From the Department of Medicine,
Division of Clinical Immunology and Allergy,
Karolinska Institutet, Stockholm, Sweden

Characterisation of eosinophil activity markers – relation to allergic inflammation and apoptosis

Anna Nopp

Stockholm 2002

ABSTRACT

An allergic inflammation is characterised by many different factors, among which eosinophils and the release of their granule content e.g. eosinophilic cationic protein (ECP) constitute main features. Activated eosinophils play an important role in both the early and late stages of the allergic inflammation and participate in the remodelling process in tissue, thus contributing to scarring and fibrosis development. Moreover, there are data showing that eosinophils in an activated state have prolonged survival, which could in part be explained by a decrease in apoptosis. This thesis focuses on identification of eosinophil activity markers of importance for the clinical evaluation of allergic inflammation, as well as the relation between activation and prolonged survival of the eosinophil granulocyte.

We have shown that when analysing activated cells, primarily eosinophils, the choice of anticoagulant is of importance for the maintenance of cell survival and function, and that citrate as anticoagulant is preferable to EDTA. These studies were designed to allow detection of eosinophil activity markers, both *in vitro* (intracellular expression of the EG2-epitope on ECP (EG2), ECP release as well as CD69 expression) and *in vivo* (eosinophil number, ECP, EG2 and CD69). When examining the kinetic relation between EG2 expression, ECP release, CD69 upregulation and apoptosis, we found that EG2 expression and ECP release precede CD69 upregulation and apoptosis, but we were not able to establish any relation between CD69 upregulation and level apoptosis susceptibility. However, one must take in account that the presence of the natural ligand for CD69, which was still unknown at the time of the investigation, may play a role in induction of apoptosis. To further examine the apoptosis process in eosinophils, we analysed eosinophils from allergic and non-allergic individuals, and we found an increased survival in cells from allergic individuals as compared to non-allergic individuals. In addition, we examined the possible role of the mitochondrial membrane potential, release of cytochrome c and caspase 3 activation. When we used the apoptosis inducer tributyltin (TBT), an extremely fast induction of apoptosis was seen (measured by caspase 3 activity and Annexin binding) without the prior mitochondrial changes generally associated with apoptosis (dissipation of the mitochondrial membrane potential and release of cytochrome c).

In conclusion, we have identified different markers, which can be used for detection of activated eosinophils in relation to the allergic inflammation. We have also described the kinetic relation between markers for activation, degranulation and apoptosis in eosinophils. Our hope is that these results may eventually contribute to the identification of a drug that can selectively induce apoptosis in eosinophils without causing activation and degranulation, thus decreasing the pathophysiological effect of eosinophils both in the acute inflammation, as well as in the process of tissue remodelling after injury.

LIST OF PUBLICATIONS

This thesis is based on following papers, which are referred to by their Roman numerals:

[I]   Halldén G, Nopp A, Ihre E, Peterson C, Lundahl J.

Conditions in blood sampling procedures extend the *ex vivo* stability of eosinophil activity markers in peripheral blood from allergic patients and healthy controls.

Annals of Allergy, Asthma, & Immunology 1999;83(5):413-421.

[II]   Nopp A, Lundahl J, Halldén G.

Quantitative, rather than qualitative, differences in CD69 upregulation in human blood eosinophils upon activation with selected stimuli.

European Journal of Allergy and Clinical Immunology 2000;55:148-156.

[III]   Nopp A, Halldén G, Lundahl J, Johansson E, Vrtala S, Valenta R, Grönneberg R, van Hage-Hamsten M.

Comparison of inflammatory responses to genetically engineered hypoallergenic derivatives of the major birch pollen allergen Bet v 1 and to rBet v 1 wild type in skin chamber fluids collected from birch pollen allergic patients.

Journal of Allergy and Clinical Immunology 2000;106:101-109.

[IV]   Nopp A, Lundahl J, Stridh H.

Caspase activation in the absence of mitochondrial changes in granulocyte apoptosis.

Clinical and Experimental Immunology, In Press.

[V]   Nopp A, Stridh H, Grönneberg R, Lundahl J.

Differences in neutrophil and eosinophil survival between allergic and non-allergic individuals.

Submitted.

CONTENTS

LIST OF ABBREVIATIONS

APAF	apoptotic protease-activating factor
AN	Annexin V
ANT	adenine nucleotide translocator
BA	bongkrekic acid
BAL	bronchoalveolar lavage
C5a	complement factor a
Caspase	cystein dependent aspartate specific protease
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
CD	cluster of differentiation
ECP	eosinophil cationic protein
EDTA	ethylene diaminetetraacetic acid
EG2	monoclonal antibody to ECP
ELISA	enzyme linked immunosorbent assay
EPO	eosinophil peroxidase
EPX/EDN	eosinophil protein X / eosinophil derived neurotoxin
FITC	fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FOG	fixation and permeabilisation method
GM-CSF	granulocyte macrophage-colony stimulating factor
IL	interleukin
IFN-γ	interferon-gamma
IU	international units
LPR	late-phase reaction
LPS	lipopolysaccharide
MBP	major basic protein
MFI	mean fluorescence intensity
MPT	mitochondrial transition pore
OG	n-octyl-β-D-glucopyranoside
PAF	platelet activating factor
PBE	peripheral blood eosinophils
PBN	peripheral blood neutrophils
PBS	phosphate-buffered saline
PE	phycoerythrin
PI	propidium iodide
PFA	paraformaldehyde
PMA	12-phorbol myristate 13-acetate
PS	phosphatidylserine
RAST	radioallergosorbent test
RIA	radioimmunoassay
SPT	skin prick test
TBT	tributyltin
TMRE	tetramethylrhodamine
w/v	weight/volume
VLA-4	very late antigen 4 (CD29/CD49d)

1 INTRODUCTION

Allergic disease is a health problem that is increasing in the industrialised world. As early as 1873, Blackely described the increasing prevalence of hay fever. A lot of different theories have been proposed to explain this phenomenon among them genetic factors, the use of broad-spectrum antibiotics, pollution, changes in the indoor environment, diet and changed intestinal flora to name a few *[Bonini 1994]*. Inflammation is an important part in allergy, and one main player in the inflammatory response is the eosinophil. This thesis focuses on identification of eosinophil activity markers of importance for the clinical evaluation of allergic inflammation, as well as the relation between activation and prolonged survival of the eosinophil granulocyte.

1.1 GENERAL ASPECTS OF INFLAMMATION

Inflammation is a generalised (non-specific) response to infection, wounding, or trauma. The existence of an adaptive immune response was already known when the Smith Papyrus was written in 1600 BC, but the theory presented there is believed to be derived from a source written between 3000 and 2500 BC. The general characteristics of inflammation have been known since Celcus catalogued the major features of an inflammatory reaction. These are carried down even today as the four cardinal signs of inflammation: rubor (redness), tumor (swelling), calor (heat), and dolor (pain). These reactions can be followed by impaired function (functio laesa) of the affected tissue. The events of inflammation can be divided in two phases: a vascular phase, and a cellular phase. During the vascular phase, local blood flow and vascular permeability are increased. These contribute to the redness, heat, and swelling associated with inflammation. During the cellular phase, chemotactic factors released by responding cells in the damaged tissue attract appropriate effector cells. These cells gain entry to the extravascular space, through rolling, adhesion and migration. Among the migrating cells eosinophils, neutrophils, and lymphocytes can be found. Lymphocytes can be divided into $CD4^+$ (helper) and $CD8^+$ (cytotoxic) cells, and the $CD4^+$ sub-population can further be divided into T helper 1 (Th1) and T helper 2 (Th2) cells.

1.2 THE ALLERGIC INFLAMMATION

Allergy is a hypersensitivity reaction initiated by immunological mechanisms, and can be divided in IgE-mediated reactions and non IgE-mediated reaction. IgE-mediated reactions can be further subdivided into atopic or non-atopic reactions. The definition of atopy is a personal or a familial tendency to produce IgE antibodies in response to low doses of allergens, and to

develop typical symptoms such as asthma, rhinoconjunctivitis, or eczemal dermatitis *[Johansson 2001]*. Antigens normally enter the body at very low doses by diffusion across mucosal surfaces, and many antigens are proteins that possess enzymatic activity that facilitates the diffusion. Naive allergen-specific T cells are induced to develop into Th2 cells in the presence of IL-4, which is released from activated mast cells and basophils. The allergen-specific Th2 cells produce more IL-4, which in turn drives allergen-specific B cells to produce IgE *[Chung 1999]*. The antigen binds to the high-affinity receptor for IgE (FcεR1) on the surface of mast cells and basophils. Mast cells are strategically distributed beneath the mucosal surface and in connective tissue. When an antigen binds to FcεR1, the cell becomes activated and starts the allergic reaction. The resulting inflammation can be divided into early (vascular) and late events (cellular). The early phase follows from the activity of histamine, prostaglandins and other rapidly synthesised molecules, which cause a rapid increase in vascular permeability and the contraction of smooth muscles. The late phase of the inflammatory response starts after approximately 8-12 hours and is caused by induced synthesis and release of mediators including leukotrienes, chemokines, and cytokines from the activated mast cells and basophils, and recruitment of inflammatory cells: eosinophils, neutrophils and lymphocytes *[Lichtenstein 1991]*. Although this reaction is clinically less dramatic than the immediate response, it can evolve into chronic inflammation, like that seen in allergic asthma.

1.3   CYTOKINES AND CHEMOKINES IN ALLERGY

Cytokines are usually extracellular signalling proteins, often less than 80 kDa in size, and many of them are glycosylated. They are produced in various types of cells, and act through specific receptors on the surface of target cells. Cytokines evoke a wide range of cellular responses including activation, proliferation, chemotaxis, immunomodulation, release of other cytokines or mediators, growth and cell differentiation, and apoptosis. Cytokines themselves are able to induce the expression of receptors that may change the responsiveness of both source and target cells. Some cytokines may also stimulate their own production in an autocrine manner, whereas others stimulate the synthesis of different cytokines, which exert a feedback stimulatory or inhibitory effect on the initial cytokine. Cytokines play an integral role in the co-ordination and persistence of the allergic inflammation, since they are capable of inducing several of the pro-inflammatory effects characteristic of this condition. Chemokines are small cytokines that are involved in the migration and activation of cells, and they play a central part in the inflammatory response. Several cytokines/chemokines are involved in the allergic inflammation. Some of the most important are interleukin-3 (IL-3), IL-4, IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF), and eotaxin *[Chung 1999]*.
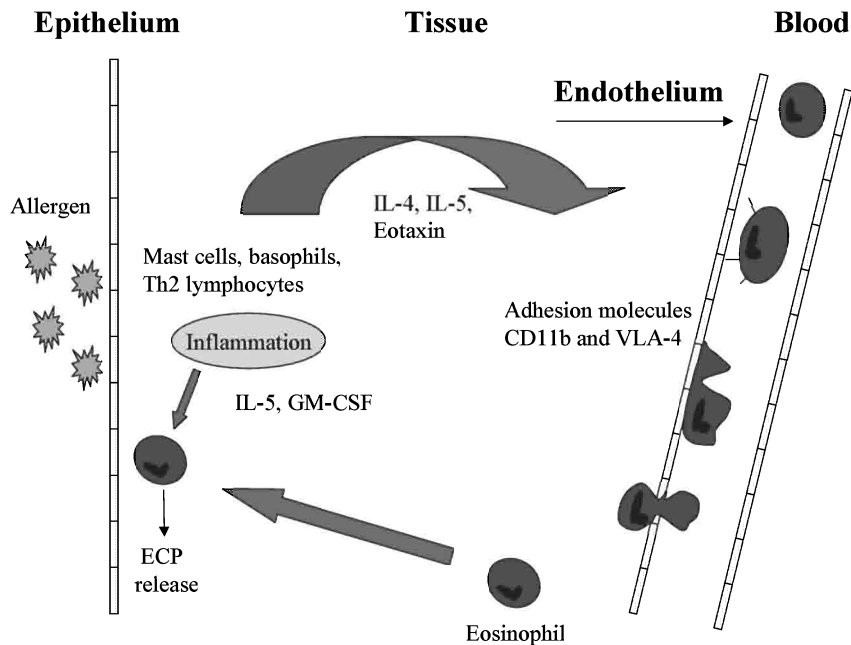
Figure 1. A simplified illustration of the recruitment of eosinophils from the blood stream to the inflammatory focus in an allergic inflammation.

