphorylation of several intracellular proteins with a similar pattern to that seen after TCR/CD3 stimulation [120]. Co-cross-linking of DPP IV and CD3 antigens induced prolonged and increased tyrosine phosphorylation in comparison with CD3 alone, indicating that DPP IV is a true co-stimulatory entity [120]. In addition to T cell activation, anti-DPP IV stimulated T cells show enhanced proliferative responses, increased CD3ζ phosphorylation and increased $p56^{lck}$ activity [121]. One possible mechanism for the enhanced response of T cells to perturbation of DPP IV was suggested by the demonstration that CD45, a tyrosine phosphatase that positively regulates TCR signaling, co-precipitates with DPP IV

[122]. Thus, DPP IV antibodies may stimulate T cell proliferation in part by decreasing CD45-mediated dephosphorylation of key substrates.

Inhibition of DPP IV activity results in reduced DNA synthesis as well as reduced production of IL-2, IL-10, IL-12, IL-13, and interferon (IFN)-γ of pokeweed mitogen (PWM)-stimulated purified T cells [123, 124]. Most importantly, DPP IV inhibition increased mRNA synthesis and secretion of transforming growth factor (TGF)-β, and a neutralizing antibody directed against TGF-β abolished the DPP IV-inhibitor-induced suppression in cytokine production [124]. In a rat study, repeated subcutaneous injection of DPP IV inhibitors reduced serum DPP IV activities to levels less than 30% of the normal level [125]. When primary, secondary or tertiary immune responses to bovine serum albumin (BSA) were evoked in these animals, they showed reduced anti-BSA antibody production. In normal rats, immunization with BSA was followed by a temporary decrease in serum DPP IV activity and then by enhanced serum enzyme activity after several days [125]. These results suggest that DPP IV plays an important role in immune responses *in vivo*.

Memory T cells have been shown to increase their antigen sensitivity gradually with time after re-stimulation, an effect that is accompanied by increased cell-surface expression of DPP IV [126]. Using antibodies directed against DPP IV, it has been shown that DPP IV directly contributed to this increased antigen sensitivity of late-memory T cells. As mentioned above, this effect may be explained by the co-stimulatory capacity of DPP IV [120]. Increasing the antigen-sensitivity via antigen-nonspecific molecules may be a physiologic mechanism for maintaining T cell memory in face of decreasing antigen concentrations, and may ensure preferential activation of memory T cells upon repeated antigen challenge.

DPP IV is also found to be associated with adenosine deaminase (ADA), and this complex is thought to serve as an important immunoregulatory mechanism [127-129]. Released ADA may bind to cell surface DPP IV, and the DPP IV/ADA complex subsequently binds adenosine, thereby reducing its local concentration [130, 131].

DPP IV may also function as an auxiliary adhesion factor. DPP IV was found to bind to components of the extracellular matrix, such as fibronectin and collagen [132-134]. Binding of human CD4-positive T cells to collagen produced a co-stimulatory signal in anti-CD3-mediated T cell activation, resulting in increased proliferation [121]. An anti-DPP IV antibody inhibited this effect [121].

Finally, DPP IV may be involved in the pathogenesis of the acquired immuno-deficiency syndrome (AIDS) [135]. DPP IV may act as one of the co-receptors for human immunodeficiency virus (HIV) [135, 136]. Furthermore, the HIV Tat antigen has been shown to inhibit the enzymatic activity of DPP IV, resulting in the inhibition of T cell responses to antigen and anti-CD3 antibodies [137-139]. Thus, the immunosuppressive effects of the HIV-1 Tat protein may be mediated by DPP IV inhibition.

3.4. OTHER PEPTIDASES

In addition to the three peptidases described above, other peptidases are involved in the degradation of (neuro)peptides. These include angiotensin-converting enzyme (ACE), endothelin-converting enzyme (ECE), aminopeptidases, and carboxypeptidases.

Angiotensin-converting enzyme

ACE, also known as peptidyl peptidase A or kinase II, is a type II integral membrane endopeptidase belonging to the superfamily of metallopeptidases (reviewed in [140]). Two isoforms of ACE are present within the human body: a somatic form with a molecular weight around 150 Kd, which is found in endothelial, epithelial and neural cells, and a smaller isoform (90-110 Kd) found in germinal cells. Both forms are transcribed from a single gene by the use of two separate functional promoters, a somatic and a testicular form [141]. The somatic form is composed of two highly homologous domains, probably arisen by gene duplication in the course of evolution [142]. The germinal isoform only contains one of the two homologous domains. Somatic ACE comprises 1306 amino acids with 17 potential N-linked glycosylation sites [142]. Each domain has a catalytic site, containing zinc, which functions independently [143].

ACE is widely distributed in human tissues: it is present on vascular endothelial cells, in the brush border of absorptive epithelia of the small intestine and the renal proximal tubuli, and in monocytes, macrophages, and T lymphocytes [144-147]. Nevertheless, its major location is considered to be the vascular endothelial surface of the lung [146, 147]. The enzyme preferentially cleaves peptides containing an aromatic residue in the P_1 position (Table 1), but the enzyme is far less selective than NEP. It is capable of inactivating bradykinin [148, 149] and enkephalins, and hydrolyzes angiotensin I to yield the vasoconstrictor peptide angiotensin II [150]. ACE appears to play a major role in controlling blood pressure and water and salt metabolism. In addition, ACE hydrolyzes intravascular substance P, but neurokinin A is not a good substrate [151].

Endothelin-converting enzyme

ECE is a type II integral membrane protein homologous with NEP [152, 153]. Unlike NEP, however, ECE exists as a highly glycosylated disulfide-linked dimer of subunit molecular weight 120-130 Kd [154-156]. ECE converses big-endothelin to its biologically active product ET-1 (Table 1), which is a potent broncho- and vasoconstrictor that may regulate vascular tone and blood pressure [157, 158]. Three isoforms of ECE can be distinguished: ECE-1α, ECE-1β (resulting from alternative splicing of a single gene [159, 160]), and ECE-2 [152, 153, 161].

In the human lung, ECE has been found in airway epithelium, pulmonary endothelium, airway and vascular smooth muscle, and serosal bronchial glands [162]. Although ECE may play a role in modulating biologically active peptides, it remains to be determined whether it is involved in the pathogenesis of asthma [163-165]. Nevertheless, in asthmatic patients increased levels of ET-1 have been found in bronchoalveolar lavage fluid [166-168], plasma [169], and bronchial epithelial cells [170] compared to healthy controls.

Aminopeptidases

Human tissues contain an array of cytosolic and membrane-bound aminopeptidases. The best-characterized, aminopeptidase N, is described above. Other aminopeptidases are aminopeptidase A, which is specific for N-terminal Glu and Asp residues, and aminopeptidase P, which will release an N-terminal residue adjacent to a proline (Table 1) [1, 77]. The role of these peptidases in the metabolism of susceptible peptides has been little investigated, but it may be hypothesized that these enzymes are involved in the final hydrolysis of a variety of substrates, with or without initial cleavage by an endopeptidase. A role for aminopeptidase A in modulating the potency of peptides binding to the neurokinin $(NK)_2$ receptor has been

suggested [171, 172]. Aminopeptidases may also be involved in the regulation of CC chemokine activities, as deletion of the NH_2-terminal residue converts monocyte chemotactic protein-1 from an activator of basophil mediator release to an eosinophil chemoattractant [173].

Carboxypeptidases

Carboxypeptidase N (CPN, kininase I) cleaves the C-terminal arginine and lysine of peptides such as bradykinin [174]. One of the functions of CPN is to protect the body from potent vasoactive and inflammatory peptides containing COOH-terminal Arg or Lys which are released into the circulation. In the human lung, CPN has been detected in alveolar type I cells, in the glycocalyx of the epithelium, in some vessels, and in gland ducts near the epithelial basement membrane [175, 176]. CPN activity in nasal lavage fluid has been shown to be enhanced after histamine challenge [176]. This CPN originated in plasma, suggesting that plasma extravasation and interstitial fluid exudation across the epithelium are the primary processes regulating its appearance in nasal secretions. CPN has also been found in BAL fluid [177]. Since increased CPN activity was found in patients with lung disease (pneumonia or lung cancer), it was hypothesized that CPN activity in BAL fluid may be an indicator of type I cell injury [177].

3.5. SOLUBLE COUNTERPARTS OF MEMBRANE-BOUND PEPTIDASES

Although the above mentioned peptidases are integral membrane glycoproteins, soluble peptidases with comparable enzymatic activity can be detected in body fluids. These soluble counterparts may either be derived from shedding of membrane-bound peptidases, or may be formed by post-translational cleavage of the membrane-bound form.

Serum *neutral endopeptidase* activity probably arises from shedding of the membrane-bound peptidase [178]. Increased serum activity of NEP has been observed in underground miners exposed to coal dust particles [178] and in patients with adult respiratory distress syndrome (ARDS) [179], rheumatoid arthritis [180] or sarcoidosis [181]. Although the source of the increased NEP levels remains to be determined, it has been suggested that increased NEP levels may reflect local tissue damage with subsequent shedding of membrane-bound NEP. Furthermore, serum activity of NEP is increased in acute renal graft rejection [182], in patients with end-stage renal failure [183], and in cholestatic liver disease [184].

Human serum contains an array of *aminopeptidase* activities, including alanine aminopeptidase and leucine aminopeptidase [185]. Serum alanine aminopeptidase activity predominantly comprises a circulating isoform of CD13 [186, 187]. Increased activity of leucine aminopeptidase has been observed in BAL fluid of patients with pulmonary tuberculosis and it was shown that this increase could be attributed to lung tissue damage [188].

Dipeptidyl peptidase IV is present in several forms in human serum and may enhance antigen-induced T cell proliferation [118, 189]. Recent studies indicate that serum DPP IV is a monomer of 175 kDa and that this molecule, which is a potent T cell co-stimulator, is not a breakdown product of membrane-bound CD26 [190]. Furthermore, the 175 kDa form of DPP IV found in normal serum is identical with a similarly-sized molecule, DPPT-L, found to be rapidly expressed on the surface of activated T cells [94]. CD45RO- CD4-positive T cells appeared to be the major source of serum DPP IV activity [94]. DPP IV activity in serum is decreased in patients with major depression, and a correlation was observed be-

tween DPP IV activity and CD4-positive T cells in blood of depressed subjects, but not of normal controls [191]. There were no significant relationships between serum DPP IV activity and plasma cortisol or immune-inflammatory markers, such as serum IL-6 or soluble IL-2 receptor (CD25) [192]. Reduced serum DPP IV activity has also been described in patients with systemic lupus erythematosus [193] and in oral cancer patients [194, 195]. In the latter study a significant correlation between serum DPP IV activity and peripheral blood lymphocytes or CD26-positive T cells was found [195].

3.6. MODULATION OF (NEUROGENIC) INFLAMMATION

In addition to the two well-known autonomic nervous systems (parasympathetic and sympathetic) that innervate the airways, a non-adrenergic non-cholinergic (NANC) neural pathway is present (see chapter 2). While inhibitory NANC (i-NANC) effects are bronchodilatory through the activity of vasoactive intestinal peptide (VIP) and nitric oxide (NO) released from cholinergic nerves, excitatory NANC (e-NANC) effects are bronchoconstrictor and mediated through the release of neuropeptides (especially tachykinins and calcitonin-gene related peptide (CGRP)) from sensory nerves [196-198]. Stimulation of sensory nerves, either by chemical or physical triggers, results in an axon reflex and subsequent release of neuropeptides from the peripheral endings of the sensory nerves [199]. Following release, these neuropeptides exert a variety of effects through activation of specific neurokinin receptors, including vasodilation, increased microvascular permeability, leukocyte recruitment and adhesion, submucosal gland secretion, smooth muscle contraction, cough, and facilitation of cholinergic neurotransmission. This sequence of events is now known as 'neurogenic inflammation' [200]. Since the neurogenic inflammatory response mimics many of the pathophysiological features of asthma, a role for neuropeptides in the pathogenesis of asthma has been implicated. In the asthmatic airways, the effects of bronchoconstrictor peptides (including tachykinins and bradykinin) may be enhanced, whereas the effects of bronchodilator peptides (including VIP) may be reduced [201, 202].

After it became apparent that neuropeptides were responsible for the neurogenic inflammatory responses, it was hypothesized that degradative mechanisms existed which may limit the effects of neuropeptides, comparable to the role of cholinesterase in limiting the effects of acetylcholine [200]. Several studies now have demonstrated that peptidases play a major role in the modulation of peptide-mediated effects in the airways (reviewed in [203]). Much research has focussed on the degradation of the tachykinins, like SP and NKA, and the enzyme NEP.

The physiologic relevance of tachykinin inactivation by enzymatic hydrolysis has been deduced from studies of the effects of enzyme inhibition on the physiologic action of exogenously administered or endogenously released peptides. In the first study, it was shown that selective inhibition of NEP potentiated the secretagogue effect of SP on submucosal gland secretion in the ferret trachea *in vivo* [204]. Several other reports subsequently demonstrated that inhibition of NEP potentiated the effects of SP on cough, vascular permeability, cholinergic neurotransmission, and smooth muscle contraction [203]. In guinea pigs, it was shown that both NEP and ACE participate in the metabolism of SP when administered intravascularly, whilst SP administered by aerosol was degraded by NEP only [205-208]. In addition, the ACE inhibitor captopril did not affect TK-induced bronchial smooth muscle contraction

in man. Therefore, ACE is thought to play an important role in modulating the biological activity of intravascular peptides, whereas NEP is also involved in the hydrolysis of peptides present within lung tissue or within the bronchial lumen. The importance of NEP in modulating tachykinin-mediated effects is further supported by the observation that administration of other peptidase inhibitors (including inhibitors of aminopeptidases, serine proteases, and carboxypeptidases), did not potentiate tachykinin-induced effects in the airways [209-214]. The involvement of NEP in the breakdown of tachykinins has also been shown in *in vivo* studies in humans. These studies showed that both NKA- and SP-induced bronchoconstriction could be potentiated by NEP inhibition [215-217]. Furthermore, these studies indicated that SP, but not NKA, increased the airway responsiveness to methacholine, suggesting that inflammatory processes are contributing to SP-induced airway narrowing [218].

In contrast to the studies above, in which the effects of neuropeptides were increased due to the inhibition of peptidases, some studies have shown that administration of recombinant NEP may prevent neurogenic inflammation. Thus, administration of aerosolized NEP inhibited the SP-induced cough and ozone-induced hyperreactivity to SP in guinea-pigs [200, 219].

Biochemical and immunohistochemical studies have shown that NEP is present on airway epithelial cells [34, 35, 200]. Removal of the epithelium was further shown to result in increased responses to exogenously applied or endogenously released tachykinins [209, 213, 220-225]. However, NEP is also present at other sites within the airways, and also after removal of the epithelium NEP inhibitors potentiate tachykinin-mediated effects [34, 209, 220]. Nevertheless, NEP expressed by epithelial cells may more easily be modulated by inhaled agents than NEP located at other sites.

Several environmental agents may modulate peptidase activity, thereby exaggerating responses to tachykinins (and other peptides) and increasing airway inflammation. These agents include viruses, ozone, cigarette smoke, chemical irritants, and possibly antigen challenge. In contrast, inhaled steroids may exert their anti-inflammatory actions in part by upregulating NEP activity.

Viruses

Viral infections may potentiate neurogenic inflammatory responses through inhibition of NEP activity. In laboratory animals, infection with Influenza virus or Sendai virus was shown to result in enhanced bronchoconstrictor responses to tachykinins, an effect that was mediated by decreased epithelial NEP activity [211, 214, 226, 227].

Ozone

In humans, guinea pigs as well as many other species, exposure to ozone results in the recruitment of neutrophils to the airways and increased responsiveness to inhaled bronchoconstrictor agents [228, 229]. Ozone-induced airway hyperreactivity can be blocked by capsaicin-pretreatment, which depletes TK from sensory nerves [230]. Exposure to ozone also results in increased responsiveness for SP, and this effect could not be enhanced by inhibition of NEP [231]. This suggests that ozone exposure inactivated NEP, which is supported by the observation that the tracheal NEP activity in ozone-exposed animals was significantly lower than the NEP activity in air-exposed animals [231].

Toluene diisocyanate

Toluene diisocyanate (TDI) is a widely used plasticizer that may cause occupational asthma [232]. In guinea pigs it was shown that TDI, albeit at rather unrealistic doses, increased airway responsiveness to SP and decreased airway neutral endopeptidase [233].

Cigarette smoke

Inhalation of cigarette smoke enhances the bronchoconstrictor response to inhaled SP in guinea pigs [234]. Inhibition of NEP by phosphoramidon increased the bronchoconstriction induced by SP in control animals, but not in animals exposed to cigarette smoke. NEP activity in homogenates of guinea pig trachea was inhibited by cigarette smoke. However, in another study no effect of cigarette smoke on airway NEP activity *in vivo* could be observed [235]. A possible explanation for this discrepancy may be that the NEP inhibited by cigarette smoke only represents a small fraction of the total amount of NEP in the airways.

Cigarette smoke is an important factor contributing to the development of small-cell lung carcinomas of the lung. As already mentioned (see paragraph 3.1.2.), NEP activity is decreased in lung cancers [43, 47]. Therefore, one may speculate that cigarette smoke contributes to the development of lung cancers in part by inhibiting NEP, thereby enhancing the mitogenic effects of peptides (like SP and BLP) on bronchial epithelial cells [43-46, 236].

Allergen

Airway inflammation may be linked to the clinical features of asthma by an effect on peptidase activity. In guinea pigs, chronic antigen exposure results in airway inflammation and hyperreactivity to SP [237]. It was shown that lungs with chronic allergic inflammation were more sensitive to the bronchoconstrictor effects of SP and less sensitive the bronchodilator effects of VIP than lungs from healthy animals. In addition, the effects of enzyme inhibitors on physiological responses and peptide cleavage profiles were consistent with decreased NEP and enhanced tryptic activity [237].

In a recent human *in vivo* study, no effect of inhaled thiorphan (a NEP inhibitor) on allergen-induced airway responses in asthmatic subjects was observed [238]. This suggests that either neuropeptides do not play a predominant role in allergen-induced airway responses, or that allergen challenge induces NEP-dysfunction in humans *in vivo*. However, in guinea pigs it has been shown that tachykinins contribute to allergen-induced bronchoconstriction, an effect that probably is mediated via the release of bradykinin and histamine [239-241].

Glucocorticoids

Glucocorticoids have potent anti-inflammatory effects and therefore are widely used in the treatment of asthma [242]. The anti-inflammatory effect may be caused, in part, by an upregulation of NEP activity, thereby reducing neurogenic inflammatory responses. Indeed, NEP activity by a transformed human tracheal epithelial cell line was shown to be increased after stimulation with glucocorticoids [243]. However, no effect of glucocorticoids was observed in another study using a bronchial epithelial cell line [244]. In guinea pigs, glucocorticoids were shown to reduce capsaicin-induced microvascular permeability, which might be due to elevated NEP expression [245]. This was supported by the observation that treatment of rats with combined NEP and ACE inhibitors prevented the effect of glucocorticoids [245]. The effect of glucocorticoid treatment *in vivo* on NEP expression in the human airways has recently been reported [246]. In that study it was shown that NEP expression in the bronchial

epithelium of steroid-treated asthmatics was significantly greater than the expression in non-steroid-treated asthmatic patients [246].

As shown above, many of the agents that lead to exacerbations of asthma appear to reduce the activity of NEP at the airway surface, thus leading to exaggerated responses to tachykinins and neurogenic inflammation (Fig. 2). However, most of these studies have been performed in laboratory animals, especially the guinea pig, and have not been confirmed in humans yet. Furthermore, in many studies the NEP inhibitor phosphoramidon was used. This inhibitor, however, not only inhibits NEP but was later also shown to inhibit ECE [247-250]. If it appears that ECE can cleave tachykinins the surety of the conclusions drawn about NEP from experiments using phosphoramidon is somewhat tempered.

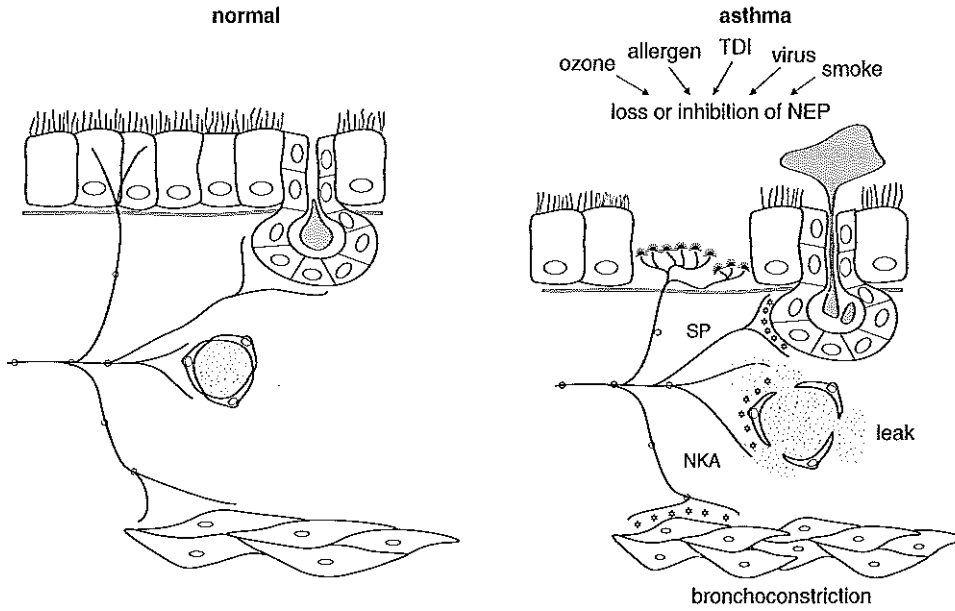

Figure 2. Neurogenic inflammation in asthmatic airways. Neuropeptides (☆) released from sensory nerves are normally rapidly degraded by peptidases. Therefore the effects of these neuropeptides are limited. In the asthmatic airways, several factors may result in a decreased peptidase activity, thereby exaggerating the neuropeptide effects.

3.7. NEUROPEPTIDES AND PEPTIDASES: IMPORTANT IN ASTHMA?

Although neuropeptides and peptidases have been shown to be present in the human airways, their role in asthma still remains to be elucidated. However, several observations may support the hypothesis that neuropeptides and peptidases are involved in the pathogenesis of asthma.

SP and NKA have been shown in several *in vivo* studies to cause bronchoconstriction, and these effects could be potentiated by inhibition of NEP (reviewed in [251]). Furthermore, these studies demonstrated that TK-mediated bronchoconstriction is greater in allergic

asthmatics compared to healthy subjects. However, the thiorphan-induced leftward shift of the NKA dose response curve was similar in asthmatic patients and healthy subjects, suggesting that the activity of NEP does not differ between both groups. Nevertheless, patients used in the latter study were stable asthmatics and it can be argued that reduced NEP activity may occur during exacerbations of asthma.

Increased amounts of SP can be detected in bronchoalveolar lavage fluid of allergic asthmatics [252] and in sputum [253] after allergen challenge. The possibility that tachykinins are endogenously released *in vivo* has also been supported by the observation that bradykinin-induced bronchoconstriction in asthmatics can be blocked by a tachykinin receptor antagonist [254] and be potentiated by NEP inhibition [255]. Bradykinin, which is present in the asthmatic airways [256] and is released after relevant aeroallergen challenge in allergic individuals [257], can stimulate sensory nerves to induce retrograde release of tachykinins [258].

Inhibition of NEP, either in healthy subjects or asthmatics, has been shown to potentiate the bronchoconstrictor effects of mediators known to be released after allergen challenge (such as LTD_4 and bradykinin) [255, 259]. However, inhibition of NEP at doses shown to enhance the bronchoconstrictor effect of NKA did not affect the early and late-phase response in mild asthmatics following allergen challenge [238]. This may suggest that endogenously released neuropeptides do not play a role in antigen-induced airway responses. Alternatively, antigen challenge may result in a dysfunction of NEP activity. Future studies, using selective tachykinin antagonists in combination with specific peptidase inhibitors, will be required to determine whether tachykinins play any role in asthma.

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Chapter 4

Bronchial epithelium
Morphology, function, and pathophysiology in asthma

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Chapter 4

Bronchial epithelium
Morphology, function, and pathophysiology in asthma

Bronchial epithelial cells have long been regarded as a passive barrier between the environment and the internal milieu of the lung. However, in addition to this barrier function, bronchial epithelial cells may also play a role in the initiation, perpetuation and modulation of inflammatory and immunological reactions within the airways [1-3]. In this chapter, the morphology of the bronchial epithelium, its function with regard to host defense, and its immunological potential will be reviewed. Alterations associated with asthma will be emphasized.

4.1. MORPHOLOGY OF THE BRONCHIAL EPITHELIUM

The bronchial epithelium forms the interface between the respiratory system and the inspired air. The epithelial layer rests upon a connective tissue substratum consisting of a basement membrane, lamina propria and submucosa, containing smooth muscle, glands and cartilage (Fig.1) [4]. The bronchial epithelium is composed of three main cell types, which together form a pseudostratified ciliated layer (Fig. 1).

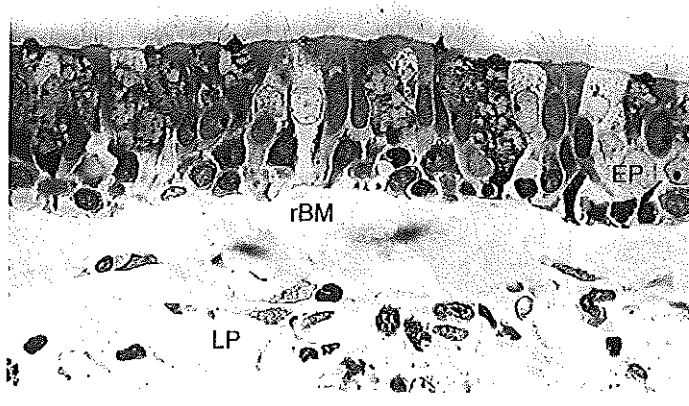

Figure 1. Light-microscopic photomicrograph of the human bronchial epithelium (magnification: 63x). EP = epithelium, rBM = reticular basement membrane, LP = lamina propria (from reference [5]).

Ciliated cells are terminally differentiated columnar cells which are thought to originate from basal or secretory cells [6, 7]. Their main function is to remove particulate matter by means of the mucociliary stairway.

Secretory cells, which comprise 15-25% of the bronchial epithelium, are present in several forms. Mucous or goblet cells are the main producers of airway mucus, in which inhaled particles, including viruses and bacteria, can be trapped [8, 9]. Clara cells produce the surfactant apoproteins A and B and secretory leukoprotease inhibitor. In addition, these cells may participate in the clearance of noxious agents via the detoxification of inhaled agents [10-12]. Serous cells also produce antiproteases [13], whereas neuroendocrine cells contain amines and peptide hormones [14-16]. The mucous cell is the predominant secretory cell in the larger airways, whereas the Clara cell is predominant in the bronchioles [6, 17, 18].

Basal cells are considered as the stem cell of the bronchial epithelium and are pyramidal-shaped cells with a small cytoplasmic/nuclear ratio [2, 6, 19].

4.2. BARRIER FUNCTIONS

Bronchial epithelial cells are part of the non-specific immune system and defend the airways against the entry of noxious substances [20]. This defense is mediated via the integrity of the epithelium that contributes to the physical barrier, the secretion and ciliary function leading to effective mucociliary clearance, and the secretion of mediators which provide protection against a wide range of potentially injurious agents.

Integrity of the epithelium

The bronchial epithelium forms a continuous layer, thereby preventing the underlying tissue from the entry of noxious agents. The integrity of the epithelium is maintained by several adhesion mechanisms (Fig. 2) [21].

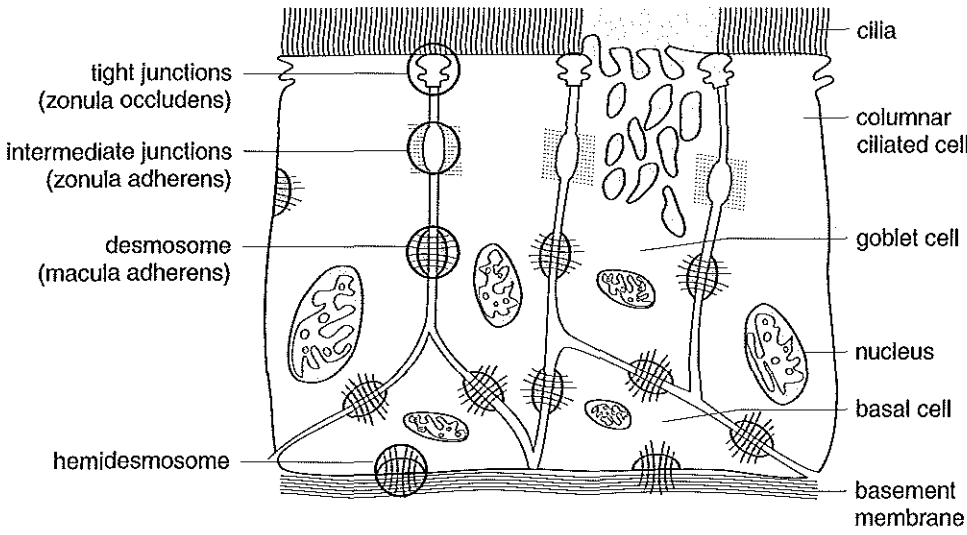

Figure 2. Schematic representation of the adhesion mechanisms which maintain the integrity of the bronchial epithelium (from reference [22]).

The desmosome (macula adherence) and the intermediate junction (zonula adherence) are involved in maintaining a strong cell-to-cell adhesion. The tight junction (zonula occludens) is a narrow belt-like structure surrounding each cell at the apical pole. It provides a physical barrier, thereby preventing 'leakage'. The epithelial cells are all anchored to the basement membrane by hemidesmosomes.

Mucociliary clearance

Inhaled particles, including bacteria and viruses, are cleared from the airways by trapping of the particle in mucus, and subsequent clearance of the mucus by the coordinated beating of cilia. The clearance of particles is facilitated by the secretion of surfactant (by alveolar epithelial type II cells and Clara cells), which changes the surface charge properties, making the particles less sticky. The mucociliary function is regulated by a complex interaction with the cells and mediators of the immune system (Table 1) [23-27].

Table1. Factors that modulate mucociliary function.

Factors	Sources	Motility	Velocity
β_2-agonists	nerves, drugs	↑	↑
Bradykinin	plasma	↑↑	↑
Histamine	mast cells	±	↑
Nitric oxide	epithelium, macrophages	↑	?
Substance P	nerves	↑	↑
Interleukin-1	epithelium, macrophages	↑	?
Major Basic Protein	eosinophils	↓	?
Oxidants	granulocytes, macrophages	↓	↑

Secretion of protective mediators

To provide protection against potentially injurious agents, the bronchial epithelium secretes a number of mediators, including antibacterial substances (lactoferrin and lysozyme), antiproteases (α_1-protease inhibitor, secretory leukoprotease inhibitor, α_1-antichymotrypsin, α_2-macroglobulin, tissue inhibitors of metalloproteases), and anti-oxidant systems (glutathione redox cycle, superoxide dismutase, and catalase) [28-32]. The bronchial epithelium produces components of the complement system, which act as opsonins allowing efficient phagocytosis by macrophages [33]. In addition, bronchial epithelial cells transport secretory immunoglobulin A (sIgA) into the bronchial lumen [34]. In lung cells of patients with asthma, a reduced expression of superoxide dismutase has been found compared to healthy controls [35, 36]. In contrast, sIgA and lactoferrin are increased in bronchoalveolar lavage (BAL) fluid of asthmatics [37]. The release of broncho-active and immunomodulatory mediators, such as cytokines, arachidonic acid metabolites, and chemokines, will be discussed further on.

Loss of barrier function in asthma

The barrier function of the bronchial epithelium seems to be disturbed in asthmatics, since epithelial shedding and loss of integrity are recognized features both in fatal asthma

and in biopsy specimens of even mild asthmatics [38-42]. Epithelial shedding or damage probably is due to the release of cationic granule proteins by activated eosinophils, which are highly toxic to the respiratory epithelium [43-47]. Indeed, asthmatic airways are characterized by increased numbers of activated eosinophils and elevated levels of eosinophil-derived mediators [38, 48, 49]. Several studies have identified an association between epithelial damage and the degree of bronchial hyperresponsiveness [40-42, 49]. This association may be caused by several mechanisms. First, epithelial damage will result in loss of a permeability barrier and enables noxious agents or allergens to directly penetrate the airway wall and reach the submucosa. Second, loss of ciliated cells will result in impaired transport of mucus. Third, epithelial damage may expose nonmyelinated afferent nerve endings. As a consequence, these nerves may more easily be stimulated by inflammatory mediators or inhaled particles, leading to an axon reflex and subsequent release of sensory neuropeptides that in turn evoke neurogenic inflammation ([50]; discussed in more detail in chapter 2). Fourth, the epithelium secretes factors that suppress airway contraction, like prostaglandin (PG) E_2, prostacyclin, nitric oxide (NO), and a putative epithelial-derived relaxing factor (EpDRF) [1]. Loss of these factors may contribute to bronchial hyperresponsiveness. Fifthly, bronchial epithelial cells contain neutral endopeptidase (NEP), which is involved in the metabolism of a variety of peptides with contractile effects on smooth muscle ([51, 52]; discussed in chapter 3). Epithelial damage and loss of NEP activity may diminish peptide breakdown and thereby enhance bronchoconstriction. Finally, epithelial damage may trigger the production and release of mediators, such as $PGF_{2\alpha}$, 13-hydroxy-linoleic acid (HODE) and endothelin-1, which can affect airway responsiveness [53-56].

4.3. IMMUNOLOGICAL PROPERTIES OF THE BRONCHIAL EPITHELIUM

Bronchial epithelial cells not only form a passive barrier but also play an active role in the immune response [1, 2]. They are able to produce a variety of mediators that may act either pro- or anti-inflammatory. In addition, bronchial epithelial cells may express adhesion molecules for many different cell types, thereby contributing to their recruitment [57].

4.3.1. Pro-inflammatory potential

Bronchial epithelial cells may initiate and perpetuate inflammatory reactions by recruitment of inflammatory cells, cell-cell adhesion and interaction of epithelial cells with inflammatory cells, and modulation of the activity of inflammatory or parenchymal cells.

Recruitment of inflammatory cells

The recruitment of inflammatory cells into the airways is dependent upon the presence of chemoattractants. It has been demonstrated that bronchial epithelial cells can synthesize and release a wide range of such chemoattractants, including arachidonic acid metabolites and chemokines, both spontaneously and after stimulation (Table 2).

Table 2. Mediators produced by human bronchial epithelial cells and their changes in asthma (see text for details).

Mediator		Main effect	Changes in asthma
Lipid mediators			
	LTB_4	Recruitment of neutrophils	↑
	LTC_4	Microvascular leak, mucus secretion, bronchoconstriction, vasoconstriction	?
	PGD_2	Bronchoconstriction	?
	PGE_2	Bronchodilation/bronchoconstriction, vasodilation	↑
	$PGF_{2\alpha}$	Bronchoconstriction	?
	12/15-HETE	Recruitment of neutrophils, mucus secretion	↑
	9/13-HODE	Recruitment of neutrophils	?
Chemokines			
	IL-8	Recruitment of neutrophils	↑
	Gro-α	Recruitment of neutrophils	?
	Gro-γ	Recruitment of neutrophils	?
	MCP-1	Recruitment/activation of monocytes, lymphocytes, basophils	↑
	Eotaxin	Recruitment of eosinophils	↑
	RANTES	Recruitment of T cells, monocytes, eosinophils, basophils	=
	MIP-2	Recruitment of neutrophils	?
Cytokines			
	IL-1α/β	Pro-inflammatory	↑
	IL-3	Growth/survival of eosinophils	=
	IL-6	Pro/anti-inflammatory, B cell activation	↑
	IL-10	anti-inflammatory	?
	IL-11	Neuropoietic	?
	IL-16	Recruitment of $CD4^+$ T cells	↑
	TNF-α	Pro-inflammatory	?
	GM-CSF	Survival/activation of eosinophils and neutrophils	↑
	G-CSF	Survival/activation of granulocytes	?
	TGF-β	Pro-fibrotic, anti-inflammatory	↑
Other			
	PAF	Recruitment of eosinophils	?
	NO	Bronchodilation, Th1 skewing, plasma exudation	↑
	Endothelin	Bronchoconstriction, smooth muscle cell proliferation	↑
	EpDRF	Bronchorelaxation	?
	Fibronectin	Epithelial cell migration and repair	↑
	PDGF	Pro-fibrotic	=
	IGF	Pro-fibrotic	?
	EGF	Epithelial growth and differentiation	↑

Bronchial epithelial cells may secrete the *arachidonic acid metabolites* 15-hydroxyeicosatetranoic acid (15-HETE) and possibly leukotriene B_4 (LTB_4), which are potent attractants for eosinophils, neutrophils and monocytes, and also increase mucus secretion [58-66]. The production and release of these mediators is up-regulated in asthma, and there is a clear correlation between the release of 15-HETE and the clinical status of the patient [62].

Human bronchial epithelial cells are able to produce several *chemokines*, including RANTES (Regulated upon Activation, Normal T cell Expressed, and presumably Secreted) [67], growth regulated oncogen (Gro)-α [68], monocyte chemotactic protein (MCP)-1 [68, 69], MCP-4 [70], interleukin (IL)-8 [71-73], and eotaxin [74]. Chemokines are a group of chemotactic and pro-inflammatory cytokines and can be divided into at least four groups depending on the number and position of cysteine residues [75, 76]. C-X-C chemokines predominantly are chemotactic for neutrophils, and include IL-8, neutrophil-activating peptide (NAP)-2, Gro-α, β, and γ, and macrophage inflammatory protein (MIP)-2. C-C chemokines, on the other hand, preferentially attract monocytes, eosinophils and T lymphocytes. Members of this subgroup include MCP-1, MCP-2, MCP-3, MCP-4, eotaxin and RANTES. Two small subgroups of chemokines are the C chemokines (with at present only one member: lymphotactin, also known as SCM-1 or ATAC [77-80]) and the CX3C chemokines (also with just one member [81]). Chemokines act through binding to the chemokine receptors, which are GTP-coupled seven-transmembrane domain receptors [82]. At present, four CXC chemokine receptors (CXC R1 through 4), at least eight CC chemokine receptors (CC R-1 through 8), and one CX3C chemokine receptor (CX3C R) have been cloned and characterized [76, 83].

Bronchial epithelial cells from asthmatics have been shown to release more IL-8 *in vitro* than epithelial cells obtained from healthy controls [73]. In addition, increased levels of IL-8 were demonstrated in BAL fluid of asthmatics [84]. Using immunohistochemical techniques, an increased expression of MCP-1 [69] and eotoaxin [85, 86] has been found in the bronchial epithelium of asthmatics. In contrast, no differences in RANTES protein or mRNA expression could be observed between healthy subjects and asthmatics [87]. Clearly, bronchial epithelial cells of asthmatic patients release increased amounts of CC and CXC chemokines and therefore contribute to the recruitment of inflammatory cells.

IL-16 is a recently discovered cytokine, which has been shown to have selective chemotactic activity for CD4-positive cells, monocytes, and eosinophils *in vitro* [88, 89]. IL-16, which shows no similarity to other cytokines or members of the chemokine family, uses CD4 as its receptor [88, 89]. In the lung, it is produced by epithelial cells, and CD4-positive and CD8-positive T lymphocytes [90]. Increased expression of IL-16 by bronchial epithelial cells has been described in asthmatics compared to healthy subjects, and the epithelial IL-16 expression was shown to correlate with the number of CD4-positive cells within the lamina propria [91]. In addition, IL-16 has been detected in BAL fluid by six hours following subsegmental allergen or histamine challenge in asthmatics, but not in atopic non-asthmatics or healthy subjects [92].

Bronchial epithelial cells have also been shown to release *platelet-activating factor* (PAF), a potent eosinophil chemoattractant [93].

Cell-cell adhesion and interaction

Bronchial epithelial cells may interact with other cells by direct contact mediated via surface membrane-bound molecules, such as adhesion molecules and major histocompatibility complex (MHC) molecules.

Adhesion molecules are glycoproteins expressed on the surface of cells, which mediate the contact between two cells or between the cell and the components of the extracellular matrix. These molecules therefore play an important role in the transmigration of leukocytes through the endothelial wall, localization of leukocytes at sites of inflammation in the epithelium, and adherence of the epithelial cells to the basement membrane. Four main families of adhesion molecules can be distinguished: the immunoglobulin-gene superfamily, the integrins, the selectins, and the cadherins [94].

The *immunoglobulin (Ig)-gene superfamily* consists of cell surface proteins characterized by a variable number of extracellular Ig-like domains [95, 96]. These molecules are involved in antigen recognition, complement binding or cellular adhesion [95]. Human bronchial epithelial cells express two members of this family: intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen-3 (LFA-3) [57]. It has been reported that the epithelial expression of ICAM-1 is increased in asthmatics compared with healthy subjects, and that the level of expression correlated with the severity of the disease [97, 98]. However, no difference in ICAM-1 expression was found in another study [99]. In the BAL fluid of asthmatics, increased levels of soluble ICAM-1 have been found after allergen challenge [100, 101]. Circulating ICAM-1 levels in the blood were elevated in patients with acute asthma compared to stable asthmatics or healthy subjects [101-103]. It has been shown that pro-inflammatory cytokines like IL-1β, TNF-α and IFN-γ, are able to increase the expression of ICAM-1 on epithelial cells *in vitro* [57, 104, 105]. Since the ligand for ICAM-1, LFA-1 (CD11a/CD18), is expressed on the surface of neutrophils, monocytes, lymphocytes and eosinophils [106], increased expression of ICAM-1 during inflammatory responses may contribute to the adhesion and subsequent maturation and activation of leukocytes in the epithelial compartment. The observation that, in primates, intravenous administration of anti-ICAM-1 antibodies attenuated both airway eosinophilia and bronchial hyperresponsiveness further supports the important role of ICAM-1 in the recruitment and adhesion of leukocytes [97, 107]. In contrast to ICAM-1, LFA-3 expression on bronchial epithelial cells could not be modulated by pro-inflammatory cytokines [57] and its role in the pathogenesis of asthma remains to be established.

Integrins are molecules composed of two non-covalently associated heterodimers, designated the α and β subunit [94, 106, 108]. β_1 integrins may associate with nine distinct α subunits and play an important role in tissue organization. Human bronchial epithelial cells have been shown to express the $\alpha_{2\text{-}6}$ integrins, both *in vivo* and *in vitro* [107, 109-112]. Recent studies have shown the expression of $\alpha_v\beta_6$ on human bronchial epithelial cells [113, 114]. The expression of this adhesion molecule is increased after epithelial injury, inflammation or exposure to EGF or TGF-β. Studies using transgenic mice indicate that $\alpha_v\beta_6$ may be involved in the down-regulation of airway inflammation [115, 116]. β_2 integrins (LFA-1 ($a_L\beta_2$), Mac-1 ($\alpha_M\beta_2$), and p150,95 ($\alpha_X\beta_2$)) are exclusively expressed on leukocytes.

The *selectin* family (consisting of E- (endothelial), P- (platelet), and L- (leukocyte) selectin) is only expressed on activated endothelial cells or leukocytes [95, 117, 118]. No expression can be found on human bronchial epithelial cells [57].

Cadherins are involved in the cellular architecture and in cell-cell adhesion. Cadherins may interact with the cytoskeleton and bind to a group of cytosolic proteins termed catenins

[119]. It has been suggested that alterations in the binding of epithelial cadherin to catenins may be involved in the desquamation and shedding of the epithelium associated with the airways of asthmatic subjects.

Human bronchial epithelial cells are also capable to express the MHC class II antigens (including human leukocyte antigens (HLA)-DR) [66, 98]. Bronchial epithelial HLA-DR expression has been shown to be increased in asthmatic patients compared to healthy subjects, and the level of expression is correlated with the severity of the disease [98]. *In vitro*, the expression of MHC class II on human bronchial epithelial cells is relatively low, but after stimulation with IFN-γ or histamine its expression is strongly increased [66, 120, 121]. Although it has been demonstrated that bronchial epithelial cells are capable of inducing T cell proliferation [122-124], it is not clear at present whether presentation of antigens to lymphocytes by bronchial epithelial cells is involved in the pathogenesis of asthma.

