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Eosinophil Inflammation in Allergic Disease

Clinical and experimental studies in allergic asthma and allergic rhinitis

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Abstract
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Allergic diseases are chronic inflammatory conditions, characterised by eosinophil inflammation systemically and in target organs, where cytotoxic granule proteins are responsible for tissue injury. Allergic rhinitis is known to be a risk factor for the development of asthma, yet not all with rhinitis develop asthma. The overall aim was to investigate the involvement of eosinophils in allergic rhinitis and allergic asthma *in vivo* and in experimental settings, with a focus on differences between rhinitis and asthma. Birch pollen allergy was used as a model and patients were studied during pollen season and after nasal and bronchial allergen challenge.

During pollen season and at baseline, allergic rhinitis and allergic asthma had the same degree of systemic eosinophil inflammation. Despite this, impairment in lung function during season and increased bronchial responsiveness at baseline were more common in the asthmatics. Systemic inflammation was more pronounced after seasonal exposure than after experimental challenge. Allergic rhinitis and allergic asthma had the same degree of eosinophil airway inflammation after bronchial challenge, but only the asthmatics had increased bronchial responsiveness measured as PD_{20} for birch allergen.

Allergen primed eosinophils were investigated *in vitro* for C3b-induced degranulation after seasonal and experimental challenge. The released amount of eosinophil granule proteins was within the same range for all three allergen challenge models with just minor differences in propensity for degranulation between rhinitics and asthmatics. Signalling through PI3K for degranulation was studied with the specific inhibitor Wortmannin. PI3K signalling for eosinophil degranulation was clearly involved in allergic rhinitis and allergic asthma irrespective of the model for allergen exposure. Asthmatics demonstrated less inhibition of degranulation through PI3K during pollen season, indicating that other pathways contribute to eosinophil degranulation in allergic asthmatics.

Conclusion: Allergic rhinitis and allergic asthma present with the same degree of systemic and local eosinophil inflammation. The eosinophils are primed for degranulation equally and follow the same pathway through PI3K for degranulation. Our data indicates that eosinophil inflammation *per se* is not sufficient for the development of asthma.

Keywords: Allergic asthma, allergic rhinitis, eosinophils, pollen season, bronchial challenge, nasal challenge eosinophil degranulation, PI3K signalling

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Not everything that can be counted counts, and not everything that counts can be counted.

Albert Einstein

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Kämpe M, Stålenheim G, Janson C, Stolt I, Carlson M. Systemic and local eosinophil inflammation during the birch pollen season in allergic patients with predominant rhinitis or asthma. *Clin Mol Allergy* 2007;(5)4:1-8.

II. Kämpe M, Janson C, Stålenheim G, Stolt I, Carlson M. Experimental and seasonal exposure to birch pollen in allergic rhinitis and allergic asthma with regard to the inflammatory response. *Clin Resp J* 2010;4:37-44.

III. Kämpe M, Stolt I, Lampinen M, Janson C, Stålenheim G, Carlson M. Patients with allergic rhinitis and allergic asthma share the same pattern of eosinophil and neutrophil degranulation after allergen challenge. *Submitted for publication.*

IV. Kämpe M, Lampinen M, Stolt I, Janson C, Stålenheim G, Carlson M. PI3-kinase regulates eosinophil and neutrophil degranulation in patients with allergic rhinitis and allergic asthma irrespective of allergen challenge model. *Submitted for publication.*

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Contents

Abbreviations

Akt	Protein kinase B, PKB
APC	Antigen presenting cell
ATP	Adenosine-5´-triphophate
BCR	B cell receptor
BHR	Bronchial hyperresponsiveness
C3b	Complement factor 3
CC	Chemokine
CCR	Chemokine receptor
COPD	Chronic obstructive pulmonary disease
CR1	Complement receptor type I, CD11b/CD18
CTL	Cytotoxic T lymphocytes, $CD8^+$
ECP	Eosinophil cationic protein
EDTA	Ethylene-diamine tetra-acetic acid
EPO	Eosinophil peroxidase
EPX/EDN	Eosinophil protein X/ Eosinophil derived neurotoxin
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
Fc	Constant part of immunoglobulin
FcεRI	High-affinity IgE receptor
FcεRII	Low-affinity IgE receptor (CD23)
FOXP3	Forkhead box P3, transcription factor
GINA	Global initiative for asthma
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HNL	Human neutrophil lipocalin
IC_{50}	Half maximal inhibitory concentration
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LT	Leukotriene
MBP	Major basic protein
MHC	Major histocompatability complex
MPO	Myeloperoxidase
PAF	Platelet activating factor
PD_{20}	Provocation dose, cumulative dose causing 20% decrease in FEV_1
PEFR	Peak expiratory flow rate

PI3K	Phospho-inositide-3 kinase
PIP3	Phosphatidylinositol (3,4,5) - triphosphate
RANTES	Chemochine ligand-5, CCL5
RIA	Radioimmunoassay
RT	Room temperature
SQ-U	Standard quality unit
TCR	T cell receptor
TGF	Transforming growth hormone
Th cell	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
T_{Reg}	T regulatory cell, $CD4^{+}CD25^{+}FOXP3^{+}$ T_{Reg}

Introduction

Background

Allergy has been increasing world-wide over the past decades, especially in the western developed countries, and is today a major health problem with a prevalence of over a third of the population in many regions (1, 2). Allergic diseases manifest as hyperresponsiveness in the target organ, whether skin, nose, lung or the gastrointestinal tract; the hyperresponsiveness may or may not be IgE-mediated. It is generally accepted that chronic inflammation underlies the manifestations of the various allergic conditions. Exposure to environmental allergens is one of the most important stimuli for the initiation of allergic inflammation, especially in the airways, and thus the most important factor associated with development of asthma and rhinitis in the western world (3, 4, 5).

Atopy, IgE and allergic disease

IgE, the fifth class of human antibodies, was discovered in the 1960s (6, 7), a major breakthrough for understanding the underlying mechanisms of allergy that has had a major impact on both diagnosis and treatment of allergic diseases. The term atopy, derived from the Greek word *atopia* (strangeness), was first introduced in 1923 by Coca and Cook to describe an inherited tendency to develop immediate-type hypersensitivity reactions against common environmental allergens (8). The definition of atopy thus is a genetic predisposition to produce IgE-antibodies against common environmental and harmless antigens and during the sensitisation period, the latent asymptomatic phase, IgE antibodies can be detected (9). However, on re-exposure to allergen the "atopic march" proceeds in some individuals to a clinically symptomatic allergic disease. In most literature, atopic sensitisation is defined as the presence of specific IgE by *in vitro* tests or skin prick test in a symptomless subject (9, 10, 11). Allergy, on the other hand, is the clinical expression of an IgE-mediated disease, the symptoms depending on the affected organs. Hence, allergy is an immunological disorder, but is often referred to by many people when meaning any uncomfortable experience.

Allergic asthma

Bronchial asthma is a widespread disease globally. Its prevalence has almost doubled over the last 50 years and is now approaching 10%, although prevalence rates differ greatly depending on geographical area, and is more frequent in childhood than in adulthood (12, 13). Asthma is clinically characterized by reversible airway obstruction, variable over time, with the cardinal sign of bronchial hyperresponsiveness associated with chronic eosinophil inflammation of the airway wall (14). Asthma is thus a disorder of the airways, which contract too much and too easily to a wide range of exogenous and endogenous stimuli (15). The typical asthma symptoms are wheeze, cough, mucus production and dyspnoea. Classification of asthma can be based on age, etiology, associated characteristics and severity. Asthma in children is mostly IgE-mediated (16) but after the age of 20 years it is more complex and more difficult to classify because of heterogeneity and overlapping entities due to gene-environment interactions. In new-onset asthma during adulthood, there appear to be entities independent of atopy in most cases (17). These non-allergic forms of the disease have been subject to careful comparative investigations, but so far no clear pathogenic cause has been identified (18, 19). The detection of IgE isotype-switching in bronchial airway biopsies in both atopic and non-atopic asthma has provided some evidence that local IgE mechanisms are involved (20). The overall pathology in atopic and non-atopic asthma seems to be quite similar (21, 22, 23). On the other hand, Amin *et al.* have reported contradictory results, with less eosinophils and more neutrophils in bronchial biopsies in non-atopic asthma than atopic asthma (24).

Thus, allergic asthma is a chronic inflammatory disease caused by repeated immediate-hypersensitivity reactions and late-phase reactions leading to intermittent and initially reversible airway obstruction. In the chronic stage the Th_2-driven disease is characterized by chronic eosinophilic inflammation, smooth muscle hypertrophy, goblet cell hyperplasia and epithelial remodelling (25). Tissue eosinophilia and eosinophil degranulation is commonly associated with fibrotic diseases and eosinophils have been identified as a significant source of transforming growth factor-β_1 (TGF-$ß_1$), one of the most fibrinogenic factors known (26). TGF-$ß_1$ is also involved in airway remodelling in asthma with increased deposition of collagen and extracellular matrix proteins (27, 28, 29, 30). Zagai *et al*. have reported from a series of *in vitro* investigations that eosinophils via released ECP mediate the remodelling of extracellular matrix by influence on human lung fibroblasts (31, 32, 33). The recognition that asthma is a highly heterogeneous disorder in terms of clinical expression, response to different therapies, natural history and association with environmental conditions has opened up the disease beyond atopic sensitization. It has been suggested that the cause of the disease may lie in the airway epithelium (34). A defective barrier function

may activate the epithelial-mesenchymal-trophic-unit (EMTU) in the airways, leading to sensitisation and ongoing disease with persistent hyperresponsiveness to irritative stimuli (35, 36).

Allergic rhinitis

Allergic rhinitis is the most frequent manifestation of allergic disease affecting the airways and its development depends on the interaction between genes, environment and immunological factors (37). The diagnosis of rhinitis is based on the report of subjective nasal complaints (nasal blockage, itching, sneezing and increased secretions), increased nasal responsiveness and increased nasal airway resistance. To date, the different tests for rhinitis have low sensitivity and specificity and the diagnosis is therefore predominately made on the basis of clinical history (38, 39). The physiological function of the nose is to condition the inhaled air, filter small airborne particles and maintain defence mechanisms against the environment (40).

Airway inflammation is also present in the upper airways, but with little collagen deposition and absence of myofibroblasts in the nasal mucosa (41). There is however, evidence of remodelling in the nasal mucosa (42). The inflammation in the nasal mucosa is dominated by esoinophils, with accumulation in the reticular basement membrane and epithelial shedding, though not to the same degree as in the bronchi of patients with allergic asthma (43, 44). It has also been suggested that neural pathways may contribute to the pathophysiology of allergic rhinitis (45). Neurotrophins, and nerve-growth-factor (NGF) expressed in the esoinophils in the nasal mucosa has been suggested as candidates for the nasal hyperresonsiveness (46). Nasal obstruction is mostly the result of dilatation of capillary vessels, whereas bronchial obstruction is mainly caused by smooth muscle contraction.

Seasonal allergic rhinitis to birch pollen is primary diagnosed by excluding a history of asthma/respiratory complaints and chronic nasal disease, with specific IgE for birch and with no need for treatment outside pollen season. But some authors claim that this is not consistent with real life and that mixtures are common (47).