1.4 THE EOSINOPHIL GRANULOCYTE

The German scientist Paul Ehrlich discovered the eosinophil granulocyte in 1879. He found that this cell had high affinity for the acidic dye eosin, and therefore called the cell "eosinophil". During the next 40 years much was learned about the eosinophil and its role in disease. It is now recognised that the eosinophil granulocyte plays an important role in the defence against helminth infections as well as in the initiation and maintenance of the allergic inflammation.

Eosinophils are non-dividing, bone marrow-derived, granule-containing cells. They are approximately 8 μm in diameter. Eosinophils, like other leukocytes, differentiate from stem cell precursors in the bone marrow. Three T-cell derived cytokines have been shown to promote eosinophil growth, differentiation and activation: IL-3, IL-5 and GM-CSF *[Wardlaw 1994]*. Eosinophils migrate into the peripheral blood where they circulate with a half-life about 18 hours before migrating into the tissue. Histological studies have shown that eosinophils are often observed in the bronchial submucosa of patients who have died of asthma attacks *[Bousquet 1990]*. It has also been shown that in asthma patients, eosinophils are present in

increased numbers in peripheral blood *[Griffin 1991, Janson 1992]*, sputum *[Hargreave 1993]* and bronchoalveolar lavage (BAL) fluid *[Kirby 1987, Wardlaw 1988]*.

Besides IL-5 and GM-CSF, other more general factors like N-formyl-methionyl-leucyl-phenylalanine (fMLP), platelet activating factor (PAF) and complement factor 5a (C5a) may activate eosinophils *[Giembcz 1999]*.

1.5 CELL SURFACE AND INTRACELLULAR MARKERS IN EOSINOPHILS

1.5.1 ECP and the EG2-epitope

The eosinophil granulocyte contains a large number of biologically active proteins. Four of the most investigated molecules are major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil protein X / eosinophil derived neurotoxin (EPX/EDN). They are localised inside secondary (specific) granules in the eosinophils. ECP is a single chain protein with a molecular weight ranging from 16 to 22 kDa. ECP has an isoelectric point of pH 10.8 *[Venge 1999]*. The basic property makes ECP a cytotoxic molecule, with the capacity to kill mammalian as well as non-mammalian cells, such as parasites and bacteria and also viruses *[McLaren 1981, Domachowske 1998, Lehrer 1989]*. The cytotoxicity is probably due to the ability of ECP to make pores in cell membranes. These pores allow the passage of water and small molecules and kill the affected cell by osmotic lysis *[Young 1986, Venge 1998]*. Activated eosinophils are known to release ECP, and high levels have been found in BAL fluid from patients with allergic asthma *[Venge 1993, Ädelroth 1990]*. The degranulation process is regulated by different interactions including receptor-ligand interaction with immunoglobulin or complement coated ligands, immune complexes and exposure to soluble factors. The immunoglobulin receptors involved in degranulation are the receptors for IgG, IgE and IgA. Depending on what receptor that is engaged, different granule content can be released. For example IgG engagement induces ECP release and IgA engagement promotes EPO release *[Wardlaw 1994]*.

One intracellular epitope of ECP is EG2. The monoclonal antibody EG2 has in both histochemical and flow cytometric studies been shown to bind to eosinophils *[Tai 1984, Jahnsen 1994]*. An increased expression of the EG2-epitope has been detected in eosinophils activated *in vitro* and in peripheral blood eosinophils (PBE) from allergic and asthmatic patients when analysed by flow cytometry *[Hed 1993, Skedinger 1998, Kuwasaki 1998]*.

1.5.2 CD69

The antigen CD69, also known as AIM, Leu-23 or MLR3, is a 60 kDa glycoprotein composed of two sub-units, 27 and 33 kDa respectively *[Hara 1988]*. The natural ligand of CD69 remains

unknown. The CD69 molecule is a type II integral protein, with a C-type lectin-binding domain, and is localised to the golgi apparatus *[Bajorath 1994]*. Cross-linking of CD69 with specific monoclonal antibodies can induce eosinophil apoptosis *[Walsh 1996]*, suggesting a role for this surface antigen in regulation of eosinophilic inflammation. PBE from healthy subjects do not express any significant amounts of CD69 on the cell-surface *[Hartnell 1993]*. However, most of the eosinophils recovered from BAL in patients with eosinophilic pneumonia *[Nishikawa 1992]* or with asthma *[Hartnell 1993]*, express this antigen on the cell surface.

1.5.3 CD9 and CD11b

The surface antigen CD9 is expressed on both eosinophils and platelets. It has been proposed that CD9 serves as a signalling molecule, since crosslinking of CD9 mediates intracellular signals in eosinophils *[Kim 1997]*. CD9 can also be involved in adhesion of eosinophils to endothelial cells. It has also been proposed that CD9 can be regarded as an activity marker on eosinophils, since an increased expression has been observed in allergic patients *[Fernvik 1996]*.

The cell surface receptor CD11b, a member of the $\beta2$-integrin family, is a well-characterised adhesion molecule. It is expressed on all granulocytes and is, like CD9, considered as an eosinophil activity marker *[Arm 1989, Beredes 1994, Kroegel 1994, Lundahl 1998]*. CD11b is the α-chain that functions together with its β-chain CD18 in the receptor complex CD11b/CD18. In their resting state, eosinophils only express a minor part of the CD11b/CD18 content on their surface. However, upon *in vitro* activation, CD11b is rapidly mobilised from an intracellular pool to the cell surface *[Lundahl 1993]*. The expression of CD11b has been shown to increase on circulating neutrophils and eosinophils in individuals with allergen-induced asthma *[Arm 1989, Fernvik 1996]*.
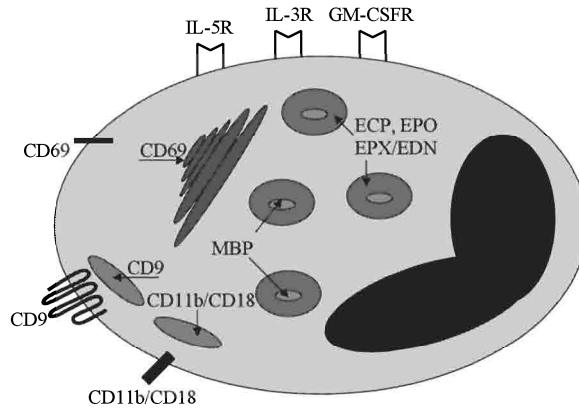
Figure 2. An illustration of selected eosinophil structures. The intracellular proteins: ECP, EPO, EPX/EDN, MBP are localised to secondary (specific) granules. The interleukin receptors (R): IL-5 R, IL-3 R, GM-CSF R. The surface receptors: CD69, an intracellular pool is localised in the golgi apparatus, CD9 and CD11b.

1.6   THE HISTORY AND DEFINITIONS OF CELL DEATH

The development of new tissue staining techniques over 100 years ago led to advances in pathology. It was now possible to study cellular pathogenesis, and cell death was one of the first phenomena described. Most studies concerned analysis of tissue after extreme and sudden injuries e.g. hyperthermia, ischaemia and direct cell trauma *[Cohen 1999]*. The term necrosis, derived from the Greek meaning "making dead" was applied. Early morphological signs of necrosis include swelling of the cytoplasm and organelles e.g. the mitochondria, followed by loss of membrane integrity and cell lysis. The destruction of cells by necrosis occurs very rapidly and the release of the intracellular contents may potentially evoke an inflammatory response leading to tissue damage and scar formation *[Wyllie 1980]*.

In 1972 Kerr, Wyllie and Currie described a type of cell death with different morphology *[Kerr 1972]*. It was seen in cells for which death was normal and predictable, and in cells dying of relatively minor injury compared with that which led to necrosis. Because this death seemed more physiological than necrosis they named it apoptosis, from the Greek meaning "falling off". Physiological cell death, or apoptosis, is an evolutionarily conserved form of cell death, prevalent in all multicellular organisms. A cells decision to undergo apoptosis can be influenced by a variety of regulatory stimuli. When misregulated, apoptosis can contribute to various

diseases including cancer and autoimmune and neurodegenerative diseases. Normally, though, the cellular corpses produced through apoptosis are recognized and their removal by phagocytosis occurs without disturbances of tissue architecture or function and without initiating inflammation. Apoptosis is regulable and is of fundamental importance to tissue development and cell homeostasis. Cellular susceptibility to apoptosis is determined by a variety of signals, of both extracellular and internal origin *[Ameisen 1994, Watanabe-Fukunaga 1992, Fisher 1995]*. Apoptosis is characterised by a series of morphological alterations including nuclear condensation, cell shrinkage, DNA fragmentation, as well as biochemical alterations including activation of proteases (caspases) and endonucleases. Apoptotic cells are rapidly removed from the tissue, thus decreasing the potential risk for tissue injury and scarring *[Cohen 1999]*. Apotosis can be induced in different ways, including engagement of death receptors (e.g. CD95) *[Gupta 2000]* or by various stimuli that activate apoptosis through the mitochondria *[Papa 1997, Petit 1997]* **Figure 3**.

1.7 CASPASES AND APOPTOSIS

One common pathway, by which many apoptotic mechanisms may function in a coordinated manner, relies on the activation of the caspase family of proteases. This group of cysteine protease enzymes is structurally related, sharing a specific ability to cleave peptides bound after aspartic acid residues (Caspase = <u>c</u>ystein-dependent <u>a</u>spartate specific prote<u>ase</u>). Caspases appear to be essential components in the apoptotic machinery. They operate by cleaving cytoskeletal and nuclear proteins critical for maintenance of cell structure, and enzymes involved in metabolism and repair *[Goreman 1998]*. At present, 14 mammalian caspases have been identified *[Deveraux 1999]*. The caspases are synthesised as pro-enzymes (zymogens) and, upon appropriate apoptotic triggering, they are converted into mature enzymes by the formation of heterodimers that contain the catalytic unit. The enzymes of the caspase family consist of three structural/functional domains, the amino-terminal, plus one large and one small subunit. During caspase activation, the small and large subunits associate, forming a heterodimer with both domains contributing important residues for substrate binding and catalytic activities *[Nicholson 1997, Gupta 2000]*. Because specific caspases are capable of activating others, it has been proposed that they operate in a cascade, with certain members responsible for upstream functions, e.g. cleavage of other caspases (initiator caspases; caspase 6, 8 and 9). Other caspases (effector caspases; caspase 2, 3, and 7) perform downstream functions, e.g. cleavage of key proteins, consequently leading to an apoptosis morphology **Figure 3**.

7

1.8 THE ROLE OF THE MITOCHONDRIA IN APOPTOSIS

The mitochondria play a central role in the initiation of apoptosis by dissipation of the mitochondrial membrane potential ($\Delta\psi_m$) and the consequent release of factors such as cytochrome c. The $\Delta\psi_m$ results from the asymmetric distribution of protons and ions across the inner mitochondrial membrane, giving rise to electrochemical gradients that are essential for many mitochondrial functions e.g. protein translocation and ATP synthesis. *[Vayssiere 1994, Zamzani 1995]*. The dissipation of $\Delta\psi_m$, followed by cytochrome c release, has in many apoptotic systems been associated with the opening of the mitochondrial permeability transition pore (MPT). The MPT pore is a protein complex formed at the inner-outer mitochondrial membrane contact sites under specific conditions. The adenine nucleotide translocator (ANT), in the inner membrane, can form a complex with several mitochondrial outer-membrane proteins, for example porin. MPT can be induced in different ways *in vitro*, one way is to use the toxic substance tributyltin (TBT), and leads to disruption of ion homeostasis, and collapse of the mitochondrial membrane potential ($\Delta\psi_m$) *[Beanrdi 1994,Martinou 2000]*.