Expression of the low-affinity IgE receptor (CD23) has been described in bronchial epithelial cells of asthmatic patients, but not of healthy controls [125]. Stimulation of bronchial epithelial cells of asthmatics with IgE/anti-IgE resulted in increased release of endothelin-1 (ET-1). This suggests that bronchial epithelial cells of asthmatic patients may be directly activated by an IgE-dependent mechanism.

Modulation of inflammatory or parenchymal cell activity

Human bronchial epithelial cells are capable of producing a wide range of mediators, which are important in modulating cellular responses in the airways, both spontaneously and after stimulation. These mediators include chemokines, lipid mediators, cytokines, endothelin, growth factors, and NO (Table 2).

As mentioned before, *chemokines* are able to recruit leukocytes to the site of inflammation [82, 126]. These mediators often also activate the attracted leukocytes. For example, it has been shown that MCP-1 is able to activate monocytes and basophils, and can induce ICAM-1 expression on endothelial and vascular smooth muscle cells [127-130]. IL-8 and LTB_4 not only attract neutrophils, but also cause neutrophil degranulation and superoxide production, at least *in vitro* [131].

Lipid mediators produced by bronchial epithelial cells include the arachidonic acid metabolites LTB_4, 15-HETE, $PGF_{2\alpha}$, and PGE_2 [65, 132-134]. As mentioned before (chapter 1), PGE_2 plays a role in skewing the Th lymphocytes toward a Th2 phenotype. In addition, PGE_2 is a vasodilator, and its release may therefore result in the formation of oedema. 15-HETE increases the secretion of mucus and enhances an early response to inhaled allergens [135], whereas $PGF_{2\alpha}$ functions as a bronchoconstrictor [136, 137]. Prostacyclin and $PGF_{2\alpha}$ can stimulate sensory nerve endings, thereby causing reflex bronchoconstriction [138].

Bronchial epithelial cells can also produce and release a wide range of *cytokines*. These include granulocyte/macrophage-colony stimulating factor (GM-CSF), TNF-α, IL-1α, IL-1β, IL-3, IL-6, IL-10, IL-11, leukemia inhibitory factor (LIF), and IL-16 [71, 73, 139-146]. GM-CSF production by the bronchial epithelium has been shown to be increased in asthmatics [73, 139]. This may contribute to a prolonged survival of neutrophils and eosinophils with concomitant cell activation [147-149]. IL-1β and TNF-α are pro-inflammatory cytokines, which may activate a large number of cells. IL-6 and IL-11 have many overlapping effects, including B cell activation and production of acute phase proteins [150-152]. In addition, IL-11 has neuropoietic properties: it is a survival factor for sensory and motor neurons, causes noradrenergic sympathetic neurons to take on a cholinergic phenotype, and induces substance P (SP), somatostatin, and vasoactive intestinal peptide-related peptide in sympathetic

neurons [153]. This raises the possibility that disregulated IL-11 production could lead to pathologic conditions characterized by cholinergic or neuropeptide excess. IL-16 not only attracts CD4-positive lymphocytes, eosinophils and monocytes but also activates these cells, resulting in cell adhesion, induction of CD25 and HLA-DR expression, and /or cytokine synthesis [89].

Bronchial epithelial cells of asthmatic patients have been shown to produce increased levels of IL-1β, IL-6, IL-8, GM-CSF, and IL-16 compared to healthy subjects [73, 91, 154]. This indicates that bronchial epithelial cells are in an activated state in the asthmatic airways. Transcription factors like NF-κB probably play an important role in the upregulation of these cytokines [155, 156]. Interestingly, a recent report showed that the allergen Der p1 induced NF-κB activation through interference with IκBα function in asthmatic bronchial epithelial cells, indicating that allergens may directly interact with transcription factors involved in the transcriptional regulation of inflammatory genes [157].

Endothelins are a family of highly homologous 21-amino acid peptides, characterized by two intrachain disulfide chains, a hairpin loop consisting of polar amino acids, and a hydrophobic C-terminal chain [158]. Human bronchial epithelial cells have been shown to produce ET-1 [54, 159, 160], which promotes the proliferation of smooth muscle cells, is a potent constrictor of both vascular and non-vascular smooth muscle cells, increases the secretion of mucus, and may activate inflammatory cells [158, 160, 161]. ET-1 also stimulates collagen gene expression and through its inhibitory actions on collagenase will promote airway wall collagen deposition, thereby contributing to airway wall thickening which underlies bronchial hyperresponsiveness [162-164]. Increased levels of ET-1-immunoreactivity were detected in airway epithelium and vascular endothelium of bronchial biopsy specimens from asthmatics compared to healthy subjects [159, 165, 166]. Furthermore, increased ET-1 levels have been detected in BAL fluid and blood plasma of asthmatics [167, 168].

Several *growth factors* can be produced by the bronchial epithelium. These include epidermal growth factor (EGF), transforming growth factor (TGF)-β, insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF) [20, 169, 170]. TGF-β is an important profibrotic growth factor, which has been implicated in airway remodeling and pulmonary fibrosis [171]. In asthma, there is an increased expression of TGF-β on epithelial cells which is correlated with the number of fibroblasts beneath the basement membrane and with the thickness of the basement membrane [3, 166, 172]. TGF-β has been shown to increase the release of fibronectin from human bronchial epithelial cells *in vitro* [173]. IGF also is a major fibroblast and epithelial cell mitogen, but a role for this growth factor in asthma has not been determined yet. However, it has been shown that airway epithelial cells express increased numbers of IGF-receptors after stimulation with eosinophil cationic protein [174]. Studies on PDGF, which has high mitogenic activity for smooth muscle cells and fibroblasts, have not demonstrated any up-regulation in the expression of this growth factor in the bronchial epithelium of asthmatics [172, 175, 176]. In contrast, epithelial cells of asthmatics do show increased immunoreactivity for EGF, which is an important factor in the regulation of epithelial growth and differentiation [175].

Nitric oxide (NO) may play an important role in regulating airway function and in the pathophysiology of asthma [177-180]. NO is produced by nitric oxide synthase (NOS), which exists in several isoforms: n (neuronal)-NOS, e (endothelial)-NOS, and i (inducible)-NOS [181, 182]. Both the inducible and constitutive form have been identified in bronchial epithelial cells [183-185] and increased expression of iNOS has been observed in response to pro-inflammatory cytokines and oxidants [179, 185, 186]. There is an increased expression

of iNOS in the epithelium of asthmatic patients and increased NO levels have been found in exhaled air of asthmatics [183, 187, 188]. Increased NO production in the airways may result in hyperemia, plasma exudation, and mucus secretion. NO also has been implicated in skewing T lymphocytes towards a Th2 phenotype, through inhibition of Th1 cells and their production of IFN-γ [189].

4.3.2. Anti-inflammatory potential

Besides the potential of human bronchial epithelial cells to recruit and activate leukocytes or parenchymal cells, bronchial epithelial cells may also down-regulate inflammatory responses. This may occur via the release of anti-inflammatory mediators, by the release of soluble receptors, or by the inactivation of pro-inflammatory mediators.

Release of anti-inflammatory mediators

Human bronchial epithelial cells are able to produce several components of the IL-1 system, including agonists, antagonists and receptors. As discussed before, human bronchial epithelial cells can release IL-1α and IL-1β, which both exert many pro-inflammatory effects. These effects are mediated via binding to the IL-1 receptor (IL-1R) type I, whereas the IL-1R type II has a short cytoplasmic domain and appears to function as a scavenger for IL-1β [190-192]. The extracellular portions of both receptors may be shed from the plasma membrane and then act as IL-1 inhibitors [193]. Three splice variants of the IL-1 receptor antagonists (IL-1RA) gene have been described thus far: secreted IL-1RA, intracellular IL-1R type I and type II. It has been shown that human bronchial epithelial cells are able to produce and release the intracellular IL-1 receptor antagonists type I, which may counteract the pro-inflammatory actions of IL-1α and IL-β [154, 194, 195]. In addition, these cells may release the IL-1R type I. Recently, a new cytokine (IL-18) with structural homology to IL-1 has been found [196, 197]. This cytokine requires cleavage by either IL-1β converting enzyme or another caspase to generate a mature bioactive molecule, and signals through IL-1 receptor-associated kinase (IRAK) to induce activation of NF-κB [198]. Clearly, the balance of the different components of the IL-1 system determines whether the overall effect will be pro- or anti-inflammatory.

TGF-β has been identified in the epithelial lining fluid of the lung and in airway epithelial cells [199, 200]. In addition to its pro-inflammatory effects (described above), TGF-β has many anti-inflammatory properties, including inhibition of IL-2 dependent proliferation of T lymphocytes, inhibition of cytokine production by macrophages, and inhibition of IL-4-induced IL-8 release by human bronchial epithelial cells [201-205]. TGF-β may also be involved in neural repair via stimulation of IL-11 production by bronchial epithelial cells [145].

PGE_2 and IL-6 produced by bronchial epithelial cells may have both pro- and anti-inflammatory properties. PGE_2 can reduce the production of neutrophil chemoattractants by macrophages, can act directly as a bronchodilator (as does prostacyclin), and inhibits fibroblast matrix production [206, 207]. IL-6 has been found to reduce inflammatory reactions in several models, including an *in vivo* model of pulmonary inflammation [205]. However, the mechanism by which IL-6 exerts this effect is not completely understood.

IL-10 is a potent regulatory cytokine that decreases inflammatory responses and T cell activation [208-210]. It reduces the production of TNF-α and IL-1β by macrophages [211-

213]. Down-regulation of IL-10 production, as has been described in patients with cystic fibrosis [144], may enhance local inflammation and tissue damage.

Interactions between epithelial cells may be of primary importance in directing repair of injury. Fibronectin, together with growth factors, is thought to have a significant role in the modulation of epithelial cell migration. Its production is increased after injury and after exposure of epithelial cells to inflammatory mediators, such as cytokines and endothelin-1 [62, 142, 214-216].

NO produced by bronchial epithelial cells may also have beneficial effects. It increases the ciliary beat frequency, thereby facilitating the clearance of mucus with trapped agents [23]. NO is also a potent bronchodilator [179, 217]. In contrast to guinea pigs, human studies have failed to demonstrate that EpDRF is identical to NO [218-221].

Release of soluble receptors

The release of soluble receptors is another mechanism to control inflammatory processes [222, 223]. Soluble receptors may bind their ligand, thereby reducing the amount of ligand able to bind membrane-bound receptors. Bronchial epithelial cells have been shown to release the IL-6 receptor and the p55 (type I) soluble TNF-α receptor (sTNF-R), which may down-regulate the effects of IL-6 and TNF-α, respectively [224-226]. In a study with stable asthmatic children, no difference in sTNF-R levels in serum could be observed compared to healthy subjects [227]. However, during asthma exacerbations serum levels of sTNF-R were significantly increased in both non-atopic and atopic asthmatics [228].

As already mentioned above, epithelial cells possibly also release the IL-1R type I. At present, no data are available on the role of this soluble receptor in asthma.

Inhibition of pro-inflammatory mediators

Bronchial epithelial cells express several enzymes which are able to degrade, and thereby often inactivate, a variety of mediators, including neuropeptides, histamine, bradykinin, and cytokines. Epithelial cells express histamine N-methyltransferase, and thus are capable of modulating histamine-mediated effects [229, 230]. The best-studied peptidase expressed by bronchial epithelial cells is NEP. A reduced activity of this enzyme has been implicated in the pathogenesis of asthma [52, 231, 232]. The characteristics and biological functions of peptidases are described in more detail in chapter 3.

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Chapter 5

Glucocorticoids
Mechanisms of action and anti-inflammatory potential in asthma

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Chapter 5

Glucocorticoids
Mechanisms of action and anti-inflammatory potential in asthma

Glucocorticoids are hormones synthesized in the adrenal cortex and secreted into the blood, where the levels of glucocorticoids fluctuate in a circadian mode. In humans, the naturally occurring glucocorticoid is hydrocortisone (cortisol), which is synthesized from its precursor cortisone.

The beneficial effects of glucocorticoids in asthmatic patients were first described in 1950 [1]. Since then on, many studies have focussed on the therapeutic potential of glucocorticoids. Several synthetic glucocorticoids, much more potent than cortisol and without the unwanted mineralocorticoid side effects, have been developed. Nowadays, glucocorticoids are powerful agents in the treatment of inflammatory diseases and are by far the most effective anti-inflammatory drugs used in the treatment of asthma.

5.1. MECHANISM OF ACTION

Although glucocorticoids have been known for a long period of time, their precise mechanism of action is still not completely understood. However, recent studies have increased our understanding of their complex mechanisms of action.

5.1.1. Glucocorticoid receptor

To exert their effects, glucocorticoids need to bind to a specific cytoplasmic glucocorticoid receptor (GR). Almost all cells of the body express the GR, but the number of receptors may vary between different cell types [2]. Cloning of the GR has revealed that the GR consists of approximately 800 amino acid residues, and that certain areas of the molecule show homology with other steroid receptors, receptors for thyroid hormones, and receptors for retinoic acid [3-7]. All members of the nuclear hormone receptor family share a characteristic three-domain structure, first described for the human GR (Fig. 1). The C-terminal domain is equal in size in all nuclear receptors studied (about 250 amino acids) and its main function is to bind the steroid [8]. It also contains the binding sites for the heat shock proteins (hsp) 90 [9, 10]. Removal of the steroid-binding domain results in a constitutively active GR molecule, indicating that this part of the molecule acts as a repressor of the transcription-activation function. The most conserved central domain is involved in direct binding of the receptor to DNA. It contains two distinct loops of protein, each bound at their base via four cysteine residues to a single zinc ion, the so-called zinc fingers [11]. These zinc clusters are

involved in binding of the GR to the major groove of the DNA double helix and play a role in dimerization of two GR molecules [12, 13]. In addition, the central DNA-binding domain has a transcription-activation function [4, 14]. The steroid-binding and DNA-binding domains are separated by the 'hinge-region', which contains sequences that are important for nuclear translocation and dimerization [9, 10]. The N-terminal domain is extremely variable in size (24-600 amino acids). Its precise role is still uncertain, but it is required in transcriptional activation [15].

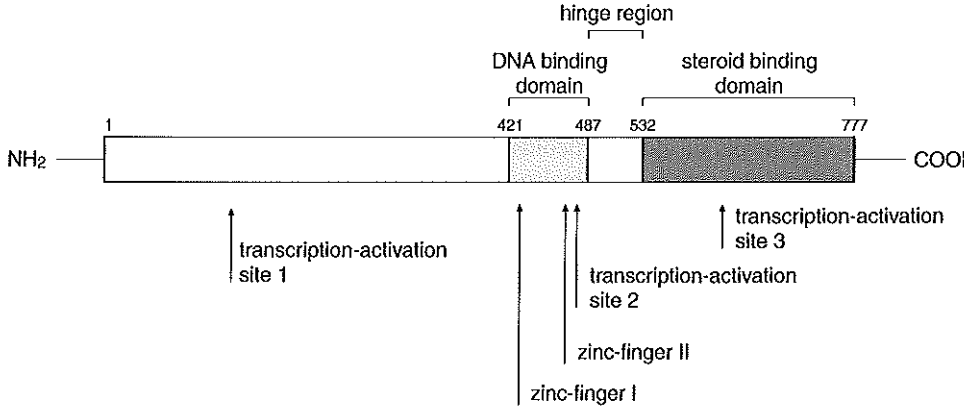

Figure 1. Linear alignment of the human glucocorticoid receptor (adapted from reference [16]).

Two different forms of the human GR have been described [3, 17]. These two highly homologous isoforms, termed GRα and GRβ, are generated by alternative splicing of the human GR pre-mRNA. The GRβ isoform differs from the GRα isoform only in its C-terminal domain, in which the last 50 amino acids of the latter are replaced by a unique 15 amino acid sequence. However, this replacement has dramatic functional consequences, since the GRβ isoform is unable to bind glucocorticoids and to transduce ligand-dependent transactivation. However, the physiological significance of the GRβ isoform remains questionable, since some recent studies indicate that this form is not conserved among species and no dominant negative inhibition of GRα activity could be found [18, 19]. Nevertheless, abundant expression of GRβ protein can be found in the epithelial cells lining the terminal bronchioli of the lung [20].

The expression of the GR may be regulated by numerous factors either at the transcriptional, translational or post-translational level [21, 22]. Glucocorticoids have been shown to down-regulate the expression of the GR, both *in vitro* and *in vivo* [23, 24]. In contrast, inflammatory mediators like interleukin (IL)-1β, IL-4, tumor necrosis factor (TNF)-α, lipopolysaccharide (LPS) and interferon (IFN)-γ have been shown to increase glucocorticoid binding *in vitro* [25-29]. However, the increase in GR numbers may be accompanied by a reduced affinity for glucocorticoids [25, 29]. Analysis of GR localization in normal and asthmatic lung has not revealed differences in the level or sites of GR expression [30].

5.1.2. Regulation of gene transcription

In the absence of glucocorticoids, the GR is present in the cytoplasm of the cell as a hetero-oligomer consisting of the GR itself, two molecules of hsp 90, one molecule hsp 70, and one molecule of hsp 56 (which probably does not interact with the GR itself, but interacts with hsp 90) [31-35]. Glucocorticoids enter the cytoplasm of the cell by passive diffusion through the cell membrane. In the cytoplasm they bind to the GR complex, which subsequently undergoes conformational changes, resulting in the dissociation of the hsp 90 and hsp 56 molecules. Upon this activation, the glucocorticoid-GR complex passes the nuclear membrane, enters the nucleus, and the hsp 70 molecule is dissociated. Furthermore, in the nucleus liganded GR form homodimers (Fig. 2).

Within the nucleus, the GR homodimers may regulate gene transcription in several ways: 1. via binding of the glucocorticoid-GR complex to specific DNA sequences, thereby directly activating or repressing genes; 2. via interaction with other transcription factors; and 3. via modulating the stability of specific mRNA molecules [36-40].

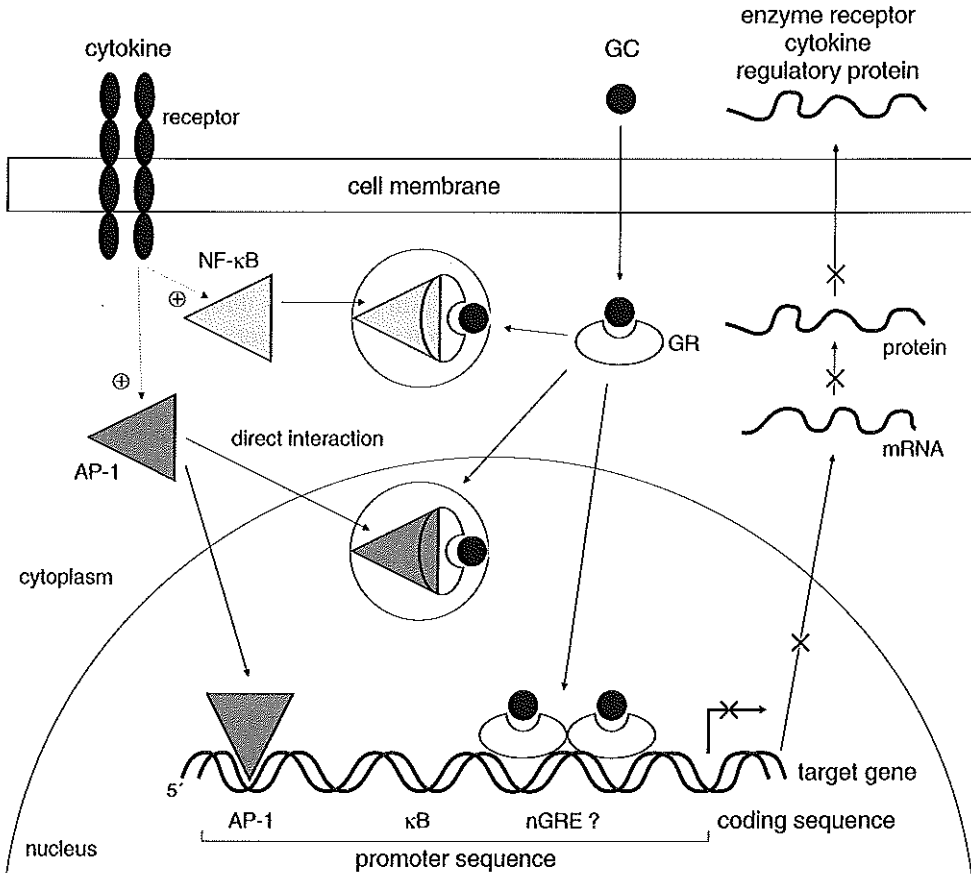

Figure 2. Cellular events after administration of glucocorticoids (adapted from reference [40]).

Binding to DNA sequences

Several steroid-responsive genes contain glucocorticoid responsive elements (GRE) in their promoter region [36, 41]. Binding of GR homodimers to GRE may either result in transcriptional activation of the gene (via a *positive* GRE) or repression of the gene (via a *negative* GRE) (Fig. 2). The consensus sequence for (positive) GRE is the palindromic 15-base-pair sequence GGTACAnnnTGTTCT, whereas the negative GRE has a more variable sequence [37]. The rate of transcriptional regulation of steroid-responsive genes is dependent on both the numbers of GRE, the affinity of the glucocorticoid-GR complex to the GRE, and the position of the GRE relative to the transcriptional start site. Binding of the complex to GRE may result in conformational changes in the DNA and exposure of previously masked areas, resulting in increased binding of other transcription factors [42-45].

Interaction with other transcription factors

Many steroid-responsive genes do not have GRE in their promoter region. However, binding sites for other transcription factors, including activating protein (AP)-1, nuclear factor (NF)-κB, and cAMP-responsive element binding protein (CREB), often can be found [46].

AP-1, which is a dimer of two proto-oncogenes (members of the c-jun and c-fos family) [47, 48], is involved in the regulation of several genes, including adhesion molecules and cytokines (reviewed in [48]). Direct protein-protein interaction between AP-1 and the glucocorticoid-GR complex results in reciprocal repression of one another's transcriptional activation by preventing binding of the AP-1 and glucocorticoid-GR complex to AP-1 sites and GRE, respectively (Fig. 2) [38, 49, 50].

Comparable to AP-1, *NF-κB* (a heterodimer of p50 and p65 subunits; [51, 52]) regulates the transcription of several genes involved in inflammatory reactions [51, 53, 54]. In unstimulated cells, NF-κB is retained in the cytoplasm of the cells through the interaction with the inhibitors IκBα and IκBβ [55-57]. Upon cell stimulation, for example by IL-1β or TNF-α, IκB are rapidly phosphorylated, ubiquitinated, and consequently proteolyzed [54, 58]. The liberated NF-κB dimers translocate to the nucleus where they can activate target genes. Glucocorticoids may inhibit NF-κB-stimulated genes by a direct interaction between the glucocorticoid-GR complex and the p65 subunit of NF-κB, resulting in transrepression (Fig. 2) [52, 56, 59, 60]. Furthermore, glucocorticoids may indirectly antagonize NF-κB mediated transcription by up-regulating the synthesis of the inhibitory protein IκBα, which traps NF-κB in inactive cytoplasmic complexes [40, 55, 56]. A large number of immunoregulatory genes, whose expression is induced by a variety of pro-inflammatory mediators, contain NF-κB sites in their promoters/regulatory regions. Therefore, it is no wonder that glucocorticoids have been found to prevent the expression of these genes, including those coding for IL-1β, IL-2, IL-6, IL-8, monocyte chemotactic protein (MCP)-1, RANTES (Regulated upon Activation, Normal T cell Expressed, and presumably Secreted), granulocyte macrophage colony-stimulating factor (GM-CSF), the IL-2 receptor, intercellular adhesion molecule (ICAM)-1, and E-selectin (reviewed in [46]). Probably, interactions between glucocorticoids and NF-κB or AP-1 will explain most of the anti-inflammatory and immunosuppressive activities of glucocorticoids.

An interaction between *CREB* and the glucocorticoid-GR complex has also been suggested [61, 62]. β-agonists, which are used as bronchodilators in the treatment of asthma, increase cAMP formation and subsequently activate CREB. Therefore, simultaneous treat-

ment of asthmatic patients with glucocorticoids and β-agonists may result in reduced responsiveness of the airways for steroids [62-64].

Modulation of mRNA stability

A third mechanism by which glucocorticoids may regulate the synthesis of proteins is via enhanced transcription of specific ribonucleases which are able to degrade mRNA containing constitutive AU-rich sequences in the untranslated 3'-region [65]. Such glucocorticoid-mediated modulation of post-translational events (resulting in decreased mRNA stability and reduced half-life time) has been observed for IL-1β, IL-6 and GM-CSF [66, 67].

5.2. GLUCOCORTICOID REGULATED GENES

Glucocorticoids are able to modulate the transcription of a variety of genes, including cytokines and chemokines, receptors, enzymes, adhesion molecules, and inhibitory proteins (Table 1). Since epithelial cells may be one of the most important targets for glucocorticoid therapy in asthma, the effects of glucocorticoids on epithelial expressed inflammatory genes will be emphasized in this review.

Table 1. Influence of glucocorticoids on the synthesis of proteins with (anti-)inflammatory effects by bronchial epithelial cells.

Protein	Glucocorticoid effect
Cytokines	
IL-1β, IL-6, IL-11, TNF-α, GM-CSF	↓
IL-10, LIF	?
G-CSF	=
Chemokines	
MCP-1, eotaxin, IL-8, RANTES, MIP-1α	↓
Receptors	
NK_1, GR	↓
IL-1R II, IL-6R, β_2-adrenergic receptor	↑
Enzymes	
iNOS, COX-2, $cPLA_2$	↓
NEP	↑
Adhesion molecules	
ICAM-1	↓
Inhibitory proteins	
Lc-1	=/↑
IL-1RA type I, SPLI	↑

Cytokines and chemokines

Glucocorticoids inhibit the transcription of most cytokines and chemokines that are relevant in asthma, including IL-1β, TNF-α, GM-CSF, IL-3, IL-4, IL-5, IL-6, IL-8, IL-11, IL-12, IL-13, RANTES, eotaxin, and macrophage inhibitory protein (MIP)-1α [46, 67]. In general, reduced synthesis of these mediators may result in a decreased recruitment and activation of leukocytes, also indirectly due to effects on adhesion molecules and cell survival. Since many cytokine gene promoters do not contain a negative GRE, the effects of glucocorticoids on cytokine and chemokine production are probably mediated via an effect on a critical transcription factor (especially NF-κB and AP-1) [68].

Bronchial epithelial cells are capable of producing a variety of cytokines and chemokines that may contribute to the initiation and perpetuation of airway inflammation. Several studies have shown that cytokine-induced expression of eotaxin, IL-6, IL-8, GM-CSF, and RANTES can be diminished by glucocorticoids *in vitro* [69-77]. In contrast, glucocorticoids did not modulate the secretion of G-CSF by human bronchial epithelial cells [77]. *In vivo* studies have shown that treatment with inhaled steroids decreases both the expression of GM-CSF [78], IL-8 [79], and RANTES [80] by the bronchial epithelium, together with the number of activated eosinophils in the epithelium.

Receptors

Glucocorticoids may modulate the expression of several receptors. The expression of the neurokinin $(NK)_1$ receptor, which mediates many effects of substance P (SP) in the airways and is believed to be up-regulated in asthma [81], is down-regulated by glucocorticoids [82]. Since the NK_1 receptor gene promoter region has no GRE but has an AP-1 response element, this effect probably will be mediated via an interaction of the glucocorticoid-GR complex with AP-1.

In contrast to NK_1 receptors, expression of the β_2-adrenergic receptor is increased by glucocorticoids [83]. Since the human β_2-adrenergic receptor gene contains three potential GRE, this effect of glucocorticoids probably is a direct one [83]. Upregulation of β_2-adrenergic receptors by glucocorticoids may be relevant in asthma as it may prevent down-regulation in response to prolonged treatment with β_2-agonists [84].

The IL-1 receptor type II, which functions as a decoy receptor [85], may also be up-regulated by glucocorticoids, thereby reducing the functional activity of IL-1 agonists [86, 87]. Soluble TNF-receptor type I (p55) release by human bronchial epithelial cells, both constitutive as well as IL-1β-induced, has been shown to be reduced by glucocorticoids [88]. In contrast, glucocorticoids up-regulate the expression of IL-6 receptors in rat hepatoma and human epithelial cells [89, 90]. Thus far little is known about this process in human bronchial epithelial cells, which constitutively express these receptors [91].

Glucocorticoids also modulate the expression of their own receptor. In a recent study it was shown that expression of the α-form (but not the β-form) of the GR in human bronchial epithelial cells was down-regulated in healthy subjects after 4 weeks of budesonide inhalation [24].

Enzymes

Glucocorticoids inhibit the synthesis of several inflammatory mediators implicated in the pathogenesis of asthma through an inhibitory effect on enzyme induction. The synthesis of inducible nitric oxide synthase (iNOS) by human airway epithelial cells is inhibited by glucocorticoids, both *in vitro* and *in vivo* [92-94]. This effect seems to be mediated via inactivation of NF-κB [95, 96]. Since nitric oxide (NO) may contribute to skewing of Th lympho-

cytes towards a Th2 phenotype, thereby promoting IgE production and eosinophil recruitment, inhibition of iNOS may be of importance in anti-inflammatory therapy in asthma [97].

Glucocorticoids also inhibit the gene transcription of a cytosolic form of phospholipase A_2 induced by cytokines [98] and inhibit the gene expression of cyclooxygenase-2, resulting in reduced formation of prostaglandins and thromboxanes [99].

In contrast to the enzymes mentioned above, glucocorticoids have been shown to increase the expression of neutral endopeptidase (NEP) [100, 101], thereby potentially limiting neurogenic inflammatory responses ([102]; see chapter 3). However, this glucocorticoid-mediated increase in NEP expression could not be confirmed in another study [103].

Adhesion molecules

Adhesion molecules play an important role in the recruitment of inflammatory cells to the inflammatory locus. Expression of adhesion molecules on endothelial, epithelial or inflammatory cells is often induced by cytokines, whereas glucocorticoids reduce surface expression of adhesion molecules. This effect may be due either to inhibition of cytokine synthesis or to a direct effect of glucocorticoids on adhesion molecule gene transcription. It has been shown that the expression of ICAM-1, endothelial leukocyte adhesion molecule (ELAM)-1, and E-selectin is down-regulated by steroids [104]. Basal and cytokine-stimulated ICAM-1 expression on human bronchial epithelial cell lines is inhibited by glucocorticoids [105, 106]. However, inhaled glucocorticoids did not modulate ICAM-1 expression by bronchial epithelial cells from asthmatics *in vivo* [107].

Eosinophil adhesion to cytokine-stimulated bronchial epithelial cells was shown to be inhibited by the synthetic glucocorticoid dexamethasone [108]. Although cytokine-activated epithelial cells showed increased expression of ICAM-1, this molecule did not seem to be involved in the decreased adhesion of eosinophils observed in the presence of dexamethasone [108].

Inhibitory proteins

The anti-inflammatory effects of glucocorticoids may be mediated by increasing the production of inhibitory proteins, such as lipocortins. Lipocortins are members of a superfamily of proteins characterized by their ability to bind calcium and anionic phospholipids, now known as the 'annexins' [109, 110]. In several cell types, but not all, glucocorticoids are inducers of lipocortins, which have an inhibitory effect on the activity of phospholipase A_2 [111, 112]. As a result, the synthesis of lipid mediators, including prostaglandins and eicosanoids, will be reduced. However, in human bronchial epithelial cells glucocorticoids do not seem to upregulate the expression of lipocortins [113]. Furthermore, no significant difference was found between lipocortin-1 concentration in BAL fluid from asthmatic patients receiving inhaled glucocorticoid therapy and those who were not treated with glucocorticoids [114].

Recently, glucocorticoids have also been shown to increase the expression of intracellular IL-1 receptor antagonist type I by human bronchial epithelial cells [115]. Increased production of this mediator may inhibit the effects of IL-1 agonists, thereby reducing inflammation.

To provide protection against potentially injurious agents, airway epithelial cells secrete a number of mediators, including antiproteases. Secretory leukocyte protease inhibitor (SLPI) is the predominant antiprotease in the airways. Its expression has been shown to be increased in airway epithelial cells after stimulation with glucocorticoids [116].

5.3. CELLULAR AND CLINICAL EFFECTS OF GLUCOCORTICOIDS IN ASTHMA

Several studies have determined the effects of inhaled glucocorticoids on bronchial inflammation, either by measurements in bronchoalveolar lavage (BAL) fluid, sputum, or exhaled air, or by performing bronchial biopsies. Although differences can be observed between different trials, these studies have confirmed that glucocorticoid treatment of asthmatic patients reduces the number and activation of inflammatory cells in the airways, together with an improvement of lung function. Nowadays, the potent anti-inflammatory actions of glucocorticoids are thought to underlie the clinical efficacy of oral glucocorticoids [117].

Effects of glucocorticoids on immunopathology

Inhaled glucocorticoids decrease the number and activation status of most inflammatory cells in the bronchus, including mast cells, dendritic cells, eosinophils, and T lymphocytes. Changes in cellular infiltration are accompanied by modulated expression of several cytokines. Inhaled glucocorticoids have been shown to decrease mRNA expression of GM-CSF, IL-13, IL-4, and IL-5, whereas mRNA levels of IL-12 and IFN-γ increased, suggesting a shift from a Th2- towards a more Th1-like environment [78, 118, 119].

Glucocorticoid treatment is associated with a reduction in *mast cell* numbers in the bronchus [79, 117, 120-123] and with reduced mast cell associated mediators in BAL fluid [123, 124]. This may be due to a reduction in IL-3 and stem cell factor production, which are necessary for the survival of mast cells in tissue. The (IgE-dependent) release of mediators from mast cells does not seem to be affected by glucocorticoid treatment [125, 126].

Dendritic cells play an important role in presenting antigens to (naive) T cells [127, 128]. Inhaled glucocorticoids have been shown to reduce the number of dendritic cells in the human bronchial epithelium [129].

Increased numbers of *eosinophils* are a prominent feature of asthmatic airways [130-136]. *In vitro* studies have shown that many eosinophil functions, including adherence and chemotaxis, are diminished following glucocorticoid treatment [126]. However, most data suggest that eosinophil responses to steroids are likely to be indirect, since eosinophil function is markedly affected by cytokines elaborated from T lymphocytes (IL-3, IL-4, IL-5, GM-CSF), endothelial cells (GM-CSF) and epithelial cells (GM-CSF) [137-141]. *In vivo* studies indicate that treatment with inhaled steroids reduces the number of eosinophils and eosinophil-related mediators in BAL fluid [79, 136, 142] and the number of (activated) eosinophils in bronchial biopsies [79, 117, 120, 121, 143]. Recently, induced sputum has been suggested as a useful tool for evaluating the effects of therapy on airway mucosal inflammation. Thus far, most studies have focussed on the presence of eosinophils and eosinophil-related mediators. In accordance with the findings in BAL fluid and bronchial biopsies, glucocorticoid treatment was associated with a reduction in sputum eosinophil numbers, eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO) [144].

Glucocorticoids also reduce the number of activated *T lymphocytes* in bronchial biopsies and BAL fluid [117, 121, 122, 143, 145]. In addition, inhaled corticosteroids reduced the number of cells expressing mRNA for IL-4 or IL-5, and increased the number of cells expressing mRNA for IFN-γ [119, 120], thereby favoring the development of Th1 cells [146].

In addition to the effects of glucocorticoids on *epithelial cells* described above, inhaled glucocorticoid therapy has been shown to reduce the shedding of epithelial cells [143, 147, 148]. No consistent effect of corticosteroids on the thickness of the basement membrane has been observed [79, 148, 149].

Besides the suppressive effects on inflammatory cells, inhaled glucocorticoids have also shown to inhibit mucus secretion and microvascular leakage (as determined by the down-regulation of plasma proteins in BAL fluid) [148, 150-154]. At present it is not clear whether this is mediated via a direct effect of glucocorticoids on endothelial or mucous cells, or via a reduction of inflammatory mediators that increase mucus secretion and vascular leakage.

Effects of glucocorticoids on lung function

Treatment with glucocorticoids has been consistently shown not only to reduce the symptoms of asthma, but also bronchial hyperresponsiveness [122, 155]. In contrast to the rapid inhibitory effects of β_2-agonists, glucocorticoids given in a *single* dose are not effective in preventing early allergen-invoked bronchoconstriction, but inhibition of the late response has been clearly demonstrated [156, 157]. In contrast, *chronic* treatment with either oral or inhaled steroids attenuates even the early bronchoconstriction to allergen [157-159], an effect that probably is mediated via the anti-inflammatory actions of glucocorticoids already described. Although inhaled glucocorticoids consistently reduce airway hyperreactivity in asthmatics [155], even after several months of treatment responsiveness fails to return to the normal range. This may reflect persistence of structural changes that cannot be reversed by steroids (such as the thickening of the basement membrane), despite of suppression of the inflammatory and immunological processes.

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ORIGINAL STUDIES

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Chapter 6

Aims of the studies

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The studies described in this thesis have focussed on three main topics: peptidases, the bronchial epithelium, and glucocorticoids. Our aim was to further define the contribution of peptidases and the bronchial epithelium to the inflammatory process characteristic for the asthmatic airways and to determine the anti-inflammatory effects of glucocorticoids on these processes. As pointed out in the first part of this thesis, several studies have demonstrated or suggested a role for peptidases and bronchial epithelial cells in the pathogenesis of asthma. However, several questions remain to be answered.

6.1. PEPTIDASES

Neurogenic inflammation mimics many of the pathophysiological features of asthma, and a role for neuropeptides in the pathogenesis of asthma has been implicated. Although the apparent upregulation of the sensory neuropeptide effects may be due to several mechanisms (see chapter 2.4), studies using laboratory animals have indicated that peptidases, especially neutral endopeptidase (NEP), play a major role in limiting neurogenic inflammatory responses. Therefore, we hypothesized that the expression and/or activity of peptidases is reduced in the airways of asthmatic patients, thereby contributing to neurogenic inflammation. Furthermore, we hypothesized that treatment with (inhaled) glucocorticoids increases the activity of peptidases in the human airways, thereby reducing neurogenic inflammation. Testing the first hypothesis can be subdivided into two phases: 1) the analysis of the expression/activity of peptidases in the human airways (both in bronchial tissue and in the bronchoalveolar lumen); and 2) the comparison of the expression/activity of peptidases between healthy subjects and asthmatic patients.

In *chapter 7* we investigate the distribution of two peptidases, aminopeptidase N (APN) and dipeptidyl peptidase IV (DPP IV), in human bronchial tissue and compared their distribution with the known distribution of NEP. Similar to NEP, APN and DPP IV are able to degrade a variety of inflammatory peptides and may therefore modulate inflammatory processes (see chapter 3). To investigate whether the expression of APN and DPP IV was altered in asthma, we determined the expression of APN and DPP IV in bronchial biopsies of asthmatic patients and compared this with the expression detected in bronchial biopsies of healthy controls.

Soluble peptidases have been found in blood, although their origin, fate, and function are still largely unknown. Increased levels of soluble peptidases have been found in blood samples of patients with pulmonary inflammation and it has been suggested that this may reflect local tissue damage (see chapter 3). In *chapter 8* we describe studies on the analysis of peptidase

activities (NEP, APN, and DPP IV) in serum and bronchoalveolar lavage (BAL) fluid, which may more properly reflect local changes. To determine whether asthma was associated with altered peptidase activities, we compared peptidase activities in serum and BAL fluid from healthy subjects and allergic asthmatics. Finally, to analyze whether glucocorticoids exert part of their anti-inflammatory actions via modulation of peptidase activities, we studied the effects of treatment with inhaled glucocorticoids on the activity of peptidases of asthmatic patients, again both in BAL fluid and in serum.

6.2. PEPTIDASES AND THE BRONCHIAL EPITHELIUM

Studies using laboratory animals have shown that NEP present on the bronchial epithelium plays a major role in the hydrolysis of neuropeptides and thereby in modulating neurogenic inflammation. It has been shown that NEP activity may be reduced by a variety of exogenous stimuli, like viral infections, ozone, and cigarette smoke (see chapter 3.6). In contrast, little is known about the modulation of NEP activity on bronchial epithelial cells by endogenously released mediators. Asthmatic airways are chronically inflamed and inflammatory mediators, such as cytokines, may affect peptidase expression and thereby modulate (neurogenic) inflammation. In *chapter 9* we aim to answer two questions: 1) what is the effect of cytokines on the activity and expression of peptidases on human bronchial epithelial cells; and 2) what is the effect of glucocorticoids on the activity and expression of these peptidases? Since it is hard to obtain large numbers of bronchial epithelial cells in primary culture, we used for this study the bronchial epithelial cell line BEAS 2B, which expresses NEP and APN. We first studied the effects of the cytokines interleukin (IL)-1β, IL-4, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and epidermal growth factor (EGF) on the peptidase activity and expression. These cytokines are abundantly present in the inflamed bronchus. Second, we studied the effects of glucocorticoids, which are widely used in the treatment of asthma, on the expression and activity of both peptidases. These studies were performed both in the absence and in the presence of cytokines, since the latter condition may more properly reflect the *in vivo* situation during glucocorticoid therapy.

6.3. THE BRONCHIAL EPITHELIUM

Until recently the bronchial epithelium was considered to be a passive barrier between the environment and the internal milieu of the lung. In addition to this barrier function, bronchial epithelial cells are now considered to play an essential role in initiating and perpetuating inflammatory reactions (chapter 4).

IL-4 is thought to be an important mediator in the development and perpetuation of allergic diseases like asthma (see chapter 1.4). Asthmatic airways show an increased number of cells expressing IL-4, predominantly Th2 lymphocytes and eosinophils, and increased levels of IL-4 can be found in BAL fluid from asthmatics compared to healthy controls. Therefore, we analyzed whether human bronchial epithelial cells express functional IL-4 receptors, both *in vivo* and *in vitro*, and whether this expression is altered in asthmatic patients. We first determined the expression of IL-4 receptor mRNA and protein in bronchial epithelial cells *in*

vivo (using *in situ* hybridization and immunohistochemistry) and *in vitro* (using RT-PCR and flowcytometry). Second, we studied the expression of IL-4 receptors in bronchial biopsies of allergic asthmatics and compared this with the expression in healthy subjects. To determine whether the IL-4R on human bronchial epithelial cells is also functionally active, we investigated whether stimulation of cultured human bronchial epithelial cells with IL-4 can modulate the release of the pro-inflammatory mediators IL-8 and monocyte chemotactic protein-1 (MCP-1) and the anti-inflammatory mediator IL-1 receptor antagonist. Finally, we studied whether IL-4 receptor expression on human bronchial epithelial cells *in vitro* can be modulated by cytokines (IL-1β, IL-6, IL-4) or pharmacological agents (dibutyryl-cAMP and phorbol myristate acetate). These studies are presented in *chapter 10.*

Accumulation of leukocytes in the lung is dependent upon the presence of chemokines and the expression of appropriate adhesion molecules. Bronchial epithelial cells are able to produce a variety of chemokines, and therefore may contribute to the increased numbers of leukocytes observed in the asthmatic airways (see chapter 4.3.1.). In addition, bronchial epithelial cells may express surface membrane molecules involved in the adhesion or activation of the recruited leukocytes, such as intercellular adhesion molecule (ICAM)-1 and the human leukocyte antigen (HLA) class II molecule. Modulation of chemokine release and surface membrane molecules may serve as an important mechanism to control the recruitment and activation of leukocytes. In *chapter 11* we describe studies regarding the release of MCP-1, the prototype C-C chemokine, and IL-8, the prototype C-X-C chemokine, by human bronchial epithelial cells. We investigated whether inflammatory agents, like cytokines (IL-1β, TNF-α, IFN-γ) and the bacterial cell wand product lipopolysaccharide (LPS), can modulate the release of these chemokines. In addition, we investigated whether stimulation of bronchial epithelial cells by these cytokines can also affect the expression of molecules involved in the adhesion and activation of the recruited leukocytes. Therefore, we analyzed the epithelial expression of ICAM-1, HLA class II, and the costimulatory molecule CD40, both in the absence and in the presence of cytokines. Finally, the effects of glucocorticoids on the cytokine-induced responses were analyzed to determine whether these drugs can exert part of their anti-inflammatory actions through inhibition of chemokine release or marker expression by human bronchial epithelial cells.

