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**The biological mechanisms in neutrophil and eosinophil
adhesion and transmigration *in vitro* and their relation to the
inflammatory process *in vivo***

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ABSTRACT

In inflammatory reactions, eosinophils and neutrophils constitute the major classes of granulocytes that emigrate from the bloodstream into the tissues. The recruitment of inflammatory cells constitutes a multi-step process that includes sequential adhesion and transmigration through, the endothelial lining, the underlying basement membrane and a variety of extracellular matrix components. This thesis examines the adhesion and transmigration properties of neutrophil and eosinophil during different inflammatory processes.

We have investigated the *in vitro* effect of eotaxin on human peripheral blood eosinophils (PBE) with respect to CD11b/CD18 expression and adhesion properties to the matrix protein fibronectin. We demonstrate that eotaxin is involved in the quantitative up-regulation of CD11b/CD18 expression and increases the adhesion properties in primed, but not resting, human eosinophils. We hypothesise that eotaxin and IL-5, but not fMLP, act synergistically in these respects.

The adhesion and transmigration assays were set up to simultaneous analysis of eosinophil and neutrophil adhesion and transmigration in mixed granulocyte cell populations, based on analysis of eosinophil cationic protein (ECP) and myeloperoxidase (MPO) as markers for eosinophils and neutrophils, respectively. These models can be useful tools in exploring the mechanisms whereby neutrophils and eosinophils integrate chemotactic signals, communicate and to evaluate the impact of this communication on the overall leukocyte accumulation at disparate inflammatory sites.

We examined the capacity of circulating neutrophils, collected before, during and after hemodialysis with cuprophan, low- and high-flux polysulfone dialyzers, to transmigrate through a fibronectin covered membrane *in vitro*. The high-flux polysulfone treatment, as opposed to low-flux and cuprophan dialyzers, ameliorates the transmigration properties of circulating neutrophils, despite similar effects on adhesion molecule phenotypes.

Eosinophils in the neonatal period possess an enhanced responsiveness against the bacterial-related peptide fMLP, as judged by enhanced transmigration capacity and CD11b up-regulation, presumably due to the ability to express the fMLP receptor. We therefore suggest that eosinophils in the neonatal period represent a first line of cellular defence that might be triggered by bacterial antigen stimulation initiated by the early colonisation of the surfaces of the mucosa.

We demonstrate that PBE in allergic, but not in healthy children possess an increased spontaneous, as well as eotaxin-induced capacity to transmigrate *in vitro*. This increased capacity is further enhanced within 2 hours after an allergen challenge *in vivo* without accompanying signs of eosinophil activation in terms of increased PBE counts or ECP levels. These observations are probably related to the phenomenon designated "priming".

LIST OF PUBLICATIONS

1. Lundahl J, **Moshfegh A**, Grönneberg R, Halldén G. Eotaxin increases the expression of CD11b/CD18 and adhesion properties in IL5, but not fMLP-prestimulated human peripheral blood eosinophils. *Inflammation* 1998;22:123-135.
2. **Moshfegh A**, Halldén G, Lundahl J. Methods for simultaneous quantitative analysis of eosinophil and neutrophil adhesion and transmigration. *Scand J Immunol* 1999;50:262-269.
3. **Moshfegh A**, Jacobson SH, Halldén G, Thylén P, Lundahl J. Impact of hemodialysis membrane and permeability on neutrophil transmigration *in vitro*. *Nephron*, in press.
4. **Moshfegh A**, Gunilla Halldén, Carina Luthian, Goanna Marchini, Hugo Lagercranz and Joachim Lundahl. Neonates eosinophils possess an efficient fMLP-induced transmigration capacity. Submitted.
5. Karin Lönnkvist*, **Moshfegh A***, Gunilla Hedlin, Gunilla Halldén and Joachim Lundahl. Eotaxin induces a time dependent transmigration of peripheral blood eosinophils from asthmatic children after allergen provocation. Submitted.

These authors have contributed equally to the manuscript

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LIST OF ABBREVIATIONS

CD	Cluster differentiation
CTAB	N-cetyl-N,N,N-trimethylammuniom bromide
EDTA	Ethylene diaminetetraacetic acid
ECP	Eosinophil cationic protein
FITC	Fluorescein isothiocyanate
fMLP	N-Formyl-methionyl-leucyl-phenylalanine
FOG	Fixation and membrane permeabilisation with OG
IL-	Interleukin-
LTC ₄	Leukotriene C ₄
MFI	Mean fluorescence intensity
MPO	Myeloperoxidase
OG	n-octyl- β -D-glucopyranoside
PAF	Platelet activating factor
PBE	Peripheral blood eosinophils
PBS	Phosphate-buffered saline
RIA	Radioimmuno-assay

1 INTRODUCTION

The inflammatory response is essential for all organisms in the defence against microorganisms, as well as for tissue repair. The definition of inflammation is based upon the classical clinical signs including pain, heat, redness, swelling and loss of function. These symptoms all reflect the effects of inflammatory mediators, e.g. cytokines and complement fragments, on local blood vessels by induction of dilation and increased permeability. Cytokines have important effects on the adhesive properties of the endothelium with initial adhesion of circulating leukocytes to the endothelial cells of the blood vessel wall. Subsequently, attached leukocytes migrate through the endothelial cells to the site of inflammation to which they are attracted by other inflammatory mediators.

The immune response to infectious challenge is divided into two constituents, the innate and the adaptive response, aimed at eliminating the intruding pathogen. The phagocytes of the innate immune system provide a first line of defence against many common microorganisms and are essential in controlling common bacterial infections. However, they cannot always eliminate the infectious organisms and there are many pathogens not recognized by phagocytes. The lymphocytes, of the adaptive immune system have evolved to provide more versatile means of defence that, in contrast to the innate immune system, provide improved protection from a subsequent re-infection with the same pathogen. Due to the delay, of 4-7 days, before the initial adaptive immune response is fully activated, the innate immune response has a critical role in controlling infections during this period.

1.1 INFLAMMATORY CELLS

The inflammatory cells arise from pluripotent stem cells through two main lines of differentiations. The myeloid lineage which consists of precursors for granulocytes and macrophages, and the lymphoid lineage which gives rise to lymphocytes.

Injury to tissue and induction of an inflammatory response may be caused by a number of factors including mechanical, physical, biological, chemical and immunological triggers, resulting in accumulation of effector cells and serum proteins at the inflammatory site (Gallin et al., 1988). It is well recognised that effector cells accumulate in a sequential order at the local inflammatory site. In the initial phase, mainly polynuclear cells, e.g. neutrophils and eosinophils, accumulate, followed by mononuclear cells, e.g. monocytes and lymphocytes (Fiebig et al., 1991; van Furth et al., 1973; Haslett et al., 1988).

