Reciprocal inhibition of binding between interleukin 3 and granulocyte–macrophage colony-stimulating factor to human eosinophils

(growth factors/receptors/hemopoiesis)

A. F. LOPEZ*, J. M. EGLINTON*, D. GILLIS*, L. S. PARK†, S. CLARK‡, AND M. A. VADAS*

*Division of Human Immunology, Institute of Medical and Veterinary Science, Frome Road, Adelaide, South Australia, 5000 Australia; †Immunex Corporation, Seattle, WA 98101; and ‡Genetics Institute Incorporated, Cambridge, MA 02140

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ABSTRACT ^{125}I -labeled recombinant human interleukin 3 (IL-3) bound, at 4°C, to a single class of high-affinity receptors on human eosinophils with an apparent dissociation constant (K_d) of 470 pM, but it did not bind to human neutrophils. ^{125}I -labeled recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) also bound to a single class of high-affinity receptors on eosinophils with an apparent K_d of 44 pM and on neutrophils with an apparent K_d of 70 pM. These binding characteristics were consistent with the biological activities of IL-3 and GM-CSF on eosinophils and with the lack of stimulation of neutrophil function by IL-3. Specificity studies under conditions shown to prevent receptor internalization showed that the binding of ^{125}I -labeled IL-3 to eosinophils was partially inhibited by GM-CSF but not by other cytokines. Reciprocal experiments with ^{125}I -labeled GM-CSF showed that IL-3 but not other cytokines partially inhibited binding to eosinophils. In contrast, the binding of ^{125}I -labeled GM-CSF to neutrophils was not inhibited by IL-3 or other cytokines tested. Quantitative inhibition binding experiments on eosinophils showed that the reciprocal inhibition between IL-3 and GM-CSF was not complete up to a concentration of heterologous ligand of 100 nM. These results show that (i) IL-3 binds to eosinophils but not neutrophils and (ii) IL-3 and GM-CSF specifically interact on the surface of eosinophils, providing a possible mechanism for the overlapping activities of IL-3 and GM-CSF on these cells.

Eosinophils are specialized hemopoietic cells found in blood and other tissues whose numbers and functional status rise in pathological situations such as parasitic infections, allergic reactions, and certain neoplasias (1–4). The production and function of eosinophils is controlled, at least *in vitro*, by a group of hemopoietic growth factors termed interleukin 3 (IL-3), granulocyte–macrophage colony-stimulating factor (GM-CSF), and IL-5 (5–11). Recent experiments showing increased levels of eosinophils after administration of IL-3 and GM-CSF suggest that these molecules also work *in vivo* (12, 13).

The existence of three hemopoietic growth factors with overlapping activities on eosinophils is puzzling and may reflect similarities in the structure of these molecules or in their mechanism of eosinophil stimulation. Computer analysis of the amino acid sequence of these molecules revealed that mouse IL-3 and GM-CSF have similarities at their N terminus (14). In humans, IL-3 and IL-5 have sequence similarity in the C terminus (5); this similarity was also noted in comparisons including GM-CSF, IL-4, and IL-6 (15). It is also noteworthy that the genes coding for IL-3, GM-CSF, and IL-5 have a similar genomic organization (16) and are closely

linked on the long arm of chromosome 5 (17, 18). In particular, the IL-3 and GM-CSF genes are only 9 kilobases apart, a finding that led to the speculation that these genes may have arisen from a common ancestral gene (19).

Despite the similarities of IL-3 and GM-CSF in the genomic organization and localization of their genes and in their primary structure, it is not known whether this apparent relatedness is manifested at the level of binding of these growth factors to the surface of eosinophils. In this report, we studied the interaction of radiolabeled IL-3 and GM-CSF with eosinophils at 4°C and the specificity of this interaction in relation to other eosinophil-activating and nonactivating cytokines. Our results show that IL-3 and GM-CSF bind to high-affinity receptors on the surface of human eosinophils and that their binding is inhibited by each other but not by other cytokines. This suggests an intimate interaction of IL-3 and GM-CSF on the eosinophil surface, which may explain their overlapping activities on these cells. In addition, we show that this interaction is absent on human neutrophils.

MATERIALS AND METHODS

Purification of Human Eosinophils and Neutrophils. Eosinophils were obtained from the peripheral blood of slightly hypereosinophilic patients after dextran sedimentation and centrifugation on a hypertonic gradient of metrizamide (Nyegaard, Oslo) as described (20). Cell purity was always >90%. In one case, eosinophils were obtained from the pleural fluid of a patient with eosinophilic pleural effusion after density gradient centrifugation on Lymphoprep (Nyegaard). This preparation contained 99% eosinophils. Neutrophils were purified from normal volunteers by centrifugation on a hypertonic gradient of metrizamide as described (20) and were always >97% pure, with no contaminating eosinophils. The cells were resuspended in RPMI 1640 medium (pH 7.4) supplemented with 20 mM Hepes, 2% (vol/vol) fetal calf serum, and antibiotics.