The united airways concept

Allergic rhinitis and allergic asthma are considered to be manifestations of "the allergic syndrome" and it has been demonstrated that allergic rhinitis is a strong risk factor for the onset of asthma (42, 43, 48, 49), even independent of allergy (50, 51). The majority of patients with allergic asthma present with symptoms of seasonal or perennial rhinitis and in epidemiological studies rhinitis were found in 70-80% of patients with asthma (52, 53). The risk

for asthma development is dependent on the allergens involved and is strongest for perennial allergens, as cat and mite (43). The lack of precise diagnostic criteria for diagnosing rhinitis is a problem when comparing epidemiological studies and in many studies there has been no clear distinction between allergic rhinitis and non–allergic rhinitis (54). In addition, bronchial hyperresponsiveness (BHR) is likely to be an intermediate factor in the process leading to asthma and in many papers the included patients with allergic rhinitis were already diagnosed with BHR (43). Almost all data concerning the association of upper and lower airways came from earlier epidemiological studies, but there is now substantial evidence from both epidemiological data and basic immunology suggesting that allergy is not a disease confined to a specific organ but rather a systemic disorder (54). The context of a systemic disorder in the respiratory airways was the reason behind the WHO-position paper ARIA (Allergic Rhinitis and its Impact on Asthma) from 2001 (50).

The respiratory tract can be considered as a single morphological-functional entity, with ciliated epithelium, mucinous glands and extensive vascularisation and innervation (54). In healthy subjects the airway mucosa has a similar structure in the nose and bronchi, although there are differences in the capillary/venous network and presence of smooth muscle (42). Embryologically the upper and lower airways also differ in origin; the nose coming from ectodermal and the bronchi from endodermal tissue. Several mechanisms have been proposed for the interaction between upper and lower airways; oral breathing due to blocked nose (55), postnasal drip (56), naso-bronchial reflexes (57) and the bone marrow-systemic route (58).

Taken together, mucosal and systemic inflammation is a main feature of allergic rhinitis and allergic asthma and eosinophils are considered to constitute a hallmark of this inflammation (39), with contribution of T-cells (59). In addition, the respiratory mucosa is rich in mast cells, the phenotyp though differing in upper and lower airways (54, 60) (Table1). Furthermore, airway remodelling is well known in both allergic asthma (27, 28, 29, 30) and allergic rhinitis (59). Immunopathologically, rhinitis and asthma share several characteristics with evidence of local IgE production in atopic as well as non-atopic disorders (20, 61).

Despite chronic inflammation of the nasal mucosa, and sometimes undiagnosed BHR and airway remodelling, patients with allergic rhinitis do not have an overt asthma (62, 63, 64). Further, allergic rhinitis is a common disorder in childhood, but in the vast majority of patients symptoms ease off and eventually disappear (39). Actually, according to Nielsen *et al.* 90% of patient with seasonal allergic rhinitis had unchanged or improved symptoms in a follow-up study after 6 years (39), which is consistent with other studies of the natural history of allergic rhinitis (65, 66, 67). In the same paper, though, they concluded that eosinophil markers in serum, but not in nasal fluids, demonstrated a high predictive value for later asthma development in

patients with allergic rhinitis (39). In conclusion, this indicates that eosinophil inflammation is not sufficient to cause asthma and that additional factors contribute to whether or not a patient with rhinitis develops asthma.

Table 1. *Similarities and differences in allergic rhinitis and allergic asthma [Modified after Braunstahl et al. (59)].*

		Nose	Bronchi
Epithelium	Shedding	0 to +	+++
	Metaplasia	0 to +	0
Basement membrane	Pseudo-thickening	0 to +	++ to +++
	Collagen deposition	0 to +	++ to +++
Submucosal cells	Eosinophils	+++	+++
	Lymphocytes (CD4+)	+ to ++	+ to ++
	Vascular network	+++	+
	Smooth muscle	0	++
	(Myo)fibroblasts	0 to +	++ to +++
	Mast cells	MC_{TC}*	MC_T**

MC_{TC}*: tryptase/chymase-positive mast cells, MC_T**: tryptase-positive mast cells

Treatment of allergic rhinitis and allergic asthma

Allergic diseases constitute a substantial global health problem with increasing socioeconomic impact and impaired quality of life. In addition to pharmacological treatment it is important to control contributing factors, i.e. environmental allergens and triggers, gastro-oesophageal reflux, sinus disease, smoking history etc. The basis for treatment of mild seasonal allergic asthma is rapid-acting β_2-agonists as reliever medication in addition to low-dose inhaled corticosteroids as monotherapy. According to GINA guidelines this therapy is recommended as first-line maintenance therapy for most mild asthmatics, reducing exacerbations and improving quality of life (68). Mild seasonal rhinitis is treated with antihistamines (per oral and topical) and nasal steroids, where the nasal steroids produce the greatest improvements in nasal symptoms in patients with seasonal allergic rhinitis according to a recent Cochrane review (69).

Leukotriene receptor antagonists may be added for further symptom improvement in both allergic rhinitis as well as in allergic asthma. For the more severe cases allergen-specific immunotherapy may be considered and occa-

sionally the novel anti-IgE therapy, that recently has been proved to be affective not only in allergic asthma but also in allergic rhinitis (70, 71).

Allergens and birch pollen allergy

Pollen allergy amounts to approximately 20 % of community allergy (72). Air pollution is known to exaggerate pollen allergen reactions, probably through acting as hapten/adjuvant or by direct toxicity and damage of the airway mucosa, facilitating the penetration of allergens (72, 73). The most common allergen sources are pollen, fungi, pet dander and house dust mite.

Allergens are antigens giving an IgE response, instead of an IgG response, to harmless peptides or proteins (74, 75), eliciting immediate hypersensitivity and late-phase reactions in different target organs. Most allergens are proteins or glycoproteins with masses ranging from 5-100 kD and as the size of the allergen increases, the number of potential epitopes increase. The major allergenic pollens (grasses, trees and weeds) are wind-pollinated rather than insect-pollinated, they are soluble and with a size of 5 - 200µm (76). Birch pollen allergies are increasing and approximately 20% of the population in the Northern European countries is sensitized to birch pollen, the proteins responsible belonging to the *Betula verrucosa* (Bet v) family. In over 90% the allergy is due to the major and most important allergen Bet v 1 (77), belonging to the pathogenesis-related protein family 10 (PR-10 family) (78).

Many common allergens are enzymes, particularly proteases, and it has been suggested that in human defence against invading helminths the host secretes proteolytic enzymes which promote Th_2 responses (79). This hypothesis is supported by the fact that the major house dust mite allergen *Dermatophagoides pteronyssinus I* (Der p I) is a cysteine protease that cleaves the intracellular tight junctions, and so gains access to the subepithelial antigen-presenting cells (80). However, even if many common allergens are enzymes, most are not and in a systematic overview of the structural biology of 40 common allergens no characteristic structural feature could be recorded (81). The structural components recognized by the innate immune system, the route of entry, particle size and adjuvants as pollutants are assumed to be important in determining if the antigen is recognized as an allergen by the host (82).

Seasonal allergen exposure vs. experimental allergen challenge

Allergen exposure during pollen season is a low-dose challenge over a long period and is more like natural course of allergy development compared to a single high-dose allergen challenge. It is known, from both experimental studies and real life, that very high doses of allergen can elicit asthma symptoms in non-asthmatics, as in epidemic asthma linked to thunderstorms during grass-pollen season and epidemic soybean asthma in Barcelona in the 1980s (83, 84). The Swedish birch pollen season is very convenient to study, as it is relatively short and defined in time, coming after a long cold winter with no pollen prevalence. Studies that depend on the natural variation of allergens are though time consuming and involve a level of uncertainty as the concentration of allergens may vary regionally and also from year to year. In contrast, studies employing experimental allergen exposure such as bronchial and nasal challenge are easier to perform (85, 86, 87). However, there are also several issues related to bronchial challenge methods such as establishing relevant doses and correct lung deposition (88, 89). In addition, the results of nasal and bronchial allergen challenge may be more difficult to interpret as the time course and dose of exposure is fundamentally different from seasonal exposure. Other experimental models such as repeated low-dose regimens for a range of days (90, 91) or different models of experimental environmental settings (92, 93) may simulate natural exposure to a larger degree.

The hygiene hypothesis

Allergic diseases are inflammatory disorders that develop on the basis of complex gene-environment interactions. The incidence is steadily increasing, which seems to be associated with a modern lifestyle where evolutionary adaptation has not had time to catch up (95). The “hygiene hypothesis”, or the “Old Friends” hypothesis, was originally proposed by Strachan in 1989, pointing out that allergies increased in society with reduction in the number of family members in the household (96). Epidemiological studies demonstrate an explosion of allergic diseases over the past decades (97, 98) parallel to a tremendous decrease in both the incidence and prevalence of bacterial, viral and helminth infections during the same time period (95, 99). This has been achieved by vaccination strategies, antibiotic treatment and improved living conditions as high standards of water supply and sewage systems.

The current view of the cellular and molecular mechanisms responsible for these phenomena includes changes in the fine balance of Th_1, Th_2 and regulatory T-cell responses, which are triggered by altered or missing innate immune cell activation (100). The fetus is exposed to a Th_2 environment in

the uterus and the immune system is therefore Th_2-polarized at birth. It is believed that immune deviation towards Th_1 responses normally occurs in response to bacterial infections in the neonate, resulting in suppression of the Th_2 skewness (89). It is well accepted that Th_2 responses, above all have evolved in order to efficiently combat helminth infections (99, 101).

Though the "hygiene hypothesis" originally was an attempt to explain the rising incidence of allergic diseases in the developed countries, today it is also suggested to be applicable to the coinciding increase of several other chronic inflammatory disorders and autoimmune diseases (102, 103, 104, 105). It has been speculated that infections of historical importance might have shaped our immune system during evolution and may have played a major role in down regulating allergic and autoimmune responses (106).

Hypersensitivity reactions

Hypersensitivity reactions are undesirable responses of the adaptive immune system to innocuous antigens sometimes causing severe disease due to tissue damage. Already in 1963 Gell and Coombs stated the scheme for classification of hypersensitivity reactions (type I-IV), requiring a pre-sensitized status of the host (107). The classification is still applicable for both allergic and other immune reactions after minor modifications. The immune system has many built-in feedback loops and amplification mechanisms and once a pathologic response starts it is often difficult to control and to terminate, which explains why hypersensitivity reactions often tend to be chronic. Allergic reactions can be divided into immediate and late-phase reactions. The immediate reaction (type I) is caused by vasoactive mediators released primarily from mast cells and the late-phase reaction (type IV) is cell-mediated due to recruitment of inflammatory cells to the target organ, the latter phase not always preceded by a detectable type I reaction. The immediate reaction can subside but generally proceeds to the late-phase reaction, giving rise to chronic inflammation with more serious long-term illness in the affected tissue (108).

Sensitisation and the allergic cascade

Dentritic cells, mast cells, basophils, esoinophils and IgE are essential components of the allergic reaction, as well as T-cells and B-cells (109). The dentric cells, mast cells and basophils together with epithelial cells are crucially located at body surfaces ensuring first defence against environmental pathogens (110). The first step in the allergic sensitisation is the uptake and presentation of the allergen/antigen by antigen presenting cells (APC), the most important being the dentritic cells. The dentritic cells are professional

APCs, i.e. they express MHC class II molecules as well as co-stimulatory factors necessary for T-cell activation and initiation of the adaptive immune response. After allergen uptake and processing, the dentritic cells mature and migrate to the lymph nodes were they present the peptide fragments for naïve T-cells, thereby directing them in favour of a Th_2-phenotype (111). The antigen-specific Th_2-cells migrate to the target tissue where they orchestrate the immune response by releasing Th_2-cytokines, starting cross-talk between immune cells and an amplification cascade takes place (112).

