Eotaxin, airway inflammation and hyperresponsiveness

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Eotaxine, luchtwegontsteking en overgevoeligheid

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Chapter 1

General Introduction

AIM OF THIS THESIS

Immediately after the discovery of eotaxin it was suggested that this protein could contribute to unravelling the mechanism of selective eosinophil migration during allergic reactions. Since eosinophils are prominent cells in the pathology of allergic airway inflammation, we hypothesised that eotaxin is an important mediator in eosinophil-mediated disease. In this thesis we aimed to assess the role of eotaxin in allergic asthma and viral respiratory infections, focussing on its role in development of airway inflammation and airway hyperresponsiveness.

EOTAXIN

Eotaxin was first isolated from the broncho-alveolar lavage (BAL) fluid of guinea pigs sensitised and challenged with ovalbumin. After purification of this protein, it was demonstrated that eotaxin induced increased intracellular calcium concentrations in eosinophils and eosinophil aggregation *in vitro*. Injection of this chemotactic factor into the dermis of guinea pigs resulted in migration of radio-labelled eosinophils to the site of injection (1). This first gave rise to the idea that eotaxin induces selective eosinophil migration and might therefore be important in eosinophil-associated diseases.

Due to its structure and its implication in eosinophil migration, eotaxin was classified as a chemokine. Chemokines are a unique group of proteins involved in migration of leukocytes to different parts in the body, both in non-inflamed and inflamed conditions. These proteins are small inducible cytokines ranging from 8-10 kDa. More than 40 chemokines have been identified today. All chemokines share common structures and have the ability to attract leukocytes. Chemokines are classified into different sub-families based on the position of their first cystein residues. The two largest and most studied groups of chemokines are CC- and CXC-chemokines. In the later group, the first cystein residues are separated by another amino acid. Eotaxin belongs to the CC-chemokine family. Nowadays, two other groups are identified as C- and CXXXC-chemokines.

A new nomenclature for chemokines has recently been established. The chemokines where numbered according to the order they were discovered (2). This means that eotaxin is addressed as CCL11. In this thesis CCL11 will be referred as eotaxin.

Human, mouse, rat and guinea pig eotaxin have been cloned so far (3-10). The cDNA for human eotaxin encodes a 97 amino acid protein with a 23 amino-acid leader sequence. The human gene of eotaxin is located on chromosome 17 (11). Eotaxin displays some homology with other chemokines such as human monocyte chemotactic protein-1 (MCP-1) (53%) and guinea pig MCP-1 (44%) (12).

Eotaxin receptor

Chemokines exert their action by binding to chemokine receptors on target cells, which are classified according to their ligands, i.e. CC-chemokine receptors (CCR) bind CC-chemokines and CXC-chemokine receptors (CXCR) bind only CXC-chemokines (table 1). Chemokine receptors are G-protein-coupled receptors. Activation leads to a cascade of cellular events of different intracellular signalling pathways resulting in migration of cells toward high chemokine concentration.

Chemokines are redundant in their action on their target cells meaning all leukocytes express more than one chemokine receptor (table 1). Furthermore, there is promiscuity among the chemokines; most chemokines activate multiple chemokine receptors and therefore are able to act on multiple leukocyte populations (13, 14). The point of this redundancy is not known, but in this way the chemokine family forms a stringently regulated network for leukocyte migration.

Although there is a lot of promiscuity among chemokines and their receptor, eotaxin only binds to the CCR3 (10, 15). This point stresses the importance of the involvement of eotaxin in migration of CCR3 expressing cells. Other chemokines also bind to CCR3, but not exclusively. For example, besides binding to CCR3 the chemokine RANTES can activate CCR1 as well, meaning that besides eosinophils this chemokine also act on monocytes and dendritic cells.

Similar to other chemokine receptors, CCR3 is a seven trans-membrane G-protein coupled receptor. Activation of CCR3 leads to a rise in intracellular Ca^{2+} concentrations resulting in eosinophil activation and migration Very little is known about the signal transduction pathway (16). After binding of eotaxin the receptor is internalised to down-regulate CCR3 from the surface of eosinophils (17).

Unlike the first believes that CCR3 is only expressed on eosinophils, it became clear that also basophils, mast cells and Th2 cells express CCR3 on their surface (18-20). Since all these cells are important in allergic asthma, might indicate that eotaxin and its receptor are involved in the pathophysiology of this disease.

Chemokine receptors (CKR)	Ligands	Cells
CC-CKR		
CCR1	MCP-3, MIP-1α, RANTES	Eosinophils, monocytes, activated T-cells, dendritic cells
CCR2	MCP-1, -2, -3, -4	Basophils, monocytes, activated T-cells, dendritic cells, NK-cells
CCR3	MCP-3, MCP-4 eotaxin-1,-2,-3 RANTES	Eosinophils, basophils, activated T-cell
CCR4	TARC, MDC	Activated T-cells, dendritic cells
CCR5	MIP-1α, MIP-1β, RANTES	Monocytes, activated T-cells, dendritic cells, NK-cells
CCR6	LARC	Dendritic cells
CCR7	MIP-3β, SLC	Activated T-cells
CCR8	I-309	Monocytes
CCR9	TECK	Activated T-cells
CCR10	CTACK, MEC	Activated T-cells, dendritic cells
CXC-CKR		
CXCR1	IL-8, GCP-2	Neutrophils
CXCR2	IL-8, GCP-2, NAP-2, GRO-α, -β, -γ	Neutrophils
CXCR3	IP-10, MiG, iTAC	NK-cells, activated T-cells
CXCR4	SDF-1	Monocytes, T-cells
CXCR5	BLC	T-cells, B-cells
CXXXC-CKR		
CXXXCR1	Fraktalkine	Monocytes, activated T-cells, dendritic cells, NK-cells
C-CKR	Lymphotactin	Dendritic cells

Table 1: Chemokine receptors and binding chemokines expressed on various cells. Table adapted from (21, 22).

Eotaxin generation

Under basal conditions eotaxin mRNA is expressed in a variety of tissues. Moreover, constitutive eotaxin mRNA levels were found in the lungs, intestines, spleen, liver, heart, thymus, testis, and kidney in guinea pigs, humans, and mice (4, 9). Finding of constitutive eotaxin mRNA expression suggests that eotaxin may play a role in normal tissue homing and turnover of eosinophils (23).

Eotaxin-2 and -3

A few years after the discovery of eotaxin, eotaxin-2 was isolated, which was quickly followed by the finding of eotaxin-3 (24, 25). The similarity between the three forms of eotaxin is that these chemokines exclusively bind to CCR3 and not to any other chemokine receptor. Structurally, eotaxin-2 and -3 share only 39 and 33 % homology, respectively, with eotaxin. In contrast to eotaxin-1, their gene is mapped on chromosome 7.

Similar to eotaxin, binding of eotaxin-2 and -3 to CCR3 results in eosinophil activation and migration. Though, eotaxin is a more potent signal compared to the other forms of eotaxin. Comparable to eotaxin, expression of isoforms-2 and -3 is increased after allergen challenge (26, 27). This thesis concentrates on eotaxin-1 and is addressed to as eotaxin.

ASTHMA

Asthma is a multi-factorial complex disease of the airways characterised by reversible airflow obstruction, bronchial hyperresponsiveness and chronic airway inflammation (28). Both genetic and environmental factors are involved in the onset of this disease. For instance, atopy is one of the most important genetic factors linked to this illness (29). However, asthma prevalence is increasing rapidly, and therefore can not only be explained by genetic factors. Environmental determinants, such as house dust mite, occupational agents, cigarette smoke, and viral respiratory infections are important in inducing this disease (30). In general, people predisposed to atopy have excessive allergic reaction to common inhaled allergens. Many cells and mediators are implicated in the pathology of asthma.

Allergic asthma

In allergic asthma upon an encounter with allergens, dendritic cells that line the airways take up the antigen and, guided by a variety of chemokines, migrate to the draining lymph nodes. Here, dendritic cells activate T and B cell. A subdivision of T cells, Th2 cells are associated with allergic diseases. Activation of Th2 cells by antigen presenting dendritic cells, leads to the production inflammatory cytokines such as interleukin (IL)-4, IL-5, and IL-13, which are important inflammatory cytokines in the cascade of events leading to allergy. Under the influence of IL-4 and IL-13 B-cells switch to production of immunoglobulin (Ig) E. IgE enters the circulation and binds to high affinity receptors (FcεR1) for IgE, which are present on mast cells and basophils. Antigen binding to IgE on mast cells results in mast cell activation, inducing the early phase reaction characterised by constriction of airway smooth muscle, vascular leakage, and mucus production. Four to six hours later, the early phase reaction is followed by the late phase reaction. This phase is characterised by excessive inflammation of the lungs resulting in airflow obstruction induced by various cytokines derived from inflammatory cells. The majority of the

recruited inflammatory cells consists of eosinophils and lymphocytes. This inflammatory infiltrate induces mucus secretion, smooth muscle hypertrophy, epithelial shedding, and thickening of the bronchial wall (28, 31, 32).

Viral respiratory infections

Besides allergens, other environmental agents can cause asthma, such as viral respiratory infections. In otherwise healthy adults and children respiratory viruses, e.g. rhinovirus, respiratory syncytical virus, and para-influenza virus, can cause wheezing and other asthma-like symptoms. It was demonstrated that in 80-85% of school-aged children with wheezing episodes tested positive for rhinovirus demonstrating the importance of viruses (33).

In general, respiratory viruses infect epithelial cells of the airways, but do not cause evident epithelial damage. Viruses induce and augment inflammatory reactions by infecting and activating epithelial cells resulting in increased cytokine and chemokine release (34, 35). Viral particles activate resident airway cells, such as macrophages and granulocytes resulting in increased cytokine production and up-regulation of adhesion molecules causing additional migration of inflammatory cells (reviewed in (36)).

Eosinophils take part in the pathology of viral respiratory infections. Increased numbers of eosinophils are present in lung tissue during viral infections. In addition increased eosinophil specific mediators were detected in infected airways, illustrating that eosinophils are activated. This increase in activated eosinophils could be induced directly via stimulating eosinophils with virus, or indirectly by activation of epithelial cells (37).

Experimental infection of human volunteers with rhinovirus resulted in airway hyperresponsiveness, which coincided with increased levels of lymphocytes and eosinophils (38). A correlation exists between lung function parameters and the number and activation of eosinophils in sputum was demonstrated (39). Respiratory viruses can directly attract and stimulate eosinophils, which might be the cause of increased airway responsiveness. In a guinea pig model for virus-induced airway inflammation, airway hyperresponsiveness and airway eosinophilia were associated (40). Eosinophils present in the airway demonstrated piecemeal degranulation (41). Furthermore, histamine was shown to be associated in the induction of airway hyperresponsiveness (42). This illustrates that during viral infections in the airway eosinophils and mast cells might play a prominent role in inducing airway hyperresponsiveness.

In asthmatic patients, viral respiratory infections are often the cause of exacerbations (43). In the majority of patients with asthma exacerbations were correlated with rhinovirus infections. In this process atopy is a major risk factor. Increased histamine levels were measured in asthmatic patients infected with rhinovirus after allergen challenge compared to non-asthmatic individuals. Hence, respiratory virus infections can intensify both the early and late phase reaction in response to allergen and is accompanied by increased mast cell mediator release, and recruitment of eosinophils to the airways (37).
Thus, mast cells and eosinophils play a prominent role in both allergic asthma and viral respiratory infections.

Mast cells

Mast cell progenitors are present in the bone marrow and in the circulation. Only after these cells have reached the tissue they undergo final differentiation resulting in the formation of different mast cell populations. This phenotype heterogeneity of mast cells is reflected in different protease expression. In general, two mast cell populations can be differentiated. First, mast cells that only express tryptase are found in lungs and intestinal mucosa. Second, mast cells that express both tryptase and chymase are found in skin, lymph nodes, and intestinal submucosa (44).
Mast cells have long been recognised as an important effector cell in initiating early phase reactions. After a second encounter with the allergen, this protein can bind to IgE labelled mast cells. Hereafter, cross-linking of the Fcε receptor by the multivalent antigen results in mast cell activation. Exocytosis of pre-formed mediators, e.g. histamine and serotonin, cause the early phase reaction (45). Besides release of pre-stored mediators, mast cells also produce newly synthesised products like eicosanoids and cytokines such as tumour necrosis factor-α (TNF-α), IL-3, IL-4. (46). This indicates that mast cells might contribute to ongoing chronic inflammation in asthma Furthermore, increased numbers of mast cells are present in the airways during the late phase reaction.
Studies with mast cells deficient mice have contributed significantly to resolve the role of mast cells in asthma. Some studies showed that airway hyperresponsiveness and mast cells are associated. Although mast cells and their products can initiate allergic reactions, it has been demonstrated that IgE independent processes can induce these airway symptoms. Further, contribution of mast cells and its products in airway eosinophilia are variable in different models.

Eosinophils

Eosinophils are granulocytes, which arise from pluripotent cell in the bone marrow. Under influence of cytokines, such as granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3 and eosinophil specific IL-5, precursor cells in the bone marrow develop into mature eosinophils (47, 48). These mature cells are released into the circulation. Under normal circumstances eosinophils leave the blood and travel to the gut were they form the host's main defence mechanism against parasites. The life span of eosinophils is very short, but after appropriate stimulus eosinophils produce GM-CSF, which makes them to survive in the tissue for more then two weeks (49). Besides the important role of eosinophils in defence against parasitic infections, these cells are involved in several recurrent disorders, for instance allergic asthma (50).

Many studies have indicated that eosinophils are an important feature in the pathology of asthma. Moreover, increased numbers of eosinophils are present in the airways of asthmatic patients. In broncho-alveolar lavage (BAL) fluid, bronchial biopsies and sputum increased numbers of eosinophils are present in mild, moderate and severe asthmatics (51, 52). The percentages of eosinophils present in asthmatic airways may rise up to 50% of the leukocytes.

To reach the airways, eosinophils have to undergo a multi-step process, which is coordinated by various cytokines, adhesion molecules and chemokines. Initially, eosinophils are released from the bone marrow and enter the circulation, resulting in blood eosinophilia (48). This pool of eosinophils is responsive to stimuli that augment their migration toward the tissue. The process of eosinophil maturation is regulated by IL-5. The importance of this cytokine was demonstrated by genetic manipulation of mice. Over-production of IL-5 induces a profound eosinophilia (47). In contrast, deletion of the IL-5 gene caused a marked reduction of eosinophils in the blood and airways after allergen challenge (53).

Ones the eosinophils are in the circulation; these cells are guided to the airways by inflammatory mediators. Before they reach the airway lumen, eosinophils have to pass the endothelium, cross the airway tissue, and the epithelium. Adhesion molecules are involved in the whole process of transmigration. Pro-inflammatory cytokines such as IL-1, IL-4 and TNF-α induce adhesion molecules on the endothelium, thereby facilitating eosinophil translocation (54, 55). The first step in initiating eosinophil translocation is rolling of eosinophils along the endothelial cells. This process is mediated through a weak binding involving selectins. This is followed by a more firm binding via integrins. VLA-4 (very late antigen-4) is

present on eosinophils and binds to VCAM-1 (vascular cell adhesion molecule-1) or LFA-1 (lymphocyte-function-associated antigen-1) and MAC-1 (complement receptor-3) can bind to ICAM-1 (inducible cell adhesion molecule-1). This adhesion is mediated by IL-3, IL-5 and GM-CSF. After this binding eosinophils move from the apical site to the basolateral site of the endothelium, a process which requires reversible adhesion. In this way, eosinophils enter the airways. To reach the airway lumen eosinophils also have to pass the epithelial layer. Most of the previous mentioned mediators are also involved in this process, with the exception of VCAM-1 and selectins (reviewed (56)).

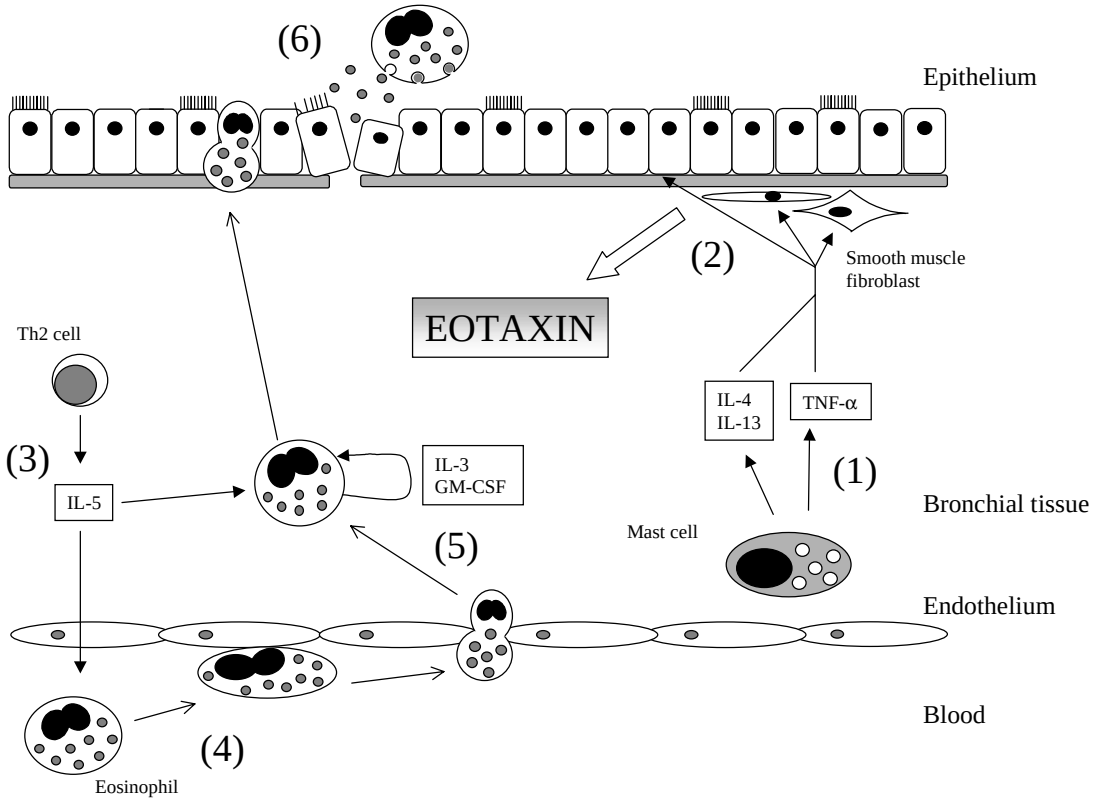

Figure 1: Proposed role of eotaxin in allergic asthma. (**1**) Mast cells become activated by allergens and pro-inflammatory cytokines and are stimulated to produce cytokines such as IL-4, IL-13, and TNF-α. (**2**) These cytokines are able to induce eotaxin production in epithelial cells, smooth muscle cells, and fibroblasts. (**3**) In the meantime Th2 cells are formed and produce IL-5. This cytokine is involved in the release of eosinophils from the bone marrow, primes eosinophils and stimulates eosinophil survival. (**4**) Eosinophils enter the circulation under influence of IL-5. Due to binding to adhesion molecules eosinophils start rolling along the epithelium. Eventually the cell will transmigrate in the direction of high eotaxin concentrations. (**5**) In the bronchial tissue eosinophils will survive for a long time under the influence of autocrine IL-3 and GM-CSF, and IL-5. (**6**) In the next step eosinophils reach the airway lumen. Here, activation of the eosinophils results in tissue damage, which eventually can lead to airway hyper-responsiveness. (Figure adapted from (62)).

Chemokines can interact with the process of leukocyte extravasation at several different steps. First of all, chemokines form a gradient across the tissue, which guides the leukocyte to the direction of high chemokine concentration (57). Secondly, chemokines interfere with binding of leukocytes to the epithelium by influencing adhesion molecules. Chemokines increase the number of integrins on leukocytes and enhances its adhesive activity (58-61). In this way, chemokines facilitate leukocyte extravasation. Thirdly, once leukocytes are present at their destination, chemokines activate these cells. Chemokines can activate the respiratory burst resulting in production of oxygen intermediates, or may initiate cytokine production (57).

Eotaxin interferes with the migration of eosinophils in similar ways (figure 1). First of all, comparable to IL-5 eotaxin rapidly releases eosinophils from the bone marrow, thus generating a pool of eosinophils in the circulation, which is available for entering the tissue (63). Second, eotaxin forms a gradient in the tissue ensuing in eosinophil migration to high eotaxin concentrations, both *in vitro* and *in vivo* (64-66). Third, eotaxin modulates the affinity and expression of adhesion molecules thereby facilitating eosinophil extravasation (60, 67). Fourth, eosinophils can be activated by eotaxin, resulting in calcium mobilisation, actin rearrangement, release of reactive oxygen species, and other eosinophil derived mediators such as IL-4 and eosinophil-derived neurotoxin (EDN) (68-70).

Once eosinophils are present in the tissue they can become activated resulting in the release of eosinophil specific basic proteins (71). Major basic protein (MBP), eosinophil peroxides (EPO), eosinophil cationic protein (ECP), and EDN are toxic mediators that are pre-stored in cytoplasmic granules. These proteins are released in purpose of destroying parasitic invaders of the host. In contrast to these pre-stored mediators, eosinophils are able to produce a wide variety of other newly synthesised mediators. Eosinophils are a prominent source of eicosanoids, for example leukotriene C_4 (LTC_4). In addition, these cells produce cytokines such as GM-CSF and IL-4 (figure 2).

Eosinophils become activated after stimulation by various products, for example through Fc receptors present on the membrane. Engagement of the Fcε, Fcα or Fcγ (receptors for immunoglobulin (Ig) E, IgA and IgG, respectively) triggers release of eosinophil granules (73). In most tissues eosinophils undergo piecemeal degranulation (74). After allergen challenge eosinophil cytolysis is a method of eosinophil degranulation (75).

There is a correlation between airway symptoms, such as airway hyperresponsiveness, and the number of eosinophils in the airway (76). A correlation was shown between the number of eosinophils present in the lungs and the magnitude of the early phase reaction and late phase reaction (77, 78). In asthma patients the number of eosinophils in the airways correlated with lung function parameters and products of activated eosinophils such as cationic proteins are able to directly induce airway hyperresponsiveness (79, 80). In addition, eosinophils can cause airway hyperresponsiveness in indirect ways, for instance by damaging airway epithelium and interactions with sensory nerves (81, 82). Frequently, eosinophils in bronchial biopsies have a partial degranulated appearance and eosinophil-derived mediators are often detectable in BAL fluid and sputum of asthma patients (51, 83). This indicates that eosinophils in the airways of allergic asthmatics are activated, and might play a role in decreased airway function.

