

Lectins interact differentially with purified human eosinophils, cultured cord blood-derived mast cells and the myeloid leukaemic cell line AML14.3D10: induction of interleukin-4 secretion is conserved among granulocytes, but is not proportional to agglutination or lectin–glycoprotein interaction

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Summary

Background Atopy is closely associated with the cellular T helper type-2 (Th2) phenotype, that is dominated by the pleiotropic cytokine IL-4. The cellular source of IL-4 has yet to be determined, although basophils have been proposed. Eosinophils and mast cells are likely contenders investigated here, and the eosinophil-like leukaemia line AML14.3D10 is compared to eosinophils as an *in vitro* culturable model for eosinophils. Lectins can cross-link-specific surface glycoproteins and are found in the ingested (processed foods) and inhaled (airborne pollen grains) human environment. Therefore it is of interest to determine whether lectins can elicit the release of IL-4 from Th2-associated granulocytes other than basophils.

Method This study investigated the ability of eosinophils, AML14.3D10 and mast cells to secrete preformed IL-4 in response to stimulation with lectins, and explored molecular mechanisms underlying the interaction.

Results Purified eosinophils and basophils, and cultured mast cells and AML14.3D10 cells were incubated with 1 µm lectin. Agglutination was scored by microscopy. IL-4 secretion was measured by enzyme-linked immunosorbent assay. Biotinylated lectins were used to determine binding to cells by flow cytometry and in lectin blots of sodium dodecyl sulphate (SDS) gels.

Discussion Purified human eosinophils, AML14.3D10 cells and cultured mast cells secrete IL-4 with a pattern similar to that found in basophils when stimulated with a panel of reactive and unreactive lectins. The lectin SNA induces IL-4 secretion from mast cells and basophils, but not from eosinophils or AML14.3D10. Eosinophils appear to secrete only pre-formed IL-4, whereas mast cells may synthesize IL-4 on ligation with the lectin LCA. Lectins that agglutinate the granulocytes investigated do not necessarily induce secretion of IL-4. Lectins that elicit secretion of IL-4 bind more to eosinophils than unreactive lectins as determined by flow cytometry and lectin blotting of SDS gels.

Conclusion As granulocytes with functions related to that of basophils, eosinophils, AML14.3D10 and cultured mast cells respond to stimulation with lectins similarly to basophils. This emphasizes the possibility that eosinophils and mast cells may be linked in their cellular heritage as the cellular partners, and lectins as ligands, may contribute to the maintenance of a Th2-favoured microenvironment that is thought to underlie the allergic march.

Keywords agglutination, AML14.3D10, ELISA, eosinophil, flow cytometry, human, IL-4, lectin, mast cell

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Introduction

IL-4 is a pleiotropic cytokine that plays an important role in the allergic response; it promotes the generation of T helper type-2 (Th2) cells, and recent results in the mouse indicate that IL-4

enhances the Th2 response initiated by IL-13 [1]. IL-4 also induces isotype switch from IgM to IgE in B cells [2] and, by enhancing expression of VLA-4 on endothelium, assists in the extravasation of granulocytes into the tissue in concert with IL-5, IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF) and TNF-α [3].

Several cell types are discussed as a source of IL-4 [4], and elucidation of the cell type that is the source of IL-4 is of considerable interest, as modulation of the secretion of IL-4 could modify Th cell profile, B cell isotype switching and granulocyte numbers in tissues. Basophils release considerable

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amounts of IL-4 *in vitro* upon antigen specific or non-specific stimulation such as lectins [5–8]. Furthermore, lectins are present in widely varying concentrations in the environment, ranging from being substantial constituents of human foods (peanuts, soy- and other beans), to airborne lectins in pollen and possibly dust from processing plants handling lectins [9]. This makes lectins interesting candidates for inducing IL-4 production from other leucocyte types such as eosinophils and mast cells and the induction of Th2 response and IgE-mediated allergy in non-sensitized individuals.

