

Identification of interleukin-2 in human peripheral blood eosinophils

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SUMMARY

Interleukin-2 (IL-2) is an essential growth factor for T cells. Previous studies have shown that human peripheral eosinophils respond to IL-2 in chemotaxis and express the IL-2 receptor (CD25). In addition, eosinophils have been shown to transcribe messenger RNA for IL-2. The aim of the present study was to determine whether eosinophils translate mRNA for IL-2 and to determine the site of intracellular localization. By immunocytochemistry, an average of 9% of cells showed cytoplasmic staining for IL-2 in freshly isolated unstimulated blood eosinophils obtained from asthmatic subjects who were not receiving oral corticosteroid treatment ($n = 5$). Freshly isolated, disrupted, highly purified eosinophils (>99%, by CD16⁻ immunomagnetic selection) contained an average of 6 pg/10⁶ cells of IL-2 measured by a specific enzyme linked immunosorbent assay (ELISA) ($n = 7$). Purified eosinophil incubated with serum-coated Sephadex beads showed an increase in the amount of intracellularly-retained IL-2 (26.2 ± 7.2 pg/10⁶ cells) with some evidence for release of this cytokine but only in three out of six eosinophil preparations (range 1.3–5.8 pg/10⁶ cells). The intracellular localization of IL-2 was determined by fractionation of the cells on a linear (0–45%) Nycodenz gradient in sucrose buffer followed by detection of IL-2 in the fractions using an IL-2-specific ELISA and dot blotting. The majority of the IL-2 detected co-eluted with known eosinophil granule markers (i.e. major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and β -hexosaminidase) but small quantities were also detected in the cytosolic (lactate dehydrogenase (LDH) associated) and membrane (CD9⁺) fractions. Immunogold labelling of intact eosinophils using an anti-IL-2 monoclonal antibody confirmed IL-2 immunoreactivity in association with the eosinophil crystalline granule cores. These data are consistent with the hypothesis that eosinophils synthesize, release and store IL-2 largely within crystalloid granules. This stored IL-2 may serve as a reservoir for rapid release of IL-2 in inflammatory reactions associated with eosinophilia.

INTRODUCTION

Recent studies have indicated that cytokines, originally considered to be derived predominantly from lymphocytes and monocytes, are also synthesized and released by a number

of other inflammatory and tissue cell types. Interleukin-2 (IL-2) is produced by activated CD4⁺ T lymphocytes and is recognized as an essential T-cell growth factor. It also participates in the growth and differentiation of B cells and natural killer (NK) cells as well as various non-lymphoid cells.^{1–4} In the mouse, IL-2, together with interferon- γ (IFN- γ), is predominantly produced by Th1-type cells.⁵ However, other than T cells, only mast cell lines have hitherto been shown to be a source of IL-2.^{6,7}

Eosinophils are prominent cells in asthma and allergic inflammation and immune reactions to parasitic helminths. They are implicated in tissue damage associated with allergic disease, especially asthma, possibly via the release of lipid mediators derived from their membrane phospholipids and cytotoxic cationic proteins from their unique crystalloid granules (reviewed by Wardlaw and Moqbel⁸). Eosinophils were shown to synthesize and translate a number of Th0- and Th2-type cytokines including IL-1 α , tumour growth factors α and β , granulocyte-macrophage colony-stimulating

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Abbreviations: APAAP, alkaline phosphatase anti-alkaline phosphatase; BSA, bovine serum albumin; ECP, eosinophil cationic protein; ELISA, enzyme-linked immunosorbent assay; EPO, eosinophil peroxidase; GM-CSF, granulocyte-macrophage colony-stimulating factor; Hex, β -hexosaminidase; IFN- γ , interferon- γ ; IL, interleukin; LDH, lactate dehydrogenase; mAb, monoclonal antibody; MBP, major basic protein; MIP1- α , macrophage inflammatory protein-1 α ; TBS, tris-buffered saline; TGF, transforming growth factor; TNF, tumour necrosis factor.

