

Interleukin 5 Synthesis by Eosinophils: Association with Granules and Immunoglobulin-dependent Secretion

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Summary

Interleukin 5 (IL-5) is the main factor that promotes the terminal differentiation of eosinophil progenitors (as indicated by colony formation assays), and enhances the effector capacity of mature eosinophils. IL-5 is produced by T lymphocytes, CD4⁻/CD8⁻ and mast cells and recently, messenger (m)RNA of this cytokine has been identified in eosinophils from patients with coeliac disease, asthma, or eosinophilic heart diseases. In this study, IL-5 mRNA and immunoreactive IL-5 protein were detected in tissue and blood eosinophils from patients with eosinophilic cystitis or hypereosinophilic syndromes but not in Crohn's disease. By electron microscopy associated to immunogold staining, immunoreactive IL-5 was identified in eosinophilic granules. After stimulation with IgA-, IgE-, or IgG-immune complexes, blood eosinophils were shown, by immunocytochemistry and by enzyme-linked immunosorbent assay, to secrete IL-5. These observations demonstrate that eosinophils, under physiological stimulation, can release significant amounts of IL-5, which may contribute to local eosinophil recruitment and activation.

T lymphocytes play a central role in eosinophil production, differentiation, and function through the release of soluble mediators. A number of cytokines with selective action on eosinophils has been cloned and sequenced. Among those, IL-5 is the main factor that promotes the terminal differentiation of the committed eosinophil precursors (1) and that enhances the effector capacity of mature eosinophils (2). Whereas IL-5 production by T cells has been well documented, mRNA for IL-5 also has been identified in mast cells (3) and more recently in eosinophils from patients with coeliac disease (4), as well as in alveolar eosinophils from asthmatic patients (5) or tissue eosinophils from patients with eosinophilic heart diseases (6). However, until now, no evidence for the subcellular localization and secretion of IL-5 protein by eosinophils was presented.

The aim of this study was to examine the presence of immunoreactive IL-5 in tissue eosinophils from patients with a massive eosinophil infiltration associated to cystitis or Crohn's disease, as well as in blood eosinophils from patients with hypereosinophilic syndromes. Immunogold staining was used to determine the subcellular localization of IL-5. Using a specific immunoenzymatic technique, the release of IL-5 was evaluated after incubation of eosinophils with various immune

complexes. Our results show that immunoreactive IL-5 protein, localized in eosinophil granules, can be secreted in pharmacologically active concentrations by highly purified eosinophils. They indicate, moreover, that *in vivo* synthesis, as well as *in vitro* secretion, might differ according to the stimulus of activation of eosinophils. The release of IL-5 by eosinophils may participate to the local recruitment and activation during inflammatory diseases associated with eosinophilia.

Materials and Methods

Eosinophils. Patients with a massive eosinophil infiltration (>30%) were selected. Bladder specimens were taken from a 47-yr-old woman who had presented several episodes of severe cystitis. Peripheral eosinophil counts were 840/mm³. Before treatment, histological examination revealed an eosinophilic cystitis.

Four patients with Crohn's disease were selected for the presence of numerous eosinophils infiltrating intestinal lesions (between 20 and 40% of the infiltrating cells), whereas blood eosinophil counts were normal.

Peripheral eosinophils were purified from the blood of six patients fulfilling the diagnostic criteria for idiopathic hypereosinophilic syndrome (HES): persistent eosinophilia (>1.5 × 10⁹/liter) for at

least 6 mo, multivisceral lesions, and absence of any recognized cause of eosinophilia. The mean blood eosinophil counts were 2.8×10^9 /liter with SD 1.4. By using discontinuous metrizamide gradients, hypodense eosinophils, which sediment in the lightest density, were isolated (7). After washing with HBSS, the degree of purity (90–99%) was evaluated on cytocentrifuged preparations stained with Giemsa. After centrifugation, eosinophils were resuspended in HBSS culture medium alone, without FCS, at a concentration of 10^6 cells/ml.