Cytochrome c is located within the mitochondria, where it constitutes an essential part of the respiratory chain. Studies have shown that during apoptosis, cytochrome c is translocated from the mitochondrion into the cytosol and that the release of cytochrome c is inhibited by Bcl-2. Once in the cytosol, cytochrome c interacts with pro-capase-9, apoptotic protease-activating factor 1 (Apaf-1) and dATP, forming a multi-protein complex, the apoptosome, that participates in the activation of the effector caspase, caspase-3 **Figure 3**.
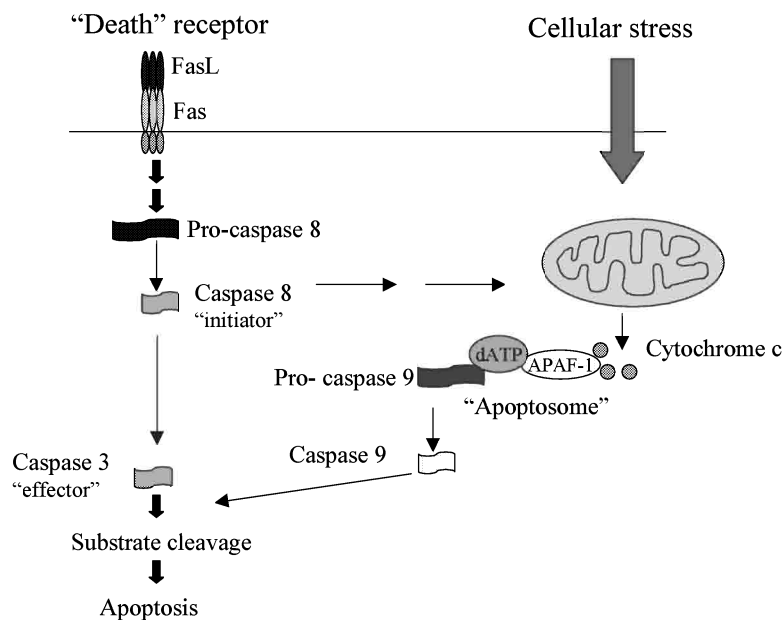
"Death" receptor   Cellular stress

FasL

Fas

Pro-caspase 8

Caspase 8
"initiator"

Pro- caspase 9   dATP APAF-1   Cytochrome c

"Apoptosome"

Caspase 3   Caspase 9
"effector"

Substrate cleavage

Apoptosis

Figure 3. Pathways of apoptosis signal transduction.

1.9   APOPTOSIS IN EOSINOPHILS AND NEUTROPHILS

To maintain cellular homeostasis, granulocyte tissue-infiltration is normally followed by elimination of eosinophils and neutrophils by induction of apoptosis. Different inflammatory mediators e.g. GM-CSF, complement factor C5a, and LPS *[Lee   1993]* can modulate the apoptotic process in eosinophils and neutrophils. Cytokines like IL-5 and IL-3 have been proposed to selectively prolong the eosinophil life span by inhibition of the apoptotic machinery and thus contribute to tissue loading of eosinophils *[Tai 1992]*. Both IL-5 and GM-CSF have been detected in bronchoalveolar lavage (BAL) fluids from allergic individuals *[Virchov 1995]*, and *in vitro*-culture of eosinophils in the presence of these cytokines suggests that IL-5 and GM-CSF up-regulate the anti-apopoptotic family member Bcl-xL, thus contributing to the delayed apoptosis *[Dibbert 1998]*. On the other hand, it has also been proposed that IL-5 delays neutrophil apoptosis by reducing the level of Bax, a pro-apoptotic member of the Bcl-2 family *[Dibbert 1999]*. These studies put forward theories of mitochondrial involvement in the regulation of granulocyte apoptosis, but currently available data are still too few and interpretation should be done with caution. Differences in apoptosis-susceptibility between eosinophils and neutrophils have previously been reported after treatment with glucocorticoids *[Meagher 1996]*, which have been employed as potent anti-inflammatory agents for many years

9

[Goulding 1993]. One reported effect of dexamethasone, is the rapid and dramatic induction of apoptosis in the eosinophils, while the number of neutrophils increased, if anything *[Meagher 1996, Schleimer 1990]*, indicating differences in the mechanisms regulating the apoptotic machinery.
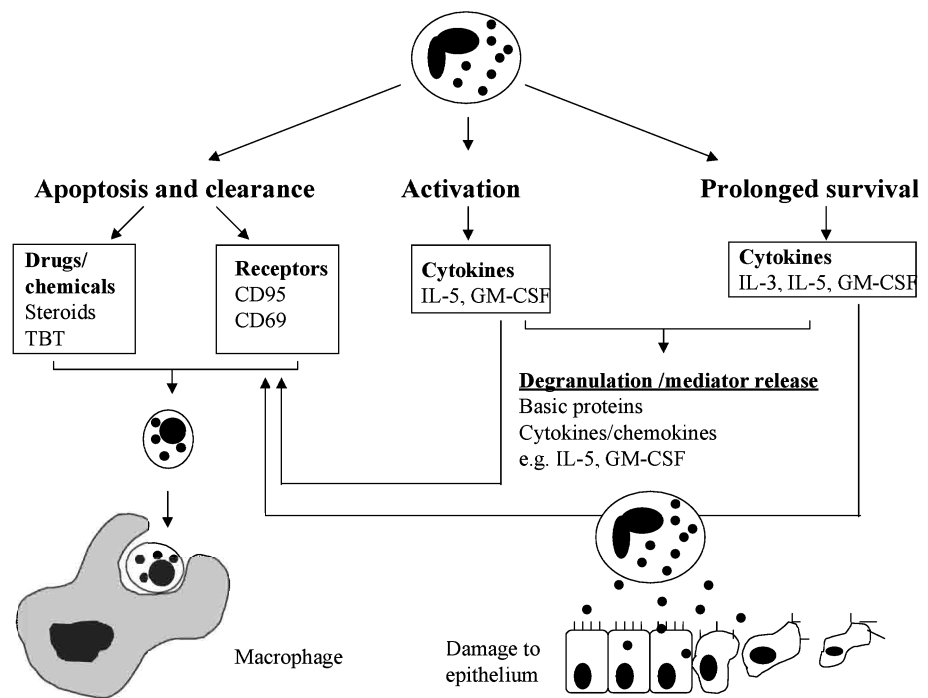

Figure 4. Illustration of different pathways of eosinophil activation, degranulation and apoptosis.

2 AIMS OF THE STUDY

The aim of this thesis was to investigate eosinophil activity markers and their relation to allergic inflammation and apoptosis. The specific aims were:

[I]   To apply a standardised sampling procedure for blood sample analysis to minimise variations and to prolong storage time without affecting cell number, selected eosinophil and neutrophil activity markers and function *in vitro*.

[II]   To investigate whether CD69 expression is subject to specific regulation by inflammatory mediators, and if so, to identify these factors in relation to eosinophil and neutrophil function.

[III]   To identify differences in the inflammatory response in terms of eosinophil accumulation and mediator release evoked by recombinant birch pollen allergen in patients with birch pollen related allergic rhinitis and mild asthma.

[IV]   To further characterise the role of mitochondria in granulocyte-apoptosis as well as to investigate potential differences in apoptosis-susceptibility between eosinophils and neutrophils by applying TBT.

[V]   To examine the kinetics of CD69 expression in eosinophils and neutrophils in relation to the rate of apoptosis with respect to the presence of GM-CSF. We also addressed the question whether there are differences between allergic patients and healthy individuals in this respect.

3 MATERIALS AND METHODS

3.1 SUBJECT CHARACTERISATION [I-V]

3.1.1 [I]

Peripheral blood was collected from ten non-allergic blood donors aged 37 (30-46) years and from ten allergic patients (Table 1) referred to the Department of Allergology, Karolinska Hospital for investigation. All patients in the study were drug-free and had a positive skin prick test (SPT) of 3+ (response equivalent to or greater than that to histamine) for at least one of the allergens included in a standard prick test protocol (Table 1). The subjects gave their informed consent to participate in the study, which was approved by the Ethics Committee of the Karolinska Hospital.

Table 1. Demographic data

Sex	Age	Symptoms	Spt 3+
M	46	RC, A	Birch, timothy, pets
M	19	RC, A	Birch, timothy, pets
F	25	RC	Pets
F	24	RC, A	Birch, timothy, pets
M	35	RC	Timothy
F	26	AD, RC, A	Birch, timothy, pets
M	25	RC, A	Birch, timothy, mugwort, pets
F	26	AD, RC, A	Pets
M	26	RC, A	Birch, timothy, pets
F	24	RC, A	Timothy, pets

AD	= Atopic dermatitis
RC	= Rhinoconjunctivitis
A	= Asthma

3.1.2 [II,IV]

In these studies, peripheral blood was collected from healthy blood donors (aged 18-65 years).

3.1.3 [III]

In the third study, the study group consisted of nine allergic patients aged 32 (18-57) years with a history of birch pollen related allergic rhinitis and mild bronchial asthma (Table 2). The patients were positive to recombinant (r) Bet v 1 in skin prick test 3+, had end-point concentrations from intradermal titration tests in 10-fold dilution steps up to 1 µg/ml rBet v 1

(7.5-15 mm wheal at 10 min), and were RAST rBet v 1 positive ≥ 2 kU$_A$/L (Pharmacia CAP RAST System, Pharmacia & Upjohn, Uppsala, Sweden) (Table 2).

All subjects were asymptomatic and drug-free, except that inhaled β_2-agonists were allowed on an as-needed basis. There was no evidence of current airway infection. The study was performed out of pollen season. The subjects gave their informed consent to participate in the study, which was approved by the Ethics Committee of the Karolinska Hospital and by the Swedish Medical Product Agency.

Table 2. Demographic data

Sex	Age	Symptoms	Duration of symptoms (years)	Total IgE (kU/L)	RAST rBetv1 (kU$_A$/L)	End point titration intradermal test rBet v 1 (μg/ml)	rBet v 1, trimer, and fragment mix installed in skin chamber (μg/ml)
F	48	R	28	55	2.2	1	10
F	28	RC	4	120	3.2	1	10
M	25	R	6	39	7.8	1	10
M	57	A	5	105	13	1	10
M	40	A, RC	22	336	26	1	10
F	27	RC	8	379	37	1	10
F	27	A, RC	25	174	73	1	1
M	18	RC	5	733	90	1	1
F	22	A, RC	13	809	190	0.1	1

RC = Rhinoconjunctivitis

A = Asthma

R = Rhinitis

3.1.4 [V]

In this study peripheral blood was collected from seven healthy blood donors (aged 18-65 years) and seven allergic patents (aged 29-46 years). All patients in the study were drug-free and had a positive skin prick test (SPT) of 3+ (response equivalent to or greater than that to histamine) for at least one of the allergens included in a standard prick test protocol (Table 3). The subjects gave their informed consent to participate in the study, which was approved by the Ethics Committee of the Karolinska Hospital.

Table 3. Demographic data

Sex	Age	Symptom	Spt ≥3+
M	36	RC, A	Birch, timothy, pets
M	29	RC	Birch, timothy
F	37	RC, A	Birch, pets
M	46	RC,	Birch, pets
F	45	RC	Mite
F	44	RC, A	Birch, timothy, pets
F	32	RC, A	Birch, pets

RC   = Rhinoconjunctivitis

A   = Asthma

3.2 SKIN CHAMBERS [III]

Two skin blisters were raised on the volar side of each forearm with gentle suction *[Fernvik1999:I]*. The blisters were covered overnight with a plastic eye chamber and the next morning the epidermal blister roofs were gently removed. A transparent plastic skin chamber with an inner volume of 1 ml was placed over each unroofed blister and secured with tape. The chambers were washed four times with sterile PBS containing 20 IU/ml heparin. Three chambers were filled in random order with one of the three recombinant products, generally at 10 x end-point concentrations for rBet v 1 wild type as previously determined by intradermal titration, and one chamber served as negative control (PBS+heparin 20 IU/ml). Total volume was 0.5 ml. Subjects exhibiting RAST rBet v 1 levels above 50 kU_A/L, were for safety reasons challenged with concentrations no higher than 1 µg/ml of rBet v 1 wild type.

After two hours of incubation, the blister fluids were harvested and the skin chambers were washed twice with PBS-heparin to avoid carry-over effect of any recombinant product. The chambers were then refilled with PBS-heparin (1 ml), and left for an additional 6 hours (a total of 8 hours after start of challenge) before final removal.

The skin chamber fluid was collected in plastic tubes after 2 and 8 hours of incubation for further analysis. All aspirated chamber fluids were centrifuged at 300×g for 5 min, and supernatants were stored at $-70^\circ C$ until further analysed.

3.3 PREPARATION OF RECOMBINANT ALLERGENS [III]

Recombinant Bet v 1 wild type and rBet v 1 fragments comprising amino acid 1-74 and 75-160 were expressed in *E. coli* and purified as described earlier *[Breitender 1989, Vrtala 1997, Hoffmann-Sommergruber 1997]* and rBet v 1 trimer was engineered by expressing three copies

14

of the Bet v 1 cDNA *[Breitender 1989]* in *E. coli* BL21 (DE3) as a stable trimer *[Vrtala 1999]*. Lyophilised recombinant Bet v 1, Bet v 1 fragments (aa 1-74 and 75-150 mixed in equal amounts) and recombinant Bet v 1 trimer, were dissolved in sterile PBS pH 7.4 to 1 mg/ml. Aliquots were stored at $-20^{\circ}C$ until the test day when they were diluted to working concentration (1-10 µg/ml) with PBS and heparin (final concentration 20 IU/ml). PBS-heparin (20 IU/ml) was used for control purposes.