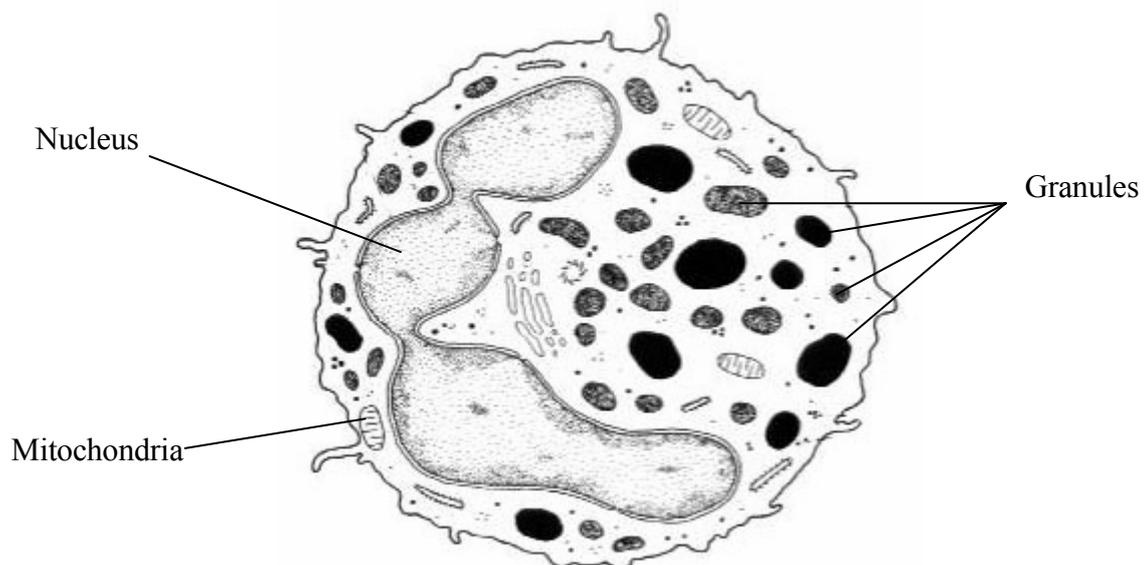
1.2 NEUTROPHILS

Neutrophils belong to the group of leukocytes designated granulocytes (figure 2). They develop and mature in the bone marrow and are then released into the blood circulation. Neutrophils circulate in the blood stream for about 10 hours before migrating into the tissue where they remain functional for 1-2 days (Gallin, 1988). After transmigration through the endothelium, the neutrophil moves directly to the site of the bacterial invasion or tissue trauma (Albelda et al., 1994). The translocation of neutrophils from the blood to the inflammatory site depends on a directed migration along a gradient of chemical mediators called chemoattractants. A number of extensively characterised molecules have been reported to function as chemoattractants. These include bacterial peptides, e.g. fMLP (Everitt et al., 1996), products of the complement cascade, e.g. C3a, C5a (Movat 1987), secreted products of stimulated phospholipid metabolism e.g. PAF and LTB₄ (Krump and Borgeat, 1999), and cytokines, e.g. IL-8 (Follin et al., 1991).

Foreign material at an inflammatory site is usually phagocytosed by neutrophils. The phagocytic process is divided into two steps. First, binding of the prey to the cell surface and secondly, engulfment followed by intracellular killing (Wright, 1992). There are several receptors on the neutrophil surface, which mediate the binding and ingestion of the intruder. These receptors are divided into two groups: non-opsonic, which interact directly with structures on the target surface (Greenberg, 1995), and opsonin-dependent, that operate when

targets are coated with opsonins; the latter permitting a more effective attachment and ingestion (Henson et al 1988). The most well known receptors mediating opsonin-dependent phagocytosis are Fc γ receptors (Fc γ R) and complement receptors (CR) which bind to the Fc part of IgG and complement factors C3b/C3bi respectively. There are predominantly two kinds of Fc γ Rs expressed on neutrophils, namely Fc γ RII (CD32) and Fc γ RIII (CD16) where the most abundantly expressed complement receptors are CR1 (CD35) and CR3 (CD11b/CD18) (Rosales and Brown, 1993). Ligation of these receptors induces the formation of an intracellular vesicle, a so-called phagosome. Various populations of granules fuse with the phagosome to form a phagolysosome (Gallin, 1988). Neutrophils contain four types of granules, which are mobilised in a strict sequence upon cell activation. Primary (azurophilic) granules, containing myeloperoxidase (MPO), serin proteases, β -glucuronidase and bacterial cationic protein and secondary (specific) granules which contain receptors for extracellular matrix proteins. Furthermore, there are tertiary granules with gelatinase and cytochrome B and finally the fourth type, or secretory vesicles, comprising CR3 and CR1.

Figure 2



1.3 EOSINOPHILS

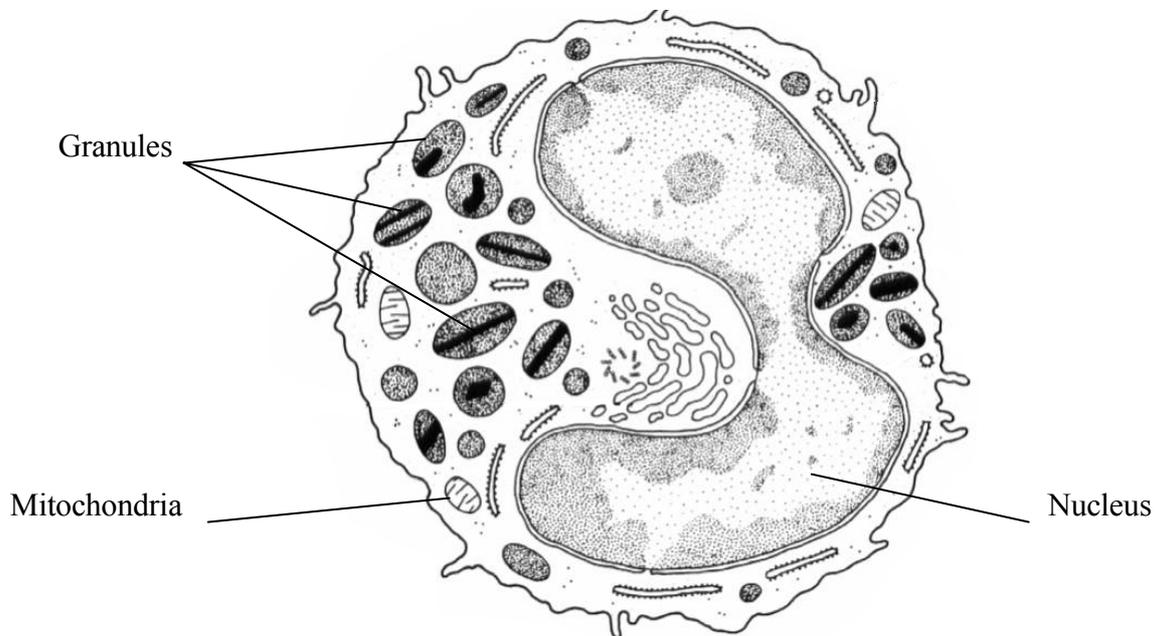
The eosinophils, (figure 2) are like to neutrophils, bone marrow-derived granulocytes. They constitute 1-5% of the leukocyte population in the circulation, and have a lifetime about 3-4 days in blood, whereas in tissue eosinophils survive for 1-3 weeks. This time span depends upon the state of activation and presence of IL-5, IL-3 and GM-CSF which prolongs survival and prevents apoptosis (Venge 1992; Tai et al., 1991; Yamaguchi et al., 1991). The majority of eosinophils reside in tissue primarily in the respiratory tract, gut, and urogenital subepithelium. This implies involvement in host defence against invading microbes especially the killing of parasites. The effector functions of eosinophils are of twofold. First, the release of highly toxic granule proteins stored preferentially in secondary granules. The primary granules contain lysophospholipase, associated with eosinophilic infiltrations (Dvorak et al., 1988), and the secondary, or specific granules, contain major basic protein (MBP), eosinophils cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil protein X or eosinophil derived neurotoxin (EPX/EDN) (Spry et al., 1988; Frigas et al., 1991).

The second effector function of the eosinophil is to produce and release inflammatory mediators such as PAF (Blom et al., 1991), LTC₄ (Blom et al., 1991), IL-5 (Dubucqoui et al., 1994, Lundahl et al., 1999) and eotaxin (Rothenberg, 1999), which amplify the inflammatory response by recruiting and activating leukocytes and epithelial cells.

The regulatory mechanisms of activation and degranulation of eosinophils occur in three stages. The first phase is the regulation of proliferation and differentiation in the bone marrow, which is consistent and occurring at a low level in the absence of immunological stimulation. When Th2 lymphocytes are activated to produce cytokines, such as IL-5, this increases the production of eosinophils in the bone marrow and promotes release of these cells into the circulation (Clutterbuck & Sanderson 1988). The second step is the migration of eosinophils from the circulation to various tissues. The migration of eosinophils depends on chemoattractants such as eotaxin, which is an eosinophil specific chemokine (Rothenberg

1999). The third step comprises the release of granule proteins, which occurs when eosinophils become exposed to soluble mediators for example PAF (Venge 1992), immunocomplexes (Takenaka et al., 1977) or solid particles (Winqvist et al 1984).

Figure 2



1.4 ADHESION MOLECULES

For the leukocytes to leave the blood stream and accumulate at the inflammatory site, they need to attach physically to the endothelium and extracellular matrix. This process involves several steps in which leukocyte transmigration starts with rolling along the endothelial lining. This is a selectin-based interaction intended to reduce the velocity relative to the blood flow (Atherton & Born 1972; McEver 1994; Tedder et al., 1995). The presence of chemotactic mediators, directed towards the rolling leukocytes, induces activation and adherence of cells to the vascular endothelium. After an allotted time period, leukocytes begin to crawl through interendothelial junctions and transmigrate into extracellular matrix, a process mediated by integrins and immunoglobulin-family receptors (Kavanaugh, 1997; Patarrayo et al., 1990; Springer, 1994; Carlos & Harlan, 1994) (Table 1).

