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Allergic inflammation in children with pet allergy and asthma

Mechanisms, markers and clinical consequences

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To my nearest
and dearest

ABSTRACT

Immunological background: Allergic inflammation is a multiple-step process with many actors, where eosinophils and the release of their granula proteins play an important role. The recruitment of eosinophils is a crucial event in the inflammatory process. What determines the direction of production and recruitment of eosinophils is still somewhat unclear but it is known that the process is driven by key molecules (cytokines/chemokines).

Clinical background: Asthma coexists with allergy, and the prevalence of asthma among schoolchildren in Sweden is 8%. Half of them have an associated pet allergy. Exposure to allergens triggers asthma in the sensitized child and is one of the reasons why children with asthma and pet allergy require continuous therapy with inhaled glucocorticosteroids (GC). This also makes asthma a "hidden" handicap for the pet-allergic child.

Objective: To investigate the usefulness of markers of inflammation in predicting asthma deterioration caused by allergen exposure, in untreated children and children undergoing GC therapy.

Methods: The five papers included in this thesis, are primarily based on three clinical studies: study of allergen exposure at school, a double-blinded prospective study of inhaled budesonide and an allergen challenge study. As a complement two *in vitro* studies have been performed based on the same material.

Results: Exposure of asthmatic children to animal dander in school dust during one week causes recruitment of eosinophils to the airways and increased bronchial reactivity - both possible signs of mild inflammation.

Peripheral blood eosinophil count (PBE) and S-ECP levels can be used to estimate the risk for deterioration and the need for corticosteroid treatment in cases of mild to moderate asthma. Furthermore exhaled NO (ENO) in addition to PBE and S-ECP can serve as a marker for increased inflammation after withdrawal of inhaled GC.

Children treated with inhaled GC have decreased expression of the IL-5 receptor on the eosinophil, suggesting a steroid mechanism by which cell-surface receptors are downregulated.

Allergen challenge of allergic children *in vivo* promotes an increased eosinophil migration capacity *in vitro* (within 2 hours), which can be further enhanced by eotaxin. The results are compatible with the initial stage of allergic inflammation.

General conclusion: We have been able to show that allergic inflammation is a dynamic process involving eosinophils. Many markers and methods are available, but no single marker can provide the answers to all questions. Yet, as this thesis shows, by selecting suitable markers and by combining the information the markers provide, it is possible to draw conclusions about the process of allergic inflammation and the inflammatory state of an individual patient.

Key words: asthma, animal dander allergy, children, eosinophils, ECP, EPO, EPX/ EDN, ENO, bronchial hyperresponsiveness, sputum, nasal lavage, budesonide, withdrawal.

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LIST OF ORIGINAL PAPERS

This thesis is based upon the following papers, which will be referred to by their Roman numerals:

- I. **Lönnkvist K**, Halldén G, Dahlén SE, Enander I, van Hage-Hamsten M, Kumlin M, Hedlin G. Markers of inflammation and bronchial reactivity in children with asthma, exposed to animal dander in school dust. *Pediatr Allergy Immunol* 1999; 10: 45-52

- II. **Lönnkvist K**, Hellman C, Lundahl J, Halldén G, Hedlin G. Eosinophil markers in blood, serum and urine for monitoring the clinical course in childhood asthma: Impact of budesonide treatment and withdrawal. *J Allergy Clin Immunol* 2001; 107: 812-7

- III. Hellman C, **Lönnkvist K**, Hedlin G, Halldén G, Lundahl J. Down-regulated IL-5 receptor expression on peripheral blood eosinophils from budesonide-treated children with asthma. *Allergy* 2002; 57: 1-7

- IV. **Lönnkvist K**, Anderson M, Hedlin G, Svartengren M. Levels of exhaled nitric oxide (ENO), bronchial hyperresponsiveness, lung function and eosinophil activity in blood, nasal lavage (NAL) and sputum in children with allergic asthma after withdrawal of inhaled steroids: A randomized, double blind controlled trial. *In manuscript*

- V. **Lönnkvist K**, Moshfegh A, Pedroletti C, Hedlin G, Halldén G, Lundahl J. Increased eosinophil transmigration after nasal allergen challenge in children with allergic asthma and rhinitis. *Submitted for publication*

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ABBREVIATIONS AND TERMS

BHR: bronchial hyperresponsiveness
BU: budesonide
CD: cluster of differentiation
EAR: early allergic reaction
ECP: eosinophil cationic protein
EG2: monoclonal antibody to ECP
ELISA: enzyme linked immuno sorbent assay
ENO: exhaled nitric oxide
Eo/B-CFU: eosinophil/basophil-colony forming unit
EPO: eosinophil peroxidase
EPX/EDN: eosinophil protein X/ eosinophil derived neurotoxin
FEV1: forced expiratory volume in one second
FOG: fixation and permeabilization method
FP: fluticasone proprionate
GC: glucocorticosteroids
GM-CSF: granulocyte macrophage-colony stimulating factor
HSA: human serum albumin
IgE: immunoglobuline E
IHS: inhaled steroids
IL: interleukin
iNOS/eNOS: inducible NO synthase/endothelial NOS
LAR: late allergic reaction
LT: leukotriene
LT-R: leukotriene receptor
Mab: monoclonal antibody
MBP: major basic protein
MFI: mean fluorescence intensity
MPO: myeloperoxidase
NAL: nasal lavage
PBE: peripheral blood eosinophils
PBS: phosphate buffered saline
PD10-20 : provocative dose that causes a 10-20 % fall in FEV1
PG: prostaglandin
OR: odds ratio
RIA: radio immuno assay
RPMI: Rosewell Park Memorial Institute culture media
SPT: skin prick test
Th: T-helper cell

INTRODUCTION

Allergic disease is a health problem that is increasing in the industrialized world. Nearly 50% of the children of school age suffer from atopy or other types of hypersensitivity [1]. Hypersensitivity refers to any adverse reaction after exposure to an otherwise harmless substance. If such a reaction involves the immune system, the condition is called allergy [2]. The term atopy, derived from the Greek “out of place”, refers to a hereditary predisposition to produce IgE antibodies [3] or to show adverse immune reactions involving IgE antibodies.

Asthma coexists with allergy and the prevalence of asthma among schoolchildren in Sweden is 8%. Half of them have an associated allergy to furred pets [4, 5]. Exposure to allergen triggers asthma in the sensitized child, and indirect exposure to animals plays a role in maintaining the allergic inflammation in the airways [6]. The concentration of animal dander allergen in school dust frequently exceeds the amount measured in homes where there are no furred pets [7, 8]. Experimental studies suggest that repeated exposure to low doses of allergen increases bronchial hyper reactivity in individuals with allergy and asthma [9, 10]. This may explain why children with asthma and pet allergy often require continuous therapy with inhaled corticosteroids to control symptoms, even when they avoid all direct contact with furred animals.

Asthma is an inflammatory chronic disease of the airways. In the last 20 years, treatment with inhaled corticosteroids has dramatically reduced asthma-related morbidity, even though the prevalence continues to increase. This makes asthma a “hidden” handicap for the pet-allergic child. The number of households with pets is also increasing leading to an increase in the exposure to animal dander allergens even in environments where pets are not kept and a consequence for the allergic child is that her or his need for continuous treatment with corticosteroids increases. [11]

Nevertheless this is not only a problem for the child but also an increasing cost for society. Thus, additional studies are needed to increase our understanding of the fundamental mechanisms of the pathophysiology of the disease as well as to find objective markers for diagnosis and evaluation of treatment and severity. My studies focus on the growing child of school age with pet allergy and asthma, causing a chronic inflammation in the lower airways.

My thesis is based on five studies focusing on eosinophil function and on identification of activity markers of importance for the clinical evaluation of allergic inflammation in children with pet allergy and asthma, as well as the health effects of exposure to allergens in the daily surroundings.

EARLY AND LATE ALLERGIC REACTIONS (EAR AND LAR)

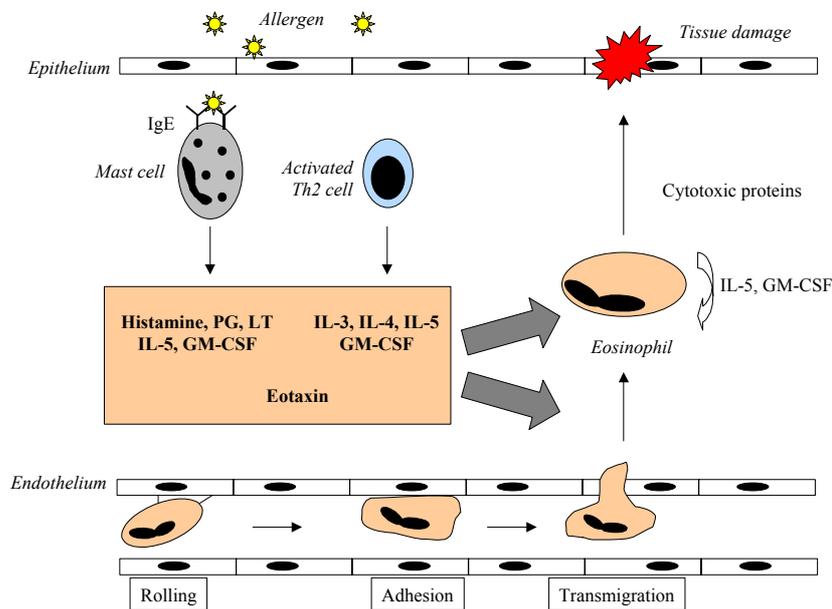


Fig 1. The dynamic development of the EAR and the LAR (C.Hellman)

Most antigens/allergens are either inhaled or ingested. These normally non-pathogenic antigens, which are constantly in contact with our respiratory tract or gastrointestinal mucosa, are largely ignored by the immune systems of non-allergic individuals. However, when atopic individuals are first exposed to an allergen (e.g. animal dander) they produce large amounts of IgE antibodies directed against this specific allergen. Mast cells have high affinity receptors on their surfaces that can bind to the Fc region of the IgE antibodies, so that after the initial exposure, mast cells will have large numbers of specific IgE attached to their surfaces. On a second exposure, the allergens can crosslink the IgE antibody's Fab-site, on the mast cell, dragging the Fc receptors together. This clustering of Fc receptors signals to mast cells to degranulate [12] and release histamine and leukotrienes [13, 14]; this causes acute symptoms in minutes: the early allergic reaction (EAR). Recently, the mast cell has also been shown to contain both interleukin 5 (IL-5) and eotaxin [15].