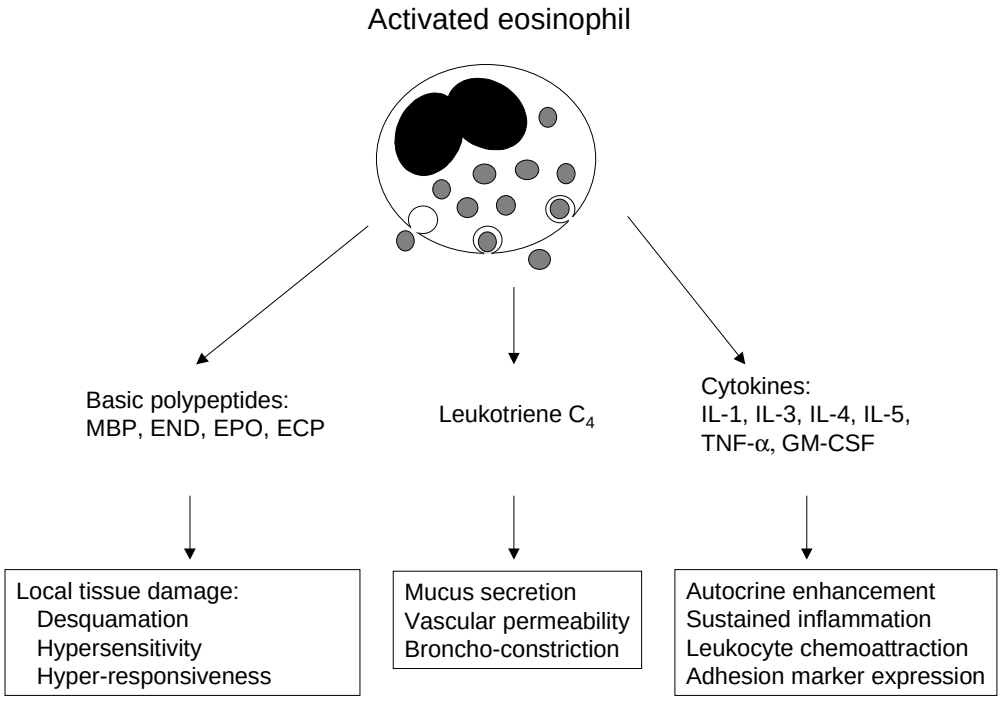

Figure 2: Several eosinophil products implicated in establishing allergic response. (Figure adapted from Pearlman (72)).

Despite clear evidence that eosinophils and their toxic products can induce airway hyperresponsiveness, the role of eosinophils in the pathogenesis of asthma is currently under consideration. Numerous clinical and animal studies have shown

that airway hyperresponsiveness can exist without the presence of airway eosinophils (84-86). This may support the idea that airway hyperresponsiveness and airway eosinophilia are independently regulated but closely interrelated.

Eotaxin and inflammation

The importance of eotaxin in allergic asthma still needs to be elucidated. Increased eotaxin protein levels were measured in the BAL fluid of atopic asthmatic people compared to healthy controls. Furthermore, increased levels of eotaxin mRNA and protein were detectable in epithelium and submucosa of asthmatic patients (87). Additionally, the levels of eotaxin correlated with the number of eosinophils in the lung (88). Allergen inhalation resulted in elevated levels of eotaxin and this increase correlated well with eosinophil migration into sputum (89). Moreover, CCR3 mRNA is increasingly expressed in asthmatic humans compared to non-asthmatic controls (90). Interestingly, eotaxin levels in plasma are also elevated in asthmatics and correlate with the severity of the disease (91, 92).

The major cell source of eotaxin are epithelial and endothelial cells, but also smooth muscle cells, mast cells, fibroblast and eosinophils can produce eotaxin (93-96). Up-regulation of eotaxin is dependent on the transcription factors nuclear factor-κB and STAT-6 (signal transducer and activator of transcription-6) (97). Allergen challenge results in the formation of pro-inflammatory cytokines, like TNF-α, IL-1β, IL-4, IL-13 or bradykinin, which in their turn stimulate epithelial and endothelial cells to produce eotaxin (figure 1, ref (98, 99). Viral respiratory infection can directly activate epithelial cells to produce eotaxin (34).

Actions of eotaxin in animal models

As a tool to study the mechanisms behind the pathological features of asthma, animal models are regularly used. Usually, mice or guinea pigs are sensitised with allergen such as ovalbumin, aspergillus, or house dust mite, with or without the presence of an adjuvant. After sensitisation the animals are challenged in the airways once or repeatedly with allergen. This treatment gives rise to several measurable parameters characteristic of allergic asthma, such as formation of antigen specific IgE, airway hyperresponsiveness, cellular infiltration in BAL fluid, histological changes, and alterations in cytokine levels.

A great number of studies were undertaken to study the relationship between eosinophil migration and eotaxin in animal models for allergic inflammation. A

consistent feature of animal models for allergic asthma is airway eosinophilia. Interestingly, this increase in eosinophils in the airways correlates well with the expression of eotaxin mRNA. As quick as three hours after allergen challenge eotaxin gene expression is increased with 25-fold in the lungs of guinea pigs (100). Often, eosinophil influx into the airways is proceeded by increased levels of eotaxin protein (101, 102). This indicates that eotaxin might be involved in eosinophil migration to the airways in response to allergen challenge.

To show the importance of eotaxin in allergen induced airway symptoms; many investigators tried to block the allergic response using eotaxin-neutralising antibodies. Treatment of sensitised mice with neutralising antibodies during allergen challenge period resulted in decreased eosinophil migration to the airways in response to the allergen (103). This reduction in airway eosinophilia contributed to a reduction of airway hyperresponsiveness (104). Another tool to investigate the role of eotaxin in allergic inflammation is offered by the construction eotaxin knockout mice. Two studies have investigated eosinophil trafficking in these mice, resulting in conflicting evidence. One study showed a reduction in eosinophil migration in response to allergen challenge (105), while in the other study airway eosinophilia existed without the presence of eotaxin (106).

Eotaxin and IL-5

In both guinea pigs and mice eotaxin-induced eosinophil migration is greatly enhanced by pre-treatment of mice with IL-5 (107, 108). It is generally believed that during an inflammatory response both eotaxin and IL-5 are produced. IL-5 promotes eotaxin responsiveness and prolongs eosinophil survival, thereby enhancing the eotaxin-induced eosinophil migration.

In an elegant study, it was shown that in eotaxin/IL-5 knockout mice no peripheral blood eosinophilia or tissue eosinophilia could be induced by allergen challenge (109). In contrast, in IL-5 knockout mice and in eotaxin knockout mice the eosinophilia induced by antigen was reduced but still present. Thus, eotaxin and IL-5 co-operate to selectively regulate tissue eosinophilia.

Effects of eotaxin on Th2 cells, basophils and mast cells

Basophils accumulate in allergic sites, and are an important source of inflammatory mediators. *In vitro,* basophils migrate towards high eotaxin concentrations. Eotaxin elicited a slight release of histamine and leukotrienes by in IL-3 primed basophils (110). Mast cell migration is induced by CCR3 agonists (111). Although, eotaxin

induced an influx of intracellular calcium, mast cell degranulation has not been demonstrated (19, 112). Additionally, eotaxin is involved in growth and differentiation of mast cell progenitor (113).
T-cells express a wide variety of chemokines on their surface (114). Some of these chemokines are markers for a subset of T-cells; for instance CCR3 is exclusively expressed on Th2 cells (20). Eotaxin is able to induce cell migration and activation of Th2 cells (114). In contrast, some studies had difficulties in demonstrating expression of this receptor on T-cells (19).

OUTLINE OF THIS THESIS

In brief, since eotaxin is involved in migration and activation of eosinophils, basophils, Th2 cells, and mast cells, which all play a prominent role in allergic reactions and viral respiratory infections, we hypothesis that eotaxin is implicated in the development of asthma. Data presented in this thesis focus on the role of eotaxin in airway eosinophilia and airway hyperresponsiveness, in mice, guinea pigs and humans.
Basically the investigations addressed three questions:
1. Does eotaxin instillation in the airways induce airway eosinophilia and airway hyperresponsiveness?

This was done in naïve mice (chapter 2) and human volunteers (chapter 6). In the first chapter, we aimed to assess whether eotaxin directly induced airway hyperresponsiveness in mice. Tracheas of mice were incubated with eotaxin and direct contraction of airway smooth muscle and the tracheal reactivity to muscarin receptor agonists was determined. Furthermore, it was investigated whether eotaxin installation in the airways, either alone or in combination with IL-5, induced eosinophil trafficking to the airways and airway hyperresponsiveness (chapter 2). In chapter six, the results of a randomised placebo-controlled crossover study, in which mild asthmatic volunteers inhaled eotaxin, is discussed. After inhalation of eotaxin, the percentage of sputum eosinophils, exhaled nitric oxide, and lung function was determined.
2. Is antigen- or virus-induced airway inflammation and airway hyper-responsiveness regulated by eotaxin?

As a tool to investigate this we used a mouse model for allergic asthma (chapter 3) and a guinea pig model for viral respiratory infections (chapter 4) In these models

we attempted to block the airway response to these agents by blocking eotaxin or its receptor. In chapter 3, mice sensitised and challenged with ovalbumin showed a considerable degree of airway eosinophilia and airway hyperresponsiveness (115). In airways of these mice we measured eotaxin concentrations. Further, we tried to increase asthma symptoms by instilling additional eotaxin in the airways of ovalbumin treated mice. Moreover, consequences of treating ovalbumin-sensitised mice with anti-eotaxin antibodies during the challenge period were investigated. Likewise, viral respiratory infections can cause airway eosinophilia and airway hyperresponsiveness (116). In chapter 4 we assessed the role of eotaxin in a guinea pig model for viral respiratory infections.

3. Can eotaxin activate mast cells and thereby induce airway inflammation or hyperresponsiveness?

Since mast cells express CCR3 as well, we examined the effect of eotaxin on murine bone marrow-derived mast cells (117). It was investigated whether these cells express CCR3 and whether stimulation of the CCR3 receptor resulted in mast cell activation (chapter 5).

The final conclusions are summarised in chapter 7.

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Chapter 2

Eotaxin *alone* is not responsible for airway inflammation and airway hyperresponsiveness

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SUMMARY

Airway eosinophilia and airway hyperresponsiveness are two characteristic features of asthma. Eotaxin is a chemokine involved in trafficking of eosinophils to the site of inflammation. In this study we aimed to assess whether eotaxin can induce airway hyperresponsiveness in mice. The direct effect of eotaxin and the tracheal reactivity to methacholine after pre-incubation with eotaxin was measured in naïve mice. Moreover, airway inflammation and airway responsiveness *in vivo* was assessed in mice treated intratracheally with eotaxin with or without an extra dose of intravenous administered interleukin-5. Eotaxin had no direct effect on mouse tracheas *in vitro*, and the airway reactivity to methacholine was not modulated by eotaxin pre-incubation. Furthermore, intratracheal treatment of mice with eotaxin did not effect the number of eosinophils and airway responsiveness *in vivo*. Similar results were found in the presence of an additional dose of interleukin-5. In conclusion, eotaxin alone does not induce airway hyperresponsiveness or eosinophil migration to the airways, with or without the presence of interleukin-5 in naïve mice.

INTRODUCTION

Airway hyperresponsiveness is a prominent feature of asthma, characterised by increased responsiveness of airway smooth muscle to non-specific stimuli. In the majority of asthmatic patients airway hyperreactivity coincides with an influx of inflammatory cells to the airways. The infiltrate predominantly consists of eosinophils. An increased number of these cells are found in the airway wall, broncho-alveolar lavage (BAL) fluid, and sputum of asthmatic patients (1, 2). The number of eosinophils in the airways correlates well with the severity of asthma (3, 4). Activated eosinophils in the airways release toxic proteins. These toxic mediators can induce airway hyperactivity directly or cause epithelial damage resulting in airway hyperresponsiveness (5). In this way, eosinophils could be important in the pathophysiology of asthma.

Eotaxin is one of the most potent chemokines involved in eosinophil migration. This chemokine was first isolated from the BAL fluid of guinea pigs sensitised and challenged with ovalbumin (6). This protein is able to induce eosinophil migration, *in vitro* and *in vivo* (6, 7). Besides its chemotactic activity, eotaxin can activate

eosinophils, resulting in an increase of intracellular calcium concentrations (8, 9). Eotaxin-induced eosinophil migration is enhanced in presence of interleukin (IL)-5 (10). During inflammation increased levels of eotaxin are detectable in BAL fluid of asthmatic patients (11-13). This increase in eotaxin concentration coincides with trafficking of eosinophils to the airway (14). In a mouse model for allergic asthma, treatment with anti-eotaxin antibodies resulted in a decrease in eosinophil migration to the airways and reduced airway hyperresponsiveness compared to control-treated mice (15). This suggests that, besides involvement of eotaxin in eosinophil trafficking, eotaxin might be involved in induction of airway hyperresponsiveness as well.

The aim of the present study was to examine whether eotaxin is able to induce airway hyperreactivity. The direct effect of eotaxin on a mouse trachea was studied. Airway responsiveness was determined *in vitro* after eotaxin incubation or *in vivo* after intratracheal administration of eotaxin in mice. In these mice, the numbers of eosinophils in the airways and peripheral blood were enumerated, and airway responsiveness was measured. The experiments were also performed in combination with an intravenous injection of IL-5.

MATERIALS AND METHODS

Animals

In these experiments male specified pathogen free BALB/c mice of 6-8 weeks of age were obtained from the Central Animal Laboratory at Utrecht University, The Netherlands. They were housed under controlled condition in macrolon cages containing 12 mice per cage. Water and standard chow was presented *ad libitum*. Dutch committee of animal experiments approved the experiments described in this paper.

Isometric measurements of tracheal reactivity

Tracheal reactivity was measured as previously described (16). In short, mice were killed by an overdose of sodium pentobarbital (Nembutal, 0.2 ml, 60 mg/ml). The tracheas were carefully dissected and cleared from connective tissue under a binocular microscope. One piece of nine cartilage rings of the trachea, from just below the larynx, was placed in an organ bath coupled to an isometric transducer. The organ bath contained 10 ml Krebs' solution (118 mM NaCl, 4.7 mM KCl, 2.5

mM $CaCl \cdot 6H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 25.0 mM $HaHCO_3$, 1.0 mM $NaHPO_4 \cdot H_2O$, 11.1 mM glucose), was aired with carbogen (95% O_2 and 5% CO_2) and kept at a constant temperature of 37°C. The pre-tension of the trachea was kept on 1,000 mg. The tracheas were allowed to stabilise for one hour before tracheal reactivity was measured. Tracheal reactivity was assessed by measuring the contraction to eotaxin (10^{-9}–10^{-12}M) or to methacholine (10^{-9}–10^{-3}M). Isometric measurements were expressed as changes in mg force. In stated experiments tracheas were incubated with eotaxin (10^{-8} M, dissolved in Krebs' solution) for half an hour prior to placement in the organ bath.

Treatment of mice with eotaxin and IL-5 in vivo

Treatment with IL-5: Two treatment protocols were used. One group of mice received one injection of IL-5 (4 μg/mouse), intravenously, blood samples were taken on different time-points (0, 15, 30, 60 or 120 minutes) after the injection. Another group of mice was treated on three consecutive days with IL-5 intravenously (1 or 10 μg/mouse/day). Blood was retrieved for analysis 24 hours after the last IL-5 injection. Control treated animals were injected with saline.

Treatment with eotaxin: Mice were anaesthetised with a short lasting inhalation anaesthetic (Halothane). Eotaxin (50 μl, 10 μg/ml saline 0.1% BSA, Peprotech, Heerhugowaard, The Netherlands) was applied intratracheally. Control animals were treated in the same manner with saline 0.1% BSA. In stated experiments, this was done 18 hours after IL-5 injection. Several time-points (three or 18 hours) after eotaxin mice were sacrificed

Airway responsiveness in vivo

Airway responsiveness was measured in mice using whole body plethysmography (Buxco, Sharon, CT, USA). Unrestrained, unanaesthetised mice were placed in a whole-body chamber. Airway responsiveness was measured by aerosolising increasing concentrations of metacholine (1.6 mg/ml saline to 50 mg/ml saline) for three minutes in the chamber. Airway responsiveness was expressed as enhanced pause (Penh) (17).

Blood cell differentiation

Blood was retrieved by heart punction and 40 μl was dissolved in 20 μl heparin (1000 U/ml). This was used for enumerating the number of leukocytes. One drop of

blood was smeared on a slide and stained with Diff Quick (Merz & Dale, Dudingen, Switzerland) for differential cell counts.

Broncho-alveolar lavage

A canula was inserted in the trachea. The lungs were lavaged 4 times with 1 ml phosphate buffered saline (PBS, 37°C). The fractions were stored on ice. Thereafter, the samples were centrifuged for 10 minutes, 300 g. Supernatant of the first fraction was stored at -70°C for further analysis. The cells of all four fractions were pooled and resuspended in 100 µl PBS. These cells were enumerated and cytospins were prepared. These cytospins were stained with Diff Quick (Merz & Dale, Dudingen, Switzerland) for differential cell counts.

Data analysis and statistics

Data are represented as mean ± SEM (standard error of the mean). Differences in dose-response curves were analysed with repeated measures ANOVA (analysis of variance) followed by a LSD post-hoc test for multiple comparison. Differences in data on cell number after eotaxin were analysed with Student's t-test. Other data were analysed with a one-way ANOVA followed by a LSD post-hoc test. Correlation was demonstrated by way of linear regression. Data were considered as statically different if $p < 0.05$.

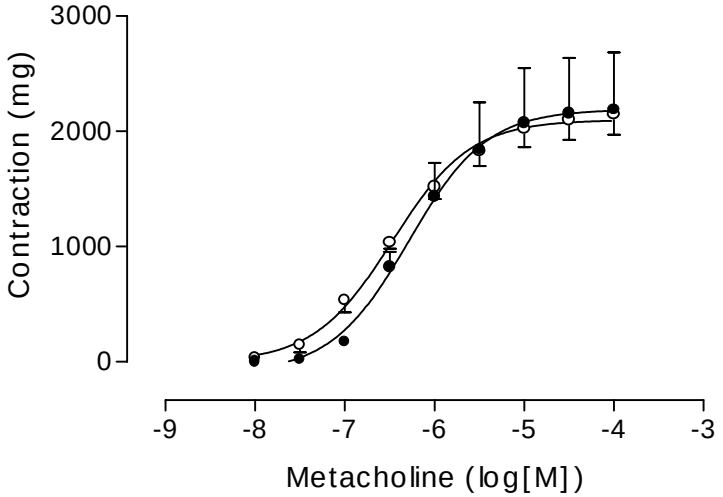

Figure 1: Tracheal reactivity to metacholine (10^{-8}-10^{-9} M) 30 minutes after incubation with 10^{-8} M eotaxin (open circles), or control solution (con, closed circles) in an organ bath set-up. Results are expressed as mean ± SEM.

RESULTS

Direct effect of eotaxin

To investigate the effect of eotaxin on mouse trachea, increasing concentrations of eotaxin (10^{-12} - 10^{-9} M) were added to the mouse trachea in an organ bath set-up. Eotaxin by itself did not induce contraction of the tracheal smooth muscle.

To examine whether eotaxin was able to influence airway responsiveness, mouse tracheas were incubated with eotaxin (10^{-8} M) for 30 minutes in a 96-wells plate. In an organ bath set-up eotaxin incubation did not alter the responsiveness of mouse tracheas to methacholine (figure 1).

In vivo effects of eotaxin

Intratracheal injection of eotaxin did not result in changes in the number of eosinophils in the BAL fluid three hours after injection (figure 2a). Furthermore, this treatment did not alter airway responsiveness to methacholine (figure 2b). Similar results were obtained with 18 hours after treatment with eotaxin.

IL-5 [a]	Blood eosinophils [b]
0	1.19 ± 0.232
1.0	0.86 ± 0.41
10	0.90 ± 0.41

Table 1: Number of eosinophils in blood ([b]x10^4) of mice treated three days IL-5 ([a] 0, 1, or 10 μg/mouse/day). No increase in blood eosinophils was demonstrated. Results are expressed as mean ± SEM.

Co-operation with interleukin-5

In order to increase the number and responsiveness of eosinophils, mice were treated with 4 μg IL-5/mouse intravenously. This treatment did not result in increased blood eosinophils at 15, 30, 60 or 120 minutes after injection (data not shown). Therefore, another set of mice was treated for a longer period of time. These mice received three injections of IL-5 (1 or 10 μg/mouse/day) intravenously. This treatment did not result in blood eosinophilia either (table 1).

Despite the fact that IL-5 was not able to induce blood eosinophilia, it might be that IL-5 primed the circulating eosinophils. Therefore, mice were treated with PBS or IL-5 (10 μg/mouse, intravenously) 18 hours prior to saline or eotaxin (500 ng/mouse, intratracheally) administration. Three hours after eotaxin treatment airway responsiveness, number of eosinophils in blood and BAL, were assessed. Treatment with IL-5 did not result in significantly increased numbers of eosinophils (table 2).

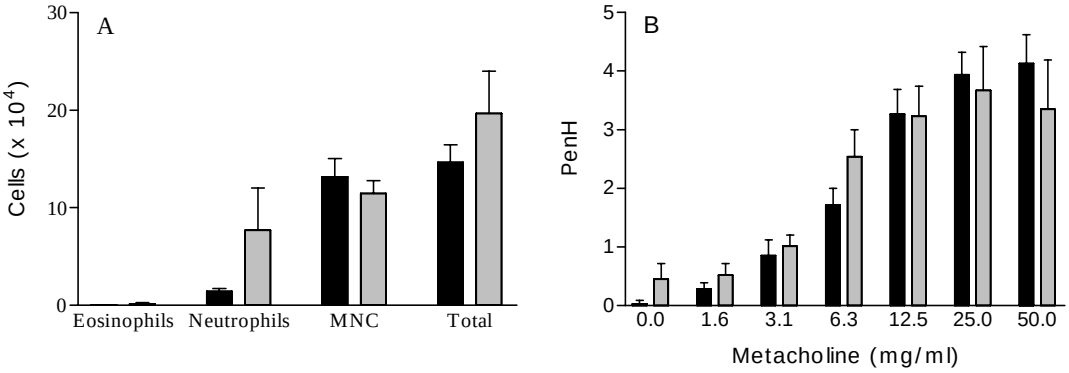

Figure 2: A Differential cell counts and total number of broncho-alveolar lavage cells in mice 3 hours after intratracheal injection with eotaxin (grey bars) or saline (black bars). BAL cells are differentiated into eosinophils, neutrophils, mononuclear cells (MNC). Eotaxin did not change cell numbers. B Airway responsiveness (Penh) to methacholine *in vivo* 3 hours after intratracheal injection with eotaxin (grey bars) or saline (black bars). Eotaxin treatment did not alter airway responsiveness. Similar results were obtained after 18 hours (data not shown). Results are expressed as mean ± SEM.

Treatment	Blood eosinophils [a]	BAL eosinophil [b]	E_{max} [c]
PBS-sal	1.15 ± 1.26	0.5 ± 0.1	4.78 ± 0.42
PBS-eotaxin	3.95 ±2.31	0.0 ± 0	5.44 ± 0.59
IL-5-sal	3.85 ± 1.25	0.0 ± 0	4.51 ± 0.24
IL-5-eotaxin	4.15 ± 1.15	0.0 ± 0	6.04 ± 1.1

Table 2: Mice treated with IL-5 or PBS (intravenously) received an intratrachal injection with eotaxin or saline (sal) 18 hours later. Three hours after eotaxin treatment airway responsiveness to methacholine (*in vivo*), number of eosinophils in blood and BAL, and eosinophil peroxidase levels in lung homogenates were assessed. [a] Number of eosinophils in blood ($x10^4$). [b] Percentage of eosinophils in the BAL fluid of total number of leukocytes. [c] Maximal response to the highest dose of methacholine (Penh). Data are expressed as mean ± SEM.

DISCUSSION

The action of eotaxin is restricted to eosinophils, basophils, Th2 cells, and mast cells (18-21). The present study focussed on airway eosinophilia and airway hyperresponsiveness. No effect of eotaxin was demonstrated both *in vitro* and *in vivo*. Eotaxin did not effect tracheal reactivity *in vitro*, nor did it induce eosinophil migration or alter airway responsiveness *in vivo*, even in combination with IL-5.