3.4 PREPARATION OF LEUKOCYTES [I-V]

3.4.1 [I]

Peripheral blood was either collected in tubes containing EDTA (Vacutainer, 10 ml, with 120 µl of 15% EDTA) (Becton Dickinson, Meylan-Cedex, France) or citrate (Vacutainer, 5 ml, with 1 ml 0.129 M 9NC, Becton Dickinson), or in SST test tubes (Vacutainer, 2.5 ml, Hemogard SST, Becton Dickinson). The blood samples containing EDTA and citrate were stored at $+4^{\circ}C$ for 1, 5 or 24 hours, before use. Three blood samples collected in SST test tubes were allowed to coagulate at room temperature $(+20-22^{\circ}C)$ for 1 hour before storage at $+4^{\circ}C$ for 4 or 23 hours. The blood was haemolysed in 150 µl portions by dilution in 3 ml $+4^{\circ}C$ isotonic NH_4Cl-EDTA lysing solution (154 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) and incubation for 5 min at $+15^{\circ}C$. The leukocyte suspensions were then centrifuged at 300×g for 6 min at $+4^{\circ}C$ and washed with 2 ml $+4^{\circ}C$ PBS supplemented with 0.1 mM EDTA and 0.02 % NaN_3 (PBS-EDTA).

3.4.2 [II,III]

Blood was collected in tubes containing citrate (as described above), and haemolysed in 150 µl portions (as described above).

3.4.3 [III]

Leukocytes in skin chamber fluid were obtained by centrifugation of the chamber fluids at 300xg for 5 min.

3.4.4 Purification of eosinophils and neutrophils [II,IV-V]

Citrate blood was collected and purified with Ficoll-Hypaque *[Boyum 1984]* or Percoll centrifugation. The mononuclear cells were removed from the inter phase and used for further analysis. The erythrocytes in the granulocyte pellet were haemolysed with lysing solution (as described above). To obtain purified eosinophils, a magnetic cell separation system MidiMACS

(Miltenyi, Biotec, Bergisch, Gladbach, Germany) was used *[Hansel 1991]*. Briefly, the enriched granulocytes were incubated with magnetic anti-CD16 antibodies for 20-25 min at +4°C, before being layered on top of a separation column in a magnetic field. Neutrophils labelled with the magnetic CD16 were trapped in the column, whereas CD16-negative eosinophils were collected. The column was then removed from the magnetic field and the neutrophils were eluated. All cells were washed in PBS at 300×g for 8 min. Both the purity and viability of eosinophils and neutrophils were >95 %.

3.4.5 Purification of lymphocytes [IV]

Blood was collected as described above, and purified by Percoll centrifugation. The mononuclear cells from the interface were collected and washed twice with 40 ml PBS and centrifuged for 12 min at 300×g and than for 12 min 400×g. The mononuclear cells were then resuspended in hepes (10 mM)-buffered RPMI 1640 medium (Gibco Ltd, Paisly, Renfrewshir, U.K.) supplemented with 40 % heat inactivated calf serum at a concentration of 1 million cells per ml. The cells were then incubated in a tissue culture flask for 40 min at +37°C. Under these conditions the monocytes adhere to the plastic and the lymphocytes can be collected after incubation. The lymphocytes were washed once in 40 ml PBS and centrifuged for 12 min at 300×g. Both the purity and viability of lymphocytes were >95 %.

3.5 IN VITRO ACTIVATION OF LEUKOCYTES [II]

Mixed leukocytes or purified eosinophils were resuspended in 200 µl hepes (10 mM)-buffered RPMI 1640 medium supplemented with 10 % heat inactivated calf serum (RPMI) in the presence of the following activators: phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co, St. Louis, USA) (10^{-7} M), N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma Chemical Co) ($5×10^{-7}$ M), lipopolysaccharide (LPS) (Sigma Chemical Co) (100 µg/ml), recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF) (Nordic BioSite AB, Täby, Sweden) (10^{-9}-1.0 µg/ml), recombinant human eotaxin (Prepro Tech Inc., Rocky Hill, NJ, USA) (200 ng/ml) or recombinant human interleukin-5 (IL-5) (Immunokontact, Frankfurt, Germany) (100 ng/ml).

Incubation times were as follows: GM-CSF, LPS, fMLP and IL-5 120 min, IL-5+eotaxin 105 min +15 min, and PMA 0-120 min. All incubations were performed at +37°C. Mixed leukocytes or purified eosinophils were incubated, in parallel, with medium alone at +4°C and at +37°C for the periods of time stated above. After the *in vitro* activation, the cells were washed once with 2 ml PBS-EDTA by centrifugation at 300×g for 5 min.

3.6 CULTURE OF EOSINOPHILS AND NEUTROPHILS [V]

Purified eosinophils or neutrophils (10^6 cells/ml) were cultured in RPMI with or without GM-CSF 2 ng/ml for up to 72 hours at $+37^\circ$C in 5% CO_2. Cells were then washed once with 2 ml PBS by centrifugation at 300×g for 5 min before further use.

3.7 INDUCTION OF APOPTOSIS [IV]

Purified eosinophils or neutrophils were incubated with RPMI in the absence (negative control) or presence of tributyltin (TBT) (2 μM) (Sigma-Aldrich Chemical Co, St. Louis, MO, USA) for up to 4 hours at $+37^\circ$C, 5 % CO_2.

3.8 CASPASE 3 ACTIVITY [IV-V]

Caspase 3 activity in eosinophils or neutrophils was measured by using the cell permeable substrate PhiPhiLux-G2D2 (OncoImmunogen, Inc., Gaithersburg, MD, USA) containing the prototypical sequence DEVDG.
A minimum of 100 000 cells/tube were incubated with PhiPhiLux (10 μM) and 10 % foetal calf serum at $+37^\circ$C, 5 % CO_2, for 60 min according to the manufacturer's instructions. Fluorescence was measured in the FL-2 channel of a flow cytometer (see below).

3.9 CELL MEMBRANE FIXATION AND PERMEABILISATION
(THE FOG METHOD) [I-III,IV]

The peripheral blood leukocyte preparations were treated according to a cell membrane permeabilisation technique, the FOG method *[Halldén 1989, Halldén 1993]*. This procedure was done to obtain a clear differentiation between eosinophils and neutrophils in a scatter plot histogram employing flow cytometry, and to be able to analyse staining of intracellular antigens. Cells were incubated at $+20$-22°C in 200 μl phosphate-buffered 4% (w/v) paraformaldehyde (PFA) (Sigma Chemical Co) and then washed in 3 ml $+4^\circ$C PBS-EDTA (centrifuged at 400×g, 7 min). The fixed leukocytes were then permeabilised by incubation in 200 μl 0.74% *n*-octyl-β-D-glucopyranoside (OG) (Sigma Chemical Co) for 6 min at $+20$-22°C. The permeabilised cells were finally washed once in 3 ml $+4^\circ$C PBS-EDTA (at 400×g, 7 min), resuspended in PBS-EDTA and kept on ice.

3.10 IMMUNOFLUORESCENCE STAINING OF CELL SURFACE AND INTRACELLULAR ANTIGENS [I-III,V]

Cell surface expression of CD69 (clone: L78, Becton Dickinson), CD9 (clone: P1/33/2, DAKO A/S, Denmark) and CD11b (clone: 2LPM19c, DAKO) was detected by using fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal antibodies to surface antigens. Antibodies were added to untreated mixed leukocytes (from 150 µl of peripheral blood) or purified eosinophils prior to the FOG treatment. To determine the non-specific binding, an isotype-matched IgG1 control antibody was used in parallel.

Intracellularly localised CD69 and ECP was detected by adding FITC conjugated monoclonal CD69 (Becton Dickinson) or EG2 (Pharmacia & Upjohn, Uppsala, Sweden) antibody to FOG-treated mixed leukocytes. All incubations with monoclonal antibodies were performed for 30 min on ice, followed by two washes in 2 ml PBS-EDTA at +4°C (300-400×g, 5 min). The different leukocyte preparations were finally resuspended in 500 µl PBS-EDTA before flow cytometric analysis (see below).

3.11 ANNEXIN-V AND PROPIDIUM IODIDE STAINING [IV-V]

Granulocytes were analysed for phosphatidylserine (PS) exposure by Annexin V (AN) binding and propidium iodide (PI) incorporation. The double staining with Annexin V and PI gives an opportunity to distinguish between early apoptotic (AN+/PI-) and late apoptotic/necrotic cells (AN+/PI+).

A minimum of 100 000 eosinophils or neutrophils was incubated on ice with 100 µl ice cold binding buffer, 5 µl PI (Beckman Coulter, Inc., Fullerton, CA, USA) and 1 µl Annexin V (Beckman Coulter) for 10 min in the dark. The incubation was stopped by adding 400 µl binding buffer. The cells were then analysed by flow cytometry (see below).

3.12 MEMBRANE POTENTIAL [IV]

To measure the mitochondria membrane potential ($\Delta\psi_m$) in granulocytes, we employed the fluorescent dye tetramethylrhodamine (TMRE) that accumulates in the inner mitochondrial membrane according to the $\Delta\psi_m$. A loss of $\Delta\psi_m$ can be monitored by flow cytometry, as a decrease in FL-2, expressed in a log scale. Briefly, eosinophils or neutrophils (10^6 cells/ml) were pre-incubated with TMRE (20 nM) at +37°C, 5 % CO_2 for 15 min before being exposed to TBT (2 µM). The $\Delta\psi_m$ was measured every 5 min until 20 min. TBT-treated purified lymphocytes were used as positive control cells, as it has previously been described that TBT reduces the $\Delta\psi_m$ in these cells *[Stridh 1998]*. The granulocytes were also tested for their

response to the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), a well characterised agent known to dissipate $\Delta\psi_m$ in many cell types. Eosinophils, neutrophils and lymphocytes were exposed to CCCP (10 μM) and the $\Delta\psi_m$ was monitored every 5 min until 20 min by flow cytometry.

In some experiments, granulocytes were exposed to both TBT and CCCP. The membrane potential was measured as mentioned above.

3.13 ANALYSIS BY FLOW CYTOMETRY [I-V]

The different leukocyte preparations were analysed in an EPICS XL flow cytometer (Beckman Coulter, Inc., Hialeah, FL, USA). Leukocyte subpopulations are distinguished by their different light scattering properties; forward scatter (FS) reflects the cell size and side scatter (SS) reflects the complexity/granularity. In the present study, three different leukocyte clusters were distinguished in untreated blood samples: lymphocytes, monocytes and granulocytes (neutrophils+eosinophils) **Figure 5**.

The FOG method causes a decrease in light scatter properties of all leukocyte populations, with the exception of eosinophils. The altered light scattering properties permit the detection of three separate leukocyte populations designated lymphocytes, neutrophils (neutrophils+monocytes) and eosinophils *[Halldén 1993]* **Figure 5**. Eosinophil and neutrophil analyses were based on a minimum of 1000 or 10 000 cells, respectively, within the defined gate. The monocytes in untreated leukocyte preparations were identified by their characteristic scatter properties and CD14 expression. A minimum of 2000 cells was analysed within the monocyte cluster, which contained >90% CD14 positive cells.

The FS-SS histogram was used to divide the cells into separate populations of living and dead cells *[Sandström 2000]*. The upper population in each histogram is composed of living cells, confirmed as caspase 3 negative, and the lower population consists of apoptotic (caspase 3 positive) as well as necrotic cells. When data refer to the whole population, both the upper and lower populations are included **(Figure 6)**.