Table 1. Example of adhesion molecules receptors and their counter receptors

Receptor	Counter receptor
Selectins	
L-selectin (CD62L)	mannose/fructose-6-phosphate
P-selectin (CD62P)	Sialyl-Lewis ^x /Lewis ^x (CD15)
Integrins	
$\beta_1\alpha_4$ (CD49d/CD29, VLA-4)	Fibronectin, VCAM-1
$\beta_2\alpha_M$ (CD11b/CD18, CR3)	ICAM-1
Immunoglobulin super family	
ICAM-1	CD11b/CD18, CD11a/CD18
VCAM-1	CD49d/CD29

1.5 SELECTINS

The selectin family consists of three lectin-binding domain carbohydrates molecules designated L- (CD62L), P- (CD62P), and E-selectin (CD62E).

L-selectin is expressed on most circulating leukocytes (Jutilla et al., 1989; Kishimoto et al., 1991; Spertini et al., 1991) and on granulocytes it is involved in the initial attachment, e.g. rolling, along the endothelium (Ley et al., 1991; Zimmerman et al., 1992). Following cell activation, L-selectin is rapidly shed from the cell surface (Jutilla et al., 1989; Kishimoto et al., 1991).

P-selectin is stored in endothelial cells and platelets (McEver et al., 1989). A few minutes after stimulation with inflammatory mediators such as histamine, P-selectin is upregulated on the surface of endothelial cells and is thereafter down-regulated partly through a shedding mechanism (Hattori et al., 1989; Geng et al., 1990). In certain studies soluble P-selectin has been shown to prevent neutrophil adhesion to endothelium (Gamble et al., 1990).

E-selectin is involved in adhesion of granulocytes and monocytes and is expressed on endothelial cells. The expression is induced by TNF- α , IL-1- β or LPS and reaches a maximum of expression at 4 to 6 hours (Cotran et al., 1986; Leeuwenberg et al., 1992).

1.6 INTEGRINS

Integrins are large heterodimeric proteins composed of one α -subunit in noncovalent linkage with one β -subunit, 1100 and 750 amino acids respectively (Zimmerman et al 1992; Pardi et al 1992; Imhof and Dunon 1995; Luscinskas and Gimbrone 1996; Pigott and Power 1993; McEver 1991; Gamberg et al 1997; Chothia and Jones 1997 Diamond & Springer 1994; Carlos & Harlan 1994). These adhesion receptors are usually expressed on cell surfaces and their ligands are preferentially expressed on extracellular matrix proteins. Integrins have been categorised, according to their β -subunit composition, and to date, at least 8 different β -subunits have been identified (Carlos & Harlan 1994).

The β_1 (CD29) integrin (VLA) can be found in almost all cell types. The integrin $\alpha_4\beta_1$ (CD49d/CD29, VLA-4), which is not expressed on neutrophils (Helmer & Lobb 1995), may be involved in leukocyte adhesion to the endothelium, since its counter receptor is VCAM-1 which is expressed on endothelial cells (Kitayama et al 1997).

The β_2 integrins (CD18) exclusively found on leukocytes, have three members designated $\alpha_L\beta_2$ (CD11a/CD18, LFA-1), $\alpha_M\beta_2$ (CD11b/CD18, Mac-1, CR3) and $\alpha_X\beta_2$ (CD11c/CD18, p150,95). CD11a/CD18 is predominately expressed on lymphocytes whereas CD11b/CD18 is expressed on neutrophils and eosinophils, and CD11c/CD18 on monocytes (Rampart 1994).

It has been proposed that integrins play a major role in allergic disease especially CD11a/CD18 and CD49d/CD29. These receptors are involved in adhesion and transendothelial migration of T-lymphocytes and eosinophils (Springer 1990; Albelda & buck 1990; Mackay & Imhof 1993; Carlos & Harlan 1994). T-lymphocytes are proposed to be important conductors in allergic disease and eosinophils seem to play a more direct pathophysiologic role by their release of basic proteins. Moreover, the integrins also act as co-stimulatory molecules for T-lymphocytes which provide secondary signals amplifying a more specific and pronounced immunological response (Kavanaugh 1997).

1.7 GLYCOPROTEINS OF THE IMMUNOGLOBULIN SUPER FAMILY

Members of this family of adhesion receptors are mainly expressed on endothelial cells and their counter receptors are preferentially members of the integrin family, e.g. ICAM-1, VCAM-1 etc. (Stanson et al., 1989; Elices et al., 1990).

1.8 CYTOKINES AND CHEMOKINES

Cytokines are soluble proteins, which are released from a vast number of cells. Most of the cytokines exert local actions, which synergise with those of membrane-bound effector molecules resulting in combinatorial effects.

Cytokines can be grouped into families based on their structure:

1. Hematopoietins
2. Interferons
3. Chemokines
4. Tumor necrosis family

Chemokines are thought to provide the directional signals for the movement of leukocytes in tissue development, homeostasis and inflammation. Accumulation at inflammatory loci requests leukocytes to interact in a sequential order with the endothelium, the basement membrane and a variety of extracellular matrix components. Chemokines provide the signals that convert low-affinity, selectin mediated interaction leading to extravasation of leukocytes.

Chemokines are 8 to 10 kd proteins with 20 to 70 percent homology in amino acid sequences. They have been subdivided into families on the basis of the position of cysteine residues (Baggiolini et al 1997 Idem 1997). There are at least four families of chemokines, where two have been more characterised, the α and β chemokines (table 2).

Table 2. Example of chemokine family, chemokine and their receptor

Chemokine family	Chemokine	Receptor
CC (α)	Eotaxin, RANTES	CCR3
C (β)	Lymphotactin	?
CXC (β)	SDF-1	CXCR4
CXXXC (β)	Fracalkinet	CX ₃ CR1

2 AIM OF THE STUDY

The main purpose of these studies was to investigate biological mechanisms of neutrophil and eosinophil adhesion, transmigration *in vitro*, and to relate these findings to their function in inflammatory processes *in vivo*.

Specific aims:

- [I] To study the *in vitro* effect of recombinant human (rh) eotaxin on human peripheral blood eosinophils with respect to CD11b up-regulation and adhesion properties of resting as well as *in vivo* and *in vitro* primed eosinophils.
- [II] To describe *in vitro* methods for simultaneous analysis of eosinophil and neutrophil adhesion and transmigration in mixed granulocyte cell populations. Analysis were based on detection of eosinophil cationic protein (ECP) and myeloperoxidase (MPO) as markers for eosinophils and neutrophils, respectively.
- [III] To evaluate the influence of various biocompatible dialyzers, serving as a model for systemic inflammation, on the capacity of neutrophils and eosinophils, collected during hemodialysis, to transmigrate *in vitro*. Furthermore, we also addressed the question whether *in vivo* changes in quantitative expression of adhesion molecules correlated to the transmigration capacity *in vitro*.
- [IV] To study the *in vitro* transmigration capacity of neonatal and adult eosinophils in response to the bacterial-related peptide fMLP, as well as chemoattractants related to allergic inflammation, namely interleukin-5 and eotaxin. Moreover, we also assessed the ability to up-regulate CD11b upon fMLP-stimulation and the surface expression of the fMLP-receptor.

- [V] To determine whether eosinophils from cat-sensitised children possessed an increased state of activation by comparing PBE from both allergic and non-allergic children and analysing the eosinophil transmigration capacity *in vitro* before and after nasal challenge with cat allergen.

3 MATERIALS AND METHODS

3.1 SUBJECTS AND STUDY DESIGNS [I, II, III, IV, V]

All studies included healthy individuals enrolled at the Blood Donation centre, at the Karolinska Hospital, except for study V in which healthy children from Astrid Lindgren Hospital participated.