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factor, IL-3, IL-5, IL-6, IL-8 and tumour necrosis factor- α and the chemokine macrophage inflammatory protein- α (MIP- α) (reviewed in ref. 9), all of which are believed to possess important auto- and paracrine regulatory roles in inflammation. However, the ability of eosinophils to produce Th1-type cytokines has not yet been evaluated, especially in relation to allergic or asthmatic inflammatory reactions. Eosinophils express functional IL-2 (CD25) receptors¹⁰ while IL-2 has been shown to be a potent chemoattractant for eosinophils from normal and eosinophilic individuals.¹¹

In the present study we have attempted to identify IL-2 in human peripheral blood eosinophils and to determine its subcellular localization.

MATERIALS AND METHODS

Materials

Di-isopropyl fluorophosphate (DFP), leupeptin, aprotinin, *N*-*p*-tosyl-L-arginine methyl ester (TAME), 4-methylumbelliferyl-sulphate, 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide, *o*-phenylenediamine, Coomassie Brilliant Blue G-250, β -nicotinamide adenine dinucleotide, reduced form (NADH), sodium pyruvate, were all purchased from Sigma (Poole, Dorset, UK). Adenosine triphosphate (ATP) was obtained from Boehringer Mannheim (Mannheim, Germany). Nycodenz was purchased from Nycomed AS Pharma, Oslo, Norway.

Eosinophil purification

A volume of 100 ml of peripheral blood was obtained from mild atopic asthmatics with eosinophilia >10% who were not receiving oral corticosteroids. After dextran sedimentation of the erythrocytes, a granulocyte pellet was obtained from the remaining leucocytes by density centrifugation on Percoll. Eosinophils were purified by immunomagnetic selection¹² using the MACS system (Becton Dickinson, Cowley, UK). This method is based on the observation that resting eosinophils, unlike neutrophils, do not express surface CD16. In brief, the granulocyte pellet was incubated (40 min, 4 degree) with anti-CD16 monoclonal antibody (mAb) bound to micromagnetic beads (Miltenyi Bio-Tech, Bergisch-Gladbach, Germany). In order to eliminate any contamination of the eosinophils with mononuclear cells, anti-CD14- and anti-CD3-coated micromagnetic beads (Lab Impex, Teddington, Middlesex, UK) were also added to the anti-CD16/granulocyte mixture. By negative selection, highly-purified CD16⁻ eosinophils (>99%) depleted of magnetically-positive neutrophils (CD16⁺) and any contaminating mononuclear cells (CD14⁺/CD3⁺) were obtained after passage of the granulocytes through a ferrous matrix column held in the field of a permanent magnet. The few contaminating cells were neutrophils.

IL-2 content of freshly isolated eosinophils

Freshly isolated and purified eosinophils (5×10^6 – 10×10^6 cells) were washed once by sedimentation (5 min, 120 g) in phosphate-buffered saline (PBS) and resuspended in the same buffer at a concentration of $1 \times 10^6/100 \mu\text{l}$. Eosinophils were disrupted with three cycles of freeze-thawing or by continuous sonication in a bath sonicator (30 seconds, output 5, 50% Duty cycle, Heat Systems Ultrasonics) and their IL-2 content was evaluated by the Quantikine enzyme-linked immunosorbent assay (ELISA) kit (British Biotechnology, Oxford, UK) and

adjusted to $\text{pg}/10^6$ cells. Eosinophils were also incubated with serum-treated Sephadex G-15 beads for 45 min at 37° as described¹³ and IL-2 was measured both in the cell pellet and in the supernatant (collected by centrifugation for 10 min at 1000 g) using the same ELISA kit.