In *Chapter 12*, the experimental findings of chapters 7 through 11 are discussed in the context of the present literature. In this chapter also future research directions will be given.

Chapter 7

Expression of aminopeptidase N and dipeptidyl peptidase IV in the healthy and asthmatic bronchus

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ABSTRACT

BACKGROUND: Asthma is characterized by reversible airway obstruction, airway hyperresponsiveness, and chronic inflammation of the airways. Since peptides are able to produce many of the pathophysiological features characteristic of asthma, peptide-mediated inflammation is thought to play a role in this disease. The effects of peptides are modulated by peptidases, which are able to degrade peptides, mostly resulting in their inactivation.

OBJECTIVE: In this study, we investigated the distribution of two peptidases, aminopeptidase N and dipeptidyl peptidase IV, in the human bronchus and determined whether their expression was altered in allergic asthmatics.

METHODS: We first determined the distribution of aminopeptidase N and dipeptidyl peptidase IV in the human bronchus using immuno- and enzymehistochemistry and compared this with the distribution of neutral endopeptidase. Second, the expression of aminopeptidase N and dipeptidyl peptidase IV was determined in bronchial biopsies of healthy subjects (n=8) and allergic asthmatics (n=12).

RESULTS: Aminopeptidase N was localized in connective tissue, blood vessels, gland ducts, perichondrium, nerves and leukocytes (mainly mononuclear phagocytes, dendritic cells, and eosinophils). Dipeptidyl peptidase IV was localized in serosal glands, blood vessels, and T cells. Immunohistochemistry and enzymehistochemistry gave similar results. Comparison of the expression of aminopeptidase N and dipeptidyl peptidase IV in bronchial biopsies of healthy controls and atopic asthmatics revealed no significant differences in the lamina propria. In contrast, in the bronchial epithelium of atopic asthmatics an increased number of aminopeptidase N-positive cells could be found. Double-staining identified these cells as $L25^+$ dendritic cells and eosinophils.

CONCLUSION: We conclude that expression of aminopeptidase N and dipeptidyl peptidase IV is restricted to specific sites within the human bronchus. Furthermore, in the bronchial epithelium of allergic asthmatics an increased number of aminopeptidase N-expressing dendritic cells and eosinophils can be found.

INTRODUCTION

Asthma is clinically characterized by reversible airway obstruction and airway hyperresponsiveness [1]. Nowadays, it is thought that these symptoms result from a chronic inflammation of the airways, characterized by an influx of leukocytes and increased levels of inflammatory mediators [2]. This inflammation is caused, at least partially, by peptides like cytokines and neuropeptides. Degradation of peptides by peptidases is an important mechanism to modulate peptide-mediated inflammation. It has been demonstrated that inhibition of peptidases, either by drugs or by environmental factors such as ozone, results in potentiation of neuropeptide-induced effects in the airways [3-7]. In contrast, administration of an aerosolized recombinant peptidase prevented neuropeptide-mediated cough [8]. Based on these results, it is thought that peptidases also play an important role in the modulation of peptide-mediated inflammation in asthma (reviewed in [9]). Until now, most attention has been given to neutral endopeptidase (NEP, identical to CD10 [10]). In the human bronchus, this peptidase has been identified in the epithelium, smooth muscle, submucosal glands, and endothelium [11]. However, other membrane-bound peptidases, such as aminopeptidase N

(APN) and dipeptidyl peptidase IV (DPP IV) may also be involved in the modulation of peptide-mediated inflammation.

APN, which is identical to CD13 [12], preferentially cleaves neutral amino acids from the N-terminus of peptides, including enkephalins, fMLP, tachykinins, and cytokines like IL-1β, IL-2, IL-6 and IL-8 [13-15]. Its general function is to reduce cellular responses to peptides, but APN may also be involved in processing MHC-bound peptides [16] and in the degradation of type IV collagen [17]. APN is expressed on myeloid cells (granulocytes, monocytes and macrophages), in the intestinal and renal epithelium, endothelium, placenta, brain, kidney, breast, and liver (reviewed in [18]). On many cells, APN is co-expressed with NEP and it is thought that initial cleavage by NEP may precede APN activity.

DPP IV (which is identical to CD26 [19]), is a serine protease which preferentially cleaves Xaa-Pro and less frequently Xaa-Ala dipeptides from the NH_2-terminus of polypeptides [20]. Among the possible substrates for DPP IV are substance P (SP) and bradykinin [21, 22]. DPP IV may also be able to degrade cytokines, like IL-1β, IL-2 and IL-6, although preceding cleavage by an endopeptidase may be required [14]. In addition, DPP IV may also function as an adhesion molecule to fibronectin [23], as a co-receptor for HIV [24], and is involved in T-cell activation [25]. DPP IV is expressed by a variety of cell types, most abundantly in epithelia of the small intestine, liver and kidney [26, 27], but also by activated T-lymphocytes [28].

Given the known characteristics of APN and DPP IV, these two peptidases may be involved in the modulation of peptide-mediated inflammation in the airways. Therefore, our aims were to determine the expression and activity of APN and DPP IV in the human bronchus and to compare this distribution with the recently established distribution of NEP. In addition, the expression of APN and DPP IV in bronchial biopsies of healthy controls and atopic asthmatic patients was studied to determine whether APN and DPP IV are involved in bronchial inflammation.

MATERIALS AND METHODS

Patients and control subjects

Bronchial mucosal biopsy specimens were obtained from twelve non-smoking allergic asthmatic patients (9 men, 3 women, median age 32 years, range 20 - 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 87% of the predicted value (range 59 - 108%); the median of the ^{2}logs of the provocative concentrations of inhaled methacholine required to reduce their FEV_1 by 20% (PC_{20}) was 0.29 mg/ml (range -3.89 - 3.43 mg/ml). Allergy was defined by one or more positive skin-prick tests to extracts of 16 common aeroallergens. All patients were receiving inhaled β-agonists, and none had taken oral or inhaled corticosteroids in the month prior to the study.

A control group was composed of 8 non-allergic non-asthmatic subjects (5 men and 3 women, median age 24 years, range 23 - 52 years). All controls had a PC_{20} histamine of more than 8 mg/ml and a median FEV_1 of 100 (88 - 109)% of the predicted value. Characteristics of patients and controls are shown in Table 1. The study was approved by the local Ethics Committee and all participants gave their written informed consent.

Table1. Characteristics of patients and healthy subjects.

	Patient number	Sex	Age (years)	FEV_1 (% predicted)	$^2log\ PC_{20}$ (mg/ml)
Allergic asthmatics	1	M	51	59	1.47
	2	M	41	83	-1.83
	3	M	55	60	-1.80
	4	M	23	104	2.85
	5	M	56	79	1.93
	6	F	21	105	-3.42
	7	M	32	91	0.64
	8	F	20	91	-0.06
	9	M	43	67	3.43
	10	F	26	92	2.86
	11	M	47	81	-1.05
	12	M	23	108	-3.89
Healthy subjects	1	M	23	109	
	2	F	24	103	
	3	F	23	96	
	4	M	23	88	
	5	M	52	88	
	6	F	33	97	
	7	M	24	104	
	8	M	26	109	

Bronchial biopsy

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. 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.

Tissue

Samples of human bronchus were obtained from patients undergoing pneumonectomy or lobectomy. Tissue distinct from the tumor and having a normal appearance was embedded into Tissue-Tek, frozen in liquid nitrogen, and stored at -80°C.

As control tissues for the enzymehistochemical studies, murine kidney and placenta, and guinea-pig trachea were used. These tissues were treated in the same way as the human samples.

Antibodies

The following mouse monoclonal antibodies were used: CLB-CD13 (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands) and WM-47 (gift of dr. E. Favaloro, Westmead, Australia), both specific for APN; Ta-1 (Coulter Clone, Hialeah, FL, USA), specific for DPP IV [28]; L25 (kindly provided by Dr. T. Takami, Gifu, Japan), directed against B cells and dendritic cells (DC) [29]; OKT6 (American Type Culture Collection, Rockville, MA, USA), directed against the CD1a antigen of DC [30]; S100 (DAKOpatts,

Glostrup, Denmark) directed against DC; anti-CD3 (Leu-4), anti-CD4 (Leu-3) and anti-CD8 (Leu-2), all from Becton Dickinson (San Jose, CA, USA); anti-CD14 (My-4; Coulter Clone); anti-CD19 (B4; Coulter Clone); EG1, recognizing eosinophil cationic protein (ECP) in resting and activated eosinophils (Pharmacia, Uppsala, Sweden); BMK13, recognizing Major Basic Protein (MBP) in resting and activated eosinophils (Genzyme, Cambridge, MA, USA); and EG2, (Pharmacia) recognizing the cleaved form of ECP in activated eosinophils.

Immunohistochemistry

Sections (6 μm) were cut using a cryostat and collected on poly-L-lysine (Sigma, St. Louis, MO, USA) coated slides. The sections were air-dried for at least one hour, and stored at -20°C until use. Before immunohistological staining, frozen tissue sections were brought to room temperature and fixed in acetone for 10 minutes.

Immunohistochemical staining of bronchial biopsies was performed with the immuno-alkaline phosphatase anti-alkaline phosphatase (APAAP) method [31], using new fuchsin (Chroma-Gesellschaft, Stuttgart, Germany) as the chromogen. For tissue obtained after lung surgery, the avidin-biotincomplex (ABC) method (DAKOpatts) was performed with diaminobenzidine (DAB; Sigma) as substrate [32]. Staining of bronchial tissue with the APAAP or ABC method did not reveal significant differences in staining pattern and relative intensity.

Double-stainings were performed essentially as described earlier [33]. However, sections were fixed in acetone for 10 min and saponin was not added to the washing-buffer.

Enzymehistochemistry

Enzyme activities for APN, DPP IV and NEP were determined according to Lojda [34] with some small modifications. Briefly, frozen tissue sections were brought to room temperature and either fixed in Cafoma (1 g $CaCl_2$, 10 ml formalin, 90 ml macrodex (dextran 60 g/l + NaCl 9 g/l)) for one minute or used immediately. Similar results were obtained with both methods. Substrates used are ala-4-methoxynaphtylamide (ala-MNA; Sigma) for APN, gly-pro-MNA (Bachem, Bubendorf, Switzerland) for DPP IV, and glut-ala-ala-phe-MNA (Sigma) for NEP. Enzymehistochemistry using prolyl-MNA (pro-MNA; Sigma) was used as a control for the specificity of APN activity, since this substrate is resistant to cleavage by APN. Three milligrams of substrate were dissolved in 0.5 ml N,N-dimethylformamide (Merck, Darmstadt, Germany). For APN and DPP IV, this solution was mixed with 9.5 ml PBS containing 10 mg Fast Blue B salt (Sigma), and filtered. Sections were incubated in a moist chamber at room temperature for two hours, followed by a short wash in PBS, and embedded in Aquamountant (BDH Laboratory Supplies, Poole, UK).

For detection of NEP activity, a two-step reaction was used. Three milligrams of the substrate were dissolved in 0.5 ml N,N-dimethylformamide, mixed with 9.5 ml 50 mM TRIS-HCl pH 7.4 containing 10 mg Fast Blue B, and filtered. Subsequently, alanine-aminopeptidase (3 units; Sigma) was added. Alanine-aminopeptidase cleaves the phe-MNA bond after the initial cleavage of the ala-phe bond by NEP. To determine specific NEP activity, parallel sections were incubated with the same substrate, with the addition of phosphoramidon (Sigma), a specific NEP inhibitor (final concentration: 1 μM). Sections were incubated in a moist chamber at 37°C for 24 hours, washed shortly in 50 mM TRIS-HCl pH 7.4, and embedded in Aquamountant.

Sections were analysed immediately using a light microscope. Negative controls included: 1. omission of Fast Blue B in the incubation medium, 2. heating the sections at 90° C for 5 min in buffer prior to incubation, and 3. omission of the substrate in the incubation medium.

Since no counter-staining was used in the enzymehistological stainings, serial sections were stained using the periodic acid Schiff (PAS) method.

Quantification

Biopsies were coded and two sections were counted in a blinded fashion for each antibody and each biopsy at a magnification of 10x40. With an eye piece graticule the number 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 of cells per mm of basement membrane. Since the majority of APN in the lamina propria was expressed on permanent structures within the bronchus (*i.e.* not confined to infiltrating cells), APN expression in the lamina propria was scored semi-quantitatively on a 0 - 3 scale (0= negative; 1= weak; 2= moderate; 3= strong).

Statistical analysis

Median cell counts of biopsies of allergic asthmatics were compared with median cell counts of the control subjects using the Mann-Whitney *U*-test. Correlation coefficients were obtained by Pearson's rank method. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Distribution of aminopeptidase N

The distribution of APN in the human bronchus was investigated using WM-47 and CLB-CD13 antibodies. The two antibodies displayed identical reactivities in all tissues. As shown in Figure 1A/B and Table 2, APN was observed in connective tissue (especially just beneath the basement membrane of the bronchial epithelium and submucosal glands), secretory epithelium of bronchial glands, perichondrium, nerves, and endothelial cells. Some positively staining leukocytes, mainly in the lamina propria, could be observed. Using double-stainings with CD14, the majority of these cells was identified as mononuclear phagocytes. In addition, APN was expressed by eosinophils (double-staining with BMK13) and certain dendritic cells (double-staining with L25 but not with CD1a).

Using the enzymehistochemical staining for APN activity, a pattern similar to the immunohistochemical staining was obtained (Fig. 1C/D and Table 2). No activity could be observed using pro-MNA as a substrate (for incubation periods up to 48 hours; data not shown).

Distribution of dipeptidyl peptidase IV

DPP IV expression could be detected in submucosal glands and leukocytes (Fig. 2A/B and Table 2). In submucosal glands, DPP IV seemed to be located intracellularly. Blood vessels, in particular venules but also capillaries, expressed DPP IV weakly. To determine whether serosal or mucosal glands displayed DPP IV expression, serial sections were stained using the PAS-method. This revealed that DPP IV was only present in serosal glands. No staining of fibroblasts, bronchial epithelium and smooth muscle cells could be observed. Double-stainings with CD3 revealed that the majority of DPP IV-positive leukocytes were T cells.

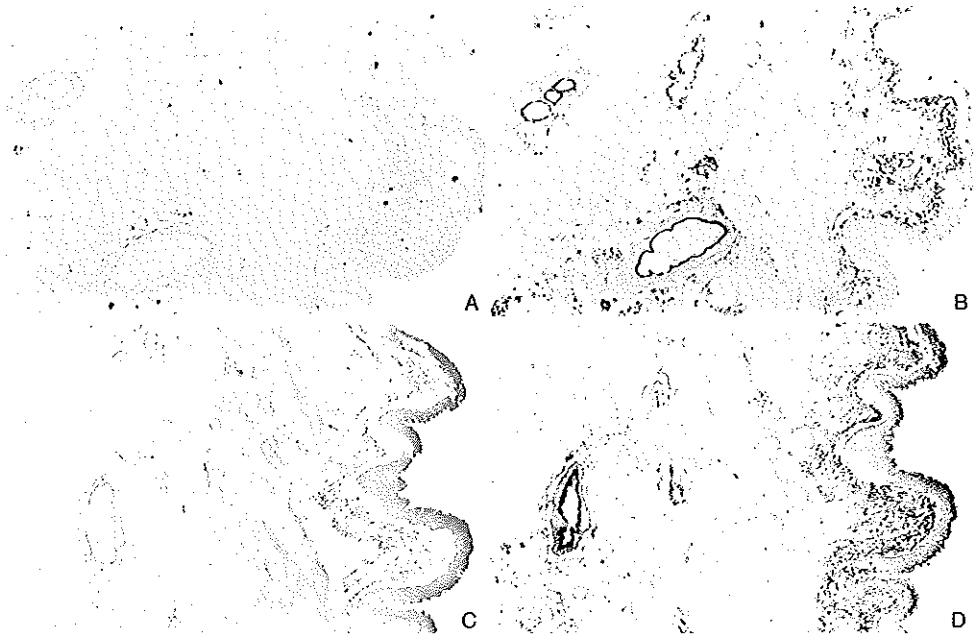

Figure 1. Distribution of APN in human bronchial tissue. Expression of APN was determined by immunohistochemistry (A and B) and enzymehistochemistry (C and D). A and C: negative control (omission of primary antibody or substrate, *respectively*); B: CLB-CD13, an antibody specific for APN; D: ala-MNA. Original magnification: 160 x.

Enzymehistochemistry revealed strong DPP IV activity in submucosal serosal glands, blood vessels, and leukocytes (Fig. 2C/D and Table 2).

Distribution of neutral endopeptidase

To compare the distribution of APN and DPP IV with NEP, the activity of NEP was determined using enzymehistochemistry. In the human bronchus, a very weak NEP activity was observed, but attribution of this activity to a certain cell type was difficult. Faint staining of the bronchial epithelium, submucosal glands, smooth muscle and blood vessels could be observed (Table 2). In all cases, no activity could be observed in the presence of phosphoramidon (1 μM), indicating that indeed NEP activity was measured. In contrast to the human bronchus, in the guinea-pig trachea NEP activity could easily be detected, especially within the epithelium (data not shown).

APN and DPP IV in bronchial biopsies of healthy controls and allergic asthmatics

Analysis of the type of cell infiltrate revealed no statistically significant difference in the numbers of T cells, B cells, monocytes, or dendritic cells between healthy subjects and allergic asthmatics. In contrast, the number of (activated) eosinophils in the lamina propria of allergic asthmatics (as determined by staining with EG1, EG2 and BMK13) was significantly increased compared to healthy subjects (Table 3).

No difference was observed in APN expression of the lamina propria of healthy subjects and allergic asthmatics (Fig 3B). In contrast, in the bronchial epithelium of allergic asthmatics an increased number of APN-positive cells could be observed compared to healthy controls (Fig. 3A). These APN-positive cells morphologically appeared to be infiltrating leukocytes rather

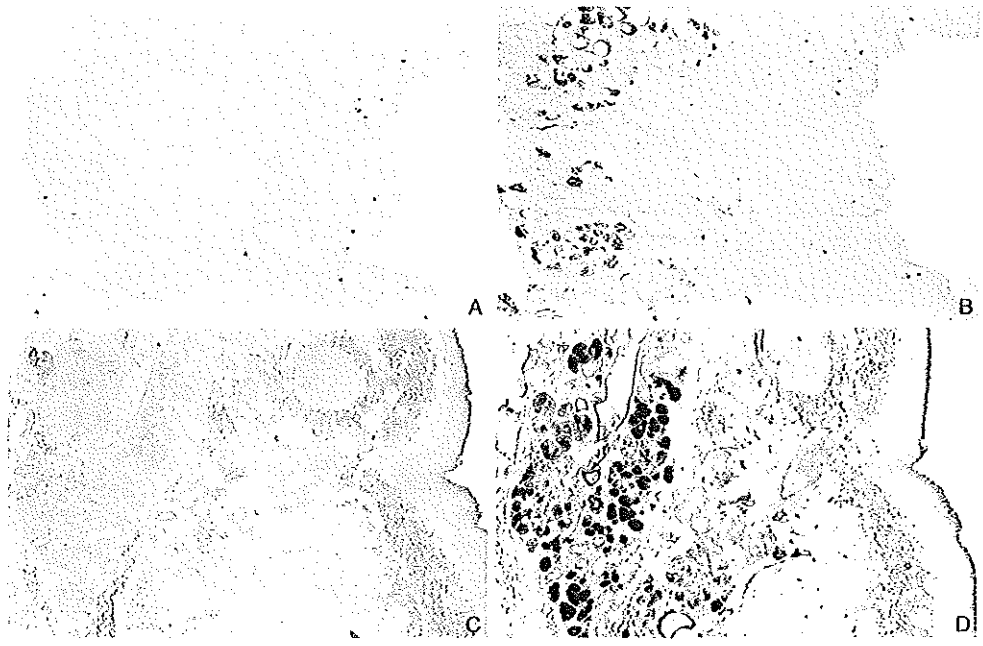

Figure 2. Localization of DPP IV in human bronchial tissue. Expression of DPP IV was determined by immunohistochemistry (A and B) and enzymehistochemistry (C and D). A and C: negative control (omission of primary antibody or substrate, *respectively*); B: Ta-1, an antibody specific for DPP IV; D: gly-pro-MNA. Original magnification: 63 x.

Table 2. Distribution of aminopeptidase N (APN), dipeptidyl peptidase IV (DPP IV), and neutral endopeptidase (NEP) in the human bronchus[1].

	APN	DPP IV	NEP
Bronchial epithelium	-	-	±
Smooth muscle	-	-	±
Connective tissue	++	-	-
Blood vessels	++	+/±[2]	±
Serosal/mucosal glands	-/-	++/-	±/±
Gland ducts	++	-	-
Nerves	+	-	-
Leukocytes[3]	+	+	+

[1] Staining intensity (as determined by immunohistochemistry and enzymehistochemistry) was arbitrarily graded as negative (-), weak (±), moderate (+), and intense (++).

[2] Staining intensity of venules > capillaries ≈ arteries.

[3] See text for details.

than bronchial epithelial cells themselves. In addition, weak but significant correlations were found between the APN score in the bronchial epithelium and the number of BMK13$^+$ eosinophils ($r_s = 0.582$; $p < 0.05$), EG2$^+$ eosinophils ($r_s = 0.569$; $p < 0.05$) or L25$^+$ dendritic cells ($r_s = 0.473$; $p < 0.05$) in the bronchial epithelium. Double-stainings using CLB-CD13 and L25 or BMK13

confirmed the presence of APN on L25$^+$ dendritic cells and eosinophils. Although L25 may also be present on B cells, the expression of APN seems to be restricted to L25$^+$ dendritic cells, since B cells (CD19$^+$) were hardly observed in the bronchial biopsies. In addition, double-staining with CD19 and CLB-CD13 (APN) revealed no double-positive cells.

The number of DPP IV-positive cells in the bronchial epithelium or lamina propria did not differ between healthy controls and allergic asthmatics (Fig. 4A and 4B).

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

Marker	Epithelium				Lamina propria			
	Controls		Asthmatics		Controls		Asthmatics	
CD3	11.0	(3.4-34.1)	13.8	(0.4-59.1)	33.8	(8.8-52.9)	16.2	(6.3-127.2)
CD4	3.2	(0.0-5.0)	0.0*	(0.0-1.1)	13.6	(4.2-38.5)	16.5	(1.3-37.6)
CD8	0.4	(0.0-9.5)	5.3	(0.0-18.2)	4.0	(0.0-18.1)	10.7	(0.0-18.2)
CD19	0.0	(0.0-0.0)	0.0	(0.0-0.7)	0.0	(0.0-2.7)	0.0	(0.0-4.5)
CD14	0.0	(0.0-1.7)	0.0	(0.0-0.0)	1.2	(0.0-3.8)	0.0	(0.0-1.6)
ECP	0.0	(0.0-0.0)	0.0	(0.0-1.0)	0.0	(0.0-0.0)	3.8*	(1.0-14.4)
MBP	0.0	(0.0-0.0)	0.0	(0.0-12.2)	1.7	(0.0-3.8)	4.5*	(1.7-16.0)
$ECP_{cleaved}$	0.0	(0.0-0.0)	0.0	(0.0-1.0)	0.0	(0.0-1.7)	1.9*	(0.0-29.3)
CD1a	0.0	(0.0-3.6)	0.9	(0.0-4.0)	0.0	(0.0-1.7)	0.9	(0.0-5.4)
L25	0.7	(0.0-3.2)	0.6	(0.0-5.8)	1.7	(0.0-9.0)	4.0	(0.0-13.8)
S100	0.0	(0.0-0.0)	0.0	(0.0-2.0)	0.4	(0.0-2.1)	1.2	(0.4-5.2)

* $p < 0.05$ compared to controls.

DISCUSSION

In this study, we show that APN and DPP IV are expressed at specific and distinct sites within the human bronchus. APN was localized in connective tissue, blood vessels, gland ducts, perichondrium, nerves and leukocytes (mainly mononuclear phagocytes, dendritic cells, and eosinophils). DPP IV was localized in serosal glands, blood vessels, and T cells. Comparison of the expression of both peptidases in bronchial biopsies of healthy controls and atopic asthmatics revealed a significantly increased number of APN-positive cells in the bronchial epithelium of atopic asthmatics. These cells were identified as L25$^+$ dendritic cells and eosinophils.

Asthma is characterized by reversible airway obstruction, airway hyperresponsiveness and chronic inflammation of the airways, characterized by an influx of leukocytes and increased levels of inflammatory mediators [1, 2]. *In vitro* and animal studies have shown that peptides, especially neuropeptides, are able to produce many of the pathophysiological features characteristic of asthma [35]. Therefore, it is thought that (neuro)peptides play an important role in the pathogenesis of asthma. The effects of bio-active peptides are modulated by peptidases. Most attention has been given to NEP and several studies have indicated that NEP plays an important role in the modulation of peptide-mediated effects, like neurogenic inflammation [3-7]. In addition to NEP, other peptidases, such as APN and DPP IV, may be involved in the modulation of peptide-mediated inflammation. However, at present little is known about their function and distribution within the human bronchus. Therefore, we determined the distribution

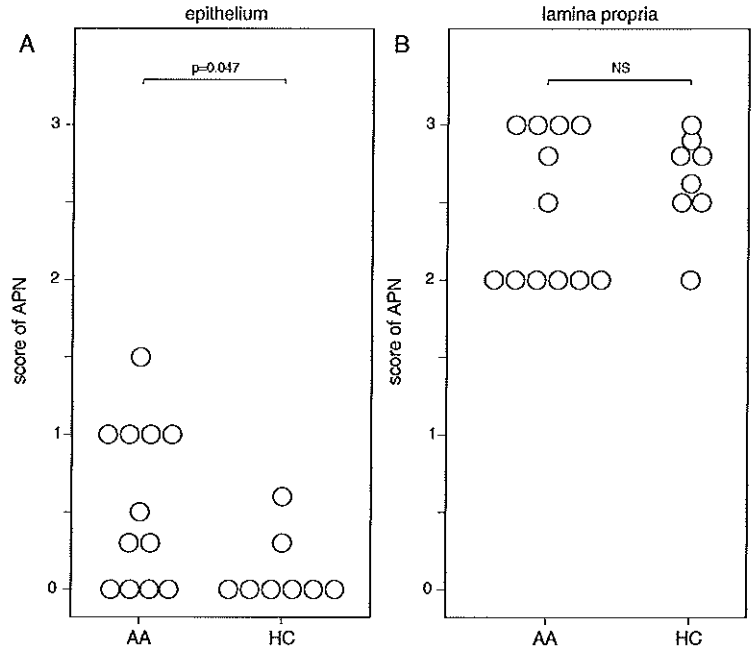

Figure 3. APN expression in bronchial biopies obtained from healthy controls and alergic asthmatics. Individual cell counts for cells expressing APN in the bronchial epithelium (A) and individual score of APN expression in the lamina propria (B) of bronchial biopsies obtained from healthy controls (HC) and allergic asthmatics (AA). NS: not significant.

of APN and DPP IV within the healthy human bronchus and investigated whether their expression was modified in asthmatic airways.

APN expression and activity was observed in connective tissue just beneath the basement membrane of the bronchial epithelium and submucosal glands. At these sites, APN is in a perfect location to degrade neuropeptides released by sensory nerves, since many of these nerves end beneath the bronchial epithelium [36]. APN, but also DPP IV, was present on blood vessels indicating that it may be involved in the processing of intravascular peptides, such as SP. APN expression could be observed in arteries, capillaries and venules, whereas aminopeptidase A activity (as determined by the cleavage of glut-MNA) was confined to capillaries (data not shown) and DPP IV was mainly present in venules. The latter location is of interest, since SP-induced plasma leakage occurs in these postcapillary venules [37]. We speculate that DPP IV expressed on these post-capillary venules is involved in the regulation of SP-induced plasma leakage and that the site-restricted presence of different peptidases in blood vessels may represent a mechanism to control blood flow and plasma leakage at specific locations. However, the exact physiological function of these peptidases is still unknown and needs further study.

Comparison of the expression of APN in bronchial biopsies of healthy controls and allergic asthmatics revealed a significantly increased number of APN-positive cells in the bronchial epithelium of allergic asthmatics. In accordance to the known distribution of APN among cells of myeloid origin [38], these cells were shown to be dendritic cells and eosinophils.

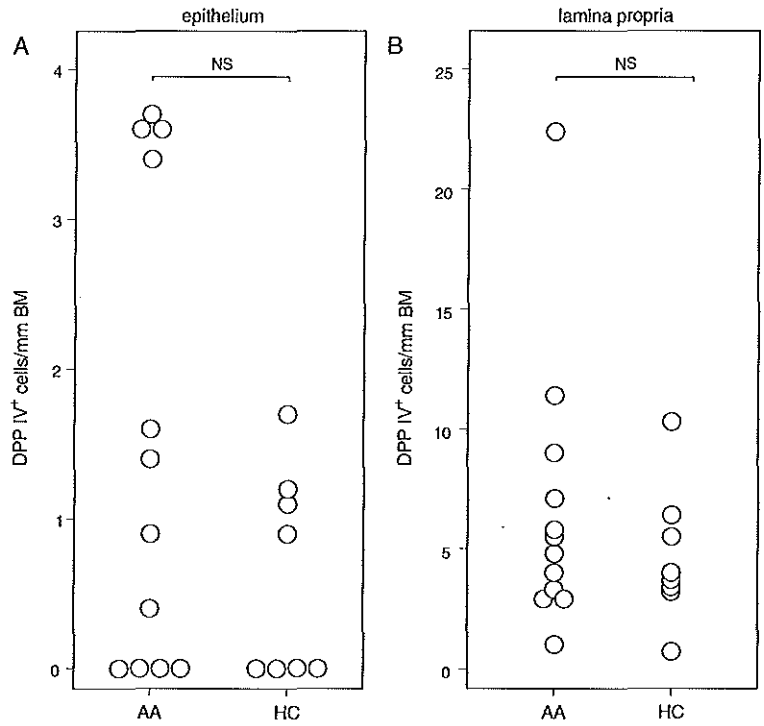

Figure 4. DPP IV expression in bronchial biopsies obtained from healthy controls and allergic asthmatics. Individual cell counts for cells expressing DPP IV in the bronchial epithelium (A) and lamina propria (B) of bronchial biopsies obtained from healthy controls (HC) and allergic asthmatics (AA). NS: not significant.

Other studies have shown an increased number of dendritic cells in bronchial biopsies of patients with asthma [33] and during inflammatory responses in the rat lung [39]. Furthermore, recent studies have indicated a role for APN in processing MHC-bound peptides [40]. Recruitment of APN-positive dendritic cells may therefore serve as a mechanism to effectively take up and process foreign antigens. However, since in our study the number of dendritic cells and eosinophils in the bronchial epithelium did not differ significantly between healthy subjects and allergic asthmatics, it can not be excluded that the increase in the number of APN-positive cells within the bronchial epithelium of allergic asthmatics is due to an upregulation or induction of APN on the surface of these cells. In previous studies, we have shown that IL-4 is able to upregulate APN expression on mononuclear phagocytes [41]. Although we did not observe a difference in the number of IL-4-positive cells in bronchial biopsies of allergic asthmatics compared to healthy controls (data not shown), other reports have indicated that IL-4 may be increased in asthma [42, 43]. Therefore, it may be possible that the increase in the number of APN-positive cells is a result of increased APN expression due to elevated IL-4 production in asthmatic airways.

Recruitment of APN-positive cells in the bronchus of asthmatics may also result in a more rapid degradation of bronchodilating peptides, like vasoactive intesinal peptide and peptide histidine methionine, released by nonadrenergic nerves [44]. This may result in exaggerated bronchial responsiveness, thereby contributing to the pathology of asthma [45].

The APN-score in the lamina propria of allergic asthmatics did not differ significantly from healthy subjects. However, since the majority of APN was expressed on permanent structures (*e.g.* connective tissue, glandular ducts, endothelium) in the bronchus, it was not possible to

determine the expression of APN quantitatively. Therefore, changes in the number of APN-positive leukocytes within the lamina propria will be hard to detect.

DPP IV activity and expression were strongly present in serosal glands and seemed to be located intracellularly. This may indicate that glandular DPP IV is not involved in the modulation of peptide-mediated effects on glandular cells, but rather is a product of these cells. This suggests that DPP IV may be secreted into the epithelial lining fluid, thereby being able to degrade intraluminal peptides, such as substance P and bradykinin [21, 22]. Indeed, DPP IV activity can be detected in bronchoalveolar lavage fluid (data not shown).

In patients with chronic obstructive pulmonary diseases (COPD), a submucosal gland hypertrophy has been observed [46]. This may result in an increased DPP IV activity, and thus in an increased (neuro)peptide-degradation in the lumen of the bronchus. To our knowledge, no data concerning a role for DPP IV in degrading peptides in the human lung *in vivo* or a role for DPP IV in COPD are available yet.

In the lamina propria of the bronchus, T cells appeared to be a major site for DPP IV activity. Comparison of healthy subjects and allergic asthmatics did not reveal significant differences in DPP IV expression, indicating that the number of activated T cells was not changed. A limitation of our study is that the bronchial biopsies hardly contained submucosal glands, one of the most prominent locations for DPP IV.

The expression of NEP protein and mRNA in the human bronchus has been described recently [11]. In that study, NEP could be found in the bronchial epithelium, smooth muscle, submucosal glands, and endothelium. In our study, we used enzymehistochemistry to detect NEP activity in the human bronchus. Although NEP activity in the guinea-pig trachea could easily be detected, NEP activity in the human bronchus was low and attribution of NEP activity to a certain cell type was difficult. Nevertheless, weak activity could be observed within the bronchial epithelium and submucosal glands. Comparison of the distribution of NEP, APN and DPP IV indicates that these peptidases are localized at specific and often distinct sites within the human bronchus which are also known to possess receptors for many peptide mediators. This colocalization suggests that the cellular response to a peptide can be modulated by peptidases on the surface of the same cell.

In conclusion, peptidases are widely distributed in the human bronchus. The peptidases studied have a distinct distribution, with APN expressed by blood vessels, nerves, gland ducts, perichondrium, connective tissue and leukocytes (mononuclear phagocytes, eosinophils, dendritic cells), and DPP IV predominantly expressed by submucosal glands, blood vessels and T cells. In bronchial biopsies of allergic asthmatics an increased number of APN-positive cells (mainly dendritic cells and eosinophils) can be found in the bronchial epithelium, whereas no differences are apparant for DPP IV. The distribution and characteristics of APN and DPP IV suggest that these peptidases are involved in the modulation of peptide-mediated inflammatory reactions in the human bronchus.

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Chapter 8

Peptidase activities in serum and bronchoalveolar lavage fluid from healthy non-smokers, smokers, and allergic asthmatics

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ABSTRACT

Neuropeptides may be involved in the pathogenesis of asthma by evoking neurogenic inflammation. The effects of neuropeptides are limited by peptidases. We investigated peptidase activities in bronchoalveolar lavage (BAL) fluid and serum of healthy non-smokers, smokers, and allergic asthmatics, and studied the effect of inhaled glucocorticoids on peptidase activities in the asthmatic patients.

Serum and BAL fluid was obtained from non-smoking and smoking volunteers and from allergic asthmatics both before and after treatment with placebo or inhaled fluticasone propionate. Activities of neutral endopeptidase (NEP), aminopeptidase N (APN) and dipeptidyl peptidase IV (DPP IV) were determined using colorometric assays.

Reduced DPP IV activity in serum and reduced NEP and APN activities in BAL fluid were found in healthy smokers compared to non-smokers. In allergic asthmatics, reduced APN activity was observed in BAL fluid compared to healthy non-smokers. Fluticasone propionate did not affect peptidase activities in the asthmatic patients.

We conclude that reduced peptidase activities in serum or BAL fluid can be found in healthy smokers and allergic asthmatics and that inhaled glucocorticoids do not affect peptidase activities in BAL fluid or serum of asthmatics. Reduced peptidase activities may result in impaired degradation of neuropeptides and thereby contribute to the inflammatory process.

INTRODUCTION

Neuropeptides like substance P (SP) and neurokinin A (NKA) have been demonstrated in sensory airway nerves of animals and man and are thought to be neurotransmitters of local axon reflexes [1-3]. Activation of sensory nerves may occur after exposure to a variety of stimuli, such as bradykinin, viral infections, and cigarette-smoke. This activation results in the release of neuropeptides, which subsequently exert a variety of effects, including the contraction of smooth muscle cells, secretion of mucus, vasodilation, increased microvascular leak, and the recruitment and activation of leukocytes. This sequence of events is now known as 'neurogenic inflammation' [4]. Since neurogenic inflammation mimics many of the pathophysiological features of asthma, neuropeptides have been implicated in the pathogenesis of this disease. Several studies have shown that asthmatic airways are more responsive to neuropeptides [5] and increased amounts of SP can be detected in BAL fluid of allergic asthmatics [6] and in serum during asthmatic exacerbations [7].

The effects of neuropeptides are normally limited by rapid degradation by peptidases [4, 8]. Thus far, most peptidase studies have focussed on the role of NEP, which, in the human lung, is expressed on the bronchial epithelium, submucosal glands, smooth muscle cells, endothelial cells, and alveolar epithelial cells [9-11]. It has been demonstrated that inhibition of NEP, either by drugs or by environmental factors such as ozone, results in increased responses to exogenously applied or endogenously released peptides [8]. In contrast, neuropeptide-mediated cough can be prevented by administration of an aerosolized recombinant peptidase [12]. Based on these results, it has been hypothesized that peptidases play an important role in the modulation of peptide-mediated inflammation in asthma.

In addition to NEP, other peptidases may be involved in modulating peptide-mediated effects in the human airways. APN preferentially cleaves neutral amino acids from the N-terminus of peptides, including enkephalins, fMLP, tachykinins, and cytokines like interleukin (IL)-8. APN is widely distributed in the human lung, being present on endothelial cells, glandular ducts, fibroblasts, and alveolar epithelial cells [11, 13]. DPP IV is a serine protease cleaving peptides like SP and bradykinin. In the human lung, DPP IV is mainly present on serosal submucosal glands and endothelial cells, but can also be found on activated T lymphocytes [11, 14].

Although peptidases are normally membrane-bound enzymes, soluble forms can be detected in body fluids. These soluble counterparts may either be derived from shedding of membrane-bound peptidases or may be formed by post-translational cleavage of the membrane-bound form [15]. NEP activity in serum probably arises from shedding of the entire membrane-bound peptidase [16]. Increased serum activity of NEP has been observed in underground miners exposed to coal dust particles [16] and in patients with adult respiratory distress syndrome (ARDS) [17] or sarcoidosis [18]. Although the source of the increased NEP levels remains to be determined, it has been suggested that increased NEP levels may reflect local tissue damage with subsequent shedding of membrane-bound NEP [16, 17]. Alternatively, NEP might be released from activated granulocytes sequestered in the lung and leak into the bloodstream [17, 19]. DPP IV activity in serum has recently been shown to originate, at least in part, from the DPPL-T antigen expressed on the surface of activated T cells [20], whereas serum APN activity predominantly comprises a circulating isoform of the CD13 antigen [21]. There is evidence that serum DPP IV activity is decreased in patients with malignancies and in auto-immune and inflammatory disorders [22-26]. Thus far, little is known about the presence of NEP, APN, and DPP IV in BAL fluid and the activities of these peptidases in serum and BAL fluid of subjects with airway inflammation. In asthma, peptidases may act as central modulators of neurogenic inflammation and may therefore serve as an important therapeutical target to control asthmatic symptoms. Additionally, one of the working mechanisms of glucocorticoids, which are widely used in the treatment of asthma, may be upregulation of peptidase activity [27-30]. However, to our knowledge no data are currently available on the effects of inhaled glucocorticoids on peptidase activities in serum and BAL fluid.

In this study, we aimed to investigate the activity of NEP, APN, and DPP IV in serum and BAL fluid from healthy non-smokers, smokers, and allergic asthmatics. We also studied whether treatment with inhaled glucocorticoids could alter the activity of these peptidases.

MATERIALS AND METHODS

Patient characteristics

Thirty-one allergic patients (8 women, 23 men; Table 1), all non-smokers, participated in this study. The diagnosis of asthma was based upon a history of attacks of breathlessness and wheezing without chronic (i.e. for more than 3 months per year) cough or sputum production, according to the criteria of the American Thoracic Society [31]. Reversible airway obstruction was defined by an increase in forced expiratory volume in one second (FEV_1) of $\geq 9\%$ after inhalation of 1000 μg terbutaline. Allergy was defined by one or more positive skin prick tests to extracts of 16 common aeroallergens. All patients were receiving inhaled

β_2-agonists, and none had taken oral or inhaled glucocorticoids in the month prior to the study. At entry of the study, all patients showed airway hyperresponsiveness defined as a 20% decrease in FEV_1 caused by inhalation of a histamine concentration (PC_{20}) of less than 8 mg/ml. After a run-in period of two weeks, patients were treated double-blind with the inhaled glucocorticoid fluticasone propionate (500 µg twice daily; n=15) or placebo (n=16) for 12 weeks [32]. Venous blood samples and BAL were taken before and after this treatment period and at both visits a methacholine dose-response curve was determined. Patient characteristics before and after treatment are shown in Table 1. The study protocol was approved by the Medical Ethics Committee of the Erasmus University/University Hospital Dijkzigt, Rotterdam, and all participants gave their written informed consent.

Healthy subject characteristics

Nineteen healthy subjects (7 women, 12 men; 10 non-smokers, 9 smokers; Table 1), who denied symptoms of pulmonary diseases and did not use any steroidal or nonsteroidal anti-inflammatory drugs, participated in this study. All controls had a PC_{20} of more than 8 mg/ml. Venous blood samples were collected and BAL was performed as described below. In addition, venous blood samples were collected from 11 other healthy non-smoking subjects (3 women, 8 men; median age 29 years, range 22 – 53 years).

Bronchoalveolar lavage

BAL was performed after premedication with inhaled terbutaline (2 puffs of 250 µg via Nebuhaler) and atropine 0.5 mg intramuscularly. The nose, throat and vocal cords were anaesthetized with topical lidocaine spray (2% w/v). The bronchoscope (Olympus BI IT 10, Tokyo, Japan) was placed in wedge position in the middle lobe, and four aliquots of 50 ml sterile phosphate-buffered saline (PBS) solution were infused and aspirated immediately in a siliconized specimen trap placed on melting ice. Immediately after collection, the BAL fluid was strained through a sterile nylon gauze to trap large mucous particles. Subsequently, the BAL cells were separated from the fluid by centrifugation at 4°C and 400 g for 5 min. Cytocentrifuge preparations were prepared and stored at -80°C until use. Differential cell counts (analyzing at least 500 cells) were done after May-Grünwald Giemsa staining. Supernatants were stored at -80°C until biochemical analysis.

Blood samples

Venous blood samples were collected in heparinized tubes and clotting tubes. Blood was either allowed to clot at room temperature, after which the serum was separated by centrifugation, or used for differential cell counts (after May-Grünwald Giemsa staining and by counting at least 500 cells). Previous experiments indicated that clotting time (30 min - 24 h) did not affect peptidase activities in serum (data not shown). Serum was aliquoted and stored at -80°C until use.

Neutral endopeptidase activity

NEP activity was determined in a two-step reaction using the substrate succinyl-alanyl-alanyl-phenylalanyl-para-nitro-anilide (Suc-Ala-Ala-Phe-*p*NA; Sigma, St. Louis, MO). One hundred and fifty µl BAL fluid or 100 µl 10-fold diluted serum (in saline) was incubated with Suc-Ala-Ala-Phe-*p*NA (final concentration: 4 mM in TRIS-HCl pH 7.4) and 1 µg aminopeptidase (Sigma), in the presence or absence of phosphoramidon (final concentration: 1 µM; Sigma). The reaction (total volume: 250 µl for BAL, 200 µl for sera) was performed

Table 1. Characteristics of healthy non-smokers, smokers and allergic asthmatics.