Cytokines. Recombinant human (rh) IL-3 and rhIL-6 produced in *Escherichia coli* and rhGM-CSF and recombinant human macrophage colony-stimulating factor (rhM-CSF) produced in COS cells were obtained from Genetics Institute (Cambridge, MA). rhIL-4 (in binding medium) and rhIL-5 produced in yeast were obtained from Immunex Corporation (Seattle). Recombinant human tumor necrosis factor α (rhTNF- α) produced in *E. coli* was obtained from Genentech, and recombinant human granulocyte colony-stimulating factor (rhG-CSF) produced in *E. coli* was obtained from L. Souza (Amgen, Thousand Oaks, CA). All cytokines were

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Abbreviations: IL-*n*, interleukin *n*; GM-CSF, granulocyte–macrophage colony-stimulating factor; TNF- α , tumor necrosis factor α ; ADCC, antibody-dependent cell-mediated cytotoxicity; rh, recombinant human; G-CSF, granulocyte colony-stimulating factor; M-CSF, macrophage colony-stimulating factor.

>99% pure as specified by the suppliers. For binding studies, an rhIL-3 produced in yeast and modified by the addition of a tyrosine-containing N-terminal octapeptide was used. This rhIL-3 has unaltered biological activity (21).

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Assay. This assay was performed with ^{51}Cr -labeled P815 cells coupled to trinitrophenyl as targets as previously described (22). The percent cytotoxicity was determined according to the following formula: $100 \times (\text{test} - \text{control}) / (\text{total} - \text{control})$, where total was the ^{51}Cr released by the addition of 2% (wt/vol) Triton X-100 (Ajax Chemicals, Auburn, New South Wales, Australia) and control was the amount of label released by P815 cells alone. Results shown are the mean of triplicate determination, with the SD always <15% of the mean.

Radioiodination of Cytokines. IL-3 and GM-CSF were radioiodinated to high specific radioactivity by an iodine monochloride method (23). The specific radioactivities of IL-3 and GM-CSF varied between 1.3×10^6 and 3.5×10^6 (mean = 2.4×10^6) cpm/pmol and 4.5×10^5 and 6.2×10^5 (mean = 5.2×10^5) cpm/pmol, respectively. The radiolabeled cytokines were separated from iodide ions by Sephadex G-25 chromatography (Pharmacia) with phosphate-buffered saline containing 0.02% polyoxyethylene sorbitan monolaurate (Tween 20) as elution buffer. ^{125}I -labeled IL-3 (^{125}I -IL-3) was stored at 4°C and ^{125}I -GM-CSF was stored at -20°C for up to 4 weeks. Prior to use, radiolabeled protein was purified from non-protein-associated radioactivity by cation-exchange chromatography on a carboxymethyl-Sepharose CL-6B column and stored at 4°C for up to 5 days.

Binding Assays. For binding assays, $2-4 \times 10^6$ cells were incubated in 0.15 ml of binding medium [RPMI 1640 (pH 7.4) supplemented with 20 mM Hepes, 0.5% bovine serum albumin, and 0.1% NaN_3] containing radioligand in siliconized glass tubes on a rotating table. Eosinophils were incubated for 16 hr at 4°C. Neutrophils tended to aggregate at 4°C, and, for this reason, binding was performed at 24°C as described (24). Cell suspensions were cooled on ice, overlaid onto 0.2 ml of fetal calf serum at 4°C, and centrifuged for 30 sec at maximum speed in a Beckman Microfuge 12. The visible cell pellet was removed by cutting, and the radioactivity in the pellet was determined in a Packard Auto-Gamma 5650. Nonspecific binding was determined by incubation in the presence of a 100-fold excess of unlabeled cytokine. Specific binding was calculated by subtracting nonspecific binding from total binding. Free radioligand was the difference between total and specifically bound radioactivity.

To determine the amount of receptor-ligand internalization under these conditions, acid-resistant radioactivity was measured at the conclusion of the assay (25). Briefly, cell suspensions were centrifuged through 0.5 ml of ice-cold fetal calf serum; the cell pellet was resuspended in binding medium and divided into two identical samples. Each aliquot was diluted in two volumes of 0.5 M acetic acid/0.5 M NaCl (pH 2.0) or binding medium (pH 7.4) for 5 min on ice and then centrifuged through fetal calf serum as described. Dissociable radioactivity was defined as the difference between total and acid-resistant specific radioactivity bound.

Binding data were analyzed by the EBDA and LIGAND computer programs (Elsevier-BIOSOFT, Cambridge, England) (26). For Scatchard analysis (27), free radioactivity was corrected for maximal binding capacity (28, 29), and the specific radioactivity of the radioligand was determined by self-displacement analysis (30) after correction for maximal binding capacity.