The instrument was calibrated daily with standardised 10 μm fluorospheres, Flow-Check (Beckman Coulter). Flow-set (Beckman Coulter), another fluorosphere with controlled fluorescence intensity, was used to standardise the mean fluorescence intensity (MFI) before each experiment. The proportions and absolute numbers of immunostained peripheral blood eosinophils and neutrophils were determined by the use of isotype matched control antibodies to define the cut-off for positively labelled cells. Quantification of each respective antigen was obtained by measuring the MFI-units of the corresponding positively labelled cell population.
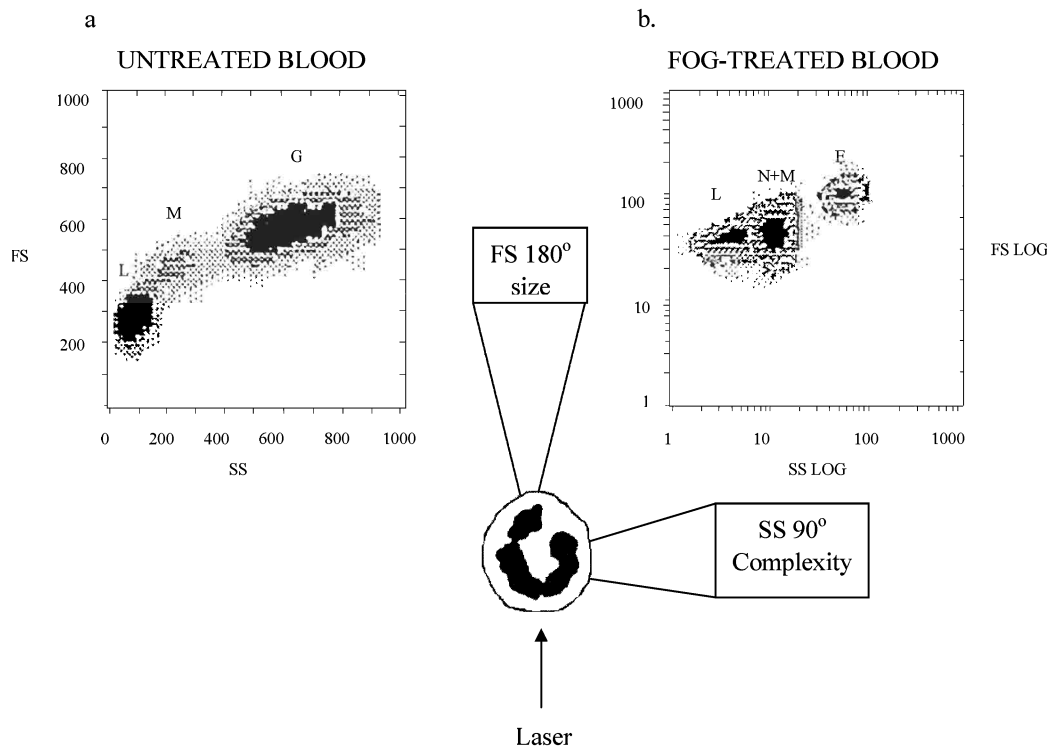
a.   b.

UNTREATED BLOOD   FOG-TREATED BLOOD

Figure 5. The distribution of peripheral blood leukocytes by their scatter properties. The leukocyte populations are detected in 2-parameter scatter plot histograms with linear (a) or logarithmic amplification (b).
L=lymphocytes, M=monocytes, G=granulocytes, N=neutrophils, E=eosinophils,
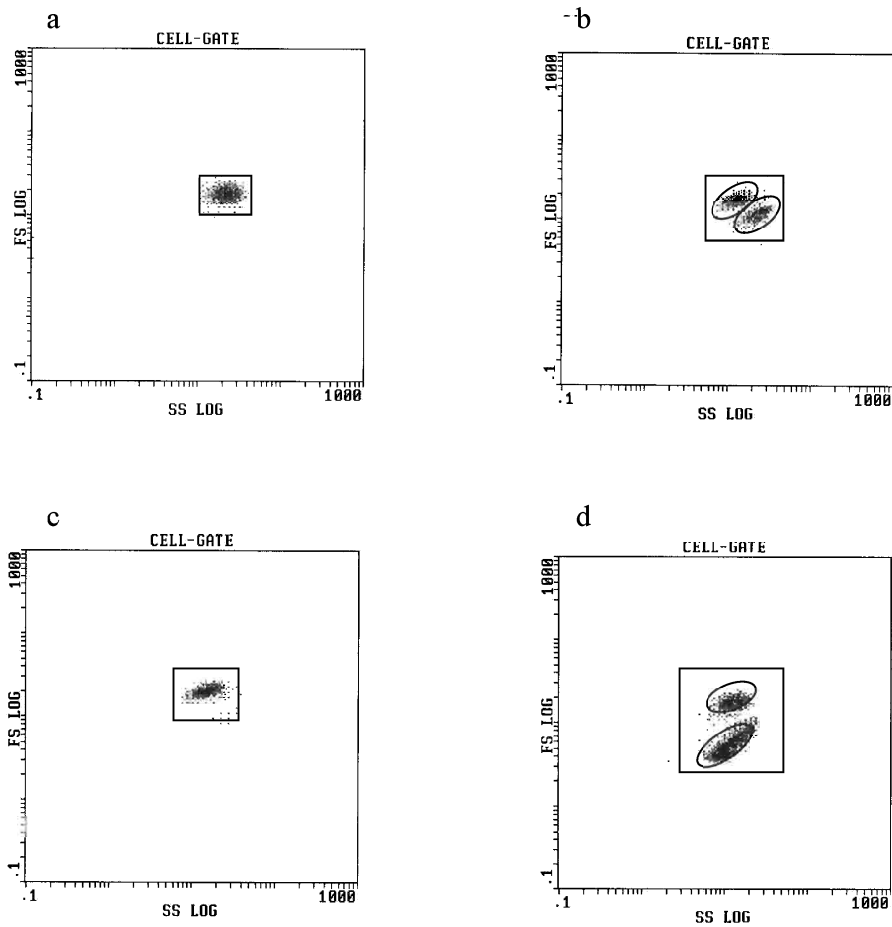FS=forward scatter, SS=side scatter.

Figure 6. Eosinophils (**a,b**) and neutrophils (**c,d**) were distinguished by their different light scatter properties. The FS-SS histogram was used to divide the cells into separate populations of living and dead cells. Figure **a** and **c** show untreated living cells, whereas figure **b** and **d** show cells treated with an apoptotic stimulus (TBT). The upper population is composed of living cells (**b,d**), in this study confirmed as caspase 3 negative, and the lower population consists of apoptotic (caspase 3 positive) as well as necrotic cells (**b,d**). Cells in the gate placed around both cell populations are designated as the whole cell population (**a-d**).

3.14 MORPHOLOGICAL ANALYSIS OF EOSINOPHILS [IV-V]

The cells were stained with May-Grünwald Giemsa and the morphology was analysed with light microscopy (Nikon Eclipse E400).

21

3.15 MITOTRACKER RED CMX ROS AND CYTOCHROME C DOUBLE-LABELLING [IV]

Eosinophils, neutrophils and lymphocytes (control cells) were purified and stained with Mitotracker CMX Ros (75 nM) (Molecular Probes) for mitochondrial localisation for 45 min at +37°C, 5 % CO_2, washed, followed by exposure to TBT (2 μM) for 0, 10, 30 or 60 min at +37°C, 5 % CO_2. The cells were then separated in different test tubes and treated according to a fixation and permeabilisation technique, the FOG-method (as described above). Cells were then incubated with 100 μl 0.1 % BSA-c (Aurion, Seligenstadt, Germany) for 30 min on ice before incubated with an antibody to cytochrome c (Mouse IgG1) (Pharmigen, San Diego, CA, USA) or an irrelevant control antibody (Mouse IgG1) (Dako A/S, Denmark) for 30 min on ice. Cells were washed again and incubated with a secondary fluorescent antibody Alexa fluor 488 (goat-anti-mouse) (Molecular Probes) for 30 min on ice. Finally, cells were washed and smears for fluorescent studies were prepared by cytocentrifugation as described above and mounted with fluoromount (Dako A/S, Denmark).

Fluorescence studies were done by confocal laser microscopy (Leica DM IRBE).

3.16 STATISTICAL ANALYSIS [I-V]

The results are presented as medians (interquartile range). When less than five experiments were performed results are presented as mean (min-max). Statistical evaluation was performed within and between groups, using non-parametric methods, Wilcoxon paired test and Mann Whitney U-test, as appropriate. Differences were considered statistically significant at $p < 0.05$.

4 RESULTS AND DISCUSSION

4.1 PAPER [I]

4.1.1 The distribution and number of eosinophils

The total number of leukocytes as well as the number and proportion of eosinophils in blood samples from the patient and healthy control groups, were stable during the different storage times (1, 5 and 24 hours), regardless of anticoagulant used (citrate or EDTA). The leukocyte sub-populations — lymphocytes, monocytes and granulocytes — preserved their scatter properties under the different storage conditions, with the exception of monocytes in EDTA-blood. When blood collected in tubes with EDTA was stored for 5 hours or more the forward scatter of the monocytes decreased. This result indicates that citrate could be a better alternative as anticoagulant when differential counts are to be calculated by techniques based on scatter properties.

The number of eosinophils, and eosinophil markers EG2 and serum-ECP, have previously been used to detect eosinophil activity in asthmatic patients *[Griffin 1991, Skedinger 1995, Roquet 1996]*. In the present study, all three markers were stable in blood samples from both healthy and allergic individuals regardless of sampling condition, and there were significant differences in all markers between the two study groups at all time points. These results indicate that eosinophils from the patients are activated, which could reflect ongoing allergic inflammation. The increased expression of the EG2-epitope confirms previous studies showing that EG2 can be used as an intracellular activity marker for eosinophils in patients with allergic inflammation and asthma *[Halldén 1993, Fernvik 1996, Kuwasaki 1998]*. The results also indicate that the choice of anticoagulant has no significant impact on the analysis of eosinophil number and EG2 expression.

4.1.2 Surface expression of CD9 and CD11b

Two other markers used to detect eosinophil activity are CD9 and CD11b. In our study we did not find any differences between patients and controls in the context of CD9 and CD11b expression on eosinophils at any time point or sample condition. However, there was a significantly increased basal expression of CD11b on eosinophils, in both controls and patients, after 5 and 24 hours storage when EDTA-blood was used, with the highest expression after 24 hours of storage. This result indicates that citrate could be a better alternative as anticoagulant when CD11b is to be analysed.

4.1.3 Functional testing of metabolic activity and CD11b mobilisation

In this study the functional state of monocytes and granulocytes, evaluated in terms of metabolic activity and *in vitro* mobilisation of CD11b on neutrophils, was unaltered during storage. Irrespective of storage condition used, both granulocytes and monocytes preserved their metabolic activity, as shown by their ability to produce H_2O_2 in response to *in vitro* stimulation with PMA or fMLP. Different levels of CD11b expression were observed in EDTA- and citrate-blood. The initial, basal expression of CD11b on neutrophils was significantly greater in EDTA-blood, at all time points, as compared to citrate-blood, and the neutrophils in citrate-blood had greater ability to respond to fMLP, in both the control and patient group. Our results indicate that citrate is preferable to EDTA as anticoagulant when mobilisation of CD11b is to be analysed.

4.2 PAPER [II]

4.2.1 Surface and intracellular expression of CD69 in eosinophils and neutrophils

In this study we found that approximately 5 % of the resting eosinophils and 1 % of resting neutrophils from healthy individuals express CD69 on the surface. These figures are in line with previously reported data *[Gavioli 1992, Hartnell 1993, Luttmann 1996]*. When measuring the surface expression of CD69 on eosinophils and neutrophils after *in vitro* stimulation with the cytokines GM-CSF and IL-5, we observed an increased expression of CD69 in the majority (>70 %) of eosinophils, but not in neutrophils.

In another series of experiments, we found that eosinophils and neutrophils have a preformed intracellular pool of CD69, and we detected a decrease in the intracellular pool of CD69 in eosinophils after stimulation with GM-CSF, whereas the preformed pool in neutrophils was not significantly changed. These results indicate that there are different mechanisms regulate the cell surface mobilisation of CD69 in eosinophils as compared to neutrophils.

To address the question whether additional stimuli, more related to infections, also may up-regulate CD69, we used the bacterial related products LPS and fMLP. Approximately 50 % of the eosinophils responded with CD69 surface upregulation after LPS stimulation, and approximately 17 % after fMLP stimulation. These results indicate that stimuli related to bacterial infection are less efficient up-regulators of CD69 than the eosinophil-specific stimuli, and that the observed differences between these families of stimuli are quantitative, rather than qualitative, in nature. When we used LPS as stimulus, a significantly lower (p<0.01) degree of CD69 expression was measured on purified eosinophils as compared to eosinophils in mixed leukocytes. This might be due to a cascade effect where LPS activates the monocytes, which in

turn produce a factor or factors that promote CD69 upregulation. A plausible factor could be GM-CSF, which is released from monocytes upon LPS stimulation *[Sieff 1988]*.

The neutrophils did not respond with CD69 upregulation under the experimental conditions used in the present study, despite a preformed CD69 pool. Our results do not exclude that CD69 can be mobilised to the neutrophil cell surface under different *in vitro* conditions. However, the physiological role of CD69 in neutrophils is currently not fully understood.