The crucial role in the sensitisation process is played by the dentritic cells (110), but the B-cells are also important for allergen capture and processing when small allergens are involved (113). In the presence of co-stimulation the Th_2-cells upregulate the expression of IL-4 and IL-13, which are necessary for initiation of class-switching to IgE synthesis (114). Class-switch is generally thought to occur in the lymph nodes, but has recently been reported to take place locally in nasal and bronchial mucosa and also in the gastrointestinal tract in patients with food allergy (115). The Th2-cytokines IL-4 and IL-13 also act on epithelial cells, smooth muscle cells and goblet cells in the airways and are suggested to be responsible for airway hyperresponsiveness (112). The released Th_2-cytokines are involved in recruitment of mast cells (IL-4, IL-9 and IL-13), basophils (IL-3 and IL-4) and eosinophils (IL-3, IL-5 and GM-CSF), the main mediator cells involved in the allergic tissue response (116). Once sensitised to an allergen, on re-exposure the allergen cause cross-linking of the IgE bound to high-affinity IgE-receptors on mast cells, stimulating release of preformed histamine and newly generated lipid mediators responsible for the immediate allergic reaction. In addition, the released mediators and cytokines from the mast cells recruit eosinophils, basophils and macrophages responsible for the late allergic reaction (109).

Toll-like receptors

The first line of defence against pathogens is the constitutive innate immune system, immediately mobilised upon infection or any tissue damage. The immune cells have Pattern Recognition Receptors (PRRs) on their surface, which recognise the highly conserved set of molecular structures specific for microbes, the Pathogen Associated Molecular Patterns (PAMPs) (117). The best understood and perhaps the most important subgroup of PRRs is the family of Toll-like receptors (TLRs), natural ligands for the PAMPs of bacteria, viruses and fungi (118). Signalling through Toll-like receptors, expressed on the epithelium of all body barriers, initiates acute inflammatory responses and plays a crucial role for the early shaping of the immune system and the suppression of Th_2-driven allergic immune responses (118). Recent evidence suggests that the TLRs play an important role in controlling

the adaptive immune responses through “the IgE-Toll like receptor network” (115, 119, 120, 121, 122).

Mast cells and basophils

Mast cells and basophils share many features and they are thought to have similar functions in protecting against helminth infections and in IgE-mediated allergic inflammation. Both cells are highly conserved through evolution, traditionally considered to be cells of the innate immune system, but have proved to be able to modulate adaptive immune responses as well (123,124,125). Mast cells and basophils express TLRs, MHC class II molecules and the high-affinity IgE receptor (FcεRI) and can act as professional APCs (108,123,126,127). Both cells are considered to maintain the sensitised state of the mucosa and to be initiators of the allergic reaction (125).

Mast cell precursors circulate in the blood and mature when entering the tissue, localizing near blood vessels and at epithelial surfaces (128), whereas basophils are primary encountered in the circulation (129). The homing propensity of mast cells to mucosal tissues is essential for the allergic reactions to take place in particular organs and the microenvironment of mucosal tissues favours the local synthesis of IgE ahead of IgG in atopic subjects (126). The human mast cells are divided into two types according to the content of tryptase (MC_T cells) or tryptase and chymase (MC_{TC} cells) (108).

Basophils constitute less than 1% of the peripheral blood leukocytes and have previously gained very little interest, even though it has previously been unravelled that they play a critical role both in chronic allergic inflammation and in systemic anaphylaxis (125, 129, 130). Basophils are involved in IgG-mediated anaphylaxis, often requiring larger amounts of allergens than IgE-mediated, by releasing the mediator Platelet-activating factor (PAF) (125). In addition, the activated basophils release histamine, serine proteases and leukotriens, but not prostaglandins, as well as a cytokines, chemokines and complement receptors (108).

B cells and isotype class-switch to IgE

Adaptive immunity is mediated through numerous genetic and cellular processes to generate antigen-binding immunoglobulins and T-cell receptors (TRCs) through recombination of variable (V), diversity (D) and joining (J) segments (131). All antibody molecules share the same basic structure with two identical light chains, two identical heavy chains and a constant C-region, the Fc-region, on the heavy chain. The light and heavy chains have variable regions that participate in antigen recognition and the constant region determines Ig-class isotype; different isotypes (IgA, IgD, IgE, IgG and

IgM) performing different effector functions when antigen is bound to their Fc-receptor (131). B-cells can act as professional APCs and via their receptor (BCR) internalize and process small antigens for Th-cell presentation (113).

All naïve B cells express both IgM and IgD but on encountering antigen, depending on the cytokine signals, a class-switch to either IgG, IgA or IgE will take place in the secondary lymph nodes (132, 133). The Th_2-derived cytokines IL-4 and IL-13 specify class switch in the lymph nodes and in the target tissue basophils are responsible for class-switch, which protect and re-sensitise FcεRI on mast cells in a positive feedback mechanism giving further amplification of the IgE production (108, 128). The process of mast cell and APC recruitment and IgE production in the mucosal tissues is central to the function of the IgE network (89), and also in protecting the host against systemic anaphylaxis (115). The B-cells express two types of IgE-receptors on their surface: the high- and the low-affinity IgE-receptors (FcεRI and FcεRII/CD23). IgE is usually measured as total serum IgE, but there are also *in vitro* assays for detection of free IgE (108).

T cells

Naïve T lymphocytes migrate from the bone marrow to the thymus were they traditionally are considered to mature into two main lineages: T-helper cells and T-cytotoxic cells (CTL). The lineage choice goes by way of double thymocytes, i.e., $CD4^+CD8^+$ cells expressing a fully developed T-cells receptor composed of α and ß chains with immense variability (134,135). The basis for selection of TCRs in the thymus is their ability to discriminate self from non-self, i.e., the TCR is MHC-restricted and self-tolerant (136). T-helper cells express CD4 protein on their cell surface and are activated by antigen bound to MHC class II molecules, whereas CTLs are $CD8^+$ and activated by antigen associated to MHC class I (137). The division of T cells into two functional subsets based on their cytokine production was already described in 1986, though recently this concept has been re-evaluated pointing in the direction of functional plasticity of the T cell subsets with ability to convert to another phenotype (138, 139). CTLs are potent in defence against pathogens but can also be directed against self-tissues; in autoimmune diseases, graft rejection and graft-versus-host-disease (GVHD) (140). T-helper cells play critical roles in adaptive immune responses and the diverse functions are determined by their cytokine secretion patterns and tissue location, though it is known today that they rather represent polarised forms of the highly heterogeneous $CD4^+$ T cell-mediated immune responses (140, 141). So far, the generally recognized $CD4^+$ lineages are Th1, Th2, Th17 and T_{Reg} cells, but further subunits have been proposed (142, 143, 144).

$CD4^+$ T cell lineage

Th1 cells are characterised by INF-γ production and are primarily involved in cellular immunity against intracellular bacteria (142). Th17 cells are characterised by expression of IL-17 and IL-22, mainly located at barrier surfaces (145). The regulatory T cells are crucial for the maintenance of self tolerance and immune homeostasis, dysfunction causing autoimmune diseases, immunopathology and allergy (146). T_{Reg} cells, expressing $CD4^+CD25^+$ and the transcription factor FOXP3 (forkhead box P3) are naturally present in the immune system (nTregs) and developmentally determined in the thymus (147). In addition, there are induced T regulatory cells (iTreg) generated in the periphery in the presence of antigen and cytokines, especially TGF-ß (146). At mucosal surfaces the iTregs are in close connection with Th17 cells suggesting overlapping development and dynamic interplay in peripheral tolerance (148, 149). The Th17 cells have especially been considered to have a crucial roll in the development of allergy (144).

The Th2 cells control immunity to extracellular parasites and all forms of allergic responses by way of class switch to IgE (112). The products of allergens and helminths are strong activators of Th2 responses locally in the airways, sensing these products and activating the Th2 response in the tissue (112). However, as mentioned, not only Th2 cells decide the outcome of immune responses to allergen encountered in the tissues, but also the interplay and cross-regulation with other T cells, above all Tregs and possibly Th17 cells (150, 151).

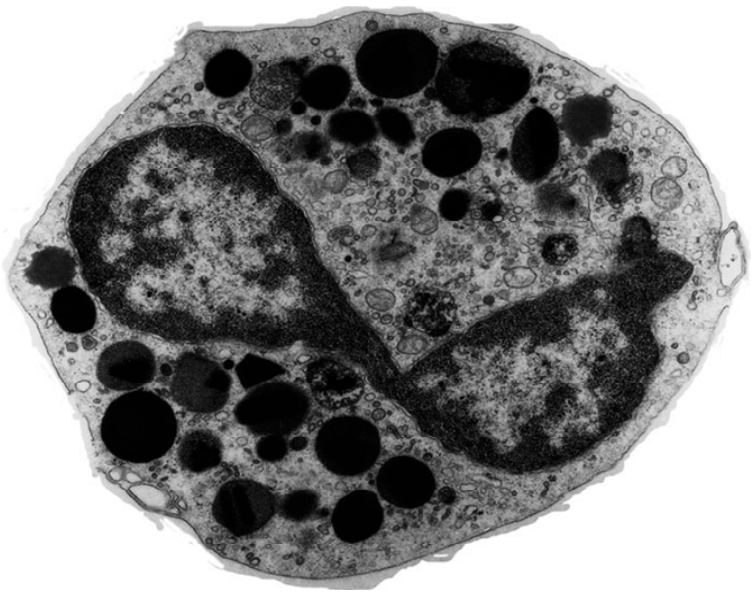

Figure 1. *Electron microscopic picture of an eosinophil granulocyte, with the characteristic bi-lobated nucleus and cytoplasmic granules. (Published with kindly permission from Malgorzata Karawajczyk).*

Eosinophils

Paul Ehrlich, winner of the Nobel Prize in 1908 for work in immunity, also discovered a new dye to visualise granulocytes (152). He described in 1878 both the mast cell and the basophil in his thesis and in 1879 he identified a bi-lobated nucleated cell that he called "*eosin*" on the basis of the cell's granular uptake of the dye (153).

The eosinophil is a multifunctional leukocyte involved in inflammatory reactions, parasite defence and in immune modulating responses (154). A hallmark of allergic disease is infiltration of the target tissue with increased numbers of eosinophils besides a variety of chronic changes due to remodelling (155). The migration of eosinophils to the site of inflammation, where they perform their end-phase effector functions, is mediated by Th_2 cytokines, chemokines and adhesion molecules.

The human eosinophils have highly condensed nuclear chromatin and two major types of granulae, specific and primary. Specific granulae have a distinct core and contain cationic proteins, the primary granulae are formed early in the development and are enriched with Charcot-Leyden Crystal protein (CLC). In addition, the eosinophils contain cytoplasmic lipid bodies, synthesising eicosanoids. The major cationic proteins in the specific granule

are major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil protein X [(EPX)/eosinophil derived neurotoxin (EDN)] all of which are extremely toxic to tissues (156, 157).

The eosinophils express an array of cell-surface proteins, including Ig-receptor for IgG, IgA, complement receptors, leukotriene receptors, prostaglandin receptors, PAF receptor and TLRs as well as several inhibitory receptors. In addition, the eosinohpils express receptors for cytokines (IL-3, IL-5, GM-CSF and IL-1α, IL-4, INF-α, TNF- α), chemokines (CCR1 and CCR3) and for adhesion molecules (VLA-4, α4ß7 and siglec-8) (108). Furthermore, when activated for degranulation the eosinophils express both high-affinity IgE-receptors (FcεRI), low-affinity IgE-receptors (FcεRII/CD23), high-affinity IgG-receptors and complement receptors (154, 158). FcεRII/CD23 facilitates antigen presentation in the highly activated eosinophil (154). They also express MHC class II molecules and co-stimulatory factors, e.g. they can act as professional APCs (159). Development and differentiation of eosinophils is promoted by IL-3, IL-5 and GM-CSF, but only IL-5 is considered specific for eosinophils. Eosinophils are released from the bone marrow to the circulation after stimulation with IL-5, produced at the site of allergic inflammation, and migrating to the inflammatory tissue (160). The half-life of eosinophils in the circulation is 8-18 hours, but in the tissues they can survive for up to several weeks (161).