Fractionation of human eosinophils

Highly purified human eosinophils (99% pure, 5×10^7 cells) were treated with 2 mM DFP, a serine proteinase inhibitor, for 5 min at room temperature and then sedimented by centrifugation at 240 g for 5 min. The pellet was resuspended in ice-cold 0.25 M sucrose buffer (containing 10 mM HEPES, 1 mM EGTA, and 5 mg/ml each of leupeptin, aprotinin and TAME, pH 7.4) and the cells were pelleted again at 4°. Cells were resuspended at 10 – $15 \times 10^6/\text{ml}$ in the above sucrose buffer supplemented with 2 mM MgCl₂ and 1 mM ATP.

For homogenization, the cells were subjected to 8–12 passages through a high precision ball bearing device (EMBL, Heidelberg, Germany) with a clearance of 11 μm . The post-nuclear supernatant, (3–4 ml) obtained by centrifugation at 400 g for 10 min was layered gently onto an 8 ml linear Nycodenz gradient (0–45% Nycodenz dissolved in HEPES-buffered sucrose) in a Beckman (High Wycombe, UK) Ultra-Clear centrifuge tube (14 \times 89 mm). Nycodenz is a non-ionic tri-iodinated density gradient medium which, unlike sucrose, exhibits relatively low toxicity throughout the gradient. The supernatant was centrifuged to equilibrium density at 100 000 g for 1 hr at 4°. Fractions (24 \times 0.4 ml) were collected from each preparation. These were stored at 4° for no longer than overnight, and later at –80° until used. The density of each fraction was later determined by use of a refractometer. Density (in g/ml) was calculated by the equation $3.410\eta - 3.555$, given by the Nycodenz specification sheet for a solution of Nycodenz using a sucrose diluent, and where η represents the refractive index.

Enzyme assays

Hexosaminidase activity, a marker for granular and lysosomal compartments, was measured as previously described.¹⁴ To measure eosinophil peroxidase (EPO) activity we adapted the method of White *et al.*¹⁵ for microtitre plates. The cytosolic enzyme lactate dehydrogenase (LDH) was measured by a previously described method,¹⁶ but adapted for use with a microtitre plate reader. Absorbance (340 nm) was recorded over 20 min at 1-min intervals and the rate of enzyme reaction was calculated by regression analysis over the 20-min period for each sample.

Antibodies

For detection of IL-2 by dot blot, a polyclonal goat anti-human IL-2 antibody was used in parallel with its negative control of a goat anti-mouse polyclonal antibody (British Bio-Technology, Abingdon, UK). Eosinophil granule major basic protein (MBP) was detected by an in-house mouse mAb BMK-13 (Supernatant IgG1) which has been carefully validated.¹⁷ Anti-CD9 mAb (purified IgG1) was purchased from Becton Dickinson. A monoclonal mouse IgG1 anti-human IL-2 antibody was used for immunogold staining. Normal mouse IgG, anti-CD3 mAb (supernatant IgG1) and anti-CD5 mAb (purified IgG1) were used as irrelevant negative controls for the above mAb (Becton Dickinson).

Immunocytochemistry

The alkaline phosphatase anti-alkaline phosphatase (APAAP) method was performed on cytopins. These were washed in Tris-buffered saline (TBS), anti-human IL-2 (either polyclonal or monoclonal) antibodies was added at a final concentration of 100 mg/ml and allowed to incubate overnight at 4°. Slides were briefly washed in TBS, APAAP-conjugated rabbit anti-goat (Dako, High Wycombe, UK) was added at 1:40 dilution and developed with Fast Red. Appropriate negative controls (TBS only, an irrelevant antibody raised in the same species, anti-IL-2 in the absence of the second layer and second layer alone) were included. The anti-IL-2 antibodies were validated by the manufacturers.

IL-2 ELISA

IL-2 was detected in the fractions using the Quantikine ELISA kit (R&D, MN, USA). The assay was performed in duplicates of undiluted fractionated material, according to the manufacturer's instructions. The sensitivity limit of this ELISA was 6.0 pg/ml.