3.1.1 [I]

Six patients with pollen-related asthma, five non-smokers and one former smoker were included in the study (Table 3). They had a positive history of allergic rhinitis and occasional asthma symptoms. All exhibited a positive skin prick test (SPT) and RAST test (Pharmacia CAP system, Uppsala, Sweden) to birch and / or timothy pollen and the majority also to cat dander. Bronchial inhalation was routinely performed with histamine and relevant allergen. The patients were symptom-free before the allergen provocation, which was performed outside season. Only inhaled β 2-agonists were used for therapeutic intervention when necessary but totally withdrawn during the examination periods. None received immunotherapy. Blood samples were collected before and 24 hours following the allergen inhalation.

Table 3. Patient characterisation

	Sex	Age (year)	Spt Birch	Spt Timothy	Spt Cat	Histamin-inhalation (μ g)	Allergen-inhalation (SQ)
1	female	24	2+	3+	3+	220	1760 (timothy)
2	female	40	4+	-	2+	440	1760 (birch)
3	female	27	5+	5+	4+	440	880 (birch)
4	female	42	3+	2+	4+	220	1760-3520 (birch)
5	female	19	1+	3+	1+	110	1760 (timothy)
6	Male	30	-	3+	-	880	880 (timothy)

3.1.2 [III]

Nine hemodialysis patients, five men and four women (mean age 69 ± 10 years) undergoing maintenance hemodialysis three times a week with polysulfone dialysis membrane (F6HPS or

F7HPS Fresenius AG, Germany participated. Informed consent was obtained from all participants and the study was approved by the Ethics Committee of the Karolinska Hospital. Patients suffering from infectious diseases, diabetes mellitus, chronic lung disease or inflammatory diseases as well as patients receiving antibiotics, corticosteroids or nonsteroidal anti-inflammatory agents were excluded. The patients were treated with cuprophan, low-flux polysulfone or high-flux polysulfone dialyzers in random order (altogether 27 treatments) during this investigation as part of the routine dialysis program. Blood collected from healthy donors served as controls and run in parallel (n=9). Hemodialysis were performed for four hours with bicarbonate (34-36 mmol/L) dialysate using cuprophan (GFE 18, area 1.8m², Gambro, Sweden), low-flux polysulfone (F7HPS, 1.6 m², Fresenius AG,) or high-flux polysulfone (F60S, 1.3 m², Fresenius AG) hollow fibre dialyzers. Dialyzers were not reused. Heparin doses were individualised and given as an initial bolus dose followed by a continuous intravenous infusion until one hour before termination of treatment. Blood samples were collected prior to dialysis, at 15 minutes and at the end of treatment (four hours). Dialysis adequacy was calculated as urea reduction ratio (URR) and Kt/V.

3.1.3 [V]

Fourteen children, 8 girls and 6 boys aged between 9 and 18 (median age 13) participated in the study (Table 4). They had positive SPT for cat and a normal lung function at start. None of them had symptoms of ongoing asthma or rhinitis. After nasal challenge with cat allergen all developed symptoms of rhinitis and one girl also showed signs of bronchial obstruction and symptoms of asthma. The post-challenge symptoms were reversible within 6 hours in all patients. Four healthy non-atopic children participated in a similar procedure and none showed any symptoms from the nose or the lungs. Blood samples were drawn prior to and again at 2 and 24 hours following challenge. The study was performed out of the pollen season.

Table 4. Patient characterisation.

Patients					Control subjects			
	Sex	Age (year)	IHS (μg)	Cat Allergy	Sex	Age (Year)	IHS (μg)	Cat Allergy
1	Female	9	FL 100	Yes	Female	11	No	No
2	Female	13	FL 100	Yes	Female	11	No	No
3	Female	14	FL 100	Yes	Female	18	No	No
4	Female	13	FL 500	Yes	Male	9	No	No
5	Female	13	BU 100	Yes				
6	Female	10	BU 400	Yes				
7	Female	9	No	Yes				
8	Female	16	No	Yes				
9	Male	15	BU 100	Yes				
10	Male	16	BU 100	Yes				
11	Male	11	No	Yes				
12	Male	13	No	Yes				
13	Male	13	No	Yes				
14	Male	18	No	Yes				

3.2 PREPARATION OF BLOOD LEUKOCYTES [I, II, III, IV, V]

3.2.1 [I, III-V]

Peripheral blood from healthy non-allergic donors and asthmatics was collected in tubes containing EDTA (Vacutainer, 5 mL, with 50 μL of 21% EDTA) (Terumo, Leuven, Belgium) [I] or citrate (Vacutainer, 5 mL, with 500 μL of 0.129M Citrat-Na) (Becton-Dickinson, NJ, USA) [III]. Erythrocytes were specifically hemolysed by adding 3 mL NH_4Cl -EDTA (154 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA, pH 7.2) to 150 μL blood. The suspensions were incubated for 5 min at +15°C and centrifuged at $300 \times g$ for 5 min at +4°C. The leukocyte preparations were washed in 2 mL +4°C 0.15 M phosphate-buffered-saline (PBS), pH 7.4, supplemented with 0.1 mM EDTA and 0.02% NaN_3 (PBS-EDTA). The leukocyte pellets were kept on ice until further treatments.

3.3 GRANULOCYTE PURIFICATION [II, III, IV, V]

To purify peripheral blood granulocytes, blood was collected from patients and healthy blood donors in tubes containing citrate (Becton-Dickinson) and diluted in PBS (pH 7.4) and layered

onto isotonic Percoll solution (1.082 g/ml) (Pharmacia & Upjohn, Uppsala, Sweden). After centrifugation ($1000 \times g$, 30 minutes, $+20-22^{\circ}\text{C}$), the mononuclear cells at the interface were removed and the erythrocytes, neutrophils and eosinophils in the pellet were collected. Erythrocytes were hemolysed by adding 40 mL $\text{NH}_4\text{Cl-EDTA}$, incubated for 7 min at $+15^{\circ}\text{C}$ and centrifuged at $300 \times g$ for 12 min at $+4^{\circ}\text{C}$. The granulocyte preparations were thereafter washed in 2 mL PBS ($+4^{\circ}\text{C}$) and finally resuspended in 2 mL PBS. The number of granulocytes was determined by flow cytometry and adjusted to 0.10×10^6 granulocytes/mL for adhesion and 0.15×10^6 granulocytes/mL for transmigration. The FOG-method was used to calculate the number of eosinophils and neutrophils in mixed granulocyte populations by flow cytometry.

3.4 EOSINOPHIL PURIFICATION [I]

To purify eosinophils, blood was collected from healthy blood donors in EDTA tubes. The first step of purification were performed as above. The purified granulocytes were washed twice ($300 \times g$, at $+4^{\circ}\text{C}$) in PBS-EDTA before addition of anti-CD16 magnetic particles (Miltenyi, Biotec). After incubation for 25 min at $+4^{\circ}\text{C}$, the cell and particle suspension was layered on top of a separation column in a magnetic field. Magnetically CD16-labelled cells (neutrophils) were trapped into the column and unlabelled cells (eosinophils) were collected and washed in PBS-EDTA ($300 \times g$, 6 min, $+4^{\circ}\text{C}$) prior to the adhesion procedure.

3.5 IN VITRO STIMULATION [I, III, IV, V]

3.5.1 In vitro incubation of blood leukocytes with human IL-5, human eotaxin [I, IV, V] and fMLP [I, III, IV]

First we evaluated the effect of eotaxin alone on eosinophils. Peripheral blood leukocytes (150 μL blood/test tube) from healthy donors and asthmatics were resuspended in 200 μL hepes (10 mM)-buffered RPMI 1640 medium (Gibco Ltd, Paisly, Renfrewshire, U.K.) supplemented with 5% heat inactivated fetal calf serum (RPMI) alone or in the presence of 50, 100 or 200 ng rh

human eotaxin/mL (Prepro Tech Inc., Rocky Hill, NJ, USA). The cell suspensions were incubated for 15 min at +37°C and then washed with 2 mL +4°C PBS-EDTA (300 × g, 5 min). The cells were kept on ice until surface immunostaining. In the second stage, we wanted to investigate if there is any synergy between IL-5 and eotaxin and/ or between bacterial peptide fMLP and eotaxin. Peripheral blood leukocytes from non-allergic healthy donors were incubated for 60 min at +37°C in rh human IL-5 (Immunokontakt, Frankfurt, Germany) at a concentration of 100 ng/mL or for 15 min at +37°C in *N*-Formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma Chemical, Co, st. Louis, MO, USA) at 10⁻⁸ M and 10⁻⁹ M, respectively. The selection of IL-5 and fMLP concentrations used was based on previous reports of the effect on CD11b expression on human eosinophils (Lundahl et al., 1993). Leukocytes incubated in RPMI alone for 15 and 60 min respectively, served as control cells. Thereafter, prestimulated cells were incubated for another 15 min at +37°C in RPMI alone or RPMI supplemented with rh eotaxin at a final concentration of 50, 100 or 200 ng/mL.