As basophils, eosinophils and mast cells have a common progenitor, related function and similar expression, we speculated that human peripheral blood eosinophils and cultured cord blood-derived mast cells (CBDMC) would also synthesize IL-4 in response to lectins known to elicit IL-4 secretion from basophil granulocytes.

Eosinophils are known to contain pre-formed IL-4 [10, 11] and have recently been shown to secrete it rapidly in response to physiologic stimuli [12]. Tissue mast cells are known to contain IL-4 [13], and the expression and secretion of IL-4 by the CBDMC, cultured by a new protocol that offers significantly increased yields of mast cells [14], is demonstrated here. The myeloid leukaemia cell line AML14.3D10 has dominating eosinophil features [15], although it can be redifferentiated into a cell with a more neutrophil-like phenotype within 1 week [16]. Here the response in AML14.3D10 is compared to purified eosinophils to elucidate the degree of this relationship.

We wanted to see whether the mechanisms inducing secretion of IL-4 are similar to those found in basophils [7]. Accordingly, we investigate the ability of a panel of lectins with known ability to induce secretion of IL-4 from basophils, to induce secretion from purified human eosinophils, CBDMC and the myeloid cell line AML14.3D10. We further compared binding of lectins to the cells by estimating agglutinative ability, and lectin binding by flow cytometry. Previous thoughts addressed the possibility that lectins cross-link IgE [7] or IgE receptors [17]. To investigate the heterogeneity of lectin ligands in the cell types studied, patterns of lectin binding to total cell lysates were compared by western blotting with biotinylated lectins of the three cell types.

Methods

Purification of eosinophils

Eosinophils were purified from 29 consenting donors by gradient centrifugation and negative selection on a MACS column as described [18]. The eosinophil preparations were more than 95% pure.

Purification of basophil granulocytes

The basophil leucocytes were purified using dynabeads (Dyna Oslo, Norway) by negative selection [19]. Blood from consenting healthy volunteers was layered on a two-step Percoll gradient with densities of 1.070 and 1.080 g/mL. The gradient was centrifuged at 400 *g* for 20 min at room temperature. The remainder of the separation procedure was performed at 4 °C. The interface was harvested, washed and the basophil leucocytes counted. Non-basophil cells were removed by negative selection of major lineages (CD3⁺, CD4⁺, CD8⁺, CD14⁺, CD16⁺, CD19⁺). Only samples with more than 80% basophil

leucocytes were used for experiments. Basophils were stimulated with 1 µM lectin or a 1/1000 dilution of anti-IgE (Behring, Marburg, Germany).

Culture of AML14.3D10

AML14.3D10 [16], a gift of C. Paul and M. Baumann, were maintained at between 0.2·10⁶/mL and 10⁷/mL in AML medium [RPMI-1640 with 10% fetal bovine serum (FBS), 50 µM β-mercaptoethanol and 1 mM sodium pyruvate].

Culture of mast cells

The culture of mast cells has been described [14]. After informed consent was obtained, human umbilical cord blood samples from normal full-term deliveries were obtained into heparinized syringes. The blood was diluted three times with phosphate-buffered saline (PBS) and layered over Ficoll-Paque PLUS (Amersham Pharmacia Biotech, Buckinghamshire, UK) and centrifuged at 400 *g* for 30 min at room temperature.

CD34⁺ cells were separated after collecting the mononuclear cell layer using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) following manufacturer's instructions. We used LS+ columns on a midi-MACS after incubating the mononuclear cells with microbeads (AC133 cell isolation kit, Miltenyi Biotec).

The CD34⁺ cells were collected and incubated at 3 × 10⁵ cells/mL in StemSpanTM SF Expansion Medium (Stemspan, Vancouver, Canada), supplemented with human stem cell factor (SCF, 100 ng/mL, Serotec, Oslo, Norway), human IL-6 (50 ng/mL, Serotec) and human IL-3 (5 ng/mL, Serotec). IL-3 was added for the first 2 weeks only. Penicillin/streptomycin (PSF) at 1% was added to the culture (Life Technologies, Grand Island, NY, USA).