	Patient Number	Sex	Age	Before treatment		After treatment	
				FEV_1 (% predicted)	^{2}log PC_{20} (mg/ml)	FEV_1 (% predicted)	^{2}log PC_{20} (mg/ml)
Healthy non-smokers	1	M	19	102			
	2	M	24	100			
	3	M	21	99			
	4	M	23	109			
	5	M	23	109			
	6	F	24	108			
	7	F	23	96			
	8	M	23	88			
	9	M	52	88			
	10	F	35	110			
Healthy smokers	11	M	20	104			
	12	M	18	124			
	13	F	33	97			
	14	M	26	109			
	15	F	45	118			
	16	M	24	104			
	17	F	21	91			
	18	M	23	110			
	19	F	27	89			
Allergic asthmatics							
Placebo	20	F	44	80	-0.67	81	0.58
	21	M	52	63	-0.33	59	1.47
	22	M	41	87	-1.62	83	-1.83
	23	M	55	60	-1.80	62	-2.34
	24	F	17	89	-3.44	93	-1.87
	25	M	55	72	3.57	72	1.06
	26	M	26	95	0.74	88	1.45
	27	M	22	92	-0.52	87	-1.53
	28	M	56	84	3.4	79	1.93
	29	M	25	86	2.57	76	3.21
	30	F	21	105	-3.42	106	-1.80
	31	F	26	97	-2.56	93	-0.58
	32	M	24	47	0.04	21	1.19
	33	M	32	91	0.64	102	1.41
	34	F	26	95	7.98	92	2.86
	35	M	23	108	-3.89	85	-1.10
Fluticasone propionate	36	F	25	90	0.77	115	5.50
	37	M	51	84	4.12	96	4.99
	38	M	21	93	2.40	98	> 8
	39	M	24	90	-0.91	93	3.4
	40	M	22	78	0.12	84	2.06
	41	M	24	100	-0.99	86	4.02
	42	M	23	63	-0.04	81	2.53
	43	M	23	104	2.85	108	6.72
	44	M	21	99	-5.18	103	0.15
	45	M	42	60	0.25	64	-0.18
	46	M	20	61	-0.68	90	1.18
	47	F	17	104	-0.41	107	1.23
	48	F	20	91	-0.06	91	2.08
	49	M	43	67	3.43	63	> 8
	50	M	47	81	-1.05	87	6.85

in duplo in a 96-well microtiter plate at 37°C. The increase in specific absorbance at 405 nm (as a result of the accumulation of free *p*-nitroanilide) was determined using a Titertek Multiskan MCC plate reader (I.C.N. Biomedicals B.V., Amsterdam, The Netherlands). NEP activity was determined as the activity that could be inhibited by phosphoramidon and is expressed as nmoles/mg protein/min.

Aminopeptidase N(-like) activity

APN-like (APL) activity was determined by incubating 150 µl BAL fluid or 50 µl 10-fold diluted serum with alanyl-para-nitro-anilide (ala-*p*NA; 4 mM; Sigma) at 37°C and measuring the increase in specific absorbance at 405 nm. To determine specific APN activity, BAL fluid or diluted serum was first incubated with the CD13 monoclonal antibody WM-15 (Pharmingen, San Diego, CA), which specifically inhibits the enzymatic activity of APN [33]. As a control, BAL fluid or serum was incubated with PBS, an isotype-matched control antibody, or WM-47 (an antibody that binds to APN but does not inhibit the enzymatic activity; generous gift of dr E. Favaloro, Westmead, Australia). After incubation at room temperature for 15 min, ala-*p*NA (final concentration: 4 mM) was added (total volume: 300 µl for BAL, 200 µl for sera) and the increase in absorbance at 405 nm was measured. APL and APN activity are expressed as nmoles/mg protein/min.

Dipeptidyl peptidase IV activity

DPP IV activity was determined by incubating 150 µl BAL fluid or 100 µl 10-fold diluted serum with glycyl-prolyl-para-nitro-anilide (gly-pro-*p*NA; 4 mM; Sigma) at 37°C and measuring the increase in specific absorbance at 405 nm. The reaction (total volume: 250 µl for BAL, 200 µl for sera) was performed in duplo in a 96-well microtiter plate. DPP IV activity is expressed as nmoles/mg protein/min.

Protein analysis

Total protein levels in serum and BAL fluid were determined according to Bradford [34]. Albumin levels were determined by routine biochemical assessment.

Statistical analysis

Data are presented as mean and SEM. For comparisons between groups, an Analysis of Variance (ANOVA) was used where multiple conditions were compared. Variables for which significant differences were found or significant trends observed were also analyzed by simple comparisons between groups using unpaired Student's *t*-test. Data which were statistically significant with unpaired *t*-tests were also significant in a Mann-Whitney *U*-test. The effects of treatment in the asthmatic patients were analyzed using the paired Student's *t*-test. Relationships between parameters were examined by the Spearman Rank Correlation Coefficient. Statistical significance was taken as $p < 0.05$.

RESULTS

Study 1. Comparison between healthy non-smokers, smokers, and allergic asthmatics

Cellular composition of blood samples

Total protein levels in serum and total white blood cell numbers in blood samples did not differ significantly between healthy non-smokers, smokers and allergic asthmatics (Table 2). The percentage of eosinophils in blood samples was significantly increased in allergic asthmatics compared with healthy non-smokers, whereas relative numbers of other cell types did not differ between the three groups (Table 2).

Table 2. Mean (± SEM) total white blood cell count, relative cell numbers, and protein content of serum samples of healthy non-smokers, smokers, and allergic asthmatics.

	Non-smokers	Smokers	Allergic asthmatics
Total cell number (10^9/l)	5.2 ± 0.2	6.2 ± 0.5	5.6 ± 0.2
Lymphocytes (%)	35 ± 2	34 ± 3	33 ± 1
Monocytes (%)	7 ± 1	7 ± 1	7 ± 0
Neutrophils (%)	56 ± 3	55 ± 2	54 ± 2
Eosinophils (%)	2 ± 1	3 ± 1	5 ± 1 †
Basophils (%)	1 ± 0	1 ± 0	1 ± 0
Total protein (mg/ml)	53.1 ± 0.9	52.8 ± 2.0	50.3 ± 0.8

†: $p<0.05$ compared to healthy non-smokers

Peptidase activities in serum

NEP, APL and APN activity did not differ significantly between healthy non-smokers, smokers, and allergic asthmatics (Fig. 1). In contrast, DPP IV activity was significantly decreased in serum of smokers, compared to both healthy non-smokers and allergic asthmatics (Fig. 1).

Cellular composition of BAL fluid

Percentage recovery of BAL fluid did not differ significantly between healthy non-smokers, smokers and allergic asthmatics (Table 3). In BAL fluid of smokers, a significant increase in total cell numbers was found, but the cellular composition did not differ compared to healthy non-smokers (Table 3). In contrast, total cell numbers in BAL fluid of allergic asthmatics tended to be reduced compared to healthy subjects (p=0.051). Furthermore, BAL fluid of asthmatic patients showed increased relative numbers of eosinophils and lymphocytes, whereas the relative number of macrophages was reduced (Table 3). However, the absolute numbers of lymphocytes, eosinophils and macrophages did not differ between healthy non-smokers and allergic asthmatics (data not shown). BAL fluid of smokers showed a reduced percentage of epithelial cells compared to allergic asthmatics, but the absolute numbers did not differ. Total protein and albumin levels in BAL fluid did not differ significantly between the three groups studied (Table 3).

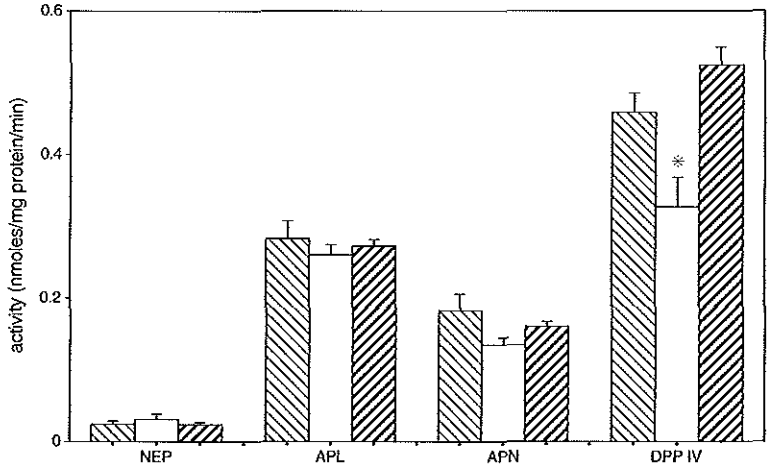

Figure 1. NEP, APL, APN, and DPP IV activity in serum. Peptidase activities were measured in serum from healthy non-smokers (shaded bars), smokers (white bars), and allergic asthmatics (heavily shaded). *: $p < 0.05$ compared to healthy non-smokers.

Table 3. Recovery, total white blood cell count and relative cell numbers in BAL fluid of healthy non-smokers, smokers, and allergic asthmatics (mean ± SEM).

	Non-smokers	Smokers	Allergic asthmatics
Recovery (%)	68 ± 2	57 ± 6	56 ± 3
Total cell number (10^6)	16.0 ± 2.2	39.2 ± 6.1 †*	11.2 ± 1.1
Lymphocytes (%)	4.5 ± 1.3	4.1 ± 1.0	11.6 ± 1.3 †‡
Macrophages (%)	90.1 ± 2.5	92.6 ± 1.4	80.3 ± 1.4 †‡
Neutrophils (%)	1.9 ± 1.2	2.1 ± 0.6	2.2 ± 0.6
Eosinophils (%)	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.2 †
Epithelial cells (%)	3.5 ± 1.4	1.2 ± 0.5 *	5.0 ± 0.9
Total protein (µg/ml)	63 ± 8	103 ± 16	97 ± 10
Albumin (µg/ml)	23 ± 5	30 ± 6	36 ± 5

†: $p<0.05$ compared to healthy non-smokers; ‡: $p<0.05$ compared to smokers; *: $p<0.05$ compared to allergic asthmatics

Peptidase activities in BAL fluid

All four peptidase activities were significantly higher in BAL fluid than in serum (Fig. 1 and 2). The BAL fluid:serum ratios of the peptidase activities in healthy non-smokers were 62.7 (NEP), 5.3 (APL), 5.1 (APN), and 2.3 (DPP IV).

Comparison of DPP IV activity in BAL fluid of healthy non-smokers, smokers, and allergic asthmatics did not reveal significant differences (Fig. 2). NEP activity (either expressed per ml or per mg protein) was significantly reduced in BAL fluid of smokers (Fig. 2). APL activity (expressed in nmoles/mg protein/min) in BAL fluid of smokers and allergic asthmatics was reduced compared to healthy non-smokers, but these differences did not reach statistical significance (p=0.070 for both comparisons). These reductions were completely due to a decreased APN activity in BAL fluid of both smokers and allergic asthmatics ($p<0.05$;

Fig.2). However, APL and APN activity per ml BAL fluid were not significantly different between the three groups (data not shown).

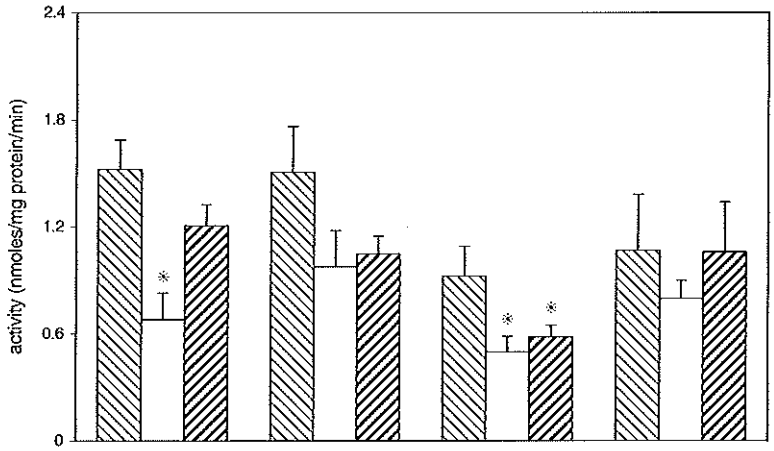

Figure 2. NEP, APL, APN, and DPP IV activity in BAL fluid. Peptidase activities were measured in BAL fluid from healthy non-smokers (shaded bars), smokers (white bars), and allergic asthmatics (heavily shaded). *: $p < 0.05$ compared to healthy non-smokers.

Correlations between peptidase activities and cell numbers

NEP activity in BAL fluid showed a strong correlation with APL and APN activity (Fig. 3A and C). These correlations were not due to lack of specificity of the assays since phosphoramidon did not affect APN activity, and NEP activity could not be inhibited by the CD13 monoclonal antibody WM-15 (data not shown). NEP activity in serum also correlated with serum APL activity ($r_s = 0.3466$; $p < 0.01$) and APN activity ($r_s = 0.2798$; $p < 0.05$), although these correlations were less clear. APL activity correlated significantly with APN activity, both in BAL fluid and in serum (Fig. 3B and D). There were no significant correlations between peptidase activities in BAL fluid and serum. Furthermore, there were no significant correlations between peptidase activities in BAL fluid or serum and relative or absolute cell numbers (data not shown).

Study 2. Effect of inhaled fluticasone propionate on peptidase activities in BAL fluid and serum of allergic asthmatics

Clinical parameters and cellular composition of blood samples and BAL fluid

Patients receiving inhaled fluticasone propionate for three months showed improved lung function as determined by an increase in FEV_1 and PC_{20} values (Table 1). No improvement was observed in the patients receiving placebo.

BAL fluid recovery, cell numbers, total protein levels, and albumin levels did not differ before and after treatment with either fluticasone propionate or placebo (data not shown). Relative numbers of lymphocytes in BAL fluid were increased after treatment with fluticasone

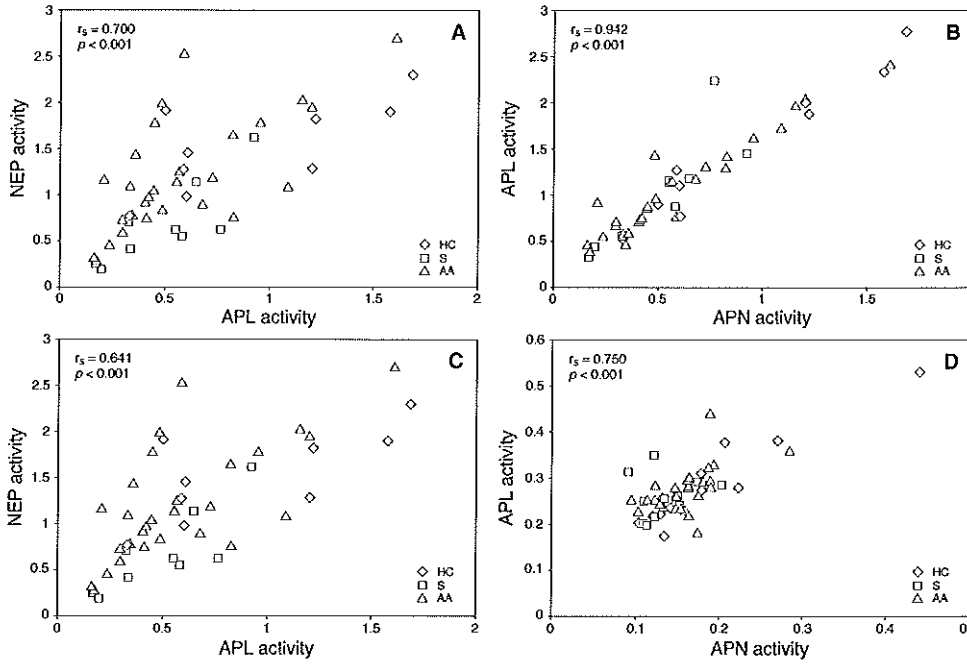

Figure 3. Relationship between peptidase activities in BAL fluid (A, B, C) and serum (D). Diamonds: healthy non-smokers (HC); Squares: smokers (S); Triangles: allergic asthmatics (AA). Activities are expressed as nmoles/mg protein/min.

propionate (before: 11.0 ± 2.0%. after: 16.9 ± 3.1%), whereas other cell numbers were unchanged. Total leukocyte numbers in blood samples were significantly increased after treatment with fluticasone propionate (before: 5.4 ± 0.3, after: 6.7 ± 0.5). This was accompanied by a reduction in the relative number of eosinophils (before: 6.1 ± 0.8%, after: 3.5 ± 0.4%) and an increase in the relative numbers of neutrophils (before: 53.8 ± 1.8%, after: 58.9 ± 1.9%). No differences in total and relative cell counts were observed in BAL fluid or blood samples of allergic patients treated with placebo (data not shown).

Peptidase activities in serum and BAL fluid

Treatment with inhaled fluticasone propionate for three months did not significantly affect peptidase activities in serum (Fig. 4). Peptidase activities in BAL fluid were also not affected by fluticasone propionate treatment (Fig. 5). However, in contrast to the reduced APN activity in BAL fluid observed before treatment of asthmatics, APN activity after treatment with either placebo or fluticasone propionate did not differ significantly compared to healthy controls (data not shown). In the asthmatic patients treated with placebo, no significant differences in peptidase activities in serum or BAL fluid were observed (data not shown).

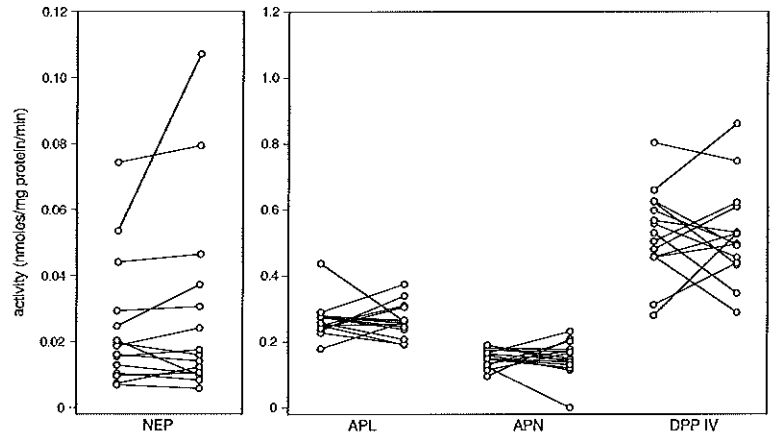

Figure 4. Effect of fluticasone propionate on peptidase activities in serum. Allergic asthmatics were treated for 12 weeks with inhaled fluticasone propionate. Before and after this period peptidase activities were analyzed in serum.

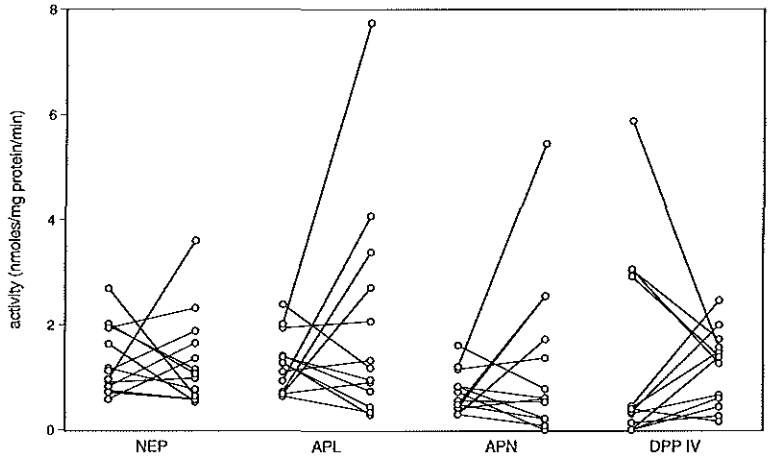

Figure 5. Effect of fluticasone propionate on peptidase activities in BAL fluid. Allergic asthmatics were treated for 12 weeks with inhaled fluticasone propionate. Before and after this period peptidase activities were analyzed in serum.

DISCUSSION

In this study, we present data on the activity of peptidases in BAL fluid and serum of healthy non-smokers, smokers, and allergic asthmatics, and show for the first time that NEP activity can be detected in human BAL fluid. Our results indicate that DPP IV activity is significantly reduced in serum of smokers, whereas NEP and APN activity are reduced in BAL fluid as compared with healthy non-smokers. In allergic asthmatics, the activity of APN was reduced in BAL fluid as compared with healthy non-smokers. Treatment of allergic

asthmatics with inhaled glucocorticoids for three months improved their FEV_1 and PC_{20} values but did not affect peptidase activities in serum or BAL fluid.

Comparison of serum and BAL fluid revealed that activities (expressed as nmoles/mg protein/min) of all peptidases studied were considerably higher in BAL fluid and that there was no correlation between peptidase activities in BAL fluid and serum. These findings suggest that the presence of peptidases in these two compartments is regulated independently of each other and suggest local release of the enzymes in the airways. Although alveolar macrophages [35] and granulocytes [36] express NEP, we could not detect any correlation between NEP activity and neutrophil or macrophage numbers in BAL fluid. Therefore, NEP activity in BAL fluid most likely results from shedding of NEP from epithelial cells [16, 37]. This is supported by the observation that NEP activity can be detected in culture supernatants of human bronchial epithelial cells (V.H.J. van der Velden, unpublished data). DPP IV activity in BAL may, comparable to DPP IV activity in serum, be released from activated CD4-positive T lymphocytes [20, 38, 39], but numbers of these cells in normal BAL fluid are low. Alternatively, DPP IV may be secreted by serosal submucosal glands, as these are major sites for DPP IV activity in the human bronchus [11], or may be released by alveolar macrophages [40]. DPP IV activity on alveolar macrophages is, however, much lower than on activated T cells (V.H.J. van der Velden, unpublished data). APN activity in serum predominantly comprised an isoform of CD13, since the inhibitory monoclonal antibody WM-15 inhibited the majority (65%) of APL activity in serum. This is in accordance with the results described by Favaloro and colleagues [21]. In BAL fluid, CD13 activity comprised more than 60% of the APL activity and there was a strong correlation between APL and APN activity. This suggests that in the human lung, the release of both activities is regulated in a similar manner. In the human lung, APN may be shed from granulocytes, dendritic cells or macrophages [11, 39]. However, we and others [39] did not observe a significant relationship between cell numbers and APN(-like) activity in BAL fluid, suggesting that APN(-like) activity may rather be derived from non-hematopoietic cells in the airways. Since APN (-like) activity also showed a strong relation with NEP activity in BAL fluid, both enzymes may be derived from the same source, possibly alveolar epithelial cells which express both NEP and APN [10, 13].

Cigarette smoke has been shown to inhibit NEP activity in laboratory animals. This effect is thought to be due to oxydation of the enzyme by hydroxyl radicals [41-43]. Our study shows that in humans, cigarette smoke reduces NEP activity in BAL fluid. Since NEP modulates the growth and differentiation of bronchial epithelial cells by hydrolyzing bombesin-like peptides (BLP), reduced NEP activity may promote BLP-mediated proliferation and facilitate the development of small-cell carcinomas of the lung [44-46]. In accordance to our observation, increased levels of BLP have been found in the lower respiratory tract of asymptomatic smokers [47]. Furthermore, recent studies indicate that human lung cancers show low or absent NEP activity [45]. We hypothesize that cigarette smoke facilitates the development of small-cell carcinomas of the lung at least in part by inhibiting NEP and APN activity. Further studies need to be performed to demonstrate that cell surface NEP activity in humans is also inhibited by cigarette smoke and to prove that the reduced NEP activity is due to inactivation of the enzyme rather than decreased presence of the peptidase itself.

DPP IV activity in serum of smokers was significantly decreased compared to both healthy non-smokers and allergic asthmatics. In BAL fluid, a comparable reduction in DPP IV activity was observed, but this did not reach statistical significance. DPP IV activity in serum or BAL fluid may have an important immunoregulatory function, as it is able to act as a costimu-

lating molecule for T lymphocytes [20, 38, 48]. Reduced DPP IV activity in serum of smokers may therefore contribute to the down-regulated immune responsiveness observed in smokers [49]. DPP IV may also interfere with the processing of cytokines, such as IL-1β, IL-2, and IL-6, which have an essential role in the proliferation or activation of helper T cells and B cells [50].

In BAL fluid from stable allergic asthmatic patients, no significant difference in NEP activity was observed compared to healthy subjects. However, APN activity was significantly reduced in BAL fluid of asthmatics. The lack of reduced NEP activity is in accordance with the observation that thiorphan (an inhibitor of NEP) reduced NKA-induced bronchoconstriction in asthmatics, suggesting the presence of endogenous NEP activity [51]. Down-regulation of NEP activity in asthma may be prevented by the apparent chronically enhanced release of neuropeptides providing increased amounts of substrate for NEP upregulation [6, 42]. In addition, our previous studies have shown that IL-1β and TNF-α, which are abundantly present in the inflamed asthmatic airways, are able to increase the activity of NEP on human bronchial epithelial cells [27]. In contrast to NEP, APN activity per mg protein was significantly reduced in allergic asthmatics (and smokers) compared to healthy subjects, but APN activity per ml BAL fluid was not changed. This reduction appears therefore to be due to the elevated total protein levels in the BAL fluid of allergic asthmatics and not to a reduction in the amount of APN itself.

Several studies have shown an increased NEP activity in serum from patients with pulmonary inflammatory diseases, such as sarcoidosis and ARDS [17, 18]. Increased enzyme activities in serum may reflect local tissue damage with subsequent shedding of membrane-bound enzymes [16]. In this study, we did not observe significant increase in NEP or APN activities in serum from patients with stable allergic asthma, indicating that there probably is little acute tissue damage in the lungs. One could speculate that NEP and APN activities in serum could be altered during or shortly after acute asthmatic exacerbations. However, preliminary results indicate that peptidase activities in serum during and up to five days after exacerbations do not differ from those in healthy subjects (V.H.J. van der Velden, unpublished data).

Treatment of allergic asthmatic patients with inhaled fluticasone propionate for three months resulted in an improvement of lung function but did not significantly affect peptidase activities in BAL fluid or serum. In contrast, several studies have shown that glucocorticoids upregulate the surface expression of peptidases on human bronchial epithelial cells, both *in vivo* [28] and *in vitro* [27, 29, 30]. Thus, glucocorticoids may exert part of their anti-inflammatory actions by increasing the surface expression of peptidases. However, they do not alter soluble peptidase activities in BAL fluid or serum of stable allergic asthmatics. Finally, fluticasone propionate treatment resulted in an increase in total white blood cells in blood, indicating systemic effects of inhaled glucocorticoids.

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Chapter 9

Cytokines and glucocorticoids modulate human bronchial epithelial cell peptidases

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ABSTRACT

Peptidases play an important role in the regulation of peptide-mediated effects. Modulation of peptidase activity may therefore be a major mechanism to control peptide actions. Our aim was to analyse the effects of cytokines and glucocorticoids on peptidases expressed by human bronchial epithelial cells, which have been shown to be an important site for peptidase activity.

The effects of cytokines (IL-1β, TNF-α, IL-4, IFN-γ, and EGF) and/or dexamethasone (DEX) on both expression and activity of neutral endopeptidase (NEP) and aminopeptidase N (APN) by BEAS 2B cells were determined using flow cytometry and activity assays, respectively.

IL-1β and, to a lesser extent, TNF-α and IL-4 increased NEP activity and expression, whereas IFN-γ decreased NEP. The effect of IL-1β was mediated, at least in part, via a cAMP-dependent pathway which did not involve prostaglandin E_2 synthesis. APN was increased after 24 h stimulation with IFN-γ, whereas other stimuli had no effect. DEX strongly increased NEP and APN expression and activity, both in the absence and in the presence of cytokines.

We conclude that cytokines and glucocorticoids are able to modulate the activity of NEP and APN on BEAS 2B cells. Our results suggest a role for the human bronchial epithelium in the control of inflammation and indicate that one beneficial effect of glucocorticoids on asthma may be upregulation of peptidases expressed by bronchial epithelial cells.

INTRODUCTION

The bronchial epithelium is considered to play an important role in the regulation of inflammatory and immunological reactions in the airways. Bronchial epithelial cells are able to produce a variety of pro-inflammatory mediators, like cytokines, chemokines and arachidonic acid metabolites [1]. Release of such mediators may result in the initiation and perpetuation of inflammation. In contrast, bronchial epithelial cells may down-regulate inflammatory and immunological responses by the release of anti-inflammatory mediators, like interleukin (IL)-1 receptor antagonist [2], soluble tumor necrosis factor (TNF)-receptor [3], and lipocortins [4], and by inactivation of pro-inflammatory peptides by epithelial cell-bound peptidases [5]. Although peptidases are present on a number of cell types within the lung, several studies have indicated that neutral endopeptidase (NEP, E.C.3.4.24.11) expressed by the bronchial epithelium plays a major role in limiting peptide-mediated inflammation [6, 7].

NEP (identical to common acute lymphoblastic leukemia antigen (CALLA) or CD10 [8]) is a membrane-bound metalloenzyme which cleaves peptide-bonds at the amino side of hydrophobic amino acids, thereby being able to inactivate a variety of small peptides, including substance P, neurokinins, bradykinin, endothelin, and bombesin-like peptides [9]. In the human lung, NEP is expressed in the bronchial epithelium, but can also be found in smooth muscle, endothelium, and submucosal glands [5]. Loss of NEP activity, for example as a result of viral infection, has been shown to prolong the actions of neuropeptides released by sensory nerves, thereby resulting in neurogenic inflammation [10]. This neurogenic inflammation is characterized by mucus secretion, cough, vasodilation, increased vascular permeability, infiltration of leukocytes, and bronchoconstriction [10], findings that are comparable with the pathophysiological features characteristic of asthma. Therefore, it has been implicated that peptidases play a role in the pathogenesis of asthma.

Recent studies indicate that even small changes in NEP activity affect peptide-mediated events in the human lung. The activity of NEP may be modulated by external and internal factors. With regard to external factors, several studies have shown that NEP activity is reduced after exposure to viruses [11], cigarette smoke [12], ozone [13] or chemicals, such as toluene-2,4-diisocyanate [14]. In each case, this reduction resulted in increased responses to exogenously applied or endogenously released peptides. In contrast, little is known about the effect of endogenously released mediators, such as cytokines, on the activity of NEP. Cytokines are increasingly recognized to be important in chronic inflammation and play a critical role in orchestrating inflammatory responses. Multiple cytokines, including IL-1β, TNF-α, IL-4, interferon (IFN)-γ, and epidermal growth factor (EGF), are present during inflammatory responses in the lung [15] and may control peptide actions by modulating the activity of peptidases.

Glucocorticoids are widely used in the treatment of asthma and are able to reduce inflammatory reactions in the airways. The bronchial epithelium is an actual target for inhaled glucocorticoid therapy, since the greater part of inhaled glucocorticoids precipitate on the epithelia of the larger airways [16], and bronchial epithelial cells possess functional glucocorticoid receptors [17]. Glucocorticoids are potent inhibitors of cytokine production by a variety of cells, thereby suppressing inflammatory responses. In addition, the anti-inflammatory actions of glucocorticoids may be mediated by modulation of peptidase activity by bronchial epithelial cells. Data concerning the effects of glucocorticoids on NEP activity are contradictory, since some studies indicate that NEP is upregulated by steroids [18, 19], whereas other investigators did not observe any effect [20]. Furthermore, little is known about the modulation of peptidase activity by glucocorticoids in the presence of cytokines, which may more properly reflect the *in vivo* situation during glucocorticoid therapy.

In this study we investigated the effects of cytokines, glucocorticoids, and their combination, on the expression and activity of peptidases by the human bronchial epithelial cell line BEAS 2B. In comparison with the data on primary cultures of human bronchial epithelial cells, the BEAS 2B cell line has been shown to be an appropriate model [21, 22]. In addition to NEP, BEAS 2B cells also express aminopeptidase N (APN, identical to CD13, E.C.3.4.11.2 [23]). Comparable to the role of NEP, it is hypothesized that APN plays an important role in modulating the activity of bioactive peptides [24]. Accordingly, APN may play a role in the regulation of inflammatory and immunological responses. Although human bronchial epithelial cells *in vivo* normally do not express this peptidase [25], they do have APN-like activity (unpublished observations). Therefore, we also investigated the effects of cytokines and glucocorticoids on APN activity and expression to clarify whether the expression and activity of this peptidase was regulated in a way similar to NEP.

MATERIALS AND METHODS

Cytokines, steroids and chemicals

Cytokines used in this study were IL-1β (10 U/ng, UBI, Lake Placid, NY), TNF-α (5 U/ng, UBI), IL-4 (5 U/ng, UBI), and IFN-γ (20 U/ng, Boehringer Ingelheim, Germany). A stock solution (10 μg/ml) of EGF (Collaborative Research Inc., Lexington, MA) was prepared in 10 mM phosphate-buffered saline, pH 7.4 (PBS)/0.1% bovine serum albumin (BSA) and stored at -20°C.

A 10 mM stock solution of the synthetic glucocorticoid dexamethasone micronisatum (DEX; Duchefa b.v., Haarlem, The Netherlands) was prepared in ethanol and stored at -20°C. The glucocorticoid antagonist RU 38486 was kindly provided by Roussel Uclaf (Romainville, France). A stock solution of 10^{-2} M RU 38486 in ethanol was prepared and stored at 4°C. The metabolically stable testosteron analogue R1881 (10^{-3} M in ethanol) was kindly provided by dr. H. Bruggenwirth (Rotterdam, The Netherlands) and stored at 4°C. The concentration of ethanol during culture was less than 0.05% in each experiment.

Dibutyryl-cyclic adenosine monophosphate (db-cAMP) and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma (St. Louis, MO). Solutions were freshly made in milli Q-filtered water before each experiment.

Cell culture

The SV-40 transformed human bronchial epithelial cell line BEAS 2B was kindly provided by dr. J. Lechner (Inhalation Toxicology Research Institute, Albuquerque, NM) [26]. The cells were cultured in a 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 (DMEM/F12)(Gibco Ltd., Paisley, Scotland), supplemented with insulin (0.01 mg/ml; Sigma), hydrocortisone (0.1 µg/ml; Pharma Chemie, Haarlem, The Netherlands), transferrin (0.01 mg/ml; Behring, Marburg, Germany), EGF (10 ng/ml), fetal calf serum (FCS; Gibco) (1%), Na_2SeO_3 (50 nM), glutamine (1 mM; JT Baker, Deventer, The Netherlands), penicillin G sodium (100 U/ml; Gist-Brocades, Delft, The Netherlands) and streptomycin sulfate (0.1 mg/ml; Biochrom KG, Berlin, Germany) (complete medium). Plastic cell culture plates (Becton Dickinson, Plymouth, UK and Nunclon, Roskilde, Denmark) were precoated as described by Lechner et al. with a mixture of human fibronectin (10 µg/ml; Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands), collagen (Vitrogen 100, 30 µg/ml; Collagen Corp., Palo Alto, CA) and BSA (10 µg/ml; Boehringer, Mannheim, Germany) in PBS [27]. Medium was replaced three times weekly and cultures were passaged when the monolayers were 80 to 90% confluent. Passages 16 through 34 were used in this study.

The human promyelocytic leukemic cell line HL60 [28] and T-leukemia cell line DND41 [29] served as controls for the detection of peptidase expression and activity. Under the conditions used, the HL60 cell line is NEP^- APN^+, whereas DND41 cells are NEP^+ APN^-.

Stimulation of cells with cytokines, glucocorticoids and/or other agents

To ensure similar cell densities in the different assays, cells were seeded in fixed numbers per cm^2 (20×10^3 cells/cm^2). Using these cell numbers, cells reached ~90% confluency during the standard time of the experiments (5 days). After 24 hours, the complete medium was replaced by a basal medium consisting of DMEM/F12 (1:1) supplemented with 1% FCS and antibiotics. After 24 hours, cytokines, glucocorticoids and/or other agents were added to the medium in the following doses (unless indicated otherwise): IL-1β, TNF-α, and IL-4: 20 ng/ml; IFN-γ and EGF: 5 ng/ml; DEX 10^{-6} M; db-cAMP: 1 mM; IBMX: 100 µM. These doses were widely shown to be effective *in vitro*. If the effect of IBMX was to be studied, this inhibitor was added 30 minutes prior to cytokine administration. After the addition of the stimuli the culture was continued for an additional 24 or 48 hours, unless indicated otherwise.

Proliferation assay

The number of viable cells in proliferation was determined using a colorimetric method following the instructions of the manufacturer (CellTiter 96™ AQ_{ueous} Non-Radioactive Cell Proliferation Assay, Promega). Briefly, cells (5×10^3) were seeded in a coated 96-well plate and

stimulated as described above. Cell proliferation was determined by the addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) and the increase in absorbance at 490 nm was measured after 30 min.

Detection of surface peptidase expression

For immunofluorescence staining of human bronchial epithelial cells, the following monoclonal antibodies (mAbs) were used: J5 (Coulter Clone, Hialeah, FL), BMA-CALLA (Behring), OKB-CALLA (Ortho Diagnostic Systems, Raritan, NJ), VIL-A1 (dr. W. Knapp, Vienna, Austria), all directed against NEP; CLB-CD13 (CLB, Amsterdam, The Netherlands) and WM-15 (generous gift of dr. E. Favaloro, Westmead, Australia), both directed against APN; and BBA-4 (anti-ICAM-1, ITK Diagnostics, Uithoorn, The Netherlands). Epithelial cells, cultured in 80 cm^2 culture flasks, were rinsed with PBS and detached using 0.02% EDTA. Cells were harvested in PBS/0.5% BSA, washed and resuspended in PBS/BSA to a final concentration of 2 x 10^6 cells/ml. Fifty µl of this bronchial epithelial cell suspension was incubated with 50 µl of one of the optimally titrated mAb at 4°C for 30 min. Irrelevant mouse isotype-matched primary antibodies were used as a control for nonspecific binding. After two washings with PBS/BSA the cells were incubated with FITC-labelled rabbit-anti-mouse antibody for 30 min at 4 °C. After another two washings the cell pellets were resuspended for analysis of the fluorescence intensity by means of a FACScan (Becton Dickinson, San Jose, CA).

Fluorescence intensities were quantified using calibrated fluorescence standards (FCSC Quantum 26, Research Triangle Park, NC) which were measured in each experiment. Using the standard curve obtained by plotting the median fluorescence intensity of the standards against the Molecules of Equivalent Soluble Fluorescence (MESF) for each peak, the MESF of the membrane antigens was calculated. After subtraction of the MESF of cells incubated with isotype-matched control antibody, the antigen-specific MESF was obtained.

Neutral endopeptidase activity

NEP activity was measured as previously described with some small modifications [30]. BEAS 2B cells, cultured in 6-well culture dishes, were rinsed with 50 mM Tris-HCl pH 7.4. A volume of 600 µl of N-dansyl-D-alanyl-glycyl-*p*-nitro-phenylalanyl-glycine (DAGNPG, a synthetic NEP substrate; 25 µM in Tris-HCl; Sigma) was added to each well. The dishes were incubated at 37°C for 2 hours. Subsequently, a 500 µl aliquot was transferred to a microcentrifuge tube, 500 µl DMSO were added, and the mixture was spun in an Eppendorf microcentrifuge at 15,000 rpm for 5 min. The fluorescence of the supernatant was measured in a Perkin-Elmer spectrophotofluorometer (type LS50B) with excitation at 329 nm and emission at 531 nm. These wave lengths appeared to be optimal under the conditions used. A standard curve was measured in each experiment to determine the amount of product formed. In some experiments, the specificity of the reaction was confirmed by the addition of the NEP inhibitor phosphoramidon (dissolved in Tris-HCl; final concentration: 1 µM; Sigma). All assays were performed in duplo at least. After measuring NEP activity, the cells were trypsinized and counted using a hemacytometer (Coulter). NEP activity was calculated as pmoles/min/10^6 cells.

Aminopeptidase N-like activity

APN-like activity was determined on adherent cells in 6-well culture dishes. After rinsing the BEAS 2B cells with PBS (pH 7.4), 600 µl L-alanine-*p*-nitroanilide (an APN substrate; 8 mM in PBS; Sigma) were added and the dishes were incubated at 37 °C for 30 min. Subsequently,

the reaction mixture was transferred to a microcentrifuge tube and spun in an Eppendorf microcentrifuge at 15,000 rpm for 5 min. An aliquot of 200 µl of the supernatant was transferred into a 96-well microtiter plate (in duplo) and the increase in specific absorbance at 405 nm (as a result of accumulation of free *p*-nitroanilide) was determined immediately by using a Titertek Multiskan MCC plate reader (I.C.N. Biomedicals B.V., Amsterdam, The Netherlands). All assays were performed in duplo at least. After measuring APN-like activity, the cells were trypsinized and counted using a hemacytometer. APN-like activity was expressed as the production of *p*-nitroanilide in 1 min by 10^6 cells (nmoles/min/10^6 cells), using a standard curve which was determined in each experiment.

Specific aminopeptidase N activity

In some experiments the specific APN activity was determined using the monoclonal antibody WM-15, which specifically blocks the enzymatic activity of APN [31]. BEAS 2B cells, cultured in 24-well cell culture dishes, were rinsed with PBS and pre-incubated with WM-15 (1:50) for 15 min. As control, cells were pre-incubated in PBS/0.5% BSA, with an isotype-matched control antibody, or with WM-47 (an antibody that binds to APN but does not inhibit the enzymatic activity; generous gift of dr. E. Favaloro). After this pre-incubation, cells were rinsed with PBS and assayed for APN activity essentially as described using 250 µl L-alanine-*p*-nitroanilide, transferring 100 µl for absorbance measurements, and incubating at room temperature for 100 min. All measurements were performed in duplo at least. APN activity was expressed as the production of *p*-nitroanilide in 1 min by 10^6 cells (nmoles/min/10^6 cells).

Statistical analysis

In experiments where the effects of cytokines and dexamethasone were studied, data are expressed as the relative expression or activity compared to unstimulated control cells. Data are expressed as mean ± SEM, and were subjected to nonparametric statistical analysis, using the Mann-Whitney *U* test for between-group comparison. A *p*-value of <0.05 was considered significant.

RESULTS

Activity and expression peptidases on unstimulated BEAS 2B cells

The bronchial epithelial cell line BEAS 2B expresses NEP and APN. NEP activity, APN-like activity, NEP expression and APN expression of unstimulated BEAS 2B cells, which are shown in Table 1, did not change significantly during the time period used to perform the experiments. When cells were grown in complete medium, NEP and APN activity increased with growing cell densities from approximately 0.19 x 10^6 cells/cm^2 and up (data not shown). Below this density, NEP and APN activity remained largely unaltered. During the standard time period used to perform the experiments (5 days), cell densities consistently did not exceed 0.17 x 10^6 cells/cm^2.

Effect of cytokines, growth factors and dexamethasone on cell numbers

Cell numbers of unstimulated cells increased in time (0.52 ± 0.04 (0 h), 1.07 ± 0.08 (24 h), and 1.63 ± 0.15 (48 h) x 10^6 cells/well of a 6-well culture dish). After 24 hours of stimulation, IFN-γ and the combination of IFN-γ and DEX resulted in significantly decreased cell numbers

Table 1. NEP activity, APN-like activity, NEP expression, and APN expression of unstimulated BEAS 2B cells[a].

	0 h	24 h	48 h
Neutral endopeptidase activity[b]	9.4 ± 1.3	9.2 ± 1.5	8.6 ± 1.7
Aminopeptidase N-like activity[c]	4.7 ± 0.3	3.8 ± 0.4	3.4 ± 0.6
Neutral endopeptidase expression[d]	2.12 ± 0.84	2.03 ± 0.77	1.68 ± 0.66
Aminopeptidase N expression[d]	34.1 ± 10.3	29.8 ± 10.7	23.8 ± 8.6

a. BEAS 2B cells were passaged in complete medium at day -2. At day -1, the medium was replaced by the basal medium. At day 0, 1 (24 hours) and 2 (48 hours) NEP and APN-like activity and NEP and APN expression were determined using activity assays and flow cytometry, respectively. Values represent the arithmetic mean ± SEM ($n \geq 6$).

b. Neutral endopeptidase activity is expressed as pmoles/min/10^5 cells.

c. Aminopeptidase N-like activity is expressed as nmoles/min/10^6 cells.

d. Expression was determined by flow cytometry and is expressed as 10^4 MESF.