Activation

The activation of eosinophils is strictly regulated; an inappropriate activation would be harmful to the individual and in healthy conditions the eosinophils are inactive with a high threshold for release of their granule proteins (162,163). There is no consensus on the major signalling mechanism for eosinophil activation, but they are known to be activated by cross-linking of IgG and IgA Fc-receptors and a number of mediators (IL-3, IL-5 and GM-CSF, CC chemokines and PAF), the role of FcεRI is however unclear (108).

The granule proteins can be released by exocytosis, compound exocytosis, piecemeal exocytosis by transport vesicles or cytolysis (164, 165). *In vitro* studies have demonstrated selective release of the individual granule proteins (166, 167) and interestingly, different eosinophilic diseases are characterised by a marked heterogeneity in degranulation levels (168). Previous studies have implicated that the priming degree of the blood eosinophils is related to the degranulation status of the tissue-residing eosinophils and corresponds to the activity of the eosinophilic disease (169). Hence, the eosinophil can be activated in different ways and by different antigens and furthermore there are four ways of eosinophil degranulation reported, the outcome of activation can thus be variable.

Effector functions and released mediators

The eosinophil stores a vast array of mediators, cytokines and chemokines with different target activities. Depending on the triggering stimuli a differential release of proinflammatory mediators takes place in the target tissue (170). At the site of inflammation the primed eosinophil rapidly secretes its preformed granule proteins (MBP, ECP, EPO, EPX/EDN) in addition to different cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, Il-16 and IL-18), chemokines (eotaxin-1 and RANTES), growth factors (TGF-β) and newly synthesised eicosanoids [platelet-activating factor (PAF) and leukotriene C4 (LTC4)] (161). These molecules have pro-inflammatory effects in up-regulation of adhesion systems, modulation of cellular trafficking and in inducing tissue damage; above all ECP is involved (160, 161). MBP accounts for more than half of the granule protein mass, is highly cationic but lacks enzymatic activity and is believed to act through enhanced membrane permeability (161). It has in vitro activity against parasites but its role in allergy is unclear (108). EPO, also highly cationic and localised to the matrix of the specific granule, makes up approximately 25% of the granule proteins (161) and is considered to be the most specific of the eosinophil granule proteins (171) and also the most potent, on a molar basis, to kill helminths (172).

In many papers it has been observed that EPO is more difficult to mobilize than ECP (168,173,174) and it has been postulated that the very low release in degranulation assays depends on the sticky nature of the protein, due to a very high cationic charge and therefore EPO is adhering to the laboratory tubes (175, 176). This difference in degranulation could also be explained by selective granule release in response to different stimuli for degranulation (168).

ECP and EPX/EDN have RNAse activity and are localized to the matrix of the specific granule. Although both have *in vitro* toxicity against pathogens, the RNAse activity of EDN is much more potent than that of ECP (156, 161). Genes coding for ECP and EPX/EDN show extremely high mutation rates, suggesting extraordinarily selective pressure due to rapid evolution of pathogens (108). The gene family expressing ECP has one of the highest rates of mutations in the primate genome, suggesting specialised biological activities for the subgroups (177, 178) and involvement in adverse reactions eliciting allergic diseases (180). ECP in particular has gained interest in the pathogenesis of allergic diseases, and being a ribonuclease it facilitates entry of other cytotoxic molecules as well (181, 182). Eosinophils are highly cytotoxic to human airway epithelial cells and are considered to have a major impact on airway remodelling by promoting fibrotic processes and increased vascularity, as well as modulating mesenchymal functions directly (61, 183, 184). It has been shown that ECP stimulates migration of human

lung fibroblasts, stimulates TGF-ß1 release from the fibrobalsts and also interact with mesenchymal cells *in vitro* (31, 32, 33).

Taken together, there is much evidence suggesting that eosinophils have an important effector role in chronic allergic inflammation.

Neutrophils

Neutrophils are the most abundant leukocytes in circulation and play a fundamental role in the innate immune response against pathogens. They are normally not present in healthy tissue, but are rapidly recruited to the site of inflammation. Neutrophils are phagocytic cells and a major source of pro-inflammatory cytokines, contributing to the onset and early orchestration of the inflammatory response in many diseases. Recently it has been unravelled that neutrophils also are important in shaping immune responses and are able to suppress T-cell activation (185). Furthermore, the neutrophils do not only destroy tissue, they are also involved in tissue repair (185).

Neutrophil granulae are divided into primary (azurophilic), secondary (specific) and tertiary granulae (gelatinase) (186,187,188), though a modern view is to regard granulae as a continuum over time as the neutrophil precursors mature in the bone marrow (187, 188). The primary source of the highly cytotoxic MPO is from the primary neutrophil granule (186, 189) and after activation it will be secreted together with other inflammatory mediators (169). Human neutrophil lipocalin, HNL, a recently discovered unique neutrophil mediator, is stored in the secondary granule and measurement of this protein seems to be of particular clinical relevance for studying neutrophil involvement (190, 191).

The eosinophil's role in inflammation is well established in the pathogenesis of asthma, but the role of the neutrophils is less understood in allergic airway inflammation except in more severe forms of chronic asthma and COPD (192, 193, 194, 195). It has recently been reported that neutrophils are the first cells recruited to the site of the allergic reaction where they also may take part in the resolution process of the allergic response (196). This is in line with studies of remodelling in mild atopic asthma after allergen challenge (94) and the finding of neutrophils in induced sputum of non-atopic asthmatic children (197). Additionally, the recent advances in studies of anti-IL-5 therapy also indicate an involvement of other inflammatory cells than just the eosinophils (198).

Altogether, this implies that there might not be a clear-cut difference between mild and severe asthma with regard to the neutrophil involvement, and thus eosinophilic and neutrophilic asthma might not be mutually exclusive subtypes of asthma.

Release of granule proteins and signalling through PI3K

Effector functions of the eosinophils are based on secretory pathways for release of pre-stored pools of cationic granule proteins, cytokines and chemokines. The eosinophil is known to have four different forms of degranulation, although piecemeal degranulation have been demonstrated to be the pathway of choice for selective granule release in allergic responses and also for IL-4 release (164, 165, 168, 169). Recently, piecemeal degranulation has been reported to be the secretory pathway for release of MBP from eosinophils after stimulation with eotaxin in healthy donors and in patients with hypereosinophilic syndrome (HES) (199, 200).

There is no consensus as to the major signalling mechanism for eosinophil activation, but they are known to be activated by cross-linking of IgG and IgA Fc-receptors. It has long been known that binding of eosinophils to a surface by complement receptors induces a strong signal for degranulation, i.e. involving the receptor for complement factor 3 (C3b-receptor, CR1, CD11b/CD18) (201, 202, 203). Experimental settings adjusted to elucidate the degranulation process in more detail in eosinophils and neutrophils have been developed (67, 203, 204, 205). Using serum opsonised Sephadex particles *in vitro* enhances this C3b-induced degranulation of the eosinophils in allergic diseases as well as in infection (203).

The mechanism behind ECP release after addition of serum opsonised Sephadex-particles is that of frustrated phagocytosis, as the opsonised surface is non-phagocytable, representative for the mechanism involved in extracellular killing of parasites (202). Furthermore the cationic proteins are only cytotoxic at high local concentrations, i.e. in close contact with the parasite, for which reason granule must be released in a closed compartment onto the surface of the parasite (202). This implies that soluble secretagogues, such as cytokines, are of less importance for degranulation in eosinophils. The main signal for degranulation comes from cross-linked Fc-receptors and C3-derivates, and proinflammatory cytokines (IL-3, IL-5 and GM-CSF) enhance rather than act themselves in the degranulation process. This indicates a role for complement receptors (mainly CR1) and the eosinophil IgG receptor in ECP release induced by serum opsonised Sephadex particles (202, 205).

The chemotaxis and recruitment of the eosinophils to the site of inflammation is fairly well studied, but the downstream signalling leading to the degranulation of eosinophils in allergic diseases is less understood and still much unexplored. The phosphoinositide-3-kinase family (PI3-kinases, PI3K) regulates membrane trafficking and controls many fundamental biological functions such as cell growth, survival and proliferation by downstream activation (206, 207). PI3Ks are divided into three major classes based on structural features and lipid substrate preference, of which class I is the most studied (206, 208). Little is also known about the tissue distribution of the

different PI3Ks, as there are no available antibodies for immunohistochemistry to date (208). PI3Ks generate 3-phosphorylated phosphoinositide lipids and for class I the end-product is PIP3 [Phosphatidylinositol (3,4,5)-trisphosphate, PtdIns(3,4,5)P_3]. Class I generated PIP3 acts as a second messenger and promote downstream activation through a phosphorylation cascade of pleckstrin homology domain-containing proteins, e.g. activation of primarily the Akt pathway which in turn phosphorylates numerous protein targets (206, 209, 210). Class I PI3Ks can be activated by external stimuli (211) and are thought to be involved in mast cell and basophil degranulation in allergy (212, 213), but the role in eosinophil degranulation in answer to allergic reactions is so far not elucidated.

Inhibitors have been useful for characterising the PI3Ks and Wortmannin, a fungal metabolite, has been widely used for studying the PI3K pathway in leukocytes, although implications mainly have been drawn to class I PI3Ks (214). Wortmannin is a very potent universal PI3K inhibitor with IC_{50} of ~2-10nM depending on the stimulus activating PI3K (215, 216). It has been regarded as a selective PI3K inhibitor, although recent evidence suggests that Wortmannin can compete with additional kinases and ATP, but only at much higher concentrations than are usually used in assays (211, 216). In recent years the biology and signalling through the PI3K axis and further downstream has been the subject of intense investigations and the basic framework of PI3K signalling has been unravelled, especially for class I, although less is known of the dynamic regulation and stimuli needed for their relative functional output.

Aims of the present investigations

Overall aim

The overall aim of the present thesis was to study eosinophil inflammation in allergic rhinitis and allergic asthma with regard to the systemic and local tissue inflammation as well as similarities and differences between the two allergic disorders. The investigations were performed during birch pollen season and after bronchial and nasal allergen challenge for assessing differences in long-term low-dose allergen exposure compared to single high-dose allergen exposure. The neutrophil inflammation was studied concurrently for comparison.

Specific aims

- To study differences in systemic and local inflammation in allergic rhinitis and allergic asthma during birch pollen season (Paper I).

- To examine differences in systemic and local inflammatory responses after experimental allergen challenge and seasonal allergen exposure in allergic rhinitis and allergic asthma (Paper II).

- To assess stimulated *in vitro* degranulation from systemically allergen primed eosinophils in allergic rhinitis and allergic asthma after experimental allergen challenge and seasonal allergen exposure (Paper III).

- To examine signalling through PI3-kinase for *in vitro* degranulation of systemically allergen primed eosinophils in allergic rhinitis and allergic asthma after experimental allergen challenge and seasonal allergen exposure (Paper IV).

Subjects

Subjects

Seventeen birch pollen allergic patients in total were selected for the studies in Papers I-IV, where not everyone completed all the investigations in the different studies. All the patients ere diagnosed with seasonal allergic rhinitis or allergic asthma by a lung physician and allergologist at the Allergy out-patient clinic at Uppsala University Hospital. All patients were skin prick test positive to birch pollen and none of the patients had symptoms or were on any regular treatment outside birch pollen season. Eight patients were diagnosed with allergic asthma, having a history of respiratory symptoms (wheeze and dyspnoea) during birch pollen season and denying nasal symptoms. They were thus categorised as having asthma as the predominant symptom. Nine patients were diagnosed with allergic rhinitis, having eye and nose symptoms and denying respiratory symptoms. They were categorised as having rhinitis as the predominant symptom. Topical steroids were not allowed during pollen season or outside season, and none of the patients were on any regular medication during pollen season. None of the patients had smoked for the past ten years. Out of season forced expiratory volume in one second (FEV_1) was more than 75% of predicted and FEV_1/forced vital capacity (FVC) more than 70% in all patients (Table 2).

