

Allergen extracts directly mobilize and activate human eosinophils

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Allergic diseases are characterized by the presence of eosinophils, which are recruited to the affected tissues by chemoattractants produced by T cells, mast cells and epithelium. Our objective was to evaluate if allergens can directly activate human eosinophils. The capacity of purified allergen extracts to elicit eosinophil chemotaxis, respiratory burst, degranulation and up-regulation of the adhesion molecule complement receptor 3 (CR3) was determined in eosinophils isolated from healthy blood donors. Eosinophils stimulated with an extract from house dust mite (HDM) released the granule protein major basic protein (MBP) and up-regulated the surface expression of CR3. Cat allergen extracts also induced the up-regulation of CR3, but not the release of MBP; instead cat, as well as birch and grass allergens, elicited the release of eosinophil peroxidase (EPO). In addition, grass pollen extract caused the secretion of MBP. None of the allergens stimulated eosinophilic cationic protein release, nor production of free oxygen radicals. Both HDM and birch extracts were chemotactic for eosinophils. These findings establish that common aeroallergens can directly activate eosinophils *in vitro*. We propose that eosinophil activation *in vivo* is not exclusively mediated by cytokines and chemokines of the allergic inflammatory reaction, but could partly be the result of direct interaction between allergens and eosinophils.

Key words: Eosinophil / Allergen / Degranulation / Chemotaxis / Endotoxin

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1 Introduction

Eosinophilic granulocytes are tissue-dwelling white blood cells, mainly found in the lung, gastrointestinal tract and lower genitourinary tract [1]. Their name is derived from the fact that the dye eosin stains eosinophils red by binding to highly basic proteins — major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN/EPX) and eosinophil peroxidase (EPO) — that are stored in membrane-enclosed cytoplasmic compartments called specific or secondary granules [1].

The physiological role of these cells in healthy people is poorly understood. Since eosinophilia is a common feature of parasitic infections, the nearly universally

accepted idea regarding the normal function of these cells is that they destroy large, non-phagocytosable parasites, although the experimental data to support this concept are scant [1]. Eosinophils accumulate at sites of allergic inflammation [1], but also in tissues afflicted by inflammatory bowel disease [2], graft-versus-host disease [3], transplant rejection [4] and various forms of malignancies [5].

Eosinophils are produced in the bone marrow, briefly transit through the bloodstream, and leave the circulation first by rolling on the endothelium, mainly through the interaction of L-selectin on the eosinophil with E- and P-selectins on the endothelial cells [6]. The next step, firm adhesion, is mediated by eosinophil–endothelial interactions via complement receptor 3 (CR3)–ICAM-1 and VLA-4–VCAM-1 interactions [6]. Various chemotactic substances attract eosinophils into tissues affected by allergic inflammation: Eotaxin and monocyte chemotactic protein-3 (MCP-3) are important in the early recruitment phase of eosinophils, whereas chemokines such as RANTES and MCP-4 are important later on [7].

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Abbreviations: **CR3:** Complement receptor 3 **ECP:** Eosinophil cationic protein **EPO:** Eosinophil peroxidase **fMLF** N-formyl-Met-Leu-Phe **HDM:** House dust mite **MBP:** Major basic protein **PAF:** Platelet-activating factor

The current dogma regarding the pathophysiology of allergic diseases is that allergens are processed, at the site of allergen delivery, by professional antigen-presenting cells, such as dendritic cells and macrophages, and presented to T lymphocytes bearing the cognate TCR in draining lymph nodes. The activated allergen-specific T cell clones produce cytokines such as IL-4 and IL-13, which promote the differentiation of allergen-specific B cell clones and their switch to IgE production [8]. These IgE molecules bind via their Fc moiety to high-affinity IgE-receptors (Fcε) on tissue-bound mast cells. Upon renewed allergen exposure, allergen crosslinks the prebound IgE antibodies, thus triggering the release of histamine, tryptase, cytokines and leukotrienes from the mast cells, which constitutes the early phase of the allergic reaction [8]. The secreted mast-cell products attract T cells, eosinophils and other leukocytes into the microenvironment within hours, which is termed the late phase of the allergic reaction [9].

In the present study, we demonstrate that environmental allergens may directly activate and elicit migratory behavior in eosinophils isolated from healthy blood donors. Furthermore, allergen extracts from house dust mite (HDM), birch pollen, grass pollen and cat dander gave rise to different activation patterns in human eosinophils.