(85 ± 3% and 88 ± 5% of control, resp., $p<0.05$). This decrease was even larger after 48 hours of stimulation (70 ± 5% and 78 ± 5% of control, resp., $p<0.05$). In contrast, cell numbers were increased after 24 and 48 hours stimulation with EGF (114 ± 5% and 131 ± 5% of control, resp., $p<0.05$) or EGF and DEX (117 ± 7% and 134 ± 4% of control, resp., $p<0.05$). The effects of IFN-γ and EGF on cell number were dose-dependent (data not shown). Other stimuli used in our experiments had no significant effect on cell number (data not shown).

When cell proliferation was determined after 48 hours of stimulation with the various mediators, comparable results were obtained (*i.e.* decreased proliferation after stimulation with IFN-γ and increased proliferation after stimulation with EGF) (data not shown).

Effect of dexamethasone on peptidase activity

Incubation of BEAS 2B cells with 10^{-6} M DEX resulted in a time-dependent increase in NEP and, to a lesser extent, APN-like activity (Fig. 1A). Changes in activity could be observed as early as 6 hours after the addition of DEX and lasted at least 5 days. The effect of DEX on NEP and APN-like activity were dose-dependent (Fig. 1B) with ED50 values of ~10 nM for both enzymes.

To further characterize the DEX-mediated effects, specific inhibitors of NEP or APN were added during the activity assays. NEP activity of unstimulated cells could be inhibited completely by the NEP-specific inhibitor phosphoramidon (1 μM), indicating that all activity could be attributed to NEP. After stimulation with DEX, all the activity could still be inhibited by phosphoramidon (data not shown), indicating that the DEX-mediated increase in activity is completely due to an increased activity of NEP.

APN-like activity of unstimulated cells could be inhibited by the CD13 monoclonal antibody WM-15 to 67 ± 4% compared to inhibition by an isotype-matched control antibody, *i.e.* 33% of the APN-like activity is due to APN ($p<0.05$). After stimulation with DEX, WM-15 was able to reduce the APN-like activity to a similar level of APN-like activity detected in WM-15 treated control cells (Fig. 2). Therefore, the DEX-mediated increase in APN-like activity was completely due to an increase in APN (to 221 ± 30% of control; 24 h, $p<0.05$).

The DEX-mediated effects on NEP and APN-like activity could be inhibited completely by the addition of a 10-fold excess of the glucocorticoid receptor antagonist RU38486 (data not shown). Furthermore, the stable testosterone analogue R1881 (10^{-7} M) did not modify the NEP or APN-like activity (data not shown).

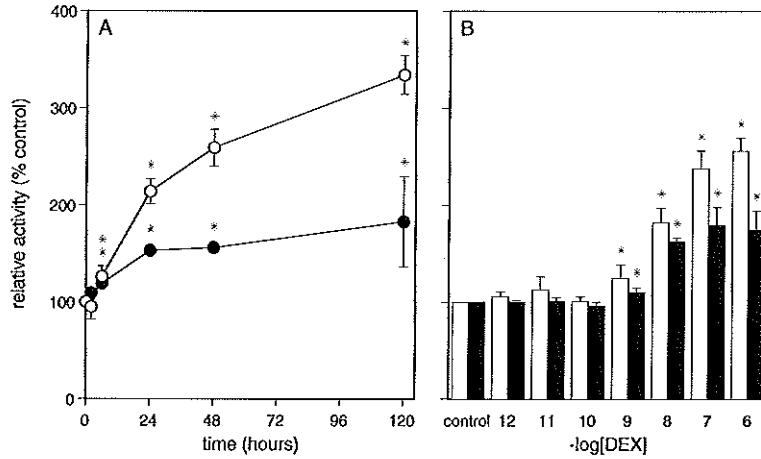

Figure 1. Effect of DEX on NEP and APN activity. (A) DEX (10^{-6} M) stimulated NEP (open circles) and APN-like (closed circles) activity in a time-dependent manner (mean ± SEM; n=2-10). (B) DEX stimulated NEP (open bars) and APN (solid bars) in a dose-dependent manner (mean ± SEM; n=4; 24 h incubation). Activity of unstimulated cells = 100%. *$p<0.05$ compared to control.

Effect of cytokines on peptidase activity

The effects of IL-1β, TNF-α, IFN-γ, IL-4, and EGF on NEP activity are shown in figure 3A. NEP activity was significantly increased after 48 hours by IL-1β (155 ± 7%), TNF-α (122 ± 4%) and IL-4 (119 ± 6%). EGF did not modulate NEP activity significantly. In contrast, IFN-γ decreased NEP activity after 24 hours (91 ± 3%) and 48 hours (83 ± 5%). All effects were dose-dependent (data not shown). Changes in NEP activity induced by IL-1β, TNF-α, IL-4 or IFN-γ were not observed after 2 or 6 hours, peaked around day 2 and lasted at least 5 days (data not shown).

APN-like activity was not significantly modified by stimulation (24 and 48 h) with IL-1β, TNF-α, IL-4, or EGF (Fig. 4A). Even after 5 days of stimulation with these mediators, no changes in APN-like activity were observed (data not shown). Stimulation with IFN-γ increased APN-like activity after 24 hours (121 ± 4% of control), whereas no effect was observed at 2, 6, 48, or 120 h after the addition of IFN-γ.

Effect of DEX and cytokines on peptidase expression

To determine whether the changes in activity were paralleled by changes in membrane expression, the effects of DEX and cytokines on membrane-expression of NEP and APN were determined by flow cytometry. The expression of ICAM-1 served as a positive control for the effects of the cytokines IL-1β, TNF-α and IFN-γ. In all experiments, ICAM-1 expression was strongly increased after stimulation with these cytokines (data not shown).

NEP expression (determined using the mAb J5) was significantly increased by DEX (200 ± 22% (24 h) and 217 ± 5% (48 h)), IL-1β (151 ± 13% (48 h)), TNF-α (145 ± 11% (48 h)), and IL-4 (111 ± 6% (48 h)). EGF had no significant effect on NEP expression (101 ± 18% (48 h)), whereas IFN-γ significantly decreased NEP expression, both after 24 (84 ± 2%) and after 48 hours (62 ± 4%). Comparable results were obtained using other monoclonal antibodies directed against NEP (data not shown).

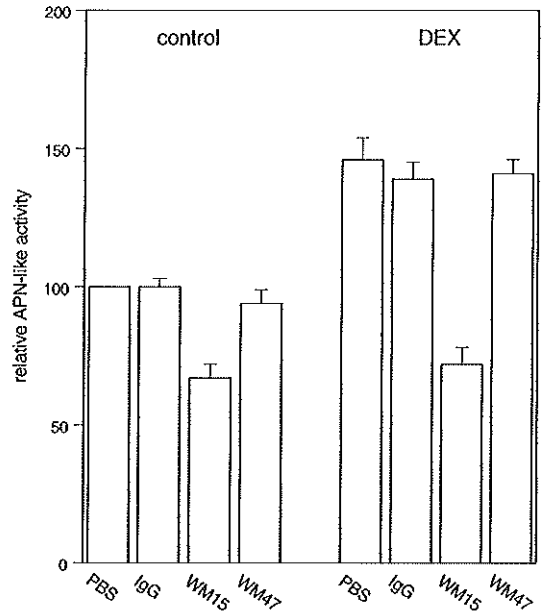

Figure 2. Characterization of APN-like activity and effect of DEX. APN-like activity (mean ± SEM; n=5) of BEAS 2B cells was determined after pre-incubation with PBS/BSA, an IgG1 isotype control antibody, the CD13 mAb WM-15, or the CD13 mAb WM-47. It is assumed that after pre-incubation with WM-15 the detectable aminopeptidase activity is not mediated by APN. WM-47 is a CD13 mAb that binds to APN but does not affect its activity. After stimulation with DEX (10^{-6} M, 24 h), WM-15 reduced APN-like activity to a similar level of APN-like activity detected in control cells pre-incubated with WM-15, indicating that the DEX-mediated increase in APN-like activity is completely due to an increased APN activity. Activity of unstimulated cells pre-incubated with PBS/BSA = 100%.

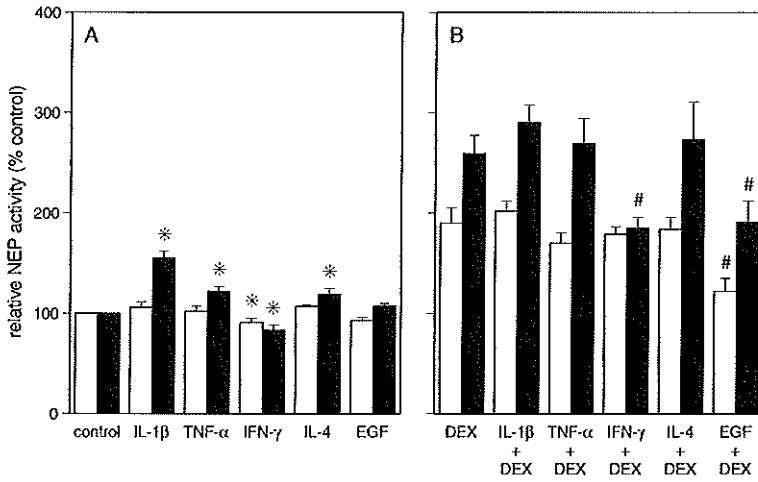

Figure 3. Effects of cytokines on NEP activity in the absence (A) or presence (B) of DEX. BEAS 2B cells were stimulated for 24 (open bars) or 48 (solid bars) hours with IL-1β (20 ng/ml), TNF-α (20 ng/ml), IFN-γ (5 ng/ml), IL-4 (20 ng/ml) or EGF (5 ng/ml) in the presence (A) or absence (B) of DEX, after which NEP activity was determined (mean ± SEM; n=5-11). Activity of unstimulated cells = 100%. $^{*}p<0.05$ compared to control. $^{\#}p<0.05$ compared to DEX.

APN expression was significantly increased by DEX (259 ± 24% (24 h) and 332 ± 6% (48 h) of unstimulated cells). Stimulation with IFN-γ consistently resulted in a slightly increased APN expression after 24 hours (116 ± 6%), whereas no significant changes were observed after 48 hours. Other stimuli did not significantly modify APN expression (data not shown).

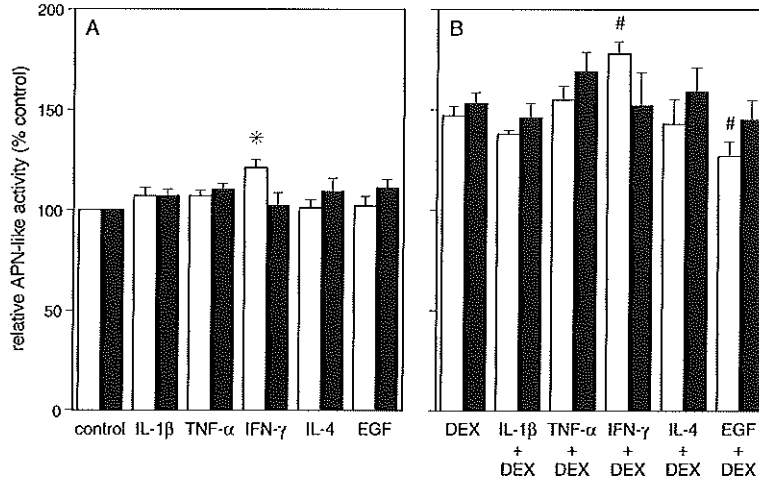

Figure 4. Effects of cytokines on APN-like activity in the absence (A) or presence (B) of DEX. BEAS 2B cells were stimulated for 24 (open bars) or 48 (solid bars) hours with IL-1β (20 ng/ml), TNF-α (20 ng/ml), IFN-γ (5 ng/ml), IL-4 (20 ng/ml) or EGF (5 ng/ml) in the absence (A) or presence (B) of DEX, after which APN activity was determined (mean ± SEM; n=5-11). Activity of unstimulated cells = 100%. *p<0.05 compared to control. #p<0.05 compared to DEX.

Effect of glucocorticoids on peptidase activities in the presence of cytokines

Simultaneous stimulation of BEAS 2B cells with DEX and IL-1β, TNF-α, or IL-4 resulted in increased NEP activities that were comparable to the increases observed with DEX alone (Fig. 3B). When cells were stimulated with DEX and IFN-γ, also an increase in NEP activity was observed, although this effect was less than the effect observed in the presence of DEX alone. Costimulation of cells with DEX and EGF resulted in an increased activity of NEP that was less than the increases observed after stimulation with DEX alone (Fig. 3B).

Simultaneous stimulation of BEAS 2B cells with DEX and IL-1β, TNF-α, IL-4 or EGF resulted in an increased APN-like activity that was similar to the increase observed after stimulation with DEX alone (Fig. 4B). After 24 hours of stimulation with DEX and IFN-γ an approximately additive effect was observed.

Involvement of secondary messengers

To investigate the possible involvement of secondary messengers in the IL-1β-mediated increase in NEP activity, we analyzed the effect of the cyclic-AMP analogue db-cAMP and the phosphodiesterase inhibitor IBMX on NEP activity. Db-cAMP time-dependently increased NEP activity: after 6, 24 and 48 hours NEP activity was increased to 121 ± 5%, 140 ± 8% and 149 ± 14%, *respectively* (Fig. 5A). Db-cAMP did not modulate APN-like activity (data not shown). IBMX did not affect NEP activity after a 48 h incubation time (Fig. 5B). However,

stimulation with IL-1β in the presence of IBMX resulted in a small but significantly enhanced effect of IL-1β (Fig. 5B).

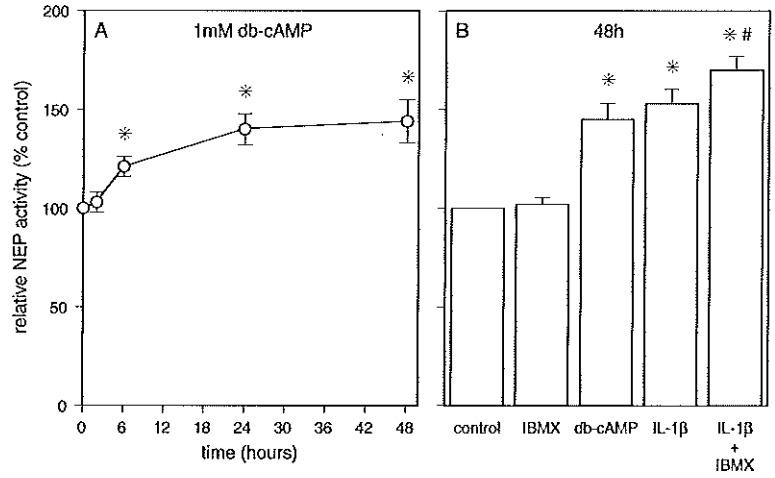

Figure 5. Role of secondary messengers in IL-1β-mediated effects on NEP activity. (A) Effect of the cyclic-AMP analogue db-cAMP (1 mM) on NEP activity (mean ± SEM; n=3-6). (B) Effect of phosphodiesterase inhibition by IBMX (0.1 mM) on IL-1β-mediated effects on NEP activity (48 h stimulation) (mean ± SEM; n=6). The activity of unstimulated cells = 100%. *$p<0.05$ compared to control. #$p<0.05$ compared to IL-1β.

DISCUSSION

Peptidases play an important role in the regulation of peptide-mediated events in the human lung, such as neurogenic inflammation [10]. Modulation of peptidase activity or expression may therefore be a major mechanism to control peptide actions, and thereby inflammatory responses. Several studies have shown that the activity of NEP can be reduced by a variety of external factors, including viruses [11], cigarette smoke [32], ozone [13], and chemicals [14]. In contrast, little is known about the effects of cytokines on NEP activity and expression. Therefore, we investigated the effects of the cytokines IL-1β, TNF-α, IFN-γ, IL-4 and EGF, which are known to be present during inflammatory reactions in the human airways [15], on the activity and expression of peptidases by human bronchial epithelial cells, since these cells have been shown to be a major site for peptidase activity [6, 7].

NEP activity and expression were increased after 48 hours of stimulation with IL-1β and, to a lesser extent, with TNF-α or IL-4. After 24 hours no significant increase could be observed, raising the possibility that these cytokines had an indirect effect, involving the release of a secondary mediator. It has been shown that lung fibroblasts increase NEP activity after stimulation with IL-1α, IL-6, and TNF-α and that this upregulation, which could already be detected after 6 h of stimulation, is dependent upon prostaglandin synthesis and elevation of cAMP [33]. We therefore aimed to investigate whether IL-1β, which in our study was the cytokine with the most potent effect on NEP activity, acted via similar mechanisms in bronchial epithelial cells. Stimulation of BEAS 2B cells with the cyclic-AMP analogue db-cAMP resulted in an increased NEP activity which was of similar magnitude but had a more rapid onset (~ 24 h earlier)

compared with the effect observed after stimulation with IL-1β. In addition, inhibition of phosphodiesterases with IBMX significantly enhanced the effect of IL-1β. These data strongly suggest that the effect of IL-1β on NEP activity is mediated, at least partially, via a cAMP-dependent pathway. In contrast to fibroblasts, prostaglandin-dependent mechanisms did not seem to be involved in BEAS 2B cells, since these cells hardly produced prostaglandins under the culture conditions used (data not shown). Thus, IL-1β-mediated upregulation of NEP activity by BEAS 2B cells involves a cAMP-dependent pathway, which seems to be independent of prostaglandin E_2 synthesis. Since the promotor region of NEP contains potential binding sites for the NF-IL6 transcription factor [34] and IL-1β is known to stimulate the release of IL-6 by bronchial epithelial cells [35], it may be that the effect of IL-1β is mediated via release of IL-6. Further studies will be necessary to test this possibility.

The increase in NEP activity and expression observed after stimulation with IL-1β and, to a lesser extent, TNF-α or IL-4 may result in an increased capacity to degrade peptides like substance P, kinins, neutrophil chemoattractants, and possibly cytokines [9]. As a consequence, the biological effects of these peptides (*e.g.* increased secretion of mucus, vasodilatation, increased microvascular permeability, recruitment of neutrophils, release of inflammatory mediators) will be decreased, resulting in reduced inflammatory responses. Thus, upregulation of NEP on bronchial epithelial cells by cytokines, or in general during inflammatory reactions, might limit peptide-mediated inflammation in the human bronchus, either by paracrine or autocrine mechanisms.

In contrast to NEP, APN-like activity and APN expression were not markedly modulated after stimulation of BEAS 2B cells with IL-1β, TNF-α, IL-4, or db-cAMP for up to 5 days. Since no change in APN-expression and no change in APN-like activity was found, specific APN-activity was not determined. Other reports, using human glomerular epithelial cells [36], endothelial cells or monocytes [37], have shown an increased expression and activity of APN after stimulation with IL-4. IFN-γ has been shown to decrease APN expression on monocytes in culture [37], whereas it increased APN activity in glomerular epithelial cells [36]. In our study we also found an increased APN activity after stimulation with IFN-γ. The difference in response between monocytes and epithelial cells may be due to the presence of alternative promoters in myeloid and epithelial cells [38]. The IFN-γ-induced increase in APN activity and expression is of great interest, since a recent report demonstrated a role for APN in trimming MHC class II-associated peptides [39]. Since IFN-γ also increases HLA-DR expression by epithelial cells [40], this may represent a mechanism to process and present (viral) peptide antigens.

Glucocorticoids are widely used in the treatment of pulmonary diseases characterized by inflammation. The anti-inflammatory action of glucocorticoids may be mediated, in part, by modulating the activity and expression of peptidases by bronchial epithelial cells. In our study, we found a strong increase in NEP and APN activity and, as determined by flow cytometry, expression. Using the inhibitory CD13 monoclonal antibody WM-15, we were able to show that the DEX-mediated increase in APN-like activity was completely due to an increased activity and expression of APN. The DEX-mediated increase in NEP and APN activity was reversed by the glucocorticoid receptor antagonist RU38486, indicating that these effects were mediated by the glucocorticoid receptor. Furthermore, the effect seemed to be specific for glucocorticoids and not for other steroid hormones, since the stable testosteron analogue R1881 had no effect on NEP or APN activity. DEX-mediated changes in NEP and APN activity/expression were time- and dose-dependent and could be observed with concentrations likely to occur around the epithelium *in vivo* after the inhalation of glucocorticoids [41]. Therefore, one beneficial effect

of inhaled glucocorticoids may be an increased peptidase activity by bronchial epithelial cells, thereby limiting the effects of pro-inflammatory peptides. In accordance with our results, an increased NEP expression was observed in the epithelium of glucocorticoid-treated asthmatics compared to non-treated asthmatics [42].

We subsequently determined the effects of DEX on peptidase activities in the presence of cytokines, since this may reflect the *in vivo* conditions during glucocorticoid therapy more properly. Although the effect of DEX on NEP activity was reduced in the presence of IFN-γ or EGF, stimulation of BEAS 2B cells with DEX in the presence of cytokines allways resulted in increased NEP and APN activities. It should be noted that the DEX-mediated increase in NEP activity was much higher than the cytokine-mediated increases in NEP activity, and that the IFN-γ-mediated decrease in NEP activity was counteracted by DEX. The reduced effect of DEX on NEP and APN activity in the presence of EGF suggests that stimulation of BEAS 2B cells with EGF decreases the responsiveness of NEP and APN for DEX, an observation that merits further study. Our data indicate that also in the presence of cytokines, DEX is able to increase NEP and APN activity by human bronchial epithelial cells. This suggests that even during inflammatory reactions *in vivo*, when several cytokines are simultaneously present [15], glucocorticoids are able to increase peptidase activities on human bronchial epithelial cells. In addition, cytokines released during inflammatory reactions may increase the activity of the hypothalamo-pituitary-adrenocortical axis, resulting in the release of the natural glucocorticoid hydrocortisone [43]. Our results suggest that hydrocortisone may prevent the body's defense reactions against stress from overshooting and needless tissue damage, at least partially, by upregulating peptidases which are able to inactivate pro-inflammatory peptides.

In conclusion, IL-1β, TNF-α and IL-4 are able to up-regulate NEP activity and expression by human bronchial epithelial cells, whereas IFN-γ results in a decreased activity and expression. In contrast, APN activity and expression were upregulated by IFN-γ, whereas other cytokines used in our experiments had no effect. DEX strongly increased NEP and APN activity and expression, both in the presence as in the absence of cytokines. Our results support a role for the human bronchial epithelium in the control of inflammation and indicate that an important anti-inflammatory effect of glucocorticoids in the treatment of asthma may be upregulation of peptidases expressed by bronchial epithelial cells.

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Chapter 10

Interleukin-4 receptors on human bronchial epithelial cells

An *in vivo* and *in vitro* analysis of expression and function

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ABSTRACT

Asthma is considered a Th2-like disease, characterized by locally increased levels of interleukin (IL)-4. The bronchial epithelium plays an important role in the initiation and perpetuation of inflammatory reactions within the airways. However, little is known about the presence of IL-4 receptors on human bronchial epithelial cells, or the effects of IL-4 on these cells.

In this report, we present definitive evidence of IL-4 receptor expression on human bronchial epithelial cells using several methods. IL-4 receptor expression on human bronchial epithelial cells *in vivo* was demonstrated using *in situ* hybridization and immunohistochemistry. No difference in IL-4 receptor protein expression was observed between bronchial biopsies of healthy subjects compared to allergic asthmatics. Cultured human bronchial epithelial cells also expressed IL-4 receptor mRNA and protein (as determined by RT-PCR analysis and flowcytometry, respectively). IL-4 receptor protein expression by bronchial epithelial cells could be increased by stimulation with PMA + calcium ionophore, whereas IL-1β and IL-6 decreased IL-4 receptor expression. A cyclic AMP analogue and IL-4 had no effect. Finally, we show that the IL-4 receptor is functionally active as IL-4 stimulates the release of IL-8, monocyte chemotactic protein-1, and particularly IL-1 receptor antagonist by human bronchial epithelial cells.

We conclude that human bronchial epithelial cells express IL-4 receptors both *in vivo* and *in vitro*. Stimulation of human bronchial epithelial cells by IL-4 may result in the release of both pro- and anti-inflammatory mediators known to be upregulated in asthmatic airways.

INTRODUCTION

Asthma is clinically defined by reversible airway obstruction and hyperresponsiveness of the airways. Chronic inflammation of the airways is a prominent feature of asthma and is generally believed to underly the clinical symptoms. This inflammation is characterized by an influx of eosinophils, mast cells, mononuclear phagocytes and T cells, and by increased levels of inflammatory mediators [1, 2]. Recent studies indicate a predominance of Th2 cells and Th2 cell-derived cytokines, such as interleukin-4 (IL-4) and IL-5, in the pathogenesis of bronchial asthma (reviewed in [3]). Bronchial biopsies of asthmatic patients show an increased number of cells containing IL-4 mRNA and protein, compared to healthy controls [4, 5]. In addition, increased levels of IL-4 have been found in bronchoalveolar lavage fluid of asthmatic patients [6].

IL-4, which may also be released by basophils, mast cells, and eosinophils [7, 8], exerts a wide range of effects on several cell types. These effects include proliferation of T and B cells [9], isotype switching to IgE in B cells [10], adhesion molecule expression on endothelial cells [11], and induction of major histocompatibility complex class II, CD13 and CD23 expression on mononuclear phagocytes [12, 13]. In addition, anti-inflammatory properties of IL-4 have been described. For example, IL-4 inhibits the release of prostaglandin E_2, IL-1β, tumor necrosis factor-α, IL-6, and IL-8, and upregulates the release of IL-1 receptor antagonist by monocytes [14-16].

The activity of IL-4 is mediated through binding to the IL-4 receptor (IL-4R). On many cells, the IL-4R is a heterodimeric complex comprising an α chain and a second chain. The

α chain, which is shared with the IL-13 receptor [17], is a 130 kD transmembrane protein consisting of a 220 amino acid extracellular domain including two pairs of cystein residues and the typical WSXWS motif of the hematopoietin or type I cytokine receptor superfamily. The second subunit of the IL-4R is, at least in some cells, the common γchain, which is also used by the IL-2R, IL-7R, IL-9R, and IL-15R [18, 19]. However, some recent reports have indicated that in certain cell types IL-4 may signal via the IL-4R in the absence of the common γ chain [20-23].

The bronchial epithelium has long been regarded as a passive barrier between the environment and the internal milieu of the lung. Currently, the bronchial epithelium is also considered to play an important role in the regulation of inflammatory and immunological reactions in the airways [24, 25]. Bronchial epithelial cells are able to produce a variety of inflammatory mediators, such as cytokines, chemokines and arachidonic acid metabolites [24]. Release of such mediators may result in the initiation and perpetuation of inflammation. Bronchial epithelial cells recovered from asthmatics show increased expression of inflammatory mediators, including IL-8 [26] and monocyte chemotactic protein-1 (MCP-1) [27], and increased levels of these mediators have been found in bronchoalveolar lavage fluid of asthmatic patients [28, 29]. In addition, human bronchial epithelial cells may produce anti-inflammatory mediators, including IL-1 receptor antagonist (IL-1RA). Increased levels of IL-1RA immunoreactivity have been found in the bronchial epithelium of asthmatics [30].

No IL-4 receptors (IL-4R) have been demonstrated on human bronchial epithelial cells yet. Therefore, we analyzed the mRNA and protein expression of IL-4R α chain by bronchial epithelial cells both *in vivo* and *in vitro*. We subsequently determined whether the expression of the IL-4R by bronchial epithelial cells differed between healthy subjects and allergic asthmatics and analyzed the modulation of IL-4R expression by human bronchial epithelial cells *in vitro*. Finally, we studied the effect of IL-4 on the release of the pro-inflammatory chemokines IL-8 and MCP-1 and the anti-inflammatory cytokine IL-1RA by human bronchial epithelial cells.

MATERIALS AND METHODS

Materials

Recombinant cytokines used in this study were IL-1β (10 U/ng, UBI, Lake Placid, NY, USA), IL-4 (5 U/ng, UBI), and IL-6 (1000 U/ng, kindly donated by prof. dr. L. Aarden, Amsterdam, The Netherlands). Stock solutions were prepared in 10 mM PBS, pH 7.4/0.1% BSA and stored at -20°C.

Dibutyryl-cyclic adenosine monophosphate (dibutyryl-cAMP) was obtained from Sigma (St. Louis, MO, USA). Solutions were freshly made in milli Q-filtered water before each experiment. PMA (Sigma) and the calcium ionophore A23187 (Sigma) were disolved in dimethylsulphoxide and stored at -20°C until use.

Antibodies

Monoclonal antibodies (mAb) used are MR6, which is directed against gp200-MR6, a 200 kDa molecule functionally associated with the human IL-4R complex ([31], kindly donated by dr. M. Larché, London, United Kingdom); M-57, directed against the human IL-4R α chain (CD124; [32], kindly provided by Immunex, Seattle, WA, USA); MAB284, directed against the common γchain (CD132; R&D Systems, Abingdon, United Kingdom); BBA-4 (anti-ICAM-

1 (CD54), British Biotechnology Products Ltd., Oxon, United Kingdom); Ta-1 (anti-CD26; Coulter Clone, Hialeah, FL, USA); CK-1, directed against a panmarker of epithelial cytokeratins (DAKOpatts, Glostrup, Denmark); OKT6, directed against the CD1a antigen of dendritic cells (American Type Culture Collection, Rockville, MA, USA); Leu-4 (anti-CD3), Leu-2 (anti-CD8), and Leu-3 (anti-CD4), all from Becton Dickinson (San Jose, CA, USA); My-4 (anti-CD14) and B4 (anti-CD19) (both from Coulter Clone); EG1 recognizing eosinophil cationic protein in resting and activated eosinophils (Pharmacia, Uppsala, Sweden); BMK13, recognizing major basic protein in resting and activated eosinophils (Genzyme, Cambridge, MA, USA); and EG2, recognizing the cleaved form of eosinophil cationic protein in activated eosinophils only (Pharmacia). For double-stainings (see below) FITC-labeled Leu-4 (CD3) and FITC-labeled My-4 (CD14) were used.

Bronchial tissue

Samples of human bronchus were obtained from patients undergoing pneumonectomy or lobectomy (University Hospital Dijkzigt, Rotterdam, the Netherlands). Tissue distinct from the tumor and having a normal appearance was dissected and either used for bronchial epithelial cell culture (see below) or directly embedded into Tissue-Tek (Miles Inc., Elkhart, IN, USA), frozen in liquid nitrogen, and stored at -80°C.

Bronchial mucosal biopsy specimens were obtained from eleven non-smoking allergic asthmatic patients (8 men, 3 women, median age 32 years, range 20 - 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 $\geq 9\%$ of the initial value after inhalation of 1000 µg terbutaline. The asthmatic subjects had a mean FEV_1 of 91% of the predicted value (range 60 - 108%). The median of the ^{2}logs of the provocative concentrations of inhaled methacholine required to reduce their FEV_1 by 20% (PC_{20}) was -0.06 mg/ml (range -3.89 - 3.43 mg/ml). Allergy was defined by one or more positive skin-prick tests to extracts of 16 common aeroallergens. All patients were receiving inhaled β-agonists only, and none had taken oral or inhaled corticosteroids in the month prior to the study.

The control group consisted of 6 non-allergic non-asthmatic subjects (4 men and 2 women, median age 24 years, range 23 - 52 years). All controls had a PC_{20} histamine of more than 8 mg/ml and a median FEV_1 of 104 (88 - 109)% of the predicted value. The study was approved by the local Ethics Committee and all participants gave their written informed consent.

Immunohistochemistry

Immunohistochemical (double) stainings were performed as described previously [33]. To evaluate IL-4R expression between healthy subjects and asthmatics, biopsies were coded and two sections per staining were analyzed in a blinded fashion for each biopsy at a magnification of 10x40. IL-4R expression by the bronchial epithelium or lamina propria was scored semi-quantitatively on a 0 - 4 scale (0= negative; 1= weak; 2= moderate; 3= strong; 4 = very strong). In addition, the number of positively stained cells were counted in a zone covering 100 µm of the the lamina propria along the length of the epithelial basement membrane (BM), covered with epithelium over at least 500 µm. Cells were counted if they stained red and contained a nucleus. Each section was evaluated by at least two independent investigators. The cell counts are expressed as the number of cells per mm of BM. Composition of the cellular infiltrate was determined using specific leukocyte markers (see *Antibodies*) and counted as described above.

In situ hybridization (ISH)

Anti-sense and sense oligonucleotides were synthesized with a DNA Synthesizer 380B (Applied Biosystems, Foster City, CA, USA). The IL-4R oligos were designed using the Oligo Primer Analysis Software (MedProbe, Oslo, Norway), and displayed no homology to known complementary cDNA sequences (except human IL-4R cDNA), as verified by searching EMBL database. Anti-sense oligonucleotides used are (5'to 3') GTG GAC GCA GAG GCT GAT GTA CTC GTA GAG; TCT TTC TTA CCA CCC TAG TCT AAG GGT TGG; CGG ACA AGG ACC TGG ACG AGC CTC TCC TCT; TGA CAC GGG GTT GGA CTC GGT CTT TGG ACC. The sense oligonucleotides overspanned the same regions in the gene. Oligonucleotides were 3'-tailed with digoxigenin (DIG) using the DIG Oligonucleotide Tailing Kit (Boehringer Mannheim, Mannheim, Germany). Labeling efficiency was verified using hybridization and comparison with a DIG-tailed control oligonucleotide (supplied with the kit) according to the instructions of the manufacturer. Prior to use for ISH, a mixture of the four sense or the four anti-sense oligonucleotides was prepared.

DIG-labeled oligonucleotides for α-actin (British Biotechnology Products Ltd.) and DIG-labeled oligo-dT (R&D Systems) were used as control for the preservation of cellular RNA.

ISH and immunological detection were performed essentially as described previously [34]. The negative controls included omission of oligonucleotides or antibody and hybridization with the sense probe mixture. Human skin biopsies of psoriatic patients were used as positive control tissue [35].

Culture of human bronchial epithelial cells and cell lines

Human bronchial epithelial cells were cultured from bronchial tissue (obtained from non-allergic patients undergoing lung surgery) as described previously [36]. Cells were characterized using a mouse monoclonal antibody directed against a number of human cytokeratins (CK-1). At least 99% of the isolated cells stained positive for cytokeratin. When cells were 70-90% confluent, they were used for experiments.

The human bronchial epithelial cell line BEAS 2B [37] was kindly provided by dr. J. Lechner (Inhalation Toxicology Research Institute, Albuquerque, NM, USA) and cultured as described previously [38]. Medium was replaced three times weekly and cultures were passaged when the monolayers were 80 to 90% confluent. Passages 16 through 35 were used in this study.

The human histiocytic lymphoma cell line U937 [39] was cultured in RPMI 1640 medium (Gibco) supplemented with 10% FCS and antibiotics. The THP-1 cell line [40] was obtained from the American Type Culture Collection and maintained according to their instructions.

Stimulation of bronchial epithelial cells

When cultures reached 70-90% confluency, the medium was replaced by a basal medium consisting of DMEM/F12 (1:1) supplemented with antibiotics and IL-1β (20 ng/ml), IL-4 (20 ng/ml), IL-6 (0.1 ng/ml), dibutyryl-cAMP (1 mM), or PMA (10 ng/ml) and calcium ionophore A23187 (1 μg/ml) were added to the medium. These doses were widely shown to be effective *in vitro*. For studies on the effect of IL-4 on cytokine release by human bronchial epithelial cells, the cultures were continued for an additional 24 h, after which culture supernatants were collected and cells were trypsinized and counted using a haematocytometer (Coulter). For studies on the modulation of IL-4R expression by human bronchial epithelial cells, cells were stimulated for 24 h, after which the cells were analyzed by flowcytometry.

Flowcytometry

For immunofluorescence staining of human bronchial epithelial cells, the following mAb were used: M57, MAB284, BBA-4, and Leu-4 (control for nonspecific binding). Bronchial epithelial cells were rinsed with PBS and detached using 0.02% EDTA. Cells were harvested in PBS/0.5% BSA, washed and resuspended in PBS/BSA to a final concentration of 2 x 10^6 cells/ml. Flowcytometry was performed as described previously [38]. Fluorescence intensities were quantified using calibrated fluorescence standards (FCSC Quantum 26, Research Triangle Park, NC, USA) and expressed as Molecules of Equivalent Soluble Fluorescence (MESF) [38].

RNA isolation and cDNA synthesis

Total cellular RNA was isolated from human bronchial epithelial cells and BEAS 2B cells according to Chomczynski and Sacchi [41]. The integrity of the RNA was assessed by electrophoresis of the RNA samples on a 1% ethidium bromide-stained agarose gel and observation of intact S28 and S18 ribosomal bands. The RNA was stored at -80°C until use. RNA isolated from the HTLV-1 infected human T cell line MT-1 [42] was kindly donated by dr. M.C.M. Verschuren from the department of Immunology (Erasmus University Rotterdam, The Netherlands).

Prior to amplification, a 1 μg aliquot of total cellular RNA was treated with RNase-free DNase (Gibco) to remove contaminating genomic DNA. RNA was reversed transcribed to cDNA as described by previously (total volume: 20 μl) [34]. The cDNA were stored at -20°C until use.

Polymerase chain reaction (PCR) analysis

For PCR analysis 1 μl cDNA solution was used. The reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 pM sense and anti-sense oligonucleotide primers, and 20 U/ml Taq polymerase (Amplitaq, Perkin-Elmer Cetus, Norwalk, CT, USA) in a total volume of 50 μl. Analysis of cDNA levels of hypoxanthine phosphatidyl ribosyltransferase (HPRT) was used as an internal control to standardize for total cellular mRNA. The following primers (synthesized with a DNA Synthesizer 380B (Applied Biosystems)) were used (5' to 3'): IL-4R sense: CTG GAG CAC AAC ATG AAA AGG; IL-4R anti-sense: AGT CAG GTT GTC TGG ACT CTG; common γ chain sense: GAT TAT AGA CAT AAG TTC TCC; and common γ chain anti-sense: GAT GAT TAT CAA CAG AAA CTT; HPRT sense: GTG ATG ATG AAC CAG GTT ATG ACC TT [43]; HPRT anti-sense: CTT GCG ACC TTG ACC ATC TTT GGA [43]. The amplified cDNA products are 510, 831, and 454 base pair in size, respectively. Samples were amplified in a DNA Thermocycler 480 (Perkin-Elmer Cetus) for 35 cycles, each cycle consisting of denaturation at 94°C for 30 sec (IL-4R and HPRT) or 1 min (common γ chain), annealing at 55°C for 30 sec (IL-4R and HPRT) or 1 min (common γ chain), and extension at 72°C for 1 min. These conditions appeared to be optimal as determined in preliminary experiments. Aliquots of PCR products were run on 1% agarose gels and visualized by ethidium bromide staining. HPRT was used as an internal control for total cellular mRNA. Each experiment included positive controls (cDNA from a THP-1 cell line (IL-4R) or MT-1 and U937 cell line (common γ chain)) and two negative controls (water and DNA treated with DNAse).

Radioactive hybridization of PCR products

Agarose gels containing the amplified cDNA were blotted to Nytran N nylon membranes (Schleicher and Schuell, Dassel, Germany) and fixed to the membrane with a 254 nm UV

crosslinker. Oligonucleotide probes used are (5' to 3') TGG CCA GAG AGC ATC AGC GT (IL-4R α chain) and GTG AGG TGA GTA TGA GAC GCA GGT G (common γ chain). The oligonucleotides, being complementary to internal sequences of the PCR products, were end-labeled with ^{32}P-γ-ATP (ICN Pharmaceuticals Inc., Irvine, CA, USA) by T4 polynucleotide kinase (Pharmacia LKB, Piscataway, NJ, USA). Blots were hybridized in 5x SSPE (50 mM SodiumPyrophosphate, 0.9 M NaCl and 5 mM EDTA, pH 7.0), 0.6% SDS and 50 μg/ml salmon sperm DNA for 2 h at 55°C. Subsequently, blots were rinsed twice for 20 min with 5x SSPE with 0.1% SDS at 55°C and exposed to phosphor screens, after which the intensities of the signals were measured using a PhosphorImager (type Storm 820; Molecular Dynamics, Sunnyvale, CA, USA) and analyzed using ImageQuaNT software (Molecular Dynamics).

ELISA for IL-8, MCP-1 and soluble IL-4R α chain

IL-8 was measured with a commercially available sandwich ELISA (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands) with a detection limit of 1 pg/ml. Levels of immunoreactive MCP-1 were quantified in a previously described sandwich ELISA [44], using a novel, highly specific monoclonal antibody against MCP-1 (5D3-F7 mAb, IgG_1) and a polyclonal rabbit anti-MCP-1 serum. The sensitivity of this assay was 30 pg/ml. Human soluble IL-4R α chain levels in the culture supernatants were measured using a commercially available sandwich ELISA (R&D Systems; detection limit: 5 pg/ml). IL-1RA levels were determined using a commercially available sandwich ELISA (Medgenix, Etten-Leur, The Netherlands) with a detection limit of 10 pg/ml.

Statistical analysis

Data are expressed as mean ± SEM (cytokine levels) or median with range (immunohistochemical analyses). The Mann-Whitney *U* test was used to assess significant differences in cytokine-release and IL-4R expression between stimulated and unstimulated cultures of human bronchial epithelial cells and IL-4R expression between bronchial biopsies of asthmatics and healthy subjects. Correlation coefficients were obtained by Pearson's rank method. A *p*-value less than 0.05 was considered significant.

RESULTS

Expression of IL-4R mRNA and protein in vivo

Although some recent reports indicate an effect of IL-4 on human bronchial epithelial cell function, no IL-4R have been demonstrated on these cells yet. Therefore, we determined the expression of IL-4R mRNA and protein in bronchial tissue. This tissue was obtained from non-allergic patients undergoing lung surgery. *In situ* hybridization (ISH) of bronchial tissue showed that IL-4R mRNA was strongly present in the bronchial epithelium and in smooth muscle cells (Fig. 1). In addition, IL-4R mRNA was detected in blood vessels and infiltrating cells (Fig. 1). ISH using the DIG-labeled β-actin or oligo-dT oligonucleotides showed strong and roughly equal hybridization signals in all cells.

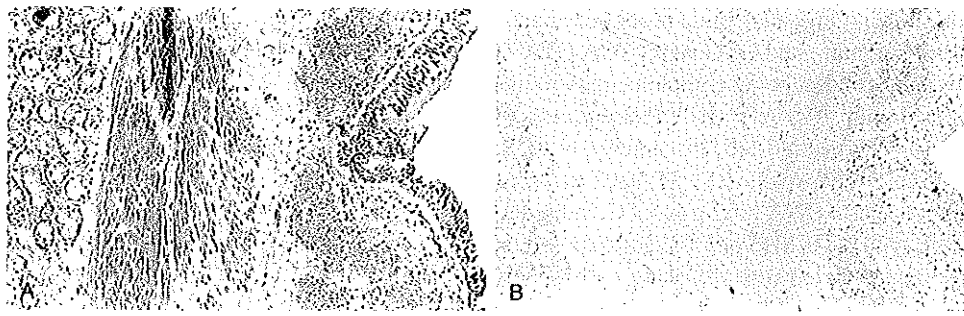

Figure 1. *In situ* hybridization (ISH) of IL-4R mRNA in bronchial tissue. ISH of IL-4R mRNA using the DIG-labeled anti-sense oligonucleotide mixture (A). The IL-4R sense oligonucleotide mixture gave no hybridization signal (B). Magnification: 160x.

The ISH data were substantiated by immunohistochemical stainings of bronchial tissue, using the mAb MR6 and M57. Strong IL-4R protein expression was demonstrated in the entire bronchial epithelium and in smooth muscle cells (Fig. 2). The expression of IL-4R protein was particularly restricted to the membrane of these cells. Less intense staining was observed in blood vessels and infiltrating cells within the lamina propria. Using double-stainings with anti-CD3 and anti-CD14 mAb, the majority of the IL-4R-positive inflitrating cells could be identified as T-lymphocytes and mononuclear phagocytes (data not shown). The MR6 and M57 mAb (which recognize distinct parts of the IL-4R) showed similar staining patterns. These results indicate that in the healthy human bronchus, IL-4R expression can be found in the bronchial epithelium, smooth muscle, blood vessels, and infiltrating cells (predominantly T lymphocytes and mononuclear phagocytes).