Control group

The control group consisted of five healthy, non-atopic subjects who never had smoked and who did not have allergic symptoms outside or during the birch pollen season. They were skin prick test negative to all nine standard allergens, had no serum IgE antibodies to birch pollen, and had normal lung function with an FEV_1 >80% of predicted. The control group completed investigations only during the pollen season (Table 2).

Table 2. *Clinical data of the study population (mean, range).*

	Allergic rhinitis n= 9	Allergic asthma n=8	Controls n=5
Gender	8/1	3/8	2/3
(Male/female)	43 (24-66)	41 (19-56)	38 (27-58)
Age	2	1	0
Ex-smoker (>10 yrs)	4.0 (2.4 -4.9)	3.5 (2.6 – 4.0)	3.6 (3.0–4.0)
FEV_1 (L)	615 (415-826)	504 (347-652)	571 (348-854)
PEFR (L/min)			

Methods

Study design

The overall study design in Papers I-IV included altogether seven visits (Table 3) to our out-patient clinic: inclusion, baseline, during pollen season, bronchial challenge and day after, nasal challenge and day after. Paper I includes visits 1-3, Paper II visits 1-7 and Paper III-IV visits 1-5. When the airborne pollen counts had reached 4 000 grains/m^3, patients were instructed to start recording in the diary (see below) and two to three weeks later the season visit was conducted. Pollen grains were counted by the Palynological Laboratory, Swedish Museum of Natural History, Stockholm, Sweden. The study was performed during the birch pollen seasons in the year 2000 and 2002; the pollen season 2001 was excluded due to low pollen counts. After inclusion patients were investigated consecutively. Thus, all patients were included pre-season and studied during the forthcoming pollen season the same year. Bronchial and nasal allergen challenges were performed during a four week period in January and February the following year (Table 3). The subjects were told to avoid short-acting bronchodilators and anti-histamines for 24 hours before the visits and nasal decongestants for four hours before the visits.

Total pollen count

Pollen grains were counted by the Palynological Laboratory, Swedish Museum of Natural History, Stockholm, between 1 April and 31 May in the year 2000 and 2002 (Papers I-IV) (217). Pollen recordings were made using a Burkhard seven-day recording volumetric spore trap, placed on the roof of the Arrhenius Laboratory at Stockholm University in the centre of Stockholm. The pollen count was expressed as the mean number of pollen grains per day and per cubic meter of filtered air at two-hour intervals during the day. The pollen counts during the two seasons were comparable in terms of both pollen peak and duration of the season.

Table 3. *Study design demonstrating the individual visits and investigations performed.*

	Visit 1 Inclusion	Visit 2 Baseline	Visit 3 Pollen season	Visit 4 Bronchial challenge	Visit 5 Nasal challenge	Visit 6 Induced sputum	Visit 7 Nasal lavage
Blood sampling	√	√	√	√	√	√	√
Spirometry	√	√	√	√	√	√	√
Skin prick test	√						
Specific IgE	√						
Induced sputum		√	√			√	
Nasal lavage		√	√				√
Diary			√*			√**	

*two weeks
**one day

Diary

In Paper I, results from diary recordings during birch pollen season are reported. When pollen counts reached 4 000 grains/m^3 the subjects were told to start recording their morning and evening PEFR, symptoms and daily symptomatic medication. Symptoms were graded from 0 to 3 (none to severe) for each symptom of rhinitis, conjunctivitis and respiratory complaints (shortness of breath, chest tightness, cough) during day and night, respectively. Medication categories used were: oral antihistamines, topical treatment for nose and eyes (anti-histamines and/or chromones) and inhaled short-acting β_2-agonists during day and night in the asthmatic group. In Paper II patients were told to record symptoms, medication and PEFR for one day after the bronchial allergen challenge. PEFR was recorded every two hours until night and also the next morning. Late allergic reactions were measured as FEV_1 decrease in per cent from bronchial pre-challenge FEV_1 to the value the next day when induced sputum was sampled.

Skin prick tests

Skin prick tests (SPT) were performed with nine standard aeroallergen extracts (birch, timothy, mugwort, cat dander, dog dander, horse dander, *Dermatophagoides pteronyssinus, Cladosporium herbarum* and *Alternaria* using Soluprick SQ ALK (Hørsholm, Denmark). The results were read after 15

minutes measuring the largest diameter of the wheal and its perpendicular diameter, and the product was expressed in mm^2. Skin reactions were considered positive when larger than 9 mm^2.

Spirometry

Lung function tests were performed with a Vitalograph-Compact spirometer (Vitalograph Ltd., Buckingham, England). FEV_1, FVC, FEV_1/FVC% and PEFR were recorded. The reference values were those from the European Community for Coal and Steel (218). Spirometry was performed before and 90 sec after inhalation of physiologic saline. FEV_1 was measured before and after inhalation of hypertonic saline solution and the magnitude of the FEV_1 decrease was used as a marker of bronchial responsiveness. Morning and evening PEFR were measured during pollen season (Paper I) and for one day after bronchial challenge (Paper II), using a mini-*Wright* Peak Flow Meter (Clement Clarke International Ltd., Essex, England) and recorded in the diaries, respectively.

Nasal lavage

Lavage of the nasal mucosa was performed according to Wålinder *et al* (219) with a 20 mL syringe attached to a nose olive; the subjects standing with their heads flexed 30° forward. Each nostril was lavaged with 5 mL of 0.9% sterile saline solution at RT and flushed back and forth five times via the syringe at intervals of three seconds. The recovered fluid was weighed and the amounts obtained were comparable in all subjects. The fluid was transferred into 10 mL polypropylene centrifuge tubes, kept on ice and within 30 min centrifuged at 337 *g* (1500 rpm) for 10 min. The supernatant was immediately frozen in small aliquots at -70°C for later analyses of HNL. Differential cell counts were calculated on the remaining suspension, using a cytospin preparation (Cytospin, Shandon, Southern Instruments, Sewickley, PA, USA), stained with May-Grünewald and Giemsa and examined under light microscope (Paper I-II).

Induced sputum

Sputum samples were obtained by inhalation of hypertonic saline according to Pizzichini *et al.* (220) except that the subjects were not pre-treated with inhaled bronchodilators. An ultrasonic nebuliser (OMRON U 1, Sonesta Tamro no 28 36 06, Stockholm, Sweden) was used for the inhalations. After inhalation of physiologic saline solution for negative control the subjects inhaled (4.5%) hypertonic saline solution in five inhalation steps; 0.5, 1, 4, 8

and 16 min. After each inhalation step the subjects were instructed to "huff" and cough into the container. The mucus clods were aspirated and collected with a 2 mL syringe, then weighed and immediately transported to the laboratory. The sputum sample was kept on ice, incubated at 22°C for 15 min with equal amounts of 0.2% dithiothreitol (DTT) in phosphate buffer and sputolysin (CalbioChem, SputolysinReagent, art no 56000) before centrifugation. The supernatant was then frozen at -70°C for subsequent analysis of ECP and HNL (Paper I-II).

Nasal allergen challenge test

The experimental nasal challenge test was performed by instillation in the same nostril of 0.3 mL diluent for negative control followed by instillation of birch pollen extract (Aquagen ® SQ, ALK-Abelló, Hørsholm, Denmark) every 15 min in three steps: 1 000 SQ-U/mL, 10 000 SQ-U/mL and 100 000 SQ-U/mL. The symptom score was estimated; if pronounced local symptoms and sneezing occurred the challenge test was stopped. The response to the allergen challenge was categorized into four groups: no response or response to each of the three allergen doses used. Blood samples and nasal lavage were taken 18 hr (±1 hr) after the challenge test was completed (Paper II-IV).

Bronchial allergen challenge test

The experimental bronchial challenge test was performed using a DeVilbiss-40 nebuliser [(particle size 0.5 to 5.5 μm, output 0.175 ± 0.3 ml/min, mean ±SD) (Devillbiss Co, Somerset, PA)] (221). Bronchial challenge with birch pollen extract (Aquagen ® SQ, ALK-Abelló, Hørsholm, Denmark) was performed in three steps with the doses 1 000 SQ-U, 10 000 SQ-U and 100 000 SQ-U, starting with inhalation of a diluent for negative control. The response to the allergen provocation was calculated as the cumulative dose that caused at least 20% decrease in FEV_1 (allergen provocation dose, PD_{20}). In cases when no significant fall in FEV_1 occurred, allergen PD_{20} was arbitrarily given the value 150 000 SQ-U. Blood and sputum samples were taken after 18 hr (±1 hr) after the challenge test was completed (Paper II-IV).

Inflammatory cell counts and preparation of serum samples

Four ml of blood was collected in EDTA tubes for routine laboratory tests of eosinophil and neutrophil counts (Cell-Dyn 4000, Abbott Laboratories, Ab-

bot Park, Illinois, USA) at the accredited laboratory at the Department of Clinical Chemistry, Uppsala University Hospital. Differential cell counts were obtained using a cytospin preparation (Cytospin, Shandon, Southern Instruments, Sewickley, PA, USA) stained with May-Grünewald and Giemsa and examined under light microscope. For analyses of serum ECP and HNL four ml of blood was collected in SST tubes (Becton Dickinson AB), kept for 60 min in RT and then centrifuged for 10 min at 1942 *g* (3600 rpm). The serum was frozen to -70°C. Measurements were performed in duplicates of 50 µl of the supernatants. Inter- and intra assay coefficients of variation were less than 10% for all tests.

Specific IgE

Specific IgE was determined by RAST (ImmunoCAP, Pharmacia Diagnostics AB, Uppsala, Sweden) at the Department of Clinical Immunology, Uppsala University Hospital (normal <0.35 kU/L).

Isolation of blood granulocytes

Granulocytes were isolated from heparinised blood. The mononuclear leukocytes were separated by percoll gradient centrifugation (222). The granulocyte mixture obtained by this procedure had a purity of 99.8% ± 0.2% (SD) (Paper III-IV).

Measurement of eosinophil and neutrophil degranulation

The release assay for C3b-mediated degranulation by Sephadex particles, was performed according to Winquist *et al.* (223), with some minor modifications previously described (169). The final concentration of granulocytes in the assay was 1.0 x 10^9/L. The cells were pre-incubated for 10 min with assay buffer. Incubation was then performed at 37ºC for 0 and 20 min with either assay buffer for spontaneous granule release or with washed, serum-treated Sephadex G-15 particles (83.5 g/L) [GE Healthcare (formerly Amersham Biosciences) NJ, USA] for stimulated release. Hank´s solution supplemented with 0.74 mM Ca^{2+} and 0.1% human serum albumin (HSA) was used as assay buffer. All incubations were performed in duplicate. For measurement of total cell content of granule proteins; 300 mL of granulocytes (3.0 x 10^9/L) was mixed with 1.5mL of 0.5% N-acetyl-N,N,N-trimethylammonium bromide [cetyl-trimethyl- ammoniumbromide (CTAB)] in 0.15 mM NaCl and incubated for 1 hr at RT followed by centrifuga-

tion at 600 *g* for 10 min at 4ºC. A volume of 1.5 mL of supernatant was removed and stored for later measurement of granule proteins (Paper III).

Inhibitor

The PI3K pathway inhibitor Wortmannin (Calbiochem-Novabiochem Corp, La Jolla, CA, USA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Company, St. Louise, Mo, USA) and kept in the dark at -18ºC. For negative controls, granulocytes were incubated with DMSO in dilutions corresponding to the stated concentrations of the inhibitor. On the day of use, dilutions from the stock material were performed in assay buffer [Hank´s solution supplemented with 0.74 mM Ca^{2+} and 0.1% human serum albumin (HSA)] to the stated concentrations of 10^{-6} to 10^{-9}M of Wortmannin (Paper IV).

Inhibition of PI3K pathway

Granulocytes were preincubated with Wortmannin (10^{-6} to 10^{-9}M) for 10 min at 37°C, before induction of granule protein release. The cell viability after this procedure was 99.0-99.5%, determined by Tryptan blue staining. All incubations were made in duplicates (Paper IV).