3.6 IMMUNOSTAINING AND FLOW CYTOMETRY

3.6.1 Immunofluorescence staining of cell surface CD11b [I, III, IV], CD62L [I, III] and EG2 [V]

Untreated and *in vitro* stimulated leukocytes were incubated with phycoerythrin (PE) conjugated monoclonal antibodies to CD11b (DAKO A/S, Glostrup, Denmark) at a final concentration of 50 µg/mL for 30 min on ice and then washed once in 3 mL cold PBS-EDTA (300 × g, 6 min). In *study III*, leukocytes were incubated with FITC-conjugated monoclonal antibodies to CD62L (Becton Dickinson) at a final concentration of 12.5 µg/mL, and in *study V* the leukocytes were incubated with FITC-conjugated monoclonal antibodies to EG2 (MIAB, Uppsala, Sweden) at a final concentration of 40 µg/mL for 30 min on ice and then washed once in 3 mL +4°C PBS-EDTA (300 × g, 6 min). EG2 staining was done after cell membrane permeabilisation (see below).

3.7 CELL MEMBRANE PERMEABILISATION (FOG METHOD) [I, III, IV, V]

The surface and intracellular immunostained leukocytes were treated according to a previously described cell membrane permeabilisation method, the FOG method (Halldén & Hed 1993, Hed & Halldén 1993). This permeabilisation procedure was performed in order to obtain the eosinophils as a distinctly-separated population identified by their scatter properties in the flow cytometer. Briefly, the leukocyte pellets were resuspended and incubated in 200 mL phosphate-buffered 4% paraformaldehyde (PFA) (Sigma, St. Louis, MO, USA) (paraformaldehyde, 40 g/L; $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$, 16.7 g/L; NaOH, 3.86 g/L and D-glucose, 5.4 g/L; prepared at +65°C, pH 7.4) for 10 min at +20-22°C and washed in 3 mL +4°C PBS-EDTA (400 × g, 7 min). The fixed leukocytes were then permeabilized by incubation in 200 µL 0.74% *n*-octyl-D-glucopyranoside (OG) (Sigma, St. Louis, MO, USA) (dissolved in PBS, pH 7.4) for 6 min at +20-22°C. The permeabilised cells were finally washed once in 3 mL +4°C PBS-EDTA (400 × g, 7 min), resuspended in 500 µL PBS-EDTA and kept on ice until analysed.

3.8 ANALYSIS BY FLOW CYTOMETRY [I, III, IV, V]

The final preparations of immunostained FOG-treated mixed leukocytes were analysed and the non-adherent purified eosinophils were counted in an EPICS XL (Coulter, Inc., Hialeah, FL, USA) flow cytometer. In the flow cytometer the cells 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 the leukocyte populations were detected as separated cell clusters in two-parameter scatter plot histograms with logarithmic amplification. The FOG-treated eosinophils were gated by setting a frame around the leukocyte population with preserved high scatter signals. A minimum of 500 cells was accumulated within the eosinophil gate for analysis.

The instrument was calibrated daily with standardised 10 µm fluoropheres, Immuno-Check, (Coulter, Inc., Hialeah, FL., USA). Standard-Brite (Coulter), another fluorosphere with

controlled fluorescence intensity, was used to standardise the mean fluorescence intensity (MFI) before each experiment. If needed, the amplification of the photomultiplier tube voltages (PMT) was adjusted in order to obtain correct MFI. The fluorescent signals were expressed in logarithmic scaled histograms and a quantitation of the surface expression of CD11b, CD62L and EG2 was obtained by measuring the MFI of the positive cell population.

3.9 ADHESION ASSAY [I, II]

3.9.1 [I]

The adhesion assay was performed with purified eosinophils in 12-well culture plates (Costar, Cambridge, UK). The culture plates were coated with human plasma fibronectin (20 µg/mL) (Sigma, St. Louis, MO, USA) for two hours at +37°C, 5% CO₂ and 95% air-atmosphere. The culture wells were then washed twice with 500 µL PBS at +20-22°C and blocked with 500 µL 0.5% bovine serum albumin (BSA) for one hour at +37°C, 5% CO₂ and 95% air-atmosphere. Finally, the wells were rinsed with PBS before being air-dried at +20-22°C and thereafter eosinophils were added. Purified eosinophils (7.5×10^4) were prestimulated with either rh IL-5 or fMLP followed by incubation with rh eotaxin, as described above for the procedure of mixed blood leukocytes. The prestimulated eosinophils were resuspended in 500 µL PBS and incubated in fibronectin coated culture wells at +37°C, 5% CO₂ and 95% air-atmosphere for 5, 15 and 30 min respectively. Non-adherent cells were collected, by carefully rinsing the wells with 500 µL +37°C-RPMI 1640-medium followed by centrifugation and suspension in 500 µL PBS before being counted in the flow cytometer. The percentage adherent cells were calculated from the initial number of cells added to the wells and the number of non-adherent cells obtained. The cell recovery rate (number of adhered cells + number of non-adhered cells / number of initial cells added) in this experimental setting was > 95%.

3.9.2 [II]

The adhesion assay was performed in 24-well culture plates (Becton-Dickinson, NJ, USA), coated with human plasma fibronectin, as described above. Prepared granulocytes from non-allergic healthy donors were mixed with rh IL-5 (100 ng/mL) (Immunokontakt), and rh eotaxin (100 ng/mL) (Prepro Tech Inc.), or fMLP (10^{-8} M) (Sigma Chemical) to a final volume of 250 μ L. The cell suspensions were incubated in the fibronectin coated wells at +37°C, 5% CO₂ and 95% air-atmosphere for different time periods (15, 30, 45 and 60 min for neutrophils and 60, 90, 120 and 150 min for eosinophils). Granulocytes, incubated in RPMI 1640-medium (RPMI) alone, were used as controls. Two cell fractions were collected as follows; non-adherent cells were transferred into test tubes by careful aspiration followed by rinsing of the wells with 250 μ L RPMI (+37°C) whereas adherent cells were left in the wells and 200 μ L RPMI was added. Non-adherent cells in the test tubes and adherent cells in the culture wells were stored at -80°C until further treatment with detergent and analysis of MPO and ECP (see below). In order to measure whether release of MPO and ECP occurs during the adhesion the cell-free supernatant from the non-adherent cell fraction was collected after centrifugation at $300 \times g$ for 10 minutes at +4°C.

3.10 TRANSMIGRATION ASSAY [II, III, IV, V]

Transmigration studies were performed by using a 24-wells microchemotaxis chamber with a polyethylene terephthalate treated membrane (Becton Dickinson) which has a high pore density membrane (2.0×10^6 pores/cm², 3.0 μ m in diameter) for maximum permeability. Insert filters were coated with human fibronectin (20 μ g/mL) (Sigma, Chemical) dissolved in PBS with 0.2% BSA overnight at +20-22°C, then washed twice with 200 μ L PBS at +20-22°C and blocked with 200 μ L 0.5% HSA for one hour at +37°C, 5% CO₂ and 95% air-atmosphere. Finally, the wells were rinsed with PBS and air-dried at +20-22°C. Prepared granulocytes from non-allergic healthy donors were mixed with 200 μ L RPMI alone or supplemented with IL-5

(100 ng/mL) (Immunokontakt) and added to the upper chamber. Eotaxin (100 ng/mL) (Prepro Tech Inc) or fMLP (10^{-8} M) (Sigma Chemical) was added to the lower chamber. The cell suspensions were incubated on the fibronectin coated membrane at +37°C, 5% CO₂ and 95% air-atmosphere for different time periods (15, 30, 60, 90 and 120 min for neutrophils and 30, 60, 120, 240 and 360 min for eosinophils). To prevent fluid permeation due to hydrostatic pressure, the volumes in the upper and lower compartments were selected to avoid a pressure gradient across the filter. Three fractions of cells were collected at specific time intervals as follows. Cells in the upper chamber (non-transmigrated cells) were collected by careful aspiration and rinsing with 200 µL RPMI (+37°C). Cells which had transmigrated to the lower chamber (transmigrated cells) were transferred to test tubes. These two cell-fractions were centrifuged $300 \times g$ for 10 minutes at +4°C in order to collect the cell-free supernatants. The third cell fraction was comprised of cells trapped in the membrane and was designated “adherent cells”. Finally the three cell fractions (two in suspensions and one in the membrane) and the two cell-free supernatants were stored at -80°C before treatment with detergent and MPO and ECP (see below). The levels of total MPO and ECP content in the original granulocyte population were measured in parallel.