After 8 weeks of culture, the culture medium was changed to StemSpan supplemented with SCF, IL-6 and PSF as before but also 10% fetal bovine serum (FBS, Life Technologies). Cells were passaged to new medium every week, and were 12 weeks old when used for these experiments.

Lectin stimulation and assay for IL-4

Cells were cultured with 1 µM lectin in RPMI-1640 (Gibco, Grand Island, NY, USA) with 10% FBS and 1% PSF (eosinophils, approximately 3 × 10⁵ cells/well), in AML medium (AML14.3D10, approximately 4 × 10⁵ cells/well) or StemSpan supplemented with growth factors (mast cells, approximately 5 × 10⁴ cells/well) for 4 h. The panel of lectins consisted of *Artocarpus integrifolia* agglutinin (AIA, L3515), *Canavalia ensiformis* agglutinin (Con A, L7647, biotinylated C2272), *Helix pomatia* agglutinin (HPA, L7760, biotinylated L6512), *Lens culinaris* agglutinin (LCA, L92667), *Phaseolus vulgaris* erythroagglutinin (Pha-E, L8629, biotinylated L3509), *Pisum sativum* agglutinin (PSA, L5380) and *Sambucus nigra* agglutinin (SNA, L6890) all from Sigma (St Louis, MO, USA), with specificities as described before [7]. Individual wells of eosinophils were photographed on an Olympus IX50 for comparison of agglutination. Photographs were scored for agglutination indices on an arbitrary scale from 1 to 4 (most agglutination). The supernatant was stored at –80 °C until analysis. IL-4 was measured in duplicate with a high-sensitivity enzyme-linked immunosorbent assay (ELISA) kit (detection limit 0.05 pg/mL, R&D Systems, Minneapolis, MN, USA) according to the

manufacturers instructions. The internal variation was below 10%. Eosinophil preparations were tested for secretion in a median of nine experiments (PSA was only tested on seven preparations, HPA on 11), mast cells with a median of 4, and AML4.3D10 were tested in two experiments.

Flow cytometry

Cells (10^6 per analysis) were incubated with 70, 200 and 700 nM biotinylated lectin for 10 min, washed with PBS and incubated with 10 μ L fluorescein-conjugated streptavidin for 2 min. After addition of propidium iodide to identify dead cells, 50 000 events were acquired on a BD FACSCalibur for analysis. Data were analysed with FCS Express. Their characteristic forward- and side scatter identified granulocyte populations, and propidium iodide-negative events were selected for the analysis. The mean fluorescence intensity of the fluorochrome conjugated to streptavidin was determined as a measure of the extent of binding of the biotinylated lectin to the cell population.

Lectin blotting

Protein samples representing 5×10^4 cells were separated on SDS gels and transferred to nitrocellulose filters as described [20]. The filters were blocked and probed as described [7], and lectin binding of protein bands was visualized with horseradish peroxidase (HRP). Individual filters were developed for optimized times as indicated.

Results

Human eosinophils, mast cells and the myeloid leukaemic cell line AML14.3D10 secrete IL-4 when stimulated with lectins.

In order to confirm the results of a previous report, that lectins can induce secretion of IL-4 from human basophils, basophils from three normal donors were purified and stimulated with Con A or with IgE/anti-IgE (Fig. 1). The preparations secreted marked amounts of IL-4 in response to stimulation with Con A, and two of three preparations