4.2.2 Time course of eosinophil surface and intracellular expression of CD69 and EG2-epitope in relation to ECP release

We compared the expression of CD69 with two other activity markers: the intracellular expression of the EG2-epitope on ECP and the release of ECP. Stimulation with GM-CSF resulted in a pronounced upregulation of CD69 that peaked late during the actual incubation period. This kinetic pattern was in contrast to the EG2 and ECP pattern, which showed a more gradual and moderate increase, without any pronounced peak during late incubation. These results indicate that increased expression of the EG2-epitope and the ECP release precedes the main up-regulation of CD69, upon GM-CSF stimulation. What these results mean is not clear, but they indicate that a kinetic order exists between degranulation and CD69 upregulation, and that the physiological role for surface CD69 is primarily after the initiation of the degranulation process. In line with this hypothesis is the notion that ligation of anti-CD69 induces apoptosis in eosinophils *[Walsh 1996]*. One may hypothesise, that an insufficient upregulation of CD69 may prevent apoptosis, and thus lead to prolonged survival of inflammatory cells in a state that permits degranulation. The consequence of this might be increased tissue damage, observed in e.g. asthma and lung fibrosis.

4.3 PAPER [III]
4.3.1 Levels of histamine in skin blisters

In this study we investigated the levels of histamine in skin blister chambers applied on patients with allergic rhinitis, after challenge with rBet v 1 wild type and the hypoallergenic derivatives. We found a significantly reduced release of histamine in the skin blister fluid, collected after 2 hours, in chambers challenged with rBet v 1 derivatives compared to rBet v 1 wild type. These results are in agreement with previous *in vitro* and *in vivo* studies which show that the derivatives generate a reduced basophil histamine release and have a 1000 x lower potency in inducing wheal-flare-reactions compared to rBet v 1wild type in atopics *[Vrtala 1997, van Hage-Hamsten 1999]*. The levels of histamine collected in the rBet v 1-provoked chambers were comparable to the levels obtained with crude allergens of birch and timothy pollen

[Fernvik 1999:II]. In the present study we found no correlation between the recruitment of eosinophils to rBet v 1 challenged chambers at 8 hours and the early histamine release. This finding is not unexpected considering the presence of additional factors, other than histamine, which may attract eosinophils in a complex tissue microenvironment *[Kay 1995].* Moreover, theoretically, when the challenge is prolonged, activated mast cells and invading basophils may contribute to a sustained release of IL-4 and IL-5, which in turn can activate eosinophils *[Kay 1995].*

4.3.2 Number of eosinophils, levels of eotaxin, ECP, and the expression of the EG2-epitope, obtained from skin blisters

The number of eosinophils and levels of the chemoattractant eotaxin *[Rothenburg 1999]* was the same in all challenged chambers after 8 hours, which is in agreement with previously reported data *[Fernvik 1999:II].* As markers for eosinophil activation, the expression of the EG2 epitope on intracellular ECP and the release of ECP were measured. All recombinant molecules had the same ability to upregulate the expression of EG2, but the ECP release was significantly lower in the chambers challenged with the derivatives as compared to rBet v 1 wild type. The upregulation of EG2 is in agreement with previous *in vitro* and *in vivo* results, which show that once eosinophils are recruited to an inflammatory focus the EG2 epitope is upregulated, probably due to the adhesion and/or transmigration process *[Fernvik 1999:II, Fernvik 2000].*

4.3.3 The expression of CD69 on eosinophils, and release of GM-CSF, IL-4 and IFN-γ in skin blister fluids

To further study eosinophil activation we measured the CD69 expression on eosinophils, after 8 hours of challenge. We detected CD69 expression on eosinophils in all skin blister chambers, with a significantly lower expression in chambers challenged with rBet v 1 derivatives as compared to the rBet v 1. We hypothesised that the upregulation of CD69 and increased release of ECP found in the rBet v 1-challenged chamber could be attributed to activation of eosinophils, maybe through eosinophil-bound Bet v 1-specific IgE. In this study the hypoallergenic derivatives had a reduced ability to activate eosinophils, measured in terms of ECP-release and CD69 expression. Our hypothesis is in line with earlier reports showing that the hypoallergenic rBet v 1 derivatives exhibited a reduced capacity to activate basophils and mast cells via crosslinking of Bet v 1-specific IgE *[van Hage-Hamsten 1999].*

Besides T-cells, eosinophils from blood, nasal mucosa, bronchoalveolar lavage and skin are reported to be important sources for GM-CSF and IL-4 *[Chung 1999, Moqbel 1995, Vowels 1995, Kita 1991]*. Our finding that IL-4 and GM-CSF appeared in the blister fluid collected after 8 hours, is in agreement with previous studies reporting expression of mRNA for IL-4 in late phase reaction biopsies *[Vowels 1995]* and for GM-CSF in blister fluid after 5 hours challenge with allergen *[Zweiman 1997]*. Our data show that the trimer, fragment mix and the control challenges evoked significantly lower levels of GM-CSF and IL-4 than the rBet v 1 challenged chamber. The finding that IL-4 and GM-CSF levels were higher in the chambers with elevated CD69 expression and ECP-release, supports the idea that both cytokines originate from activated eosinophils. While influx of basophils into skin blister fluids can be registered already within 6 hours *[Irani 1998]* most studies report a late recruitment (9-24 hours after challenge) *[Charlesworth 1991, Ying 1999]* and therefore argue against basophils being the major source of IL-4 in our study. However, the levels of IL-4 were just above cut-off detection limit, and the interpretation should be done with caution. Furthermore, we did not detect any IFN-γ, which is consistent with the previously reported lack of IFN-γ expression in mite allergen-exposed skin biopsies taken 2-48 hours after allergen challenge *[Yamada 1995]*.

4.4 PAPER [IV]

4.4.1 TBT-induced apoptosis in eosinophils and neutrophils measured by Annexin binding and activation of caspase 3

In this study, we used a potent mitochondrial toxin to further characterise the molecular mechanisms of granulocyte apoptosis. Low concentrations of TBT induced caspase-3-like activity and apoptosis in human blood granulocytes within 1-2 hours of treatment. In eosinophils, the caspase activation was extremely rapid, with a 14-fold increase already after 10 minutes of TBT-exposure, whereas similar treatment of neutrophils resulted in a 4 to 5-fold increase a the same time-point. In both cell types, typical apoptotic morphology succeeded the caspase activity. However, the neutrophils appeared less sensitive to TBT-induced apoptotic death. In these cells, typical morphological markers of apoptosis, such as phosphatidylserine (PS) exposure and condensed nuclei were detected after 120 min of exposure, 110 min after the initiation of caspase activity. In the eosinophils on the other hand, PS exposure was detected at 30 min, 20 min after the initiation of caspase activity. Apparently, there are differences in the kinetic between eosinophils and neutrophils.

4.4.2 The role of mitochondria in eosinophil and neutrophil apoptosis

The potential role of the mitochondrion in eosinophil apoptosis is obscure. In a recent study, it was demonstrated that eosinophils do contain a small number of mitochondria (24-36 per cell) with a maintained $\Delta\psi_m$, but without significant respiration. The $\Delta\psi_m$ was demonstrated to be generated by hydrolysis (performed by a reversed ATP synthase) of cytosolic-generated ATP rather from respiration *[Peachman 2001]*.

In contrast to lymphocytes, the $\Delta\psi_m$ remained stable up to 20 min in TBT-exposed eosinophils and neutrophils. A slight decrease in $\Delta\psi_m$ was observed at 20 min, a time point at which the caspases already have been activated. Mitochondrial effects in granulocytes were also investigated upon exposure to the mitochondrial uncoupler, CCCP. In eosinophils and neutrophils no loss of $\Delta\psi_m$ was detected after 20 min of exposure. However, a strong mitochondrial hyperpolarisation, initiated at 5 min, was observed. In energised mitochondria, CCCP uncouples respiration from the ATP synthesis by translocation of protons from the matrix to the cytosolic side. In the granulocytes, CCCP did not immediately reduced the $\Delta\psi_m$, indicating that the effect of CCCP may be strongly compensated by an increased rate of import and hydrolysis of cytosolic ATP in order to maintain the $\Delta\psi_m$. Exposure to TBT alone on the other hand, inhibits the ATP hydrolysis, the source of $\Delta\psi_m$, but does not reduce $\Delta\psi_m$. When cells were exposed both to an agent that dissipated the $\Delta\psi_m$ (CCCP) and to an agent that inhibits the ATP hydrolysis (TBT), we detected a significant reduction of $\Delta\psi_m$ in both the eosinophils and neutrophils. These data support the theory that $\Delta\psi_m$ in arises from the hydrolysis of cytosolic-derived ATP, and not primarily from respiration *[Peachman 2001]*.

4.4.3 The role of ANT and MPT in eosinophil apoptosis

The mitochondrial action of TBT in granulocytes definitely seems to differ from its action in lymphocytes, where adenine nucleotide translocator (ANT) plays a crucial role in the initiation of apoptosis. The ANT is one of the key proteins involved in mitochondrial transition pore (MPT) and, possibly, in changes leading to the release of mitochondrial proteins. It has previously been shown that TBT-induced loss of $\Delta\psi_m$, cytochrome c release, and apoptosis in lymphocytes are blocked with bongkrekic acid (BA), an agent that inhibits MPT *[Stridh 1999, Lenartowicz 1991]*. In eosinophil mitochondria, the ANT is assumed to carry cytosolic ATP to the mitochondrial matrix, as incubation with the ANT-inhibitor BA alone reduced $\Delta\psi_m$, suggesting that eosinophils do contain ANT *[Peachman 2001]*. However, the potential role of the ANT and the induction of classical MPT in eosinophil mitochondria, lacking respiration, still awaits experimental proof.

4.4.4 Release of cytochrome c from eosinophils and neutrophils

To exclude the possibility that cytochrome c might be released by an alternative mechanism than induction of MPT in granulocyte apoptosis, we performed immunohistochemistry and confocal analysis of cytochrome c release in TBT-treated eosinophils and neutrophils. In all donors, cytochrome c was localised in the mitochondria in both neutrophils and eosinophils at the time point of initiation of caspase activation (10 min), and release of cytochrome was not observed until the late stage of the TBT-induced apoptotic process. The late dissipation of $\Delta\psi_m$ and release of cytochrome c after TBT-treatment raises the question of whether apoptosis in eosinophils and neutrophils can be induced through a different pathway than via the mitochondria. However, we cannot role out the possibility that a small amount of mitochondrial cytochrome c (not detectable by immunohistochemistry) may be released into the cytosol by an alternative mechanism than induction of MPT, and that this small amount of cytochrome c may be sufficient to activate the caspases.

4.5 PAPER [V]

4.5.1 GM-CSF modulation of cell death in eosinophils

We found an overall good *in vitro* survival for eosinophils, which could be further improved by GM-CSF. Exposure to GM-CSF also resulted in a lower activation of caspase 3, indicating that GM-CSF exerts its effect up-streams of this point in the apoptosis cascade. Indeed, one proposed effect of GM-CSF has been to increase expression of anti-apoptotic genes *[Dibbert 1998]*. The eosinophil viability decreased during the initial 24 hours of *in vitro* culture and remained thereafter almost unchanged. Based on this observation, we subjected freshly isolated cells and cells incubated for 24 hours either with or without GM-CSF to caspase 3 analysis. When analysing cells in the lower cell population in the flow cytometric histogram, we found that 70-80 % stained positive for active caspase 3, indicating that they had died by apoptosis and not necrosis. We also noted a significantly lower caspase 3 activity in non-stimulated eosinophils from allergic individuals as compared to non-allergic individuals. One might speculate that eosinophils have a strong tendency to go through apoptosis to minimise the risk of uncontrolled release of basic proteins with subsequent cytotoxic effect on surrounding tissue.

4.5.2 CD69 upregulation on eosinophils

Several studies have shown that CD69 expression increases on peripheral blood and BAL eosinophils in allergic individuals during the pollen season *[Kita 1999]* and after allergen provocation *[Julius 1999]*. In this study, we addressed the question whether CD69, regarded as

an eosinophil activity marker, could be linked to atopic status. We found that surface CD69 was expressed to the same extent on all living eosinophils from both non-allergic and allergic individuals. However, we were not able to detect any difference in the capability to upregulate CD69 neither before nor after GM-CSF stimulation. Together these data suggest that differences in CD69 expression on eosinophils could not be attributed to the atopic status per se and should merely be regarded as a consequence of exposure to factors like GM-CSF.