3.11 SELECTION OF DETERGENTS FOR ECP AND MPO ANALYSIS [II]

To select a detergent for detection of intracellular stored ECP and MPO in eosinophils and neutrophils, respectively, we prepared purified granulocytes (0.30×10^6 cells/mL) which were subjected to either one freeze-thaw cycle followed by treatment with 0.8% n-octyl--D-glucopyranoside (OG) (Sigma Chemical), 1% Triton X-100 (Sigma Chemical) or 0.2% N-cetyl-N,N,N-trimethylammuniom bromide (CTAB). All detergents were dissolved in PBS with 0.2% BSA (Sigma Chemical) for 30 or 60 min at +20-22°C. ECP was measured by ECP-CAP-FEIA (Pharmacia & Upjohn). ECP-CAP-FEIA is a vitro in test system for quantitative measurement of ECP. Briefly, the principle of the procedure is that anti-ECP, covalently coupled to

ImmunoCAP, reacts with ECP in the lysed eosinophils suspension. After washing, an enzyme labelled with antibodies against ECP is added to forming a complex. After incubation, unbound enzyme-anti-ECP is washed away and the bound complex is incubated with a developing agent. After stopping the reaction, the fluorescence of the eluate is measured in fluorocount. The fluorescence intensity is directly proportional to the concentration of ECP in the eosinophils lysed in the suspension. MPO was measured by competitive RIA (Pharmacia & Upjohn). The detection limit was < 2 ng/mL for ECP and < 8 µg/mL for MPO. In the following experiments, treatment was selected with 0.2% CTAB (Sigma Chemicals) for 60 minutes at +20-22°C. ECP was measured by ECP-CAP-FEIA (Pharmacia & Upjohn) and MPO was assessed by competitive RIA (Pharmacia & Upjohn).

3.12 STATISTICAL ANALYSIS [I-V]

3.12.1 [I, II]

The results are expressed as median and interquartile range (IQR). Data were analysed by using a non-parametric method (Wilcoxon paired test). Correlation with 95% confidence bands. NS = non-significant.

3.12.2 [III]

Results are given as mean ± SEM and median and interquartile range (IQR). Comparisons were made, by using Analysis of variance, Mann-Whitney U test and Wilcoxon matched paired test.

3.12.3 [IV, V]

Results are given as mean ± SEM. Comparison was made by using analysis of variance and T-test. Interactions between groups have been calculated. As *post-hoc* analysis, the Tukey-test, was used.

4 RESULTS AND DISCUSSION

4.1 THE EFFECT OF EOTAXIN ON THE EXPRESSION OF CD11B ON EOSINOPHILS PREINCUBATED IN VITRO WITH IL-5 AND FMLP AS WELL AS ON EOSINOPHILS PRIMED IN VIVO [I]

The CD11b/CD18 antigen is an inducible adhesion-promoting complex involved in the different steps leading to eosinophil accumulation and subsequent degranulation (Walker et al., 1993; Horie and Kita, 1994). In our study the quantitative level of CD11b on eosinophils, expressed as MFI-units, did not differ between eosinophils incubated in RPMI buffer alone (n=7) and eosinophils incubated with eotaxin at different concentrations (50, 100, and 200 ng/mL), (NS, n=7). Furthermore, to evaluate if eotaxin has an effect on primed eosinophils in terms of CD11b up-regulation, leukocytes were preincubated with either IL-5 or fMLP, followed by incubation with eotaxin 100 ng/mL for 15 minutes. The CD11b expression was not significantly altered by preincubation with fMLP (NS, n=10) but the expression of CD11b on eosinophils preincubated with IL-5 was significantly higher than eosinophils preincubated with RPMI alone ($p < 0.05$, n=10).

To evaluate if peripheral blood eosinophils acquire an increased responsiveness, in terms of eotaxin induced up-regulation of CD11b *in vivo*; the following experiment was performed. Peripheral blood leukocytes from asthma patients, harvested before and after an inhalation provocation with a relevant allergen, were incubated with rh human eotaxin (100 ng/mL) for 15 minutes at +37°C. A significant increase in CD11b expression was obtained when eosinophils were harvested 24 hours after provocation and subsequently incubated with eotaxin ($p < 0.05$, n=6) whereas eosinophils harvested prior to inhalation did not significantly increase their expression of CD11b when incubated with eotaxin (NS, n=5). These observations could be related to the phenomenon designated "priming". This state of activation is considered to be the result of exposure to an agent lacking intrinsic effector function but induces a more pronounced response to a subsequent, second stimuli.

Our findings, that eotaxin only has an effect on eosinophils, either pre-treated *in vitro* with IL-5 or primed *in vivo*, are supported by the observation in an animal model that the eotaxin induced eosinophil infiltration is further enhanced if IL-5 is given intravenously (Collins et al., 1995). In line with these findings is also the finding that IL-5 exposed eosinophils from healthy subjects attain characteristics similar to eosinophils harvested from atopic subjects (Warringa et al., 1992, Moser et al., 1992). Given these observations, accumulated data indicate an existing synergy between IL-5 and eotaxin in the context of selective eosinophil accumulation *in vivo* (Mould et al., 1997, Collins et al., 1995),

4.2 THE EFFECT OF EOTAXIN ON ADHESION PROPERTIES OF EOSINOPHILS PREINCUBATED WITH IL-5 OR FMLP [I, II]

Purified eosinophils from healthy blood donors were preincubated *in vitro*, either with rh IL-5 (100 ng/mL) or fMLP (10^{-9} M) followed by an additional incubation with RPMI buffer alone or supplemented with rh eotaxin (100 ng/mL). The purified eosinophils were thereafter allowed to adhere for 15 minutes, since the eosinophil adhesion was optimal at this time point. The effect of eotaxin on the eosinophil adhesion to fibronectin was significantly higher for eosinophils prestimulated with IL-5 than for eosinophils preincubated with RPMI buffer alone ($p < 0.05$, $n = 5$). IL-5 alone had no effect on the adhesion properties. There was no additional effect of eotaxin on eosinophil adhesion when the cells were preincubated with fMLP. These results further support the hypothesis that IL-5 and eotaxin co-operate in several aspects of eosinophil function.

4.3 SELECTION OF DETERGENT FOR OPTIMAL DETECTION OF ECP/MPO [II]

To select a detergent for optimal detection of intracellularly stored ECP and MPO in eosinophils and neutrophils, respectively, we treated the cells with 0.8% OG, 1% Triton X-100 or 0.2% CTAB for 30 or 60 minutes as described in Materials and Methods. The quantitative levels of ECP and MPO were significantly higher for granulocytes treated with detergent

compared to when detergent was omitted. The granulocytes treated with 0.2% CTAB had significantly higher ECP and MPO levels than granulocytes treated with 0.8% OG or 1% Triton X-100. Furthermore, granulocytes treated with 0.2% CTAB for 60 minutes had significantly higher ECP and MPO levels than granulocytes treated with 0.2% CTAB for only 30 minutes. To evaluate the method, we compared cell count obtained by flow cytometry (FOG-method, with cell count based on MPO and ECP analysis. We obtained significant correlation for both neutrophils ($r = 0.84$) and eosinophils ($r = 0.91$), with 95% confidence bands. CTAB has been successfully used by other investigators (Xu X & Håkansson 1999). Our data emphasises the importance of evaluating optimal detergent conditions for protein recovery.