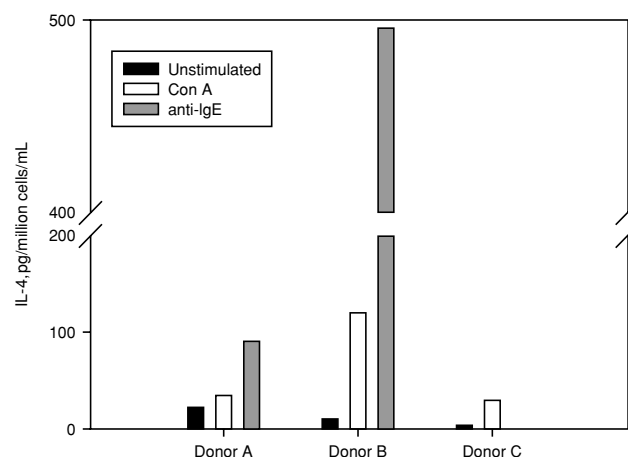


Fig. 1. Human basophils secrete IL-4 when stimulated with Con A or anti-IgE. Purified basophils were incubated with Con A or anti-IgE for 4 h at 37 °C in 5% CO₂. Cultures were assayed in serial dilutions to determine the amount of IL-4 secreted by the cell preparations. Both Con A and anti-IgE treatment resulted in secretion of IL-4 from donors A (purity 97%) and B (purity 95%), but not from donor C (purity 85%), who was a non-responder for anti-IgE.

secreted IL-4 in response to stimulation with anti-IgE. The third preparation was non-responsive, as is known to occur in previous work [7].

In order to determine the magnitude of the response to be expected from eosinophils, a number of preparations were stimulated with a range of concentrations of ionomycin (not shown). There was dose-dependent secretion of IL-4 in response to ionomycin, though to a much lower extent than was observed with basophils.

To investigate whether eosinophils, mast cells and AML 14.3D10 could, like basophils, secrete IL-4 when stimulated with lectins, preparations of the cells were stimulated with a panel of lectins known to stimulate secretion of IL-4 from basophils (LCA, Pha-e, PSA, SNA) or to be inert (CFA, AIA, HPA [7]; Fig. 2). Lectins that induce secretion from basophils (LCA, Pha-e, PSA) also induced secretion from eosinophils and mast cells, whereas lectins inert towards basophils could not induce secretion of IL-4 from eosinophils or mast cells. SNA induces secretion from basophils, but did not have appreciable activity when tested with eosinophils or AML14.3D10. It induced marked secretion of IL-4 from mast cells. The response of eosinophils and mast cells was more similar to that of basophils, whereas the cell line AML14.3D10 was less restrained and secreted IL-4 when stimulated with all lectins except SNA. Con A was included in the panel of stimulatory lectins, but at the level of sensitivity used, Con A in the supernatant contributed unspecifically to the IL-4 ELISA assay. Other lectins in the panel did not interfere with the assay.

A kinetic experiment was designed to approximate the rate of release of IL-4 from eosinophils and mast cells (Fig. 3). Eosinophils released all IL-4 within the first 2 h of exposure, whereas the release of IL-4 from mast cells was biphasic; there was early release of IL-4 up to 2–4 h, and at 6–8 h, IL-4 secretion from mast cells increased again. This may be newly synthesized IL-4, but was not pursued.

Different lectins bind to eosinophils and mast cells with different affinity, and recognize different patterns of proteins on the cell surface

Agglutination was estimated as a measure of the interaction of a given lectin with granulocytes (Fig. 4). Although all lectins that effected secretion also caused agglutination, AIA also caused marked agglutination in the absence of appreciable secretion of IL-4. Thus, there was no absolute correlation between the extent of agglutination of eosinophils or mast cells and release of IL-4.

The extent of binding of two biotinylated lectins was compared by flow cytometry (Fig. 5). These lectins are representative of the group of reactive and agglutinative lectins (Pha-E) and the non-reactive, non-agglutinative lectins (HPA). The lectins bound to cells in proportion to the amount of IL-4 secreted and the extent of agglutination; HPA did not bind appreciably, whereas Pha-E bound well.