In Situ Hybridization. Bladder and intestinal specimens were immediately fixed in paraformaldehyde 4% in cacodylate buffer and embedded in paraffin. In situ hybridization was performed as previously described (4). Briefly, the cDNA for human IL-5 was subcloned into the Blue Script vector by standard techniques. Linearized plasmid was used as the template for the synthesis in vitro of a ^{35}S -labeled RNA probe (Amersham-France, Les Ulis, France) complementary to the cellular IL-5 mRNA (antisense probe). RNA was also transcribed in the opposite direction and used as a negative control (sense probe). Antisense or sense probes ($4,150$ cpm/mm 2) were hybridized with bladder and intestinal samples as previously described (4). To inhibit nonspecific binding of ^{35}S , tissues were acetylated in triethanolamine 0.1 M, then in acetic anhydride 0.25% triethanolamine for 10 min before hybridization. Further, to avoid nonspecific binding to eosinophils, prehybridization was carried out with a solution containing a nonradiolabeled S-UTP irrelevant probe for at least 2 h at 42°C, dithiothreitol (DTT) was added to the hybridization buffer, and RNase A was used for posthybridization washings. After development of the emulsion, tissue sections were then stained with May Grünwald Giemsa for examination by light microscopy.

Immunohistochemistry. To detect the presence of immunoreactive IL-5 in eosinophils, tissue paraffin sections or cytocentrifuged preparations of purified blood eosinophils were incubated with a mouse mAb (IgG1 isotype) directed against human IL-5. This mAb (5A5) has a neutralizing effect on human IL-5, did not crossreact with mouse IL-5, and has been purified on protein A-Sepharose (Van der Heyden, J., T. Tuypens, R. Devos, G. Plaetinck, Y. Guisez, and J. Tavernier, manuscript in preparation). Bladder or intestinal sections and cytocentrifuged preparations were washed in Tris-buffered saline (TBS), and anti-IL-5 mAb was added at a final concentration of 10 $\mu\text{g}/\text{ml}$. After incubation for 45 min at room temperature, slides were briefly washed in TBS, and enzymatic complex, alkaline phosphatase anti-alkaline phosphatase (APAAP; Dako, Glostrup, Denmark) was added at 1:50 dilution, and developed with ASB1 phosphate substrate and neofuscin (Sigma Chemical Co., St. Louis, MO) as previously described (8). As control, a mAb of the same isotype but with a different specificity (anti-*Echinococcus granulosus*) was used with the same conditions.

Immunogold Staining. Bladder specimens were fixed in glutaraldehyde 2% in cacodylate buffer, dehydrated, and embedded at -35°C in Lowicryl K4M. Lowicryl sections were obtained as previously described (9). In brief, after saturation with heat-inactivated normal rabbit serum, sections were incubated overnight at 4°C with anti-human IL-5 mAb at 50 $\mu\text{g}/\text{ml}$ and diluted in TBS containing OVA (TBS-O). After washing with TBS-O and TBS, sections were transferred on 15 nm gold-conjugated rabbit anti-mouse IgG solution (diluted 1:30; Janssen Life Science Division, Beerse, Belgium). After a 1-h incubation at room temperature, grids were washed with TBS and distilled water successively. Lowicryl sections were stained with uranyl acetate and lead citrate. As controls, two mAbs of the same isotype were used in the same conditions: EG2, which recognizes the secreted form of eosinophil cationic protein (ECP) (10) (kindly given by Dr. P. C. Tai, St. George Hospital Medical

School, London, UK), was used as positive control, and anti-IgA mAb (Immunotech, Marseille Luminy, France) was used as a negative control. The observations were made at 60 kW with a Zeiss EM 10 electron microscope.

Eosinophil Activation. Highly purified eosinophils (>95% purity) from patients with hypereosinophilic syndromes were incubated in HBSS without serum for 24 h at 37°C and 5% CO $_2$ with recombinant human IL-3 (kindly donated by Sandoz Ltd., Basel, Switzerland) and recombinant human GM-CSF, a generous gift of Dr. J. Banchereau (Schering Plough, Dardilly, France) at a final concentration of 10^{-9} M.