In an earlier study, Walsh et al. demonstrated that cross-linking of CD69 resulted in apoptosis induction in eosinophils, which indicated a functional role of CD69 in eosinophil apoptosis *[Walsh 1996]*. In the present study we did not find any parallel between the rate of CD69 upregulation and the level of cell death, indicating that CD69 upregulation on eosinophils does not necessarily promote apoptosis within the time frame studied. However, one must take into account that in the presence of the still unknown physiological ligand for CD69, the upregulation of CD69 may play an important role in apoptosis induction.

4.5.3 GM-CSF modulation of cell death in neutrophils

We found that neutrophils from allergic individuals, when stimulated with GM-CSF, had a lower survival rate as compared to neutrophils from non-allergic individuals. Another observation was that neutrophils had a general tendency to die by necrosis (50-60 %) as determined by absence of caspase 3 activity. However, it has previously been discussed that isolated apoptotic neutrophils are unlikely to remain inert for long periods and rather undergo secondary necrosis after releasing their own intracellular content *[Haslett 1999]*. This observation could be one explanation for the low level of caspase 3 positive neutrophils detected in our study since we cannot rule out the possibility that the neutrophils might have undergone secondary necrosis, and subsequently leaked the substrate that indicates active caspase 3.

4.5.4 CD69 upregulation on neutrophils

We have previously shown that neutrophils contain a preformed intracellular pool of CD69 *[Nopp 2000]*, which can be mobilised to the cell surface upon exposure to relevant stimuli *[Gavioli 1992]*. We detected a time-dependent upregulation of CD69 on all viable neutrophils, and no CD69-expression on dead cells, indicating a role for CD69 primarily in the activation process. This finding was most pronounced in allergic individuals, and supports the view of pre-activated neutrophils in allergic subjects *[Caroll 1985]*.

5 CONCLUSIONS

[I]   We conclude that standardisation of blood sampling procedures could extend the *ex vivo* stability of eosinophil activity markers up to 24 hours in samples from both asthmatics and healthy controls. Furthermore, *in vitro* functional tests of both neutrophils and monocytes could be performed on blood after 24 hours of storage at +4°C. In addition, our results indicate that citrate is preferable to EDTA in terms of preservation of scatter properties and functional state of stored leukocytes.

[II]   Our results show that both eosinophils and neutrophils from healthy blood donors have a preformed intracellular pool of CD69, and that almost no eosinophils and neutrophils in the circulation express CD69 on the cell surface. The intracellular pool can be mobilised to the surface in eosinophils, but not neutrophils, by GM-CSF, IL-5, and LPS and to a lesser extent by the bacterial related product fMLP. The neutrophils did not respond under these conditions. The response to various stimuli indicates that there is a quantitative, rather than a qualitative difference in the ability of various stimuli to up-regulate CD69. Moreover, our data show a specific kinetic order of the degranulation process and CD69 upregulation. The activity marker CD69 appears later during activation than the increased expression of the EG2-epitope and the ECP release.

[III]   The results of our skin blister study demonstrate that genetically engineered hypoallergenic rBet v 1 derivatives induce significantly lower activation of eosinophils, as measured by means of surface expression of CD69, ECP-release and levels of GM-CSF. The levels of histamine and IL-4 were also lower after the challenge with the derivatives as compared to rBet v 1 wild type. The previously reported reduced allergenic activity of the rBet v 1 derivatives together with our results obtained in this study encourage us to further explore the feasibility of using rBet v 1 derivatives for immunotherapy.

31

[IV]   We have demonstrated an extremely rapid induction of caspase-3 activity and apoptosis in human blood granulocytes without prior mitochondrial changes, including loss of mitochondrial membrane potential and release of cytochrome c. Our results opens for the possibility for a mitochondrial-independent activation of caspase 3 and subsequent apoptosis in granulocytes.

[V]   The main finding is that the kinetics of CD69-upregulation in neutrophils, but not in eosinophils, differs between non-allergic and allergic individuals, without any relation between the level of expression and the apoptosis rate. To understand the implications of these data in the concept of asthma pathophysiology, we will have to do further studies. However, the data on differences in survival rates and CD69 expression between allergic and non-allergic individuals suggest a potential role for neutrophils in the allergic reaction.

6 ACKNOWLEDGEMENTS

7 REFERENCES

Ameisen JC. Programmed cell death (apoptosis) and cell survival regulation: Relevance to AIDS and cancer. AIDS 1994;8:1197-1213.

Arm JP, Walport MJ, Lee TH. Expression of complement receptors type 1 (CR1) and type 3 (CR3) on circulating granulocytes in experimentally provoked asthma. J Allergy Clin Immunol 1989;83:649-655.

Bajorath J, Aruffo A. Molecular model of the extracellular lectin-like domain in CD69. J Biol Chem 1994;269(51):32457-32463.

Benardi P, Vasanelli S, Veronese P, Colonna R, Szabo I, Zoratti M. Modulation of the mitochondrial permeability transition pore. Effects of protones and divalent cations. J Biol Chem 1992;267:2934-2939.

Berends C, Dijkhuizen B, Demonchy JG, Gerritsen J, Kauffman HF. Induction of low density and up-regulation of CD11b expression of neutrophils and eosinophils by dextran sedimentation and centrifugation. J Immunol Methods 1994;167:183-193.

Bonini S, Magrini L, Rotiroti G, Ronchetti MP, Onorati P. Genetic and environmental factors in the changing incidence of allergy. Allergy 1994;49:6-14.

Boyum A. Separation of lymphocytes, granulocytes and monocytes from human blood using iodinated density gradient media. In: Di Sabato G, Langone JJ, van Vunakis H, eds. Methods in Enzymology. Academic Press, 1984;108:88-102.

Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I, Venge P, Ahlstedt S, Simony-Lafontaine J, Godard P, Michel FB. Eosinophilic inflammation in asthma. N Engl J Med 1990;323:1033-1039.

Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, Breitenbach M. The gene coding for the major birch pollen allergen, Bet v 1, is highly homologous to a pea disease resistance response gene. EMBO J 1989;8:1935-19638.

Caroll M, Durham SR, Walsh GM, Kay AB. Activation of neutrophils and monocytes after allergen- and histamine-induced bronchoconstriction. J Allergy Clin Immunol 1985;75:290-296.

Charlesworth EN, Kagney-Sobotka A, Schleimer RP, Norman PS, Lichtenstein LM. Prednisone inhibits the appearance of inflammatory mediators and the influx of eosinophils and basophils associated with the cutaneous late-phase response to allergen. J Immunol 1991;146:671-676.

Chung KF, Barnes PJ. Cytokines in asthma. Thorax 1999;54:825-857.

Cohen JJ. Apotosis: Mechanisms of life and death in the immune system. J Allergy Clin Immunol 1999;103:548-554.

Deveraux QL, Stennicke HR, Salvesen GS, Reed JC. Endogenous inhibitors of caspases. J Clin Immunol 1999;19.388-389.

Dibbert B, Daigle I, Braun D, Schranz D, Weber M, Blaser, K, Zangemeister-Wittke U, Akbar AN, Simon H-U. Role of Bcl-x_L in delayed eosinophil apoptosis mediated by granulocyte-machrophage colony-stimulating factor and interleukin-5. Blood 1998;92(3): 778-783.

Dibbert B, Weber M, Nikkkolaizzik W.H, Vogt P, Schöni MH, Blaser K, Simon H-U. Cytokine-mediated Bax deficiency and consequent dalayd neutrophil apoptosis: A general mechanism to accumulate effector cells in inflammation. Proc Nat. Acad Sc. USA 1999;96(23):13330-13335.

Domachowske JB, Dyer KD, Adams AG, Leto TL, Rosenberg HF. Eosinophil cationic protein/Rnase 3 is another A-family ribonuclease with direct antiviral activity. Nucl Acids Res 1998;26:3358-3363.

Fernvik E, Grönneberg R, Lundahl J, Hed J, Andersson O, Johanson SGO, Halldén G. The degree of natural allergen exposure modifies eosinophil activity markers in the circulation of patients with mild asthma. Allergy 1996;51:697-705.

Fernvik E, Halldén G., Lundahl J, Raud J, Alkner U, van Hage-Hamsten M. and Grönneberg, R. Allergen induced accumulation of eosinophils and lymphocytes in skin chambers is associated with increased levels of IL-4 and sVCAM-1. Allergy 1999:I;54:455-463.

Fernvik E, Grönneberg R, Lundahl J, Raud J, Zetterström O, van Hage-Hamsten M, Halldén G. Characterisation of eosinophils and detection of eotaxin in skin chamber fluid after challenge with relevant allergen in patients with mild asthma. Clin Exp Allergy 1999:II;29:1515-1525.

Fernvik E, Lundahl J, Halldén G. The impact of eotaxin- and IL-5-induced adhesion and transmigration on eosinophil activity markers. Inflammation. 2000;24(1):73-87.

Fisher GH, Rosenberg FJ, Dale JK, Middleton LA, Lin AY, Strober W, Lenardo MJ, Puck JM. Dominant interfering Fas gene mutations impair apoptosis in human autoimmune lymphoproliferative syndrome. Cell 1995;81:935-946.

Gavioli R, Risso A, Smilovich D, Baldissarro I, Capra CM, Bargellesi A, Cosulich EM. CD69 molecule in human neutrophils. Its expression and role in signal-transducing mechanisms. Cell Immunol 1992;142:18-96.

Giembycz MA, Lindsay M. Pharmacology of the eosinophil. Pharmacological reviews. 1999;51(2):213-339.

Goreman AM, Orrenius S, Ceccatelli S. Apoptosis in neuronal cells: role of caspases. NeroReport 1998;9(10):49-55.

Goulding NJ, Guyre PM. Glucocorticoids. Lipocortins and the immune response. Curr Opin Immunol 1993 Feb;5(1):108-113.

Griffin E, Håkansson L, Formgren H, Jörgensen K, Peterson C, Venge P. Blood eosinophil number and activity in relation to lung function in patients with asthma and with eosinophilia. J Allergy Clin Immunol 1991;87:48-57.

Gupta S. Molecular steps of cell suicide: An insight into immune seneescence. J Clin Immunol 2000;20(4):229-239.

Halldén G, Andersson U, Hed J, Johansson SGO. A new membrane permeabilization method for the detection of intracellular antigens by flow cytometry. J Immunol Methods 1989;124:103-109

Halldén G, Hed J. The FOG-method. A new approach to detecting activated eosinophils. ACI News 1993;5:139-143.

Hansel T, de Vries J, Iff T, Rihs S, Wandzilak M, Betz S, Blaser K, Walker C. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. J Immunol Methods 1991;415:105-110.

Hara T, Jung LKL, Bjorndahl JM, Fu SM. Human T cell activation III. Rapid induction of phosphorylated 28 kd/32 kd disulfide-linked early activation antigen (EA 1) by 12-0-tetradecanoyl phorbol-13-acetate, mitogens and antigens. J Exp Med 1988;164:1988-2005.

Hargreave FE, Popov T, Kidney J, Dolovich J. Sputum measurements to assess airway inflammation in asthma. Allergy 1993;48:81-83.

Hartnell A, Robinson DS, Kay AB, Wardlaw AJ. CD69 is expressed by human eosinophils activated *in vivo* in asthma and *in vitro* by cytokines. Immunology 1993;80:281-286.

Haslett C. Granulocyte apoptosis and its role in resolution and control of lung inflammation. Am J Respir Crit Care Med 1999;160:5-11.

Hed J, Halldén G. Counts of activated blood eosinophils for monitoring asthma. Allergy 1993;48:87-93.

Hoffmann-Sommergruber K, Susani M, Ferreira F, Jertschin P, Ahorn H, Steiner R, et al. High-level expression and purification of the major birch pollen allergen, Bet v 1. Protein Expr Purif 1997;9:33-39.

37

Irani AM, Huang C, Xia HZ, Kepley C, Nafie A, Fouda ED, Craig S, Zweiman B, et al. Immunohistochemical detection of human basophils in late-phase skin reactions. J Allergy Clin Immunol 1998;101:354-362.

Jahnsen FL, Brandtzaeg P, Halstensen TS. Monoclonal antibody EG2 does not provide reliable immunohistochemical discrimination between resting and activated eosinophils. J Immunol Methods 1994;175:23-36.

Janson C, Herala M. Blood eosinophil count as riskfactor for relapse in acute asthma. Resp Med 1992;86:101-104.