The glycosylation patterns of eosinophils and mast cells were compared on a lectin blot (Fig. 6). Similar numbers of cells (5×10^4 cells) were separated on an SDS gel, transferred to nitrocellulose and probed with biotinylated lectins. The lectins were visualized with HRP. Three preparations of eosinophils and of mast cells were compared with each other and with AML14.3D10. There was no detectable difference between the

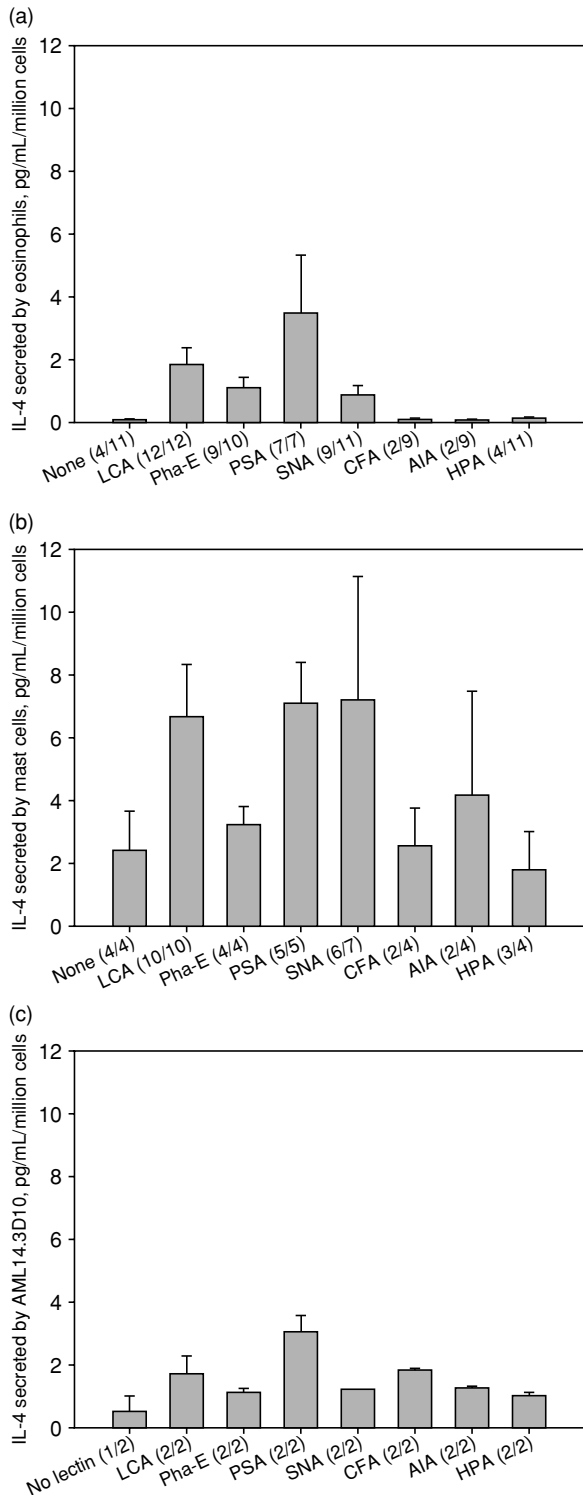


Fig. 2. The pattern of lectin-induced secretion of IL-4 from eosinophils, mast cells and AML14.3D10 is similar to that from basophils. Eosinophils, mast cells or AML14.3D10 were cultured for 4 h at 37 °C in 5% CO₂ with a panel of lectins (1 µM) of which some have been shown previously to elicit IL-4 secretion from basophils or not to do so [7]. Numbers in brackets after the lectin name indicate the fraction of experiments in which IL-4 secretion with a given lectin could be measured. LCA, Pha-E and PSA stimulated secretion of IL-4 from all cell types tested. CFA, AIA and HPA elicited no detectable response from eosinophils, a marginal response from mast cells and a response comparable to the one obtained by responder lectins above from 3D10. Ligation with SNA did not elicit degranulation of eosinophils or AML14.3D10, but marked degranulation of mast cells.