Highly purified eosinophils from the six HES patients were also incubated with serum IgA, secretory IgA (both purchased from Sigma Chemical Co.) and myeloma IgE protein (PS myeloma; a kind gift of Dr. H. L. Spiegelberg, The Scripps Research Institute, La Jolla, CA), or with human IgG (Sigma Chemical Co.) at a final concentration of 15 $\mu\text{g}/\text{ml}$ in HBSS without serum. After a 1-h incubation at +4°C, and without washing, eosinophils were then stimulated at 37°C and 5% CO $_2$ with the corresponding anti-IgA, anti-IgE, or anti-IgG mAb (Immunotech) at a concentration of 20 $\mu\text{g}/\text{ml}$ for a period of time varying between 15 min and 18 h.

The experiments were stopped by centrifugation of the tubes at 500 g for 10 min at 4°C. Supernatants were collected and frozen at -20°C until the ELISA could be performed. The cell pellets were resuspended at a concentration of 0.8×10^6 cells/ml of HBSS and loaded onto gelatin-coated slides (0.8×10^5 cells/slide) by cytocentrifugation. The cytopreparations were immediately fixed in sterile acetone for 10 min and stored until analyzed for the presence of immunoreactive IL-5 by immunocytochemistry.

Measurement of IL-5 in Supernatants. IL-5 levels were determined by ELISA as previously described (11). Briefly, serial dilutions of culture supernatants were incubated for two h at room temperature in microwell modules (Maxisorps; Nunc, Roskilde, Denmark) coated with 0.5 $\mu\text{g}/\text{ml}$ of the H30 rat anti-human IL-5 mAb IgG2b (provided by Dr. J. Van der Heyden, Roche Research). After washing, 0.1 μg of a mouse monoclonal anti-human IL-5 mAb IgG1 (mAb7; kindly provided by Glaxo Group Research Ltd., Greenford, Middlesex, UK) was added. Finally, the binding of the latter mAb was revealed by horseradish peroxidase-conjugated rat anti-mouse IgG1 mAb (LO MG1-2; Experimental Immunology Unit, Université Catholique de Louvain, Brussels, Belgium) followed by the appropriate substrate solution. Optical densities at 450 nm were converted in pg/ml of IL-5 using a standard curve constructed with the recombinant human IL-5 kindly provided by Roche Research.

Results and Discussion

Presence of IL-5 mRNA. When bladder sections performed in a patient with eosinophilic cystitis were submitted to in situ hybridization, a strong positive signal was observed on numerous cells with the radiolabeled IL-5 antisense RNA probe (Fig. 1 A). Intestinal sections with massive eosinophilic infiltration, performed in the four patients with Crohn's disease, were submitted to the same procedure. In contrast with bladder sections of the patient with eosinophilic cystitis, only few cells showed a positive signal (Fig. 1 C). No signal was observed with the respective control sense probes (Fig. 1, B and D).

Immunostaining with Anti-human IL-5 mAb To further characterize the cell population involved in the synthesis of