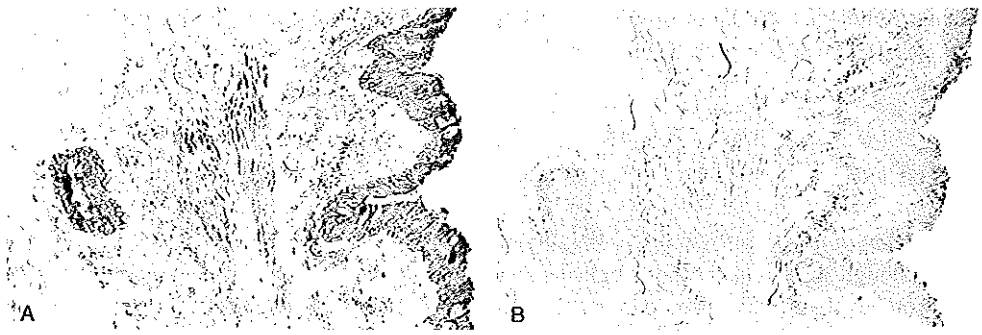

Figure 2. IL-4R immunoreactivity in human bronchial tissue. Human bronchial tissue was stained using the M57 mAb (A). Mouse isotype-matched control antibody showed no reactivity (B). Magnification: 160x.

IL-4R expression in bronchial biopsies of asthmatics

To determine whether the expression of IL-4R was altered in bronchial tissue of asthmatic patients, we evaluated the expression of IL-4R protein in bronchial biopsies of healthy non-allergic subjects and allergic asthmatics. Bronchial biopsies of asthmatic patients showed an increased number of total eosinophils (EG1/BMK13 positive) and activated eosinophils (EG2

positive) in the lamina propria compared to healthy subjects. In addition, the number of CD1a-positive dendritic cells in the bronchial epithelium was increased in the asthmatic patients. No significant differences were observed for the number of T cells (CD3, CD4, or CD8 positive), B cells, or mononuclear phagocytes (data not shown).

Comparison of the bronchial epithelium between bronchial biopsies of healthy non-allergic subjects and allergic asthmatics (using the MR6 mAb) did not reveal significant differences in the IL-4R expression *in vivo* (Fig. 3, left part). In addition, IL-4R expression in the lamina propria, as determined by semi-quantitative analysis, did not differ between both groups (Fig. 3, right part). Quantitative analysis of the number of IL-4R-positive cells in the lamina propria also revealed no significant differences between healthy controls (median: 8.4 cells/mm BM (range: 4.5-47.5)) and allergic asthmatics (median: 7.5 cells/mm BM (range: 0.8-94)). Semi-quantitative and quantitative analysis showed strong correlation with each other (Spearman's rank correlation coefficient 0.88 ($p<0.01$)).

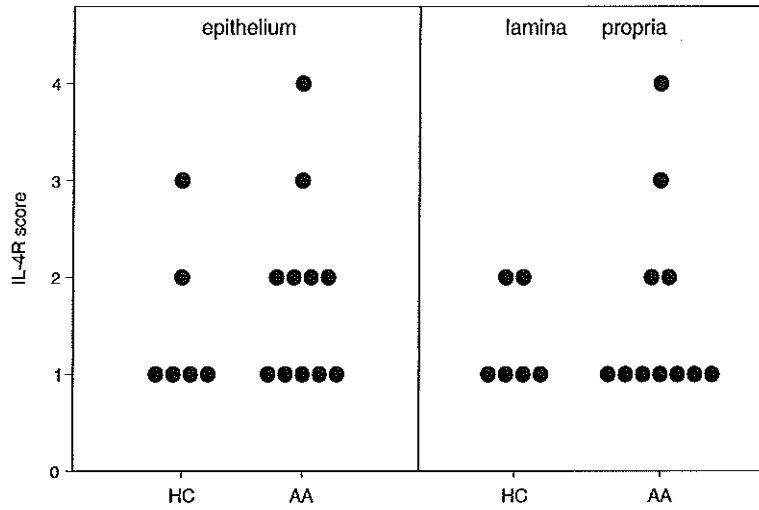

Figure 3. Score for IL-4R expression in bronchial biopsies of healthy controls (HC) and allergic asthmatics (AA). IL-4R immunoreactivity of the bronchial epithelium and lamina propria was semi-quantitatively scored on a 0-4 scale. See text for details.

IL-4R expression in the lamina propria (using either semi-quantitative or quantitative data of both healthy controls and allergic asthmatics) showed a correlation with the number of activated (EG2-positive) eosinophils in the lamina propria ($r_s= 0.8995$; $p<0.001$; Fig. 4). IL-4R expression in the lamina propria showed a tendency to correlate with the number of activated (CD26-positive) T cells in the lamina propria (semi-quantitative analysis: $r_s= 0.5369$; $p<0.05$; quantitative analysis $p=0.068$).

These results suggest that the expression of IL-4R on the bronchial epithelium is rather constitutive and is not significantly altered in the asthmatic airways. Furthermore, the expression of IL-4R in the bronchus significantly correlates with the number of activated eosinophils, and less clearly with activated T cells, within the lamina propria.

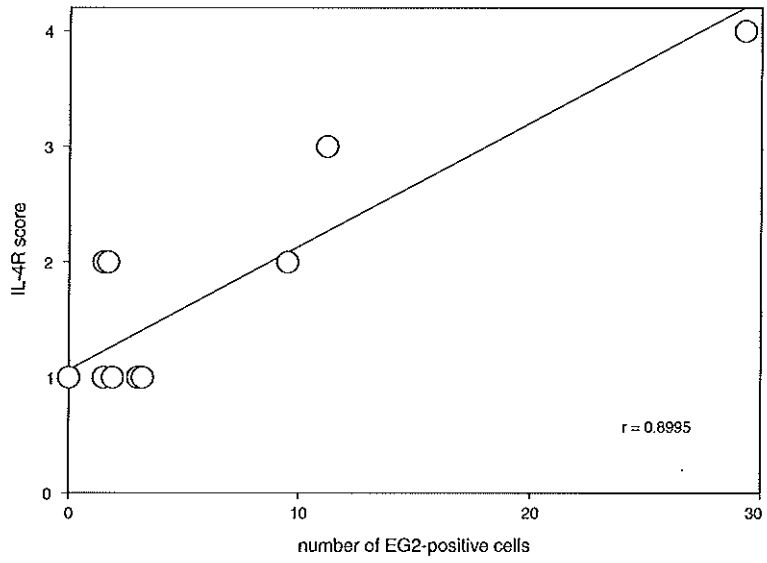

Figure 4. Correlation between IL-4R expression and activated eosinophils in the lamina propria. IL-4R expression (quantitative) and the number of activated (EG2-positive) eosinophils in the lamina propria was determined as described in the Materials and methods section.

Expression of IL-4R mRNA and protein in cultured human bronchial epithelial cells

We subsequently determined whether cultured human bronchial epithelial cells expressed the IL-4R mRNA and protein. Using RT-PCR analysis, IL-4R mRNA could be observed both in primary cultures of human bronchial epithelial cells and in the established BEAS 2B cell line (Fig. 5A). In addition, IL-4R protein expression (as determined by flowcytometry using the M57 mAb), could be observed in cultured human bronchial epithelial cells (Fig. 5B). Basal expression of IL-4R and ICAM-1 was 0.88 $\pm$ 0.22 x 10^4 MESF and 13.8 $\pm$ 5.1 x 10^4 MESF, respectively (n=7). The expression of IL-4R mRNA and protein seemed to be rather independent of the time of culture and the confluency of the cultures (data not shown). The IL-4R expression by cultured human bronchial epithelial cells was further demonstrated by the observation that soluble IL-4R α chain immunoreactivity could be detected in the culture supernatants of these cells (mean $\pm$ SEM: 41.9 $\pm$ 6.8 pg/ml; n=17; range 7.8 - 116.6 pg/ml).

Since the common γ chain (CD132) is, at least in certain cell types, a functional component of the IL-4R, we aimed to analyze the expression of this molecule in cultured human bronchial epithelial cells. RT-PCR analysis and subsequent hybridization of the common γ chain cDNA showed expression of common γ chain mRNA in cultured human bronchial epithelial cells and BEAS 2B cells (Fig. 5A). In addition, expression of the common γ chain protein on human bronchial epithelial cells and BEAS 2B cells was demonstrated by flowcytometry (data not shown). The expression levels of the common γ chain (0.66 $\pm$ 0.15 x 10^4 MESF; n=8) were almost similar to the expression levels of the IL-4R α chain .

These results demonstrate that cultured human bronchial epithelial cells express both mRNA and protein for the IL-4R α chain and the common γ chain.

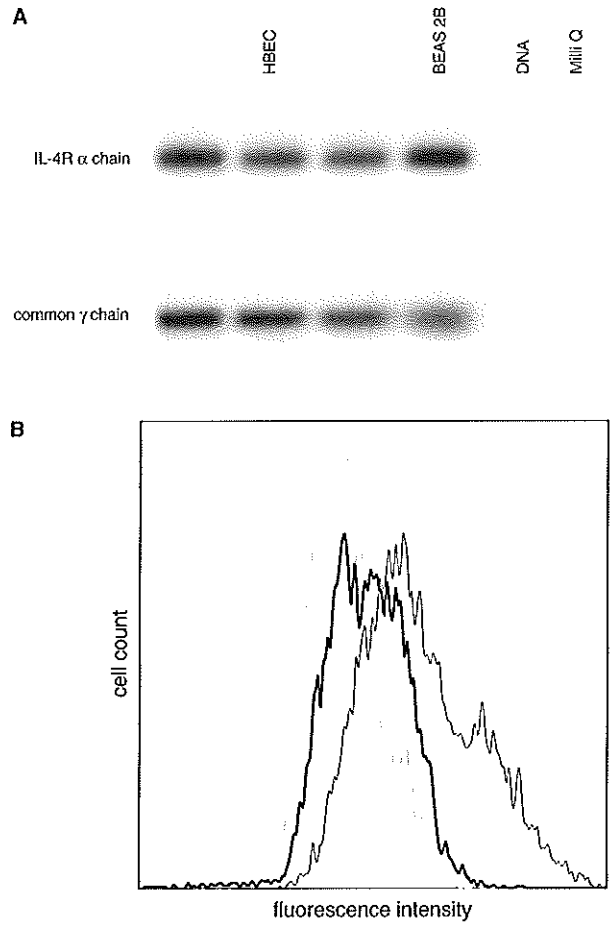

Figure 5. IL-4R expression in cultured human bronchial epithelial cells. A: IL-4R α chain mRNA and common γ chain mRNA expression were determined using RT-PCR analysis and subsequent hybridization. HPRT mRNA levels were comparable in all samples (data not shown). B: IL-4R α chain protein expression on cultured human bronchial epithelial cells was determined by flowcytometry using the M57 mAb (thick line). ICAM-1 (thin line) and an isotype-matched antibody (dotted line) served as positive and negative controls, respectively. One representative experiment out of seven is shown.

Modulation of bronchial epithelial IL-4R protein expression

To analyze whether the expression of the IL-4R could be modulated *in vitro*, we stimulated human bronchial epithelial cells with IL-1β, IL-4, IL-6, dibutyryl-cAMP, or PMA + A23187 for 24 h. Stimulation with PMA + A23187 consistently increased IL-4R expression on human bronchial epithelial cells (Fig. 6). In contrast, a small but consistent decrease in IL-4R expression was observed after stimulation with IL-1β or IL-6 ($p < 0.05$ for both cytokines). IL-4 and dibutyryl-cAMP did not significantly affect on IL-4R expression by human bronchial epithelial cells. Comparable results were obtained after stimulation for 48 or 72 hours (data not shown). ICAM-1 expression was consistently increased after stimulation with IL-1β (297 ± 54% of unstimulated cells) or PMA + A23187 (672 ± 560%), whereas IL-4 (115 ± 10%), IL-6 (92 ± 6%) and dibutyryl-cAMP (124 ± 25%) had no significant effect.

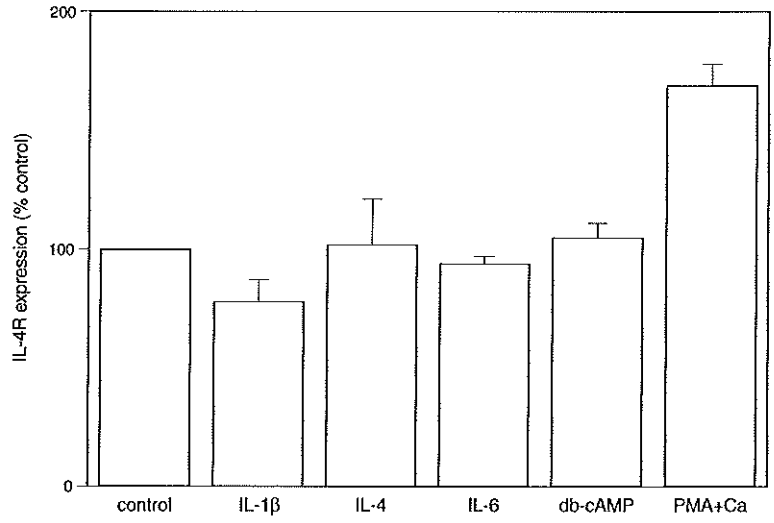

Figure 6. Modulation of IL-4R expression on human bronchial epithelial cells. Human bronchial epithelial cells were stimulated for 24 h with IL-1β, IL-4, IL-6, dibutyryl-cAMP (db-cAMP) or PMA + calcium ionophore A23187 (Ca). IL-4R expression was determined by flowcytometry (using the M57 mAb). Data represent the mean ± SEM (n=3-5).

Effects of IL-4 on mediator release by human bronchial epithelial cells

To study whether the IL-4 receptors expressed by human bronchial epithelial cells were functionally active, human bronchial epithelial cells were stimulated with IL-4. For comparison, we also stimulated human bronchial epithelial cells with IL-1β. As shown in figure 7, IL-1β increased the IL-8 and MCP-1 release. IL-4 also consistently increased the release of IL-8 and MCP-1 release, although to a lesser degree (Fig. 7). IL-1RA production by human bronchial epithelial cells was strongly increased by stimulation with IL-1β and IL-4. Thus, IL-4 stimulates the release of both IL-8, MCP-1 and particularly IL-1RA from cultured human bronchial epithelial cells.

DISCUSSION

This paper shows for the first time that IL-4R are expressed by human bronchial epithelial cells and that this IL-4R is functionally active. Since IL-4 is thought to be an important cytokine in asthma, our findings are of importance for our understanding of the pathogenesis of this disease. Increased numbers of cells containing IL-4 mRNA and protein have been found in bronchial biopsies of asthmatics [4, 5] and increased levels of IL-4 have been found in bronchoalveolar lavage fluid of asthmatic patients compared to healthy individuals [6]. T lymphocytes and eosinophils have been shown to be an important source of IL-4 in the inflamed bronchus, and both cell types can be found in close proximity to the bronchial epithelium. Therefore, it is very likely that bronchial epithelial cells will be exposed to IL-4 during inflammatory and immunological responses within the lung. Our findings indicate that human bronchial epithelial cells express the IL-4R. Furthermore, we show that this IL-4R is functionally

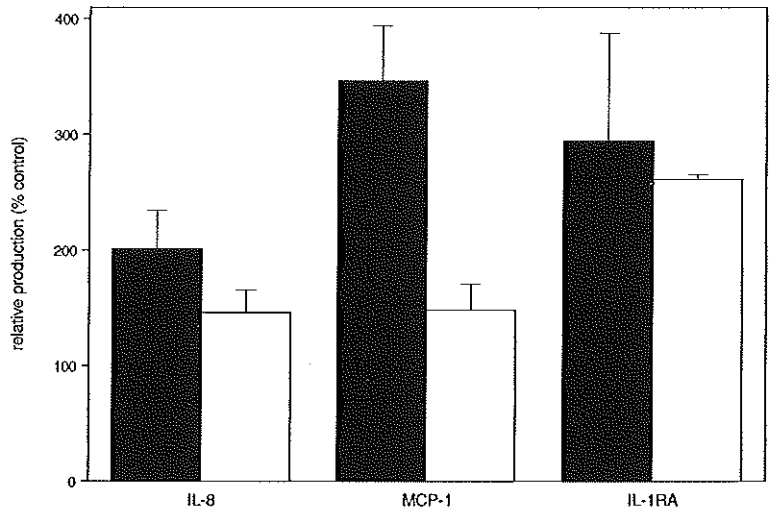

Figure 7. Effect of IL-1β and IL-4 on IL-8, MCP-1, and IL-1RA production by human bronchial epithelial cells. Human bronchial epithelial cells were stimulated with IL-1β (20 ng/ml; closed bars) or IL-4 (20 ng/ml; open bars) for 24 h. IL-8, MCP-1, and IL-1RA levels in the supernatant were analyzed using ELISA. Data are expressed as percentage production compared to unstimulated cells (mean ± SEM; n ≥ 3).

active, since exposure of human bronchial epithelial cells to IL-4 stimulated the release of IL-8, MCP-1, and particularly IL-1RA, which are mediators known to be upregulated in the asthmatic bronchial epithelium [26, 27, 30].

Using *in situ* hybridization and immunohistochemistry, we show that the human bronchial epithelium expresses IL-4R mRNA and protein *in vivo*. IL-4R expression was also observed in endothelial cells, smooth muscle cells and infiltrating cells (T cells and mononuclear phagocytes), all of which are known to possess IL-4R [45, 46]. Expression of the IL-4R has also been described for other epithelial tissues, like intestinal epithelium [47], epidermal cells [35], and breast epithelium [48], suggesting that expression of IL-4R is a general feature of epithelial cells.

IL-4R expression in the lamina propria of healthy controls and allergic asthmatics showed a strong correlation with the number of activated eosinophils and a weak correlation with the number of activated T cells. Eosinophils are known to express IL-4R [49], however, it is not known at present whether these receptors are upregulated on activated eosinophils. Unstimulated T cells express low levels of IL-4R, but the number of IL-4R is upregulated upon activation [50]. Therefore, one may speculate that the correlation between IL-4R expression and number of activated eosinophils or activated T cells is a reflection of the increased IL-4R expression on these cells. On the other hand, activated T cells or activated eosinophils may secrete mediators (for example IL-4) which subsequently increase the IL-4R expression in a paracrine or autocrine manner.

Expression of IL-4R mRNA and protein was also demonstrated in cultured human bronchial epithelial cells and in the BEAS 2B cell line. In addition, protein and mRNA expression of the common γ chain was observed in these cells. The common γ chain (CD132) is believed to be a functional component of the IL-4R [18, 19], but recent reports have shown that in certain cell types IL-4 may signal via the IL-4R in the absence of the common γ chain [20, 21]. Although

we did not directly determine the subunit structure of the IL-4R, it can be assumed that in human bronchial epithelial cells IL-4 signals, at least in part, via binding to an IL-4R composed of the IL-4R α chain and the common γ chain.

Modulation of IL-4R expression *in vitro* was studied using several cytokines, the cAMP analogue dibutyryl-cAMP, and stimulators of protein kinase C (PMA + calcium ionophore). Stimulation with PMA + calcium ionophore consistently increased the IL-4R expression, whereas IL-4 and dibutyryl-cAMP had no effect. IL-4R expression on T cells and B cells has been shown to be upregulated by IL-4 and calcium ionophore [51-53]. In contrast, IL-4R expression on monocytes has been shown to be upregulated by PMA or stimulation of intracellular cAMP levels, whereas IL-4 and calcium ionophore had no effect [51]. It was hypothesized that this discrepancy might be caused by the presence (T cells) or absence (monocytes) of the common γ chain [51]. Our results do not support this hypothesis since we did not observe an increased IL-4R expression after IL-4 stimulation of human bronchial epithelial cells, expressing the common γ chain. After stimulation with IL-1β or IL-6, a small but consistent decrease in IL-4R expression was observed. The physiological role of this decrease, however, remains to be established and it would be of interest to determine whether changes in receptor numbers are accompanied by changes in receptor affinity.

Comparison of IL-4R expression by the bronchial epithelium of asthmatics and healthy subjects did not reveal significant differences. In addition, no difference was found in IL-4R expression in the lamina propria. Although IL-4 levels are increased in asthmatic patients [4-6], our *in vitro* data show that this does not affect IL-4R expression on the bronchial epithelium. In fact, from our *in vitro* data one might expect a decreased IL-4R expression due to elevated IL-1β and IL-6 levels in asthmatics [54]. On the other hand, our *in vivo* data should be interpreted with caution, since our method may not detect small changes in receptor numbers, and the number of patients was relatively small. Nevertheless, the lack of difference in bronchial epithelial IL-4R expression between asthmatics and healthy subjects and the only minor effects of stimuli on bronchial IL-4R expression *in vitro* suggest that the expression of IL-4R on human bronchial epithelial cells is rather constitutive.

Recent reports have demonstrated an effect of IL-4 on human bronchial epithelial cells. In one study, it was shown that IL-4 upregulated the release of granulocyte/macrophage colony-stimulating factor, thereby stimulating eosinophil survival [55]. In contrast, other studies have shown that IL-4 may exert anti-inflammatory properties on human bronchial epithelial cells by inhibiting cytokine-induced RANTES expression [56] or inducible nitric oxide synthase expression [57]. In the latter studies, IL-4 was added 30 min prior to stimulation of the cells by a mixture of IL-1β, tumor necrosis factor-α, and interferon-γ. Anti-inflammatory effects of IL-4 on human bronchial epithelial cells have also been described by Levine and colleagues [58], who demonstrated that IL-4 increased the release of IL-1RA. Our results substantiate these data and show that IL-4 may act both pro-inflammatory (by increasing IL-8 and MCP-1 release) and anti-inflammatory (by increasing IL-1RA release) on human bronchial epithelial cells. However, the strongest effect of IL-4 was observed on the production of IL-1RA. It may be speculated that in the healthy bronchus, IL-4 is involved in controlling inflammatory reactions by stimulating the release of IL-1RA by bronchial epithelial cells.

In conclusion, bronchial epithelial cells express IL-4 receptor mRNA and protein both *in vivo* and *in vitro*. The functionality of the receptor was demonstrated by the ability of IL-4 to stimulate IL-8, MCP-1, and particularly IL-1RA release by cultured human bronchial epithelial cells. Enhanced levels of IL-4 in the asthmatic airways may therefore, together with other cytokines, result in increased responses of bronchial epithelial cells, thereby resulting in an

increased recruitment and activation of leukocytes. Our results further support a role for the human bronchial epithelium in the control of inflammation and indicate that IL-4 may stimulate bronchial epithelial cells to produce inflammatory mediators.

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Chapter 11

Interleukin-1β and interferon-γ differenially regulate release of monocyte chemotactic protein-1 and interleukin-8 by human bronchial epithelial cells

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ABSTRACT

Airway inflammation is characterized by an accumulation of activated leukocytes. Bronchial epithelial cells may contribute to this process by releasing chemokines and by expressing surface membrane molecules involved in the adhesion and activation of the recruited leukocytes. In this study, we analyzed the effects of cytokines and glucocorticoids on the release of monocyte chemotactic protein-1 (MCP-1), a potent chemoattractant for predominantly monocytes and luymphocytes, by human bronchial epithelial cells and compared this with the release of interleukin-8 (IL-8), which potently attracts neutrophils. In addition, we analyzed the effects of cytokines and glucocorticoids on the epithelial expression of intercellular adhesion molecule (ICAM)-1, CD40, and human leukocyte antigen (HLA) class II molecules.

Primary cultures of human bronchial epithelial cells constitutively released MCP-1 and IL-8. IFN-γ strongly increased MCP-1 release, which was accompanied by increased expression of MCP-1 mRNA and an increased monocyte chemotactic potential. In contrast, IFN-γ had no effect on the release of IL-8, but it did increase the epithelial expression of ICAM-1, CD40, and HLA class II molecules. IL-1β increased both MCP-1 and IL-8 release, and increased the expression of ICAM-1 and CD40, but not HLA class II molecules. Dexamethasone partially inhibited the cytokine-induced release of MCP-1 and IL-8 and the expression of ICAM-1, CD40, and HLA class II molecules by human bronchial epithelial cells.

Our results indicate that IFN-γand IL-1β differentially regulate the MCP-1 and IL-8 release by human bronchial epithelial cells. In addition, IL-1β and particularly IFN-γ increase the expression of ICAM-1, HLA class II and/or CD40 molecules, which are involved in the adhesion and possibly activation of the recruited leukocytes. Finally, the beneficial effect of glucocorticoid therapy in airway inflammatory diseases may be mediated in part by inhibition of chemokine release and ICAM-1, CD40, and HLA class II expression by bronchial epithelial cells.

INTRODUCTION

Asthma is a chronic inflammatory disease of the airways. The inflammatory process is characterized by an accumulation of activated leukocytes, predominantly macrophages, T lymphocytes and eosinophils, in the bronchial wall [1, 2]. The recruitment of peripheral blood leukocytes into the airways is mediated through several signals, including adhesion molecules and chemotactic factors produced at the inflammatory focus. MCP-1, a member of the C-C branch of the chemokine family, has chemotactic properties for monocytes, basophils, T lymphocytes and NK cells [3]. In addition, MCP-1 may activate monocytes and basophils, and can induce leukocyte adhesion molecules on endothelial and vascular smooth muscle cells [4-6]. IL-8 is a member of the C-X-C branch of the chemokine family and is *in vitro* predominantly chemotactic for neutrophils [7]. The composition of the cellular infiltrate will therefore be determined, at least in part, by the chemokines present during inflammatory reactions in the airways.

The bronchial epithelium is considered to play an important role in the regulation of inflammatory and immunological reactions in the airways [8]. Bronchial epithelial cells have the capacity to recruit inflammatory cells via the release of chemokines, to direct inflammatory cell adhesion via the expression of cell surface molecules, and to regulate inflammatory cell activity both via the release of cytokines and via expression of cell surface molecules. Bronchial

epithelial cells are known to produce MCP-1 [9] and IL-8 [10]. Increased expression of MCP-1 and IL-8 protein has been found in the bronchial epithelium [11, 12] and in bronchoalveolar lavage fluid [13, 14] of asthmatic subjects compared to normal subjects. IL-8 release by human bronchial epithelial cells has been shown to be increased after stimulation with pro-inflammatory cytokines [10], but at present little is know about the regulation of MCP-1 release by these cells. Human bronchial epithelial cells can also express surface molecules like adhesion molecules, costimulatory molecules, and HLA class II molecules. The bronchial epithelium of asthmatics shows increased expression of ICAM-1 and HLA class II molecules [15, 16], which may contribute to the retention and activation of recruited leukocytes in the asthmatic airways [8].

The release of chemokines and the expression of surface molecules is thought to be modulated by cytokines, which are increasingly recognized to be important in chronic inflammation and play a critical role in orchestrating inflammatory responses. In bronchoalveolar lavage fluid of asthmatics, increased levels of IL-1β, TNF-α, and IFN-γ have been detected compared to healthy controls [17, 18]. Bacterial cell wall products, such as lipopolysaccharide (LPS), may also contribute to the inflammatory process in the airways [19]. In contrast, glucocorticoids, which are the most effective drugs in the treatment of asthma [20], reduce the number of mast cells, eosinophils, lymphocytes and monocytes in the bronchial wall [21]. These anti-inflammatory effects of glucocorticoids may be mediated, at least in part, by inhibition of the MCP-1 and IL-8 release by bronchial epithelial cells and by a reduced epithelial expression of ICAM-1, HLA class II, and CD40 molecules.

The present study was designed to investigate the effect of cytokines (IL-1β, IFN-γ, TNF-α), LPS and glucocorticoids on the release of MCP-1 by human bronchial epithelial cells. The effects of these stimuli were compared with the effects on IL-8 release by human bronchial epithelial cells to determine whether the release of these chemokines was regulated differentially. In addition, we investigated the effects of IL-1β and IFN-γ, which were the most potent stimulators of MCP-1 release, on the expression of ICAM-1, HLA class II, and costimulatory CD40 molecules by human bronchial epithelial cells, to determine whether these mediators could also be involved in the modulation of leukocyte adhesion and possibly leukocyte activation. Finally, we investigated the effects of glucocorticoids on the cytokine-induced effects on chemokine release and surface marker expression.

MATERIALS AND METHODS

Reagents

Cytokines used in this study were IL-1β (10 U/ng, UBI, Lake Placid, NY, USA), TNF-α (5 U/ng, UBI), and IFN-γ (20 U/ng, Boehringer Ingelheim, Germany). A 10 mM stock solution of the synthetic glucocorticoid dexamethasone micronisatum (DEX; Duchefa b.v., Haarlem, The Netherlands) was prepared in ethanol and stored at −20°C. LPS (E. coli 0127:B8) was purchased from Difco Laboratories (Detroit, MI, USA).

Cell culture

Bronchial tissue was obtained from patients undergoing surgery for lung cancer and used immediately for culture of the epithelial cells by a cell culture method described previously [22]. Cells were characterized as epithelial cells by immunofluorescence staining using a mouse

monoclonal antibody directed against a number of human cytokeratins (CK-1; DAKOpatts, Glostrup, Denmark). At least 99% of the isolated cells stained positive for cytokeratin (data not shown).

Stimulation experiments with cytokines and/or dexamethasone

To study the effects of cytokines and dexamethasone, human bronchial epithelial cells were plated onto 6-well culture dishes (0.5 x 10^6 cells/well). After the cells reached 80 to 90% confluence, the culture medium was replaced by a basal medium (DMEM/F12 (1:1) with penicillin G sodium and streptomycin sulfate), and IFN-γ, IL-1β, TNF-α, LPS and/or DEX were added. Supernatants were collected after 24 h (unless stated otherwise), centrifuged and stored at –80°C for subsequent analysis. In some experiments, cells were used for RNA isolation or flowcytometry as described below.

Analysis of MCP-1 and IL-8 release

Levels of immunoreactive MCP-1 in the culture medium were quantified in a previously described sandwich ELISA (sensitivity: 30 pg/ml) [23].

Levels of immunoreactive IL-8 in the culture medium were quantified using a commercially available ELISA (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands), according to the manufacturer's instruction (sensitivity: 1 pg/ml).

RNA isolation and semi-quantitative polymerase chain reaction (PCR) analysis

Total cellular RNA was isolated from human bronchial epithelial cells according to Chomczynski and Sacchi [24] with some small modifications [22]. For cDNA synthesis a mixture containing 1 µg of total cellular RNA and 10 µg/ml oligo$(dT)_{15}$ (Pharmacia, Uppsala, Sweden) was heated at 70°C for 5 min, cooled down to room temperature and the RNA was subsequently reversed transcribed to cDNA as described previously [25].

Semi-quantitative PCR analysis, using serially diluted cDNA samples, was performed essentially as described by Horikoshi [26]. Hypoxanthine phosphatidyl ribosyltransferase (HPRT) was used as an internal control for total cellular mRNA. The reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 pM sense and 1 pM antisense oligonucleotide primers (synthesized with a DNA Synthesizer 380B (Applied Biosystems, Forster City, CA)), and 20 U/ml Taq polymerase (Amplitaq, Perkin-Elmer Cetus, Norwalk, CT) in a total volume of 50 µl. Primers used are (5' to 3'): MCP-1 sense: CTG GAG CAC AAC ATG AAA AGG; MCP-1 antisense: AGT CAG GTT GTC TGG ACT CTG [9]; HPRT sense: GTG ATG ATG AAC CAG GTT ATG ACC TT; HPRT antisense: CTT GCG ACC TTG ACC ATC TTT GGA [27]. The amplified cDNA products are 510 and 454 basepairs, respectively. Samples were amplified in a DNA Thermocycler (Perkin-Elmer Cetus) for 30 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 55°C (HPRT) or 65°C (MCP-1) for 30 sec, and extension at 72°C for 1 min. These conditions were shown to be optimal in preliminary experiments. Ten µl aliquots of PCR products were electrophoresed on 1% agarose, blotted to Nytran N nylon membranes (Schleicher and Schuell, Dassel, Germany) and fixed to the membrane with a 254 nm UV crosslinker. Oligonucleotides, being complementary to internal sequences of the PCR products, were end-labeled with ^{32}P-γATP (ICN Pharmaceuticals Inc., Irvine, CA, USA) by T4 polynucleotide kinase (Pharmacia LKB, Piscataway, NJ). Oligonucleotide probes used are (5' to 3'): TGG CCA GAG AGC ATC AGC GT (MCP-1) and GAA GAG CTA TTG TAA TGA CCA GTC A (HPRT). Blots were hybridized according to standard procedures, and subsequently exposed to phosphor screens (Molecular Dynamics,

Sunnyvale, CA, USA). The intensity of the radio-active signals was measured using a PhosphorImager (type Storm 820; Molecular Dynamics) and analyzed using ImageQuaNT software (Molecular Dynamics). The resulting intensity values were plotted against the amount of RNA originally present in the samples, and a line was fitted in the linear part of the graph. The amount of RNA was calculated as the slope of the MCP-1-curve divided by the slope of the HPRT-curve [26].

Chemotaxis

Monocyte chemotaxis was measured in a microchemotaxis assay as described by Falk [28]. A 48-well microchemotaxis chamber (Neuro Probe Inc., Cabin John, MD, USA) was used. The bottom wells were filled with bronchial epithelial cell culture supernatants, culture medium (negative control), or 10^{-8} M N-formyl-Methionyl-Leucyl-Phenylalanine (fMLP; positive control). The upper chambers were filled with 50 µl cell suspension containing 20,000 monocytes. The test was always performed in triplo. The wells were separated by a polycarbonate filter with 5 µm pores (Nuclepore, Pleasanton, CA, USA). After 30 min, migrated cells were swept of using a windscreenwiper. Subsequently the filters were air dried and stained with Coomassie blue. Migrated cells were counted in a blinded fashion either automatically using a VIDAS-RT image-analyzing computer (Kontron Elektronik GmbH, Neufarn, Germany) or under a microscope at magnification of 10x40. Chemotaxis was calculated from the mean number of migrated cells in the presence of culture supernatant minus the mean number of migrated cells with basal medium (to which corresponding cytokines were added).

To evaluate specific MCP-1-mediated chemotaxis, a neutralizing monoclonal antibody directed against MCP-1 (MAB279, 5 µg/ml; R&D Systems, Abingdon, United Kingdom) was added to the culture supernatants, after which monocyte chemotaxis was determined as described above.

Flowcytometry

For immunofluorescence staining of human bronchial epithelial cells, the following mAb were used: anti-CD40 (Immunotech, Marseille, France), anti-HLA class II (HLA-DR; Becton Dickinson, San Jose, CA, USA), BBA-4 (anti-ICAM-1 (CD54), British Biotechnology Products Ltd., Oxon, United Kingdom), and Leu-4 (CD3, control for nonspecific binding; Becton Dickinson). Bronchial epithelial cells were rinsed with PBS and detached using 0.02% EDTA. Cells were harvested in PBS/0.5% BSA, washed and resuspended in PBS/BSA to a final concentration of 2×10^6 cells/ml. Flowcytometry was performed as described previously [29]. Fluorescence intensities were quantified using calibrated fluorescence standards (FCSC Quantum 26, Research Triangle Park, NC, USA) and expressed as Molecules of Equivalent Soluble Fluorescence (MESF) [29].

Statistical analysis

Data are expressed as mean ± SEM. The Mann-Whitney *U* test was used to assess significant differences in chemokine release, chemotactic potential, or marker expression between stimulated and unstimulated cultures of human bronchial epithelial cells. A p-value of less than 0.05 was considered significant.

RESULTS

Modulation of MCP-1 and IL-8 protein release by cytokines and LPS

To investigate the modulation of MCP-1 release, primary cultures of human bronchial epithelial cells (>99% pure) were stimulated with IL-1β, TNF-α, IFN-γ, or LPS. Unstimulated human bronchial epithelial cells released small amounts of MCP-1 (0.58 ± 0.12 ng/10^6 cells/24 h). As shown in figure 1 (left part), MCP-1 release was significantly increased when human bronchial epithelial cells were stimulated for 24 h with IL-1β, TNF-α, or particularly IFN-γ. In contrast, LPS did not significantly modulate the MCP-1 release. Simultaneous stimulation with IFN-γ, IL-1β and/or TNF-α resulted in approximately additive effects (data not shown).

The effect of inflammatory mediators on the release of MCP-1 was compared with the effect on the release of IL-8, a neutrophil chemotactic cytokine. Unstimulated human bronchial epithelial cells released 55.8 ± 6.8 ng IL-8/10^6 cells/24 h. In contrast to MCP-1, IL-8 release by human bronchial epithelial cells was not modulated by IFN-γ at concentrations up to 200 U/ml (Fig. 1 and data not shown). IL-1β dose- and time-dependently increased the release of IL-8 by human bronchial epithelial cells (Fig. 1 and data not shown). LPS also increased the IL-8 release, whereas TNF-α did not have a significant effect (Fig. 1).

Since IFN-γ and IL-1β were the most potent stimulators of MCP-1 release, the effects of both cytokines were analyzed in more detail. As shown in figure 2A, both IFN-γ and IL-1β increased MCP-1 release in a concentration dependent manner. However, the IFN-γ-induced increase was stronger and could already be observed at lower doses. The IFN-γ-induced increase in MCP-1 production could be detected as early as 4 h after stimulation and increased with time up to 24 h (Fig. 2B). IL-1β also induced a time-dependent increase in MCP-1 levels, with the earliest effect measurable 6 h after stimulation (Fig. 2B). Thus, both IFN-γ and IL-1β strongly increased MCP-1 release in a time- and dose-dependent manner, but only IL-1β increased the release of IL-8 by human bronchial epithelial cells.

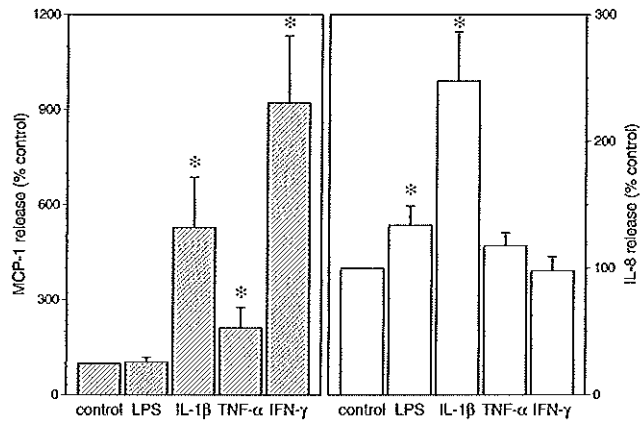

Figure 1. Modulation of MCP-1 and IL-8 release by LPS and cytokines. Human bronchial epithelial cells were stimulated with LPS (1 μg/ml), IL-1β (200 U/ml), TNF-α (100 U/ml), or IFN-γ (200 U/ml) for 24 h, after which the MCP-1 (left) and IL-8 (right) release were determined by ELISA. Data are expressed as percentage release compared to unstimulated cells (mean ± SEM; n≥3). * : $p < 0.05$ compared to unstimulated cells.

Modulation of MCP-1 mRNA by IL-1β and IFN-γ

To investigate whether the effects of IFN-γ and IL-1β on MCP-1 protein release were paralelled by increased MCP-1 mRNA levels, RNA was isolated 6 h and 24 h after stimulation with IFN-γ or IL-1β and mRNA levels were determined using a semi-quantitative PCR analysis. Stimulation with IFN-γ resulted in increased MCP-1 mRNA levels. After 6 h, a 2 fold increase was observed (data not shown); after 24 h MCP-1 mRNA levels were increased 3-fold (Fig. 3). IL-1β slightly increased MCP-1 mRNA levels after 6 h (1.4 fold), but after 24 h levels had returned to control values (data not shown).

Effect of IL-1β and IFN-γ on monocyte chemotaxis

To determine whether the changes in MCP-1 release were reflected in the capacity to attract monocytes, supernatants of stimulated bronchial epithelial cells were tested in chemotaxis assays. As shown in figure 4, conditioned media derived from human bronchial epithelial cells stimulated

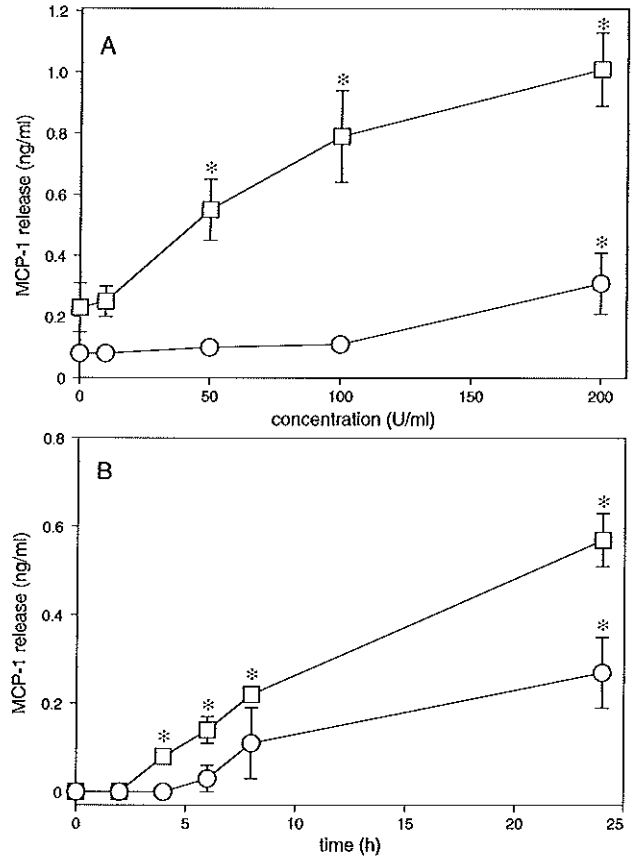

Figure 2. IL-1β and IFN-γ increase MCP-1 release by human bronchial epithelial cells in a concentration-and time-dependent manner. A: Human bronchial epithelial cells were stimulated with IL-1β (0-200 U/ml; circles) or IFN-γ (0-200 U/ml; squares) for 24 h, after which MCP-1 levels were determined by ELISA (mean ± SEM; n≥3). B: Human bronchial epithelial cells were stimulated with IL-1β (200 U/ml; circles) or IFN-γ (200 U/ml; squares) for 0 to 24 h, after which MCP-1 levels were determined using ELISA. Data are expressed as mean ± SEM (n≥3) after subtracting basal release of MCP-1 at each individual time-point. * : $p < 0.05$ compared to unstimulated cells.

with IFN-γ for 24 h, consistently increased monocyte chemotaxis ($p<0.05$). The monocyte chemotaxis induced by conditioned media derived from human bronchial epithelial cells stimulated with IL-1β was increased compared to conditioned media of unstimulated cells, but the increase was more heterogeneously and did not reach statistical significance (Fig.4; p =0.081). In three experiments the specific contribution of MCP-1 to the monocyte chemotaxis was determined by using a neutralizing antibody directed against MCP-1. Addition of this antibody to conditioned medium derived from unstimulated cells reduced chemotactic activity by 19±19%. Chemotaxis induced by conditioned medium derived from IL-1β- or IFN-γ-stimulated cells was inhibited by 31±16% and 49±9%, respectively.

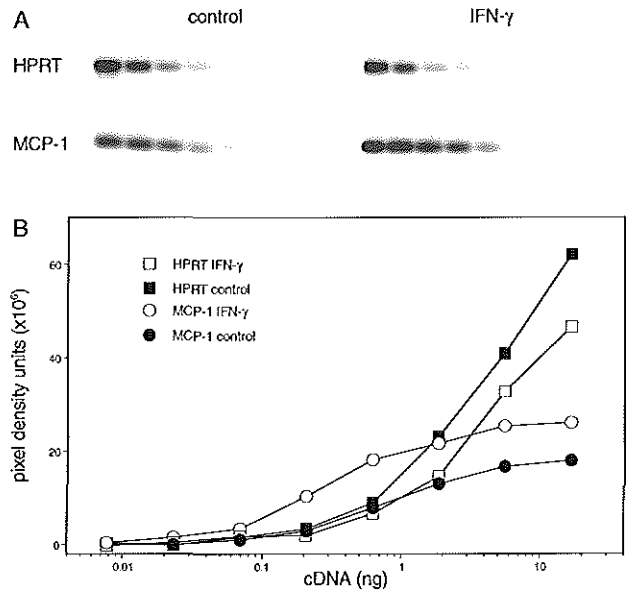

Figure 3. IFN-γ increases mRNA levels for MCP-1 in human bronchial epithelial cells. Human bronchial epithelial cells were incubated for 24 h in the absence (left) or presence (right) of IFN-γ (200 U/ml), after which RNA was isolated. RT-PCR analysis was performed as described in the Materials and Methods section. Hybridization signals (A) were quantified using ImageQuant software and plotted against the amount of RNA originally present (B). A representative experiment out of four is shown.