The fact that eotaxin application into the airways did not result in eosinophil migration is conflicting with recent published data. It is often shown that eotaxin is involved in eosinophil migration. Application of eotaxin into the skin and airways resulted in eosinophil migration from the blood to the skin or airways, respectively (22, 23). There are some explanations why eotaxin did not induce eosinophil migration in our experimental set-up. First, in other papers guinea pigs or IL-5 transgenic mice were used to show eosinophil migration in response to eotaxin. After intranasal administration of eotaxin an eosinophil influx was detected in IL-5 transgenic mice while in their normal littermates only a small increase was detected (23). Both guinea pigs and IL-5 transgenic mice have more circulating eosinophils compared to BALB/c mice used in this study. Therefore, it was decided to treat animals with IL-5 in order to increase the number of eosinophils in the circulation. Furthermore, IL-5 primes eosinophils to respond to eotaxin (10). Surprisingly, treatment with IL-5 did not alter the response to eotaxin administration. It might be that the concentrations of IL-5 were too low or a more sustained basal expression is necessary.

Second, the eotaxin concentration used for these experiments is known to induce eosinophil migration in guinea pigs and IL-5 transgenic mice. Maybe, in the experiments discussed here, higher concentrations of eotaxin are necessary, since there are fewer circulating eosinophils. It is certain that eotaxin reaches the airways using intratracheal administration. However, maybe eotaxin is broken down very fast by proteases and is therefore not able to exert its action.

A third explanation why eotaxin did not induce eosinophil migration might be that eotaxin is not able to induce eosinophil migration by its self. During an inflammatory reaction, like asthma, lots of mediators are produced. Eosinophil exudation is co-ordinated by many mediators. For example, adhesion molecules need to be activated or upregulated so they can interact with eosinophils which role along the endothelium, and eventually extravagate (24, 25). Other mediators co-operate with eotaxin, which results in homing of eosinophils.

Our findings indicate that eotaxin does not directly influence airway responsiveness. The involvement of eotaxin in airway hyperresponsiveness is probably indirect by attracting and activating eosinophils. The fact that we could not induce eosinophil migration to the airways might explain why these mice do not display airway hyperresponsiveness. Gonzalo and colleagues showed that blocking eotaxin in a murine model for allergic inflammation resulted in decreased airway responsiveness compared to mice treated with control antibody (15). However, this effect could be

indirect since in these mice also the number of eosinophils in BAL is decreased, meaning that in this model eosinophils are involved in the induction of airway hyperresponsiveness, rather then eotaxin itself.

Activated eosinophils are able to release toxic products, which can cause epithelial damage, eventually leading to airway responsiveness (26). The mere presence of eosinophils in the airways of mice does not necessarily mean that airway hyperresponsiveness is initiated. Only activated eosinophils could be involved in increased responsiveness of the lungs. In a model for eotaxin and IL-5 over-expression in the airways of mice, it was shown that eotaxin and IL-5 induced airway eosinophilia, but not airway hyperresponsiveness. In those experiments, antigen inhalation in combination with IL-5 and eotaxin was required for eosinophil degranulation and airway hyperresponsiveness to occur (27). Apparently, other factors potentate the effect of eotaxin during an allergic reaction.

Since eosinophils play an important role in the pathophysiology of asthma, it is important to investigate the events leading to tissue eosinophilia. One of the striking things is that the influx of inflammatory cells predominantly consists of eosinophils. This suggests that specific chemo-attractants, like eotaxin are indeed involved. Whether or not the eosinophil is involved in the induction of airway responsiveness, it is still worthwhile to investigate the events leading to airway eosinophilia and its importance in asthma.

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Chapter 3

Eotaxin protein levels and airway pathology in a mouse model for allergic asthma

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SUMMARY

Eotaxin is a chemokine involved in eosinophil migration. Eosinophils are a prominent feature in allergic airways. In this paper, we investigated the role of eotaxin in a mouse model for allergic asthma. The response to ovalbumin aerosols in ovalbumin sensitized mice was modulated by either treatment with eotaxin antibodies or additional eotaxin, to suppress or promote the development of both airway hyperresponsiveness or airway inflammation. Further, eotaxin levels were measured in lung and broncho-alveolar lavage fluid after challenge of mice with ovalbumin or saline. Finally, the numbers of eosinophils in the BAL fluid and airway hyperresponsiveness were related to eotaxin concentrations. Repeated ovalbumin challenges in sensitized mice resulted in airway hyperresponsiveness and increased numbers of eosinophils in the broncho-alveolar lavage fluid compared to saline challenged animals. Administration of eotaxin antibodies during the challenge period did not prevent development of these asthma-like symptoms. Furthermore, administration of additional eotaxin after the last challenge did not result in increased airway responsiveness or airway eosinophilia in saline or ovalbumine challenged animals. In contrast, eotaxin concentrations were markedly increased in lungs and broncho-alveolar lavage fluid of ovalbumin challenged mice. Although increased levels of eotaxin were detected, there was no correlation with the number of eosinophils in lavage fluid or airway hyperresponsiveness. From these experiments it was concluded that although eotaxin is up-regulated in a murine model for allergic airway inflammation, this chemokine is not involved in the development of airway eosinophilia or airway hyperresponsiveness in this mouse model for allergic asthma.

INTRODUCTION

Airway inflammation is a key feature of allergic asthma (1). The late asthmatic phase reaction to an allergen often coincides with an increase of inflammatory cells in the airways. This influx of cells predominantly consists of eosinophils. In asthmatic patients increased numbers of these cells are detectable in the submucosa of bronchial biopsies, and in broncho-alveolar lavage (BAL) fluid (2). Whether the number of eosinophils is associated with disease severity is currently under debate. Although some studies showed that there is a relationship between the number of

eosinophils in the airways and the degree of airway hyperresponsiveness (another key feature of asthma), this association is weak and might indicate that these phenomena might not be dependent on each other (3, 4). Nevertheless, an influx of eosinophils into the airways is unmistakably associated with allergic asthma.
The mechanism underlying eosinophil migration to the airways remains intriguing. Chemokines are small inducible cytokines involved in trafficking and activation of leukocytes. Since different types of inflammatory cells express particular chemokine receptor, chemokine binding to a receptor provides a specific signal for restricted cell migration (5). Eotaxin is a chemokine, which binds to the CC-chemokine receptor (CCR3) (6). This receptor is present on eosinophils, Th2 cells, basophils and mast cells (7-9). As all these cells are important in asthma, it is believed that eotaxin is involved in the pathogenesis of this disease. Indeed, eotaxin and CCR3 mRNA and protein are up regulated in airways and blood of asthmatic patients (10-13). Eotaxin induces eosinophil migration in the direction of high eotaxin concentrations, both *in vitro* and *in vivo* (14). Animal models indicated that eotaxin injection resulted in trafficking of these leukocytes to the site of injection (15). Interleukin-5 enhances the responsiveness of eosinophils to eotaxin (16).
The objective of the present study was to assess whether eotaxin is implicated in allergen induced airway inflammation and airway hyperresponsiveness. As a tool to investigate the development of these two phenomena, a murine model for allergic asthma was used. Mice were sensitized and challenged with ovalbumine, which lead to formation of asthma like-symptoms, e.g. increased levels of IgE, airway hyperreactivity to methacholine and serotonin, and influx of eosinophils to the airway lumen (17). The first question to address was whether blocking of eotaxin with anti-eotaxin antibodies prevented airway hyperresponsiveness and inflammation. Second, does eotaxin administration worsen asthma features? Third, is eotaxin expressed in the airways of mice challenged and sensitized with ovalbumin?

MATERIALS AND METHODS

Animals

In these experiments male specified pathogen free BALB/c mice of 6-8 weeks of age were obtained from the Central Animal Laboratory at Utrecht University, The Netherlands. They were housed under controlled condition in macrolon cages

containing 12 mice per cage. Water and standard chow was presented ad libitum. Dutch committee of animal experiments approved the experiments described in this paper.

Treatment

At 6-8 weeks of age mice received intraperitoneal injection of 10 μg ovalbumin (grade V, Sigma, Zwijndrecht, The Netherlands) in 0.5 ml saline every other day, 7 times. Four weeks after the last sensitization, mice were challenged with ovalbumin aerosols (2 mg/ml, 5 min) on 8 consecutive days. Vehicle treated animals were challenged with saline. Twenty-four hours after the last challenge, airway reactivity was measured, and blood and BAL were analyzed.

In stated experiments mice were treated with neutralizing anti-eotaxin antibodies (R&D, Abingdon, UK) during the challenge period 30 minutes before the challenge on day 0, 3, and 7 or days 5, 6, 7. The antibodies were injected intravenously (20μg/mouse/ time). Rat IgG (rIgG) was used for control antibody treatment.

In some experiments additional eotaxin (500 ng/mouse, R&D, Abingdon, UK) was injected intravenously 24 hours after the last ovalbumin challenge. Thirty minutes, 5 hours and 24 hours after eotaxin injection airway responsiveness was measured. Twenty-four hours after eotaxin treatment blood and BAL were taken.

Airway responsiveness in vivo

Airway responsiveness was measured in mice using whole body plethysmography (Buxco, Sharon, CT, USA). In short, unrestrained, unanaesthetised mice were placed in a whole-body chamber. Airway responsiveness was measured by aerosolising increasing concentrations of metacholine (1.6 mg/ml saline to 50 mg/ml saline, Sigma) for three minutes in the chamber. Airway responsiveness was expressed as enhanced pause (Penh) (18).

Blood

At several time points (2, 6, 18 or 24 hours) after the last ovalbumin challenge mice were killed with an overdose of pentobarbital (0.5 g/kg bodyweight). Blood was retrieved by heart punction. Blood (40 μl) was dissolved in 20 μl heparin (1000 U/ml). This was used for enumerating the number of leukocytes. One drop was smeared on a slide and stained with Diff Quick (Merz & Dale, Dudingen, Switzerland) for differential cell counts.

Broncho-alveolar lavage

A canula was inserted in the trachea. The lungs were lavaged 4 times with 1 ml PBS (37°C). The fractions were stored on ice. Thereafter, the fractions were centrifuged for 10 minutes, 300 g. The supernatant of the first fraction was stored at -70°C for further analysis. The cells of all four fractions were pooled and resuspended 100 µl phosphate buffered saline (PBS). These cells were enumerated and cytosins were made. These cytosins were colored with Diff Quick (Merz & Dale, Dudingen, Switzerland) for differential cell counts.

Lung isolation

Lungs were perfused by injection of 5 ml PBS into the right ventricle of the heart. Lungs were dissected and stored on ice. One ml of 1.5M KCl was added per 0.10 gram lung tissue. Thereafter, lungs were grained with a mixer and centrifuged at 580g for 10 minutes. The supernatant was stored at -70°C until further analysis.

Eosinophil peroxidase measurement

Samples were diluted once in 0.05 M Tris/HCl. In a 96-wells plate 50 µl sample and 100 µl substrate solution (10 mM o-phenylenediamine dihydrochloride, 4 mM H_2O_2 in 0.05 Tris/HCL) were added. The plate was incubated at room temperature for 30 minutes in the dark. Thereafter the reaction was stopped with 50 µl 4M H_2SO_4. The absorbency was determined at 490 nm.

Eotaxin ELISA

Eotaxin was measured by ELISA using matched-paired antibodies (R&D; Abingdon, UK). A 96-wells plate was coated with 4 µg/ml capture antibody (100 µl/well) in PBS and incubated overnight. The next day the plate was washed three times with wash buffer (PBS, 0.05% Tween-20 (sigma), pH 7.4). The wells were blocked for one hour with 300 µl blocking buffer per well (PBS, 1% BSA, 5% Sucrose, 0.005% NaN_3). The samples were diluted once in assay diluent (tris-buffered saline, 0.1% BSA, 0.05% tween-20, pH 7.3). One hundred µl of the diluted samples was pipetted into the wells after washing three times. Also, 100 µl of standard (1000-15.6 pg/ml human eotaxin R&D in assay diluent) was added. After two hours of incubation the plate was washed again followed by adding biotinylated detection antibody (100 µl/well, 180 ng/ml) to the wells. Again, after incubation of two hours the plates were washed with wash buffer. One hundred µl of streptavidine-HRP (horseradish peroxidase) (0.125 mg/ml) was added to each well

and incubated for 20 minutes. Following this, the plate was washed and 100 µl of substrate solution (reagents A and B, Pharmingen; Heidelberg, Germany) was added per well. After an incubation period of 20-30 minutes in the dark the reaction was stopped by adding 50 µl of H_2SO_4 in each well. The absorbency was read at 450 nm using a plate reader. The whole assay was performed at room temperature.

Statistical analysis

Data are represented as mean ± SEM (standard error of the mean), unless stated otherwise. Differences in airway responsiveness were analysed using repetitive measures ANOVA (analysis of variance) followed by a LSD post hoc test for multiple comparison. Data were analysed for normal distribution using Kolmogorov-Smirnov test. Normally distributed data were analysed with a one-way ANOVA using an LSD post hoc test; otherwise Kruskal-Wallis test was used. Correlation was demonstrated by way of linear regression. Differences were considered statistically significant if $p<0.05$.

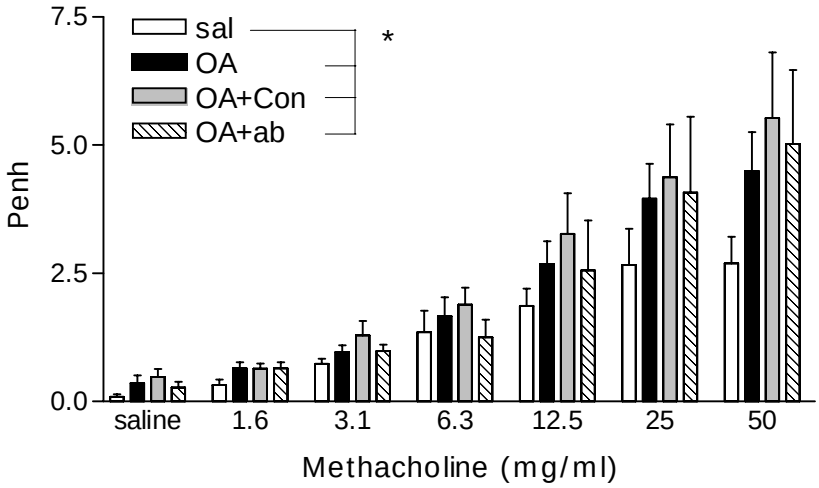

Figure 1: Airway responsiveness to methacholine as measured in mice sensitizes with ovalbumine challenged with saline (Sal, white bars) or ovalbumine (OA, other bars) treated with rIgG antibodies (Con, gray bars) or antibodies directed against eotaxin (ab, hatched bars). Challenge with ovalbumin resulted in airway hyper-responsiveness. This was not blocked with treatment of mice with anti-eotaxin antibodies. Data are represented as mean (± SEM) (* $p<0.05$ compared to ovalbumin challenged mice, ANOVA).

RESULTS

Effect of anti-eotaxin antibodies

To answer the question whether eotaxin is involved in induction of airway eosinophilia in this model, we treated these mice with neutralizing anti-eotaxin antibodies. Rat IgG (rIgG) served as control treatment.

Treatment	Total cells[a]	Eosinophils[b]	EPO[c]	Total IgE[d]
Sal	17.0 ± 2.30	0.10 ± 0.10	267 ± 96.0	163.8 ± 37.0
OA	55.7 ± 10.2*	49.4 ± 9.6$^{\#}$	309 ± 55.2	1736.0 ± 484.5*
OA + Con	45.0 ± 7.13	29.3 ± 2.9$^{\#}$	389 ± 81.7	1534.7 ± 479.0*
OA + Ab	63.3 ± 13.3**	52.3 ± 6.5$^{\#}$	361 ±26.6	2405.5 ± 482.9**

Table 1: Effect of ovalbumin challenge and antibody treatment on the number of BAL cells, percentage of eosinophils, eosinophil activation and total IgE. Data are represented as mean (± SEM). Sal is ovalbumin sensitized mice and challenged with saline. OA are mice sensitized and challenged with ovalbumin. rIgG was used as a control antibody (Con) and Ab is used as a neutralizing antibody directed against eotaxin. [a] $x10^4$ cells in BAL fluid; [b] percentage of eosinophils in BAL fluid [c] Eosinophil peroxidase (ng/ml) in lung homogenates; [d] Total IgE in ng/ml. (* $p<0.05$ ** $p<0.01$compared to saline challenge (ANOVA). # $p<0.01$ compared to saline (Kruskal-Wallis) No significant differences were detectable between ovalbumin challenged groups).

Challenge of sensitized mice with ovalbumin resulted in increased airway responsiveness compared to mice challenged with saline ($p<0.05$). Treatment with antibodies directed to eotaxin did not result in a decrease in antigen-induced airway responsiveness to methacholine (figure 1).

Treatment	0.5 hours	6 hours	24 hours
Sal-vehicle	4.43 ± 0.88	4.57 ± 0.836	4.14 ± 0.24
Sal-eotaxin	4.52 ± 0.449	4.132 ± 0.831	3.17 ± 0.69
OA-vehicle	7.143 ± 1.640 *	6.13 ± 0.99 *	3.37 ± 0.23
OA-eotaxin	7.443 ± 1.54 *	8.11 ± 1.27 *	7.92 ± 1.92$^{\#}$

Table 2: Airway reactivity to highest dose of methacholine (50 mg/ml) in ovalbumin sensitized mice challenged with saline (Sal) or ovalbumin (OA) with additional treatment of intravenous injection of vehicle or eotaxin (500 ng/animal) 24 hours after the last challenge. At 0.5, 6, and 24 hours airway reactivity was measured. Data are represented as mean ± SEM (* $p<0.01$ compared to saline challenged animals, ANOVA on the whole methacholine curve (1.6-50 mg/ml), # $p<0.05$ compared to sal challenged animals and ovalbumin receiving vehicle, ANOVA on maximal response to methacholine (not significant if whole methacholine curve is considered)).

In addition, the number of cells in the BAL fluid was increased in ovalbumin challenged mice compared to saline challenged mice. This increase was mainly due

to an influx of eosinophils (table 1). Treatment with antibodies did not result in a decreased number of eosinophils. To investigate whether the eosinophils present in the airways were more activated after ovalbumin challenge, eosinophil peroxidase (EPO) activity was measured in lung homogenates of these mice. No difference in EPO activity could be demonstrated in the four different groups of mice (table 1). As expected an increase in total IgE and ovalbumin specific IgE was measured in serum of mice challenged with ovalbumin compared to mice challenged with saline which was not altered by treatment with anti-eotaxin antibody treatment (table 1).

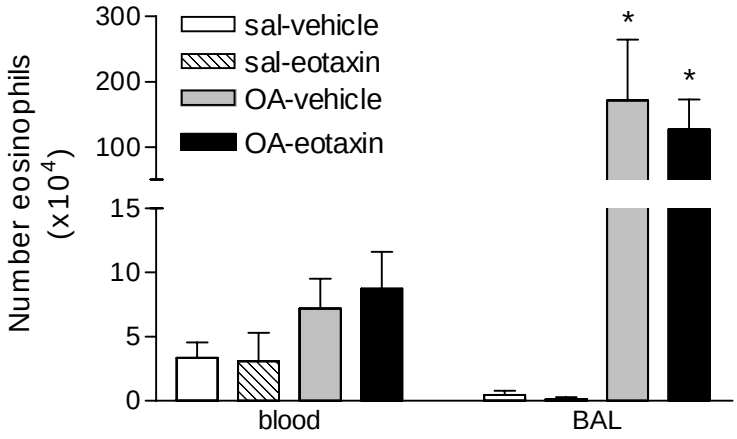

Figure 2: Number of eosinophils in blood and BAL of ovalbumin sensitized mice 24 hours after eotaxin treatment, and 48 hours after the last challenge with saline (Sal, white or hatched bars) or ovalbumin (gray or black bars) with an additional treatment with vehicle (white and gray bars) or eotaxin (500 ng/mouse, intravenously, hatched or black bars). Challenge with ovalbumin results in increased numbers of eosinophils in blood and BAL. However, intravenous injection with eotaxin did not increase the number of eosinophils in these compartments. Data are represented as mean ± SEM. (* $p<0.005$ compared to sal-vehicle and sal-eotaxin, ANOVA).

Eotaxin administration

Since eotaxin can activate eosinophils and might therefore influence airway responsiveness, it was investigated whether an additional dose of eotaxin could worsen and enhance the asthma-like symptoms induced by ovalbumin. Eotaxin was administered intravenously to mice 24 hours after the last challenge. Eotaxin injection did not result in increased airway responsiveness in saline challenged mice at 30 minutes, 5 hours and 24 hours after eotaxin injection. In addition, airway hyperresponsiveness in ovalbumin challenged mice was not enhanced by eotaxin administration (table 2). However, ovalbumin challenged mice were hyper-responsive compared to saline challenged mice 30 minutes and 5 hours after eotaxin

administration. Airway hyperresponsiveness was abrogated 24 hours after eotaxin administration (which is 48 hours after the last ovalbumin challenge). At this time point ovalbumin challenged mice with an additional injection of eotaxin, had a tendency for high values of airway responsiveness to methacholine. This might suggest that eotaxin administration into these mice prolongs the period of airway hyperresponsiveness. Ovalbumin challenge did not result in significant changes in blood eosinophils compared to saline challenged mice.
Eotaxin injection did not have an additional effect on the number of eosinophils in the blood. Although, ovalbumine challenge resulted in increased numbers of eosinophils in the airways, additional eotaxin treatment did not alter this response (figure 2).

Eotaxin protein levels

In naïve mice a fair amount of protein was detected in BAL fluid and serum (403 ± 17.1 pg/ml eotaxin, 361 ± 13.6 pg/ml eotaxin, respectively).
Already at 2 hours after the last challenge eotaxin levels were increased in ovalbumin challenged mice compared to saline challenged mice. This difference got larger in time at 6 and 18 hours after the last challenge (figure 3).

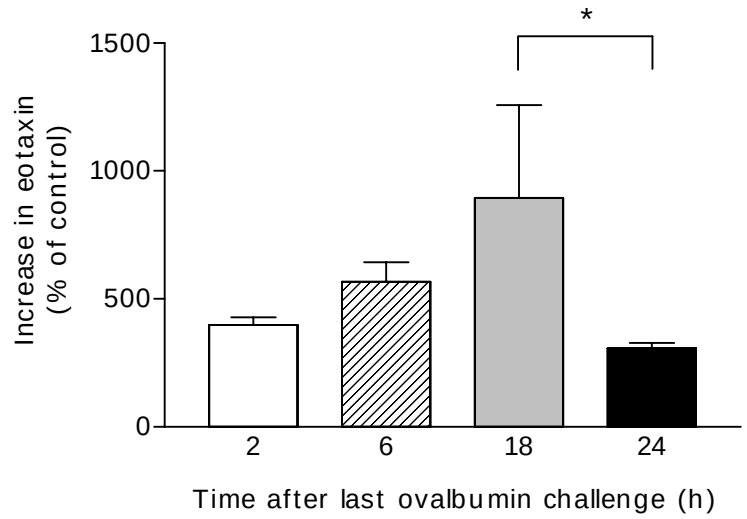

Figure 3: Increase of eotaxin concentrations in lung homogenates at different time points (2, 6 and 18 hours) after the last ovalbumin challenge compared to saline challenged mice. As early as two hours after the challenge eotaxin levels are increased in ovalbumin challenged mice compared to saline challenged mice. Eotaxin levels in lung homogenates increased for up to 18 hours after the challenge. At 24 hours the increase in eotaxin levels decrease compared to 18 hours (* $p<0.05$, ANOVA). Data are represented as mean ± SEM. All values were statistical significant compared to saline challenged animals ($p<0.05$ Student's t-test).