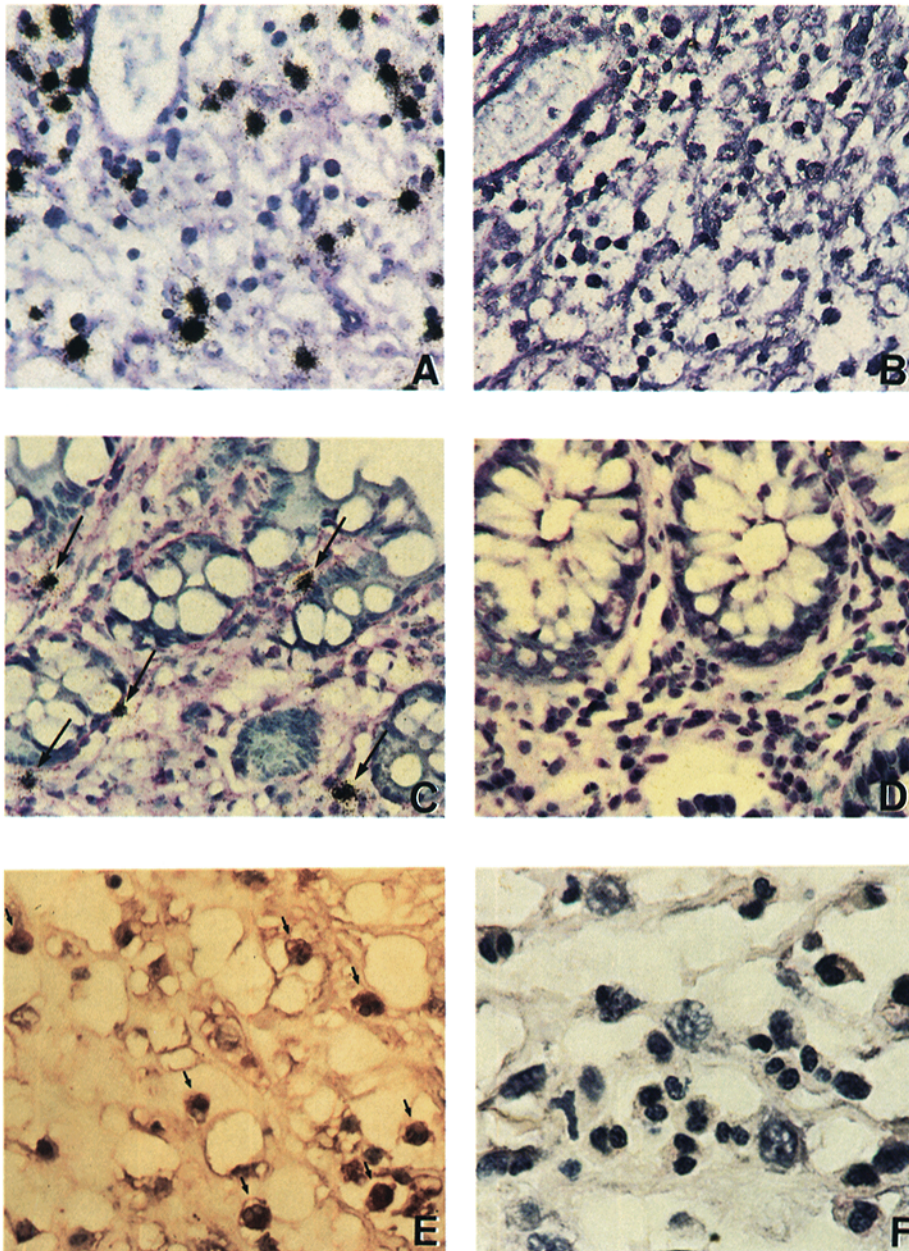


Figure 1. Detection of IL-5 mRNA and protein in human eosinophils by in situ hybridization and immunochemistry. (A and B) Bladder sections of one patient with an eosinophilic cystitis. (C and D) Intestinal sections of patients with Crohn's disease. (A) Strong positive in situ hybridization with a ^{35}S -labeled antisense IL-5 RNA probe on bladder sections (exposure time, 10 d). (C) Weak positive in situ hybridization with the same antisense probe (arrows) on intestinal sections (exposure time, 10 d). (B and D) With the sense control probe, no signal is observed on bladder sections (B) or intestinal sections (D). (E and F) IL-5 immunostaining on tissue sections. (E) Immunophosphatase localization of IL-5 protein in the eosinophilic cystitis. Note the presence of positively labeled cells with bilobed nuclei (arrows). (F) With the irrelevant antibody (anti-*Echinococcus granulosus*), no labeling is obtained. ($\times 260$).

immunoreactive IL-5, immunostaining of bladder sections with anti-human IL-5 mAb was performed. Using the APAAP procedure, typical binucleated cells were positively stained (Fig. 1 E), whereas no staining was observed with the isotype control antibody (Fig. 1 F). Eosinophils were the only cell type with a polylobulated nucleus in these sections. A similar procedure performed on intestinal sections from patients with Crohn's disease revealed a positive immunostaining of mononuclear cells only (data not shown).