4.4 KINETICS OF NEUTROPHIL AND EOSINOPHIL ADHESION AND TRANSMIGRATION [II]

To describe the time course of neutrophil and eosinophil adhesion to fibronectin, granulocytes were allowed to adhere to fibronectin-coated wells or transmigrate through fibronectin coated inserts for different time periods in the presence of either fMLP (10^{-8} M) (neutrophils) or IL-5 (100 ng/mL)/eotaxin (100 ng/mL) (eosinophils). We found a significant peak in neutrophil adhesion at 30 minutes and a similar peak in eosinophil adhesion at 90 minutes. We also observed a gradual increase in both neutrophil and eosinophil transmigration, which reached a plateau at 90 minutes and 240 minutes, respectively. The spontaneous transmigration, when respective stimuli was omitted, was $< 5\%$ for both neutrophils and eosinophils.

The median cell recovery values in adhesion and transmigration assays were $>95\%$. The neutrophil and eosinophil viability, as judged by trypan blue exclusion, exceeded over 95% in all experimental settings.

In this study we used fMLP exclusively for evaluation of neutrophil adhesion and transmigration. The kinetics of neutrophil transmigration, reaching a plateau at 90 minutes, is in

line with our previously reported data when using a confluent monolayer of endothelial cells as a barrier and fMLP as chemotactic stimulus (Thorlacius et al., 1997).

To evaluate eosinophil adhesion and transmigration, we selected a combination of IL-5 and eotaxin. The rationale for this is based on the proposed synergistic action of these two substances in regulating blood and tissue eosinophilia (Collins et al., 1995), as well as the reported roles of IL-5 and eotaxin in mediating a transfer of eosinophils from the bone marrow to the lung in response to allergen challenge (Mould et al., 1997).

4.5 EXPRESSION OF CD11B AND CD62L ON NEUTROPHILS DURING HEMODIALYSIS [III]

To evaluate the influence of the dialysis treatments to induce quantitative changes in expression of adhesion molecules on neutrophils we set up experiments with three different dialyzers. At start of dialysis, there were no significant differences in CD11b expression on neutrophils between the three treatments. The expression of CD11b on neutrophils increased significantly after 15 minutes of dialysis with all three dialyzers but was significantly more pronounced during cuprophan treatment. The CD11b expression approached pre-dialysis values at the end of dialysis in patients treated with low-flux as well as high-flux dialyzers. In contrast, the CD11b expression remained significantly higher than before dialysis in patients on cuprophan dialysis. In eosinophils, we observed a slight, albeit significant, increase in the expression of CD11b at 15 minutes in patients treated with low-flux ($p < 0.05$ in both instances, $n=9$) as well as high-flux dialyzers ($p < 0.05$, $n=9$). However, by the end of the dialysis values did not differ from pre-dialysis expression regardless of the dialyzer used.

The cell surface CD62L expression on circulating neutrophils was significantly lower in patients on cuprophan membranes dialysis at four hours than in patients treated with both low-flux and high-flux dialyzers. In eosinophils the CD62L expression was unchanged at all time points regardless of which membrane was utilised.

Other investigators have also demonstrated that these antigens are altered on circulating neutrophils and monocytes during, and after hemodialysis, and that the magnitude of alteration could partially be referred to the biocompatibility properties of dialyzer employed (Thylén et al., 1995, Chenoweth et al., 1983).

4.6 TRANSMIGRATION CAPACITY OF CIRCULATING NEUTROPHILS DURING HEMODIALYSIS IN VITRO [III]

To determine the influence of biocompatibility on the capacity of neutrophils, collected from patients before, during and after hemodialysis, to transmigrate *in vitro* and to separate this effect from that caused by differences in dialysis membrane permeability. In this study we used fMLP exclusively for evaluation of neutrophil transmigration.

Transmigration index for neutrophils did not differ at the start of dialysis between the three dialyzers used and was significantly lower than in healthy subjects. At 15 minutes the transmigration capacity was significantly impaired in patients treated with cuprophan ($p < 0.01$, $n = 9$) and low-flux polysulfone ($p < 0.05$, $n = 9$) membranes as compared to pre-dialysis values. In contrast, transmigration capacity increased gradually in patients treated with high-flux polysulfone and was higher than in patients treated with both cuprophan and low-flux membrane at 15 minutes. At end of dialysis, neutrophils in high-flux treated patients had significantly higher transmigration index than neutrophils from patients treated with low-flux membranes ($p < 0.01$, $n = 9$) and values approached those of healthy controls. Transmigration index for eosinophils, analysed as control cells, was not altered at any time point during the three different dialysis regimens and equalled the transmigration index for eosinophils from healthy blood donors. We did not observe any over-all significant correlation between CD11b expression and transmigration index.

Previous studies have reported that granulocytes harvested at the end of a cuprophan dialysis session adhered less to unstimulated, but equally to stimulated HSVECs when compared to cells

collected pre-dialysis (Thylén et al., 1997). It has also been shown that granulocytes collected after 15 minutes of cuprophan dialysis have an impaired capacity to adhere and transmigrate in an experimental animal model (Thorlacius et al., 1997). In the present paper, we extend these observations by including data that show that the transmigration capacity is reverted at the end of cuprophan dialysis. An interesting finding in this study was that the neutrophil transmigration capacity is impaired during dialysis with cuprophan and low-flux polysulfone while enhancement occurs when patients are treated with high-flux polysulfone. Moreover, at end of dialysis, neutrophils in the high-flux group transmigrated significantly more efficiently, than neutrophils in the low-flux group. These observations were noted regardless of existing differences in adhesion phenotype or cell number, before, during and after the dialysis treatments, which implies that the observed differences are not solely related to a shift in the circulating pool of neutrophils.

High-flux treatment possess an advantage in that the middle range molecules, defined as molecules that are larger than urea and, consequently have kinetics different from that of low molecular weight compounds, are more efficiently eliminated. A number of these molecules have toxic features e.g. granulocyte inhibitory properties (Hörl et al., 1990, Haag-Weber et al., 1993). One might speculate that an efficient elimination of these potentially toxic molecules from plasma during high-flux hemodialysis constitutes a plausible mechanism for our findings, that the transmigration capacity of circulating neutrophils was enhanced following high flux treatment but not after low flux or cuprophan treatments which approached levels found in healthy subjects.

4.7 EXPRESSION OF CELL SURFACE ADHESION MOLECULE CD11B ON EOSINOPHILS IN NEONATES AND ADULTS [IV]

The potential physiological role of eosinophils in the neonatal period is largely unknown, despite the observation that a majority of premature infants develop eosinophilia and the existence of abnormalities in eosinophil trafficking during the first period of postnatal life. We

therefor assessed the ability of neonatal eosinophils to up-regulate CD11b upon fMLP- or IL-5/eotaxin stimulation compared to adult eosinophils.

The expression of CD11b was significantly higher in eosinophils preincubated with IL-5 than eosinophils preincubated with RPMI alone, both in adults and neonates. Addition of eotaxin (0.1 $\mu\text{g/ml}$) further increased the expression of CD11b on IL-5 preincubated eosinophils ($p < 0.0001$ in both neonates and adults). Significant differences were observed between adult and neonatal eosinophils, with respect to CD11b expression, when the cells were incubated at $+37^\circ\text{C}$ with RPMI ($p < 0.006$) with IL-5/Eotaxin ($p < 0.005$) or with fMLP ($p < 0.0003$).

Eosinophil adhesion and transmigration have been attributed to several adhesion pathways. The integrins constitute an adhesion family extensively involved in different aspects of eosinophil function and the α_M subunit, also designated CD11b, has been claimed to be essential for both transmigration and degranulation (Horie and Kita 1994; Kato et al 2000). In this study we demonstrate that neonatal eosinophils have an increased capacity to up-regulate CD11b in comparison to eosinophils in adults. There are several pathways which constitute prime candidates since the eosinophil is equipped with several adhesion molecules that can bind fibronectin, namely VLA-4 and CD54 (Yuan Q et al 1997; Seminario and Bochner 1997; Higashimoto et al 1999). In contrast to neutrophils, eosinophils transmigrate to inflammatory sites in patients with leukocyte adhesion deficiency syndrome, indicating that eosinophils can use CD18 independent pathways for transmigration *in vivo* (Anderson et al 1981).