Additionally, twenty-four hours after the last challenge, when ovalbumin challenged mice display airway hyperresponsiveness and airway eosinophilia, eotaxin levels are significantly increased after ovalbumin challenge compared to saline challenge (figure 4).

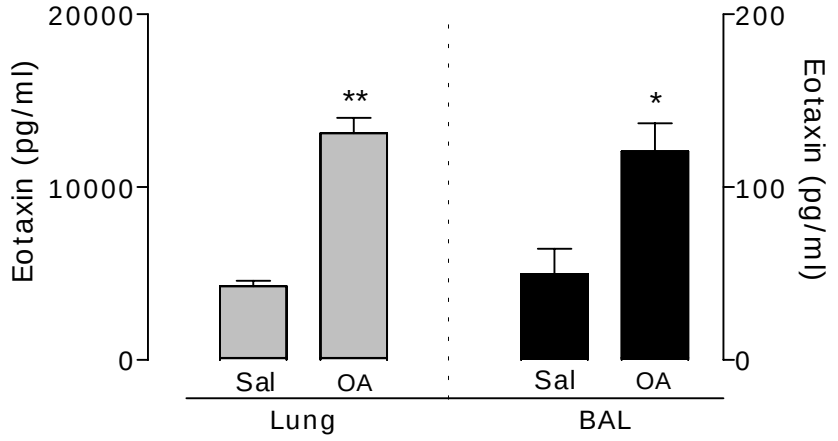

Figure 4: Eotaxin concentrations in lung homogenates (gray bars) and BAL fluid (black bars) of mice sensitized with ovalbumin and challenged with saline (Sal) or ovalbumine (OA). Challenge of mice with ovalbumin resulted in increased levels of eotaxin in both lung and BAL fluid. Data are represented as mean (± SEM) (*p=0.0085; ** p<0.0001 compared to saline challenged animals, Students t-test).

Later time points were not measured. Serum concentrations were comparable between saline and ovalbumin challenged mice. There was no correlation in ovalbumin treated mice between eotaxin concentration in BAL fluid and the number of eosinophils in the airways or airway responsiveness to methacholine (r^2=0.017 (p=0.804); r^2=0.051 (p=0.67), respectively; figure 5).

DISCUSSION

Eotaxin is a known chemokine involved in eosinophil trafficking. The objective of this study was to investigate the role of eotaxin in allergy and airway hyperresponsiveness. As a tool to investigate this a mouse model for allergic asthma was used. Mice sensitized with ovalbumin were challenged with saline or ovalbumin leading to the formation of asthma-like symptoms in ovalbumin challenged mice, e.g. airway hyperresponsiveness, increased number of eosinophils in the BAL and production of antigen specific IgE (17). Using this model we demonstrated that (a) treatment with anti-eotaxin antibodies did not prevent ovalbumin-induced airway

hyperresponsiveness and lung inflammation, (b) intravenous eotaxin installation did not worsen or enhance airway pathology although there was a tendency for prolonged airway hyperresponsiveness in ovalbumin challenged mice after eotaxin administration, and (c) eotaxin levels were increased in the airways after ovalbumin challenge.

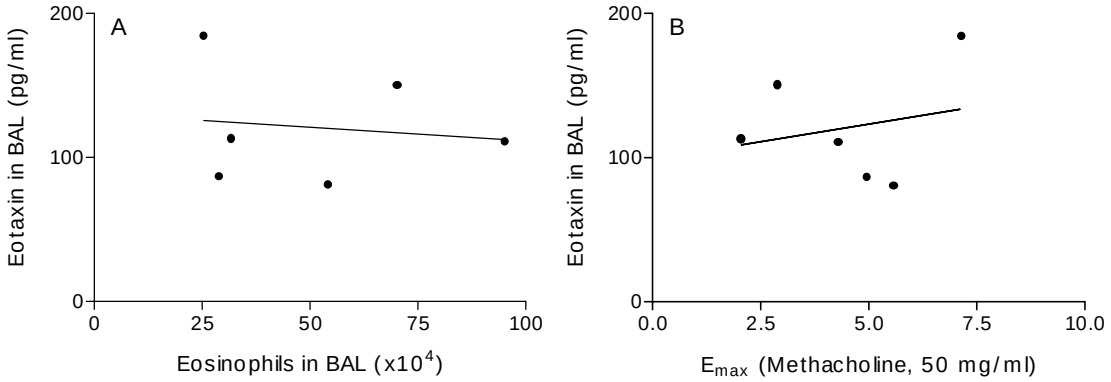

Figure 5: A No correlation between the number of eosinophils in the BAL fluid and eotaxin concentrations in the BAL fluid at 24 hours after the last challenge (r^2=0.017, p=0.80, linear regression). **B** No correlation between eotaxin concentrations in BAL fluid and the maximal airway response (E_{max}) to methacholine (50 mg/ml), 24 hours after the last ovalbumin challenge (r^2=0.051, p=0.67, linear regression). Dots represent individual data points.

Treatment with anti-eotaxin antibodies did not have any effect on eosinophil migration to the airway lumen, EPO activity, airway hyperresponsiveness, and IgE production induced by ovalbumin challenge. This might suggest that eotaxin is not involved in the development of these characteristics in this model. In contrast, other researchers have shown that anti-eotaxin antibodies were able to reduce airway eosinophilia in ovalbumin treated mice (19, 20). The ovalbumin model and time and way of antibody administration used by Gonzalo et al were similar to ours. An explanation for this contradiction might be that they used a different strain of mice, C75/bl6. Presumably this strain is more dependent on eotaxin to induce eosinophil migration.

From recent publications it appeared that eotaxin is involved in eosinophil recruitment early after allergen challenges and not in later stages. In an ovalbumin model for allergic asthma antibodies directed against eotaxin resulted in a decreased eosinophil accumulation in the airways at day four of repeated antigen challenge, but not at day 7 of ovalbumin challenge. In the same model blocking of monocyte-derived chemokine (MDC, a CCR4 agonist) resulted in a decreased airway

inflammation at day 7 and not at day 4, indicating that different stages of eosinophil recruitment are regulated by distinct chemokines (21). Moreover, in a cutanous model for allergic reactions eotaxin concentrations correlated with the number of eosinophils at 6 hours after allergen provocation, while at a later time point other chemokines were responsible for eosinophil migration (22). This illustrates that trafficking of eosinophils in response to antigen is a multi-step process in which more chemokines are involved. In the present study, we looked at eosinophil recruitment after several allergen challenges. At this time point other chemokines might have attracted eosinophils to the airways. We did not measure at earlier time points. Moreover, eotaxin knockout mice have the same number of eosinophils in their airways after ovalbumin sensitization and challenge compared to their normal littermate (23). This indicates that other factors contribute to eosinophil migration. Other chemokines can bind to CCR3. Eosinophils express other chemokine receptors, for example CCR1, which can be activated by different chemokines. This redundancy and promiscuity of the chemokine system questions whether blocking one chemokine or one chemokine receptor is sufficient for abrogating migration of one cell type.

Although eotaxin can induce eosinophil activation *in vitro* (14), antigen stimulation is required for eosinophil activation in vivo (24). In our model ovalbumin challenge did not result in increased levels of EPO in the airways, indicating that eosinophils are not increasingly activated after ovalbumin challenge at this time point. Therefore, eotaxin was administered to see whether an additional effect on the airways could be obtained. However, this treatment did not result in increased airway responsiveness and inflammation. This could be another indication that eotaxin is not involved in both of these parameters. However, these cells might have released their mediators at earlier stages before the time of measurement. Importantly, investigating eosinophil degranulation in mice, one has to keep in mind that mice eosinophils hardly degranulate in response to allergen, *in vivo* (25).

Although eotaxin is produced under basal conditions, allergen challenge results in up-regulation of this protein production (26). Our finding is consistent with other data that have shown before that allergen inhalation results in increased eotaxin mRNA and protein expression in mice, guinea pigs and humans (19, 27-30). The increase in eotaxin levels after allergen challenge is a rapid response, since two hours after the last challenge we detected high eotaxin levels in ovalbumin challenged mice. In our model, twenty-four hours after allergen challenge eotaxin concentrations in lung homogenates and BAL fluid were still more then doubled.

This increase in protein coincides with an influx of eosinophils into the airway lumen. However, no correlation was observed between eotaxin level and the number of eosinophils in the BAL fluid or airway hyperresponsiveness, questioning the function of eotaxin in these two parameters.

The fact that we could not detect a direct correlation between airway responsiveness and eotaxin levels in the airways at 24 hours after the last challenge might depend on the time of measurement. In humans, a correlation was found between eotaxin protein and the level of airflow obstruction at four hours after allergen challenge (31). Possibly, at earlier time points eotaxin might have correlated with the degree of airway responsiveness in our animal model. For example, 18 hours after the last challenge eotaxin levels were maximal increased.

Previously, it was demonstrated that treatment of ovalbumin challenged mice with anti-IL-5 antibodies completely inhibited airway eosinophilia in this model (32). IL-5 is a central cytokine regulating eosinophil function; this mediator primes eosinophils, promotes eosinophil survival and releases precursor cells from the bone marrow. Eotaxin and IL-5 synergistically act on eosinophils (15). Since we could not demonstrate an effect of eotaxin in this model, IL-5 might be responsible for migration of eosinophils (33). Treatment with anti-IL-5 decreased airway eosinophilia in this model but did not influence airway responsiveness. In contrast, anti-IFN-γ did not effect the degree of airway inflammation, while airway hyper responsiveness was completely inhibited (32). This indicates that airway hyperresponsiveness and airway eosinophilia are two independent processes, which are differentially regulated.

In conclusion, blockade of eotaxin with anti-eotaxin antibodies did not have any effect on airway hyperresponsiveness or airway eosinophilia. Additional increase in eotaxin by installing this protein into the airways did not effect airway eosinophilia, eosinophil activation or airway responsiveness in both saline and ovalbumin challenged mice. Furthermore, eotaxin levels were increased in mice sensitized and challenged with ovalbumin compared to mice challenged with saline. However, this increase did not correlate with eosinophil migration to the lungs or airway hyperresponsiveness. From these experiments we conclude that although eotaxin levels are increased in the airways of mice sensitized and challenged with ovalbumin, this protein is not involved in airway eosinophilia or airway hyperresponsiveness.

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Chapter 4

Eotaxin levels and eosinophils in guinea pig BAL-fluid are increased at the onset of a viral respiratory infection

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SUMMARY

In previous studies we found that guinea pigs demonstrate an increase in airway reactivity and eosinophil numbers 4 days after a respiratory infection with parainfluenza-3 virus. Binding of eotaxin to CC-chemokine receptor-3 present on eosinophils results in eosinophil migration. Therefore, we hypothesised that eotaxin is involved in eosinophil migration to the airways in virally infected guinea pigs. In this study, eosinophil numbers were counted in blood and broncho-alveolar lavage fluid and related with eotaxin concentrations in broncho-alveolar lavage fluid 1, 2, 3 and 4 days after intratracheal parainfluenza-3 virus administration. On day 1, blood eosinophils increased by more than 200% ($p<0.01$). The number of eosinophils were only slightly enhanced from day 2-4 (40-70%). In contrast, broncho-alveolar lavage fluid eosinophils were elevated on day 2 (180%) and remained high on days 3-4 (>300%, $p < 0.05$). This increase in lung eosinophils corresponded with increased eotaxin levels measured in broncho-alveolar lavage fluid. This indicates a role for eotaxin in recruitment of eosinophils to the airways. However, treatment of virally infected guinea pigs with a CC-chemokine receptor-3 receptor antagonist did not result in a decreased eosinophil migration to the airways. In conclusion, although eotaxin up-regulation coincides with increasing eosinophil migration to the airways, blockade of its receptor does not influence eosinophil trafficking. This indicates that other factors are involved in eosinophil migration.

INTRODUCTION

Viral respiratory infections can cause airway hyperresponsiveness in healthy humans and are a frequent cause of exacerbations in asthma patients (1, 2). It has been reported that 80% of patients with asthma exacerbations tested positive for viral respiratory infections (3).

Experimental infection with respiratory viruses in human results in increased airway responsiveness, increased sub-mucosal lymphocytes and epithelial eosinophils, which seem to persist even after convalescence (4). A correlation exists between changes in PC_{20} and changes in the number and activation of eosinophils in sputum in asthma patients experimentally infected with a respiratory virus (5). Therefore, there might be a relationship between viral respiratory infections, accumulation of eosinophils in the lung, and airway responsiveness.

Chemokines are involved in orchestrating leukocyte migration (6). Recently, the C-C chemokine eotaxin, discovered in broncho-alveolar lavage (BAL) of guinea pigs sensitised and challenged with ovalbumin, was found to be a potent eosinophil attractant (7). Eotaxin induces a selective accumulation of eosinophils when administered to the airways or injected intradermally (8, 9). The receptor for eotaxin, CC-chemokine receptor 3 (CCR3), is expressed in high numbers on eosinophils (10) and also on basophils, Th2 cells, and mast cells (11, 12). The role of eotaxin in the development of inflammation during allergic reactions is extensively studied. Eotaxin expression correlates with eosinophil influx in sensitised guinea pigs challenged with ovalbumin (13). Moreover, an increase in eotaxin levels in BAL fluid from asthmatics has been observed (14).

In our laboratory an animal model was developed as a tool to investigate the role of viral respiratory infections on airway inflammation and airway responsiveness. Therefore, guinea pigs were infected intratracheally with parainfluenza-3 (PI3) virus. Four days after the initiation of viral infection, guinea pigs expressed airway hyperresponsiveness to histamine both *in vitro* and *in vivo* (15, 16). Furthermore, the number of BAL cells, especially eosinophils, was increased (17). Airway eosinophilia and airway hyperresponsiveness was still present 8 and 16 days after infection (16).

In the present study the role of eotaxin in a time course study of airway eosinophilic influx in guinea pigs treated with PI3 virus was investigated. Migration of eosinophils was observed and was related to eotaxin levels in BAL fluid. To further investigate the role of eotaxin in this model, virally infected guinea pigs were treated with a CCR3 receptor antagonist.

METHODS

Animals

Specified-pathogen-free male Dunkin-Hartley guinea pigs (350-450 g, Harlan Olac Ltd, England) were housed under controlled conditions in cages under filter-tops. Commercial chow and water were supplied *ad libitum*. Dutch committee of animal experiments approved the experiments described in this paper.

Treatment of animals

Viral infection: Animals were treated with PI3 virus as described previously (16). In short, animals were anaesthetised with halothane. Thereafter, 0.1 ml of the virus

suspension (ID-DLO, Lelystad, The Netherlands, tissue culture infective $dose_{50}$ = $10^{8.9}$/ml) was administered intratracheally. Growth medium was subjected to a similar procedure in order to serve as a control solution.

Treatment with human recombinant RANTES: In stated experiments guinea pigs were treated with human RANTES which functions as a CCR3 receptor antagonist in guinea pigs (18). On days 0 (just before infection), 1, 2, and 3 guinea pigs were injected with human recombinant RANTES (intraperitoneally, 1.25 μg/kg/day). As a control treatment guinea pigs were injected with saline. Animals were sacrificed on day 4.

Broncho-alveolar lavage

Animals were sacrificed 1, 2, 3, or 4 days after virus inoculation by an overdose of Euthesate (sodium pentobarbital, 300 mg/kg). BAL was performed as described previously (15). From the first 10 ml only 7 ml was retrieved after one minute, and stored in a plastic tube on ice. After centrifugation 1 ml of cell-free supernatant was stored for measurement of guinea pig eotaxin with a radio-immuno assay (RIA) as previously described (13). After recovery of the first 7 ml, the lungs were lavaged 3 times with 10 ml saline. The cells were sedimented by centrifugation at 580 g for 10 minutes at 4°C. Cells were resuspended in 1 ml saline and cytospins were prepared. Cytospin preparations were stained with Diff Quick (Merz & Dale, Dudingen, Switzerland) for differential cell counts.

Blood cell differentiation

Blood was obtained by a cardiac puncture. Blood smears were stained with Diff Quick (Merz & Dale, Dudingen, Switzerland) for differential cell counts. Leukocytes were enumerated after staining with Turk solution using a heamocytometer.

Statistics

Difference in cell number between guinea pigs treated with virus and medium treated guinea pigs were tested using a Student's unpaired t-test. P values < 0.05 were considered statistically different.

RESULTS

Time course study

Blood and BAL eosinophils: Blood and BAL cells were differentiated 1, 2, 3, and 4 days after treatment with PI3 virus (figure 1). One day after virus administration, the number of eosinophils in peripheral blood was significantly increased by more than 200% ($p < 0.01$). The percentage of blood eosinophils two, three, and four days after viral infection was only slightly increased when compared with medium-treated guinea pigs. In contrast to the observations in blood, BAL fluid eosinophils were not increased at day 1 after PI3 infections. On day 2 following PI3 virus infection, the percentage of eosinophils increased by more then 180% and remained significantly elevated on day 3 and 4 (>300%, $p < 0.05$).

Eotaxin concentrations in BAL: Eotaxin concentrations were measured in BAL fluid of medium (control) and virus-treated guinea pigs (figure 2). On day 1 eotaxin levels started to increase. There was a 4-6 fold increase in eotaxin concentrations on day 2-4 ($p < 0.05$) after viral infection compared to medium-treated animals.

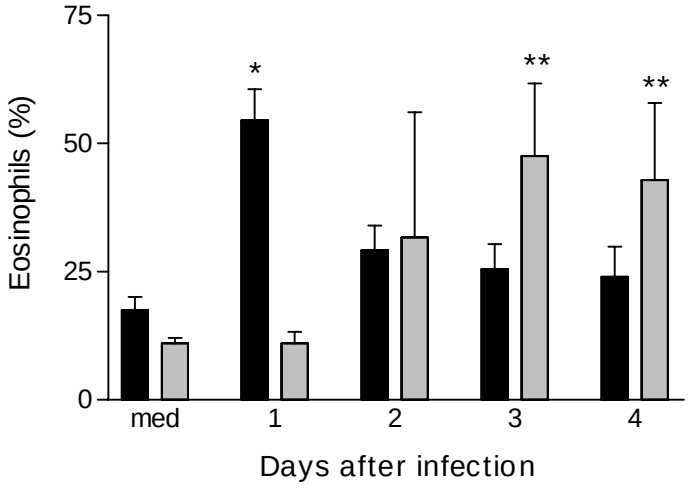

Figure 1: Percentage of eosinophils in blood (black bars) and BAL (grey bars) day 1, 2, 3, and 4 after intratracheal administration of PI3 virus or medium-treated animals (med). Increased number of blood eosinophils is present at one day after infection compared to medium treated animals. Significant increased percentage of lung eosinophils is present at day 3 and 4 of infection. Results are expressed as mean ± SEM * $p < 0.01$, ** $p < 0.05$, compared with medium-treated animals (student's T-test).

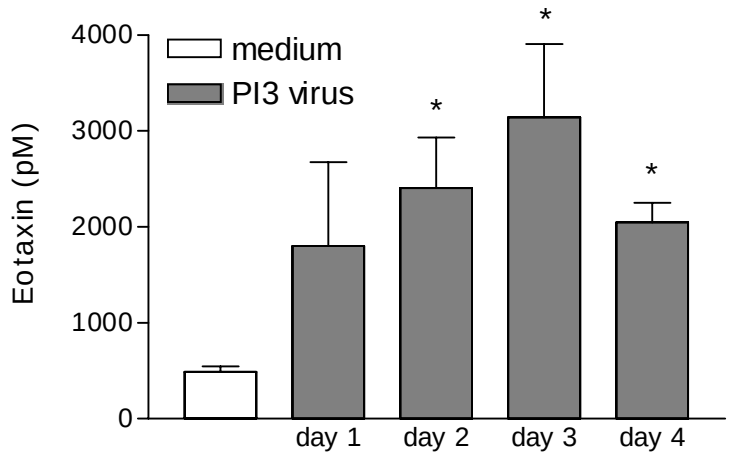

Figure 2: Eotaxin concentrations in BAL fluid of guinea pigs on days 1, 2, 3, or 4 after intratracheal administration of PI3 virus (grey bars) and medium-treated animals (white bar). Results are expressed as mean ± SEM (n=3-4). Asterisks indicate $p<0.05$ compared to medium-treated animals.

Treatment with CCR3 receptor antagonist

Medium or virus inoculated guinea pigs were treated with human recombinant RANTES that acts as a CCR-3 receptor antagonist in guinea pigs (18). After four days, the degree of inflammation was assessed. In the BAL fluid of guinea pigs treated with PI3 virus the number of eosinophils were increased, compared to medium treated animals (virus treated animals 2.9 x 10^6±1.38 vs. sham treated guinea pigs 1.4 x 10^6±0.65 eosinophils). Treatment with human RANTES did not effect the number of eosinophils in the BAL of virus or medium treated guinea pigs (virus treated animals 2.2 x 10^6±0.88 vs. sham treated guinea pigs 1.1 x 10^6±0.15 eosinophils).

DISCUSSION

Infection of guinea pigs with PI3 virus results in airway hyperresponsiveness and an influx of inflammatory cells, mainly eosinophils (15). The eosinophil influx in the lung and the degree of airway hyperresponsiveness are associated (17, 19). Therefore, it is interesting to investigate which mediators are involved in eosinophil trafficking.
From the time course study it appeared that guinea pigs infected with PI3 virus showed a rapid increase in blood eosinophils. Already at one day after virus infection there was a marked increase in circulating eosinophils. Two days after infection, eosinophil numbers decreased again to levels of those observed in medium treated guinea pigs. In

contrast, eosinophils in the BAL fluid started to increase two days after viral infection. From this study it appears that eosinophils are rapidly released from the bone marrow resulting in an increase in blood eosinophils on day one. Thereafter, it seems that these eosinophils migrate to the airways, eventually ending up in the airway lumen.

There are a number of mediators involved in eosinophil migration. In literature, it is shown that eotaxin is a potent eosinophil chemo-attractant, which might be a likely candidate to induce eosinophil trafficking in this model. Therefore, we determined eotaxin levels in the BAL fluid of guinea pigs at different days of virus infection. Eotaxin levels were detectable in BAL fluid of medium inoculated guinea pigs. One day after virus administration eotaxin levels started to increase, and reached significant higher levels compared to medium treated guinea pigs on days 2, 3, and 4. In view of the fact that increased eotaxin levels proceeded the increase in BAL eosinophils indicates that eotaxin might be involved in eosinophil migration.

To investigate whether eotaxin is indeed involved in eosinophil migration guinea pigs were treated with human RANTES. From previous studies it appeared that human RANTES acts as a CCR3 receptor antagonist for guinea pig eosinophils, both *in vitro* and *in vivo*. In this study injection of human RANTES into the skin of guinea pigs resulted in a decreased eotaxin-induced eosinophil migration (18). Treatment of guinea pigs with human recombinant RANTES blocked the CCR3 receptor in a way that eotaxin (and other chemokines) can not bind to the CCR3. Nevertheless, in our hands this treatment did not alter the response of guinea pig toward the virus infection with respect to eosinophil migration. This might be explained by a redundancy in the chemokine system. Eosinophils do not only migrate in response to CCR3 agonists, but activation of other chemokine receptors expressed by eosinophils can cause migration of these cells as well. RANTES is known to bind to both CCR1 and CCR3. Whether human RANTES also antagonises CCR1 is unknown. Blocking eosinophil migration might be more effective if multiple chemokine receptors are antagonised.