Highly purified blood eosinophils could also be positively stained with anti-IL-5, but only after priming with IL-3 and GM-CSF (Fig. 2 A), whereas no staining was observed with medium alone or on resting eosinophils (Fig. 2 B). These results indicate that tissue eosinophils present in eosinophilic cystitis as well as ex vivo activated blood eosinophils could

translate IL-5 mRNA, whereas eosinophils in Crohn's disease could not, confirming the results of in situ hybridization. These findings confirm and extend the recently published results (4-6) showing that eosinophils can transcribe IL-5 mRNA in coeliac disease, HES, asthma, or eosinophilic heart disease. However, they suggest that IL-5 expression by eosinophils is not observed in all diseases associated with massive eosinophilic infiltration.

A careful examination of eosinophils stained with the anti-IL-5 mAb revealed a heterogeneous labeling of the cytoplasm (Fig. 2 A), differing from the previously described immunostaining for GM-CSF (12) but resembling the intracytoplasmic staining for IL-8 (13). These findings led us to explore the subcellular localization of IL-5 in eosinophils.

Association of IL-5 with Eosinophil Granules. To localize IL-5

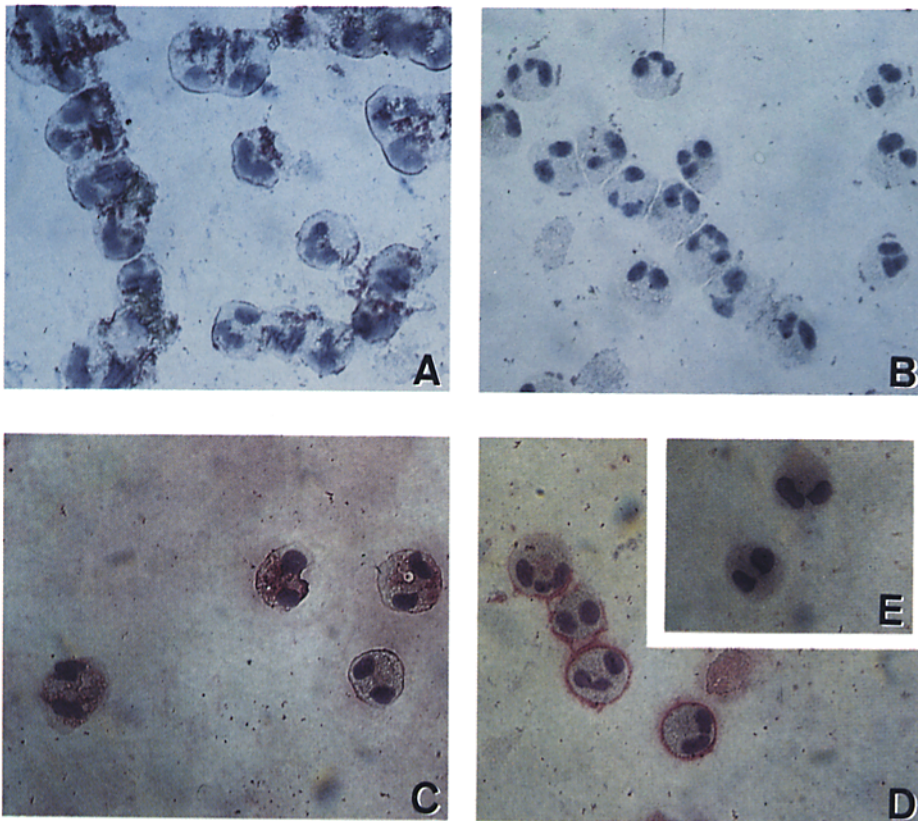


Figure 2. IL-5 immunostaining on cytopreparations of purified blood eosinophils from patients with hypereosinophilic syndromes. (A) Positive IL-5 immunostaining of peripheral binucleated cells incubated with IL-3 and GM-CSF, note the heterogeneous cytoplasmic immunophosphatase staining. (B) No signal is obtained when eosinophils were incubated in culture medium alone. (C-E). Kinetic studies of detection of immunoreactive IL-5 after IgA-dependent activation. (C) Intracytoplasmic immunostaining