Later, or maybe simultaneously, in the predisposed allergic person, the late allergic reaction (LAR) starts to develop. The cells recruited at the time of the LAR are mainly eosinophils [16] and basophils [17, 18]. The eosinophil state of activation increases, probably due to an increased production of cytokines/chemokines, mainly IL-5 and eotaxin [18] (fig. 1).

THE ALLERGIC EOSINOPHIL INFLAMMATION

For more than 80 years, eosinophils have been suggested to be part of the pathological process that characterizes allergic diseases and asthma. Studies have demonstrated a strong association between eosinophilic airway inflammation and the development of bronchial asthma. The presence of pulmonary leukocytes, especially eosinophils, has been linked to the development of several features of asthma including airway hyperresponsiveness, epithelial damage, and airway remodeling [19, 20, 21]. In human lung tissue, levels of eosinophils and their toxic granule constituents correlate strongly with asthma severity. However, recent studies have shown that the presence of eosinophils in the lung is not sufficient for the induction of bronchial hyperresponsiveness [22, 23, 24] indicating that multiple signals are necessary for eosinophil activation and/or hyperresponsiveness.

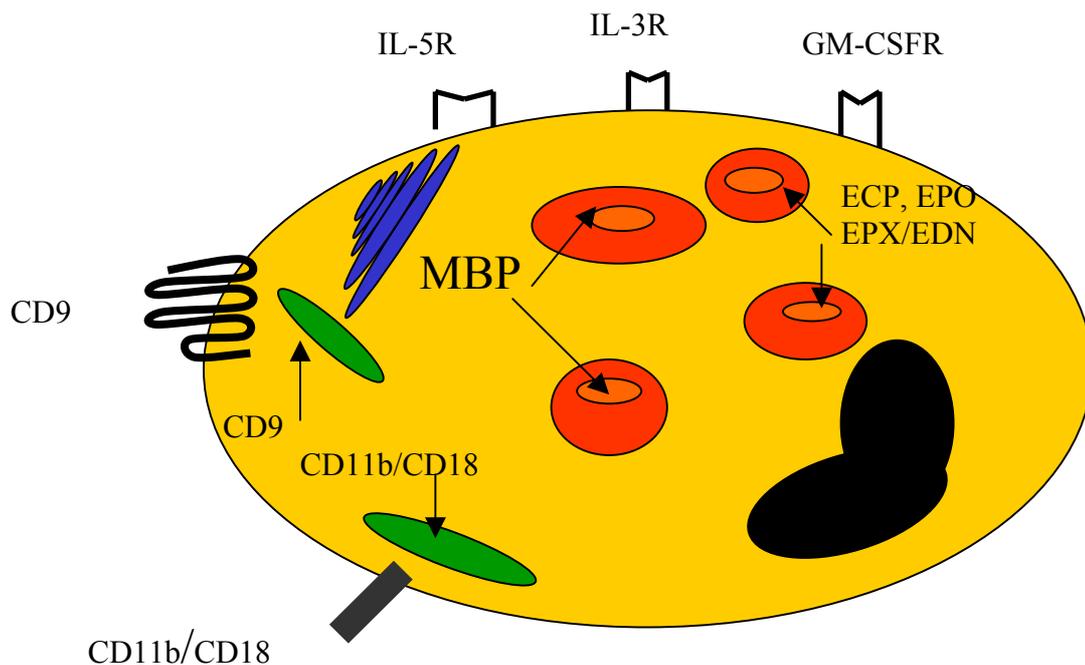


Fig 2. A schematic picture of the eosinophil, contents and receptors (A. Nopp)

CYTOKINES AND CHEMOKINES IN ALLERGY

Cytokines are signalling proteins, key molecules, that usually act extracellularly. They are produced in various types of cells [18] and act through specific receptors (R)(fig. 2) on the

surface of target cells [25]. Cytokines evoke a wide range of cellular responses [18] including activation, proliferation, chemotaxis, immuno-modulation, release of other cytokines or mediators, growth and cell differentiation and apoptosis [26]. Cytokines can be grouped into families based on their structure and function. One sub-group includes the chemokines (e.g. eotaxin). Different cytokines play an integral role in the coordination and persistence of the allergic inflammation in the airways. The most important cytokines for the allergic inflammation are IL-3, IL-4, IL-5, GM-CSF and eotaxin [18]. IL-4 is essential in regulating IgE synthesis: IL-4 gene-depleted mice have a strongly reduced capacity to synthesize IgE [27]. IL-4 also takes part in eosinophil recruitment by upregulating vascular endothelial molecule 1 (VCAM-1) [28] expression on endothelial surfaces. The cytokines IL-3, IL-5 and GM-CSF have all been shown to promote eosinophil growth, differentiation and activation. Chemokines are thought to provide the directional signals for the movements of leukocytes in tissue inflammation and the most important chemokine for the eosinophil migration capacity is eotaxin. However, eotaxins effect on eosinophil transmigration is known to be potentiated by other cytokines, as for example IL-5 [18, 29].

It is well known that cytokines exert their function by binding to certain specific cell-surface receptors (R) and that circulating cytokine receptors tend to neutralize cytokines in the circulation. The expression of cell-bound IL-5R has been shown to increase following allergen challenge in dual (EAR+LAR) but not in isolated early (EAR) responders [30, 31]. In study III, results suggest that budesonide-treatment down-regulates the IL-5R on PBE from asthmatic children. This might be one of the effects of treatment with corticosteroids which prevents the allergic subject from developing eosinophil inflammation and LAR.

THE ROLE AND THE LIFE CYCLE OF THE EOSINOPHIL

Paul Ehrlich discovered the eosinophil leukocyte /granulocyte in 1876 and found that this cell had high affinity for the acidic dye eosin, and therefore called the cell eosinophil [32]. Since then much has been learned about the eosinophil and its role in defence against parasite infections as well as in the initiation and maintenance of the allergic inflammation. The eosinophils are bone marrow-derived. They normally constitute 1-5% of the leukocyte population in peripheral blood and have a life time about 3-4 days in blood, whereas in tissue, eosinophils may survive for 1-3 weeks. This time span depends upon the state of activation and the presence and concentration of cytokines e.g. IL-3, IL-5 and GM-CSF of which especially IL-5 is known to prolong survival and to prevent apoptosis.

Eosinophils generate a variety of mediators that augment the inflammatory responses. Most prominent among these molecules are the secondary granule proteins: eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), eosinophil protein X/eosinophil derived neurotoxin (EPX/EDN) and major basic protein (MBP) [33, 34, 35] (fig.2). When released, these mediator induce a variety of inflammatory and destructive effects including toxicity to tissue. The eosinophils also generate large amounts of leukotrienes [36].

Cord blood contains more mature eosinophils as well as more progenitor cells than adult peripheral blood: thus neonates appear to have a high capacity to produce high eosinophil counts [37]. It is also known that premature infants develop eosinophilia during the neonatal period [38, 39].

THE EOSINOPHIL MIGRATION

Eosinophil migration can be divided into three phases: first migration from the bone marrow, as precursors (Eo/B-CFU) or mature eosinophils into the circulation; second through the vessel wall, the transvascular stage, which requires rolling, adhesion and transendothelial migration; and third, the tissue stage, where the eosinophils migrate on a gradient of chemokines through the tissue matrix to the target site to accumulate and cause local inflammation [40, 41, 42]. (fig. 3)

Eotaxin and IL-5 together provide the signal essential for the eosinophil transmigration to airway tissue in allergic inflammatory reactions. Mice deficient in IL-5 still develop tissue eosinophilia in the lung despite marked reductions in bronchoalveolar lavage eosinophil numbers. However, mice deficient in both eotaxin and IL-5 have a synergistic decrease of eosinophils in tissue (especially in the lungs) [43, 44]. These results are in line with ours in study V, in which peripheral blood eosinophils significantly increased their capacity to transmigrate through a fibronectin coated membrane, in an vitro system with an upper and a lower chamber, after allergen challenge in atopic, but not in non-atopic children. This phenomenon is probably due to that atopic children have an increased ability to produce IL-5. In addition, we were able to show a further significantly increased capacity of PBE to transmigrate through the membrane if eotaxin was added in the lower chamber.