The detectable increase in eotaxin levels might be a direct effect of the virus on epithelial cell without functional implications. It was demonstrated before that viral respiratory infection result in increased expression of chemokines (20). Human epithelial cells infected with Influenza A virus expressed higher levels of eotaxin mRNA and protein compared to non-infected cells (21). This increase in eotaxin production was induced by the virus itself rather than via an indirect mechanism involving other inflammatory mediators. Whether the increasing levels in BAL fluid eotaxin of the present study is caused by de PI3 infection itself or involves other mediators is not clear.

Since eotaxin is not involved in eosinophil migration in this model, other mediators could be candidates to induce eosinophil migration. IL-5 has been shown to be important in eosinophil migration in many animal models of inflammation. This mediator displays several actions on eosinophils. It proliferates and differentiates precursor eosinophils in the bone marrow, it prolongs eosinophil survival, it primes eosinophils, and it can be directly involved in eosinophil migration (22). In previous studies it was demonstrated that treatment with antibodies to IL-5 of guinea pigs treated with PI3 virus did result in a significant drop in blood eosinophils (23). This might indicate that IL-5 is involved in quickly releasing precursor eosinophils from the bone marrow into the circulation. Eotaxin was shown to be important in releasing blood eosinophils into the circulation of guinea pigs, as well. However, this chemokine is probably not involved in inducing blood eosinophils in our experiments, since blood eosinophilia existed on day one, while eotaxin levels start to increase at day two of viral infection. Anti-IL-5 treatment had almost no effect on eosinophil influx into the lung (23). Moreover, it was shown before that bradykinin was not involved in eosinophil migration in this model (24).

Since both treatment with antibodies to IL-5 (23) and treatment with anti-eotaxin antibodies did not result in a decreased airway eosinophilia in virally infected guinea pigs, it might indicates that the migration of eosinophils is a multi-factorial process, which is not easily declined. Blockade of only one mediator involved in this cell trafficking is apparently not sufficient. Treatment that is directed to block multiple stage in the process of eosinophil migration might be more effective in reducing airway eosinophilia after a viral respiratory infection.

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Chapter 5

Expression and function of CCR3 on murine bone marrow-derived mast cells

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SUMMARY

Eotaxin is a chemokine involved in leukocyte migration and acts via the CC chemokine receptor-3. Recently, it was demonstrated that human mast cells express this receptor. This study aimed to asses whether murine bone marrow-derived mast cells express CC-chemokine receptor-3 and whether stimulation of this receptor with eotaxin results in mast cell degranulation. Bone marrow cells were cultured in IL-3 containing medium to obtain bone marrow-derived mast cells of the mucosal-like phenotype. Using flowcytomerty it was demonstrated that bone marrow-derived mast cells stained positive for CC-chemokine receptor-3 (mean fluorescence intensity 24), compared isotype control (mean fluorescence intensity 9.73). However, stimulation of this receptor by eotaxin (10-2000 ng/ml) did not result in granule exocytosis, as measured by the release of β-hexosaminidase. Although cross-linking of the IgE receptor by antigen resulted in dose-dependent release of leukotriene C_4, eotaxin did not trigger mast cells to produce this lipid mediator. Due to environmental differences responsiveness of mast cells can vary between *in vitro* and *in vivo* situations. For that reason, mast cell responsiveness to eotaxin was tested *in vivo*. Intravenous injection of mice with 500 ng of eotaxin did not alter mouse mucosal mast cell protease levels in serum of these mice, indicating that eotaxin does not activate mast cells *in vivo*. Collectively, CC-chemokine receptor-3 is expressed on murine bone-marrow-derived mast cells and stimulation of this receptor does not result in mast cell degranulation or initiation of leukotriene C_4 production, both *in vitro* and *in vivo*.

INTRODUCTION

Chemokines are small inducible cytokines involved in leukocyte migration. Eotaxin is a chemokine acting via the CC-chemokine receptor-3 (CCR3) which is believed to play a prominent role in eosinophil migration during allergic diseases, such as asthma (1, 2). During allergic responses eotaxin expression is increased in airways of asthmatic individuals, resulting in migration of predominantly eosinophils to the site of inflammation (3). Recently, it was discovered that CCR3 is also expressed by mast cells (4). Therefore, in addition to the effect on eosinophils, eotaxin might augment inflammatory reactions by acting on mast cells.

Mast cells play a prominent role in the initiation and development of allergic diseases, such as asthma (5). Mast cells arise in the bone marrow and travel to mucosal or submucosal sites where they undergo terminal stages of differentiation. Cross-linking of membrane-bound IgE by allergen results in activation and degranulation. Upon activation mast cells release pre-stored mediators, such as histamine, which is an important initiator of allergic responses. Other than the release of pre-formed mediators, mast cells activation can also initiate the formation of arachidonic acid metabolites, or production of pro-inflammatory cytokines. In this way, mast cells contribute to ongoing, chronic inflammation.
Using immuno-histochemistry and flowcytometry it was demonstrated that a proportion of human mast cells in skin, gut and lungs express CCR3 (6). *In vitro*, CCR3 receptor agonists elicit mast cell migration (6, 7). Furthermore, eotaxin is involved in differentiation of embryonic mast cells into mature cells (8). Although it was shown that eotaxin elicited increases in intracellular calcium concentrations in mast cells (9), activation of these cells by eotaxin has never been demonstrated.
Since mast cells express CCR3 on their surface and eotaxin expression is increased during inflammation, we speculate that eotaxin augments inflammatory reactions by directly attracting and activating mast cells. To elaborate on the possible interactions between mast cells and eotaxin we used bone marrow-derived mast cells (BMMCs) to study CCR3 expression and functional activity of this receptor *in vitro*. In addition, it was assessed whether eotaxin administration *in vivo* resulted in mast cell activation.

MATERIALS AND METHODS

Mouse Bone marrow cultures

Conditioned medium: As a growth factor source for the culture of BMMCs pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM) was used. This was obtained from spleen cells from BALB/c mice (Charles River). These were cultured in complete RPMI (RPMI 1640 medium containing 4 mmol/l L-glutamine, $5x10^{-5}$ mol/l 2-mecaptoethanol, 1 mmol/l sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycine, and 0.1 mmol/l non essential amino acids) plus 10% fetal calf serum (FCS), containing pokeweed mitogen (8 mg/ml, Sigma; St Louis, USA) at a density of $2x10^6$ cells/ml for seven days in an incubator at 37°C in

a 5% CO_2 humidified atmosphere. After seven days the medium was centrifuged (10 minutes, 400g) and filtered through a 0.45-μm Millipore filter (Corning, USA).
Bone marrow-derived mast cells were cultured as described before (10). In short, femoral bones were obtained from 8-10 weeks old BALB/c mice. These bones were aseptically flushed with complete RPMI. The cell suspension was washed twice in complete RPMI. The cells were cultured at a density of $1x10^5$ cells/ml in complete RPMI containing 10% (v/v) Fetal Calf Serum (FCS) in an incubator at 37°C in a 5% CO_2 humidified atmosphere. PWM-SCM was added to the culture medium (20%, v/v). Cells were resuspended in fresh culture medium at a density of $1x10^5$ cells/ml every week. After 3-6 weeks, mast cells represented >98% of the total cells as determined by toluidine blue staining.

Immunofluorescent flowcytometry

At 26 days of culture BMMCs were stained with purified rabbit anti-mouse CCR3 polyclonal antibody (Pharmingen, Beckton Dickinson, Heidelberg Germany) for 30 minutes at 4°C. For the isotype control purified rabbit polyclonal antibody Gαi-3 (Pharmingen) was used. This was followed by incubation with anti-rabbit IgG for 30 minutes at 4°C. Staining was done with streptavidin PE (Beckton Dickinson, Heidelberg Germany) for 30 minutes at 4°C. After each incubation, cells were washed with phosphate buffered saline (PBS) with 1% (v/v) FCS and 0.01% (w/v) sodium azide. Samples were analysed using a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany).

Mast cell activation

β-Hexosaminidase: BMMCs were cultured for 15 till 20 days under conditions described above. In stated experiments, approximately $2\text{-}3x10^6$ cells were resuspended in culture medium and incubated with anti-DNP IgE (26.82) for 1 hour at 37°C, 5% CO_2. BMMCs were washed twice with Tyrodes buffer (GIBCO life technologies, Paisley Scotland) containing 10 mM Hepes (Merck, Amsterdam, The Netherlands)(pH 7.2) and supplemented with 0.1% (w/v) bovine serum albumin (BSA, Sigma). The cells were resuspended at a density of $0.6x10^6$ cells/ml, aliquoted in a 96 wells plates (Costar Corning, NY, USA) ($3x10^4$ cells per well), and activated with eotaxin (10-2000 ng/ml; PBS, 0.1% BSA; R&D Systems, USA) or DNP-HSA (0.8-25 ng/ml; in case of IgE labelled cells). After 30 minutes, supernatants from activated cells were collected. Total release of granule content was established by adding 10 μl 10% (v/v) Triton X-100 (Sigma) per well to get

complete lysis. Fifty µl of the supernatants were incubated with 4-methylumbelliferyl glucosaminidine (Sigma) in 0.1 M citrate buffer (0.1 M sodium citrate (Merck) in bidest, pH 4.5) for 1 hour at 37°C. The reaction was stopped by adding glycine buffer (0.2 M Glycine (Merck), 0.2 M NaCl (Merck), pH 10.7). Fluorescence was measured using a multi-well plate reader (Millipore Cytofluor 2350) at an emission wavelength λ=360 nm and excitation wavelength λ=450 nm. The percentage of degranulation was calculated as: ((a-b)/(t-b))x100, were *a* is the amount of β-hexosaminidase released from stimulated cells, *b* is the β-hexosaminsidase released from non-stimulated cells and t is total cellular content.
Leukotriene C_4 measurements: Cells were stimulated as described above. Supernatants of 4 wells were pooled for the measurements of leukotriene C_4 (LTC_4). This was done using a LTC_4 enzyme-immuno assay system (Biotrak, Amersham Pharmacia biotech, Roosendaal, Netherlands) according to manufacturer's instructions.

In vivo mast cell activation

Male BALB/c mice (Charles River) were injected with eotaxin (20 µg/ml PBS, 0.1% BSA, 25 µl/mouse, R&D Systems) or with control solution (PBS/0.1% BSA) in the tail vein. Thirty minutes after this injection blood was taken by a cardiac puncture. Blood was placed at room temperature for one hour. Subsequently, samples were centrifuged and serum aliquots were stored at -20°C. To further assess mast cell degranulation mouse mucosal mast cell protease-1 and LTC_4 were measured in these serum samples.

Mouse mucosal mast cell protease-1 ELISA

NUNC-immuno plates were coated with 50 µl capture antibody (2 µg/ml, 0.1M carbonate buffer, pH 9.6) and were incubated for 12-24 hours at 4°C. After rinsing with wash buffer (PBS/0.05% Tween 20), plates were blocked with 50 µl of 1% BSA in PBS/0.05% Tween 20 for thirty minutes at 4°C. Thereafter plates were washed with wash buffer and 50 µl of standard (4-0.25 ng/ml) and samples (diluted in 4% BSA in PBS/0.05% Tween 20) were loaded into the wells. Plates were incubated for 24 hours at 4°C. After washing, conjugate (streptavidine-HRP 1:10.000) was added to each well (50 µl/well) for 2 hours at 4°C. Plates were washed with wash buffer. To obtain colour development, 50 µl OPD/H_2O_2 (5 mg o-phenylenediamine dihydrochloride (Sigma) in 11ml PBS and 5µl H_2O_2) was added

for 30 minutes at room temperature. The reaction was stopped by 2.5M H_2SO_4 (25 µl/well). Optical density was measured at 490 nm.

Statistics

Data are represented as mean ± SEM (standard error of the mean). Differences were analysed using Student's t-test.

RESULTS

CCR3 expression by BMMCs

Development of mast cells from bone marrow cells was assessed by toluidine blue staining and flow-cytometric analysis for the expression of FcεRI and CD117 (data not shown (10)). Fully maturated BMMCs were analysed for CCR3 by flow cytometry. As shown in figure 1, BMMCs showed moderate expression of CCR3 on their cell surface.

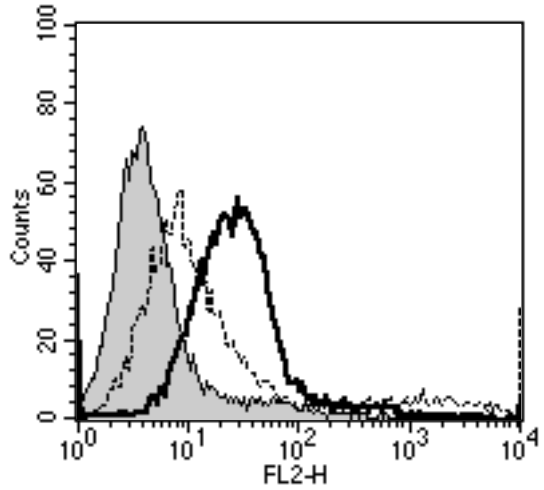

Figure 1: CC-chemokine-3 expression on bone marrow derived mast cells. Grey histogram represents the non-stained cells (mean fluorescence intensity 3.75). Black dotted line represents isotype control cells (mean fluorescence intensity 9.73) and the black line are cells stained with CCR3-PE (mean fluorescence intensity 24).

BMMC in vitro activation

β-Hexosaminidase release: β-hexosaminidase release of mast cells was used as a tool to measure mast cell granule exocytosis. To assess whether CCR3 activation on BMMCs could lead to mast cell degranulation, cells were incubated with increasing concentrations of eotaxin. Stimulation of BMMCs with eotaxin (10-2000 ng/ml) did

not resulted in significant release of β-hexosaminidase (table 1), while stimulation of the IgE receptor with IgE and antigen (0-33 ng/ml DNP-HSA) resulted in a dose dependent release of β-hexosaminidase.

LTC_4 production: In addition to granule exocytosis mast cells are able to produce arachidonic acid metabolites after stimulation. To investigate whether eotaxin is able to initiate LTC_4 production, BMMCs were stimulated with eotaxin or antigen. Antigen stimulation resulted in a dose dependent release of LTC_4 under all culture conditions. However, eotaxin did not induce production of LTC_4 (figure 2).

In vivo mast cells activation

In order to test whether eotaxin could activate mast cells *in vivo*, mice were injected with eotaxin (500 ng/mouse), and thirty minutes later mast cell activation was assess by measuring mucosal mast cells protease-1 in serum, which is a marker for mast cells activation. Treatment with eotaxin did not result in increased levels of this protein (eotaxin: 5.72 ± 0.746 ng/ml, control: 5.64 ± 0.859 ng/ml; p=0.933). In addition, no significant changes in LTC_4 levels were present in serum of mice treated with eotaxin (eotaxin 79.29 ± 38.1 pg LTC_4, control: 132.0 ± 19.8 pg LTC_4; p=0.249).

Treatment	Concentration (ng/ml)	Degranulation (%)	
Eotaxin	0	0.0	(± 0.121)
	100	0.485	(± 0.254)
	500	0.242	(± 0.360)
	1000	-0.307	(± 0.272)
	2000	0.081	(± 0.116)
DNP-HSA	0	0.0	(± 0.121)
	1.2	10.96	(± 1.372)
	3.7	18.49	(± 2.803)
	11	30.15	(± 3.072)
	33	32.48	(± 2.326)

Table 1: Percentage of β-hexosaminidase release by BMMCs in response to eotaxin (0-2,000 ng/ml) or DNP-HSA (0-33 ng/ml). Data is represented as mean of 4 wells (± SEM).

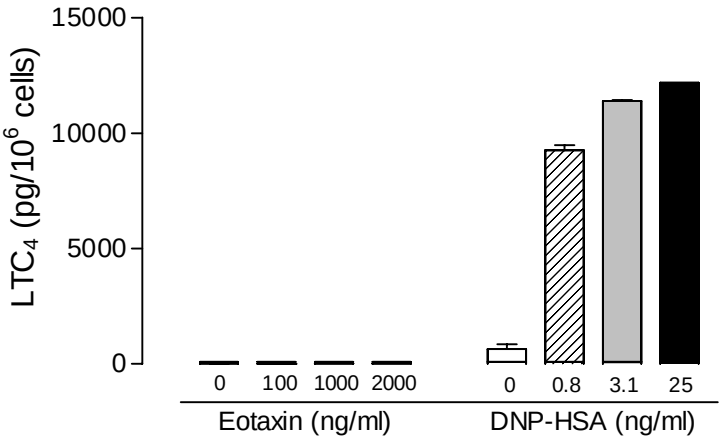

Figure 2: Stimulation with DNP-HSA (0.8, 3.1 and 25 pg/ml) resulted in increased production of LTC_4 by BMMCs. Stimulation with eotaxin does not induce production of LTC_4. Data are represented as mean (± SEM) of quadruplicates.

DISCUSSION

In this study we report that BMMCs express CCR3 on their surface. Other studies showed before that human mast cells in skin, gut, and lung tissue express this receptor (6, 11). To assess the functional properties of CCR3, BMMCs were stimulated with eotaxin, a potent ligand of the CCR3 receptor. Increasing concentrations of eotaxin do not lead to granule exocytosis by BMMCs. These results are concordant with other studies, showing that eotaxin does not induce mast cells activation. Human mast cells isolated from lungs do not release histamine after stimulation with eotaxin (11). Moreover, RANTES, which binds to both CCR1 and CCR3, does not induce histamine or β-hexosaminidase release by BMMCs (7).

Although other studies have looked at mast cell exocytosis by eotaxin, our study is the first to investigate whether stimulation with eotaxin leads to the production of arachidonic acid metabolites. Previous studies in our lab showed that BMMCs are capable of producing lipid mediators, also after non-immunological stimuli (12). Leukotrienes are potent bronchoconstrictors, and are involved in the pathology of asthma. However, no LTC_4 formation can be detected after stimulation of BMMCs with eotaxin.

Other chemokines can activate mast cells leading to mast cell exocytosis. For example, monocyte chemotactic protein-1 (MCP-1), a ligand for CCR2, can dose-dependently induce histamine and serotonin release from rat peritoneal and pulmonary mast cells *in vitro* (13, 14). Surprisingly, the same dose of MCP-1 does

not lead to activation of BMMCs. This may reflect maturational differences between different population of mast cells resulting in altered responsiveness of these cells. In the present study we used BMMCs which are often used as a tool to investigate mast cell activation *in vitro*. Different culture conditions influence the maturation of mast cells and alter the responsiveness of these cells (12). *In vivo,* the microenvironment is different from *in vitro* circumstances. Therefore, in the present study the effect of eotaxin on mast cell activation is tested *in vivo*. No increase in mouse mucosal mast cell protease-1 levels in serum of mice injected with eotaxin could be detected, questioning the role of CCR3 in mast cell activation. In contrast, instillation of MCP-1 into the lungs of mice results in increased levels of histamine in the airway (14). This indicates that stimulation of CCR2 leads to mast cell stimulation while stimulation of CCR3 does not.

A rise in intracellular calcium concentrations was measured after stimulation of mast cells in response to eotaxin indicating that mast cells become activated after stimulation of the CCR3 (9), but this activation does not lead to degranulation or LTC_4 production. Binding of CCR3 ligands to the receptor results in activation of eosinophils and basophils, leading to the formation of various cytokines (15, 16). This might suggest that the signal transduction cascade is differently regulated in mast cells compared to eosinophil and basophils.

However, activation of CCR3 might lead to migration of mast cells. Indeed, CCR3 ligands are involved in mast cell migration *in vitro* (6, 7, 11). Both eotaxin and RANTES act as a chemoattractant for mast cells in a chemotaxis assay. Eotaxin is expressed under non-inflamed conditions and may therefore contribute to basal tissue homing of mast cells. Increased expression of eotaxin during inflammation can be the cause of increased numbers of mast cells in these conditions. For example, elevated numbers of mast cells are demonstrated in the lungs during the late phase reaction in asthma (17). In contrast, eotaxin injection *in vivo* leads to accumulation of mainly eosinophils at the site of injection and not mast cells. At the time of an inflammatory response other mediators might be formed which alter the responsiveness of mast cells.

Mast cells are involved in eotaxin-induced eosinophil migration. Eotaxin-induced eosinophil migration *in vivo* is dramatically decreased in absence of mast cells or stabilised mast cells (18, 19). These studies illustrate that activated mast cells have a profound function in eotaxin-induced eosinophil migration. Since direct mast cell activation in response to eotaxin is not demonstrated, the effect of mast cells in this process is likely to be indirect.

Another function of eotaxin might be its involvement in differentiation of mast cells (8). Mast cell precursors arise in the bone marrow and migrate to tissue where they undergo terminal differentiation into mucosal (tryptase positive cells in humans) or connective tissue type (tryptase/chymase positive cells in humans) cells. The final differentiation in one type or other type of cell depends on the microenvironment in the tissue. Human mast cells, which are chymase tryptase positive, primarily express CCR3 compared to tryptase positive cells (6). In the present study we used BMMCs cultured with IL-3 containing medium, which evolve into mucosal-like mast cells. Culturing BMMCs in the presence of IL-4 and SCF gives rise to connective tissue-type mast cells. This type of mast cell did not respond to eotaxin either (data not shown).

This study showed that CCR3 is present on murine BMMCs. Activation of this receptor by eotaxin does not result in mast cell degranulation or production of LTC_4. Although eotaxin does not activate mast cells directly, attraction of these cells to the site of inflammation may be important in enhancing inflammatory conditions.

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Chapter 6

Eotaxin inhalation results in eosinophil migration from blood to sputum in mild asthmatic patients

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SUMMARY

Eotaxin is a chemotactic cytokine, which is involved in eosinophil trafficking. This study aimed to assess the effect of eotaxin inhalation on migration of eosinophils to the airways. In this randomised placebo-controlled crossover trial, 5 mild asthmatic and 5 non-asthmatic volunteers were asked to inhale 20 μg of eotaxin. The effect of this treatment on sputum eosinophils, circulating eosinophils, airway reactivity to methacholine (PC_{20}), exhaled nitric oxide and carbon monoxide, and sputum eotaxin concentrations was determined for up to 72 hours after inhalation. Sputum was induced before inhalation and 5, 24 and 72 hours after inhalation. At five hours, increased concentrations of eotaxin were measurable in sputum. At the same time, a significant increase (1.7 fold) in sputum eosinophils was detectable in asthmatic volunteers. Changes in sputum eosinophils after eotaxin inhalation almost reached significance compared to placebo inhalation. Interestingly, prior to the increase in sputum eosinophilia, there was a significant drop in circulating eosinophils. Treatment with eotaxin did not effect PC_{20} or exhaled nitric oxide and carbon monoxide. In contrast to asthmatic subjects, no influx of eosinophils to sputum was detectable in non-asthmatic volunteers. This might be due to increased numbers and responsiveness of eosinophils in asthmatics. Thus, increased eotaxin levels in the airways might cause trafficking of eosinophils from the circulation to the airway lumen in mild asthmatic patients but not in healthy subjects.