Radioimmunoassay (RIA)

The released amounts of ECP and MPO from the eosinophils and neutrophils, respectively, were assayed by means of specific RIA (Pharmacia Diagnostics AB, Uppsala, Sweden) and EPO, in the supernatant, was determined using an EPO CAP-FEIA prototype (Pharmacia Diagnostics AB, Uppsala, Sweden). HNL was assayed by a double-antibody RIA (224).

Calculations of released amounts of granule proteins

The released amounts of ECP, EPO and MPO were expressed as percent of total cellular content calculated from a standard curve of serial dilutions of respective cell extracts. Results were calculated by regression analysis.

Statistical analyses

The Kruskal-Wallis, ANOVA and Mann-Whitney U test were used to evaluate statistical differences between patient groups. For paired analyses, we used Friedman's ANOVA and Wilcoxon's matched pairs test. Correlations were investigated with Spearman's test (rho). A p-value of < 0.05 was considered significant. All the calculations were performed using the statistical software package Statistica® (Statsoft Inc, Tulsa, Oklahoma, USA).

Ethical approval

The study was performed in accordance with the Declaration of Helsinki and the approval of the Ethics committee at the Medical Faculty at Uppsala University. Patients and controls were included in the study only after informed written consent was obtained.

Results

Paper I. Systemic and local allergic inflammation during pollen season

Nine birch pollen allergic rhinitics and seven with allergic asthma as well as five controls completed the investigations during birch pollen season. Patients with allergic rhinitis and allergic asthma were comparable with regard to allergic parameters. No significant differences in pre-seasonal lung function measured as FEV_1 was seen between allergic rhinitis, allergic asthma and the control group.

Clinical diary data

Patients with allergic rhinitis and allergic asthma recorded the same degree of symptoms, and medication used for rhinitis and conjunctivitis in the diary during pollen season. Both the rhinitic and asthmatic patients also reported the same rate of symptom scores for respiratory complaints, but only the asthmatics were using $ß_2$-agonists ($p=0.006$).

Lung function and bronchial responsiveness

Bronchial responsiveness at baseline, measured by inhalation of (4.5%) hypertonic saline solution was significantly higher in the asthmatic patients than in the rhinitic patients ($p=0.017$). The median decrease in FEV_1 after inhalation of hypertonic saline solution was 7.0% in the asthmatics, 0.4% in the rhinitics and 1.1% in the control group [range (-9.4 -1.6%), (-4.1 - 4.0%) and (-0.5- 8.2%), respectively].

There were no significant changes in FEV_1 during pollen season in any of the allergic groups compared to the pre-season values. However, patients with allergic asthma recorded a significantly lower morning and evening PEFR in the diary compared to the rhinitic patients ($p=0.002$ and $p=0.005$, respectively).

Eosinophil and neutrophil inflammation

At baseline no significant differences in inflammatory markers in blood, nasal lavage or induced sputum were found between allergic rhinitis, allergic asthma and the controls, except for significantly higher ECP amounts in nasal lavage in the rhinitic patients compared to the controls ($p=0.045$).

During pollen season there were significant increases in blood eosinophils and sputum-ECP in both the rhinitic and asthmatic patients, but only a sig-

nificant increase of nasal-eosinophils in the rhinitic group compared to the controls (Figure 2). During pollen season, no significant differences could be recorded in any inflammatory parameters in blood, nasal lavage or induced sputum between patients with allergic rhinitis and allergic asthma.

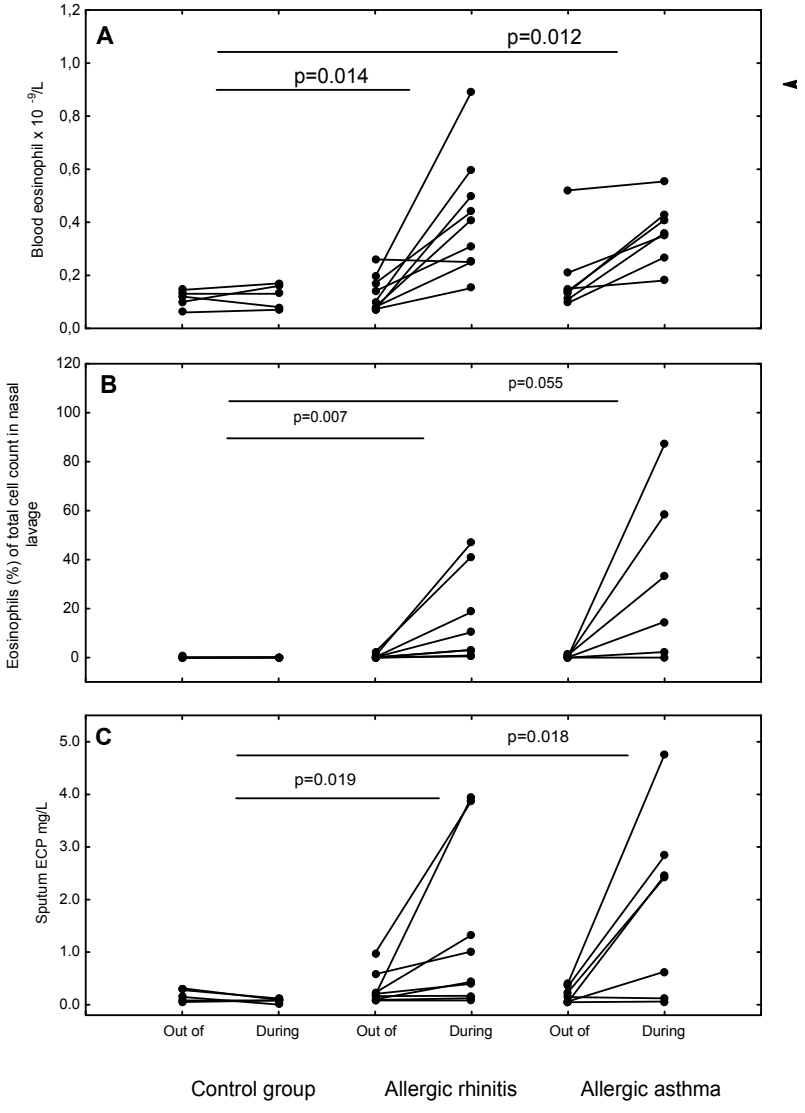

Figure 2. *Increase from baseline to pollen season in allergic rhinitis, allergic asthma and controls: A. Blood eosinophils, B. Eosinophils in nasal lavage, C. ECP in sputum.*

Paper II. Comparison of experimental and seasonal allergen exposure

Fifteen birch pollen allergic patients, eight with allergic rhinitis and seven with allergic asthma, and five controls were studied during pollen season and after nasal and bronchial allergen challenge. After bronchial challenge PEFR registration, symptoms and medication needed were recorded in a diary for one day. In this paper, we also studied the two allergic groups combined as we, in the previous paper, had observed very little differences between patients with allergic rhinitis and allergic asthma with regard to the systemic and local inflammatory response.

Clinical data

There was no significant difference in symptoms, medication needed or late allergic reactions measured as PEFR decrease in the diary between patients with allergic rhinitis and allergic asthma after bronchial allergen challenge.

Bronchial and nasal responsiveness to allergen challenge

Patients with allergic asthma were more responsive, measured by FEV_1 decline, to bronchial challenge than patients with rhinitis, and the FEV_1 decline was significantly lower in asthmatics 30 min after bronchial challenge compared to the rhinitics (p=0.018). Increased bronchial responsiveness, measured as PD_{20} for birch allergen, was also recorded in allergic asthma compared to allergic rhinitis [PD_{20} = 3 700 SQ-U (2 450-7 700) vs. 34 500 SQ-U (3 850-150 000), p=0.04] (Figure 3). After nasal challenge no differences in allergen responsiveness were found between the rhinitic and asthmatic patients (Figure 3). In the control group no reaction could be recorded after either allergen challenge test (Figure 3).

Eosinophil inflammation

The increase in blood eosinophils was significantly higher during pollen season than after bronchial and nasal allergen challenge when combining the allergic groups compared to the controls (p=0.03 and p=0.003, respectively).

After nasal challenge no significant inflammatory reactions could be found either systemically or locally in either patients with allergic rhinitis or allergic asthma, but seasonal exposure was associated with a significant increase of nasal-ECP in the allergic groups combined (p=0.04).

The eosinophil inflammatory response in the airways was low both during pollen season and after bronchial challenge. However, a significant correlation was found in the change of sputum-ECP between bronchial challenge and seasonal exposure when combining the allergic patients (rho=0.62, p=0.02).

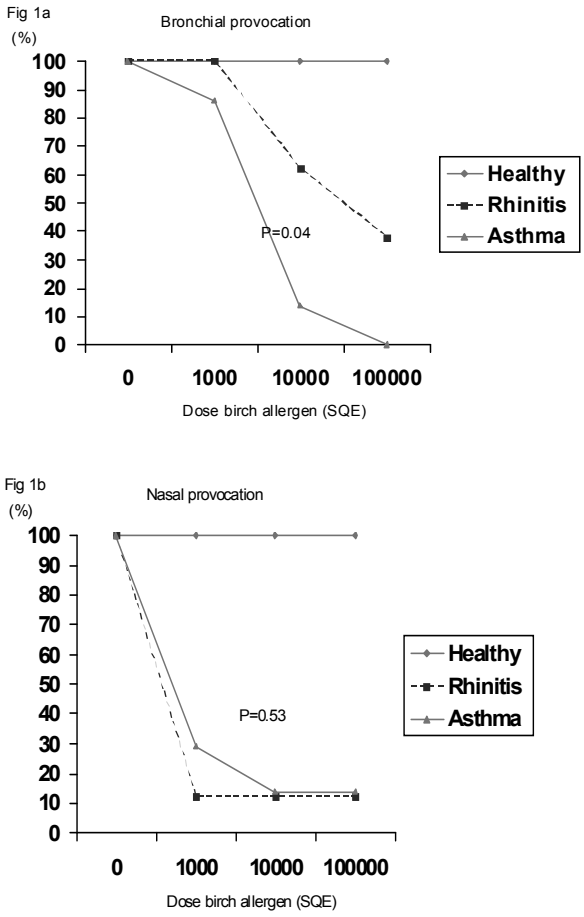

Figure 3. *Proportion of patients with allergic rhinitis and allergic asthma compared to the control group responding to bronchial (a) and nasal allergen challenge (b), respectively.*

Paper III. C3b-induced *in vitro* degranulation from primed eosinophils and neutrophils after seasonal and experimental allergen exposure

In this paper we studied the propensity of C3b-stimulated degranulation *in vitro* from blood eosinophils and neutrophils during pollen season and after bronchial and nasal allergen challenge in patients with allergic rhinitis and allergic asthma. The degranulation was studied spontaneously and after C3b-stimulation *in vitro* by opsonised Sephadex particles.

Spontaneous degranulation (0 to 20 min) of ECP, EPO and MPO

During pollen season no significant increases in degranulation of ECP, EPO or MPO were seen in allergic rhinitis, allergic asthma or the control group. After nasal challenge, ECP release increased significantly in both allergic rhinitis and allergic asthma (p=0.04 and p=0.02, respectively), but MPO release increased significantly only in allergic asthma (p=0.018). After bronchial challenge no significant increases in degranulation of ECP or EPO could be recorded in either allergic groups, but degranulation of MPO increased significantly in the asthmatic group (p=0.018).

Stimulated degranulation (0 to 20 min) of ECP, EPO and MPO

During pollen season, a significant increase of ECP and MPO release was seen in allergic rhinitis and allergic asthma (p=0.01, p=0.02 and p=0.008, p=0.018, respectively). Degranulation of EPO increased significantly only in patients with allergic rhinitis during pollen season (p=0.02). In the control group no increase in ECP, EPO or MPO release could be observed (Figure 4).