was observed when eosinophils were incubated with IgA-anti-IgA for 15 min. (D) After 2 h of incubation, IL-5 staining was located on the cell periphery. (E) No more staining was observed when eosinophils were incubated with the IgA immune complexes for 18 h. $\times 650$.

in eosinophils, immunogoldstaining with anti-IL-5 mAb revealed by means of gold-labeled anti-mouse rabbit antibodies and electron microscopy was performed on bladder sections embedded in Lowicryl. As positive control, EG2, a mAb

which binds to ECP, located in granule matrix, was used. As shown in Fig. 3 A, the binding of anti-IL-5 mAb was concentrated to granule matrix, similarly to EG2 staining (Fig. 3 C). All examined eosinophils, and more than 70%

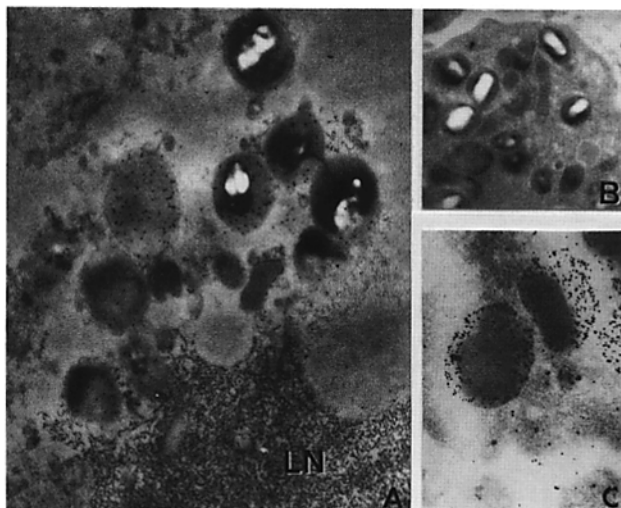


Figure 3. (A-C). Immunogold staining of Lowicryl embedded bladder sections of a patient with an eosinophilic cystitis. (A) More than 70% of the granules were labeled with the anti-human IL-5 mAb (lytic nucleus, [LN]) $\times 12,375$. (B) No staining when Lowicryl sections are incubated with an irrelevant mAb (anti-IgA). $\times 7,875$. (C) Positive control using anti-ECP mAb (EG2). $\times 10,625$.

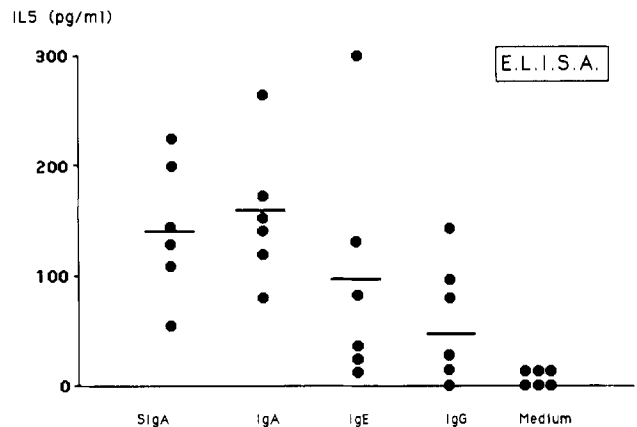


Figure 4. IL-5-ELISA procedure on eosinophil supernatants. Culture supernatants of eosinophils from six different patients, activated for 18 h with immune complexes (sIgA- or IgA-anti-IgA, IgE-anti-IgE, or IgG-anti-IgG), or with HBSS medium, were incubated in microplate wells coated with rat anti-human IL-5 mAb (H 30). After washing, a mouse anti-human IL-5 mAb (mAb 7) was added and its binding revealed by horseradish peroxidase-conjugated rat anti-mouse mAb. Bars represent mean values obtained for six different eosinophil donors.