Dot blot analysis

This technique was used to confirm the presence of IL-2 shown by ELISA and to detect granule associated proteins (MBP, using BMK-13;¹⁷ eosinophil cationic protein (ECP), using EG2; Pharmacia, Uppsala, Sweden) and the eosinophil plasma membrane marker CD9. A total of 2 µl of fraction supernatants were placed on a nitrocellulose membrane, allowed to dry and blocked in 5% milk powder (Sigma). The blotted membrane strips were incubated with the appropriate antibodies and after extensive washings in PBS/Tween 20 incubated with anti-mouse or goat biotin-conjugated antibodies followed by streptavidin-alkaline phosphatase and developed. Recombinant IL-2 was used as a positive control. Negative controls were included, as in the immunocytochemistry (see above), and the fractional activities of the markers were assessed by staining density, given arbitrary units and converted to % of total activity for each product.

Immunolabelling for electron microscopy

Pelleted, isolated eosinophils were fixed in freshly prepared formaldehyde (2% in PBS, 0.1 M pH 7.2) for 2 hr. They were then embedded in Lowicryl K4M resin using the Balzers FSU 010 preparation plant, equipped with an ultraviolet lamp, and a progressive lowering of temperature (PLT) infiltration procedure (30 min, 30% ethanol at 0°; 1 hr, 50% ethanol at -20°; 1 hr, 70% ethanol at -30°; 1 hr, 90% ethanol at -35°; 12 hr, 100% ethanol at -35°; 1 hr, 100% ethanol/resin 1:1 at -35°; 1 hr, 100% ethanol/resin 1:2 at -35°; 1 hr, pure resin at -35°; 15 hr, pure resin at -35°; changed into pure resin in gelatine capsules and polymerized using ultraviolet light for 48 hr at -35°, followed by a further 24 hr polymerization at room temperature). Silver sections were cut and picked up onto thin bar (460 TB Hex) nickel grids.

Prior to labelling, sections were immersed sequentially in the following blocking solutions: 1% acetylated bovine serum albumin (BSA), (Aurion Ltd), in 0.01 M PBS pH 7.2 (5 min); 1% BSA in PBS (5 min); 1% gelatine in PBS (10 min); 0.02 M glycine in PBS. Sections were then washed five times in 1% BSA/PBS and labelled by immersion in a solution containing mouse anti-human IL-2 antibody at 1:200 in PBS for 16 hr at

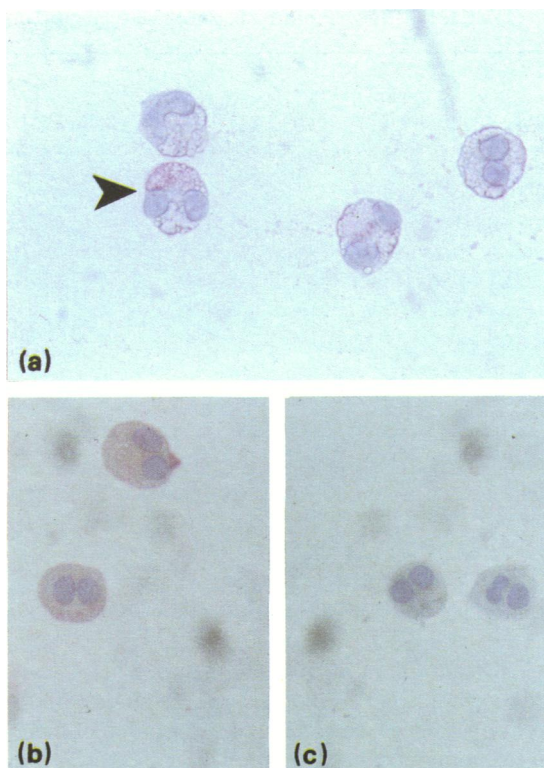


Figure 1. Light microscopy photomicrograph of human eosinophils immunostained with the APAAP technique using (a) monoclonal anti-human IL-2 antibody; (b) a polyclonal (goat) anti-human IL-2 antibody; and (c) a polyclonal (goat) irrelevant antibody ($\times 1000$).