After nasal challenge, the degranulation of ECP was significant in both allergic rhinitis and allergic asthma (p=0.04 and p=0.02, respectively). Degranulation of MPO was also significant in both allergic groups after nasal challenge (p=0.043 and p=0.018, respectively), but no significant increase in EPO release could be detected in either patient group (Figure 4).

After bronchial challenge, both degranulation of ECP and MPO increased significantly in allergic rhinitis and allergic asthma (p=0.03, p=0.02 and 0.028, p=0.018, respectively). Degranulation of EPO increased significantly only in allergic rhinitis after bronchial challenge (p=0.043) (Figure 4).

Degranulation in allergic rhinitis compared to allergic asthma

No significant differences in the degree of spontaneous degranulation of ECP, EPO or MPO were seen between allergic rhinitis and allergic asthma during pollen season or after experimental allergen challenge. After *in vitro* stimulation with Sephadex particles, there was a tendency for increased ECP release in the rhinitis patients, though only significantly after bronchial challenge (p=0.010). The tendency was similar for MPO release, but this was not significant (Figure 4).

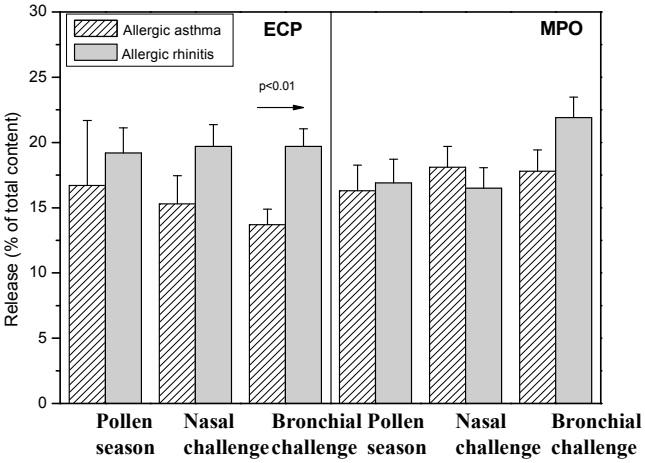

Figure 4. *C3b-induced degranulation in vitro of ECP and MPO (at 20 minutes) in patients with allergic rhinitis and allergic asthma during pollen season and after nasal and bronchial challenge.*

Paper IV. Signalling through PI3K for *in vitro* degranulation from primed eosinophils and neutrophils after seasonal and experimental allergen exposure

In this paper we studied signalling through PI3-kinase for C3b-stimulated degranulation of allergen primed blood eosinophils and neutrophils by using Wortmannin, a PI3K inhibitor, in patients with allergic rhinitis and allergic asthma during pollen season and after nasal and bronchial allergen challenge.

Inhibition of granule protein release during pollen season

Wortmannin (10^{-6} to 10^{-9}M) inhibited ECP, EPO and MPO release in a dose-dependent manner in allergic rhinitis and allergic asthma during the pollen season. However, there was a tendency towards less inhibition of ECP release in the asthmatic patients. For the suboptimal concentration of Wortmannin (10^{-8}M) this was significant compared to the control group [p=0.01, (37% inhibition in asthmatics, 70% in rhinitics and 73% in controls)] (Figure 5). Inhibition of ECP release in allergic rhinitis and the control group was in the same range during season. The same pattern with less inhibition in the asthmatic group was also seen for MPO and this was significant compared to the controls for 10^{-7}M Wortmannin (7.9% vs. 2.1%, p=0.047). EPO release

was low in all three groups in the assay buffer and inhibition with Wortmannin displayed a modest additional decrease in the release.

Inhibition of granule protein release after experimental allergen challenge

Wortmannin (10^{-6} to 10^{-9}M) inhibited ECP, EPO and MPO release in a dose-dependent manner in allergic rhinitis and allergic asthma after nasal and bronchial allergen challenge. Inhibition of granule protein release was in the same range in patients with allergic rhinitis and allergic asthma, although there was a tendency towards less inhibition in the asthmatic group. There was also a clear propensity towards less inhibition in the rhinitic patients after allergen challenge compared to seasonal exposure, especially after bronchial challenge.

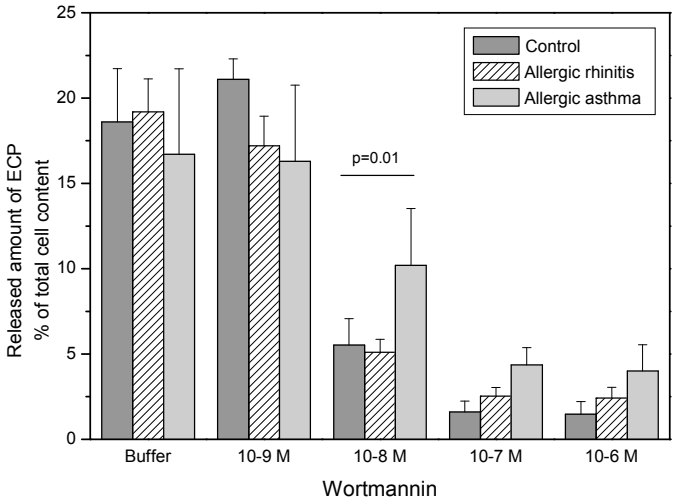

Figure 5. *PI3K inhibition with Wortmannin of C3b-induced release in vitro of ECP (after 20 min) in allergic rhinitis, allergic asthma and the control group during pollen season.*

General discussion

Classification of allergic rhinitis and allergic asthma

The primarily aim of this thesis was to study similarities and differences in eosinophil airway inflammation between patients with seasonal allergic rhinitis and patients with allergic asthma. These two conditions have traditionally been regarded as two separate diseases, but during the last decade the concept of "one airway – one disease" has been discussed (59,225). There have also been discussions about the issue of "the allergic march", e.g. whether or not rhinitis always precedes asthma and whether asthma can be prevented by early and intensive intervention for rhinitis; with allergen specific immunotherapy and environmental remediation in addition to medical treatment (15, 116, 226). Evidently, there remains a group of patients with rhinitis who do not develop asthma and patients with mild asthma who do not have nasal symptoms. In spite of difficulties categorizing patients with allergic rhinitis and allergic asthma in two separate groups, we have made a serious attempt to include only patients who have a clear predominance of symptoms of either rhinitis or asthma during the birch pollen season in the studies presented in Paper I-IV.

At baseline no significant differences in systemic and local eosinophil inflammation could be recorded between allergic rhinitis and allergic asthma, but there was a significant increase in bronchial responsiveness to inhalation of hypertonic saline in the asthmatic patients compared to the rhinitic patients (Paper I). Furthermore, patients with allergic asthma had a greater decrease in both morning and evening PEFR during pollen season (Paper I) and a greater responsiveness expressed as allergen PD_{20} for birch after bronchial challenge compared to the rhinitic patients (Paper II). Taken together, this indicates a correct classification of the two allergic patient groups in the studies.

Eosinophil inflammation

Out of pollen season no significant differences in either the systemic or local eosinophil inflammation could be recorded between patients with allergic rhinitis and allergic asthma. During pollen season the pattern between the groups was the same with comparable levels of eosinophil inflammatory

markers in blood, nasal fluids and induced sputum (Paper I). The symptom score for rhinoconjunctivitis during pollen season was within the same range in the two allergic groups, but impaired lung function was more common in the patients with asthma (Paper I). Our data is in contrast to previous reports of early impairment of lung function during pollen season in patients with allergic rhinitis, but in those studies patients with perennial allergy and established bronchial hyperresponsiveness were included (43). In Paper II a more marked systemic inflammation during pollen season compared to experimental challenge was recorded. This is in accordance with reports from other groups reporting lack of systemic inflammatory response after bronchial challenge (227, 228). However, the eosinophil inflammatory response in the nose and bronchi was within the same range after nasal and bronchial challenge as during seasonal exposure in patients with allergic rhinitis and allergic asthma.

In Paper III the hypothesis was that differences in the pattern of eosinophil degranulation accounted for the distinct clinical manifestations in rhinitis and asthma. However, the main finding was that all three allergen challenge models primed eosinophils for degranulation after *in vitro* stimulation and the release pattern was similar for ECP and EPO in allergic rhinitis and allergic asthma. The only recorded discrepancy was a significantly greater release of ECP in the rhinitic patients after bronchial allergen challenge. One interpretation could be that the eosinophils in allergic asthma are easier to activate, particularly after bronchial allergen challenge, and therefore already have released their granule proteins. This hypothesis is supported by a slightly higher amount of ECP per eosinophil cell prior to the C3b-induced granule release in the rhinitic patients and is also in accordance with results from other groups, having observed hypodense blood eosinophils after allergen exposure (164). It has been suggested that signalling through the intramembrane enzyme PI3K is involved in eosinophil degranulation (212, 213) and dysregulation of PI3K may contribute to respiratory disorders and allergies (229). Recently it has been demonstrated that the PI3K pathway is involved in eosinophil degranulation in healthy blood donors (230). In Paper IV we studied signalling through PI3K by using the inhibitor Wortmannin in order to evaluate if signalling in allergen primed eosinophils followed the same pathway in the two allergic groups. Our data clearly points toward involvement of the PI3K pathway in eosinophil degranulation in allergic rhinitis and allergic asthma irrespective of allergen challenge model. However, patients with asthma demonstrated less inhibition of ECP release through PI3K than the rhinitics during pollen season. This may indicate that pathways other than PI3K may play a larger role in eosinophil degranulation in allergic asthma than allergic rhinitis.

Neutrophil inflammation

Eosinophil inflammation is well established in the pathogenesis of asthma, but neutrophils have traditionally been connected with more severe forms of asthma and chronic obstructive pulmonary disease. In this thesis the main objective was to study eosinophil inflammation in allergic rhinitis and allergic asthma during different conditions, in order to evaluate differences between the two allergic groups. We selected the neutrophil as a comparative cell for the investigations and did not expect to discover any major influences of allergen priming on neutrophil inflammation in these patients with seasonal allergic rhinitis and mild seasonal allergic asthma. However, previous papers have reported increased degranulation of MPO after *in vitro* stimulation with GM-CSF in asthmatic patients, pointing in the direction of allergen priming of the neutrophils as well (169, 205). At baseline, an increase in neutrophil inflammatory markers was observed in nasal lavage and during pollen season a significant decrease in HNL was recorded in nasal fluids and sputum in the rhinitic patients (Paper I). In addition, in the control group there was a tendency towards an increase in systemic and nasal neutrophil inflammation during seasonal allergen exposure (Paper I). Furthermore, after nasal and bronchial challenge there was a slight tendency towards increased amounts of HNL in nasal lavage and sputum compared to pollen season in the allergic patients (Paper II). Though, we have no explanation for these somewhat surprising and opposed findings. In the study presented in Paper III it was seen that all three allergen challenge models could prime neutrophils to an increased degranulation of MPO after *in vitro* stimulation with C3b, following the same pattern as the eosinophils. In the last study, Wortmannin inhibited MPO release in a dose-dependent manner in both allergic rhinitis and allergic asthma indicating signalling through PI3 for neutrophil, as well as eosinophil, degranulation after allergen exposure (Paper IV).

HNL, which originates from the specific granule, was measured as marker of neutrophil inflammation in Paper I – II. However, in Paper III - IV MPO from the primary neutrophil granule was measured. Differences in interpretation of the data of neutrophil involvement may in part be due to the choice of marker, as there may be different physiological functions for the different granule proteins. Furthermore, in Paper I - II spontaneous neutrophil degranulation of HNL *in vivo* were measured, whereas *in vitro* stimulation of the neutrophils was performed in Paper III – IV. Taken together, the data from our studies indicate that the neutrophil is involved in the allergic inflammation.