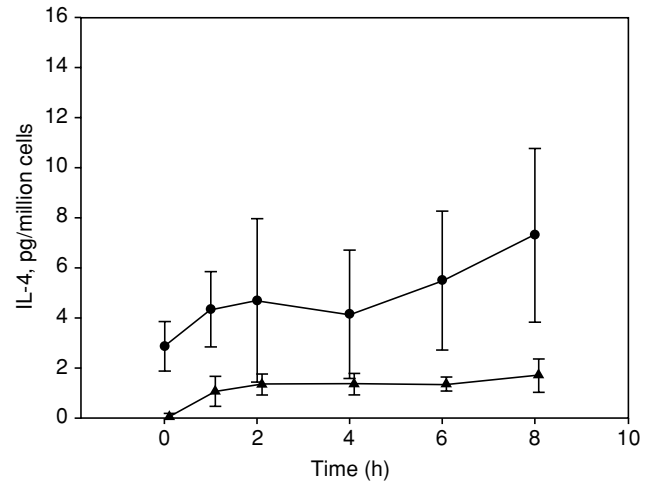


Fig. 3. Time-dependent secretion of IL-4 from blood eosinophils (▲, $n = 5$) and mast cells (●, $n = 4$) with LCA. Purified eosinophils (triangles) or cultured mast cells (circles) were incubated with 1 µM LCA for increasing amounts of time. Duplicate cultures were assayed for IL-4. Eosinophils appear to have secreted all IL-4 after 2 h, whereas there is an indication that mast cells secrete preformed IL-4 during the first 2–4 h, and secrete larger amounts of (newly synthesized?) IL-4 from 6 h onwards.

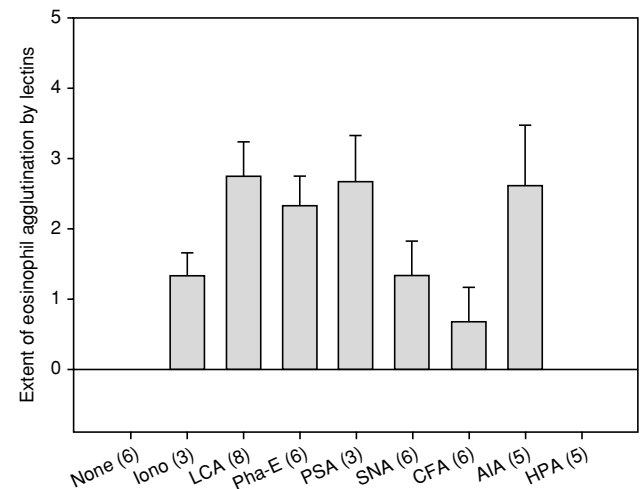


Fig. 4. Agglutination of eosinophils is not proportional to secretion of IL-4. Wells containing eosinophils were photographed 1 h after addition of lectins, and photographs were scored for agglutination on a subjective scale from 1 to 4. Eosinophils were agglutinated with all lectins stimulating secretion, as well as with ionomycin (4 µM). Although AIA did not induce secretion of IL-4 from eosinophils, it caused extensive agglutination.

different preparations (not shown). There were significant differences between the different cell types for the different lectins, and in the patterns recognized by the different lectins. HPA bound only one protein in eosinophils and two different bands in mast cells. Pha-E bound less abundantly than Con A. At the resolution offered by one-dimensional analysis of protein bands, no common epitopes could be identified.

Discussion

The ability of basophils to secrete IL-4 when stimulated with lectins was compared to the secretion achieved with IgE/