2 Results

2.1 Some allergen extracts trigger eosinophil chemotaxis and up-regulation of the adhesion molecule CR3

We investigated the ability of the four allergen extracts to elicit eosinophil migration in a serum-free, dual chamber

microwell migration system. HDM and birch allergen extracts induced significant eosinophil chemotaxis, *i.e.* a mean migratory index of 14.8 ± 2.4 and 10.7 ± 0.67 , respectively (Fig. 1A). As a comparison, the mean migratory index elicited by the two positive physiological controls, eotaxin and the formylated tripeptide N-formyl-Met-Leu-Phe (fMLF) were 30.5 ± 7.9 and 19.7 ± 2.9 , respectively. The migratory response of eosinophils exposed to cat or grass allergen extracts was similar to the spontaneous migration of eosinophils toward medium (6.0 ± 1.0) (Fig. 1A). We also established that eosinophils migrated poorly towards the HDM-derived recombinant protein Der p 2 (Fig. 1A). Finally, neither neutrophils nor monocytes shared the ability of eosinophils to migrate toward HDM and birch allergen extracts (Fig. 1B, C)

The capacity of allergen extracts to increase the expression of adhesion molecules was examined by measuring expression of CR3 — also known as the integrin CD11b-CD18 or Mac-1 — on the surface of eosinophils. Using FACS analysis, we could detect significant mobilization of CR3 to the cell surface in eosinophils stimulated for 18 h with HDM or cat allergen extract. Hence, in seven blood donors, the percentage of gated cells (M1; see Fig. 2) increased from $41.0 \pm 6.6\%$ (in medium) to $65.0 \pm 5.0\%$ ($p=0.016$) for HDM and $64.0 \pm 7.3\%$ ($p=0.046$) for cat allergen extract, respectively (Fig. 2). This may be compared to the CR3 increase to 87.0 ± 7.8 ($p=0.011$) resulting from exposure of eosinophils to LPS and heat-inactivated human serum (Fig. 2). A near-significant CR3 increase was seen in eosinophils incubated with birch allergen extract ($63 \pm 7.2\%$; $p=0.053$), whereas no alteration of CR3 levels was seen after incubation with grass allergen extract (53.0 ± 5.6 ; Fig. 2).

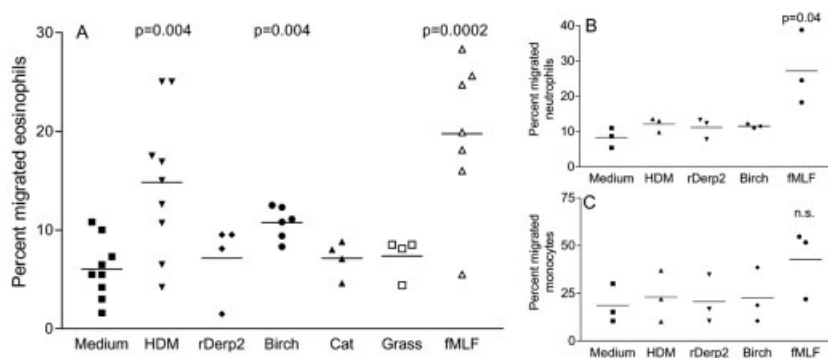


Fig. 1. Chemotactic movement of eosinophils (A), neutrophils (B) and monocytes (C) exposed to allergen extracts and the recombinant protein Der p 2. Each data point represents the percentage of cells that were derived from a healthy blood donor and that migrated towards the allergen extracts (100 µg/ml) or Der p 2 (28 µg/ml, corresponding to the quantity of protein in the HDM allergen extract) in microwell migration chambers. Spontaneous migration is indicated by cell movement towards medium. The horizontal bars denote median values for 3–9 blood donors. n.s., not significant ($p=0.11$).