Concluding remarks and future perspectives

According to our studies, with minor discrepancies, patients with allergic rhinitis and allergic asthma had the same degree of eosinophil inflammation both systemically and locally and yet they presented with different clinical pictures. In addition, no differences in eosinophil degranulation patterns or signalling through PI3K could be observed in the two allergic groups. Thus, the difference in bronchial response can not be explained by just the ongoing eosinophil inflammation. However, bronchial airway inflammation was measured by inflammatory markers in induced sputum, reflecting mainly the central airways. Previous reports have highlighted the issue of the contribution of inflammation in the small airways in asthma, since peripheral obstruction is not generally detectable by routine lung function studies (231). It has been proposed that a major difference in allergic rhinitis and allergic asthma may be due to the spread of inflammation to the small airways (232, 233). Furthermore, the recent advances using anti-IL-5 therapy did not show any efficacy in the late cell-mediated phase reaction or on non-specific hyperresponsiveness, despite depletion of eosinophils from the circulation and the basement membrane in the airways (234). This could imply that IL-5 not is as important as suggested for eosinophil recruitment (160) or that eosinophils not are as important as proposed in chronic asthma (235). On the other hand, Rådinger *et al.* have speculated in the possibility of eosinophil *in situ* differentiation in the lung tissue to mature cells, persisting in the tissue independent of IL-5 and perhaps influenced by other cytokines or chemokines, as eotaxin (236).

The former view of asthma as being simply a disease of hyperresponsive airways changed many years ago in favour of a model of chronic systemic inflammation, and extensive research during the recent decades has concentrated on allergic inflammation. However, recent studies on the concept of united airways have opened up new approaches for the underlying causes as to why not all patients with allergic rhinitis present with asthma. The studies in this thesis evaluated the two allergic groups simultaneously *in vivo* as well as *in vitro* under different conditions and for different parameters influencing eosinophil inflammation, as most previous studies have investigated either allergic rhinitis or allergic asthma separately. Airway remodelling *per se* may be one of the mechanisms that explain why some allergic patients develop asthma. Acute inflammatory reactions, regardless of the type of trigger stimuli, often tend to be exaggerated due to many feedback loops and amplification mechanisms in the immune system and can eventually give rise to tissue injury and aberrant tissue repair. Although airway remodelling has been considered to be important in the more severe forms of asthma for many years (237), defective epithelial function has only recently regained attention. The airway epithelium, being a barrier to the external environment, has been proposed to be fundamentally abnormal in asthmatics with their

increased susceptibility for allergen, pathogens and both indoor and outdoor pollutants (34). It has also been suggested that the airway epithelium drives the remodelling of the underlying mesenchyme by differentiation of fibroblasts to myofibroblasts (238). This hypothesis could explain way not all atopics and not all those with allergic rhinitis develop asthma and it takes into account the influence of the total external environmental load and not just allergen exposure. So perhaps we are back to square one and the old viewpoint that asthma is a disease of the airways; chronic eosinophil inflammation in addition to defective airway epithelium?

If we recognize the complexity of gene-environment connections behind allergic rhinitis and allergic asthma it is easy to comprehend the difficulties in obtaining effective treatment strategies. In addition to conventional treatment regimens, immunotherapy and anti-IgE treatment, biological agents for specific cytokines, cell-surface proteins and targets further downstream will likely be available in the future (239). However, the cost of presumptive future treatment with biological agents stresses the importance of a multidisciplinary approach worldwide for research and collaboration concerning asthma and allergy (240) (Figure 6).

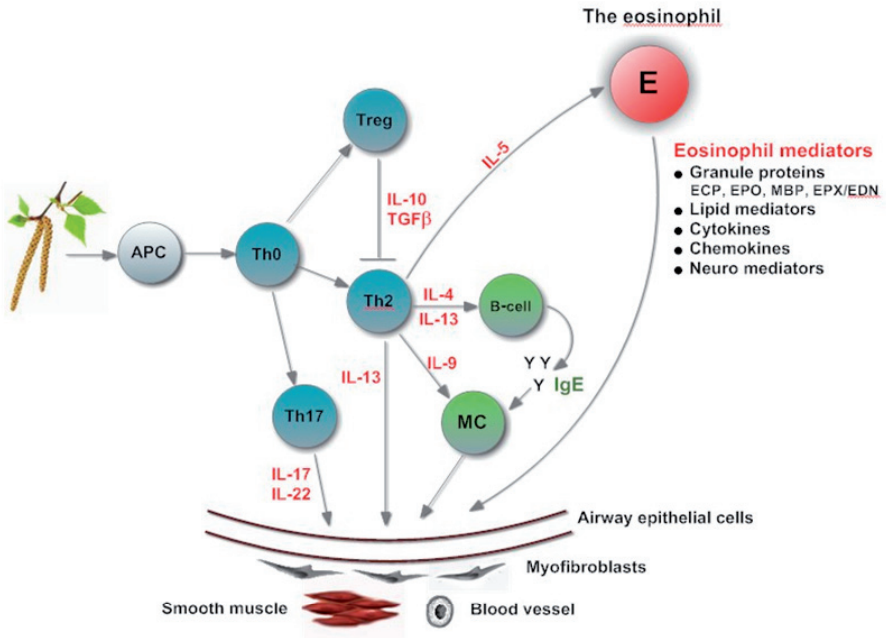

Figure 6. *The complexity and cellular cross-talk in allergic response.*

Conclusions

I. Allergic rhinitis and allergic asthma have the same degree of eosinophil inflammation systemically and locally at baseline and during pollen season. Despite this similarity, impairment in lung function during pollen season and increased bronchial responsiveness at baseline were more common in asthmatics.

II. Allergic rhinitis and allergic asthma have the same degree of airway eosinophil inflammation after bronchial allergen challenge, but allergic asthma has increased bronchial responsiveness measured as PD_{20} for birch allergen. Systemic eosinophil inflammation was more pronounced after seasonal exposure than after experimental allergen challenge in allergic rhinitis and allergic asthma.

III. Systemically allergen primed eosinophils have similar patterns of stimulated degranulation *in vitro* in allergic rhinitis and allergic asthma. The released amounts of ECP and EPO were in the same range in all allergen challenge models in allergic rhinitis and allergic asthma.

IV. Signalling through PI3K is involved in eosinophil *in vitro* degranulation of ECP and EPO in allergic rhinitis and allergic asthma irrespective of the model of allergen exposure. Patients with allergic asthma demonstrated less inhibition of ECP release via this pathway during pollen season, indicating that other pathways apart from signalling through PI3K play a greater role in eosinophil degranulation in allergic asthma than in allergic rhinitis.

Swedish summary

De allergiska sjukdomarna har kraftigt ökat de senaste decennierna i den industrialiserade delen av världen och är idag ett stort hälsoproblem med ökande socioekonomiska konsekvenser. Epidemiologiska undersökningar har visat att drygt 30 % av befolkningen i västvärlden har någon form av allergi. De allergiska sjukdomarna kan yttra sig som rhinit (hösnuva), astma, eksem och nässelutslag, men praktiskt taget alla organ kan drabbas.

Allergi är en immunologisk reaktion mot ett s.k. allergen, som är helt harmlöst och oskadligt för kroppen, men som kroppen producerar antikroppar mot. Benägenheten att producera dessa allergiantikroppar (IgE-antikroppar) är ärftlig och kallas atopi. Inte alla som är atopiker utvecklar dock allergi, utan detta beror på en kombination av olika faktorer, som t.ex. luftföroreningar, yrke, rökning men även övervikt har visat sig spela roll.

Hösnuva och astma är vanligast i barndomen och flertalet förbättras med åren, men framför allt förekomsten av astma har ökat bland vuxna på senare år . Epidemiologiska undersökningar har visat att närmare 10 % av befolkningen lider av astma i många urbaniserade geografiska områden i Sverige och andra delar av den industrialiserade delen av världen. De allergiska sjukdomarna karakteriseras som kroniskt inflammatoriska systemsjukdomar med lokalengagemang och man vet att allergisk rhinit är en riskfaktor för utvecklande av astma. Flertalet astmapatienter har vidare rhinitsymptom, men inte alla med rhinit utvecklar astma.

Den allergiska inflammationen i vävnaden kännetecknas av eosinofila celler, som är vita blodkroppar. Eosinofilerna finns i blodet, men vandrar ut i vävnaden om allergen penetrerat kroppens försvarsbarriärer. Detta är vanligt under björkpollensäsongen, då björkpollen fastnar i nässlemhinnan och i luftvägarna när vi andas. Den eosinofila cellen uppfattar björkpollenkornen som sjukdomsframkallande och bekämpar dessa genom att frisätta en rad starkt vävnadstoxiska produkter. Vid upprepad allergenexponering fås en ond cirkel med kronisk eosinofil inflammation och åtföljande vävnadsskada. Det har närmast varit vedertaget under många år att den eosinofila inflammationen i luftvägarna är en viktig orsak till astmautveckling.

Det övergripande syftet med detta avhandlingsarbete var att studera likheter och olikheter i den eosinofila inflammationen vid allergisk rhinit och allergisk astma och frågan varför inte alla rhinitiker får astma. Studierna utfördes under björkpollensäsongen och efter experimentell björkpollenprovokation i näsa respektive luftvägar. I de första två delarbetena mättes eosi-

nofila markörer i blod, nässköljvätska och luftvägar efter respektive allergenprovokationsmodell, medan däremot arbete tre och fyra var rent experimentella. I arbete tre studerades de eosinofila cellernas förmåga till frisättning av sina vävnadstoxiska produkter efter stimulering *in vitro* efter respektive allergenprovokationsmodell hos de två allergiska grupperna. I det sista arbetet studerades mekanismen för cellsignalering för frisättandet av de eosinofila markörerna.

Våra studier har visat att den eosinofila inflammationen i såväl blod, som näsa och luftvägar är av samma storleksordning hos patienter med allergisk rhinit och allergisk astma både under björkpollensäsongen och utanför pollensäsong. Trots samma grad av inflammation uppvisar endast astmatikerna en ökad känslighet i luftvägarna med försämring av lungfunktionen under pollensäsongen. Vid bronkialprovokation med björkpollenallergen ser man vidare en signifikant ökad känslighet i luftvägarna hos astmatikerna, men även här ses samma grad av eosinofil inflammation i luftvägarna hos de två patientgrupperna. Däremot är den systemiska inflammationen mer uttalad under pollensäsongen än efter både bronk- och nasalprovokation hos både rhiniker och astmatiker. I det tredje arbetet kunde vi visa att de eosinofila cellerna var likvärdigt aktiverade av björkpollenallergen för frisättning av sina inflammatoriska markörer vid *in vitro* stimulering. Detta gällde både under pollensäsongen och efter de experimentella provokationerna hos både rhinitiker och astmatiker. Vi har även visat att signalering över cellmembranet via enzymet PI3K för frisättning av eosinofila markörer ej skiljer sig åt mellan de två allergiska grupperna. Här kunde man dock se en tendens till högre frisättning hos astmatikerna vid blockering av enzymet, vilket skulle kunna tala för involvering av andra signaltransduktionsvägar hos astmatikerna.

Sammanfattningsvis kan sägas att allergisk rhinit och allergisk astma uppvisar samma grad av eosinofil inflammation i såväl blod som näsa och luftvägar. De eosinofila cellerna är vidare likvärdigt aktiverade för frisättning och följer samma signalleringsväg över cellmembranet. Våra data talar för att den eosinofila inflammationen i sig inte är tillräcklig för att patienter med allergisk rhinit ska utveckla allergisk astma, utan att det finns ytterligare bakomliggande faktorer av vikt för sjukdomsutvecklingen. Sannolikt föreligger ett komplext samspel av genetiska faktorer och miljöpåverkan bakom utvecklandet av de olika symptombilderna vid de allergiska sjukdomarna.

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