room temperature. After a 1 min wash in PBS, label was visualized by immersion in a goat anti-mouse, 10 nm gold, secondary antibody marker system, 1:20 in PBS for 75 min (Biocell Ltd, Cardiff, UK). Sections were then immersed in 1% glutaraldehyde (1 min) before being jet washed with distilled water. For negative controls, we substituted mouse IgG (1:200) for anti-IL-2 or performed labelling with 1% BSA/PBS without a primary antibody. Grids were examined unstained, as the location of gold particles was obscured by the dense crystalloid granule in stained sections.

Data presentation

The bioactivity of eosinophil granule, membrane and cytosol constituents following fractionation, including IL-2 quantitation by ELISA (in pg/ml) and dot blot, are expressed as frequency distributions according to the method of Beaufay *et al.*¹⁸ by which the fractional activities of the markers are presented as a function of the density for each fraction.

RESULTS AND DISCUSSION

Using the APAAP technique, freshly-prepared cytopins of highly purified eosinophils obtained from asthmatic subjects were examined for IL-2 immunoreactivity using both a polyclonal and a monoclonal anti-human IL-2 antibody (Fig. 1a,b). Cytoplasm- or granule-associated IL-2 immunoreactivity was observed in $6.8 \pm 0.4\%$ ($n = 5$) of the cells. The negative controls, including the irrelevant antibody of the same

Table 1. IL-2 content of freshly isolated eosinophils

Atopic asthmatic subjects	Peripheral blood eosinophils (% total leucocytes)	Purity of eosinophil (%)	IL-2 (pg/10 ⁶)* (ELISA)
1	8.8	98.6	3.8
2	12.0	97.1	4.1
3	10.0	97.1	1.7
4	13.0	98.9	7.7
5	6.0	99.6	16.0
6	7.1	99.6	4.0
7	10.0	98.5	3.0
Mean ± SEM	9.6 ± 0.9	98.5 ± 0.4	5.8 ± 1.8

* Freshly isolated eosinophils ($5-10 \times 10^6$ cells) were disrupted and IL-2 content was determined by ELISA as described in the Materials and Methods.

isotype and the omission of the primary antibody step, did not reveal any immunoreactivity (Fig. 1c).

The concentration of IL-2 in homogenates of freshly isolated (unstimulated) eosinophils was measured by a specific ELISA assay. The mean (\pm SEM) concentration of preformed IL-2 in eosinophils from seven asthmatic subjects was found to be 5.8 ± 1.8 pg/10⁶ cells (Table 1). Furthermore, purified eosinophils from six subjects, when incubated with serum-treated Sephadex G-15 beads (45 min, 37°) contained a mean of 26.2 ± 7.2 pg/ml of total extractable IL-2. Eosinophils from three out of six subjects showed variable, albeit small, amounts of released IL-2 (range 1.5–5.8 pg/10⁶ cells); the concentrations measured were near or below the sensitivity threshold of the assay (Table 2).

The subcellular localization of intracellular IL-2 was determined by homogenizing 5×10^7 freshly isolated pure (>99%) unstimulated eosinophils from four different subjects and fractionating them on a linear Nycodenz gradient. Figure 2 shows the elution profile of one representative fractionation in which we detected IL-2 immunoreactivity (by both ELISA and

Table 2. Detection of IL-2 immunoreactivity in eosinophils*

Subject	Supernatants (pg/ml)	Cell pellet (pg/ml)
1	0	15.3
2	0	10.5
3	1.3	20.1
4	0	14.3
5	2.5	44.4
6	5.8	52.4
Mean ± SEM	1.6 ± 0.9	26.2 ± 7.2

* Cells (10^5) were cultured with serum-coated Sephadex beads for 45 min at 37°. Supernatants were collected by centrifugation and IL-2 was measured by an IL-2-specific ELISA kit.