INTRODUCTION

Eotaxin is a member of the CC-chemokine sub-family (1), and binds selectively to the CC-chemokine receptor-3 (CCR3) (2), which is expressed on eosinophils, basophils, Th2 cells and mast cells (2-5). Eotaxin is involved in migration and activation of these cell types. In several animal models it has been shown that eotaxin is involved in eosinophil trafficking. Injection of eotaxin into the skin or airways of guinea pigs results in migration of eosinophils to the site of injection (1, 6). In the airways eotaxin is mainly produced by epithelial and endothelial cells (7, 8) after stimulation with pro-inflammatory cytokines, including interleukin (IL)-1, IL-4 and tumour necrosis factor-α. (7, 9).

Since eotaxin acts on eosinophils, basophils, Th2 cells, and mast cells which are key effector cells involved in asthma, it is believed that eotaxin might be important in the pathophysiology of this disease. Indeed, increased expression of eotaxin mRNA in the airways of asthmatic patients was demonstrated (10-12). In asthmatic patients, sputum eotaxin levels positively correlate with the number of eosinophils present in the airways and the severity of the disease (12, 13). Taken together, it is likely that eotaxin is involved in the pathophysiology of asthma.
Therefore, in this study we investigated whether eotaxin inhalation resulted in airway eosinophilia, a characteristic feature of asthma. In this double blind, placebo-controlled, crossover study, mild asthmatic and non-asthmatic volunteers inhaled a single dose of eotaxin. The primary objective of this study was to assess the effect of this treatment on the number of eosinophils in sputum and in the circulation. Also, the degree of airway inflammation was determined by measuring exhaled nitric oxide (NO) and carbon monoxide (CO). Furthermore, to investigate whether eotaxin is involved in inducing airway hyper-responsiveness, lung function parameters were measured.

MATERIALS AND METHODS

Patients

Five (4 female) atopic mild asthmatic (age range 23-45) and 5 (2 female) non-atopic non-asthmatic (age range 25-46) volunteers participated in this study (see table 1 for patients characteristics). All were non-smokers. Non-asthmatic volunteers did not have a history of respiratory diseases. All mild asthmatic volunteers had a history of chest tightness and wheezing. They used β_2-receptor agonist as needed, but had not used steroid treatment for at least four weeks prior to the start of the study. None of these volunteers had suffered from a respiratory infection four weeks prior to the study. Their FEV_1 (force expiration volume in 1 second) was >70% predicted. Additionally, airway responsiveness to methacholine (PC_{20}) in asthmatics was < 8 mg/ml while non-asthmatics had a PC_{20} of > 16 mg/ml methacholine. All volunteers signed an informed consent and the protocol was approved by the ethics committee of the Royal Brompton Hospital.

Subject	A/NA	Sex	Age	FEV_1 (%pred)	PC_{20} (mg/ml)	Sputum eosinophils (%)	NO (ppb)
1	NA	F	26	91.4	64	0.67	4
2	A	F	38	93.4	1.5	0.0	73.8
3	NA	M	26	77.7	64	0.17	17.5
4	A	M	27	90.9	0.45	0.83	37
5	NA	F	46	74.3	64	1.7	10.6
6	A	M	29	90.8	0.69	0.0	4.7
7	NA	F	25	95.2	64	0.0	4.6
8	A	F	24	90.0	0.22	0.17	47.9
9	NA	F	23	95.0	64	0.17	17.3
10	A	M	24	84.4	4.6	1.5	78.2
Mean (SEM)	A	2 F 3 M	23.7 (2.6)	89.9 (1.5)	0.54^{*} ($1.4^{\#}$)	0.50 (0.15)	48.3 (14.9)
Mean (SEM)	NA	4 F 1 M	29.2 (4.7)	87.1 (4.1)	64^{*} ($0.0^{\#}$)	0.53 (0.31)	10.8 (3.3)

Table 1: Characteristics of asthmatic (A) and non-asthmatic (NA) volunteers and their FEV_1 (% predicted), PC_{20} (mg/ml methacholine), precentage of sputum eosinophils and exhaled NO (parts per billion). * Geometric mean, $^{\#}$ Geometric standard error of the mean.

Study design

The study was designed as a double blind, randomised placebo-controlled, crossover study. The study continued over a period of three weeks. Baseline sputum cells and PC_{20} were determined in the week before start of the study. Baseline FEV_1, NO/CO measurements were done at the start of the study. In the first and third week, either eotaxin (R&D, 20 μg/2 ml saline 0.1% HSA) or placebo was inhaled for 5-10 minutes using a nembulizer (MEDIC-AID, Pagham, UK). Thereafter, the nembulizer was flushed with saline to ensure all the eotaxin was inhaled. FEV_1, blood eosinophils, exhaled NO/CO were determined at 0.5, 1, 2, 3, 4, 5, 24, and 72 hours after inhalation. Sputum was induced and PC_{20} to methacholine was measured at 5, 24, and 72 hours after the inhalation. Week two was considered as a washout period.

Sputum

Induction: Fifteen minutes prior to sputum induction asthmatic volunteers inhaled 200 mg Salbutamol. To induce the sputum patients inhaled 3.5 % saline solution for 15 minutes using a nembulizer (DeVilbiss Ultraneb 2000). Before sputum induction and before coughing up sputum patients were asked to spit saliva into a bowl and rinse their mouth with water to avoid saliva contamination. Sputum produced in the

first 5 minutes was regarded as an unsuitable sample. Sputum produced in the last 10 minutes was coughed up into a tube.
Processing: Part of the sputum sample was kept aside for eotaxin ELISA. This part of the sputum was weighted and dissolved in cold TFA buffer. Samples were stored at -20°C. The remainder of the sputum sample was used for cell differential analyses. Therefore, 0.25 ml 0.1% DTT (1,4-Dithio-DL-threitol; Sigma, St Louis, USA) was added. The sputum was made homogenous by repeated agitation of the sample. The sample was diluted with Hanks Balanced Salt Solution (HBSS, Sigma) to make up a volume of 5 ml bringing the final DTT concentration to 0.005%. The sputum was centrifuged (300g, 10 minutes), and supernatant was stored in -70°C. Cells were washed once with HBSS and cell pellet was resuspended in 1 ml HBSS. The number of cells was counted using a haemocytometer and kimura stain. Viability was assessed using trypan blue. Cytospins (Shandon, 6 minutes, 600 rpm) were made with 20,000 inflammatory cells per slide. Slides were stained with Diff-Quick (Merz & Dale, Dudingen, Switzerland). Differential cell counts were made from 2 slides per sample by counting 300 inflammatory cells per slide. A sample was considered to be inadequate if the percentage of squamous cells was > 80%.

Circulating eosinophils

Blood eosinophil counts were determined by the haematology department of the hospital using an automated haematology analyser (Advia, UK).

Lung function parameters

FEV_1: Forced expiratory volume in 1 second was measured using a spirometer (Vitallograph, Buckingham UK). The better of two attempts was recorded.
PC_{20}: fall of 20% of FEV_1 to methacholine was measured as described previously (14). Increasing doubling doses of methacholine (0.06-64 mg/ml) were administered via a dosimeter (Mefar, Bovezzo, Italy). Each concentration was inhaled five times for one second followed by 6 seconds non-breathing time. Two minutes after each dose FEV_1 was measured. PC_{20} was recorded as the concentration of methacholine resulting in a drop of FEV_1 by 20% compared to FEV_1 after saline inhalation. The PC20 value was calculated by interpolation of the logarithmic dose-response curve.

Exhaled nitric oxide and carbon monoxide

Exhaled nitric oxide (NO) and carbon monoxide (CO) was measured by a chemiluminescence breath analyser (Logan research, Rochester UK). Patients were

asked to take a deep breath in through their mouth and breathing out steadily maintaining constant exhalation flow for as long as possible (at least 20 seconds). Measurements were taken at the end of exhalation (15).

Mediator analyses

Eotaxin ELISA: Eotaxin was measured by ELISA using matched-paired antibodies (R&D, Abingdon, UK). A 96-wells plate was coated with 4 µg/ml capture antibody (100 µl/well) in phosphate buffered saline (PBS) and incubated overnight. The next day the plate was washed three times with wash buffer (PBS, 0.05% Tween-20 (Sigma, Dorset, UK), pH 7.4). The wells were blocked for one hour with 300 µl blocking buffer per well (PBS, 1% BSA (bovine serum albumin), 5% Sucrose, 0.005% NaN_3). The samples were diluted once in assay diluent (tris-buffered saline, 0.1% BSA, 0.05% tween-20, pH 7.3). One hundred µl of the diluted samples was pipetted into each well after washing three times. Also, 100 µl of standard (1000-15.6 pg/ml human eotaxin in assay diluent) was added. After two hours of incubation the plate was washed again followed by biotinylated detection antibody (100 µl/well, 180 ng/ml). The limit of detection for this assay is 10 pg/ml. Again, after incubation of two hours the plates were washed with wash buffer. One hundred µl of streptavidine-HRP (0.125 mg/ml) was added to each well and incubated for 20 minutes. Accordingly, the plate was washed and 100 µl of substrate solution (reagents A and B, Pharmingen) was added per well. After an incubation period of 20-30 minutes in the dark the reaction was stopped by adding 50 µl of H_2SO_4 in each well. Absorbence was measured at 450 nm using a plate reader. The whole assay was performed at room temperature.

Eosinophilic cationic protein radio-immuno assay: A kit was purchased from Pharmingen (Heidelberg, Germany). The measurements were done according to the manufacturer's instructions.

Statistics

Data are represented as individual data points or as mean ± SEM. Differences were analyzed using the Wilcoxon paired signed rank test. Between group differences (active vs placebo) were analyzed using the Mann-Whitney U-test. A sample size calculation was performed (16). We assumed that a difference of 0.5% is relevant. Sample size calculations showed that we need to include at least six individuals to detect such a difference in our study with 80% power, given a type 1 probability of 0.05%.

RESULTS

Sputum cells

The average percentage eosinophils of total cell number in sputum at baseline was 0.51 ± 0.29 in asthmatic volunteers, and 0.53 ± 0.31 in healthy subjects (table 2). In the asthmatic group, eotaxin inhalation resulted in significantly increased eosinophils in sputum after five hours (1.1 ± 0.30, p=0.031) (figure 1).

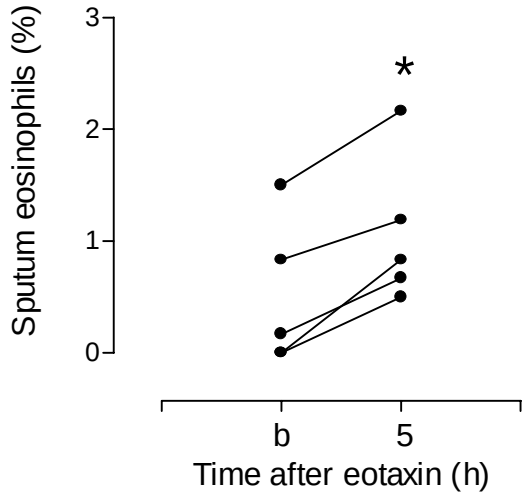

Figure 1: Percentage of eosinophils in sputum at baseline and 5 hours after eotaxin inhalation in asthmatic subjects. There is an increased percentage of sputum eosinophils 5 hours after eotaxin inhalation compared to baseline (b) levels (*p=0.031, Wilcoxon paired signed rank test).

At this time point, changes in sputum eosinophils after eotaxin inhalation tended to increase compared to placebo inhalation (p=0.055). In asthmatic volunteers, 24 and 72 hours after eotaxin inhalation no changes in sputum eosinophils were detectable compared to baseline sputum levels (24h: 0.47 ± 0.15, 72h: 0.93 ± 2.3; table 2).

Time (h)	A-eotaxin	A-placebo	NA-eotaxin	NA-placebo
B	0.50 ± 0.29	0.50 ±0.29	0.53 ± 0.293	0.53 ± 0.31
5	1.07 ± 0.30 *	2.13 ± 1.1	0.90 ± 0.78	0.17 ± 0.13
24	0.47 ± 0.15	1.42 ± 0.86	0.358 ± 0.19	0.23 ± 0.233
72	0.93 ± 0.23	0.43 ± 0.11	0.633 ± 0.40	0.40 ± 0.16

Table 2: Sputum eosinophils (% of total leukocytes) in asthmatic (A) and non-asthmatic (NA) volunteers. Sputum eosinophils were determined before (b), 5, 24 and 72 hours after eotaxin or placebo inhalation. Data are expressed as mean ± SEM. *p=0.031 compared to baseline values (Wilcoxon paired signed rank test). There are no significant changes after placebo inhalation. Differences in changes in sputum eosinophils in asthmatic patients after eotaxin or placebo inhalation almost reached significance p=0.055 (Mann-Whitney U test).

In the non-asthmatic non-atopic group no significant changes in sputum eosinophils were present at any time point after eotaxin inhalation (table 2)

The percentage of neutrophils increased in both groups following both eotaxin and placebo (figure 2). After eotaxin inhalation the percentage of neutrophils in sputum increased from 25.03 ± 4.83 to 39.53 ± 6.23 at 24 hours. After placebo treatment a similar increase in neutrophils was detected (24 hour: 40.44 ± 2.85). No significant changes in lymphocytes and macrophages were noticeable (data not shown).

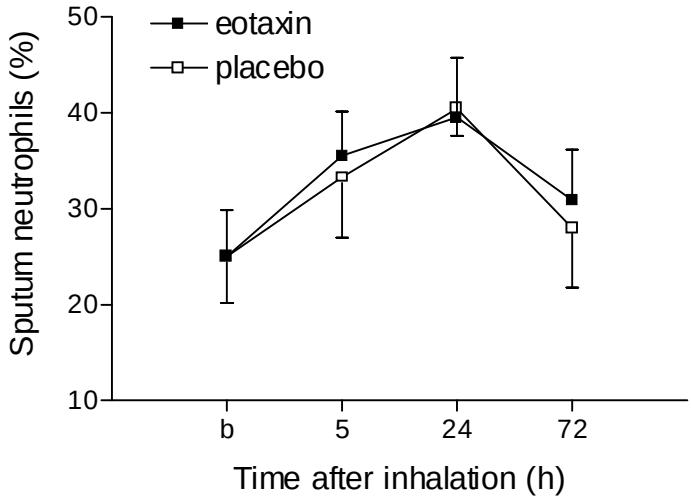

Figure 2: Percentage of sputum neutrophils after eotaxin (black squares) or placebo (open squares) inhalation. A clear neutrophilia was present 24 hours after eotaxin and placebo inhalation compared to baseline (b) levels. Data are represented as mean ± SEM.

Circulating eosinophils

Three and four hours after eotaxin inhalation a decrease in blood eosinophils was detectable (baseline: 0.25 ± 0.057, three hours: 0.19 ± 0.041 p=0.013, four hours: 0.16 ± 0.041 p=0.042). This decrease was not evident after placebo inhalation (table 3).

Eotaxin concentrations

Eotaxin was measured in sputum samples not treated with DTT and in plasma samples at different time points after inhalation. Sputum eotaxin levels were measured in 5 volunteers after eotaxin inhalation. In three of these samples eotaxin levels increased at 5 hours and returned to baseline levels at 24 hours. In two of the 5 patients eotaxin was undetectable (figure 3).

Time (hours)	Eotaxin	Placebo
Basal	0.26 ± 0.050	0.22 ± 0.033
0.5	0.26 ± 0.044	0.20 ± 0.030
1	0.233 ± 0.055	0.20 ± 0.043
2	0.21 ± 0.035	0.20 ± 0.39
3	0.19 ± 0.041*	0.15 ± 0.037
4	0.17 ± 0.040**	0.18 ± 0.032
5	0.19 ± 0.041	0.21 ± 0.039
24	0.22 ± 0.033	0.23 ± 0.036
72	0.22 ± 0.039	0.22 ± 0.042

Table 3: Number of blood eosinophils (10^9/ml) at several time points after inhalation of eotaxin or placebo. At 3 and 4 hours after eotaxin inhalation a decrease in circulating eosinophils in observed (*p=0.0133, **p=0.0431 compared with baseline values (paired t-test)) which is not present after placebo inhalation. Data are represented as mean ± SEM

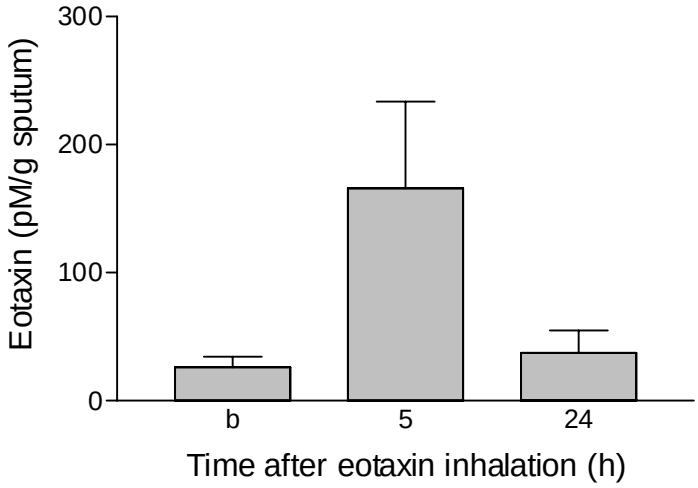

Figure 3: Eotaxin concentration (pM/g sputum) in sputum after eotaxin inhalation in five volunteers. A marked increase in sputum eotaxin concentrations is detectable in three patients. In the other two patients the eotaxin concentration was below detection limit. Data are represented as mean ± SEM.

Eosinophil activation

Eotaxin is also able to activate eosinophils; therefore we measured eosinophilic cationic protein (ECP). ECP is released by eosinophils upon activation. In asthmatic patients no significant changes were observable after eotaxin inhalation (baseline: 20.7 ± 7.0, 5 hours: 20.9 ± 4.2, 24 hours: 17.6 ± 4.9, and 72 hours: 10.8 ± 3.6) (figure 4).

Exhaled NO/CO

Exhaled NO and CO are increased in asthmatics patients and have been utilised as non-invasive markers of inflammation. We hypothesised that eotaxin inhalation would result in increasing levels of exhaled NO and/or CO. Indeed, baseline exhaled NO concentrations were increased in asthmatic volunteers compared to non-asthmatic volunteers (figure 5). Exhaled CO did not differ at baseline between the two groups. However, no significant changes in exhaled NO and CO were detectable after treatment with eotaxin.

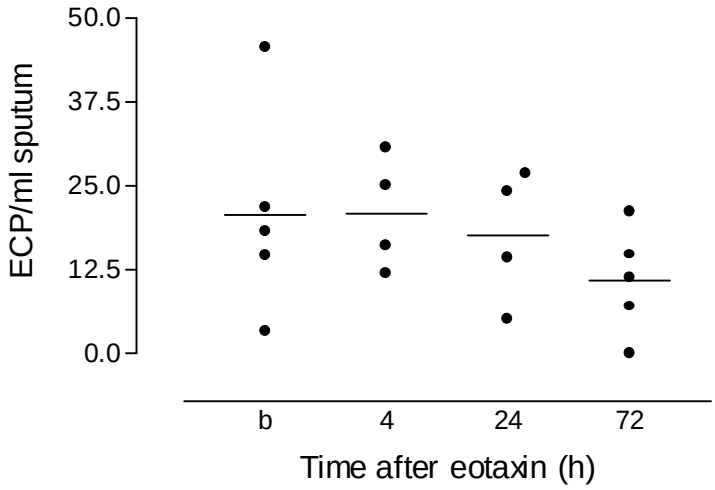

Figure 4: Eosinophil cationic protein (ECP) concentration per ml sputum at baseline, 5, 24, and 72 hours after eotaxin inhalation. No significant changes were detectable.

Lung function

The average baseline airway responsiveness to methacholine in asthmatic volunteers was 2.33 ± 1.10 mg/ml compared to more than 64 mg/ml methacholine in non-asthmatic volunteers. To investigate whether eotaxin has a direct or indirect effect on lung function, PC_{20} was determined at 5, 24, and 72 hours after treatment. The results show that eotaxin or placebo inhalation did not alter logPC_{20} values (table 4). Inhalation of eotaxin did not effect FEV_1.

	Asthmatics		Non-asthmatics	
Time	Placebo	Eotaxin	Placebo	Eotaxin
5	-5.97 ± 27.7	6.12 ± 28.7	-4.90 ± 4.35	-15.1 ± 11.7
24	10.5 ± 14.5	5.76 ± 5.80	0.0 ± 0.0	-0.941 ± 1.05
72	24.2 ± 19.0	10.7 ± 17.6	0.0 ± 0.0	-7.78 ± 6.84

Table 4: Changes (%) in airway reactivity to methacholine (log PC_{20}) in asthmatics after treatment with eotaxin or placebo. No significant changes were detectable after treatment with eotaxin or placebo. Data are represented as mean ± SEM.

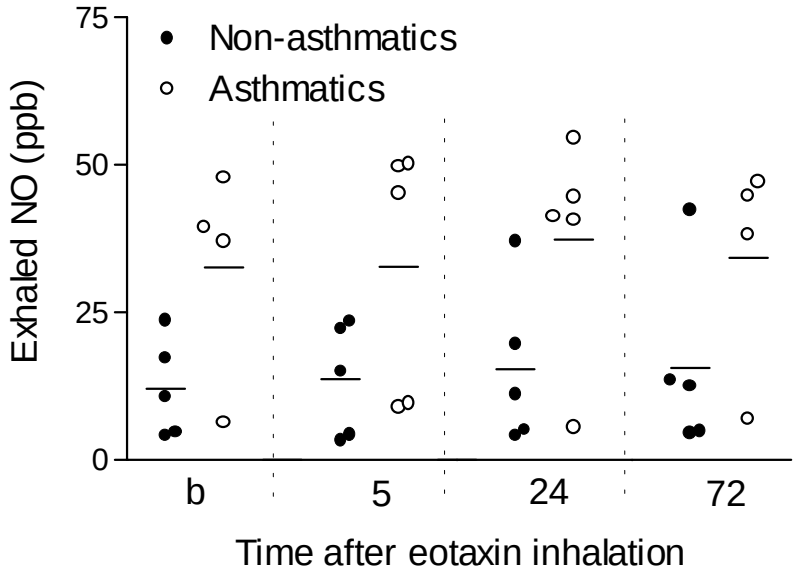

Figure 5: Exhaled NO (ppb) (A) and CO (ppm) (B) at several time points (baseline, 0.5, 1, 2, 3, 4, 5, 24, 72 hours) after eotaxin inhalation in asthmatics (closed circles) and non-asthmatics (open circles). No significant changes were detectable in exhaled gases after eotaxin inhalation.

DISCUSSION

This is the first study to show that eotaxin inhalation results in increased relative number of sputum eosinophils in mild asthmatic patients. Five hours after eotaxin inhalation the percentage of sputum eosinophils was almost doubled compared to levels of baseline sputum eosinophils in asthmatic volunteers. The increase in sputum eosinophils in the mild asthmatic group was transient, since 24 and 72 hours after eotaxin inhalation the percentage of sputum eosinophils returned to baseline levels. After placebo inhalation there were no changes in sputum eosinophils. At three and four hours after eotaxin inhalation, just before the increase in sputum eosinophils, a decrease in circulating eosinophils was observed. This might indicate that eosinophils from the circulation migrate to the airways.