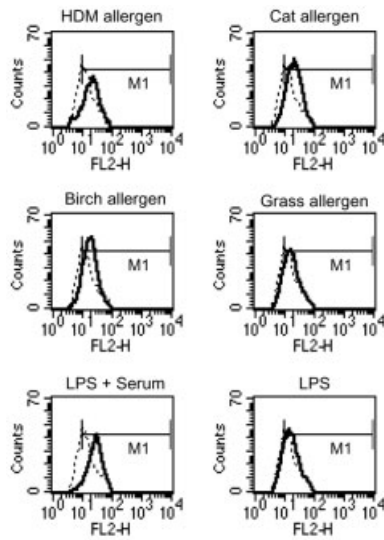


Fig. 2. Surface expression of CR3 on eosinophils after exposure to allergen extracts. Representative histograms derived from one blood donor out of seven are shown. Solid lines represent eosinophils exposed to allergen extracts (100 $\mu\text{g/ml}$) or purified LPS (1 $\mu\text{g/ml}$) with or without heat-inactivated serum; dotted lines indicate eosinophils in medium alone.

2.2 Allergen extracts trigger eosinophil degranulation

There was a significant release of the granule protein EPO from eosinophils incubated with birch, cat or grass allergen extracts compared with eosinophils incubated with medium alone (Fig. 3). HDM also tended to elicit secretion of EPO; however levels of EPO did not reach statistical significance in this case ($p=0.066$). None of the four tested allergen extracts induced any significant release of the granule protein ECP in eosinophils purified from four different blood donors (data not shown). Flow cytometry was used to test the capacity of the four allergen extracts to cause the release of MBP, through measurement of the intracellular content of MBP in eosinophils stimulated with allergen extracts *versus* medium. A significant decrease in intracellular MBP levels was seen in eosinophils incubated with grass or HDM allergen extracts, but not after incubation with birch or cat allergen extracts (Fig. 3). We also found that eosinophils stimulated with any of the four allergen extracts failed to release the cytokines IL-13, IL-5 and GM-CSF (data not shown).

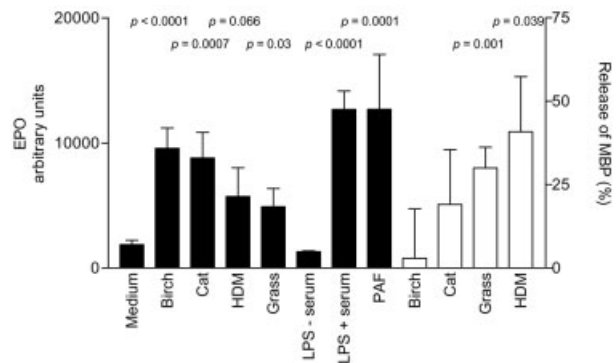


Fig. 3. Degranulation of eosinophils exposed to allergen extracts. Black bars indicate EPO release from eosinophils incubated with allergen extracts (100 $\mu\text{g/ml}$). LPS (1 $\mu\text{g/ml}$) with or without 5% heat-inactivated human serum was added to test the reactivity of human eosinophils to endotoxin. White bars indicate by how many percent intracellular MBP levels decreased in eosinophils exposed to allergen extracts compared with medium. All data represent the arithmetic mean $\pm$ SEM ($n=5-10$ different blood donors).

2.3 Allergen extracts do not trigger an eosinophil respiratory burst

To see if direct contact with allergen could induce eosinophils to generate reactive oxygen species such as superoxide anions or hydrogen peroxide, we measured the extracellular oxidative burst continuously in eosinophils exposed to allergen extracts, endotoxin, negative control (medium) or positive control (fMLF 10^{-7} M) for up to 45 min. We found that neither the allergens (HDM, birch, cat or grass allergen extracts) nor LPS could induce a respiratory burst in eosinophils (Fig. 4).

2.4 Endotoxin is not responsible for the effects observed

To exclude the possibility that the described eosinophil stimulatory effects were artifacts caused by endotoxin contamination, we measured the LPS contents in the allergen preparations and performed tests to determine the reactivity of eosinophils to purified LPS. Eosinophils were responsive to LPS only in the presence of inactivated human serum. A statistically significant release of EPO (Fig. 3) and ECP (data not shown) was seen in eosinophils exposed to the highest concentration of LPS (1 $\mu\text{g/ml}$) together with inactivated human serum. However, in our serum-free system, 1 $\mu\text{g/ml}$ of LPS alone did not cause release of either EPO or ECP above background levels (Fig. 3 and data not shown). Further, LPS did not elicit any release of MBP even in the presence of inactivated human serum. Finally, up-regulation of