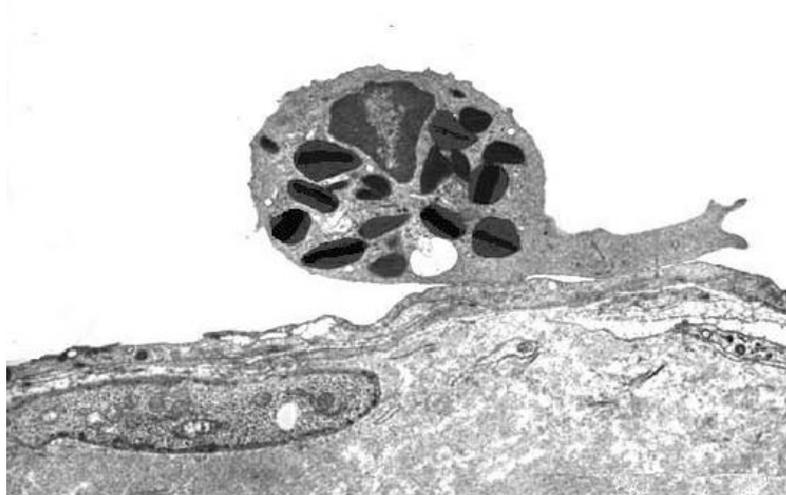


Fig 3. An electron microscopic picture of a migrating eosinophil, resting on the endothelial vessel wall, waiting for further signals for direction of movements (University of Illonoise)

An understanding of these processes gives the clinician insight into the pathogenesis of disorders involving eosinophils.

NITRIC OXIDE (NO) IN THE INFLAMMATORY PROCESS

NO in exhaled air predominantly originates from the lower airways [45, 46, 47]. Increased levels of exhaled nitric oxide (ENO) are partly due to activation of inducible NO synthase (iNOS) in epithelial [48] and inflammatory cells [49]. Endogenous NO is recognized as an important signaling molecule and may play a role in the regulation of airway function [50].

The role of NO in inflammation is complex. NO exerts a direct cytotoxic effect on lung cells and lung tissue by altering cell membrane permeability [51, 52]. NO produced by endothelial NOS (eNOS) lung capillary endothelial cells [53], as well as from eosinophils [54], may serve to prevent eosinophil adherence to endothelium in normal conditions. NO plays a pivotal role in eosinophil migration [55] and infiltration [56] in the respiratory tract in allergic conditions. NO is chemotactic for a variety of cell types including eosinophils and therefore may increase eosinophil recruitment into the lung by modulating cytokine activity [57, 58]. It has been shown that a progressive increase in ENO during the LAR is associated with iNOS activation, and eosinophil infiltration in the airway tissue. In contrast there has been no changes in ENO measured during the EAR when there is no airway inflammation [59].

ALLERGEN CHALLENGE AS A MODEL

Allergen challenge in an allergic individual increases production of IL-5 as measured in bronchoalveolar lavage (BAL). Allergen challenge also increases the number of peripheral blood eosinophils and lymphocytes containing intracellular IL-5 [60, 61].

Increase in bone marrow Eo/B-CFU has also been demonstrated in allergic asthmatic subjects after allergen challenge [31]. Subjects who develop both EAR and LAR, dual asthmatic responders, have a significantly greater number of bone marrow Eo/B-CFU, when cells are incubated with IL-5, which may reflect either a specific induction of a population from a more committed eosinophil progenitor, or an upregulation of the IL-5 receptor (R) on the surface of these cells [62, 63].

To summarize, allergen challenge can serve as a relevant model for naturally occurring allergic reactions and their consequences

PHYSIOLOGICAL ASSESSMENT OF ASTHMA

For clinical evaluation of asthma, dynamic spirometry measurements, the forced expiratory volume in one second (FEV1) and the peak expiratory flow (PEF) are used. The limitations of these tests are that they mainly reflect obstruction in the central airways and that the measurements are dependent on the effort exerted by the subject. The maximal expiratory manoeuvre itself may also have an impact on the airway caliber [64, 65]. Midexpiratory flow (MEF) measurements might reflect obstruction in the peripheral airways better, but are less reproducible than FEV1 measurements [66].

The degree of hyperresponsiveness in the airways to a stimulus (e.g. histamine, methacholine or cold dry air) has been suggested to correlate with the reactivity of the airways [67]. Bronchial hyperresponsiveness (BHR) can be measured by use of a variety of stimuli which act through different specific mechanisms [68]. The most common way of measuring airway responsiveness has been by inhalation of a mist of histamine or methacholine generated by a nebulizer [69]. Usually FEV1 is then used as a parameter of a change in lung function, and the provocative concentration (PC) or provocative dose (PD) causing 10-20% decrease in FEV1 (PC10-20 and PD10-20 respectively), determines the airway responsiveness to the given stimulus.

MARKERS OF ALLERGIC INFLAMMATION

Eosinophil proteins (ECP, EPO, EPX and MBP)

The eosinophil granulocyte has specific (intracellular) granulae, which contain different cationic proteins. These proteins are biologically active molecules. Four of the most investigated eosinophil proteins, as earlier described, are MBP, ECP, EPO and EPX/EDN. The characteristic properties and functions of the eosinophils are most likely linked to the activity of these secretory proteins (fig. 2).

ECP is one of the most basic proteins in the human body and is a very potent cytotoxic molecule with capacity to kill both human cells and parasites [70, 71, 72]. Activated eosinophils are known to release ECP, and high levels have been found in BAL fluid from patients with allergic asthma [73]. Elevated levels of ECP have also been found in serum, sputum and nasal lavage (NAL) in patients with allergic asthma and rhinitis [74].

Monoclonal antibody EG2, CD9 and CD11b/CD18

In immunohistochemical studies, the monoclonal antibody (Mab) EG2 has been shown to bind to the EG2-epitope on ECP [75]. Some investigators propose that the expression of EG2 reflects the activity of the eosinophil while others claim that the expression of EG2 merely reflects the numbers of eosinophils in peripheral blood regardless of their state of activation [76].

By the use of a cell-membrane permeabilization technique (the FOG method) followed by flow cytometry, both non-activated and activated eosinophils can be distinguished by

quantitative differences in the amount of intracellular bound EG2 [77, 78]. The FOG technique has then been used in clinical studies to measure a higher expression of EG2 in PBE in patients with asthma compared with healthy controls [79, 80]. However in clinical trials, the method has not always been shown to be superior as a mirror of disease severity in asthma compared with measurements of PBE count or ECP [81] (fig2).

Data has shown that mAb against CD11b/CD18 both *in vitro* and *in vivo* can be used as activity marker for eosinophils [82].

In addition mAb against CD9 detect the antigen on the surface of the eosinophils and CD9 can serve as an eosinophil marker [83] (fig. 2).

Nitric oxide (NO)

Elevated levels of ENO are seen in asthma [84] but also in other atopic diseases as well as in viral infections [85] and after consumption of nitrate-rich meals [86]. However, it has been demonstrated that elevated levels of ENO are strongly associated with conditions known to increase inflammation and eosinophilia in asthma. ENO is significantly elevated in acute asthma or steroid-resistant severe asthma [87] or when a maintenance dose of inhaled steroids is reduced, but ENO quickly returns down to normal levels in patients with stable asthma after steroid treatment [88].

CORTICOSTEROID THERAPY IN EOSINOPHIL AIRWAY INFLAMMATION

Glucocorticosteroids are the only class of *drug* available today with the ability to block several of the pro-inflammatory and inflammatory stages. The clinical effects have been demonstrated in numerous controlled studies in children with allergic asthma: the effects include improved lung function, reduced number of exacerbations, symptom control and less reactive airways as demonstrated by decrease of exercise induced asthma and bronchial hyperresponsiveness [89, 90, 91]. Thus treatment with glucocorticosteroids has dramatically reduced the morbidity and mortality associated with the asthma disease [90]. Inhaled glucocorticosteroids reduce airway eosinophilia in sputum and BAL but also in peripheral blood. Glucocorticosteroids inhibit mediator release from eosinophils and reduce secretion of chemokines and proinflammatory cytokines from alveolar macrophage. The effect of IL-5 and GM-CSF on eosinophil survival is a blocked by steroids. There are also effects on other cells:

epithelial cells and T-lymphocytes and on numbers of mast cells in tissue and dendritic cells in respiratory epithelium. Many of the mechanisms by which steroids work are still unknown. From a clinical point of view inhaled steroids are recommended for prophylactic purposes as well as for amelioration of symptoms. However corticosteroids have dose dependent systemic effects and the optimal dose is therefore the lowest possible effective dose. It is still difficult to evaluate the optimal dose from lung function measurements and symptom scores as the individual tolerance to symptoms varies and there are obvious difficulties to differentiate between limited physical activity due to asthma and limited physical activity due to asthma treatment. There are no clear correlation between physiological markers and airway inflammation. For this and other reasons markers of airway inflammation are needed. [92, 93]

AIMS OF THE STUDY

The overall objectives of this thesis were to study the pathophysiological role of eosinophils in allergic inflammation and to identify eosinophil markers of importance for the clinical evaluation of allergic inflammation in children with pet allergy and asthma, as well as to investigate the health effects of exposure to allergens at school.

The specific aims were:

- (I) To investigate the health effect of exposure to cat and dog allergens in school dust, in children with mild asthma and pet allergy, not treated with inhaled corticosteroids.
- (II) To relate asthma deterioration and clinical symptoms to lung function, inflammatory parameters and bronchial reactivity in children with mild to moderate allergic asthma after double-blind placebo-controlled withdrawal of inhaled corticosteroids.
- (III) To study if treatment with corticosteroids downregulates the expression of the IL-5 and GM-CSF receptor on PBE. In addition, to investigate the responsiveness of PBE to *in vitro* stimulation with IL-5 and GM-CSF in steroid-treated and in non-steroid treated children with allergic asthma.
- (IV) To evaluate the clinical usefulness of different markers of airway inflammation in children with pet allergy and asthma, by investigating levels of ENO, PBE, serum-, sputum-, and NAL-ECP in relation to bronchial hyperreactivity and lung function.
- (V) To investigate the capacity of PBE to transmigrate through a fibronectin coated membrane in an *in vitro* assay after nasal allergen challenge *in vivo*, in children with rhinitis and asthma and to test the hypothesis that eotaxin potentiates this process.