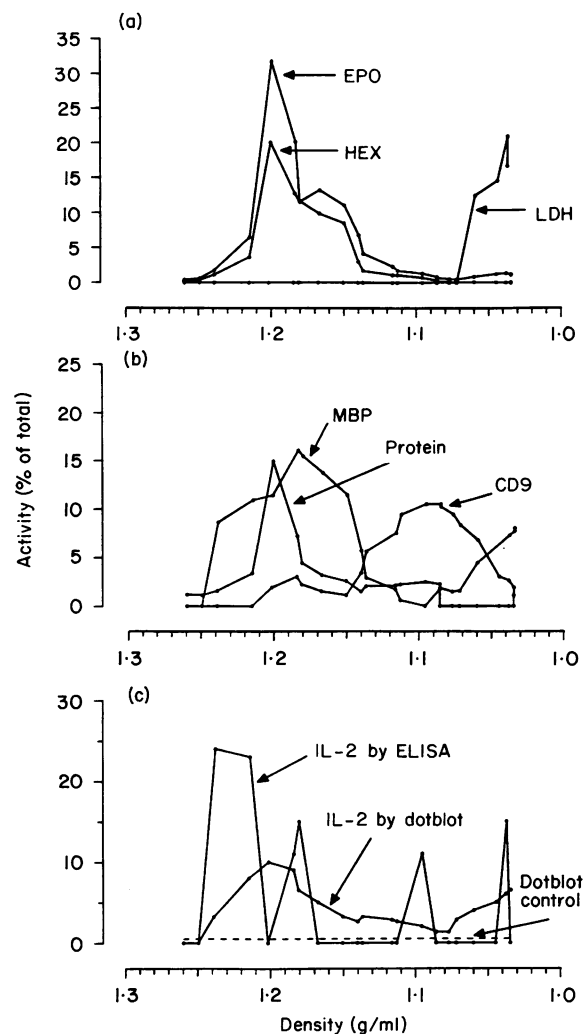


Figure 2. A representative fractionation experiment (one of four) of human eosinophils detailing all the profiles of (a) β -hexosaminidase (Hex), EPO and LDH; (b) MBP, protein and CD9; and (c) IL-2 immunoreactivity in both ELISA and dot blot analysis together with dot blot control. Eosinophil subcellular fractions were separated on the basis of their density (g/ml) on Nycodenz gradient.

dot blot) in fractions of equivalent density (1.2 g/ml) to those containing MBP (by BMK-13), EPO and β -hexosaminidase (the latter two by enzymatic assays), as well as total protein. CD9 immunoreactivity was associated with fractions having a density range of 1.04–1.17 g/ml, which is the expected range of densities for plasma membrane-containing fractions. A relatively smaller peak of CD9 immunoreactivity coeluted with MBP and EPO, β -hexosaminidase which may reflect the presence of the CD9 marker on eosinophil granule membranes. The cytosol, as identified by the presence of LDH activity was associated with the lightest fractions (density 1.03–1.07 g/ml). The majority of the IL-2, as detected by ELISA, coeluted with the granule-associated markers (EPO, β -hexosaminidase and MBP) in two distinct peaks. However, considerably smaller peaks of immunoreactivity were also detected in membrane- (CD9⁺) and cytosolic- (LDH⁺) associated fractions. Dot blot analysis of the same fractions further confirmed the ELISA