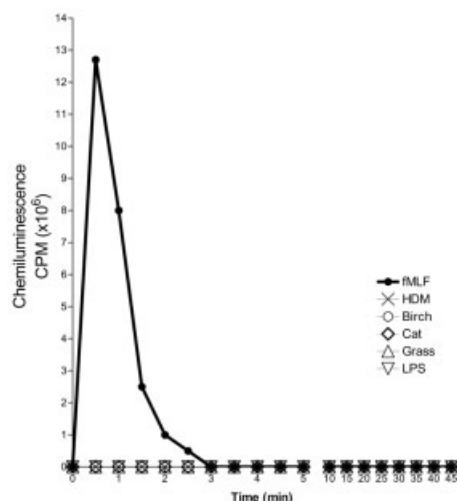


Fig. 4. Time course of respiratory burst in human eosinophils. Eosinophils were co-incubated with either allergen extracts (100 µg/ml), LPS (1 µg/ml) or fMLF as a positive control (10^{-7} M) and release of reactive oxygen specimens was continuously recorded by chemiluminescence. The plot is derived from a representative experiment out of four. CPM, counts per minute.

surface-exposed CR3 was only noted in eosinophils exposed to LPS and human serum, but not when LPS was tested in our serum-free system (Fig. 2).

3 Discussion

We found that allergen extracts up-regulated molecules of importance for eosinophil recruitment into tissues, caused direct mobilization of the cells *in vitro* and induced selective degranulation. Not all allergen extracts were able to elicit all of these effector functions. HDM was the most potent allergen extract of the four tested, whereas grass and cat allergen extracts were the weakest eosinophil activators.

Recruitment of granulocytes from peripheral blood into the tissues is in part regulated through the mobilization of intracellular organelles storing adhesion molecules, and the concomitant release of enzymes/cytokines required for firm leukocyte adhesion to the vessel wall and subsequent endothelial transmigration [10]. We found that all allergen extracts with the exception of grass allergen had the capacity to up-regulate CR3 (CD11b–CD18) on the eosinophil surface; CR3 is an integrin molecule believed to be stored in the easily mobilized secretory granules [11]. The same molecule has also been shown to bind LPS [12]. To eliminate the possibility that the up-regulation of CR3 was due to endotoxin contamination, we incubated eosinophils with

LPS and confirmed the findings of Wedi et al., that LPS does not induce up-regulation of CR3 in human eosinophils [13].

Chemotaxis is the directed movement of leukocytes towards a gradient of chemoattractant. Chemokines cause white blood cells to polarize such that they form a leading edge, which contains chemotactic receptors, and a trailing edge, containing integrins and selectins. Actin polymerization in the leading edge and depolymerization in the trailing edge keep the cell in motion, a process regulated by levels of intracellular Ca^{2+} [14]. Typical eosinophil chemoattractants are eotaxin, RANTES, platelet-activating factor (PAF), and fMLF and other peptides engaging the formyl-peptide receptor. All of these molecules bind to G-protein-coupled receptors on the eosinophil surface [15, 16]. Both HDM and birch allergen extracts could stimulate chemotactic activity in eosinophils, which was not the case for the recombinant protein Der p 2 derived from HDM. At present, we do not know which components of the allergen extracts that caused eosinophilic mobilization. Proteins other than Der p 2, lipid or carbohydrate moieties are possible candidates. Interestingly, it seems as if the ability of eosinophils to recognize and migrate toward allergen extracts is a trait particular to this cell type, not shared by monocytes or neutrophils.

All of the tested allergen extracts, with the exception of HDM, induced a significant release of EPO, and two of the four allergen extracts tested (HDM and grass) elicited the release of MBP from stimulated eosinophils. However, no allergen extract caused degranulation and release of ECP. These granule proteins are stored in membrane-enclosed intracellular compartments — named specific granules — that are composed of an electron-dense core, and a less dense surrounding matrix. Eosinophils often release their granule contents by piecemeal degranulation, a process that allows the cell to gradually secrete matrix-located proteins such as EPO and ECP, core contents such as MBP, or substances stored in both compartments [17].

Recently, Miike and Kita reported that eosinophils incubated with a purified mite allergen, Der f 1, released another matrix-located granule protein, namely eosinophil-derived neurotoxin [18]. It was most likely the cysteine protease activity of this mite allergen that mediated eosinophilic degranulation [18]. Further, Capron and co-workers have shown that eosinophils stimulated with secretory IgA could release EPO but not ECP in patients with allergies [19] or parasitic infection [20]. We extend this knowledge by showing that allergen extracts *in vitro* cause a similar selective degranulation pattern, *i.e.* release of EPO and MBP, but not ECP. Eosinophils can

thus fine-tune their responses to different stimuli by selective release of granule constituents, a phenomenon that probably encompasses bioactive substances stored in other cellular compartments as well.