of the granules were stained with the anti-human IL-5 mAb. No staining was obtained with the anti-IgA mAb used as negative control (Fig. 3 B). Eosinophils could therefore translate IL-5 mRNA and store the IL-5 protein in their granules. No precise identification of the localization of GM-CSF was given in the published study (12). However, the staining of eosinophil cytoplasm by the respective antibody suggested that GM-CSF localization or storage differed from IL-5. A similar granule localization has been reported for TNF- α in mast cells (14). Upon activation, eosinophils may release IL-5 more rapidly in the extracellular space, upon degranulation, without neosynthesis.

Synthesis and Secretion of IL-5 after Eosinophil Activation. Since immobilized or cross-linked IgA is able to induce eosinophil degranulation (15, 16), we looked further at whether IgA-anti-IgA immune complexes could lead to IL-5 secretion. A kinetic study revealed that after activation of blood eosinophils with IgA immune complexes for 15 min at 37°C, a positive intracytoplasmic staining with anti-IL-5 mAb was observed (Fig. 2 C). After 2 h of incubation, IL-5 staining was located on the periphery and the membrane of the majority of the cells, whereas few eosinophils still showed a cytoplasmic staining (Fig. 2 D). No staining could be observed after 18 h of incubation (Fig. 2 E). These results suggested that IL-5 could be secreted by eosinophils. To confirm this hypothesis, the secretion of IL-5 by purified blood eosinophils after activation with immune complexes, was investigated by measurement of IL-5 in supernatants.

The amounts of IL-5 were measured in the supernatants of highly purified eosinophils by the double sandwich ELISA procedure (Fig. 4). Significant amounts of IL-5 were measured in the supernatants of eosinophils after 18 h of activation with serum or secretory IgA. IL-5 could also be secreted after IgE- or IgG-dependent activation, but with important variations, according to the eosinophil donors.

Our results indicate that *ex vivo* activation of eosinophils with immune complexes, and principally IgA, consistently led to synthesis and secretion of the highest amounts of IL-5. They are in agreement with previous reports showing that IgA is the more potent stimulus for the release of granule proteins such as EDN (15), EPO, and ECP (16). Moreover, the localization of IL-5 associated with the role of insolubilized IgA in eosinophil degranulation might explain the detection of IL-5 in coeliac disease and eosinophilic cystitis where eosinophils are closely linked to IgA plasma cells (17, 18). In contrast, in coeliac disease with IgA deficiency, IL-5 was not detected in eosinophils (P. Desreumaux, personal communication) as well as in Crohn's disease, where IgM and IgG prevail (19).

In view of our results, we suggest that a short incubation with immune complexes induced IL-5 expression, whereas upon prolonged incubation, the secretion was initiated. Evidence for translation of IL-5 mRNA into immunoreactive IL-5 by eosinophils from patients with various diseases was obtained by immunostaining with a specific mAb to human IL-5. A kinetic study of IL-5 immunodetection after activation of eosinophils with IgA immune complexes *in vitro* confirmed the synthesis of IL-5 protein by eosinophils and ruled out a possible phagocytosis of exogenous IL-5. In addition, the identification of picogram amounts of IL-5 in the supernatants of highly purified eosinophils only after activation, provides evidence to support the fact that eosinophils can synthesize, store, and secrete IL-5.

Activated T cells remain the principal cellular source of IL-5, but the synthesis and secretion of IL-5 by eosinophils may provide an important autocrine pathway that is involved in local eosinophil recruitment and activation. The synthesis of their own activating factors might explain the chronicity of lesions in diseases associated with massive eosinophilic infiltration.

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