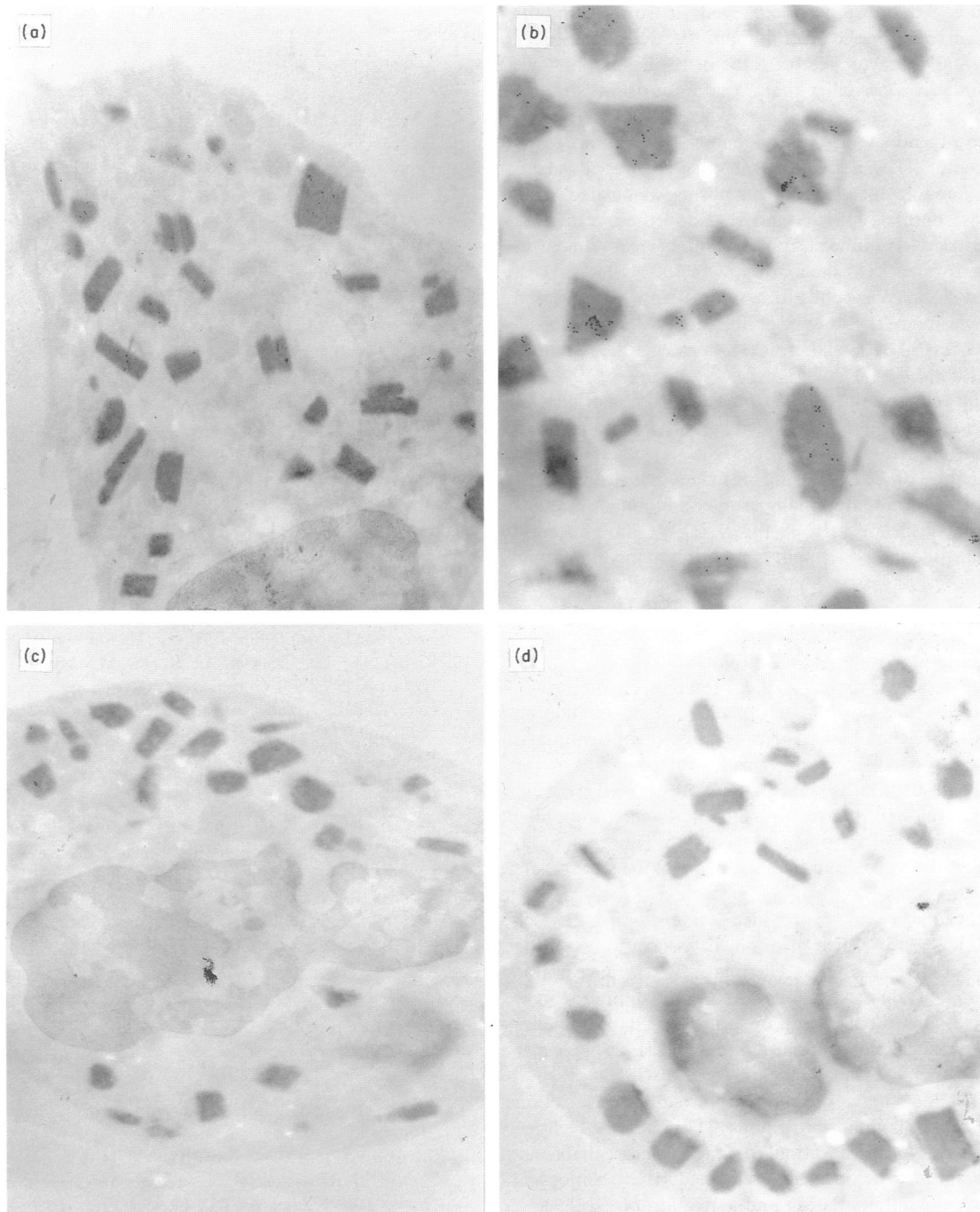


Figure 3. Immunogold labelling of embedded purified human eosinophils: (a) anti-IL-2 labelling ($\times 16\,000$); (b) high magnification of (a) ($\times 36\,000$) to show distribution of gold particles over the granules; (c) mouse IgG control ($\times 16\,000$); and (d) secondary antibody gold only control ($\times 16\,000$).

pattern of IL-2 identity, but did not identify the smaller peaks observed using ELISA.