Capron and co-workers reported that eosinophils from allergic patients released EPO upon *in vitro* exposure to specific allergens. However, only allergens related to the patient's symptoms had this capacity [19]. Here, we show that eosinophils isolated from presumably non-allergic healthy blood donors released EPO upon exposure to birch, cat and grass allergen extracts. EPO is one of the most abundant proteins in eosinophils, making up approximately 25% of the total protein mass of specific granules [21]. This peroxidase catalyzes reactions generating NO-derived oxidants and reactive halogen species [22]. Proteins that have been oxidatively modified by EPO-generated catabolites have been demonstrated in the bronchoalveolar lavage fluid of patients with severe asthma [22, 23].

We found that both HDM and grass allergen extracts could induce degranulation of MBP *in vitro*. Eosinophils and extracellular MBP are found closely associated with airway parasympathetic nerves in allergen-challenged guinea pigs and rats as well as in humans with asthma [24]. Upon stimulation, these nerves release acetylcholine, which binds to muscarine receptor 3 (M_3) on smooth muscle, leading to bronchoconstriction, mucus production and dilation of the bronchial blood vessels [25]. To control this activity, acetylcholine also acts on muscarine receptor 2 (M_2), which limits the release of acetylcholine [25]. MBP is an antagonist of M_2 , and blocking its function results in a net increase of released acetylcholine, causing airway narrowing [26]. Hence it is possible that allergen, through the direct stimulation of eosinophils at allergic sites, causes the release of MBP, which leads to the airway narrowing and mucus production typical of allergic asthma.

Many eosinophil chemoattractants, such as PAF, C5a, fMLF and other peptides engaging the formyl peptide receptor, are also activators of the NADPH oxidase [15, 27]. Activation of this enzyme complex encompasses the transfer of electrons from cytoplasmic NADPH via a specialized b-type cytochrome to molecular oxygen, a process that results in the production of toxic oxygen radicals (*i.e.* superoxide anion and hydrogen peroxide) [27]. Winqvist et al. found that there was no direct relation between degranulation and oxidative burst in eosinophils incubated with serum-treated sephadex [28]. Likewise, we found that although allergen extracts were potent eosinophil degranulators *in vitro*, they failed to elicit production of free oxygen radicals in eosinophils. Nevertheless, *in vivo*, eosinophils most likely produce

free oxygen radicals since these are substrates for the reactions catalyzed by EPO, which definitely occur in the tissues of asthmatic patients [22].

Could the described eosinophilic reactivity to allergen extracts be due to the presence of other leukocytes in our eosinophil cell suspensions? This is unlikely since, on average, our eosinophil suspensions were 95% pure. Further, several preparations consisted of 99% pure eosinophils, and these reacted in the same fashion to stimulation with allergen extracts as did the less pure cell suspensions. Neither do we believe that our results are the result of endotoxin contamination of the allergen preparations for the following reasons: (1) HDM allergen extract was the strongest eosinophil activator of all four tested allergen extracts despite containing the least amount of endotoxin (100-fold less than cat allergen extract did); (2) control experiments in which eosinophils were incubated with up to 1000-fold higher concentrations of LPS than what was found contaminating the allergen preparations revealed that LPS in the absence of human serum was unable to activate eosinophils.

Our findings establish that allergen extracts can directly activate eosinophils *in vitro*. Could eosinophils come in contact with whole, undigested allergens in the lamina propria of tissues? Actually, it has been shown that HDM, which possesses proteolytic activity, has the capacity to cross the epithelium by the disruption of tight junctions [29] and can even travel through the epithelial cells [30]. It has also been established that allergen can reach target cells, such as mast cells, dendritic cells and eosinophils, in the respiratory mucosa of asthmatic patients [30]. Interestingly, this was also seen in allergic individuals without asthma and in completely healthy individuals, although a 100-fold higher dose of allergen was required [30].