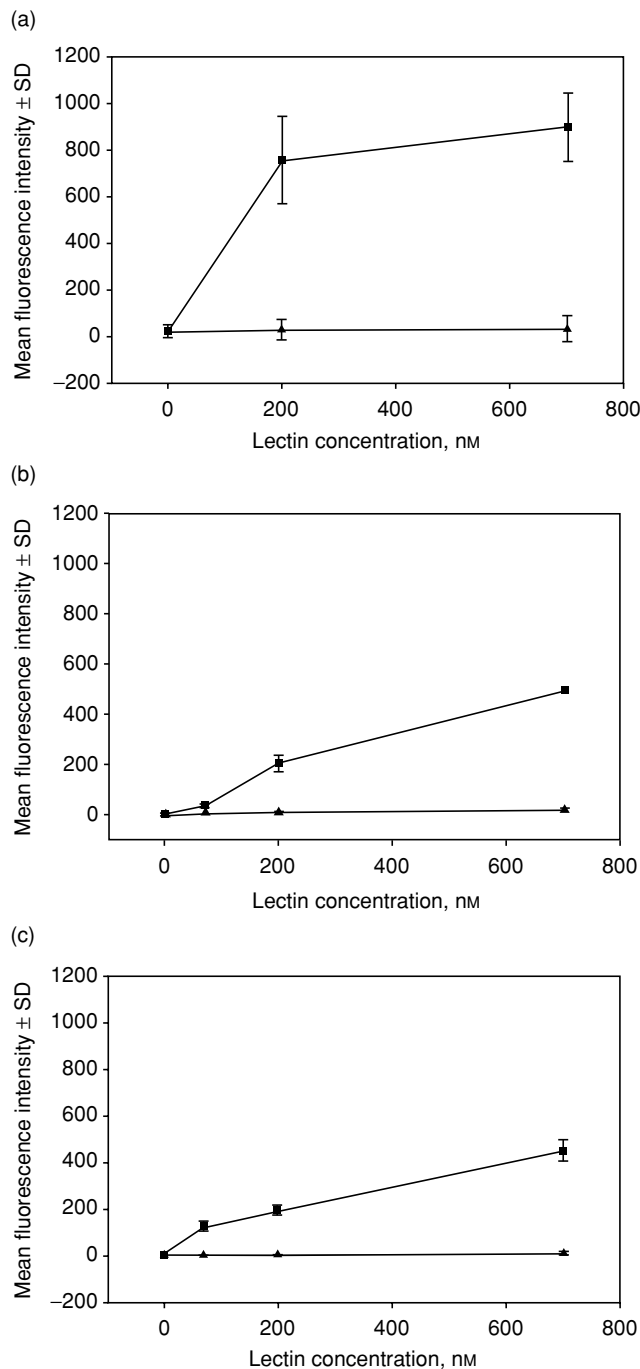


Fig. 5. Binding of Pha-E, but not of HPA to eosinophils and mast cells can be detected by flow cytometry. Purified eosinophils (a), mast cells (b) or AML14.3D10 (c) were incubated with biotinylated Pha-E (■) or HPA (▲), washed and incubated with streptavidin conjugated to either fluorescein isothiocyanate (FITC) or Alexa-633. Samples were stained with propidium iodide to exclude dead cells and analysed by flow cytometry. Cells labelled stronger with Pha-E than with HPA, in agreement with IL-4 secretion data.

anti-IgE. As shown previously, both Con A [7] and anti-IgE receptor [21] induced secretion of IL-4 from basophils. Eosinophils could secrete IL-4 when stimulated with increasing doses of ionomycin, as shown previously by us [10] and others [11, 12]. Similar results were obtained in the myeloid cell line AML14.3D10. It is unlikely that contaminating basophils

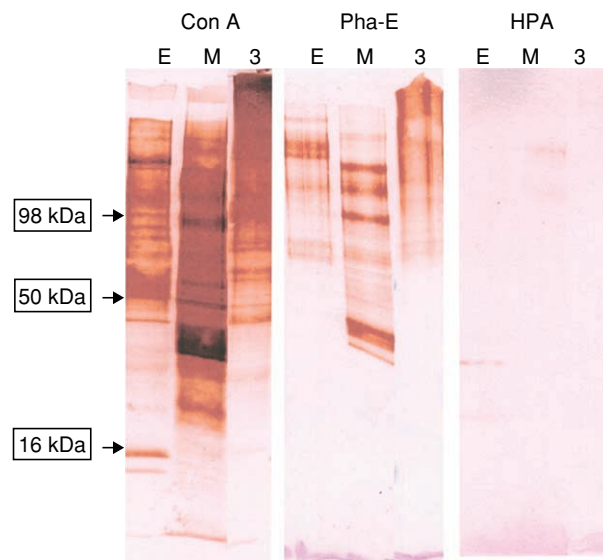


Fig. 6. The pattern of binding of Con A, Pha-E and HPA to eosinophils, mast cells and AML14.3D10 is different as detected by lectin blotting. Eosinophils, mast cells and AML14.3D10 (5×10^4 cells/lane) were separated on SDS gels, transferred to nitrocellulose, blocked and probed with biotinylated lectins as described [7]. The blot with Con A was developed for 10 min, the blot with Pha-E for 20 min and the blot with HPA for 60 min. The shown lanes are representative for more three different preparations of each cell type. The pattern of binding to proteins from AML14.3D10 is more similar to eosinophils than to mast cells. There are marked differences between cell types, and the lectins recognize widely differing spectra of glycosylated proteins.