SUBJECTS AND MATERIALS

Subject characterization

(I) The concentration of animal dander in school dust and the health effect of exposure

Ten children, six boys and four girls, aged between 9 and 14 years (median age 12 years), participated in the study. The inclusion criteria were: a history of mild allergic asthma; normal lung function; a positive skin prick test (SPT) to cat and dog; and no current treatment with inhaled corticosteroids. Exclusion criteria were house dust mite or mold allergy and ongoing symptoms of asthma. The children did not have furred pets at home. Asthma had previously been diagnosed by a physician. None of the patients included in the study had been treated with corticosteroids or cromolyn sodium for the last 2 months, and none had experienced asthmatic or allergic symptoms during the last month. Spirometric measurements confirmed normal lung function with forced expiratory volume in 1 s (FEV1) over 80% of the predicted values.

(II) The clinical usefulness of markers of inflammation after withdrawal of GC

Thirty-four children, 17 boys and 17 girls, aged between 9 and 16 (median age 13 years), participated in the study. The inclusion criteria were: a history of mild to moderate asthma; and allergy to cat and/or dog, daily use of budesonide 200 or 400 µg for more than 3 months, and FEV1 greater than 80% of predicted. The children were excluded if they were sensitized to house dust mite, mold or to a family pet or if they had ongoing symptoms of asthma despite treatment with budesonide. One child had a dog; the others had no furry pets. Asthma had previously been diagnosed by a physician.

Randomization was stratified for different doses of budesonide (400 µg/d or 200 µg/d). One girl had a severe infection on the day of the randomization and therefore never entered the study. The remaining 33 children participated according to the study protocol.

Sixteen age-matched, healthy non-atopic children served as control subjects. To exclude atopy, SPT for cat, dog, horse, house dust mite, molds, birch, grass and mugwort was done.

TABLE I. Characteristics of patients and control subjects at baseline

	Continuous treatment group	Withdrawal group	Healthy controls
n	16	17	16
Mean age (y)	12.3	11.8	13.3
Sex (F/M)	5/11	11/6	10/6
Budesonide 400/200 µg	13/3	13/4	0
Positive SPT for cat	16	17	0
Positive SPT for dog	15	15	0

(III) The role of the IL-5 and the GM-CSF receptor in asthma and the effect of GC treatment

Children, aged between 9 and 16 years, with mild to moderate asthma and pet allergy, participated in the study. All children had a FEV1 over 80% of predicted value. The budesonide treated asthmatic children (n=24) had been treated with inhaled corticosteroids (budesonide 200 or 400 µg/day) for more than 3 months. The non-steroid treated asthmatic children (n=13) had been off steroid treatment for more than 3 months. They belonged to the placebo group in study II. Age-matched healthy non-atopic children served as control subjects.

(IV) Airway related methods for evaluation of allergic inflammation after withdrawal of GC

A subgroup of 18 children from study II were investigated according to an extra protocol for markers of inflammation in blood, serum, nasal lavage (NAL), sputum and exhaled air. The control group of 16 healthy children (as in study II) served as controls.

Table I. Characteristics of patients and controls at baseline.

	Continuous-treatment group	Withdrawal group	Healthy controls
n	10	8	16
Mean-age (years)	10.7	12.2	13.3
Females/Males	3/5	7/3	10/6
Budesonide 400µg/200µg	4/4	7/3	0
Pos. SPT cat	10	8	0
Pos. SPT dog	9	7	0

(V) Eosinophil migration after allergen challenge

Fourteen cat-allergic children, aged between 9 and 18 years, with mild to moderate rhinitis and asthma, underwent nasal challenge with cat-allergen. Eight of the children had before challenge been treated daily with inhaled corticosteroids and 6 had had no steroid treatment. All of the asthmatic children had FEV1 over 80% of predicted. Four healthy children, aged between 9 and 18, served as control subjects.

Patient characteristics

	Patients	Control
N	14	4
Mean-age (y)	13,1	12,2
Females/Males	8/6	3/1
Pos SPT cat	14	0

Study design

(I)

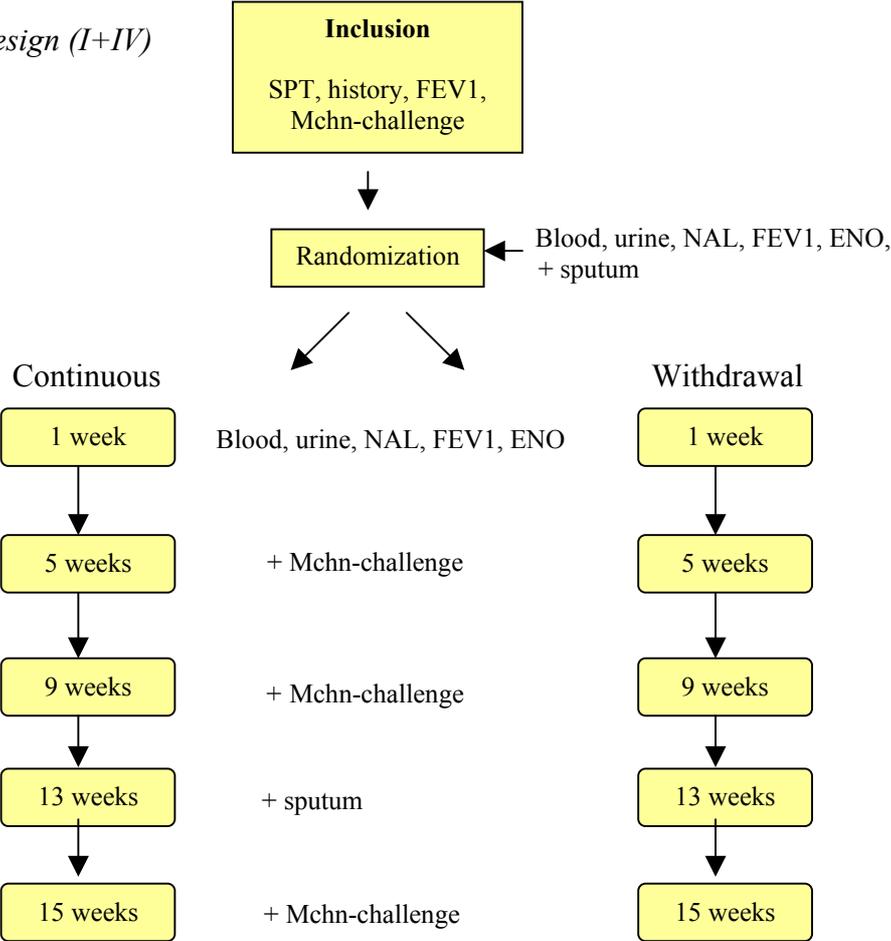
All investigations were performed on Monday and Friday of two different school weeks, before noon. In the first week, the children underwent bronchial challenge with methacholine. In the second week, NAL and induced sputum were obtained. Blood and urine samples for analysis of inflammatory markers were obtained on all occasions, before and after the school weeks and at the same time of day. FEV1 was measured at every visit. The children's contact with any furred animal and their use of bronchodilators were documented throughout the study, by means of a structured questionnaire. Dust samples were collected from the classrooms, and from every child's mattress.

(II+IV)

The study lasted 5 months and consisted of a run-in period (2-6 weeks) and an intervention period (4 months) (fig.4). Symptom control for at least 3 months during regular treatment with inhaled budesonide was required and then confirmed during the run-in period. The children were randomly grouped in a double-blind and parallel manner either to continue on budesonide (the continuous group) or to receive placebo (the withdrawal group). The children came for 6 visits during 15 weeks. They were allowed to take one oral dose (4 mg) of betamethasone as rescue medicine if worsening of asthma occurred. They were withdrawn

from the study if they experienced daily symptoms during normal activity, a need for β 2-agonist more than 4 times a day, an irreversible 20% decrease in FEV1, and/or more than a 20% decrease in PEF for more than 3 days. The study period was outside the pollen season, from October to the beginning of April. The children were assessed at each visit between 7 and 9 AM. Physical examination and lung function tests were performed and blood was drawn, and the first morning portion of urine was collected at every visit. The children underwent methacholine challenge at the run-in visit and five, nine and fifteen weeks after randomization. The children's contact with any furred animal and their use of bronchodilators were documented throughout the study, by means of a structured questionnaire (II, IV). In a subgroup of 18 children ENO was measured and NAL was obtained at every visit. Sputum was induced at randomization, and thirteen weeks after randomization (IV). The controls subjects underwent SPT, blood, NAL and urine sampling, lung function test and methacholine challenge according to the same procedure as the asthmatic children but only on one occasion (II, IV).

Fig 4. Study design (I+IV)



(III)

Blood samples were collected from children with pet allergy and age-matched healthy controls and examined within 2 hours. Three groups were identified; the first consisted of budesonide treated children with asthma, the second of non-steroid treated children with asthma and finally the third of healthy non-atopic children of the same age.

The cytokine receptor expression (e.g. IL-3R, IL-5R and GM-CSFR) of PBE and the cellular response after *in vitro* stimulation with recombinant cytokine (e.g. IL-3, IL-5 and GM-CSF) were studied in all three groups.

(IV)

The cat-allergic children came to the clinic for three visits. The initial visit served to determine inclusion in the study. On the second visit, the children underwent nasal challenge with cat (Fel d1) allergen. Nasal challenge was stopped when symptoms occurred, such as rhinorrhea, stuffy nose and/or sneezing. Blood samples were drawn before challenge and again 2 and 24 hours after. The healthy non-atopic children participated in the same procedure. Blood samples were handled within 2 hours and tested for eosinophil activity and the *in vitro* capacity of PBE to migrate through a fibronectin-coated membrane. This eosinophil transmigration capacity was tested with and without addition of eotaxin as a chemokine.