Taken together, these findings indicate that eosinophils can be activated by allergen extracts *in vitro*. Hence, it is possible that some of the eosinophil mobilization and activation that occurs *in vivo* may be directly mediated by the allergens themselves, and not necessarily by bioactive substances released by the other cellular components of the allergic inflammatory reaction. We propose that the eosinophil, a cell of the innate immune system, is not totally under the control of T cells, as has been the traditional view. In fact, it has been demonstrated that, in the airways of atopic asthmatics, it is chiefly eosinophils and mast cells that harbor IL-4 and IL-5 protein, not T cells [31, 32], and it has even been suggested that the eosinophil may be an antigen-presenting cell [33]. In future studies, we will attempt to elucidate which receptors and signaling pathways are engaged by the allergen extracts on the eosinophil. Further, we aim to determine

what fraction of the studied allergen extracts was responsible for eosinophil activation: protein, carbohydrate [34] or lipid moieties [35]. A more complete understanding of these fundamental concepts may provide the basis for the development of novel therapeutic interventions in allergy and asthma.

4 Materials and methods

4.1 Allergens and LPS

Defatted allergen extracts from cat epithelium (*Felis domesticus*), HDM (*Dermatophagoides pteronyssinus*), white birch, and timothy grass, reconstituted according to protein content, were used. All allergen extracts were purchased from Greer Laboratories, Lenoir, NC, USA. The content of LPS in the allergen extracts was checked with the Limulus amoebocyte lysate assay (Chromogenix AB, Mölndal, Sweden). Our batches of allergen extracts contained the following quantities of LPS: cat allergen 3.5 ng/ml, birch allergen 0.19 ng/ml, grass allergen 0.10 ng/ml, and HDM allergen 0.025 ng/ml. We compared allergen-mediated eosinophil activation with the effects exerted by stimulation of eosinophils with three different LPS concentrations, 1 µg/ml, 1 ng/ml and 1 pg/ml, with or without 5% heat-inactivated human serum. Purified smooth LPS derived from *Escherichia coli*, serotype 055, was a kind gift from Dr Liliana Håversen, Department of Clinical Bacteriology, Göteborg University. The recombinant protein Der p 2, a derivative of *Dermatophagoides pteronyssinus*, was a generous gift from Dr Michael Spangfort, ALK-Abelló A/S, Horsholm, Denmark.

4.2 Purification of human eosinophils, neutrophils and monocytes

Peripheral blood eosinophils were purified from fresh buffy coats obtained from healthy adult blood donors at Sahlgrenska University Hospital, Göteborg, or Kungälv Hospital, Kungälv essentially as previously described [15]. Swedish blood donors are not allowed to donate blood if they have a bout of allergic disease. After dextran sedimentation at 1×g, centrifugation on a Ficoll gradient (Pharmacia, Uppsala, Sweden) and hypotonic lysis, neutrophils were removed from the granulocyte fraction by negative depletion using magnetic beads (MACS; Miltenyi Biotec Inc., Auburn, CA, USA) coated with anti-CD16 mAb (Miltenyi). The eosinophils were washed and resuspended in either Kreb's Ringer glucose buffer (KRG) [15] or X-vivo 15 buffer lacking phenol red (BioWhittaker, Göteborgs termometerfabrik, Göteborg, Sweden). Neutrophils were isolated from the granulocyte fraction prior to negative depletion of CD16-expressing cells. Monocytes were collected from the interface of the Ficoll gradient and further separated using the countercurrent centrifugal elutriation technique [36]. The purity of the eosinophils was routinely >95%, that of neutrophils >98% and that of

monocytes >90%, determined by Diff-quick stain (Dade Behring AG, Düringen, Switzerland) of an aliquot of cytopun cells, and the viability >99%, assessed by trypan-blue stain.

4.3 Chemotaxis

Leukocyte migration was determined using 30 µl-volume 96-well microplate chemotaxis / cell migration chambers with hydrophobic filters of pore size 3 µm (ChemoTx; Neuro Probe Inc., Gaithersburg, MD, USA) as previously described [15]. In short, the purified allergen extracts, the positive controls eotaxin [10^{-8} M] (Pepro Tech EC Ltd, London, GB) and fMLF (10^{-8} M) (Sigma, St. Louis, MO, USA), or the negative control (KRG buffer) were added to wells in the lower chamber. Cell suspensions consisting of 30,000 highly purified eosinophils or neutrophils or 100,000 monocytes in KRG buffer were added on top of the filters for 90 min at 37°C, and allowed to migrate to the lower wells. To determine the number of transmigrated cells, the cells were lysed by the addition of 1% Triton-X in PBS (Sigma) and peroxidase activity was measured as described below (EPO in eosinophils, myeloperoxidase in neutrophils and monocytes). The percentage of transmigrated cells — termed the migratory index — was determined in triplicate using the following formula: absorbance in wells containing an unknown number of transmigrated eosinophils / absorbance in wells containing the maximum number (30,000 or 100,000) of cells.