The elution profile of IL-2 from three fractionations of similar numbers of eosinophils obtained from other asthmatic patients was also assessed by both ELISA and dot blot and confirmed that IL-2 was present mainly in association with the granule-rich fractions. Again, minor IL-2 immunoreactivity peaks were observed in membrane and cytosolic fractions.

Immunogold labelling of Lowicryl-embedded intact cells (fixed with 2% formaldehyde) indicated gold particles in association with eosinophil secretory granules (Fig. 3). The most abundant concentration of gold particle distribution was clearly associated with the crystalline core of the eosinophil granule with practically no labelling in the cytoplasm or nucleus. The amount of granule labelling was greater than was observed using mouse IgG or secondary gold antibody-only

controls which had little or no gold present. Bearing in mind the association of some of the IL-2 immunoreactivity by ELISA with membrane and cytosolic fractions of the eosinophils, we cannot exclude the possibility that, in addition to the granule store, a proportion of eosinophil-associated IL-2 may be a consequence of endocytosis of exogenous IL-2 bound to its specific receptor (IL-2R, CD25) on the eosinophil membrane.^{11,12} Thus, cytosolic and membrane-associated IL-2 may comprise both receptor-bound IL-2 and the internalized cytokine, respectively. Indeed, in a preliminary experiment in which we performed 18 hr incubation, with 1000 U/ml of IL-2, of peripheral blood eosinophils from two patients, we observed that after washing, these eosinophils doubled their initial content of IL-2 (F. Levi-Schaffer, R. Hohenstein and V. Barak, unpublished data).

Our results suggest that IL-2 is present as a constituent of the crystalloid granule of unstimulated human peripheral blood eosinophils from asthmatic subjects. Thus, together with the data presented by Bossé *et al.*,¹⁹ strong evidence is provided for the novel observation that human peripheral blood eosinophils transcribe and translate mRNA for IL-2. We have recently observed that following overnight incubation in medium alone (in the absence of any exogenous cytokines), eosinophils show increase in their intracellular contents of IL-2 (F. Levi-Schaffer and R. Hohenstein, unpublished observation) suggesting *de novo* synthesis and storage of this cytokine.

We have recently reported the presence of preformed granule-stored granulocyte-macrophage colony-stimulating factor (GM-CSF) in human eosinophils from asthmatic patients.²⁰ Others have also described a similar pattern for storage of tumour necrosis factor- α (TNF- α) and IL-5 in eosinophil granules from patients with the hypereosinophilic syndrome, cystitis and Crohn's disease.^{21,22} The crystalloid granule, therefore, appears to be a major cytokine storage site in human eosinophils.

In conclusion, we have provided evidence that IL-2 is largely stored as a preformed mediator in association with the eosinophil crystalloid granules. The relatively low measurable concentrations of this cytokine (both secreted and stored, Tables 1 and 2) and its colocalization to intracellular organelles (Figs 2 and 3) suggest that eosinophil-derived IL-2, like other granule-associated cytokines, including GM-CSF,¹³ IL-4,²⁰ IL-5²¹ and TNF- α ,²² may exert its pathophysiological effect on the immune and inflammatory response via juxtacrine (cell-to-cell) rather than, or in addition to, paracrine mechanisms. Thus, as an additional source of preformed IL-2, eosinophils may influence the differentiation and activation of T-cells and serve as a rapidly mobilizable source of this cytokine as well as an autocrine chemotactic factor. The precise conditions required for eosinophils to express either Th1-IL-2 and maybe IFN- γ) or Th2-(IL-4 and IL-5) type cytokine profiles are yet to be fully appreciated. It seems, however, likely that eosinophils, together with only mast cells, can and appear to store preformed TH-1 and Th2-type cytokines.

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