The pattern of eosinophil influx corresponds with eotaxin concentrations measured in sputum. At five hours after eotaxin inhalation increased eotaxin concentrations were measurable in sputum supernatant. At 24 hours after inhalation eotaxin levels in sputum were comparable to baseline values. An explanation for this transient increase in eotaxin concentrations might be that after a while eotaxin is broken down by proteases in the airways, or eotaxin is diffused further into the lungs. From this study it appears that eotaxin inhalation results in increased eotaxin levels in sputum 5 hours after eotaxin inhalation. As a result eosinophils migrate from the circulation to the airways, leading to in decreased number of blood eosinophils prior to increased sputum eosinophils.

Since asthmatic patients have an increased expression of eotaxin in their airways, eotaxin is believed to be involved in inflammation during asthma. Increased eotaxin levels were measured in sputum, and broncho-alveolar lavage fluid of asthmatic patients. Increased eotaxin levels in sputum correlate with the percentage of sputum eosinophils, (12). Allergen challenge induced eotaxin production and correlated with the number of eosinophils to in the BAL fluid (17). Our results also indicate that eotaxin is involved in airway inflammation, since increasing eotaxin concentrations in sputum trigger eosinophils to migrate to the airways, where high concentrations of eotaxin are present.

Sputum induction is a non-invasive way to determine the number of inflammatory cells in the lungs. Since the number of eosinophils in sputum correlates well with the number of eosinophils in bronchial biopsies (18), sputum is a well-accepted method of assessment of airway inflammation. In this study, 24 hours after eotaxin or placebo inhalation a prominent neutrophil influx was present in the sputum.

Repetitive sputum induction, as done in the present study, is known to cause excessive neutrophil migration to the sputum (19).

Basal sputum eosinophils did not differ between mild asthmatic and non-asthmatic patients in this study. Interestingly, inhalation of eotaxin by non-asthmatic volunteers did not result in changes in sputum eosinophils as seen in mild asthmatic volunteers. It has previously been observed that a proportion, but not all, of asthmatic patients have increased serum levels of IL-5 (20). This cytokine is known to enhance responsiveness of eosinophils to eotaxin by priming these cells (21). Intravenous IL-5 administration to humans induced peripheral mobilization of eosinophils without effecting lung eosinophils and airway responsiveness (Van Rensen, Thorax, 2001 in press). Furthermore, intravenous administration of IL-5 in humans mobilizes eosinophil progenitor cells and induces up-regulation of CCR-3 (Stirling, AJRCCM 2001, in press). Therefore, the fact that mild asthmatic volunteers responded to eotaxin inhalation while non-asthmatic participants did not, may be explained by the degree of eosinophil priming, which may well be less in non-asthmatic subjects due to low levels of IL-5.

It has been shown before that eotaxin is able to induce eosinophil migration in humans (22). In a clinical trial, eotaxin was instilled in the nose of rhinitic volunteers. Comparable to our study, eotaxin application resulted in eosinophil accumulation in nasal lavage fluid. Additionally, nasal symptom score and nasal NO were increased after treatment with eotaxin. Although previous investigators have shown correlation of exhaled NO with the number of eosinophils in the sputum (23), in the present study no changes in exhaled NO were measured after eotaxin inhalation. Certainly the influx of eosinophils into the airways was of low magnitude and may have been too low to induce changes in exhaled NO. Further, nasal application of eotaxin might be more efficient in inducing eosinophil influx since the nasal cavity is smaller then the lungs.

The role of eosinophils in inducing airway hyper-responsiveness is still unresolved. In this study eotaxin inhalation resulted in increased levels of eosinophils in the lungs, while bronchial responsiveness to methacholine was unchanged. This might demonstrate that in this case there is no association between airway responsiveness and airway eosinophilia. Recently, evidence accrued to suggest dissociation between airway eosinophilia and airway hyper-responsiveness. In a clinical trial of anti-IL-5 antibody in asthma, anti-IL-5 treatment abrogated airway eosinophilia after allergen challenge while airway hyper-responsiveness was unchanged (24) indicating that the eosinophils in the airways do not correlate with airway responsiveness.

In conclusion, eotaxin inhalation into the airways of mild asthmatic patients was associated with augmented levels of eotaxin in sputum supernatant, and resulted in an increase in relative eosinophil numbers in sputum five hours after inhalation. A significant drop of circulating eosinophils was observed prior to the increase in airway eosinophils, suggesting that eosinophils migrate from the circulation into the airways due to increased eotaxin levels in sputum. This study lends further weight to the suggestion that eotaxin and/or its receptor CCR3 may be suitable targets for anti-asthma therapies.

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Chapter 7

General Discussion

INTRODUCTION

After the discovery of eotaxin in 1993, it was suggested that this chemotactic protein might be important in eosinophil-mediated disease (1). This protein was called eotaxin since it selectively induced eosinophil migration and did not affect other granulocytes. In view of the fact that eosinophils might be important in the pathophysiology of asthma, it was put forward that eotaxin was involved in eosinophil migration in this disease. Later it became clear that eotaxin, acting via the CC-chemokine receptor-3 (CCR3), also modulated basophil, Th2, and mast cell function (2-4). All these cells are implicated in asthma.

In this thesis we aimed to assess the importance of eotaxin in the development of airway inflammation and airway hyperresponsiveness, characteristic features of asthma. The mechanism behind the development of these two phenomena is not entirely clear. It was investigated whether eotaxin or CCR3 are involved in airway inflammation and airway hyperresponsiveness in order to assess the possibilities of eotaxin or CCR3 as a potential therapeutic target.

In this thesis different methods were used to obtain insight in the relative significance of eotaxin in airway eosinophilia and airway hyperresponsiveness. This issue was addressed by administering eotaxin directly into airways of mice or humans and accordingly examined in both eosinophil migration to the lungs and airway function. Furthermore, we used existing animal models for airway inflammation and hyperresponsiveness induced by either antigen treatment or viral respiratory infections, and reviewed the role of eotaxin in these models. These experiments focussed mainly on eosinophils. Moreover, the effect of eotaxin on mast cells, which are important in initiating and augmenting inflammatory reactions and might be directly involved in inducing airway hyperresponsiveness, was investigated.

The main finding of this thesis point towards a disengagement of eotaxin and the development of airway hyperresponsiveness, and it is unlikely that eotaxin alone induces eosinophil accumulation in the airways. Our findings have been put in perspective with data recently published in scientific literature. First, the expression of eotaxin in inflammatory conditions will be discussed. Second, the role of eotaxin in airway inflammation will be addressed, distinguishing between eotaxin-induced eosinophil migration and antigen- or virus-induced airway eosinophilia. Third, the function of CCR3 expression by mast cells is discussed. Fourth, it is considered

whether eotaxin is involved in airway hyperresponsiveness. Finally the conclusions will be summarised.

EOTAXIN EXPRESSION

Under basal conditions eotaxin is expressed in the human airways and is measurable in serum (5, 6). Asthma patients display increased expression of eotaxin mRNA and protein in their airways (7-9). Furthermore, during this disease CCR3 is increasingly expressed on eosinophils in the bone marrow (10).
BALB/c mice used in our experiments express eotaxin under non-inflamed conditions. Allergen sensitisation and challenge resulted in increased levels of eotaxin in BAL fluid and lungs (chapter 2). Pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1β or IL-4 might be responsible for activation of epithelial and endothelial cells to produce increased amounts of eotaxin (11). *In vivo*, these cell types are the main producers of eotaxin during inflammation (12). Antigen-induced up-regulation of eotaxin expression is dependent on antigen specific T-cells, since anti-CD3 treatment completely abolished increased eotaxin production (13). Further, adoptive transfer of Th2 cells into mice induced antigen-dependent eotaxin expression and airway eosinophilia. In a mouse model for allergic asthma blockade of Th2 formation also abrogated airway eosinophilia (14). This suggests that in the airways, eotaxin expression and airway eosinophilia are Th2 cell-dependent, presumably via the production of Th2-types cytokines, such as IL-4 and IL-5.
As detected in other studies, this increase in eotaxin expression coincides with increasing numbers of eosinophils in the airways (9, 15). This was clearly demonstrated in the experiments done with PI3 infected guinea pigs (chapter 4). Two days after infection of guinea pigs with PI3 virus, eosinophil numbers in the broncho-alveolar lavage (BAL) fluid started to increase and was significantly elevated compared to control treated animals at day three and four after infection. Interestingly, rising eotaxin concentrations proceeded the influx of eosinophils into the airways. Two days after infection eotaxin concentrations were increased compared to non-infected guinea pigs and these levels remained high for at least four days after infection. This demonstrates that eotaxin levels rise prior to eosinophil trafficking to the airways, indicating that eotaxin is involved in the process of trans-migration of these cells. It was shown that respiratory viruses could

directly induce eotaxin expression from epithelial cells (16, 17). Whether the increased levels of eotaxin measured in virus infected guinea pigs is directly induced by respiratory viruses or is indirectly caused by immune reactions to the virus is unclear.

Thus, increased expression of eotaxin in response to viral infections or antigen coincides with eosinophil migration to the airways indicating that eotaxin might be implicated in antigen- and virus-provoked eosinophil migration. However, the fact that eosinophils start to migrate just after eotaxin levels start to increase does not necessarily mean that these two events are associated, but these two phenomena can coincidentally occur at the same time.

AIRWAY INFLAMMATION

Eotaxin-induced eosinophil migration

In order to induce eosinophil migration to the airways, eotaxin concentrations in the airways were experimentally increased in both mice (chapter 2) and humans (chapter 6).

Eotaxin instillation into the airways of mice did not induce increased numbers of eosinophils in the BAL fluid (chapter 2). This indicates that eotaxin alone is not able to induce eosinophil migration to the airways of mice. An explanation for this might be that mice do not have many eosinophils in the circulation or tissue. Other investigators showed that eotaxin induced eosinophil migration (1, 18, 19). However, in these experiments, guinea pigs, antigen sensitised mice and IL-5 transgenic mice were used. Compared to naïve mice, guinea pigs have a greater pool of mature eosinophils in their circulation and lung tissue, which are available to respond to eotaxin. The same is true for antigen sensitised animals and mice that over-express IL-5 resulting in blood eosinophilia (20). IL-5 primes eosinophils, which makes them more responsive to eotaxin (21). However, in chapter 2, intravenous injection of IL-5 did not increase the number of eosinophils or enhanced responsiveness of these cells to eotaxin. Apparently, high concentrations of IL-5 are necessary to induce eosinophilia, *in vivo*.

In chapter 6 it is clearly demonstrated that the number of eosinophils and their responsiveness might be an important factor that accounts for eotaxin-induced eosinophil migration. In human atopic asthmatic volunteers eotaxin inhalation resulted in an increase in eosinophils numbers in the sputum. Quite the opposite,

inhalation of the same amount of eotaxin in non-atopic non-asthmatics did not result in increased sputum eosinophils, which is comparable with studies of naïve mice. Evidently, asthmatic patients showed an enhanced reaction in response to eotaxin compared to healthy volunteers. Increased numbers of eosinophils in atopic, asthmatic patients and increased responsiveness of eosinophils might explain this difference in reaction. Moreover, CCR3 expression on eosinophils or the responsiveness might be enhanced in asthmatic individuals ((22), Stirling AJRCCM 2001, in press). Various cytokines mediate the increase in number and responsiveness of eosinophils, for example IL-5. Pro-inflammatory cytokines might create a suitable environment for eotaxin-induced eosinophil migration. As shown before, instillation of eotaxin into the nose of patients with allergic rhinitis induced a significant increase of eosinophils in nasal lavages, subsequently leading to increased levels of nasal NO and increased rhinitic symptoms (23). This study was performed in patients with rhinitis, under inflammatory circumstances, but not in healthy volunteers. Once more, pro-inflammatory environment might augment eotaxin-induced eosinophil migration.

In order to induce airway eosinophilia, eosinophils should be released from the bone marrow into the circulation. In virus-infected guinea pigs a clear blood eosinophilia was demonstrated at day one after infection (chapter 4). Two days after infection the number of blood eosinophils reached normal levels. This might indicate that these inflammatory cells migrated from the circulation to the airways. Eotaxin can stimulate the release of eosinophils form the bone marrow (24). The involvement of eotaxin in the initiation of blood eosinophilia in our virus model is unlikely, since eotaxin concentrations started to increase at day 2 of infection. Alternatively, IL-5 could be responsible for the induction of blood eosinophilia (21).

A disadvantage of our studies is that we counted the number of eosinophils in the BAL fluid and circulation but not in the lung interstitium. Possibly, eosinophils passed the endothelium but were not triggered to also pass the epithelium. However, other studies showed that eotaxin is able to initiate eosinophil migration from the bone marrow all the way to the airway lumen (25-27).

Collectively, eosinophils respond to increasing eotaxin concentrations *in vivo*, probably only when the eosinophils are in the right pro-inflammatory environment, as is the case with atopic asthmatic individuals. This environment might enhance the number of eosinophils and their responsiveness to eotaxin.

Antigen- and virus-induced airway inflammation

In order to investigate whether eotaxin takes part in antigen-induced airway eosinophilia a mouse model for allergic asthma was used. In this model, allergen challenge of sensitised mice resulted in airway eosinophilia (28). In chapter 3, it is described that anti-eotaxin antibodies do not influence the number of eosinophils in the BAL fluid. This indicates that: either treatment with anti-eotaxin antibodies did not completely block eotaxin, or eotaxin is not involved in eosinophil migration. However, in another study it was shown that the same amount of antibodies in a similar mouse model reduced the degree of airway inflammation (29). An explanation for the contradictory results could be differences in mouse stains used: BALB/c versus C57/bl6. It is known that different strains of mice react differently to allergen inhalation (30, 31).

From recent publications it is evident that eotaxin is involved in early stages of eosinophil migration, as opposed to late. In an ovalbumin model for allergic asthma, antibodies directed against eotaxin resulted in decreased eosinophil migration only at day four of repetitive allergen challenge, while at day seven the number of eosinophils was elevated in equal numbers compared to mice not treated with antibodies (32). In a cutanous model for late phase reaction, eotaxin expression was correlated with early rather than late stages of eosinophil recruitment (33). Furthermore, four hours after allergen provocation in humans the number of eosinophils correlated with eotaxin expression, while 24 hours later eotaxin expression declined without decreased levels of eosinophil in the airways (34). These observations suggest that eotaxin could be involved the in early phase of eosinophil recruitment while other chemokines might initiate later stages of eosinophil trafficking. We only investigated the number of eosinophils in the lungs at the time of established airway eosinophilia. It would be interesting to perform a time course study in ovalbumin treated mice injected with anti-eotaxin antibodies to see a difference in kinetics of eosinophil accumulation in the lungs for the course of eight days of challenge.

Although, eotaxin is able to induce eosinophil trafficking to the lungs, airway eosinophilia can exists without the presence of eotaxin. A decrease in antigen-induced airway eosinophilia can co-exist with unaltered eotaxin levels in the airway (35). Subsequently, airway eosinophilia can exist normally without eotaxin.

Comparable to our results, the previous mentioned studies question the importance of eotaxin in initiating airway eosinophilia. These data indicate the existence of two distinct ways for eosinophil accumulation in the airways: one way is eotaxin

dependent, the other eotaxin independent. Which way of eosinophil migration takes place depends on the model of eosinophil migration or which animal species or strains are used.

In addition, eotaxin knockout mice develop equal airway eosinophilia compared to normal littermates after allergen treatment (36). However, another study showed a reduced migration of eosinophils to the airways in response to antigen. This was prominent early after allergen treatment, while at a later time point the number of eosinophils was increased equally in knockout mice and normal littermates (37).

Investigations on the role of eotaxin in allergen induced eosinophil migration is complicated by the fact that other chemokines (RANTES, eotaxin-2 and -3, monocyte chemotactic protein (MCP)-3, -4, -5, and macrophage inflammatory protein (MIP)-1α) are also active on eosinophils and paralleled with the increase of eosinophil migration (22). Increased concentrations of all of these chemokines can lead to eosinophil migration. As a consequence, absence of eotaxin by treatment with anti-eotaxin antibodies or knockout mice could be compensated by other chemokines. Eotaxin, -2 and -3 bind selectively to CCR3 and therefore act on eosinophils, basophils, mast cells, and Th2 cells. Besides binding to CCR3, RANTES, MIP-1α and MCP-4 bind to CCR1, which is expressed by eosinophils as well. All these chemokines may contribute to infiltration of eosinophils in the airways either via activation of CCR1 or CCR3.

Nonetheless, 95% of the eosinophilic response towards these chemokines were regulated via CCR3, stressing the importance of this chemokine receptor (38). Therefore, a CCR3 receptor antagonist was used in a guinea pig model for viral respiratory infections to investigate the role of CCR3 receptor agonists on eosinophil migration (chapter 4). Surprisingly, CCR3 receptor antagonist treatment did not result in reduced eosinophil migration to the airways of virus infected guinea pigs. Once again, CCR3 is not the only chemokine receptor expressed by eosinophils. In absence of CCR3-mediated response, other chemokines and their receptor might have taken over. Eosinophil migration is a complex process regulated by many chemokines, cytokines and their receptor. Thus, blockade of only one chemokine or chemokine receptor, in this case eotaxin or CCR3, might not be enough to prevent trafficking of eosinophils in response to an allergic reaction or infection with a respiratory virus. In a previous study it was shown that antibodies to IL-5 were not able to decrease airway eosinophilia in response to PI3 virus (39). Future studies in humans have to ascertain whether impeding the eotaxin response

with anti-eotaxin antibodies or CCR3 antagonists leads to decreased airway eosinophilia after allergen provocation or a viral infection.

Other molecules involved in eosinophil recruitment

Besides eotaxin and other chemokines, other molecules have been implicated in eosinophil recruitment, such as IL-5, very late antigen (VLA)-4, platelet activating factor, and leukotiene (LT) B_4.

Since IL-5 is essential for the maturation of eosinophils, IL-5 was pinpointed as a suitable therapeutic target to reduce the number of eosinophils in allergic asthma. Evidence that IL-5 is implicated in asthma is abundant. IL-5 inhalation by asthmatic patients causes increased levels of eosinophils in sputum and increased airway responsiveness (40). IL-5 knockout mice failed to develop airway eosinophilia and airway hyperresponsiveness in response to antigen treatment (41). In a mouse model for allergic asthma antibodies to IL-5 reduced the number of eosinophils (39). In a large clinical trial, antibodies to IL-5 reduced the allergen-induced sputum eosinophilia. Surprisingly, this reduction in cells did not influence the late phase reaction in response to antigen, questioning the role of IL-5 as a therapeutic target for asthma (42). In our studies, anti-eotaxin antibodies and CCR3 antagonists were not effective in decreasing eosinophil recruitment to the airways, questioning the role of eotaxin as a new therapeutic target. However, combination with other chemokine receptors or mediators involved in airway inflammation might offer a better solution in diminishing airway eosinophilia.

MAST CELLS

Mast cells express CCR3 on their surface (3). We tried to investigate the functional properties of this receptor. Bone marrow-derived mast cells are frequently used to investigate mechanisms of mast cells degranulation *in vitro*. Culturing murine bone marrow cells in IL-3 conditioned medium leads to the formation of a homogenous mast cell population (43). We demonstrated by flow cytometry that a proportion of these cells express CCR3 on their surface (chapter 5). Stimulation of these cells with increasing doses of eotaxin did not result in mast cell granule exocytosis, both *in vivo* and *in vitro*. Furthermore, no leukotiene C_4 was formed. These results suggest that eotaxin stimulation of mast cells does not lead to mast cell activation. In contrast, eotaxin is able to activate eosinophils and basophils leading to mediator

release (44, 45). Apparently, the intra-cellular signal transduction pathway is differently regulated in these cells.
CCR3 on mast cells might be implicated in cell migration. *In vitro* studies determined that mast cells migrate to high concentrations of CCR3 ligands (3, 46, 47). Surprisingly, injection eotaxin does not lead to migration of mast cells to the site of injection *in vivo* (39). Another function of CCR3 is the development of embryonic mast cells to mature cells (48).

AIRWAY HYPERRESPONSIVENESS

Airway hyperreactivity is defined as increased reactivity of airway smooth muscle in response to non-specific stimuli and is a characteristic feature of the allergic airways. The cause of this phenomenon is unclear. In this thesis it was investigated whether eotaxin is either directly or indirectly involved in airway hyperresponsiveness.

Eosinophils and airway hyperresponsiveness

During the late phase reaction airway eosinophilia and airway hyperresponsiveness often occur together (49). Whether there is a causal relationship between the degree of airway hyperresponsiveness and the number of eosinophils in the airways remains topic of debate.
Evidence that these two characteristics correlate is numerous. Many clinical studies have found a relationship between the number of eosinophils or eosinophil-specific mediators and disease severity. For example, the numbers of eosinophils in the airways are inversely correlated with PC_{20} (50, 51). Moreover, eosinophils and their cationic products were shown to directly induce airway hyperresponsiveness (52). Further evidence that eosinophils influence airway responsiveness comes from results obtained with glucocorticoids. Treatment with anti-inflammatory drugs reduces the number of eosinophils, which goes together with a reduction of airway symptoms.
On the other hand, support that these two features coincidentally happen at the same time and do not influence each other is accumulating. Airway eosinophilia can exists without airway hyperresponsiveness, as seen in eosinophilic bronchitis, suggesting that the mere presence of eosinophils in the airways is not enough for inducing airway hyperresponsiveness (39, 53). The other way around, airway

hyperresponsiveness can take place without the presence of eosinophils in the airways (42). This implies that airway inflammation and hyperresponsiveness are independently regulated but closely interrelated (49). This was also shown in our laboratory. Although IL-5 antibodies abrogated airway eosinophilia in mice treated with ovalbumin, airway hyperreactivity could still be demonstrated (53). On the other hand, antibodies to IL-5 abolished airway hyperreactivity without affecting the number of eosinophils in a guinea pig model for allergic asthma (39).

In conclusion, airway hyperresponsiveness can take place without the presence of eosinophils. Nevertheless, airway eosinophils can influence the degree of airway responsiveness. Therefore, eotaxin might influence airway responsiveness by inducing accumulation of eosinophils to the airways.

Eotaxin and airway hyperresponsiveness

Several clinical studies support the hypothesis that eotaxin and airway hyperresponsiveness are associated. In a cross-sectional study, serum eotaxin levels were elevated in acute asthmatics compared to stable asthmatic controls (6). Additionally, plasma eotaxin levels were inversely correlated with lung function (54). The number of eotaxin positive cells is related to the degree of airway hyperresponsiveness (8).

In chapter 2, eotaxin did not directly influence airway responsiveness. This might be explained by the fact that eotaxin did not induce airway eosinophilia. On the other hand, eotaxin also acts on other cells such as basophils, Th2 cells and mast cells. These cells are not abundantly present in the airways of naïve mice. Therefore, eotaxin might not influence reactivity of naive murine airways. Although eotaxin inhalation in human mild asthmatic individuals caused an increase in sputum eosinophils no changes in airway function (FEV_1 and PC_{20} to methacholine) were detectable (chapter 6). To keep in mind, these changes in sputum eosinophils were very small and therefore might not have influenced airway responsiveness.

Injection of eotaxin in saline challenged mice did not influence airway responsiveness. Treatment with eotaxin prolonged the period of airway hyperresponsiveness in ovalbumin challenged and sensitised mice (chapter 3). This might indicate that eotaxin is not involved in the development of airway hyperresponsiveness, but it can influence existing airway responsiveness.

Some remaining questions

The last eight years a lot of research is done to clarify the role of eotaxin in airway pathology. Despite this intensive research a lot of questions remain to be elucidated.