4.4 Stimulation of eosinophils

Eosinophils (10^5) were suspended in the X-vivo 15 buffer, put in 96-well low-binding polystyrene plates (TPP; Göteborgs Termometerfabrik) and co-incubated with various allergen extracts (100 µg/ml) or various concentrations of LPS with or without 5% heat-inactivated human serum for 18 h at 37°C. Eosinophil viability after this incubation period was >97% as determined by trypan-blue exclusion. Cell-free supernatants were collected and analyzed for protein content and the cells were further assessed using flow cytometry.

4.5 Flow cytometric analysis

Measurement of the surface expression of CR3 was done with a PE-labeled mouse mAb against the CD11b moiety of human CR3 (Clone 12; Becton Dickinson Immunocytometry System, San Jose, CA, USA) and, as a control, isotype-matched PE-labeled mouse IgG2a (Becton Dickinson) was used; for further details see Svensson et al. [15]. The intracellular labeling of MBP was done by first permeabilizing the cells using Cytofix/Cytoperm (BD Pharmingen, San Diego, CA, USA) and then blocking the Fc receptors with purified human γ-globulin (1 µg / 10^5 cells; Pharmacia). Eosinophils were stained for 15 min at 4°C with a mouse anti-human-MBP mAb (BD Pharmingen) or mouse anti-human-CD22

mAb (Immunotech S.A., Marseille, France) as a control. Both antibodies were diluted in Permashield containing 1% rat serum (a kind gift of Dr Esbjörn Telemo, Department of Rheumatology and Inflammation Research, Göteborg University). Incubation with the secondary antibody, *i.e.* rat anti-mouse-IgG1 (Becton Dickinson) for 15 min at 4°C followed, before fixation in ice-cold 3.7% paraformaldehyde in PBS. Finally, the cells were resuspended in PBS and analyzed by flow cytometry using a FACScan (Becton Dickinson). Release of MBP was expressed in % and estimated according to the following formula, in which "Ic MBP" refers to intracellular levels of MBP: Ic MBP in eosinophils exposed to medium – Ic MBP in eosinophils exposed to allergen extract / Ic MBP in eosinophils exposed to medium.

4.6 Degranulation of eosinophils

EPO activity in cell supernatants or lysates was measured by the addition of H₂O₂ (4 µl of 30% H₂O₂) and o-phenylenediamine (Sigma) (10 mg) dissolved in 10 ml of a lysis buffer; see Svensson et al for further details [15]. The detection limit of the EPO assay was 0.8%, *i.e.* the lowest amount of EPO detectable in a well containing 50,000 eosinophils was that corresponding to the total amount of EPO in 400 lysed cells. Release of ECP into supernatants was determined using a semi-automated enzyme immuno-assay with fluorochrome-labeled antibodies (UniCAP 100; Pharmacia, Södertälje, Sweden). The secretagogue PAF (16 carbon atoms) (Sigma) was used as a positive control of degranulation.

4.7 Release of reactive oxygen species

An isoluminol-amplified chemiluminescence system was used to determine eosinophilic production of reactive oxygen species [37]. In short, 5 × 10⁴ eosinophils resuspended in KRG were preincubated with the chemiluminescence amplifiers isoluminol (2 × 10⁻⁵ M) and horseradish peroxidase (4 U) for 5 min at 37°C before the addition of the various allergen extracts or the positive control fMLF. The chemiluminescence activity was continuously measured in a Biolumat LB 9505 (Berthold Co., Wildbad, Germany).

4.8 Cytokine determination

IL-5, IL-13 and GM-CSF levels in supernatants were measured by sandwich ELISA using human mAb anti-IL-5, -IL-13 or -GM-CSF, respectively, for capture, and the corresponding biotinylated mAb for detection. All antibodies were purchased from BD Pharmingen.

4.9 Statistics

Statistical analyses were performed using GraphPad Prism 3.0 software (GraphPad, San Diego, CA, USA). The unpaired two-tailed Student's *t*-test was employed and a *p* value of less than 0.05 was used to indicate statistical significance.

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