Johansson SGO, Hourihane JOB, Bousquet J, Bruijzeel-Koomen C, Dreborg S, Haahtela T, Kowalski ML, Mygind N, Ring J, van Cauwenberge P, van Hage-Hamsten M, Wüthrich B. A revised nomenclature for allergy. Allergy 2001;56:813-824.

Julius P, Luttman W, Knoechel B, Kroegel C, Matthys H, Virchow JC Jr. CD69 surface ecpression on human lung eosinophils after segmental allergen provocation. Eur Respir J 1999;13(6):1253-1259.

Kay AB, Ying S, Durham SR. Phenotype of cells positive for interleukin-4 and interleukin-5 mRNA in allergic tissue reactions. Int Arch Allergy Immunol 1995;107:208-210.

Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer 1972;26:239-257.

Kim JT, Gleich GJ, Kita H. Roles of CD9 molecules in survival and activation of human eosinophils. J Immunol 1997;159:926-933.

Kirby J, Hargreave F, Gleich G, O´Byrne P. Brochoalveolar cell profiles of asthmatic and nonasthmatic subjects. Am Rev Respir Dis 1987;136:379-383.

Kita H, Ohnishi T, Okubo Y, Weiler D, Abrams JS, Gleich GJ. Granulocyte/macrophage colony-stimulating factor and interleukin 3 release from human peripheral blood eosinophils and neutrophils. J Exp Med 1991;174:745-8.

Kita H, Kaneka M, Bartemes KR, Weiler DA, Schimmimg AW, Reed AE, Gleich GJ. Does IgE bind to activated eosinophils from patients with allergy?. J Immunol 1999;162:6901-6911.

Kroegel C, Liu MC, Hubbard CW, Lichtenstein LM, Bochner BS. Blood and bronchoalveolar eosinophil in allergic subjects after segmental antigen challenge: surface phenotype, density heterogeneity, and prostanoid production. J Allergy Clin Immunol 1994;93:725-34.

Kuwasaki T, Chihara J, Kayaba H, Kamata Y, Oyamada H, Saito N, Shioya T, Sasaki M, Kagaya M, Tsuda A. Whole blood flow-cytometric analysis of eosinophil EG2 expression

as a marker of the pathological conditions of asthma. Int Arch Allergy Immunol 1998;117(suppl 1):77-80.

Lee A, Whyte BLC, Haslett C. Prolonged in vitro life-span and functional longevity of neutrophils induced by inflammatory mediators acting through inhibiting apoptosis. J Leukocyte Biol 1993;54:283-288.

Lehrer RI, Szklarez D, Barton A, Ganz T, Hamann , Gleich GJ. Anti-bacterial properties of eosinophil major basic protein (MBP) and eosinophil cationic protein (ECP). J Immunol 1989;142:4428-4434.

Lenartowicz E, Bernardi P, Azzone GF. Phenylarsine oxide induces the cyclosporin A-sensitive membrane permeability transition in rat liver mitochondria. J Bioenerg Biomembr 1991; 23: 679-688.

Lichtenstein LM, Bochner BS. The role of basophils in asthma. Annals of the New York Academy of Science 1991;629:48-61.

Lundahl J, Halldén G, Hed J. Differences in intracellular pool and receptor-dependent mobilization of the adhesion-promoting glycoprotein Mac-1 between eosinophils and neutrophils. J Leukoc Biol 1993;53:336-341.

Lundahl J, Moshfegh A, Grönneberg R, Halldén G. Eotaxin increases the expression of CD11b/CD18 and adhesion properties in IL-5 but not fMLP prestimulated human peripheral blood eosinophils. Inflammation 1998;22(2):123-135.

Luttmann W, Knoechel B, Foerster M, Matthys H, Virchow Jr JC, Kroegel C. Activation of human eosinophils by IL-13. Induction of CD69 surface antigen, its relationship to messenger RNA expression, and promotion of cellular viability. J Immunol 1996;157:1678-1683.

Martinou JC, Desagher S, Antonsson B. Cytochrome c release from mitochondria: all or nothing. Nature Cell Biol 2000;2:41-43.

McLaren DJ, McKean, Olsson I, Venge P, Kay AB. Morphological studies on the killing of schistosomula of Schistosoma Mansoni by eosinophil and neutrophil cationic proteins in vitro. Parasite Immunol 1981;3:359-373.

Meagher LC, Cousin JM., Seckl JR., Haslett, C. Opposing effect of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. J Immunol 1996;156:4422-4428.

Moqbel R, Ying S, Barkans J, Newman TM, Kimmitt P, Wakelin M, Taborda-Barata L, Meng, et al. Identification of messenger RNA for IL-4 in human eosinophils with granule localization and release of the translated product. J Immunol 1995;155:4939-4947.

Nakajima H, Loegering DA, Kita H, Kephart GM, Gleich GJ. Reactivity of monoclanal antibodies EG1 and EG2 with eosinophils and their granule proteins. J Leukoc Biol 1999;66(3):447-454.

Nicholson DW, Thornberry NA. Caspases: killer proteases. Trends Biochem. Sci 1997;22, 299-306.

Nishikawa K, Morii T, Ako H, Hamada K, Saito S, Narita N. *In vivo* expression on lung eosinophils in eosinophilic pneumonia: CD69 as a possible activation marker for eosinophils. J Allergy Clin Immunol 1992;90:169-174.

Nopp A, Lundahl J, Halldén G. Quantitative, rather than qualitative, differences in CD69 upregulation in human blood eosinophils upon activation with selected stimuli.
Allergy 2000;55(5):148-156.

Papa S, Skulachev VP. Reactice oxygene species, mitochondria, apoptosis and aging. Moll Cell Biochem 1997;174:305-319.

Peachman KK, Lyles DS, Bass DA. Mitochondria in eosinophils: Functional role in apoptosis but not respiration. Proc Natl Acad Sci USA 2001; 98: 1717-1722.

Petit PX, Zamzami N, Vayssiere JL, Mignotte B, Kroemer G, Gastedo M. Implication of mitochondria in apoptosis. Mol Cell Biochem 1997; 174:185-188

Rothenberg ME. Eotaxin. An essential mediator of eosinophil trafficking into mucosal tissue. Am J Respir Cell Mol Biol 1999;21:291-295.

Roquet A, Halldén G, Ihre E, Hed J, Zetterström O. Eosinophil activity markers in peripheral blood have a high predictive value for bronchial hyperreactivity in patients with suspected mild asthma. Allergy 1996;51:482-486.

Sandström K, Håkansson L, Lukinius A, Venge P. A new method to study apoptosis in eosinophils by flow cytometry. J Immuno Methods 2000:240;55-68.

Schleimer R.. Effects of glucocorticoids on inflammatory cells relevant to their therapeutic applications in asthma. A. Re. Respi Dis 1990;141:59-69

Schleimer R, Sterbinsky SA, Kaiser J, Bickel CS, Klunk DA, Tomioka D, Newman W, luscinskas FW, Gimbrone MA Jr, McIntyre, et al. IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1. J Immunol 1992;148:1086-1092.

Sieff CA, Niemeyer CM, Faller DV. Human colony-stimulating factors and stromal cell function. Soc Gen Physiol Ser 1988;43:47-55.

Skedinger M, Halldén G, Lundahl J, et al. Eosinophil activity reflects clinical status in patients with asthma before and during a prednisolone course. Annals Allergy Asthma Immunol 1995;75:250-255.

Stridh H, Fava E, Single B *et al.* Tributyltin-induced apoptosis requires glycolytic ATP production. Chem Res Toxicol 1999; 12: 874-882.

Tai PC, Spry CJF, Peterson C, Venge P, Olsson I. Monoclonal antibodies distinguish between storage and secreted forms of eosinophil cationic protein. Nature 1984;309:182-4.

Tai PC, Sun L., Spry CFJ. Effects of IL-5, GM-CSF and IL-3 on survival of human blood eosinophils in vitro. Clin Exp Immuno 1992; 85:312-316.

van Hage-Hamsten M, Kronqvist M, Zetterström O, Johansson E, Niederberger V, Vrtala S, Grönlund H, Grönneberg R, Valenta R. Skin test evaluation of genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1: Results obtained with a mix of two recombinant Bet v 1 fragments and rBet v 1 trimer in a Swedish population before the birch pollen season. J Allergy Clin Immunol 1999;104(5):969-972.

Vayssiere JL, Petit PX, Risler Y, Mignotte B. Commitment to apoptosis is associated with changes in mitochondrial biogenesis and activity in cell lines conditionally immortalized with simian virus 40. Proc Natl Acad Sci 1994;91:11752-11756.

Venge P. Serum measurements of eosinophil cationic protein (ECP) in bronchial asthma. Clin Exp Allergy 1993;23:3-7.

Venge P. Eosiniphils. In: Barnes P, Rodger IA, Thomson NC, eds. Asthma: basic mechanisms and clinical management, 3rd edn. London, Academic Press, 1998:141-157.

Venge P, Byström J, Carlson M, Håkansson L, Karawacjzyk M, Peterson C, Sevéus L, Trulson A. Eosinophilic cationic protein (ECP): molecular and biological properties and the use of ECP as a marker of eosinophil activation in disease. Clin Exp Allergy 1999;29:1172-1186.

Virchov JC Jr, Walker C, Hafner D, Kortsik C, Wermer P, Matthy H, Kroegel C. T cells and cytokines in bronchoalveolar lavage fluid after segmental allergen provocation in atopic asthma. Am J Respir. Crit Care Med 1995;151(4):960-968.

Vowels BR, Rook AH, Cassin M, Zweiman B. Expression of interleukin-4 and interleukin-5 mRNA in developing cutaneous late phase reactions. J Allergy Clin Immunol 1995;96:92-96.

Vrtala S, Hirtenlehner K, Vangelista L, Pastore A, Eichler HG, Sperr WR, Valenta P, Ebner C, Kraft D, Valenta R. Conversion of the major birch pollen allergen, Bet v 1, into two non-anaphylactic T cell epitope-containing fragments: candidates for a novel form of specific immunotherapy. J Clin Invest 1997;99:1673-1681.

Vrtala S, Hirtenlehner K, Susani M, Hufnagl P, Binder BR, Vangelista L, et al. Genetic engineering of recombinant hypoallergenic oligomers of the major pollen allergen, Bet v 1: candidates for specific immunotherapy. Int Arch Allergy Immunol 1999;118:218-219.

Walsh GM, Williamson ML, Symon FA, Willars GB, Wardlaw AJ. Ligation of CD69 induces apoptosis and cell death in human eosinophils cultured with granulocyte-macrophage colony-stimulating factor. Blood 1996;87:2815-2821.

Wardlaw AJ, Dunnette S, Gleich GJ, Collins JV, Kay AB. Eosinophils and mastcells in bronchoalveolar lavage in subjects with mild asthma. Am Rev Respir Dis 1988;137:62-69.

Wardlaw AJ. Eosinophils in the 1990s: New perspectives on their role in health and disease. Postgrad Med J 1994,70:536-552.

Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 1992;356:314-317.

Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980:68:386-410.

Yamada N, Wakugawa M, Kuwata S, Yoshida T, Nakagawa H. Chronologic analysis of in situ cytokine expression in mite allergen-induced dermatitis in atopic subjects. J Allergy Clin Immunol 1995;96:1069-1075.

Ying S, Robinson DS, Meng Q, Barata LT, McEuen AR, Buckley MG, Walls AF, Askenase PW, Kay AB. C-C chemokines in allergen-induced late-phase cutaneous responses in atopic subjects: association of eotaxin with early 6-hour eosinophils, and of eotaxin-2 and monocyte chemoattractant protein-4 with the later 34-hour tissue eosinophilia, and relationship to basophils and other C-C chemokines (monocyte chemoattractant protein-3 and RANTES). J Immunol 1999;163:3976-3984.

Young JD, Peterson CG, Venge P, Cohn ZA. Mechanisms of membrane damage mediated by human eosinophil cationic protein. Nature 1989;321:613-616.

Zamzani N, Marchetti P, Castedo M, Zanin C, Vayssiere JL, Petit PX, Kroemer G. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphoid death in vivo. J Exp Med 1995;181:1661-1672.

Zweiman B, Kaplan AP, Tong L, Moskovitz BS. Cytokine levels and inflammatory responses in developing late-phase reactions in the skin. J Allergy Clin Immunol 1997;100:104-109.

Ädelroth E, Rosenhall L, Johansson SÅ, Linden M, Venge P. Inflammatory cells and eosinophilic activity in asthmatics investigated by brochoalveolar lavage. Am Rev Respir Dis 1990;142:91-99.