METHODS

Assessment of atopy

Skin-prick tests (I-V)

Skin prick test (SPT) was done in duplicate in study I-IV, with extracts of cat, dog, horse, rat and rabbit. In addition the controls were tested for house dust mite, molds, birch, grass and mugwort (Soluprick 10.000 SQ; ALK, Hörsholm, Denmark). The asthmatic children were also tested for house dust mite and molds, as sensitization to those allergens was an exclusion criterion for entering those studies (I-V). The test was judged positive, if the wheal was at least 3 mm in diameter and larger than or as large as the histamine-induced wheal (positive control) after 15 minutes (=3+). In study V only cat allergy was confirmed before allergen challenge.

Circulating allergen-specific IgE-antibodies (I)

A blood sample was taken from the children in study I, to test for circulating IgE antibodies to hazel, alder, and birch pollen (Pharmacia CAP-FEIA system, Pharmacia Diagnostics AB, Uppsala, Sweden).

Assessment of asthma

History (I-V)

Asthma was diagnosed by a physician, according to the American Thoracic Society (ATS) guidelines. The asthmatic children were thereafter classified as having mild, moderate or severe asthma on the basis of the history, lung function test and physical examination.

Questionnaire (II, IV)

The children's contact with any furred animal, morning peak expiratory flow (PEF), and their use of bronchodilators were documented throughout the study, by means of a structured questionnaire.

Spirometry (I-V)

Lung function was evaluated by means of flow-volume curves (Vitalograph) measuring the forced expiratory volume in one second (FEV1), midexpiratory flow after 50% or 25% of the expiration time (MEF50, MEF25 respectively). For clinical investigations this was measured at every visits in all studies (I-V). The Vitalograph was calibrated daily and the values were obtained according to ATS standards.

Physical examination (I-V)

All children were examined by the same physician in all studies, at all visits to minimize individual differences in evaluating a patient.

Nasal lavage (NAL)

Nasal lavage (NAL) was performed (I, IV) with the patient sitting and leaning forward [94, 95]. This position prevented fluid from reaching the throat. One nasal cavity was lavaged with 6 ml of lukewarm 0.9% saline solution using a syringe (10 ml), to which a nasal cone was adapted. To avoid leakage of fluid, the cone was gently applied to the external part of the

nostril. The saline solution was injected into the nasal cavity, left there for one minute and then aspirated back into the syringe. The nasal wash was kept on ice and centrifuged at 1800 rpm, at +4°C, for 10 minutes, and thereafter the supernatant was stored at –20°C until analyzed.

Sputum induction

Sputum was induced [96] by inhalation of nebulized hypertonic saline in concentrations of first 3% and then 5%, for ten minutes each, until a sputum sample had been obtained (I, IV). The sample was examined within two hours. Salivary contamination was removed macroscopically from the specimen. The selected portion was weighed and treated with fresh dithiothreitol (DTT) in a balanced salt solution (Sputalysin 10%; Calbiochem, San Diego; CA, USA), diluted to 1% by the addition of distilled water. The selected portion was mixed with an equal volume of the DTT solution. The mixture was vortexed and placed in a 37°C shaking water-bath. After 15 minutes in the water-bath, the mixture was again vortexed for five seconds and gently aspirated in and out of a pipette to ensure mixing and strained through nylon gauze. The mixture was then centrifuged for ten minutes at 1800 rpm. Spirometry was performed before and after the 3% and 5% saline inhalation respectively. The supernatant of the sputum specimen was stored at –20°C until analyzed.

Metacholine challenge

Bronchial challenge was performed according to the protocol of Chai et al (I, II, IV) [97] with slight modifications, with increasing doses of methacholine chloride delivered using a dosimeter (Spira Elektro 2 nebulizer system; Hamenlinna, Finland). The dosimeter automatically delivers pressurized methacholine to the nebulizer during an interval shorter than the inhalation, which means that the inhaled doses can be kept constant and standardized. Forced expiratory manoeuvres were performed at start and five minutes after the start of each inhalation. Five breaths at each concentration resulted in cumulative doses of methacholine from a minimum of 7 µg (I) or 22 µg (II, VI) to a maximum of 700 µg (I) or 2830 µg (II, IV). The challenge was stopped when the fall in FEV1 exceeded 15% or the maximum dose was reached. The bronchial reactivity to methacholine (PD 10-15, provocative dose that causes a 10-15 % fall in FEV1) was determined from the log dose-response curve by interpolation.

On-line measurements of exhaled nitric oxide

Concentrations of ENO (IV) were measured using a chemiluminescence analyzer (Model 270B; Sievers, Boulder CO) connected to a vacuum pump (model RV5; Edwards, UK). The analyzer uses ozone to oxidize NO to NO₂ in an excited state. The light emitted when NO₂ returns from excited to ground state is measured. This method has a sensitivity for NO of < 2 pmol/ml of air. A pressure of 6 bar was set for the reaction chamber. The analyzer was calibrated with an NO gas certified to 90 ppm (Air Liquide, Stockholm, Sweden) and as zero filter (Sievers, Boulder, CO) for zero calibration. The analogue signal from the NO analyzer was digitized at 10 Hz (A/D-converter ADC-100, Pico Instruments, UK) and displayed simultaneously in an application developed in LabView (Ver. 4.1, National Instruments, Texas, USA). The analyzer was attached to combined mouthpiece, pneumotachograph, and flow resistance. Target exhalation flow rate for these measurements was 4 l/min and was registered simultaneously by a pneumotachograph (Fleisch Model 0, Metabo, France). Exhalations continued until an adequate plateau was obtained and the level of the plateau was determined visually. Three exhalations were registered each time and the mean value used.

Analysis of soluble markers of inflammation

Eosinophil cationic protein (ECP)

Sera and the supernatant portion of sputa and NAL were handled according to the suppliers of the ECP-kit (I, II, IV). ECP was analyzed using a commercially available fluoroimmunoassay (Pharmacia ECP CAP System FEIA, Pharmacia & Upjohn, Uppsala, Sweden). The detection limit was 2 µg/L.

Eosinophil peroxidase (EPO)

EPO (in serum and the supernatant of induced sputum and NAL) was assessed with a prototype immunofluorometric assay, utilizing the Pharmacia CAP System TM. The EPO standard curve of Carlsson et. al [98] was used, which covered the range from 0.5 to 200 µg/L. The intra- and inter-assay coefficients of variation were below 10% and the detection limit was 0.5 µg/L.

Eosinophil protein X/ eosinophil derived neurotoxin (EPX/ EDN)

EPX/ EDN (in urine, serum and the supernatant of induced sputum and NAL) was assessed with Pharmacia's commercial, specific competitive RIA [99]. The intra- and inter-assay

coefficients of variation were less than 10%. In addition, in urine, the creatine levels were adjusted for the EPX/EDN levels and expressed as ng EPX/ EDN per mmol creatinine in urine.

Myeloperoxidase (MPO)

MPO (in serum and the supernatant of induced sputum and NAL) was assessed with the Pharmacia competitive radioimmunoassay (RIA), which is a double antibody-based RIA [100]. The intra- and inter-assay coefficient of variation was below 13% and the detection limit was 8%.

The total cell count, the number of PBE and the expression of EG2, CD9 and CD11b/CD18

For flow cytometric analysis of PBEs in unseparated blood, a cell membrane permeabilization technique (the FOG method) was used as previously described [78]. The expression of the intracellular EG2 epitope on ECP, CD9 and CD11b/CD18 was measured in specific mean fluorescence intensity units (MFI).

In vitro stimulation of leukocytes

Mixed leukocytes from 150 µl of hemolyzed blood were resuspended in 100 µl of 100 ng/ml recombinant human IL-5 (Immunokontakt, Frankfurt, Germany), or 200 ng/ml recombinant GM-CSF (Immunokontakt) diluted in HEPES (10 mM)-buffered RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% heat inactivated calf serum (RPMI). As a control, leukocytes were incubated in 100 µl RPMI only. The suspensions were incubated for 60 minutes at +37°C and washed twice in PBS-EDTA (III).

Transmigration assay

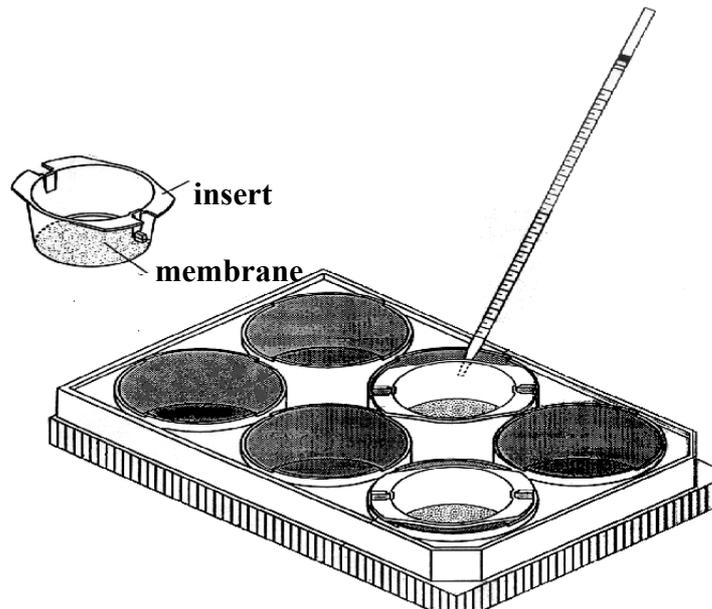


Fig 5. The Transwell model, with the upper chamber (insert) and the membrane as indicated

Transmigration studies were performed by using a 24-well microchemotaxis chamber with a polyethylene terephthalate treated membrane (BECTON Dickinson) which has a high pore density (2.0×10^6 pores/cm², 3.0 μ m in diameter) for maximum permeability. Insert filters were coated with human fibronectin (20 μ g/ml) (Sigma Chemical) dissolved in PBS with 0.2% BSA overnight at +20-22°C, then washed twice with 200 μ l PBS at +20-22°C and blocked with 200 μ l 0.5% HSA for one hour at +37°C. Finally the wells were rinsed with PBS and air dried at 20-22°C. Percoll separated mixed granulocytes from allergic or non-allergic children were mixed with 200 μ l RPMI alone and added to the upper chamber (fig. 5).