explain the results obtained with purified eosinophils, as the gradients used to separate granulocytes and mononuclear cells were optimized for purity of the granulocyte fraction (density of 1.088 g/mL).

Purified human eosinophils, CBDMC and the myeloid cell line AML14.3D10 secrete low levels of IL-4 when stimulated with lectins, compared to basophils [7]. The total amount of IL-4 in eosinophils determined here (release through ionomycin, less than 10 pg/mL/million cells) or others (37 pg/mL/million cells) [12] is much lower than that found in basophils that released more than 500 pg/mL/million cells in response to lectins or stimulation through the IgE receptor [7]. Although SNA induced secretion of IL-4 from basophils and mast cells, it was inactive with respect to eosinophils and the eosinophil cell line AML14.3D10.

LCA, Pha-E and PSA stimulated secretion of IL-4 from eosinophils, CBDMC and AML14.3D10, as well as from basophils [7]. The lectins that were inert with respect to basophils were also inert with respect to eosinophils, stimulated CBDMC slightly and AML14.3D10 cells more so.

As a more immediate index of the effect of lectins on eosinophils, agglutination was assessed 1 h after addition of the lectins. Agglutination of eosinophils did not correlate with secretion of IL-4, as AIA agglutinated eosinophils extensively, yet it did not induce secretion of IL-4, and as such would not be an accurate indicator of IL-4 secretion.

Binding of three commercially available biotinylated lectins representing the activating as well as the inert lectin group was evaluated by flow cytometry. The inert lectin, HPA, bound only marginally to cells, whereas Pha-E bound strongly to all tested cells.

As the majority of glycosylated proteins appear to be located in the membrane or extracellular [22–24], whole-cell lysates of eosinophils, mast cells and AML14.3D10 were probed with biotinylated lectins in lectin blots. The pattern of lectin binding by AML14.3D10 shown by the lectin blot was more similar to eosinophils than to mast cells, supporting the notion that AML14.3D10 is an eosinophil-like myeloid cell. There was substantial binding by Con A, less with Pha-E and hardly detectable binding with HPA. The epitopes for HPA may have been destroyed during analysis, or did not enter the SDS gel. The blot demonstrates that there are multiple interactions of the active lectins with glycosylated proteins, that may activate the cells, and that the activity thus does not need to depend on immunoglobulins.

As most of the lectins exist as multimers, the data presented here support the notion that lectins activate specific pathways by cross-linking glycosylated proteins on the cell surface. Ligation *per se* does not induce secretion of IL-4, as exemplified by the results with AIA in which binding did not elicit appreciable IL-4 secretion. Ligation could affect IL-4 secretion (Figs 1 and 3), agglutination (Fig. 4) or cell death. The observation that a panel of lectins has a similar spectrum of activity on eosinophils, mast cells, a myeloid cell line and basophils reinforces that these cell types are related, and may have similar pathways of activation. Although these results do not exclude predominant signalling through IgE and its receptors (which are present at a very low density on eosinophils [25, 26] and absent in the cell line AML14.3D10 [27]), they illustrate that this is not the only surface glycoprotein complex that can be activated to induce secretion of IL-4. Considering the relative frequency of basophils, eosinophils and mast cells (especially at sites of allergic inflammation), eosinophils and mast cells may, in spite of the low amounts of preformed IL-4 secreted, contribute significantly to the maintenance of a Th2-favoured cellular microenvironment that is thought to underlie the atopic march.

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