Why is eotaxin over-expressed during allergen challenge and how is it regulated?

During an allergic reaction eotaxin is increasingly expressed. This might illustrates the importance of eotaxin in airway pathology indicating that this expression is functional. On the other hand, eotaxin could be expressed because it is induced by pro-inflammatory cytokines and has similar signal transduction pathways compared to other inflammatory cytokines, involving NFκB for example.

Why is there so much redundancy and promiscuity in the chemokine system?

This is an intriguing question. Multiple receptors are present on one type of cell; there are multiple receptors for one ligand and one ligand for multiple receptor; and multiple ligands and receptors evoke the same cellular function. The purpose of this redundancy is not clear; however, in this way a tightly regulated network is formed. Small changes in expression can have big consequences, or big changes might have small consequences. Furthermore, one process can be regulated by various chemokines/receptors at different stages.

What are the roles of different forms of eotaxin?

This question relates to the previous question. At first eotaxin was believed to be special since it only bound to CCR3. Nowadays, eotaxin-2 and -3 are discovered which are also selective for CCR3. The specific roles of these restricted CCR3 receptor agonists remains to be elucidates. It might be that these three chemokines participate in different stages of airway inflammation (63).

Why is eotaxin expressed in organs that do not home eosinophils?

Eotaxin is expressed under basal, non-inflamed circumstances, and therefore is involved in basal tissue homing of eosinophils (37). However, organs that do not home eosinophils under basal condition also express eotaxin. This might indicate an unknown function for eotaxin.

Will CCR3 antagonists block eosinophil accumulation and thereby prevent lung pathology?

Of course this is an important question with regard to treatment of asthma. From this thesis and other data it seems unlikely that one antagonist is able to block eosinophil migration. Blockade of multiple chemokine receptor might be a better alternative to prevent airway eosinophilia. Furthermore, whether reduced airway eosinophilia will decrease airway symptoms remains to be elucidated.

In order for eosinophils to influence airway responsiveness, these cells need to be activated. Eotaxin can activate eosinophils to release toxic products and inflammatory mediators (55-57). We could not demonstrate eosinophil activation by eotaxin *in vivo* (chapter 2, and 3). Addition of eotaxin to airways of ovalbumin challenged mice did not result in increased release of eosinophil peroxidase (EPO). Surprisingly, no increased levels of EPO were detectable in mice sensitised and challenged with ovalbumin compared to mice challenged with saline (chapter 3 and (28)). Challenge of mice for 8 consecutive days might have exhausted eosinophils of EPO by the time of measurement (chapter 3). Therefore, the prolonged effect of airway hyperresponsiveness might be due to effect of eotaxin on other cells.

Similar to our data, other investigators showed, that eotaxin alone is not able to induce airway hyperresponsiveness in guinea pigs. Administration of eotaxin into the airways resulted in accumulation of eosinophils to the airways without airway hyperresponsiveness (25). Subsequently, inhalation of platelet activating factor was necessary to induce eosinophil activation resulting in airway hyperresponsiveness (25). In another study, eotaxin instillation to IL-5 transgenic mice induced airway hyperresponsiveness (58). Interestingly, LTC_4 receptor antagonists blocked the eotaxin-induced airway hyperresponsiveness. Both these studies suggest that eotaxin can only induce hyperreactivity in combination with other mediators, e.g. platelet activating factor and IL-5.

Thus, the mere presence of eosinophils in the airways is not enough to induce airway hyperreactivity. Eosinophils need to be activated and release their toxic proteins in order to induce airway hyperreactivity. Mould and colleagues demonstrated that gene transfer of IL-5 and eotaxin induced airway eosinophilia but not eosinophil degranulation and airway hyperresponsiveness. In association with IL-5 and eotaxin expression, antigen inhalation was required for eosinophil degranulation and airway hyperresponsiveness to occur (26). This suggests that other factors in association with IL-5 and eotaxin trigger eosinophil degranulation and subsequently leading to airway hyperresponsiveness. Reviewing experiments on airway hyperreactivity, one has to keep in mind that there are differences between mouse and human eosinophils. Malm-Erjefält and colleagues demonstrated that murine eosinophils do not degranulate in response to allergen while human eosinophils do (59). Furthermore, there are differences in content of eosinophil granula as well as surface molecules (60). In comparison, no eosinophil activation could be demonstrated after allergen challenge, as measured by EPO levels in chapter three.

Treatment of mice with antibodies to eotaxin and guinea pigs with CCR3 receptor antagonists did not change antigen- or virus-induced airway hyperresponsiveness, respectively. Again, this indicates that eotaxin is not directly involved in airway hyperresponsiveness. However, these results might be explained by the fact that this treatment did not alter eosinophil numbers in the BAL fluid. In another study eotaxin antibodies were able to reduce the ovalbumin-induced airway hyperresponsiveness only partly (29).

Despite the role of eosinophils, activation of mast cells can also lead to airway hyperresponsiveness. In the early phase reaction, mast cells induce airway hyperresponsiveness by IgE-dependent activation, leading to the release of broncho-constrictive mediators, such as histamine and LTC_4. Through the production of cytokines and chemokines mast cells contribute to the late phase reaction (61). From chapter 5 it is clear that eotaxin does not activate bone marrow-derived mast cells. In spite of this, eotaxin might induce and augment the inflammatory reaction by attracting mast cells to the site of inflammation in this way contributing to airway hyperresponsiveness. In the late phase reaction increased numbers of mast cells are present in the lungs (62). In the airways other inflammatory cytokines could induce mast cells activation and therefore airway hyperresponsiveness.

Taken together, it is unlikely that eotaxin is able to induce airway hyperreactivity single-handedly. In humans, eotaxin might enhance airway hyperresponsiveness by inducing airway eosinophilia, while in mice eotaxin is involved in maintaining this condition. Although eotaxin did not lead to mast cell degranulation, eotaxin might be involved in migration of these cells to the lungs and thereby augmenting airway hyperresponsiveness.

CONCLUSION

This thesis started out with addressing three questions (see page 21 and 22). The results described in this thesis lead to the following answers:

1. Does eotaxin instillation in the airways cause airway eosinophilia and airway hyperresponsiveness? From chapter 2, we conclude that eotaxin does not induce trafficking of eosinophils, and eotaxin is not involved in airway hyperresponsiveness. In contrast, in human asthmatic volunteers eotaxin induced a small increase in sputum eosinophils. This indicates that eotaxin only induces

eosinophil migration under inflammatory circumstances. However, eotaxin is not involved in airway hyperresponsiveness.

2. Is antigen- or virus-induces airway inflammation and airway hyperresponsiveness regulated by eotaxin? Although eotaxin expression increases during a viral respiratory infection or after allergen challenge, blockade of eotaxin or its receptor did not result in changes in airway eosinophilia and airway hyperresponsiveness. These studies demonstrate that eotaxin is not important in virus- or antigen-induced airway inflammation and hyperresponsiveness.

3. Can eotaxin activate mast cells and thereby induce airway inflammation and hyperresponsiveness? Although mast cells express CCR3 on their surface, stimulation of this receptor with eotaxin does not result in mast cells activation. Therefore, it is unlikely that eotaxin induces airway pathology by activating mast cells

Taken together, these results question the importance of eotaxin and its receptor as a therapeutic agent. Although eotaxin protein is increased in asthmatic patients, eotaxin is not involved in airway eosinophilia and hyperresponsiveness. There are a lot of mediators, which are able to induce eosinophil migration. In my opinion, antagonising multiple chemokine receptors might be more effective in blocking eosinophil migration, than blocking only CCR3.

Eotaxin does not induce airway hyperresponsiveness. In addition, it is unclear whether reducing airway eosinophilia will lower the degree of airway hyperresponsiveness. Therefore, eotaxin or CCR3 receptor antagonists might not be suitable targets for future asthma therapy.

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SAMENVATTING

Een karakteristieke eigenschap van astma is ontsteking van de luchtwegen. Deze ontsteking wordt gekenmerkt door een toename van ontstekingscellen in het longweefsel. De overgrote meerderheid van deze cellen bestaat uit zogenaamde eosinofiele granulocyten. Deze cellen ontwikkelen zich in het beenmerg. Onder niet-allergische omstandigheden komen deze cellen via het bloed in de darmen terecht waar zij het lichaam beschermen tegen parasieten door het uitscheiden van toxische stoffen. In het geval van astma komen er meer eosinofiele granulocyten vrij uit het beenmerg, en migreren deze cellen naar de longen. Eenmaal in de luchtwegen kunnen deze cellen toxische stoffen uitscheiden en schade berokkenen aan het longweefsel.

Een ander kenmerk van astma is luchtwegovergevoeligheid, ook wel luchtweg-hyperreactiviteit genoemd. Luchtwegovergevoeligheid is het overmatig samentrekken van glad spierweefsel in de luchtwegen als reactie op specifieke prikkels, zoals allergenen, of aspecifieke prikkels, zoals koude lucht. Hoe dit fenomeen tot stand komt is onbekend. In de literatuur wordt verondersteld dat de grote hoeveelheid eosinofiele granulocyten in de longen de luchtweghyperreactiviteit kan beinvloeden. De toxische stoffen uit deze cellen kunnen direct luchtweghyperreactiviteit veroorzaken door de gladde spieren in de trachea samen te trekken. Maar hyperreactiviteit kan ook ontstaan doordat eosinofiele granulocyten weefselschade kunnen veroorzaken. De rol van de eosinofiele granulocyten bij het ontstaan van luchtweghyperreactiviteit is echter niet onomstotelijk bewezen. Bijvoorbeeld, zonder eosinofiele granulocyten kan er luchtwegovergevoeligheid optreden en aanwezigheid van eosinofiele granulocyten leidt niet altijd tot luchtweghyperreactiviteit.

Chemokines zijn mediatoren die betrokken zijn bij migratie van cellen. Deze eiwitten leiden de cellen naar de plaats van bestemming. Cellen die hiervoor gevoelig zijn migreren naar hoge concentraties van een bepaald chemokine. Bij astmapatiënten moet er ook een signaal vanuit de longen komen die zorgen dat eosinofiele granulocyten naar de luchtwegen migreren. Eotaxine is een chemokine die in staat is om eosinofiele granulocyten aan te trekken. In de luchtwegen van astmatici wordt er veel van dit eiwit geproduceerd. Daarom wordt verondersteld dat eotaxine een belangrijke rol kan spelen bij astma.

De experimenten beschreven in dit proefschrift hebben tot doel de rol van eotaxine bij het ontstaan van ontsteking in de longen en luchtwegovergevoeligheid te bepalen. Onze hypothese is dat onder allergische omstandigheden eotaxine in de luchtwegen wordt geproduceerd en eosinofiele granulocyten naar de longen trekt en activeert. Aangezien de aanwezigheid van deze eosinofiele granulocyten kan leiden tot luchtweg-overgevoeligheid, kan eotaxine betrokken zijn bij het ontstaan van luchtweg-hyperreactiviteit. Als eotaxine inderdaad betrokken is bij het ontstaan van luchtweg-ontsteking en -overgevoeligheid dan zou eotaxine wel eens een belangrijk aangrijpings-punt kunnen zijn voor een nieuw medicijn tegen allergie en astma.
Op verschillende manieren is onderzocht of eotaxine betrokken is bij luchtweg-ontsteking en -overgevoeligheid. Ten eerste is onderzocht of verhoogde eotaxine concentraties in de luchtwegen resulteerde in een verhoging van het aantal eosinofiele granulocyten in de luchtwegen en of dit gevolgen heeft voor de luchtwegfunctie van zowel proefdieren als mensen. Vervolgens is gebruik gemaakt van bestaande diermodellen voor allergische astma en virale luchtweginfecties om te onderzoeken of eotaxine van belang was bij de migratie van eosinofiele granulocyten in deze modellen. In hoofdstuk 5 is de functionele rol van de eotaxine receptor bij de mestcel onderzocht.

Verhoging van eotaxine concentraties in de luchtwegen

Om te onderzoeken of eotaxine de trachea direct kan samen trekken werden trachea segmenten van muizen in een orgaanbad geanalyseerd. Sommige stoffen zijn in staat om het gladde spierweefsel in de trachea te doen samen trekken. Uit de resultaten beschreven in hoofdstuk 2 blijkt dat eotaxine de trachea niet direct kan samen trekken en de trachea niet gevoeliger maakt voor andere contractiele agentia. Met andere woorden, eotaxine induceert niet direct luchtweghyperreactiviteit. Vervolgens werden muizen behandeld met eotaxine. Deze mediator werd direct in de trachea geïnjecteerd. Uit deze experimenten bleek dat dit geen gevolgen had voor de longfunctie van de muizen. Een manier om de mate van luchtwegontsteking te bepalen is door de longen te spoelen en in de longspoelvloeistof, broncho-alveolaire lavage (BAL)-vloeistof, het aantal ontstekingscellen te bepalen. In de BAL-vloeistof van muizen behandeld met eotaxine werden geen eosinofiele granulocyten aangetroffen. In dit hoofdstuk wordt geconcludeerd dat eotaxine geen effect heeft op de luchtwegen wat betreft de functie en celinflux in deze muizen.
Tevens is er een klinische studie gedaan om te onderzoeken of inhalatie van eotaxine door astmatische en gezonde vrijwilligers invloed heeft op de mate van ontsteking en overgevoeligheid van de luchtwegen. Allergische astmapatiënten en

gezonde vrijwilligers werden gevraagd om eotaxine of placebo te inhaleren. Vijf, 24 en 48 uur na de inhalatie werd de mate van ontsteking van de luchtwegen bepaald en vergeleken met de basaalwaarden. De mate van ontsteking is bepaald door het percentage ontstekingscellen in sputum van patiënten te bepalen. Onderzoek heeft uitgewezen dat dit een betrouwbare manier is om de mate van luchtwegontsteking te bepalen. Inhalatie van eotaxine leidde in astmapatiënten tot een verhoging van het percentage eosinofiele granulocyten in het sputum. Dit was niet het geval bij gezonde vrijwilligers. Verhoging van de eotaxine concentratie in de luchtwegen zorgt dus voor een verhoogde mate van ontsteking van de luchtwegen bij astmapatiënten. Het verschil tussen astmatische en niet astmatische patiënten zou verklaard kunnen worden door de hoeveelheid beschikbare eosinofiele granulocyten en de mate van gevoeligheid van deze cellen. Patiënten met astma hebben een groter aantal eosinofiele granulocyten die onder invloed van ontstekingsmediatoren gevoeliger zijn en daardoor dus eerder reageren op eotaxine. Echter, de verhoging van het aantal eosinofiele granulocyten in het sputum van astmapatiënten was maar klein en had geen gevolgen voor de luchtwegfunctie.

Allergeen- en virus-geïnduceede ontsteking en luchtwegovergevoeligheid

Er werd ook gebruik gemaakt van diermodellen om de rol van eotaxine bij het ontstaan van luchtwegovergevoeligheid en -ontsteking te bepalen. In een muismodel voor allergische astma worden muizen overgevoelig gemaakt voor ovalbumine (een kippeneieiwit) door muizen te injecteren met dit eiwit. Dit gebeurt in de sensibilisatiefase. Deze fase wordt gevolgd door een provocatie, de challengefase. In deze periode worden muizen blootgesteld aan een nevel van ovalbumine. Deze behandeling leidt tot een verhoging van het aantal eosinofiele granulocyten in de BAL-vloeistof en een verhoging van de luchtweggevoeligheid. Uit de experimenten van hoofdstuk 3 blijkt tevens dat de hoeveelheid eotaxine in de luchtwegen is verhoogd in de muizen die met ovalbumine behandeld waren. Behandeling van deze muizen met antilichamen tegen eotaxine had echter geen invloed op de door ovalbumine geïnduceerde ontsteking en luchtweghyperreactiviteit. Dit duidt erop dat eotaxine niet belangrijk is voor het ontstaan van deze symptomen.

Virale luchtweginfecties veroorzaken ontsteking en luchtwegovergevoeligheid. Bij astmapatiënten kan een infectie zorgen voor verslechtering van de symptomen van astma. Om het mechanisme van het ontstaan van luchtweghyperreactiviteit tijdens een virale luchtweginfectie te onderzoeken werd er gebruik gemaakt van een caviamodel, waarin deze dieren geïnfecteerd worden met parainfluenza virus. Deze behandeling leidt tot luchtwegovergevoeligheid en -ontsteking. In hoofdstuk 4 is

onderzocht of eotaxine een rol speelt in dit caviamodel. De migratie van eosinofiele granulocyten na de infectie werd in kaart gebracht. Eén dag na de ontsteking is het aantal ontstekingscellen in het bloed verhoogd. Twee dagen na infectie verdwijnen deze cellen uit het bloed en komen de ontstekingscellen in de longen terecht. Het aantal eosinofiele granulocyten in de longen stijgt tot en met dag vier van de infectie. Het lijkt er dus op dat eerst de cellen uit het beenmerg naar het bloed migreren en vervolgens naar de luchtwegen. Vlak voor de stijging van het aantal eosinofiele granulocyten in de luchtwegen stijgt ook de hoeveelheid eotaxine in de BAL vloeistof. Dit zou erop kunnen duiden dat eotaxine het signaal is voor de eosinofiele granulocyten om te migreren naar de luchtwegen. Echter, blokkade van de eotaxine receptor heeft geen gevolgen voor de eosinofiele granulocyt migratie.
Deze experimenten laten zien dat de eotaxine productie door ovalbumine behandeling of een virale luchtweginfectie wordt verhoogd, maar dat eotaxine niet als enige betrokken is bij het ontstaan van luchtwegontsteking en luchtwegovergevoeligheid. Er zijn meerdere ontstekingsmediatoren die in staat zijn om selectief eosinofiele granulocyten aan te trekken die in deze modellen ook van belang kunnen zijn. Waarschijnlijk moeten er meerdere mediatoren geblokkeerd worden om te voorkomen dat eosinofiele granulocyten naar de luchtwegen migreren.

Mestcellen

Mestcellen spelen een belangrijke rol bij het ontstaan en onderhouden van een ontsteking. Recent onderzoek heeft uitgewezen dat de receptor voor eotaxine op mestcellen aanwezig is. Om het mechanisme van activatie van de mestcel te onderzoeken wordt er gebruik gemaakt van zogenaamde *bone marrow-derived mast cells* (BMMCs). Dit zijn beenmergcellen van muizen die onder invloed van de kweekomstandigheden uitgroeien tot mestcellen. Het doel van de experimenten beschreven in hoofdstuk 5 was om aan te tonen dat BMMCs de eotaxine receptor bezitten en te onderzoeken of eotaxine via deze receptor mestcellen kan activeren. In dit hoofdstuk laten wij zien dat een deel van deze cellen de receptor voor eotaxine bezit. Incubatie van deze cellen met eotaxine leidt echter niet tot activatie van BMMCs. Toenemende concentraties van eotaxine resulteerden niet in het uitstoten van mediatoren door de mestcellen. In muizen leidt intraveneuze injectie met eotaxine ook niet tot een verhoging van de concentratie mediatoren specifiek voor de mest cel in het bloed. Eotaxine draagt dus niet bij aan de ontstekingsreactie door activatie van mestcellen. Echter, de rol van eotaxine in het ontstekingsproces zou

kunnen zijn dat eotaxine zorgt voor de migratie van mestcellen naar de plek van ontsteking.

Conclusie

Uit dit onderzoek blijkt dat eotaxine alleen migratie van eosinofiele granulocyten veroorzaakt onder allergische omstandigheden, aangezien alleen astmatici reageren op een verhoogde concentraties van eotaxine in de luchtwegen. Eotaxine alleen is niet in staat om luchtwegovergevoeligheid te veroorzaken.

Aangezien blokkeren van eotaxine of van de receptor in onze diermodellen niet zorgt voor een vermindering van de luchtwegsymptomen, lijkt eotaxine geen goede kandidaat als aangrijpingspunt voor een nieuw medicijn. Nader onderzoek moet uitwijzen of blokkeren van eotaxine of de receptor in astmapatiënten zal leiden tot een vermindering van de klachten.

ABBREVIATIONS

ANOVA	Analysis of variance
BAL	Broncho-alveolar lavage
BLC	B-lymphocyte chemokine
BMMCs	Bone marrow-derived mast cells
BSA	Bovine serum albumin
CCR	CC-chemokine receptor
cDNA	Copy DNA
CKR	Chemokine receptor
CO	Carbon monoxide
CTACK	Cutanous T-cell attracting chemokine
DTT	1,4-Dithio-DL-threitol
ECP	Eosinophilic cationic protein
END	Eosinophil-derived neurotoxin
EPO	Eosinophil peroxidase
FCS	Fetal calf serum
FEV_1	Forced expiratory volume in 1 second
GCP	Granulocyte chemotactic protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GRO	Growth related protein
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase
HSA	Human serum albumin
ICAM	Inducible cell adhesion molecule
Ig	Immunoglobulin
IL	Interleukin
IP	Interferon-gamma inducible protein
iTAC	inducible T cell-alpha chemoattractant
LARC	Liver and activation related chemokine
LFA	Lymphocyte function associated antigen
LT	Leukotriene
MAC	Complement receptor-3
MBP	Major basic protein
MCP	Monocyte chemotactic protein
MDC	Monocyte-derived chemokine
MEC	Mammary enriched chemokine
MiG	monocyte-induced by interferon -γ
MIP	Macrophage inflammatory protein
mRNA	Messenger RNA
NAP	Neutrophil activating peptide
NO	Nitric oxide
PBS	Phosphate buffered saline
Penh	Enhanced pause
PI	Para-influenza
PWM-SCM	Pokeweed mitogen stimulated- spleen cell conditioned medium
RANTES	Regulated upon activation normal T cell expressed and secreted
RIA	Radio immuno assay
SDF	Stromal cell-derived factor
SEM	Standard error of the mean
SLC	Secondairy lymphoid tissue chemokine
TARC	Thymus and activation related protein
TECK	Thymus expressed chemokine
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren in 1972 te Amsterdam. Na het behalen van het VWO diploma aan het Waterlant College te Amsterdam in 1991 heeft zij een jaar gestudeerd aan *Creighton University* te Omaha, Nebraska (Verenigde Staten). In 1992 is zij begonnen met de opleiding medische biologie, faculteit der Biologie van de Universiteit van Amsterdam. Als onderdeel van deze studie liep zij stage bij Medische Microbiologie afdeling bacteriologie van de Universiteit van Amsterdam waarin onderzocht werd welke DNA-sequenties betrokken waren bij de deletie van het *Por A* gen van *Neisseria meningitidis*. Zij liep een tweede stage bij Farmacologie en Pathofysiologie faculteit Farmacie, Universiteit Utrecht. Tijdens deze stage werd onderzocht of mestcellen betrokken zijn bij tolueen diisocyanaat geïndiceerde beroepsastma. Na het afstuderen in 1997, begon zij als assistent in opleiding aan het project 'Is eotaxine, een nieuw chemokine, betrokken bij het ontstaan van hyperreactiviteit?' aan de afdeling Farmacologie en Pathofysiologie, faculteit Farmacie aan de Universiteit Utrecht. Dit project werd gesubsidieerd door het Nederlands Astma Fonds en uitgevoerd onder begeleiding van Dr. G. Folkerts en Prof. dr. F. P. Nijkamp. Een deel van het project werd uitgevoerd aan de afdeling *Thoracic Medicine* van het *National Heart and Lung Institute* van *Imperial College* in Londen in samenwerking met Dr. R.S. Stirling en Prof. dr. K. F. Chung.