For eosinophil transmigration, prepared granulocytes were resuspended in RPMI in the upper chambers, and eotaxin (100 ng/ml) (Prepro Tech Inc, Rocky Hill, NJ, USA) or medium only was added to the lower chambers. Eotaxin (100 ng/ml) was added to half of the lower chambers. The transmigration assay was run for 360 min, at +37°C, 5% CO₂ and 95% air-atmosphere. Cells in the upper chamber (non-transmigrated cells) were collected by careful aspiration and rinsing with 200 μ l RPMI (+37°C). Cells which has transmigrated to the lower

chamber (transmigrated cells) were transferred to test tubes. These two cell-fractions were centrifuged 300 x g for 10 minutes at +4°C and the cell-free supernatants were collected.

Cells that had transmigrated into the lower chamber (“transmigrated cells”) were, together with the original cell suspension (“total cells”), analyzed for eosinophil cationic protein ECP. A ratio between “the number of transmigrated cells” and “total cells” was calculated for each experimental setting, and expressed as per cent transmigrated cells (“transmigration index”), as previously described [101].

Sampling and processing of dust

Dust samples were collected from the children’s classrooms and bedrooms (I). In the classroom, the child’s chair, desk and textiles such as curtains and upholstered furniture. The content of cat (Fel d 1) allergens was measured with a monoclonal antibody-based ELISA (Indoor Biotechnology, VA, USA). The content of dog (Can f 1) allergens was analysed with a radioimmunoassay using partially purified anti-Can f 1 monoclonal antibodies (Pharmacia Diagnostics, Uppsala, Sweden) [102]. The detection limits of the Fel d 1 and Can f 1 assays were 55 and 80 ng/g dust, respectively, and the coefficient of variation <8% and 15%, respectively.

Ethical aspects

The studies were approved by local ethics committees in South and North Stockholm. All parents and children were supplied with information written and oral information and both children and parents gave informed consent before entering the studies.

Statistical methods

Significance was set at $p < 0.05$ (I-V). Non-parametric tests were used, Mann-Whitney U test for comparisons between the groups (I-V), Wilcoxon's signed rank test to test for differences (I-V) within the groups (II, IV) and relationships between variables were evaluated with the Spearman rank correlation coefficient (I, II, IV). Analysis of variance was tested by Anova (II, IV) and t-test (V). Multiple linear regression was also used to assess changes (II, IV). Power calculations were discussed with a statistician prior to start of study II and IV and the power was considered to be sufficient despite low numbers of participants due to the blinded 2-armed prospective design. No further statistical power calculation has been performed.

Time to deterioration of asthma status was described with the Kaplan-Meier survival curve (II), and the risk of deterioration was analyzed with the χ^2 test (II). The logistic regression model was used to analyze odds ratios (OR and 95% CI) (II).

RESULTS AND DISCUSSION

The role of eosinophils in allergic asthma (I-V)

It is clear that the eosinophil plays an important role in allergic asthma, as has already been described in detail in this thesis. All included five studies (I-V) clearly support the hypothesis that the eosinophil granulocyte is an important cell in the allergic inflammation.

Health effect of exposure to allergens in school dust in children with pet allergy and asthma (I)

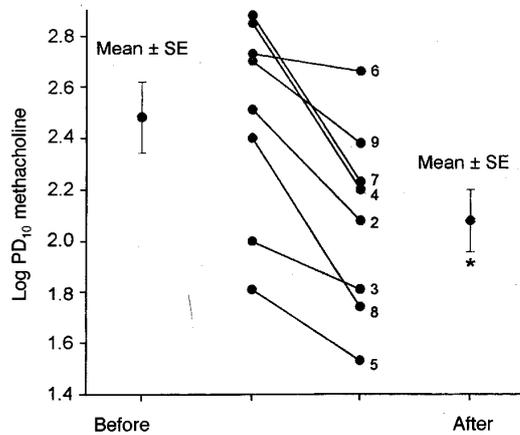


Fig 6. The PD10 change after school week

Asthmatic children with pet allergy were tested for bronchial reactivity before and after a school week. PD10 decreased after a school week ($p < 0.05$) (fig. 6). Eight out of ten children had a measurable PD10 after inhalation of 700 μg methacholine. One child (no. 1) was a non-responder, and one child (no. 10) refused to take part in more than the initial methacholine challenge and they are therefore not shown in the figure. The result indicates an increased bronchial reactivity after the school-week in these non-steroid-treated children with pet allergy and mild asthma, which might indicate that exposure to allergens triggers hyperreactivity in the sensitized child, and that indirect animal exposure plays a role in maintaining the allergic inflammation in the airways. Both the decrease in the number of PBE and the increase in bronchial reactivity are known to coincide with the onset of the LAR and consequently also with episodic and persistent asthma.

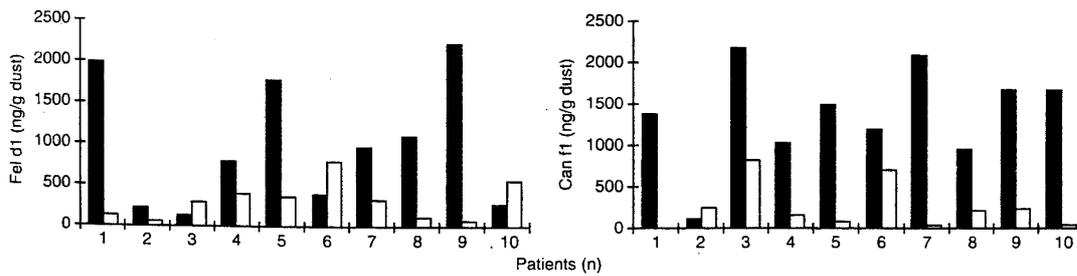


Fig 7. The concentration of Fel d1 and Can f1 in schools (■) and homes (□)

The concentrations of cat (Fel d1) and dog (Can f1) allergens were higher in school dust than in dust collected in the children's homes (fig. 7). The results showed up as a trend for Fel d1 ($p=0.07$) and as a significant difference for Can f1 ($p<0.05$). The means for the Fel d1 content in the classroom were 976 ng/g dust and in the home 306 ng/g dust and the means for Can f1 in the classroom were 1375 ng/g and in the home 255 ng/g dust. As seen in the figure above, three children had higher levels of Fel d1 at home than in school, but those still had markedly higher levels of Can f1 at school than at home; thus their total cat+dog allergen exposure was higher at school.

In summary (I), we propose that the decrease in the number of circulating eosinophils in this early stage of inflammation can be explained by the fact that airway inflammations a dynamic process and that pooling of PBE is only a transient stage before recruitment to the target organ. The observed decrease in PBE numbers and the increase in bronchial reactivity support the notion that these children get an induced mild inflammation in their airways after a school week.

Inflammatory markers to predict deterioration and need for GC treatment (II)

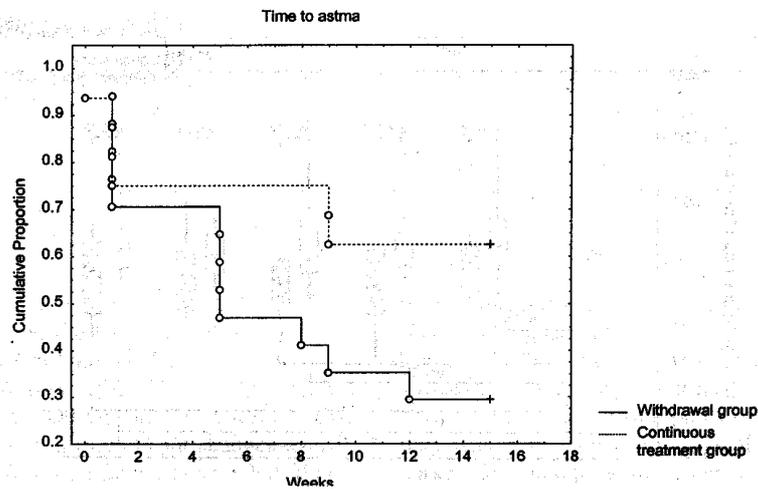


Fig 8. The time to exacerbation of symptoms of asthma

In this study, children with well-controlled asthma were withdrawn or continued on treatment; blood and urine samples were taken for monitoring of markers of inflammation. The time to exacerbation of asthma is presented in the Kaplan-Meier survival curve and all children were symptom free at entry (fig. 8). Twenty nine percent of the children in the withdrawal group and 62 % in the continuous treatment group remained symptom free throughout the study. In the withdrawal group, 71% (12/17) of the children got symptoms of asthma, and 47% (8/17) required one oral dose of rescue medication (betamethasone 4 mg), whereas in the continuous treatment group the corresponding figures were 38% (6/16) and 38% (6/16). The difference between the groups was significant ($p < 0.05$) for symptoms. Thus withdrawal of inhaled corticosteroids resulted in exacerbation in more than two thirds of these allergic children.