4.8 KINETICS OF EOSINOPHIL AND NEUTROPHIL TRANSMIGRATION IN NEONATES AND ADULTS [IV]

We also assessed the ability of neonatal eosinophils and neutrophils to transmigrate *in vitro* towards chemotactic stimuli linked to either bacterial infections, or to allergic inflammation, and to compare the results with eosinophils and neutrophils in adults. We used an *in vitro*

transmigration method and the chemotactic stimuli fMLP or a combination of interleukin (IL)-5 and eotaxin.

The chemotactic response of neonatal eosinophils towards fMLP was evident. The transmigration reached a plateau after 60 min incubation which was significantly higher than after 30 min incubation ($p < 0.02$). There was spontaneous transmigration of neonatal eosinophils, which started after 120 min incubation. We also tested the transmigration capacity towards a combination of IL-5 and eotaxin. The transmigration began at 30 min ($p < 0.02$ compared to medium only) and peaked at 240 min incubation ($p < 0.004$ compared to 120 min). Transmigration of eosinophils from healthy blood donors was initiated at 120 min and reached a maximum at 240 min incubation. Neonatal eosinophils possessed a significantly higher transmigration capacity toward IL-5/eotaxin than adult eosinophils at all time points ($p < 0.0002$ - $p < 0.002$).

A gradual increase in both neonatal and adult neutrophil transmigration was also observed when fMLP was used as stimuli and reached a maximum and remained at a constant level at 60 and 120 min respectively. The transmigration of neutrophils at 60 min was significantly higher in neonates as compared to adults ($p < 0.005$) whereas at 120 min this occurrence was reversed and the transmigration of adult neutrophils was significantly higher than that of neonatal neutrophils ($p < 0.005$). We also noted a spontaneous transmigration in neonatal neutrophils incubated with medium only, which continuously increased and reached a plateau at 240 min.

The existence of abnormalities in eosinophil trafficking in the neonatal period is suggested by several clinical observations. Neonatal exudates often contain an increased proportion of eosinophils (Bullock et al 1969; Roberts et al 1990) and an infiltrate of eosinophils constitutes a characteristic feature of erythema toxicum, a self-limited condition with unknown etiology affecting a high percentage of neonates within the first 48 hours of life (Schwartz and Janniger 1996). Moreover, eosinophils in the neonatal period have been claimed to participate in the

inflammatory process in bronchopulmonary dysplasia (BPD) (Yamamoto et al 1996; Raghavender and Smith 1997) as well as in infants with acute wheezing episodes (Council et al 1997). Eosinophilia in the neonatal period has also been linked to non-parasitic infections, and a physiological role for eosinophils during the overt colonisation of skin and mucosal surfaces that occurs during this period has been suggested (Patel et al 1994).

4.9 EXPRESSION OF fMLP RECEPTORS ON EOSINOPHILS AND NEUTROPHILS IN NEONATES AND ADULTS [IV]

To determine the responsiveness of neonatal eosinophils and neutrophils to bacterial peptide fMLP, we measured expression of fMLP receptor on the cell surface.

The surface expression of fMLP receptors on eosinophils was significantly higher in neonates as compared to adults, as judged both by MFI values and positive labelled cells. On neutrophils the fMLP receptor was more abundant expressed in adults than in neonates.

The receptor for fMLP is, so far, the only receptor that recognises an exogenous chemoattractant, the bacterially derived *N*-formyl peptides. The fMLP receptor activates heterotrimeric proteins (G-proteins) that initiate numerous elaborate signal transduction cascades, culminating in migration and activation (Uhing et al 1992). Neonatal neutrophils have been suggested to express normal numbers of fully functional fMLP receptors (Strauss and Snyder 1984; Yoshie et al 1989). Due to the well-recognised feature of re-circulation we selected an established method in which the fMLP-receptor was assessed in resting cells (Hsu et al 1997).

The question whether the fMLP-receptor in neonatal eosinophils, in addition to being abundantly expressed on the surface, is endowed with additional specific characteristics, is not addressed in this study and merits further investigations to be delineated.

4.10 TRANSMIGRATION CAPACITY OF CIRCULATING EOSINOPHILS FOLLOWING NASAL ALLERGEN CHALLENGE IN CHILDREN WITH ALLERGIC ASTHMA [V]

Local eosinophilia is characteristic for the allergic inflammation and interleukin-5 in cooperation with eotaxin is essential for the eosinophil transmigration from the peripheral blood into local tissue sites. In this study, we investigated the capacity of PBE to transmigrate through a fibronectin-coated membrane in an *in vitro* assay after cat-allergen challenge *in vivo*, of cat-allergic children with rhinitis and asthma. We also tested the additive effect of eotaxin on the transmigration capacity of PBE.

Percoll purified granulocytes were collected at prior to or 2 hours and 24 hours after allergen challenge and were then tested for *in vitro* transmigration. The spontaneous transmigration of eosinophils to the lower chamber was significantly higher 2 hours after allergen challenge as compared to transmigration before provocation ($p < 0.01$). This increased transmigration capacity returned to baseline levels 24 hours after challenge ($p < 0.02$; vs 2 hours). Eotaxin, added to the lower chamber, further significantly enhanced the transmigration capacity at 2 hours but not prior to or 24 hours after challenge ($p < 0.04$).

No spontaneous or eotaxin-induced eosinophil transmigration capacity was detected neither before nor after challenge in healthy controls.

Previously, we have shown that non-atopic adults have eosinophils with a lower capacity to adhere or express adhesion molecule CD11b/CD18, unless the cells are stimulated with IL-5 *in vitro*, when compared to asthma patients. The non-atopic children, who served as controls in our study, showed a lack of eosinophil transmigration capacity further strengthening our theory. One of our hypotheses was that atopic/allergic children already have primed PBE at baseline, which is in concordance with an enhanced *in vivo* production of IL-5 (Lampinen et al 1999, Coeffier et al 1991). In line with this, was our finding that the cat-allergic children included in this study had eosinophils at baseline with a spontaneous transmigration capacity and that this ability could be enhanced after allergen challenge. These observations are probably related to

the phenomenon designated "priming", a state of activation considered that to be the result of exposure to an agent as discussed previously

5 CONCLUSION

We demonstrate that eotaxin is involved in the quantitative up-regulation of CD11b/CD18 expression and increases the adhesion properties in primed, but not resting, human eosinophils. We hypothesize that eotaxin and IL-5, but not fMLP, act synergistically in these respects. The physiological implications of these data are not fully known but indicate that the net outcome of eotaxin exposure on eosinophil function depends on the actual state of activation.

The adhesion and transmigration assays were set up to enable simultaneous time-course analysis of eosinophil and neutrophil adhesion and transmigration in mixed granulocyte populations based on analysis of ECP and MPO. These models can be useful tools in exploring the mechanisms whereby neutrophils and eosinophils integrate chemotactic signals, communicate and to evaluate the impact of this communication on the overall leukocyte accumulation at disparate inflammatory sites.

We demonstrate in our study with hemodialysis patients that high-flux polysulfone treatment, as opposed to low-flux and cuprophan dialyzers, ameliorates the transmigration properties of circulating neutrophils. We did not find any overall correlation between adhesion phenotype and transmigration properties, which implies that a direct relationship between altered adhesion phenotype and capacity to accumulate at an inflammatory site might not exist. The transmigration assay is a useful tool to study the impact of different hemodialysis strategies on the transmigration capacity of peripheral blood neutrophil.

Eosinophils in the neonatal period possess an enhanced responsiveness against the bacterial-related peptide fMLP, as judged by enhanced transmigration capacity and CD11b up-regulation, presumably due to the ability to express the fMLP receptor. We therefore suggest that

eosinophils in the neonatal period might participate in the first line of cellular defence that can be triggered by bacterial antigen stimulation initiated by the early colonisation of the surfaces of the mucosa.

We demonstrate that PBE in allergic, but not in healthy children, possess an increased spontaneous, as well as eotaxin-induced capacity to transmigrate *in vitro*. This increased capacity is further enhanced within 2 hours after an allergen challenge *in vivo* without accompanying signs of eosinophil activation in terms of increased PBE counts or ECP levels. These observations could be related to the phenomenon designated "priming", a state of activation considered to be the result from exposure to an agent, which itself has no effect on function, but that causes a more pronounced response to a subsequent, second agent.

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