Effect of IL-1β and IFN-γ on ICAM-1, HLA class II, and CD40 expression

To determine whether the cytokines that induced an increase in chemokine release were also able to increase the expression of cell surface molecules involved in leukocyte adhesion and activation, we analyzed the expression of ICAM-1, HLA class II, and CD40 on human bronchial epithelial cells. Unstimulated human bronchial epithelial cells expressed ICAM-1 (12.2 ± 2.9 x 10^4 MESF) and CD40 (7.6 ± 0.8 x 10^4 MESF), but HLA class II expression was low or absent (0.4 ± 0.3 x 10^4 MESF). IFN-γ increased CD40 expression, and strongly enhanced ICAM-1 and HLA class II expression by human bronchial epithelial cells (Table 1). Stimulation with IL-1β increased ICAM-1 and CD40 expression, but did not affect HLA class II expression (Table 1).

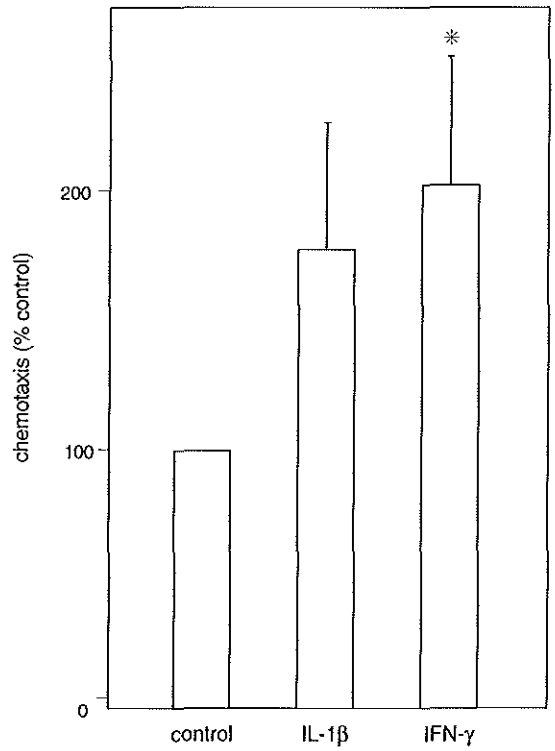

Figure 4. Effect of bronchial epithelial cell culture supernatants on monocyte chemotaxis. Data are expressed as the mean number of monocytes ± SEM which have transmigrated a membrane filter towards conditioned media derived from human bronchial epithelial cells stimulated by IL-1β (200 U/ml) or IFN-γ (200 U/ml) for 24 h (n≥4).

Table 1. ICAM-1, HLA-DR, and CD40 expression by human bronchial epithelial cells: effects of IL-1β (200 U/ml), IFN-γ (200 U/ml), and/or DEX (10^{-6} M) (24 h stimulation) ‡.

Cytokine	-		IL-1β		IFN-γ	
DEX	-	+	-	+	-	+
ICAM-1	100	69 ± 2 *	208 ± 3 *	134 ± 33 *†	980 ± 4 *	216 ± 7 *†
HLA-DR	100	259 ± 108 *	114 ± 3 *	321 ± 79 *†	2455 ± 2178*	387 ± 205 *†
CD40	100	93 ± 9	168 ± 2 *	132 ± 2 *†	138 ± 10 *	91 ± 9 †

‡ Data are expressed as relative expression compared with unstimulated cells (mean ± SEM; n ≥ 2).

* $p < 0.05$ compared to unstimulated cells.

† $p < 0.05$ compared to stimulation in the absence of DEX.

Effect of Dexamethasone on cytokine-induced chemokine release and marker expression

To investigate the effect of glucocorticoids on chemokine release and adhesion molecule expression by human bronchial epithelial cells, cells were stimulated with cytokines in the absence and presence of DEX (10^{-6} M). DEX did not affect unstimulated MCP-1 (Fig. 5) or IL-8 release (100 ± 9% compared to unstimulated cells). Costimulation of human bronchial epithelial

cells with cytokines and DEX resulted in decreased release of MCP-1 compared to stimulation with cytokines or LPS alone (Fig. 5). However, DEX never reduced MCP-1 release to basal (unstimulated) levels and thus only partially inhibited the cytokine-induced MCP-1 release. DEX also partially reduced the IL-1β-induced IL-8 release (331 ± 98% without DEX; 255 ± 80% with DEX) and completely inhibited the LPS-induced IL-8 release (134 ± 15% without DEX; 103 ± 15% with DEX).

Basal and cytokine-stimulated ICAM-1 expression was significantly inhibited by DEX (Table 1). DEX also inhibited the cytokine-stimulated expression of CD40, and slightly enhanced the low basal expression of HLA class II. IFN-γ-stimulated HLA class II expression was significantly reduced by simultaneous stimulation with DEX (Table 1).

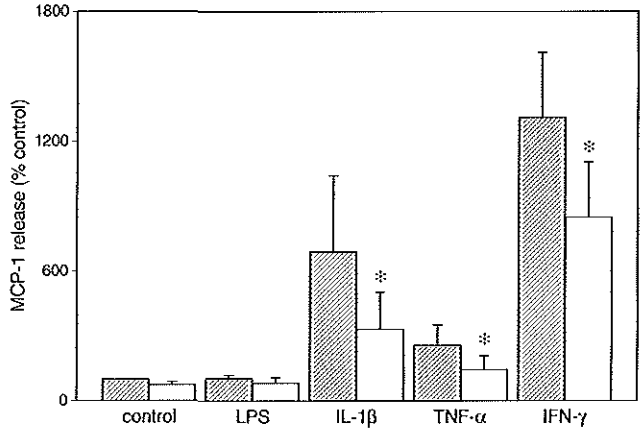

Figure 5. Effect of dexamethasone on basal and cytokine-stimulated MCP-1 release by human bronchial epithelial cells. Human bronchial epithelial cells were stimulated with LPS (1 μg/ml), IL-1β (200 U/ml), TNF-α (100 U/ml) or IFN-γ (200 U/ml) or in the absence (hatched bars) or presence (open bars) of 1 μM dexamethasone for 24 h, after which MCP-1 release was determined by ELISA. Data are expressed as percentage release compared to unstimulated cells (mean ± SEM; n≥3). * : $p < 0.05$ compared to cells stimulated in the absence of DEX.

DISCUSSION

In this study, we show that IFN-γ is a strong stimulator of MCP-1, but not of IL-8 release by human bronchial epithelial cells. The increased release of MCP-1 is parallelled by increased MCP-1 mRNA levels and increased monocyte chemotactic potential. In addition, IFN-γ upregulates the expresion of ICAM-1, HLA class II, and CD40 molecules, which may contribute to the retention and activation of the recruited leukocytes. IL-1β increased both MCP-1 and IL-8 release, and stimulated the expression of ICAM-1 and CD40, but not HLA class II molecules. DEX partially inhibited MCP-1 and IL-8 release and reduced the expression of ICAM-1, HLA class II, and CD40 molecules by human bronchial epithelial cells.

While basal MCP-1 release was low, pro-inflammatory cytokines, in particular IFN-γ, strongly increased MCP-1 release by human bronchial epithelial cells. Although cytokine-induced MCP-1 release has been observed in a variety of cell types, including mononuclear phagocytes, mesothelial cells, and epithelial cells [9, 30, 31], bronchial epithelial cells are to our knowledge unique in that IFN-γ is the most potent inducer of MCP-1. In contrast to MCP-1, IL-8 release by

human bronchial epithelial cells was not affected by IFN-γ, a phenomenon which has also been observed in human microvascular endothelial cells, renal cortical epithelial cells, and airway smooth muscle cells [32-34]. Increased levels of IFN-γ, produced by activated T lymphocytes, have been found in bronchoalveolar lavage of patients with intrinsic asthma compared to control groups [17]. In addition, increased levels of IFN-γ, together with TNF-α, were observed in bronchoalveolar lavage of allergic patients after antigen challenge [35]. IFN-γ is also produced during viral infections, which are important triggers of asthmatic attacks [36]. Therefore, viral- or antigen-induced production of IFN-γ may result in the release of MCP-1 by human bronchial epithelial cells, thereby contributing to the influx of monocytes and lymphocytes.

IFN-γ also upregulated the expression of ICAM-1 and HLA class II molecules on bronchial epithelial cells, which is in accordance with previous reports [37, 38]. In addition, we show that human bronchial epithelial cells express CD40 and that IFN-γ (and also IL-1β) upregulated the expression of this molecule. CD40, which plays a critical role in the regulation of immune responses [39], may interact with CD40 ligand expressed on T lymphocytes, thereby activating these cells. Thus, IFN-γ not only increases the release of MCP-1 by human bronchial epithelial cells, but it also stimulates the epithelial expression of molecules involved in adherence and activation of the recruited monocytes and lymphocytes. Although we did not determine the capacity of IFN-γ-stimulated bronchial epithelial cells to activate lymphocytes, previous studies have shown that human bronchial epithelial cells may function as antigen-presenting cells [37, 40]. Furthermore, preliminary data indicate that stimulation of human bronchial epithelial cells with an antibody directed against the CD40 molecule stimulates the release of IL-6 and the expression of ICAM-1, indicating that adherence of lymphocytes may also modulate epithelial cell functions (V.H.J. van der Velden, unpublished data).

IL-1β is considered as an early response cytokine [41] and is likely to be present in the lung of patients with airway inflammatory diseases such as asthma [17, 18]. The release of early response cytokines is important for induction of adhesion molecule expression, the initiation of cytokine cascades, the upregulation of specific chemokines and the recruitment of leukocyte subsets [41]. In this study, we demonstrate that IL-1β, in contrast to IFN-γ, increased the release of both MCP-1 and IL-8 by human bronchial epithelial cells. In addition, IL-1β stimulated the expression of ICAM-1 and CD40, but not HLA class II molecules. The 5'-upstream transcriptional regulatory regions of the human MCP-1, IL-8, and ICAM-1 gene all contain consensus sequences for the transcription factors NF-κB and AP-1 [42-45], which both can be activated by IL-1β. Therefore, it is very likely that these transcription factors are involved in the IL-1β-mediated increase in MCP-1 and IL-8 release and ICAM-1 expression by human bronchial epithelial cells.

Our findings and previous studies suggest that IFN-γ predominantly increases the release of C-C chemokines, and not of C-X-C chemokines by human bronchial epithelial cells [46, 47]. Although the effect of IFN-γ was not studied on the release of other C-X-C chemokines, we hypothesize that IFN-γ may be a relatively specific stimulator for the influx of monocytes and lymphocytes compared to IL-1β and TNF-α, which can stimulate the recruitment of all types of leukocytes. This effect may even be more pronounced since IL-1β, TNF-α, and LPS have recently been shown to induce a rapid down-regulation of CCR-2 expression, the main receptor for MCP-1 [48, 49].

LPS increased the release of IL-8 by human bronchial epithelial cells, whereas it had no effect on the release of MCP-1, suggesting that LPS may selectively attract neutrophils. Bacterial infections are a common characteristic of patients with chronic obstructive pulmonary disease (COPD) [50] and increased levels of IL-8 have been detected in sputum of patients with COPD

compared to healthy subjects [51]. Therefore, LPS-mediated IL-8 release may contribute to the influx of neutrophils observed in patients with COPD [1, 51].

Dexamethasone decreased the cytokine-induced release of MCP-1 and IL-8 by human bronchial epithelial cells, but in general no reduction to basal levels was observed. Thus far, no studies have been performed on the molecular mechanism of dexamethasone-mediated inhibition of MCP-1. However, the presence of AP-1 and NF-κB sites in the MCP-1 gene suggests that, similar to IL-8, the inhibitory effect of dexamethasone may be mediated, at least in part, through the repression of NF-κB activity by the glucocorticoid-glucocorticoid receptor complex and by a glucocorticoid-induced induction of IκB protein [52]. In addition to the inhibitory effects of DEX on chemokine release, DEX also inhibited the expression of ICAM-1, HLA-DR and CD40 molecules by human bronchial epithelial cells. To our knowledge, this is the first report on the effects of glucocorticoids on ICAM-1, HLA-DR, and CD40 molecule expression by primary cultures of human bronchial epithelial cells. Using bronchial epithelial cell lines, glucocorticoid-mediated inhibition of basal and IFN-γ-induced ICAM-1 expression has been described [53, 54]. Stimulation with glucocorticoids thus not only inhibits chemokine release by human bronchial epithelial cells, but also reduces the epithelial expression of molecules involved in the adhesion and activation of the recruited leukocytes. Thus, inhaled glucocorticoids may limit the recruitment as well as the adherence and subsequent activation of leukocytes in the asthmatic airways.

In conclusion, IFN-γ is a potent stimulator of MCP-1 but not IL-8 release by human bronchial epithelial cells, and simultaneously increases the expression of ICAM-1, HLA-DR, and CD40 on these cells. In contrast, IL-1β increases both MCP-1 and IL-8 release. Therefore, IFN-γ may be a relatively specific and potent stimulator of the influx, adherence and possibly activation of monocytes and lymphocytes into the human lung. Finally, part of the beneficial effect of glucocorticoid therapy in airway inflammatory diseases may be mediated via the inhibition of MCP-1 and IL-8 release and ICAM-1, HLA-DR, and CD40 molecule expression by bronchial epithelial cells.

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GENERAL DISCUSSION AND SUMMARY

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Chapter 12

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Asthma is a chronic inflammatory disease of the airways, characterized by increased numbers of eosinophils, mast cells, and activated T lymphocytes, and by increased levels of inflammatory mediators (reviewed in chapter 1). The inflammatory process in the airways is the result of complex interactions between inhaled allergens, cells of the immune system, nerves, and structural cells, like epithelial cells, endothelial cells, and fibroblasts. These interactions are mediated via cell-cell contact and via the release of a variety of mediators, including cytokines, chemokines, and neuropeptides.

Peptidases (reviewed in chapter 3) play an important role in the modulation of peptide mediated effects, like neurogenic inflammation, and may affect several cell functions, including cellular activation, antigen presentation, and cellular migration and adhesion. In the lungs, reduced expression of peptidases may contribute to the pathogenesis of asthma by enhancing (neurogenic) inflammatory reactions.

Bronchial epithelial cells (reviewed in chapter 4) contribute to the initiation and perpetuation of the inflammatory response by releasing and expressing molecules which are involved in the recruitment, adhesion, and activation of leukocytes. In addition, bronchial epithelial cells are considered an important site for peptidase activity.

Glucocorticoids (reviewed in chapter 5) are widely used in the treatment of asthma as they have potent anti-inflammatory effects. The beneficial effects of treatment of asthmatic patients with glucocorticoids may be mediated in part by modulation of peptidases or by modulation of bronchial epithelial cell functions.

This chapter briefly discusses the results of the studies described in this thesis in relation to our present understanding of asthma. Future research directions will be proposed.

12.1. Peptidases: important in asthma?

Neurogenic inflammation mimics many of the pathophysiological features of asthma, and a role for neuropeptides in the pathogenesis of asthma has been implicated [1]. Although the apparent increased effects of the sensory neuropeptide may be due to several mechanisms (see chapter 2.4), studies using laboratory animals have indicated that peptidases play a major role in limiting neurogenic inflammatory responses. Initial studies have focussed on the role of neutral endopeptidase 24.11 (NEP) in the modulation of tachykinin-induced bronchoconstriction, mucus secretion, vasodilation, and microvascular leakage. In the past few years, it became clear that neuropeptides also have many immunomodulatory functions (see chapter 2). Thus, neuropeptides not only evoke bronchoconstriction by direct effects on smooth muscle cells, submucosal glands, and blood vessels, but also may contribute to the initial and chronic phase of the airway inflammation observed in asthmatics.

In addition to NEP, several other peptidases are able to hydrolyze (neuro)peptides and therefore may be involved in the modulation of peptidergic effects. Aminopeptidase N (APN) and dipeptidyl peptidase IV (DPP IV) are of particular interest, since both enzymes are membrane-bound molecules (and thus able to cleave extracellular peptides) and both enzymes have been characterized both on non-hematopoietic cells and hematopoietic cells (where they are known as the CD13 and CD26 antigen, respectively).

Localization of APN and DPP IV in the human bronchus

Using immunohistochemistry and enzymehistochemistry we analyzed the distribution of APN and DPP IV in the human bronchus (chapter 7). APN was widely distributed, being present on blood vessels, glandular ducts, connective tissue, perichondrium, and nerves. Many of these sites also possess NEP activity [2], which is in accordance with the proposed sequential inactivation of peptides by NEP and APN [3]. Thus, NEP and APN may collaborate on the surface of these cells to modulate the cell's response towards peptide-mediated signals. In contrast to NEP, no expression of APN was found on smooth muscle cells and bronchial epithelial cells. This is in accordance with the lack of effect of APN-inhibitors on tachykinin-induced smooth muscle contraction [4-7].

In addition to structural airway cells, mononuclear phagocytes, eosinophilic granulocytes and certain dendritic cells expressed APN. APN expression on granulocytes has been shown to be involved in the modulation of chemotactic responses [3, 8], whereas APN expression on mononuclear phagocytes and dendritic cells may be involved in processing of major histocompatibility class II-bound peptides [9]. In addition, some preliminary data indicate that APN may be involved in the maturation and/or differentiation of dendritic cells (V.H.J. van der Velden, unpublished data).

Comparison of APN expression in bronchial biopsies of allergic asthmatics and healthy subjects revealed an increased number of APN-expressing cells in the bronchial epithelium of asthmatics. The number of APN-expressing cells correlated with the number of L25-positive dendritic cells and with the number of eosinophils found in the bronchial epithelium. Double-stainings showed that both cell types indeed are able to express APN, which is in accordance to the known distribution of CD13 on these cells [10-12]. Therefore, it seems likely that the increase in APN-positive cells in the bronchial epithelium reflects the increase of dendritic cells and eosinophils observed in the bronchial epithelium of asthmatics. Bronchial epithelial cells of asthmatic patients can release increased amounts of chemokines and express higher levels of adhesion molecules compared to healthy controls (chapter 11 and [13-18]). As a consequence, eosinophils and dendritic cells can be recruited by, and be retained in the bronchial epithelium.

Alternatively, the increased number of APN-expressing cells in the bronchial epithelium of asthmatic patients could be due to an upregulation or induction of APN on certain leukocytes. Previous studies have shown that APN can be upregulated on the surface of mononuclear phagocytes by interleukin (IL)-4 [19]. Asthma is considered a Th2-like disease [20], and increased levels of IL-4 have been detected in asthmatic airways [21-23]. To determine whether increased expression of APN on mononuclear phagocytes is a feature of asthma, it would be of interest to compare the expression of APN on alveolar macrophages of healthy individuals and allergic asthmatics. A recent study has indicated that APN expression can also be induced on T lymphocytes after adhesion to epithelial cells [24]. However, it is not likely that this explains the increased number of APN-expressing cells in the asthmatic patients, since there was no increase in the number of T lymphocytes in the bronchial epithe-

lium of asthmatics compared to healthy controls. The lack of difference in the number of APN-positive cells in the lamina propria is probably due to the observation that the majority of APN is expressed on non-hematopoietic cells. It was therefore not possible to accurately quantify APN expression on infiltrating leukocytes.

DPP IV expression was strongly present in serosal submucosal glands and moderately expressed on blood vessels, predominantly post-capillary venules. DPP IV expression in submucosal glands did not seem to be restricted to the cell membrane, but appeared to be located intracellularly as well. It is not clear whether the DPP IV of submucosal glands is secreted in the bronchial lumen. However, DPP IV activity can be detected in bronchoalveolar lavage (BAL) fluid (chapter 8). DPP IV has also been found in submandibular and parotid glands [25, 26], and a role for DPP IV in the secretion or reabsorption process of secretory proteins and peptides has been suggested [25]. In glandular endometrial epithelial cells from cows, a DPP IV molecule missing the signal sequence has been detected. Further studies (*e.g.* immuno-electron microscopy, sequencing of the protein and mRNA) are required to determine the characteristics of the DPP IV molecule in serosal submucosal glands. Endothelial cells were shown to express all peptidases examined (NEP, APN, DPP IV, APA), but the distribution amongst arteries, capillaries and venules showed some marked differences. The site-restricted presence of different peptidases may represent a mechanism to control blood flow and plasma leakage at specific locations. Studies using selective inhibitors are needed to determine the physiologic functions and relevance of the different peptidases expressed on endothelial cells.

DPP IV expression could also be found on T cells (double labeling with CD3). DPP IV has been shown to be a marker for activated T cells [27, 28] and plays an important role in T cell responses (see chapter 3.3.2). Comparison of DPP IV expression between bronchial biopsies of healthy controls and allergic asthmatics did not reveal significant differences, suggesting that the number of activated T cells did not differ. Several other studies have shown that the airways of allergic asthmatics contain increased numbers of activated, but not total, T cells [29-33]. This apparent discrepancy may be explained by recent studies indicating that DPP IV is predominantly expressed on Th0 and Th1 cells [34, 35], whereas many T cells in the airways of allergic asthmatics show a Th2-like phenotype [21, 22].

The expression of NEP in the human bronchus has been described by Baraniuk et al. [2]. NEP was found on epithelial cells, smooth muscle cells, submucosal glands, and endothelial cells. In our study (chapter 7), we used enzymehistochemistry to determine the distribution of NEP in the human bronchus. A very weak NEP activity was observed, but attribution of this activity to a certain cell type was difficult. Low levels of activity were observed in the bronchial epithelium and submucosal glands. In contrast, we observed strong NEP activity in the guinea pig trachea, especially in the tracheal epithelium, as has also been found previously [36]. This may indicate that (epithelial) NEP is much more important in the modulation of neurogenic inflammatory reactions in the guinea pig than in humans. Alternatively, this may suggest that peptidergic mechanisms are less prominent in humans compared to guinea pigs. Indeed, whereas a dense network of tachykinin-containing peptidergic nerves can be found in the airways of rodents, peptidergic innervation of human airways seems sparse [37-39].

Since NEP activity in the human bronchus was hard to detect, we were not able to determine whether there was a difference in NEP activity between bronchial biopsies of healthy controls and allergic asthmatics. In another study it was found that asthmatics treated with steroids expressed significantly more NEP on their bronchial epithelium than did nonsteroid-treated asthmatics [40]. However, in this study no comparison was made between non-asth-

matic subjects and asthmatic patients. Therefore, it remains to be established whether this difference was due to a reduced NEP expression in the nonsteroid-treated patients that could be reversed by the use of steroids, or that inhaled steroids increased the normal expression of NEP. The latter possibility is supported by the increased upregulation of NEP on human bronchial epithelial cells by steroids (see chapter 9) and the lack of an obvious difference in NEP activity between control subjects and mildly asthmatic subjects found in an *in vivo* study [41].

Modulation of peptidases expressed on bronchial epithelial cells

Studies using laboratory animals have shown that NEP present on the bronchial epithelium plays a major role in the hydrolysis of neuropeptides, and thereby in modulating neurogenic inflammation [4, 7, 42-47]. NEP activity may be modulated by a variety of exogenous stimuli, like viral infections, ozone, and cigarette smoke (see chapter 3.6). In contrast, at the time this study started little was known about the modulation of NEP activity on bronchial epithelial cells by endogenously released mediators. In chapter 9 we therefore addressed two questions: 1) what is the effect of cytokines (IL-1β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-4, and epidermal growth factor (EGF)) on the activity and expression of peptidases on human bronchial epithelial cells; and 2) what is the effect of glucocorticoids on the activity and expression of peptidases on human bronchial epithelial cells? Since large numbers of bronchial epithelial cells in primary culture are hard to obtain, we used in these studies the bronchial epithelial cell line BEAS 2B as a model. Our results indicate that both cytokines and glucocorticoids modulate the expression and activity of NEP and APN on human bronchial epithelial cells (chapter 9). NEP expression and activity was increased by IL-1β and, to a lesser extent, TNF-α and IL-4, whereas IFN-γ significantly reduced NEP. It has been shown that lung fibroblasts increase NEP activity after stimulation with IL-1α, IL-6, and TNF-α and that this upregulation, which could already be detected after 6 h of stimulation, is dependent upon prostaglandin synthesis and elevation of cAMP [48]. In human bronchial epithelial cells, the effect of IL-1β was mediated in part by a cAMP-dependent pathway, since a phosphodiesterase inhibitor enhanced the IL-1β-mediated increase in NEP activity. In addition, a cAMP analogue mimicked the effect of IL-1β, although this effect had a more rapid onset. Prostaglandins did not seem to be involved, and the exact mechanism by which IL-1β, and also TNF-α and IL-4, increase NEP expression in bronchial epithelial cells remains to be determined. IL-1β and TNF-α are considered to be potent pro-inflammatory cytokines. It is therefore surprising that both cytokines upregulated the expression of NEP, which can be considered as an anti-inflammatory molecule. As IL-1β and TNF-α only increased NEP expression and activity after 48 h of stimulation, one could speculate that upregulation of NEP by these cytokines may down-regulate (neurogenic) inflammatory responses.

Stimulation of human bronchial epithelial cells with IFN-γ reduced NEP activity and expression. IFN-γ is produced during viral infections, which are associated with increased bronchial responsiveness and with asthmatic exacerbations [49]. In several animal models, infection with Influenza virus or Sendai virus was shown to result in enhanced bronchoconstrictor responses to tachykinins, an effect that was mediated by decreased epithelial NEP activity [6, 50-52]. Our results suggest that the virus-induced reduction in NEP activity is mediated, at least in part, via the release of IFN-γ. Inhibition of NEP activity may result in reduced degradation of neuropeptides and thereby contribute to the airway hyperresponsiveness observed during viral respiratory infections. Studies examining the inhibitory effect

of NEP inhibition on tachykinin-induced bronchoconstriction in subjects with and without respiratory infections should clarify whether such mechanisms occur *in vivo*.

In contrast to NEP, APN expression and activity was hardly affected by cytokines. Only IFN-γ modulated APN expression and activity: a transient increase in APN expression and acti-vity was detected 24 h after stimulation. Upregulation of APN by IFN-γ has also been observed in glomerular epithelial cells [53], but not in monocytes [19], suggesting that this effect may be specific for epithelial cells. Indeed, separate promoters for APN can be found in epithelial and myeloid cells and this may account for the difference in their responsiveness [54]. Since IFN-γ also induces the expression of human leukocyte antigen (HLA) class II molecules by human bronchial epithelial cells (chapter 11 and [55, 56]), increased expression of APN may be involved in the processing of HLA class II-bound peptides [57].

Stimulation of BEAS 2B cells with the synthetic glucocorticoid dexamethasone (DEX) strongly increased NEP and APN expression and activity, both in the absence and in the presence of cytokines (chapter 9). These effects were mediated via the glucocorticoid receptor and appeared to be specific for glucocorticoids and not for other steroid hormones. Using the CD13 monoclonal antibody WM-15, which specifically inhibits APN activity, we could show that the DEX-mediated increase in APN-like activity was completely due to an increase in APN activity. These results were confirmed by flowcytometry, which showed a similar increase in APN expression. Thus, DEX does not modulate the expression of other aminopeptidases able to cleave alanyl-paranitroanilide on human bronchial epithelial cells. Furthermore, these data provide further evidence that WM-15 completely and specifically inhibits APN activity, which supports some previous reports [58, 59]. In accordance to our data, an *in vivo* study has shown increased expression of NEP on the bronchial epithelium of steroid-treated asthmatics compared to nonsteroid-treated asthmatics [40]. Part of the beneficial effects of glucocorticoid treatment in asthma may thus be mediated via upregulation of NEP by human bronchial epithelial cells, thereby limiting neurogenic inflammation.

Our study has several limitations that should be kept in mind when examining the results. First, during inflammatory processes *in vivo* several cytokines are simultaneously present, whereas in our study we did not investigate the effects of cytokine combinations on peptidase expression. Since IFN-γ decreased NEP expression and IL-1β, TNF-α, and IL-4 increased NEP expression, it would be interesting to determine the effect of IFN-γ in combination with one of the other cytokines. Second, we stimulated the cells when the monolayers were approximately 80-90% confluent. NEP expression is dependent on the confluence and proliferation of the cells (chapter 9 and [60]). In our experiments, IFN-γ decreased and EGF increased cell numbers. However, NEP and APN activity were only affected by cell numbers exceeding 0.19×10^6 cells/cm^2 and during our experiments cell numbers consistently remained below 0.17×10^6 cells/cm^2. It is therefore not likely that the effects of cytokines are due to effects on cell numbers or proliferation. Nevertheless, we can not exclude the possibility that confluent cells respond in a different way. Finally, in this study we used the bronchial epithelial cell line BEAS 2B. Although in several studies this cell line has been shown to be an appropriate model for human bronchial epithelial cells [55, 61], the results obtained in this study should be confirmed using primary cultures of human bronchial epithelial cells.

Soluble peptidases in serum and bronchoalveolar lavage fluid

Although NEP, APN, and DPP IV are normally membrane-bound enzymes, soluble counterparts can be detected in serum and BAL fluid (chapter 8). Several studies have shown that peptidase activities may be altered in serum of patients with a malignancy or inflammatory

disorder. Increased serum activity of NEP has been observed in underground miners exposed to coal dust particles [62] and in patients with adult respiratory distress syndrome (ARDS) [63] or sarcoidosis [64]. NEP activity in serum probably arises from shedding of the entire membrane-bound peptidase [62], which may reflect local tissue damage or activation of granulocytes [62, 63, 65]. DPP IV activity in serum has recently been shown to originate, at least in part, from the DPPL-T antigen expressed on the surface of activated T cells [66], whereas serum APN activity predominantly comprises a circulating isoform of the CD13 antigen [67]. There is evidence that serum DPP IV activity is decreased in patients with a malignancy and in auto-immune and inflammatory disorders [68-72]. In contrast to serum, until recently little was known about the presence of peptidases in BAL fluid. In chapter 8, we demonstrate for the first time that NEP and APN activity can be detected in human BAL fluid, and confirm the recent data on DPP IV and APN-like activity in BAL fluid [73].

Comparison of peptidase activities in serum and BAL fluid from healthy controls and stable asthmatics did not reveal significant differences in NEP and DPP IV activity, whereas APN activity (expressed per mg protein) was reduced in BAL fluid of asthmatics. However, APN activity expressed per ml BAL fluid was not significantly different between healthy controls and allergic asthmatics. In addition, after treatment of asthmatics with either placebo or fluticasone propionate (which both did not significantly affect APN activity) no difference in APN activity between asthmatics and healthy controls was observed anymore. The significance of this observation is therefore not completely clear. The lack of difference in NEP activity in serum and BAL fluid between healthy controls and allergic asthmatics may indicate that NEP activity is not altered in the airways of asthmatics. It may also indicate that there is little tissue damage in the airways of stable asthmatic patients. One could speculate that tissue damage occurs during asthmatic exacerbations and that this may cause increased peptidase activities in serum [62, 63]. Remarkably, our preliminary data suggest that also during and up to 5 days after an asthmatic exacerbation no increase in peptidase activities can be observed in serum.

In addition to increased peptidase levels in serum due to tissue damage, increased serum peptidase activities may reflect activation of granulocytes sequestered in the airways [63]. In contrast to ARDS, which is characterized by strongly increased numbers of neutrophils in the airways, the numbers of (eosinophilic) granulocytes in the asthmatic airways are relatively low. Thus, if NEP and/or APN were released from granulocytes sequestered in the airways of asthmatic airways, the absolute amounts probably will be low. Furthermore, we and others did not observe a correlation between peptidase activities in serum or BAL fluid and cell numbers of leukocytes (chapter 8 and [73]), suggesting that there is no predominant hematopoietic source of the soluble peptidases in healthy controls or asthmatic patients. During other pathological conditions (such as neoplasms, infections or sarcoidosis) increased numbers of granulocytes or lymphocytes in the airways may significantly contribute to the activities of APN and DPP IV in BAL fluid [73].

We cannot rule out the possibility that similar NEP activity in BAL fluid (and serum) from healthy controls and asthmatics is the result of a reduced membrane-bound NEP activity (either due to reduced expression or inactivation of the enzyme) together with increased shedding of the enzyme. To determine whether inactivation of NEP occurs in asthmatics, data on NEP activity in BAL fluid should be compared with ELISA data. Analysis of soluble intercellular adhesion molecule (ICAM)-1 or cytokeratin-19 levels may indicate whether increased shedding or epithelial injury occurs in the airways of asthmatics compared to healthy controls [74-76]. Finally, NEP expression and activity should be determined in bronchial

biopsies from healthy controls and allergic asthmatics.

Although we did not find major differences in peptidase activities in BAL fluid from allergic asthmatics and healthy controls, it will be of interest to determine the sources of NEP, APN, and DPP IV in BAL fluid. Comparison of serum and BAL fluid revealed that activities (expressed as nmoles/mg protein/min) of all peptidases studied were considerably higher in BAL fluid and that there was no correlation between peptidase activities in BAL fluid and serum. These findings suggest that the presence of peptidases in these two compartments is regulated independently of each other and suggest local release of the enzymes in the airways. Several cell types present in the airways express peptidases on their surface (chapters 4 and 7, and [2, 73]), but little is known about the shedding of these molecules from the membranes. Our preliminary results indicate that NEP activity can be detected in culture supernatants of human bronchial epithelial cells and alveolar macrophages. It remains to be established whether shedding of peptidases by these cells is a regulated process that can be modulated by (anti-)inflammatory stimuli. Since NEP, APN, and DPP IV may exist in several isoforms, which can be distinguished based on their molecular weight [66, 77-80], revealing the molecular weight of the peptidases present in the BAL fluid may give some indications about the source of these enzymes. Furthermore, the striking correlation between NEP and APN activity in BAL fluid strongly suggests that both enzymes are either released by the same source or that both enzymes are regulated in a similar way.

Treatment of allergic asthmatics with the glucocorticoid fluticasone propionate for 12 weeks significantly improved their lung function (chapter 9). In contrast, no effects were observed on peptidase activities in BAL fluid or serum. To our knowledge, no other studies thus far have determined the effects of glucocorticoids on peptidase activities in serum or BAL fluid. *In vitro* studies have shown that glucocorticoids upregulate the expression of NEP on human bronchial epithelial cells (chapter 9 and [81, 82]). In addition, treatment of asthmatic patients with inhaled glucocorticoids increases NEP expression by the bronchial epithelium [40]. Thus, (inhaled) glucocorticoids increase the surface membrane expression of NEP on bronchial epithelial cells, but do not affect soluble NEP activity in BAL fluid. This may indicate that NEP activity in BAL fluid is not derived from bronchial epithelial cells. Otherwise, shedding of NEP from the surface of bronchial epithelial cells may not be a random process but may be affected by glucocorticoids (which upregulate surface membrane expression but possibly reduce the relative amount of NEP shedded from the membrane). Analysis of NEP activity in culture supernatants of human bronchial epithelial cells stimulated with or without steroids will give a definite answer.

Current view on peptidases in asthma

Studies on the role of peptidases in the pathogenesis of asthma have not been able to convincingly demonstrate a dysfunction of these enzymes in the airways of stable asthmatics. Although asthmatic airways are more responsive to tachykinin-induced bronchoconstriction and nasal congestion [83-85], no apparent reduction in NEP activity could be found in stable mild asthmatic patients [41]. Our studies indicate that peptidase activities in BAL fluid and serum do not remarkably differ between healthy controls and allergic asthmatics (chapter 9). In addition, we did not observe major differences in the expression of APN and DPP IV between bronchial biopsies of asthmatics and healthy controls (chapter 7). No data are currently available on the expression of NEP in the airways of asthmatics compared to healthy subjects, although some data may suggest a reduced NEP expression in the bronchial epithelium, but not the lamina propria, from nonsteroid treated asthmatics [40]. It seems therefore

unlikely that there is a generally reduced activity of peptidases in the airways of stable asthmatic patients.

Peptidases may however be involved in exacerbations of asthma. Several stimuli that may trigger asthmatic exacerbations (including ozone [86, 87], viruses [51, 52], cigarette smoke [88], toluene diisocyanate [89], and possibly allergens [90]) have been shown to reduce NEP activity in animal models and to stimulate sensory nerves, either directly or indirectly. Increased levels of substance P and neuropeptide Y together with decreased levels of vasoactive intestinal peptide have been found in the serum of patients with an asthmatic exacerbation [91], and this may reflect reduced NEP activity and increased activity of tryptic enzymes.

To further determine whether peptidases and neuropeptides contribute to asthma, *in vivo* studies using selective neurokinin receptor antagonists should be performed both in the presence and absence of NEP or other peptidase inhibitors. Neurokinin receptor antagonists should first be tested against tachykinin-induced bronchoconstriction in order to determine the optimal dose of the antagonists. Second, the effects of these antagonists should be analyzed in allergen-induced bronchoconstriction, both in the absence and in the presence of peptidase inhibitors. Furthermore, it would be interesting to treat allergic asthmatics with appropriate neurokinin receptor antagonists (either intravascular or by inhalation) for a longer period of time, and to determine whether this affects allergen-induced bronchoconstriction and bronchial inflammation (as determined by analysis of bronchial biopsies and BAL fluid). This will give insight in the contribution of tachykinins to the (chronic) inflammatory process in the airways of asthmatic patients. Finally, the contribution of tachykinins and peptidases in asthma may be demonstrated by treating asthmatic patients with recombinant NEP, and analyzing the effects on bronchoconstriction induced by allergens or environmental agents such as cigarette smoke.

12.2. Bronchial epithelial cells: actions and reactions

The bronchial epithelium forms the interface between the respiratory system and the inspired air. Therefore, bronchial epithelial cells may be exposed to all molecules present within the inspired air, including allergens, environmental factors, and drugs. To defend the airways against the entry of noxious substances, bronchial epithelial cells form a tight and continuous layer that functions as a physical barrier. The bronchial epithelial cells also have cilia, which contribute to the mucociliary clearance, and secrete mediators that provide protection against a wide range of potentially injurious agents (chapter 2). In addition, bronchial epithelial cells may participate in the initiation and perpetuation of inflammatory reactions by releasing mediators and by expressing surface membrane molecules, which may interact with other cells or mediators.

Human bronchial epithelial cells express interleukin-4 receptors

The inflammatory process in the asthmatic airways is characterized by an increased number of leukocytes, especially eosinophils and activated T cells [92-94]. In addition, several studies have shown an increased presence of Th2 cells and Th2 cell-derived cytokines, such as IL-4, in bronchial biopsies and BAL fluid of asthmatic patients compared to healthy controls [21-23]. In addition to T lymphocytes, eosinophils are an important source of IL-4 in the in-

flamed bronchus [21, 22], and both cell types can be found in close proximity to the bronchial epithelium. Therefore, it is likely that bronchial epithelial cells will be exposed to IL-4 during inflammatory and immunological responses within the lung. However, at the time this study started little was known about the presence of receptors for IL-4 on human bronchial epithelial cells and the effects of IL-4 on epithelial cells. In the studies described in chapter 10 of this thesis, we present evidence that human bronchial epithelial cells express receptors for IL-4 (IL-4R). Expression of IL-4R mRNA and protein *in vivo* was determined using *in situ* hybridization and immunohistochemistry, respectively. Both methods demonstrated expression of IL-4R on the bronchial epithelium. Expression of the IL-4R has also been described for other epithelial tissues, like intestinal epithelium [95], epidermal cells [96], and breast epithelium [97], suggesting that expression of IL-4R is a general feature of epithelial cells. In addition to the bronchial epithelium, IL-4R expression was found on smooth muscle cells. This is in accordance with a recent report indicating that stimulation of human airway smooth muscle cells with IL-4 inhibits cytokine-induced IL-8 release [98]. In addition, IL-4R expression was observed on certain leukocytes and we observed that IL-4R expression in the lamina propria correlated with the number of activated (EG2-positive) eosinophils and the number of activated (CD26-positive) T cells in the lamina. Eosinophils are indeed known to express IL-4R [99], however, it is not known at present whether these receptors are upregulated on activated eosinophils. Unstimulated T cells express low levels of IL-4R, but the number of IL-4R is upregulated upon activation [100]. Therefore, one may speculate that the correlation between IL-4R expression and number of activated eosinophils or activated T cells is a reflection of the increased IL-4R expression on these cells. Alternatively, activated T cells or activated eosinophils may secrete mediators (for example IL-4) which subsequently increase the IL-4R expression in a paracrine manner.

Comparison of IL-4R expression between bronchial biopsies from healthy controls and allergic asthmatics did not reveal significant differences, neither in the epithelium nor in the lamina propria (chapter 10). The lack of difference in bronchial epithelial IL-4R expression between asthmatics and healthy subjects and the limited effects of stimuli on bronchial IL-4R expression *in vitro* suggest that the expression of IL-4R on human bronchial epithelial cells is rather constitutive. Increased expression of IL-4R has been found in some epithelial proliferative diseases, such as psoriasis [96], suggesting that IL-4 may be involved in the proliferation of keratinocytes. We did not observe effects of IL-4 on human bronchial epithelial cell numbers *in vitro* (chapter 9 and unpublished data) and increased epithelial cell proliferation is not a characteristic feature of asthma. Although IL-4R expression levels did not seem to differ between healthy controls and allergic asthmatics, we cannot exclude the possibility that the affinity of the IL-4R for IL-4 is changed or that there was a difference in IL-4R signalling (see below).

Analysis of IL-4R expression (both mRNA and protein) *in vitro* showed that cultured human bronchial epithelial cells also expressed IL-4R. In addition, mRNA and protein of the common γ chain was detected (chapter 10). On many cells, the IL-4R is a heterodimeric complex comprising the IL-4R α chain and a second chain. The IL-4R α chain is shared with some forms of the IL-13 receptor [101-104]. IL-4 can also bind to certain, but not all IL-13R, whereas IL-13 cannot bind to the IL-4R [101-103]. The second subunit of the IL-4R is, at least in some cells, the common γ chain, which is also used by the IL-2R, IL-7R, IL-9R, and IL-15R [105, 106]. Some recent reports have indicated that in certain cell types IL-4 may signal via the IL-4R in the absence of the common γ chain [107-110]. Although we did not directly determine the subunit structure of the IL-4R, we hypothesize that in human bronchial epithelial cells IL-4 signals, at least in part, via binding to an IL-4R composed of the IL-4R α chain and the common

γ chain. Further studies have to be performed to determine whether human bronchial epithelial cells also express one or more forms of the IL-13R α chains [102-104, 108].

Stimulation of cultured human bronchial epithelial cells with IL-4 caused an increased release of IL-8, monocyte chemotactic protein (MCP)-1, and particularly IL-1 receptor antagonist (IL-1RA) (chapter 10). Thus, IL-4 present in the asthmatic airways may contribute to the increased expression of these three molecules in the bronchial epithelium of asthmatics [13, 14, 111]. Some other recent reports have also demonstrated an effect of IL-4 on human bronchial epithelial cells. In one study, it was shown that IL-4 upregulated the release of granulocyte/macrophage colony-stimulating factor, thereby stimulating eosinophil survival [112]. In contrast, other studies have shown that IL-4 may have anti-inflammatory effects on human bronchial epithelial cells by inhibiting cytokine-induced RANTES expression [113] or inducible nitric oxide synthase expression [114]. In the latter studies, IL-4 was added 30 min prior to stimulation of the cells by a mixture of IL-1β, TNF-α, and IFN-γ ('cytomix'). Preliminary data indicate that pre-treatment with IL-4 also reduced the cytomix-induced release of MCP-1 (unpublished data). Anti-inflammatory effects of IL-4 on human bronchial epithelial cells have recently also been described by Levine and colleagues [115], who demonstrated that IL-4 increased the release of IL-1RA. Our results substantiate these data and show that IL-4 may act both pro-inflammatory (by increasing IL-8 and MCP-1 release) and anti-inflammatory (by increasing IL-1RA release), but the data obtained thus far suggest that the effects of IL-4 on human bronchial epithelial cells are predominantly anti-inflammatory.