	No symptoms		Symptoms		Healthy controls (n = 16)
	Withdrawal group (n = 5)	Continuous treatment group (n = 9)	Withdrawal group (n = 12)	Continuous treatment group (n = 6)	
Eosinophil count (μL^{-1})	235 (180-372)	645 (120-728)	528 (203-1778)	478 (294-2012)	236 (74-345)
Eosinophils (%)	4.6 (3.6-8.0)	10.2 (3.2-16.8)	7.2 (5.0-22.8)	9.9 (4.6-27.5)	4.4 (1.1-8.8)
S-ECP ($\mu\text{g/L}$)	14 (7-26)	20 (8-37)	23 (12-144)	22 (11-89)	12 (3-24)
S-EPO ($\mu\text{g/L}$)	18 (13-45)	33 (10-71)	35 (21-221)	34 (14-83)	15 (2-36)
U-EPX ($\mu\text{g}/\text{mmol creatinine}$)	104 (50-122)	161 (67-282)	191 (50-426)	135 (68-435)	68 (31-204)

Table. Inflammatory markers at baseline, in relation to symptom development in patient groups.
Data are presented as median (range).

The number of PBE and the percentage of eosinophils (PBE/total cell count) at baseline are presented in the figure above. No significant difference was found at baseline between the children randomly assigned to the continuous treatment group and those randomly assigned to the withdrawal group. Children in the withdrawal group had a significantly increased PBE count already after one week of withdrawal ($p < 0.05$). The 5 children who remained symptom free in the withdrawal group had significantly fewer eosinophils ($p < 0.05$) (median 235 PBE/ μl) than the 12 children who had symptoms after withdrawal of budesonide (median 528 PBE/ μl). Three children dropped out in the withdrawal group: two of them had PBE counts greater than 1000/ μl at baseline. The group of children who never developed asthma symptoms despite withdrawal of budesonide had PBE levels comparable to those in healthy nonatopic, non-asthmatic controls. Levels of ECP and EPO in serum as well as EPX/EDN in urine were shown to have predictive value similar to, but not better than, the number of PBE, for this clinical evaluation.

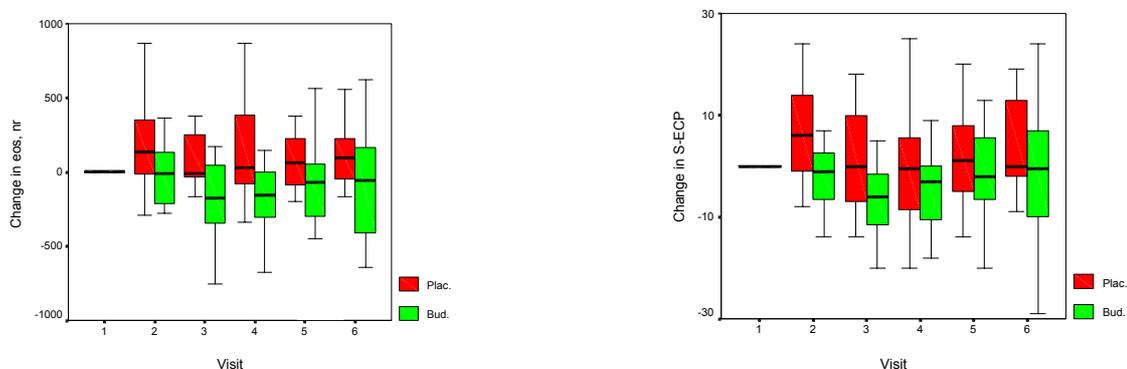


Fig 9. Change in peripheral eosinophils number and serum ECP (visit 1-6).

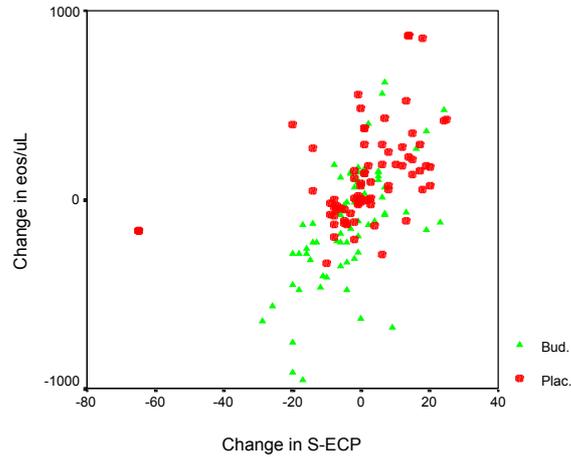


Fig 10. Correlation between PBE and S-ECP ($R=0,7$; $p<0,05$)

In summary (II), we have shown that after withdrawal of GC, the number of PBE increases, and that by measuring the levels of eosinophils in blood it is possible to predict the risk of deterioration - or rather to identify those with a low risk of worsening of asthma. We have also shown that markers of eosinophil activity in serum (fig. 9) (e.g. S-ECP, S-EPO) and in blood (EG2) increase after GC withdrawal and correlate to the number of PBE (fig. 10). Other findings in our studies supporting the unique role of the eosinophil are that the total number of leukocytes in blood or the level of MPO in serum as a marker of neutrophil activity was not increased in asthmatics compared with healthy controls, and did not increase or decrease despite other signs of increased allergic inflammation, after withdrawal of GC (data not shown).

Effects of treatment with GC on the eosinophil expression of the IL-5 and the GM-CSF receptor (III)

In this study we compared the expression of the IL-5 and GM-CSF receptors on PBE from GC-treated and non-treated asthmatic children and healthy controls.

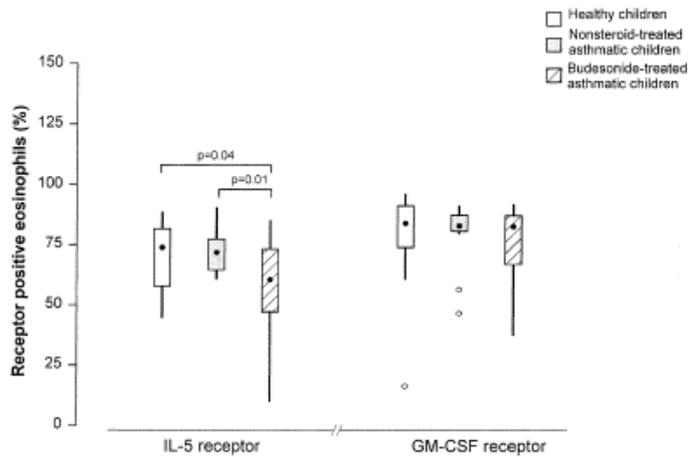


Fig 11. The proportion of IL-5 receptor (R) and GM-CSFR positive PBE from healthy children (n=16), non-steroid-treated asthmatic children (n=13) and budesonide treated asthmatic children (n=24).

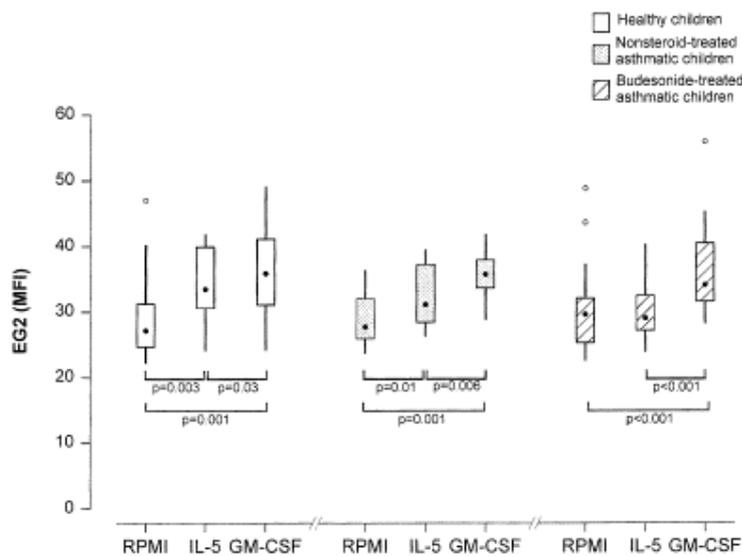


Fig 12. The expression on intracellular EG2 in PBE from healthy children (n=16), non-steroid-treated asthmatic children (n=13) and budesonide treated asthmatic children (n=24).

In summary (III), we have demonstrated that the IL-5 receptor (R) expression is decreased on PBE from GC treated asthmatic children (fig.11) and in addition, that only PBE from GC-treated children have a reduced *in vitro* responsiveness to recombinant IL-5 (fig.12). This supports further the role of the eosinophil in allergic inflammation and raises questions whether GCs act by depressing the expression of the IL-5 receptor on the eosinophil.

Exhaled NO (ENO), BHR, lung function and eosinophil activity in blood, nasal lavage (NAL) and sputum (IV)

This study was focused on the relationship between various markers of inflammation in the presence or absence of GC treatment.