The effects of IL-4 stimulation were only analyzed in cultures of human bronchial epithelial cells derived from non-asthmatic patients. One could speculate that epithelial cells derived from asthmatic patients respond differently. Interestingly, a recent report has shown the presence of a novel IL-4R α chain allele, in which guanine was substituted for adenine at nucleotide 1902 [116]. The resulting receptor protein, with a glutamine to arginine replacement at position 576, showed enhanced signalling as determined by the IL-4-induced upregulation of CD23 on peripheral blood mononuclear cells. Furthermore, a strong association of this gain-of-function mutation in the α subunit of the IL-4R with atopy was found. It remains to be established whether stimulation of bronchial epithelial cells, obtained from atopic individuals bearing the mutated allele, with IL-4 results in increased release of MCP-1, IL-8, and IL-1RA, and whether this enhancing effect is similar for all three cytokines.

Recruitment of leukocytes: chemokine release and expression of molecules involved in adhesion and activation

Accumulation of leukocytes in the lung is dependent upon the presence of chemokines and the expression of appropriate adhesion molecules. Bronchial epithelial cells are able to produce a variety of chemokines and to express surface membrane molecules involved in the adhesion or activation of the recruited leukocytes, such as ICAM-1 and HLA class II (chapter 4). Bronchial epithelial cells from asthmatic patients show an increased epithelial expression of MCP-1, IL-8, HLA-DR, and ICAM-1 compared to healthy subjects [13-16], which may contribute to the increased numbers of leukocytes observed in the asthmatic airways [92-94]. In chapter 11 we present data on the release of MCP-1, the prototype C-C chemokine, and IL-8, the prototype C-X-C chemokine, by human bronchial epithelial cells. We show that IFN-γ strongly increased the release of MCP-1, whereas it did not affect the IL-8 release. IFN-γ-stimulated MCP-1 release has also been demonstrated in mononuclear phagocytes, mesothelial cells, and epithelial cells [117-119]. Furthermore, the lack of effect of IFN-γ on IL-8 release by bronchial epithelial cells is in accordance with the lack of effect found in human

microvascular endothelial cells, renal cortical epithelial cells, and airway smooth muscle cells [98, 120, 121]. These findings and previous studies suggest that IFN-γ predominantly increases the release of C-C chemokines (*e.g.* MCP-1 and RANTES), and not of C-X-C chemokines (*e.g.* IL-8) by human bronchial epithelial cells [113, 122]. IFN-γ is present in increased amounts in the BAL fluid of patients with intrinsic asthma compared to control groups [29], especially after antigen challenge [123]. IFN-γ is also produced during viral infections, which are important triggers of asthmatic attacks [49]. Therefore, viral- or antigen-induced production of IFN-γ may result in the release of MCP-1 by human bronchial epithelial cells, thereby contributing to the influx of monocytes and lymphocytes. Since IFN-γ also increased the epithelial expression of ICAM-1, HLA class II, and CD40 molecules, the recruited leukocytes may adhere to the epithelium (via ICAM-1) and possibly be activated (via HLA class II-associated antigen peptides and CD40 molecules). Other studies have demonstrated that viral infections indeed increase the expression of ICAM-1 on airway epithelial cells [124-127]. Moreover, virus-infected epithelial cells showed increased adhesion of lymphocytes, neutrophils, and eosinophils, which could be inhibited by an ICAM-1 blocking antibody [126, 128]. Upregulation of ICAM-1 expression by viral infections may also cause an increased susceptibility to infections [124], since ICAM-1 is the receptor for the major group rhinoviruses [129]. Further studies are required to determine whether IFN-γ-activated bronchial epithelial cells show increased adhesion of monocytes and lymphocytes and whether adhesion to the bronchial epithelium results in their activation. Subsequently, the contribution of the distinct molecules in these processes should be determined using neutralizing antibodies. Our results indicate that stimulation of bronchial epithelial cells with IFN-γ may contribute to the increased epithelial expression of MCP-1, HLA-DR, and ICAM-1 found in bronchial biopsies of asthmatic patients [13, 15, 16].

IL-1β is a potent pro-inflammatory cytokine and is considered as an early-response cytokine [130]. It is able to upregulate the expression and/or release of a variety of molecules, including adhesion molecules, chemokines, and cytokines, thereby quickly but aspecifically evoking inflammatory responses [131]. In the study described in chapter 11, we demonstrate that IL-1β, in contrast to IFN-γ, increased the release of both MCP-1 and IL-8 by human bronchial epithelial cells. In addition, IL-1β stimulated the expression of ICAM-1 and CD40, but not HLA class II molecules. The increased expression of ICAM-1 on bronchial epithelial cells by IL-1β is in accordance with some previous reports [55, 56, 132]. The differences between the effects of IL-1β and IFN-γ can be explained, at least in part, by different intracellular signaling pathways. Whereas stimulation of NF-κB and/or AP-1 activity may be the most prominent signaling pathway of IL-1β [133], IFN-γ predominantly activates the JAK/STAT pathway [134, 135]. Indeed, many effects of IL-1β are mediated via activation of the transcription factors NF-κB and AP-1 and recognition sites for these transcription factors can be found in the promoter region of the MCP-1, IL-8, and ICAM-1 gene [136-139]. The exact mechanisms by which IFN-γ increases the expression of these molecules still remains to be elucidated.

Glucocorticoids partially inhibited the cytokine-induced release of MCP-1 and IL-8 and the expression of ICAM-1, CD40, and HLA class II molecules on human bronchial epithelial cells *in vitro*. These effects may be mediated, at least in part, through the repression of NF-κB and/or AP-1 activity by the glucocorticoid-glucocorticoid receptor complex and by a glucocorticoid-induced induction of IκB protein [140]. In addition, a negative glucocorticoid-responsive element has been described on the 5'-flanking region of the IL-8 gene [141]. Glucocorticoid-mediated inhibition of basal and IFN-γ-induced ICAM-1 and HLA class II

expression has also been described for epithelial cell lines [142-144]. *In vivo* studies have shown that treatment of allergic asthmatics with inhaled glucocorticoids reduces the number of activated T lymphocytes in bronchial biopsies and BAL fluid [145-149]. Our results indicate that this effect may be mediated, in part, by the inhibition of MCP-1 release by the bronchial epithelial cells, and by a reduction in the expression of ICAM-1, CD40, and HLA class II molecules. Inhaled glucocorticoids, however, did not modulate the ICAM-1 expression by bronchial epithelial cells from asthmatics *in vivo* [150], but in that particular study also no increased epithelial expression of ICAM-1 was found in asthmatics compared to healthy controls. In contrast, topical nasal glucocorticoid therapy in patients with nasal polyposis was associated with a reduced expression of ICAM-1 and HLA-DR in the epithelium [151]. To our knowledge, no data are currently available on the effects of inhaled glucocorticoids on MCP-1, IL-8, or CD40 expression by bronchial epithelial cells *in vivo*.

12.3. Conclusions

Based on the results obtained from the work presented in this thesis, we draw the following conclusions.

1. APN is widely distributed in the human bronchus, being expressed on endothelial cells, glandular ducts, connective tissue, perichondrium, nerves, and certain leukocytes (in particular granulocytes, mononuclear phagocytes, and certain dendritic cells). An increased number of APN-expressing cells can be found in the bronchial epithelium of allergic asthmatic patients. DPP IV is expressed on serosal submucosal glands and leukocytes (predominantly T cells) in the human bronchus. Expression of DPP IV is not altered in bronchial biopsies of asthmatic patients compared to healthy subjects.

The activities of NEP, APN, and DPP IV in BAL fluid and serum of asthmatic patients are not altered compared to healthy controls. In contrast, non-asthmatic smokers display reduced DPP IV activity in their serum and reduced NEP and APN activity in their BAL fluid.

2. Cytokines can upregulate (IL-1β, TNF-α, IL-4) or downregulate (IFN-γ) the expression and activity of NEP on BEAS 2B cells, but have little effects on APN expression and activity.

Human bronchial epithelial cells express receptors for IL-4 and stimulation of bronchial epithelial cells with IL-4 results in an increased release of both pro- (IL-8 and MCP-1) and anti-inflammatory mediators (IL-1RA). There is no difference in the expression of IL-4 receptors between healthy controls and allergic asthmatics.

Upon stimulation with IFN-γ, human bronchial epithelial cells increase their release of MCP-1, but not IL-8, and increase their surface expression of ICAM-1, HLA class II, and CD40 molecules. IL-1β stimulates human bronchial epithelial cells to release both MCP-1 and IL-8, and increases their surface expression of ICAM-1 and CD40, but not HLA class II molecules.

3. Glucocorticoids upregulate the expression and activity of NEP and APN by BEAS 2B cells. In contrast, glucocorticoids reduce the IL-1β or IFN-γ-mediated release of MCP-1

and/or IL-8 and inhibit the IL-1β or IFN-γ-mediated increase in ICAM-1, HLA class II, and CD40 molecule expression by human bronchial epithelial cells.

The activity of NEP, APN, and DPP IV in BAL fluid and serum is not altered by treatment with inhaled glucocorticoids.

Thus, comparison of the expression and activity of peptidases in the human airways of healthy subjects and allergic asthmatics does not support the hypothesized dysfunction of these enzymes in stable asthma. Regarding bronchial epithelial cells, our data further indicate that these cells play an important role in the inflammatory process observed in the asthmatic airways. Finally, glucocorticoids may exert their anti-inflammatory effects in part by modulation of bronchial epithelial peptidases and cell functions.

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Summary
Samenvatting

SUMMARY

Asthma is clinically defined by a reversible airway obstruction and bronchial hyperreactivity. Nowadays, a chronic inflammation of the airways is thought to underlie these clinical features. Bronchial biopsies and bronchoalveolar lavage (BAL) fluid of asthmatic patients show increased numbers of eosinophils, activated lymphocytes, and mast cells compared to healthy controls. In addition, increased levels of inflammatory mediators, such as cytokines, neuropeptides, and chemokines, can be found in asthmatic airways. In *chapter 1* a brief overview is given on asthma, with emphasis on the immunological aspects.

Autonomic nerves (reviewed in *chapter 2*) play an important role in the regulation of bronchial smooth muscle tone, secretion of mucus, and blood flow in the airways. In addition to the well-known parasympathetic (cholinergic) and sympathetic (adrenergic) nervous systems, non-adrenergic non-cholinergic (NANC) innervation can be found in the airways. The inhibitory-NANC system, which is the only neural bronchodilator pathway in the human airways, is localized in parasympathetic nerves, whereas the excitatory-NANC system is located predominantly in a subpopulation of sensory nerves. Stimulation of sensory nerves can, via a local axon reflex, result in the release of neuropeptides. These neuropeptides have a variety of effects, including the contraction of smooth muscle cells, the secretion of mucus, vasodilation, microvascular leakage, and the recruitment and activation of leukocytes. This sequence of events is now known as neurogenic inflammation. Since neurogenic inflammation mimics many of the pathophysiologic features of asthma, a role for neuropeptides in the pathogenesis of asthma has been implicated.

The effects of neuropeptides are normally limited by rapid degradation by peptidases (reviewed in *chapter 3*). A reduced activity of peptidases may therefore result in exaggerated responses to neuropeptides and thus in neurogenic inflammation. In the human bronchus, several peptidases can be found, including neutral endopeptidase (NEP), aminopeptidase N (APN), and dipeptidyl peptidase IV (DPP IV). These peptidases are not only involved in the modulation of neurogenic inflammation, but may also affect several proliferative and immunological responses. Studies using animal models have indicated that the bronchial epithelium is an important site for peptidase activity. Bronchial epithelial cells form the interface between the inhaled air and the respiratory system. These cells may therefore be exposed to an array of molecules present within the inhaled air, such as allergens, other environmental factors (including viruses, ozone, cigarette smoke, chemical irritants), and drugs. Several of these agents are known to reduce peptidase activity, thereby exaggerating the neurogenic inflammation.

Bronchial epithelial cells not only form a passive physical barrier but are also able to participate in the initiation and perpetuation of inflammatory reactions. Besides expressing peptidases, bronchial epithelial cells express several molecules on their surface that are involved in the adhesion and activation of leukocytes. In addition, bronchial epithelial cells are able to release a variety of mediators, which may recruit and activate leukocytes. The bronchial epithelium and its functions are reviewed in *chapter 4*.

Glucocorticoids are widely used in the treatment of asthma. They possess potent anti-inflammatory effects, which may underlie their clinical efficacy. In *chapter 5*, the mechanisms of action of glucocorticoids are briefly reviewed, with special attention to the effects of glucocorticoids on epithelial cell functions.

In the studies described in this thesis, we aimed to further define the contribution of peptidases and the bronchial epithelium to the inflammatory process in the asthmatic airways. In addition, we aimed to determine the effect of glucocorticoids on peptidases and the bronchial epithelium. The aims of the studies are described in *chapter 6*.

In the studies described in chapters 7, 8, and 9, we investigated the expression and activity of peptidases in the airways of healthy subjects and allergic asthmatics, both in bronchial tissue and in the bronchoalveolar lumen. In addition, we determined whether glucocorticoids could modulate the peptidase expression and/or activity. We hypothesized that the expression and/or activity of peptidases is reduced in the airways of asthmatic patients, thereby contributing to exaggerated (neurogenic) inflammatory reactions, and that glucocorticoids increase the expression and/or activity of peptidases in the airways.

In *chapter 7* we present data on the distribution of APN and DPP IV in the human bronchus. The distribution of both enzymes was determined using immunohistochemistry and enzymehistochemistry, and compared with the distribution of NEP. APN expression and activity was found on blood vessels, connective tissue, glandular ducts, perichondrium, and leukocytes, predominantly mononuclear phagocytes, dendritic cells, and eosinophils. DPP IV expression and activity was present in serosal submucosal glands, blood vessels, and T lymphocytes. DPP IV in serosal submucosal glands appeared to be localized predominantly intracellularly, suggesting that DPP IV may be secreted in the bronchial lumen. NEP activity was weak in the human bronchus and appeared to be present in the bronchial epithelium, submucosal glands, blood vessels, and smooth muscle cells. In contrast, NEP activity was easily detected in the guinea pig trachea, especially in the epithelium. Thus, APN and DPP IV are expressed at specific sites within the human bronchus, where they may be involved in the modulation of the cell's response towards peptidergic stimuli.

Comparison of the expression of APN and DPP IV in bronchial biopsies of healthy controls and allergic asthmatics revealed no significant differences in the lamina propria. In contrast, in the bronchial epithelium of the allergic asthmatics, an increased number of APN-positive cells was found. Weak but significant correlations were found between the number of APN-positive cells and the number of eosinophils and L25-positive dendritic cells. Using double-stainings, we confirmed the presence of APN on these cells.

Although peptidases normally are membrane-bound enzymes, soluble counterparts of these molecules can be found in serum and BAL fluid. In *chapter 8* we analyzed the activity of NEP, APN, and DPP IV in serum and BAL fluid of healthy non-smokers, smokers, and allergic asthmatics. In addition, we analyzed whether treatment of allergic asthmatics with the inhaled glucocorticoid fluticasone propionate for twelve weeks could alter the activity of peptidase in serum and/or BAL fluid. NEP, APN, and DPP IV activity could be detected both in serum and in BAL fluid. The activity of all three peptidases (expressed per mg protein) was higher in BAL fluid than in serum, suggesting local release of the enzymes in the airways. No correlations were found between peptidase activities and cell numbers in serum or BAL fluid, indicating no predominant hematopoietic source of the peptidases. NEP activity in BAL fluid correlated with APN activity in BAL fluid, suggesting that both enzymes are regulated in a similar manner.

Comparison of NEP and APN activity in serum did not reveal significant differences between the three groups. In contrast, DPP IV activity was significantly reduced in the serum of smokers compared with healthy non-smokers. In BAL fluid, NEP and APN activity were reduced in smokers. Reduced activity of NEP and APN in BAL fluid may enhance peptide-mediated effects in the airways and thereby promote the inflammatory process or epithelial

proliferation. APN activity (expressed per mg protein) in BAL fluid from allergic asthmatics was reduced compared to healthy non-smokers, whereas NEP and DPP IV activity did not differ. The similar levels of NEP activity in BAL fluid from allergic asthmatics and healthy subjects may indicate that there is no dysfunction of NEP in asthma. Alternatively, similar levels may be due to reduced expression/activity of membrane-bound NEP going together with increased shedding. Treatment of asthmatics with inhaled glucocorticoids improved lung function parameters, but did not affect the peptidase activities in serum and BAL fluid.

Studies using laboratory animals suggested an important role for peptidases expressed on bronchial epithelial cells in the modulation of neurogenic inflammation. Therefore, we analyzed the modulation of epithelial peptidases by cytokines present during inflammatory reactions and by glucocorticoid treatment. We used the human bronchial epithelial cell line BEAS 2B, which expresses NEP and APN, as a model for these studies. In *chapter 9* we show that interleukin (IL)-1β, tumor-necrosis factor (TNF)-α, and IL-4 increased the expression and activity of NEP (as determined by flowcytometry and a specific fluorometric assay, respectively). In contrast, interferon (IFN)-γ reduced the expression and activity of NEP, whereas epidermal growth factor (EGF) did not have an effect. The effect of IL-1β, which was the most potent cytokine in increasing NEP expression and activity, was enhanced by inhibition of phosphodiesterase and mimicked by a cyclic-AMP analogue, suggesting that the effect was mediated in part by a cyclic-AMP dependent pathway. The APN expression and activity, on the other hand, was not modulated by IL-1β, IL-4, TNF-α, or EGF. Stimulation of BEAS 2B cells with IFN-γ transiently increased the APN expression and activity after 24 h. The synthetic glucocorticoid dexamethasone strongly increased the expression and activity of NEP and APN, both in the presence and in the absence of cytokines. This effect of dexamethasone was abolished by the glucocorticoid receptor antagonist RU38486, indicating that the effect was mediated via the glucocorticoid receptor. The synthetic testosterone analogue R1881 had no effect on NEP and APN activity, suggesting that the effect is specific for glucocorticoids and not for steroid hormones in general. The anti-inflammatory effects of glucocorticoids may therefore be mediated in part through upregulation of peptidases expressed on bronchial epithelial cells.

From these studies, we conclude that peptidases are widely distributed in the human bronchus. However, no apparent dysfunction of these enzymes was found in the airways of allergic asthmatic patients. Upregulation of epithelial peptidases by inhaled steroids may, however, have clinical implications.

In chapters 10 and 11, we investigated the role of the bronchial epithelium in the initiation and perpetuation of inflammatory reactions in the airways. Asthmatic airways show an increased number of eosinophils and activated T cells, predominantly of the Th2-like phenotype. Both cell types are able to release IL-4, and increased amounts of IL-4 can be found in bronchial biopsies and BAL fluid of asthmatic patients compared to healthy controls. In *chapter 10*, we present definite evidence of IL-4 receptor expression on human bronchial epithelial cells. IL-4 receptor α chain expression on human bronchial epithelial cells *in vivo* was demonstrated using *in situ* hybridization and immunohistochemistry. No difference in IL-4 receptor protein expression was observed between bronchial biopsies of healthy subjects compared to allergic asthmatics. Cultured human bronchial epithelial cells also expressed IL-4 receptor α chain mRNA and protein (as determined by RT-PCR analysis and flowcytometry, respectively). In addition, cultured bronchial epithelial cells expressed mRNA and protein of the common γ chain, which is a functional component of the IL-4 receptor in many cell types. IL-4 receptor protein expression by bronchial epithelial cells *in vitro* could be increased by

stimulation with phorbol myristate acetate plus calcium ionophore, whereas IL-1β and IL-6 decreased the IL-4 receptor expression. A cyclic-AMP analogue and IL-4 had no effect. Finally, we show that the IL-4 receptor is functionally active as IL-4 stimulates the release of IL-8, monocyte chemotactic protein-1 (MCP-1), and particularly IL-1 receptor antagonist by human bronchial epithelial cells.

From this study we conclude that human bronchial epithelial cells express IL-4 receptors both *in vivo* and *in vitro*. Stimulation of human bronchial epithelial cells by IL-4 may result in the release of both pro- and anti-inflammatory mediators known to be upregulated in asthmatic airways.

Bronchial epithelial cells may participate in the recruitment and activation of leukocytes by releasing chemokines and by expressing molecules which can interact with leukocytes. In *chapter 11* we present data on the effects of cytokines and glucocorticoids on the release of MCP-1, the prototype C-C chemokine, and IL-8, the prototype C-X-C chemokine, by human bronchial epithelial cells. In addition, we analyzed the effects of cytokines and glucocorticoids on the epithelial expression of intercellular adhesion molecule (ICAM)-1, CD40, and human leukocyte antigen (HLA) class II molecules. These surface membrane molecules are involved in the adhesion and activation of the recruited leukocytes.

Primary cultures of human bronchial epithelial cells constitutively produced MCP-1 and IL-8. Stimulation of bronchial epithelial cells with IFN-γ strongly increased the MCP-1 release, which was accompanied by increased expression of MCP-1 mRNA and an increased monocyte chemotactic potential. In contrast, IFN-γ had no effect on the release of IL-8, suggesting that IFN-γ may selectively increase the release of chemokines that attract monocytes and lymphocytes. IFN-γ increased the epithelial expression of ICAM-1, CD40, and HLA class II molecules. IL-1β increased both the MCP-1 and IL-8 release, and increased the expression of ICAM-1 and CD40, but not of HLA class II molecules. These results indicate that IFN-γ and IL-1β differentially regulate the MCP-1 and IL-8 release by human bronchial epithelial cells. In addition, IL-1β and particularly IFN-γ increase the expression of ICAM-1, HLA class II and/or CD40 molecules.

Dexamethasone partially inhibited the cytokine-induced release of MCP-1 and IL-8 and the expression of ICAM-1, CD40, and HLA class II molecules by human bronchial epithelial cells. The beneficial effect of glucocorticoid therapy in asthma may therefore be mediated in part by inhibition of chemokine release and ICAM-1, CD40, and HLA class II expression by bronchial epithelial cells. The results described in chapters 9, 10 and 11 support the role of the human bronchial epithelium in the inflammatory process observed in the airways of asthmatic patients.

Summarizing, the results described in this thesis do not support a general dysfunction of peptidases in the asthmatic airways and substantiate the important role of the bronchial epithelium in inflammatory reactions in the airways. Finally, the beneficial effect of glucocorticoid therapy in asthma may be mediated in part by modulation of epithelial cell functions and peptidases expressed by bronchial epithelial cells.

SAMENVATTING

Astma wordt klinisch gekenmerkt door een reversibele vernauwing van de luchtwegen en een bronchiale hyperreactiviteit. Tegenwoordig wordt gedacht dat een chronische ontsteking van de luchtwegen ten grondslag ligt aan deze klinische verschijnselen. Bronchusbiopten en bronchoalveolaire lavage (BAL) vloeistof van astmapatiënten vertonen een verhoogd aantal eosinofielen, geactiveerde lymfocyten, en mestcellen vergeleken met gezonde controles. Bovendien worden verhoogde niveaus van ontstekingsmediatoren, zoals cytokinen, neuropeptiden, en chemokinen, gevonden in astmatische luchtwegen. In *hoofdstuk 1* wordt een kort overzicht gegeven over astma, waarbij de nadruk ligt op de immunologische aspecten.

Autonome zenuwen (samengevat in *hoofdstuk 2*) spelen een belangrijke rol in het reguleren van de bronchiale spierspanning, secretie van slijm, en de doorbloeding van de luchtwegen. Naast de welbekende parasympatische (cholinerge) en sympatische (adrenerge) zenuwsystemen, wordt non-adrenerge non-cholinerge (NANC) innervatie gevonden in de luchtwegen. Het inhiberende NANC systeem, dat de enige neurale bronchodilaterende route is in de humane luchtwegen, is gelocalizeerd in parasympatische zenuwen, terwijl het exciterende NANC systeem met name gelocalizeerd is in een subpopulatie van sensorische zenuwen. Stimulatie van sensorische zenuwen kan, via een locale axon reflex, resulteren in de afgifte van neuropeptiden. Deze neuropeptiden hebben een verscheidenheid aan effecten, inclusief de samentrekking van glad spierweefsel, de afgifte van slijm, vasodilatie, microvasculaire lek, en de aantrekking en activatie van leukocyten. Deze volgorde van gebeurtenissen is tegenwoordig bekend als neurogene ontsteking. Omdat neurogene ontsteking veel pathofysiologische verschijnselen van astma nabootst, wordt een rol voor neuropeptiden in de pathogenese van astma verondersteld.

De effecten van neuropeptiden worden normaliter beperkt door snelle afbraak door peptidasen (samengevat in *hoofdstuk 3*). Een verminderde peptidase activiteit zal daardoor dus kunnen resulteren in versterkte responsen op neuropeptiden en dus in neurogene ontsteking. Verschillende peptidasen kunnen worden gevonden in de humane bronchus, inclusief neutraal endopeptidase (NEP), aminopeptidase N (APN), en dipeptidyl peptidase IV (DPP IV). Deze peptidasen zijn niet alleen betrokken bij de modulatie van neurogene ontsteking, maar kunnen ook verschillende proliferatieve en immunologische responsen beïnvloeden. Studies met proefdieren hebben aangetoond dat het bronchusepitheel een belangrijke plaats is voor peptidase activiteit. Bronchusepitheelcellen vormen de interfase tussen de ingeademde lucht en het ademhalingssysteem. Deze cellen kunnen daardoor worden blootgesteld aan een verscheidenheid aan moleculen aanwezig in de ingeademde lucht, zoals allergenen, andere omgevingsfactoren (inclusief virussen, ozon, sigarettenrook, chemische irritantia), en medicijnen. Van verschillende van deze agentia is bekend dat zij de peptidase activiteit verlagen, waardoor zij neurogene ontstekingen verergeren.

Bronchusepitheelcellen vormen niet alleen een passieve fysieke barrière maar kunnen ook bijdragen aan de initiatie en instandhouding van ontstekingsreacties. Naast peptidasen brengen bronchusepitheelcellen verschillenden andere moleculen, betrokken bij de adhesie en activatie van leukocyten, op hun oppervlak tot expressie. Bovendien zijn bronchusepitheelcellen in staat tot de afgifte van een verscheidenheid aan mediatoren die leukocyten aantrekken en activeren. Het bronchusepitheel en zijn functies worden besproken in *hoofdstuk 4*.

Glucocorticoïden worden op grote schaal gebruikt in de behandeling van astma. Ze bezitten potente anti-inflammatoire effecten, hetgeen hun klinische werkzaamheid kan verklaren. In *hoofdstuk 5* zijn de werkingsmechanismen van glucocorticoïden kort samengevat, met speciale aandacht voor de effecten van glucocorticoïden op epitheliale celfuncties.

In de onderzoeken beschreven in dit proefschrift hebben wij getracht om de bijdrage van peptidasen en het bronchusepitheel aan het ontstekingsproces in de astmatische luchtwegen te ontrafelen. Daarnaast hebben wij het effect van glucocorticoïden op peptidasen en het bronchusepitheel onderzocht. De doelstellingen van het onderzoek zijn beschreven in *hoofdstuk 6*.

In het onderzoek beschreven in hoofdstukken 7, 8, en 9 hebben wij de expressie en activiteit van peptidasen onderzocht in de luchtwegen van gezonde controlepersonen en allergische astmapatiënten, zowel in bronchiaal weefsel als in het bronchoalveolaire lumen. Tevens hebben wij bepaald of glucocorticoïden de peptidase expressie en/of activiteit kunnen moduleren. Onze hypothese was dat de expressie en/of activiteit van peptidasen is verminderd in de luchtwegen van astmapatiënten, daardoor bijdragend aan versterkte (neurogene) ontstekingsreacties, en dat glucocorticoïden de expressie en/of activiteit van de peptidasen in de luchtwegen verhogen.

In *hoofdstuk 7* presenteren wij gegevens over de distributie van APN en DPP IV in de humane bronchus. De distributie van beide enzymen werd bepaald door middel van immunohistochemie en enzymhistochemie, en vergeleken met de distributie van NEP. APN expressie en activiteit werd gevonden in bloedvaten, bindweefsel, afvoergangen van klieren, perichondrium, en leukocyten, met name mononucleaire fagocyten, dendritische cellen, en eosinofielen. DPP IV expressie en activiteit was aanwezig in sereuze submucosale klieren, bloedvaten, en T lymfocyten. DPP IV in sereuze submucosale klieren leek voornamelijk intracellulair voor te komen, suggererend dat DPP IV kan worden gesecerneerd in het bronchiale lumen. NEP activiteit was zwak in de humane bronchus en leek aanwezig te zijn in het bronchusepitheel, submucosale klieren, bloedvaten, en glad spierweefsel. NEP activiteit was daarentegen gemakkelijk te detecteren in de trachea van de cavia, met name in het epitheel. Dus, APN en DPP IV komen tot expressie op specifieke plaatsen in de humane bronchus, waar ze mogelijk betrokken zijn bij de modulatie van de respons van de cel op peptiderge prikkels.

Vergelijking van de expressie van APN en DPP IV in bronchusbiopten van gezonde controles en allergische astmatici bracht geen verschillen in de lamina propria aan het licht. In het bronchusepitheel van astmapatiënten daarentegen werd een verhoogd aantal APN-positieve cellen gevonden. Zwakke maar significante correlaties werden gevonden tussen het aantal APN-positieve cellen en het aantal eosinofielen en L25-positieve dendritische cellen. Door gebruik te maken van dubbelkleuringen konden wij de aanwezigheid van APN op deze cellen bevestigen.

Alhoewel peptidasen normaliter membraan-gebonden enzymen zijn, kunnen zij ook worden gevonden in serum en BAL vloeistof. In *hoofdstuk 8* analyzeerden wij de activiteit van NEP, APN, en DPP IV in serum en BAL vloeistof van gezonde niet-rokers, rokers, en allergische astmatici. Bovendien analyzeerden wij of behandeling met het inhalatieglucocorticoïd fluticasone propionate gedurende twaalf weken de activiteit van peptidasen in serum en/of BAL vloeistof kon veranderen. NEP, APN, en DPP IV activiteit konden zowel in het serum als in de BAL vloeistof worden aangetoond. De activiteit van alle drie peptidasen (uitgedrukt per mg eiwit) was hoger in BAL vloeistof dan in serum, suggererend dat de enzymen plaatselijk in de luchtwegen worden afgegeven. Er werden geen correlaties gevonden tussen de

peptidase activiteit en de cel aantallen in het serum of de BAL vloeistof, aangevend dat er geen overheersende hematopoietische bron is van de peptidasen. De NEP activiteit in de BAL vloeistof correleerde met de APN activiteit in de BAL vloeistof, suggererend dat beide enzymen op een vergelijkbare wijze worden gereguleerd.

Vergelijking van de NEP en APN activiteit in het serum bracht geen significante verschillen aan het licht tussen de drie onderzoeksgroepen. De DPP IV activiteit was daarentegen significant verminderd in het serum van rokers vergeleken met gezonde niet-rokers. In de BAL vloeistof waren de NEP en APN activiteit verminderd in rokers. Verminderde NEP en APN activiteit kan peptide-gemediëerde effecten in de luchtwegen versterken en daardoor het ontstekingsproces of de proliferatie van het epitheel bevorderen. De APN activiteit (uitgedrukt per mg eiwit) in BAL vloeistof van allergische astmatici was verminderd ten opzichte van gezonde niet-rokers, terwijl de NEP en DPP IV activiteit niet verschilden. De gelijke niveaus van NEP activiteit in BAL vloeistof van allergische astmatici en gezonde controles kunnen erop duiden dat er geen dysfunctie van NEP is in astma. Anderzijds, gelijke niveaus zouden ook het gevolg kunnen zijn van een verminderde expressie en/of activiteit van membraan-gebonden NEP samengaand met een verhoogde shedding. Behandeling van astmapatiënten met inhalatieglucocorticoïden verbeterde de longfunctie parameters, maar had geen effect op de peptidase activiteiten in serum en BAL vloeistof.

Studies met proefdieren suggereerden dat het bronchusepitheel een belangrijke rol speelt in de modulatie van neurogene ontsteking. Daarom bestudeerden wij de modulatie van epitheliale peptidasen door cytokinen, die aanwezig zijn gedurende ontstekingsreacties, en glucocorticoïden. Wij gebruikten de humane bronchiale epitheelcelllijn BEAS 2B, welke NEP en APN tot expressie brengt, als een model in deze studies. In *hoofdstuk 9* tonen wij aan dat interleukine (IL)-1β, tumor-necrosis factor (TNF)-α, en IL-4 de expressie en activiteit van NEP (bepaald middels flowcytometrie, respectievelijk een specifieke fluorometrische testmethode) verhogen. Interferon (IFN)-γ daarentegen verminderde de expressie en activiteit van NEP, terwijl epidermale groeifactor (EGF) geen effect had. Het effect van IL-1β, wat het meest potente cytokine was in het verhogen van de NEP expressie en activiteit, werd versterkt door remming van fosfodiesterase en nagebootst door een cyclisch-AMP analoog, suggererend dat het effect deels werd gemediëerd door een cyclisch-AMP afhankelijke route. De expressie en activiteit van APN werd echter niet gemoduleerd door IL-1β, IL-4, TNF-α, of EGF. Stimulatie van BEAS 2B cellen met IFN-γ verlaagde tijdelijk de expressie en activiteit van APN na 24 uur. Het synthetisch glucocorticoïd dexamethason gaf een sterke verhoging van de expressie en activiteit van NEP en APN, zowel in de aan- als afwezigheid van cytokinen. Het effect van dexamethason werd opgeheven door de glucocorticoïd receptorantagonist RU38486, aangevend dat het effect was gemediëerd via de glucocorticoïd receptor. Het synthetisch testosteron analoog R1881 had geen effect op de NEP en APN activiteit, suggererend dat het effect specifiek is voor glucocorticoïden en niet voor steroïd hormonen in het algemeen. Dus, de anti-inflammatoire effecten van glucocorticoïden worden mogelijk deels gemediëerd door verhoging van peptidasen die op epitheel tot expressie worden gebracht.

Uit deze studies concluderen wij dat peptidasen uitgebreid voorkomen in de humane bronchus. Echter, er werd geen duidelijke dysfunctie van deze enzymen gevonden in de luchtwegen van allergische astmapatiënten. Verhoging van epitheliale peptidasen door inhalatie-steroïden zou echter klinische implicaties kunnen hebben.

In hoofdstukken 10 en 11 hebben wij de rol van het bronchusepitheel in de initiatie en instandhouding van ontstekingsrecaties in de luchtwegen onderzocht. Astmatische luchtwegen vertonen een verhoogd aantal eosinofielen en geactiveerde T cellen, voornamelijk van het Th2-achtig fenotype. Beide celtypen zijn in staat om IL-4 af te geven; verhoogde hoeveelheden IL-4 werden gevonden in bronchusbiopten en BAL vloeistof van astmatische patiënten in vergelijking met gezonde controles. In *hoofdstuk 10* presenteren wij definitief bewijs voor de expressie van IL-4 receptoren op humane bronchusepitheelcellen. IL-4 receptor α-keten expressie op humane bronchusepitheelcellen *in vivo* werd aangetoond door middel van *in situ* hybridizatie en immunohistochemie. Er werd geen verschil gevonden in IL-4 receptor eiwit expressie tussen bronchusbiopten van gezonde controles en allergische astmapatiënten. Gekweekte humane bronchusepitheelcellen brachten ook IL-4 receptor α-keten mRNA en eiwit tot expressie (bepaald middels respectievelijk RT-PCR analyse en flowcytometrie). Bovendien brachten gekweekte humane bronchusepitheelcellen mRNA en eiwit van de gemeenschappelijke γ-keten, een functionele component van de IL-4 receptor in veel celtypen, tot expressie. De IL-4 receptor eiwit expressie op bronchusepitheelcellen *in vitro* kon worden verhoogd door stimulatie met phorbol myristaat acetaat plus calcium ionofoor, terwijl IL-1β en IL-6 de expressie van de IL-4 receptor verlaagden. Een cyclisch-AMP analoog en IL-4 hadden geen effect. Tenslotte tonen wij aan dat de IL-4 receptor functioneel aktief is, daar IL-4 de afgifte van IL-8, monocyt chemotactisch proteïne (MCP-1), en in het bijzonder IL-1 receptor antagonist door bronchusepitheelcellen verhoogde.

Uit deze studie concluderen wij dat humane bronchusepitheelcellen IL-4 receptoren tot expressie brengen, zowel *in vivo* als *in vitro*. Stimulatie van humane bronchusepitheelcellen met IL-4 kan resulteren in de afgifte van zowel pro- als anti-inflammatoire mediatoren waarvan het bekend is dat zij verhoogd voorkomen in astmatische luchtwegen.

Bronchusepitheelcellen kunnen bijdragen aan het rekruteren en activeren van leukocyten door de afgifte van chemokinen en door moleculen tot expressie te brengen die een interactie kunnen aangaan met leukocyten. In *hoofdstuk 11* presenteren wij gegevens over het effect van cytokinen en glucocortcoïden op de afgifte van MCP-1, het prototype C-C chemokine, en IL-8, het prototype C-X-C chemokine, door humane bronchusepitheelcellen. Daarnaast bestudeerden wij de effecten van cytokinen en glucocorticoïden op de epitheliale expressie van intercellulair adhesie molekuul (ICAM)-1, CD40, en HLA klasse II moleculen. Deze oppervlakte membraan moleculen zijn betrokken bij de adhesie en activatie van de aangetrokken leukocyten.

Primaire kweken van humane bronchusepitheelcellen produceerden constitutief MCP-1 en IL-8. Stimulatie van bronchusepitheelcellen met IFN-γ verhoogde de afgifte van MCP-1 sterk, en dit ging samen met een verhoogde expressie van MCP-1 mRNA en een verhoogd monocyt chemotactisch vermogen. IFN-γ had daarentegen geen effect op de afgifte van IL-8, suggererend dat IFN-γ selectief de afgifte verhoogd van chemokinen die monocyten en lymfocyten aantrekken. IFN-γ verhoogde de epitheliale expressie van ICAM-1, CD40 en HLA klasse II moleculen. IL-1β verhoogde zowel de afgifte van MCP-1 als van IL-8, en verhoogde de expressie van ICAM-1 en CD40, maar niet van HLA klasse II moleculen. Deze resultaten geven aan dat IFN-γ en IL-8 de afgifte van MCP-1 en IL-8 door humane bronchusepitheelcellen verschillend reguleren. IL-1β en in het bijzonder IFN-γ verhogen bovendien de expressie van ICAM-1, HLA klasse II en/of CD40 moleculen.

De cytokine-geïnduceerde afgifte van MCP-1 en IL-8 en de expressie van ICAM-1, CD40, en HLA klasse II moleculen door humane bronchusepitheelcellen werd gedeeltelijk geremd door dexamethason. Het gunstige effect van glucocorticoïd behandeling bij astma wordt

mogelijk dus deels gemedieerd door remming van de chemokine afgifte en de ICAM-1, CD40, en HLA klasse II expressie door de bronchusepitheelcellen. De resultaten beschreven in de hoofdstukken 9, 10 en 11 ondersteunen de rol van het bronchusepitheel in het ontstekingsproces zoals dat wordt waargenomen in de luchtwegen van astmapatiënten.

Kort samengevat, de resultaten beschreven in dit proefschrift wijzen niet op een algemene dysfunctie van peptidasen in de luchtwegen bij astma en bevestigen de belangrijke rol van het bronchusepitheel in ontstekingsreacties in de luchtwegen. Tenslotte, het gunstige effect van glucocorticoïdtherapie in astma kan deels worden gemedieerd door het beïnvloeden van epitheliale celfuncties en peptidasen die door bronchusepitheel tot expressie worden gebracht.

ABBREVIATIONS

15-HETE	15-hydroxyeicosatetranoic acid
13-HODE	13-hydroxy-linoleic acid
AA	Allergic asthmatics
ABC	Avidin-biotin complex
ACE	Agiotensin-converting enzyme
Ach	Acetylcholine
ADA	Adenosine deaminase
AIDS	Aquired immuno-deficiency syndrome
ANF	Atrial natriuretic factor
AP-1	Activating protein-1
APAAP	Alkaline phosphatase anti-alkaline phosphatase
ARDS	Adult respiratory distress syndrome
BAL	Bronchoalveolar lavage
BALT	Bronchus-associated lymphoid tissue
BK	Bradykinin
BLP	Bombesin-like peptides
bp	Base-pair
BSA	Bovine serum albumin
CALLA	Common acute lymphoblastic leukemia antigen
cAMP	Cyclic adenosine monophosphate
CGRP	Calcitonin gene-related peptide
cNOS	Constitutively expressed form of nitric oxide synthase
COPD	Chronic obstructive pulmonary disease
CPN	Carboxypeptidase N
CREB	cAMP-responsive element binding protein
DAB	Diaminobenzidine
DAGNPG	N-Dansyl-D-alanyl-glycyl-*p*-nitrophenylalanyl-glycine
Db-cAMP	Dibutyryl-cyclic adenosine monophosphate
DEX	Dexamethasone micronisatum
DIG	Digoxigenin
DMEM	Dulbecco's modified Eagles medium
dNTP	Deoxynucleotide triphosphate
EAR	Early asthmatic reaction
ECE	Endothelin-converting enzyme
ECP	Eosinophil cationic protein
EGF	Epidermal growth factor
ELAM-1	Endothelial leukocyte adhesion molecule-1
e-NANC	Excitatory NANC
EpDRF	Epithelial-derived relaxing factor
EPO	Eosinophil peroxidase
ET-1	Endothelin-1
FcεRI	High affinity receptor for immunoglobulin E
FEV_1	Forced expiratory volume in one second
fMLP	Formyl-metheonyl-leucyl-phenylalanine
G-CSF	Granulocyte-colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive elements
Gro-α	Growth regulated oncogen-α
HC	Healthy controls
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigens
HPRT	Hypoxanthine phosphatidyl ribosyltransferase
Hsp	Heat shock proteins
IBMX	3-isobutyl-1-methylxanthine
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IGF	Insulin-like growth factor
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-4R	Interleukin-4 receptor
IL-1RA	Interleukin-1 receptor antagnonist
i-NANC	Inhibitory NANC
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase
ISH	In situ hybridization
LAR	Late phase asthmatic reaction
LFA-3	Lymphocyte function-associated antigen-3
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LT	Leukotrienes
LTC_4	Leukotriene C_4
LTB_4	Leukotriene B_4
mAb	Monoclonal antibody
MBP	Major basic protein
MCP	Monocyte chemotactic protein
MC_T	Mast cells containing predominantly tryptase

MC_{TC}	Mast cells containing chymase and tryptase
MESF	Mean equivalent soluble fluorescence
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein-1α
MNA	4-methoxynaphtylamide
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NANC	Non-adrenergic non-cholinergic
NAP-2	Neutrophil-activating peptide-2
NEP	Neutral endopeptidase
NF-κB	Nuclear factor -κB
NK	Neurokinin
NKA	Neurokinin A
NKB	Neurokinin B
NO	Nitric oxide
NOS	Nitric oxide synthase
NP-γ	Neuropeptide-γ
NPK	Neuropeptide K
NPY	Neuropeptide Y
PACAP	Pituitary adenylate cyclase activating peptide
PAF	Platelet-activating factor
PAS	Periodic acid Schiff
PBS	Phosphate-buffered saline pH 7.4
PC_{20}	Provocative concentration required to reduce FEV_1 by 20%
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PHM	Peptide histidine methionine
PMS	Phenazine methosulfate
*p*NA	Para-nitroanilide
PPT	Preprotachykin
RANTES	Regulated upon Activation, Normal T cell Expressed, and presumably Secreted
RT-PCR	Reverse transcriptase-polymerase chain reaction
S	Smokers
SDS	Sodium dodecyl sulphate
sIgA	Secretory immunoglobulin A
SLPI	Secretory leukocyte protease inhibitor
SP	Substance P
SSC	Standard sodium citrate
SuB	Substrate-binding
TCR	T cell receptor
TDI	Toluene diisocyanate
TGF	Transforming growth factor
Th1	T helper cell type 1
Th2	T helper cell type 2
TK	Tachykinin
TM	Transmembrane
TNF	Tumor necrosis factor
UTR	Untranslated region
VCAM	Vascular cellular adhesion molecule
VIP	Vasoactive intestinal peptide
VLA	Very late activation antigen
ZB	Zinc-binding

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