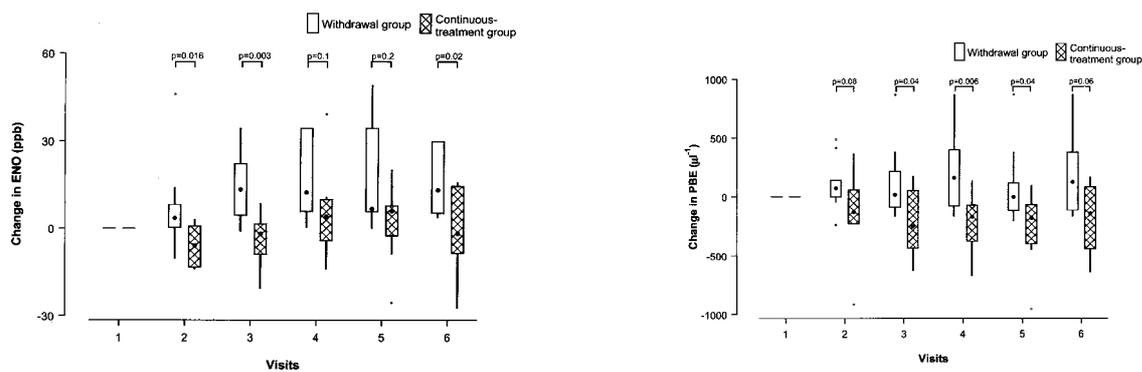


Fig 13. Change in ENO and PBE compared with baseline (visit 1=at randomization). The change from baseline values and differences between the groups are presented as median, interquartile range, min/max and outliers (dots).

In summary (IV), we have shown that levels of exhaled nitric oxide (ENO) increased after withdrawal of treatment with GC (fig 4, fig.13). This is in concordance with our earlier results concerning markers in blood, serum and urine and exhaled air (I, II) but not with measurements of inflammation markers in sputum (sputum-ECP, sputum-EPO, sputum-EPX/EDN) or nasal lavage (NAL-ECP, NAL-EPO, NALEPX/EDN) (IV). Although eosinophil markers in induced sputum have been shown by others to reflect inflammation we could not demonstrate a change in sputum markers in the respective groups that correlated to steroid therapy or the clinical course.

The transmigration capacity of circulating eosinophils following nasal allergen challenge in children with allergic asthma (V)

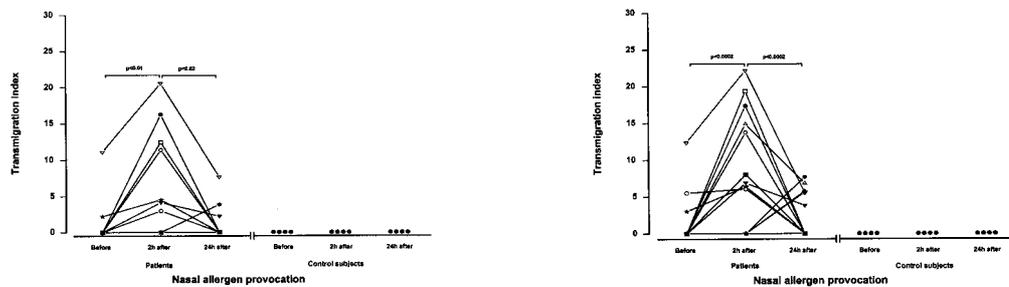


Fig 14. The spontaneous *in vitro* transmigration capacity of the PBE ($n=14$) from pet-allergic children after allergen nasal challenge with animal dander and from age-matched control subjects ($n=4$). Results, expressed as transmigration index. The additive effect of eotaxin *in vitro* transmigration of PBE.

In summary (V), we have shown that after allergen challenge and, PBEs have an increased capacity to transmigrate *in vitro* before they leave the circulation (fig.14). This is probably due to the increased level of activation of the eosinophil, a stage often referred to as “primed” in the literature. These findings are in concordance with our results from the earlier study (I), where children with pet allergy and non-steroid-treated mild asthma, showed a reproducible decrease in the number of PBE after a week of exposure to allergens at school.

We propose that the children have entered an early eosinophil recruitment phase. If the allergen exposure continues this initial step of LAR later develops into the manifest allergic inflammation in the airways. The clinical importance of our finding is that PBE has an increased capacity to transmigrate *in vitro* after an *in vivo* allergen challenge of allergic children within two hours (V) and that LAR starts after the initiations of EAR. This strengthened the indications for early intervention with anti-inflammatory drugs, such as inhaled GC.

General aspects on inflammatory markers

The sampling sites and methods of obtaining markers that we have studied all have advantages and disadvantages.

-Blood and serum eosinophil markers are always available, there are reference values, they can be used in all age-groups and have been extensively studied. On the other hand taking a blood sample is an invasive procedure, the analysis takes time and the measurement is not obtained at the target organ. Moreover, the result is affected by infections and other nonallergic mechanisms.

-Urine samples can easily be taken in all age groups and EPX in urine is stable. On the other hand there is a diurnal variation in EPX, urine is not obtained at the target organ and is also affected by nonallergic mechanisms.

-Nasal lavage is not unpleasant and the sample is obtained at the target organ. However, the procedure is difficult to standardize as there is a variable dilution effect, it is not possible to perform in infants and young children, and there is doubt about how well eosinophil markers in nasal washes reflect the state of the lower airways or bronchi.

-Sputum is obtained from the target organ and the procedure is well standardized, on the other hand the procedure is very unpleasant, time-consuming and can induce asthma. The success rate in obtaining sputum varies and in the asymptomatic children it is difficult to get a sample that is not containing saliva.

-The methacholine test is a well standardized procedure, well documented, reproducible, induces little discomfort and mirrors the state of the target organ. But the correlation between inflammation, disease severity and bronchial hyperresponsiveness is poor.

-Measurement of exhaled nitric oxide is a noninvasive method, the result is immediately available and it reflects conditions at the target organ. On the other hand the result is affected by infections, saliva, and ingested food and is strongly depressed by corticosteroid treatment. The equipment is expensive.

Suggested use of inflammatory markers

1. PBE can be used to identify patients in whom corticosteroids can be withdrawn
2. ENO can be used to follow the course of the airway inflammation
3. ECP/EPX/EPO in serum and/or urine can be used to differentiate asthma from other airway diseases
4. Biomarkers in nasal lavage and sputum are suitable only for research purposes.

CONCLUSIONS

(I)

Exposure of asthmatic children to animal dander in school dust during one week causes recruitment of eosinophils to the airways and increased bronchial reactivity to methacholine; both possible signs of mild inflammation

(II)

Measurement of PBE count and/or ECP and EPO in serum can provide information about the risk of exacerbation in allergic asthma and help to estimate the degree of airway inflammation.

(III)

Budesonide treatment induces a selectively reduced expression of the IL-5 receptor, concomitant with a reduced *in vitro* responsiveness to IL-5 stimulation in PBE. We suggest that downregulation of the IL-5 receptor might be a mechanism by which steroids inhibit the activation of eosinophils and the recruitment to inflammatory foci.

(IV)

Measurement of exhaled NO (ENO) on-line, combined with measurement of PBE is a valuable tool to avoid over-and under-treatment with corticosteroids in children with asthma. ECP in nasal lavage and sputum are not suitable for clinical evaluation.

(V)

PBE in allergic, but not in healthy children possess an increased spontaneous capacity to migrate after allergen challenge. Eotaxin added *in vitro* can further enhance this PBE migration capacity. The physiological implication of these data is currently unclear, but they indicate that PBE in allergic subjects are more activated than PBE in healthy controls and that the net outcome of eotaxin depends on the actual state of eosinophil activation.

Concluding summary

We have been able to show that allergic inflammation is a dynamic process involving eosinophils. Many markers of inflammation are available, but no single marker can provide the answers to all questions. Yet, as this thesis shows, by selecting suitable markers and by combining the information the markers provide, it is possible to draw conclusions about the process of allergic inflammation and the inflammatory state of an individual allergic patient.

“The best is to know what you are looking for before you start looking for it”

Winnie the Pooh

FUTURE PERSPECTIVES ON/FOR MODES OF INTERVENTION

For the evaluation of the effect of different drugs and the need for anti-inflammatory treatment we will continue to need markers of inflammation that correlate to the clinical consequences of airway allergy. Many new approaches for drug therapy in eosinophil-dependent diseases are of interest for the future. In this chapter I will briefly mention some examples of the possible new strategies for development of drugs for the future. [103]

The allergic process can be targeted at various steps, including anti-IgE, [104] inhibition of mast cell degranulation, interference with synthesis or activity of leukotrienes [105] (e.g. LTB₄ and LTC₄), blocking of receptors for cytokine/chemokine molecules, neutralizing antibodies against adhesion molecules (e.g. VCAM-1, ICAM-1) or very late antigen 4 (VLA-4), to mention a few. Moreover, the proliferation, survival, and activation of eosinophils can be blocked by interfering with the generation of cytokines/chemokines, for example GM-CSF, IL-3, IL-5 and eotaxin. The limited clinical effect of the IL-5 monoclonal antibody seen so far, [106] might be explained by the involvement cells other than eosinophils in these responses or by other cytokines (e.g. IL-3 and GM-CSF) getting a stronger eosinophil promoted role in the absence of IL-5. A larger scale clinical study of the efficacy of multiple doses of anti-IL-5 will provide further insight into the role of the eosinophils in the clinical pathogenesis of allergy and asthma and the clinical usefulness of anti-inflammatory treatment.

Immunotherapy is another way of targeting the anti-allergic pathway and the effect is strongly positive and obvious to the clinician, but the immunological background is still somewhat unclear. In the future, treatment with epitopes of allergens (e.g. recombinant allergens) [107] with the same immunological effect of causing the biological response to deviate from an atopic pathway to a non-atopic, but without severe side effects, might be the “treatment of choice”. Other strategies include enhancing the effect of cytokines that promote the anti-allergic pathway (e.g. IFN- γ and IL-12) [108].

Single target drugs such as anti IL-5, and anti-leukotrienes will probably be part of the new generation of multiple target drugs, in combination with a variety of other anti-target

pharmaceutical products. Nevertheless, apart from GC, there is still no other drug available on the market, able to mimic the steroids superior ability to treat allergic inflammation in asthma.

To summarize: We need markers of inflammation that correlate to the clinical consequences of airway allergy.

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