Competition assays were performed for 16 hr at 4°C (eosinophils) and 4 hr at 10–12°C (neutrophils). Eosinophils and neutrophils were mixed with a single subsaturating concentration of ^{125}I -IL-3 or ^{125}I -GM-CSF in the presence of unlabeled homologous and heterologous ligands at different

concentrations. After incubation, the cells were processed as above.

RESULTS

Stimulation of Eosinophil Function by Different Cytokines.

IL-3 and GM-CSF were tested for their ability to stimulate eosinophil function, and their activity was compared to other cytokines. Both stimulated the ADCC of tumor cells by eosinophils, and similar levels of cytotoxicity were obtained with IL-5 and TNF- α . IL-4 and IL-6 were not active in this assay (Fig. 1). Titration experiments showed that, although IL-3 and GM-CSF stimulated similar levels of cytotoxicity at high concentrations, GM-CSF was more potent on a molar basis. Thus the concentrations required for 50% stimulation of eosinophil ADCC were 1–10 pM for GM-CSF and 10–100 pM for IL-3 (range of three different experiments).

Binding Characteristics of IL-3 and GM-CSF on Eosinophils. A saturation binding experiment with eosinophils (98% pure) using 2×10^6 cells per point was performed at 4°C for 16 hr with ^{125}I -IL-3. ^{125}I -IL-3 bound specifically to human eosinophils and reached saturation at an approximate concentration of 1.0 nM. Scatchard analysis revealed the presence of 940 receptors per cell and an apparent K_d of 500 pM (Fig. 2 A and B). A second saturation binding curve of ^{125}I -rhIL-3 on eosinophils (99% pure) using eight different concentrations of ligand showed similar results, with Scatchard analysis indicating the presence of 370 receptors per cell and an apparent K_d of 440 pM. In contrast, ^{125}I -IL-3 did not specifically bind to human neutrophils. In a typical experiment with a ^{125}I -IL-3 concentration of up to 700 pM, neutrophils (97% pure with no eosinophils) demonstrated superimposable total and nonspecific binding curves, indicating that IL-3 receptors were not detectable on neutrophils. Similarly no specific ^{125}I -IL-3 binding was detected on the promyelocytic leukemic cell line HL-60 (data not shown).

Eosinophils were also found to bind ^{125}I -GM-CSF over a range of concentrations, with saturation achieved at a radioligand concentration of approximately 200 pM (Fig. 2C). Scatchard analysis demonstrated the presence of about 840 receptors per cell and an apparent K_d of 53 pM (Fig. 2D). A second experiment with eosinophils (99% pure) and eight different concentrations of radioligand indicated the presence of 520 receptors per cell and an approximate K_d of 36 pM. In parallel experiments with neutrophils (96% pure), ^{125}I -

FIG. 1. Antibody-dependent cytotoxicity of tumor cells by human eosinophils stimulated with GM-CSF, IL-3, IL-5, TNF- α , IL-4, and IL-6 at 1 nM. The eosinophil purity in this experiment was 94%. The bars indicate the SEM.

FIG. 2. Specific binding of ^{125}I -IL-3 and ^{125}I -GM-CSF to human eosinophils. Binding curves (A and C) and Scatchard analyses (B and D) of specific binding of ^{125}I -IL-3 (A and B) and ^{125}I -GM-CSF (C and D). Each point represents the mean of duplicate determinations.

GM-CSF bound specifically to about 200 receptors per cell, with an approximate K_d of 70 pM (data not shown).

Under these experimental conditions, most of the radioactivity remained surface bound and very little was internalized as revealed by acid elution experiments. In a typical experiment with eosinophils incubated with ^{125}I -GM-CSF for 16 hr at 4°C , the specific cpm obtained at pH 7.4 was 2238 ± 50 (mean $\pm$ SEM) and at pH 2.0 was 291 ± 36 , indicating that about 87% of the cytokine was dissociable (i.e., surface bound).

Specificity of IL-3 and GM-CSF Binding. To determine whether the binding of IL-3 and GM-CSF to human eosinophils was specific for these growth factors, competition binding experiments with other eosinophil-stimulating and nonstimulating cytokines were performed. In these experiments, eosinophils were incubated with a nonsaturating concentration of radiolabeled IL-3 or GM-CSF based on the respective saturation binding curves (see Fig. 2) and different cytokines at >100 -fold mass excess. We found that the

binding of ^{125}I -IL-3 to eosinophils (99% pure) was inhibited not only by unlabeled IL-3 but also partially by GM-CSF (Fig. 3). IL-4, IL-5, IL-6, TNF- α , G-CSF, and M-CSF did not inhibit binding at this concentration (Fig. 3). Conversely, the specific binding of ^{125}I -GM-CSF to the eosinophils from the same donor could be inhibited partially by IL-3 but not by other cytokines (Fig. 3). In contrast to eosinophils, the binding of ^{125}I -GM-CSF to neutrophils (98% pure) was not inhibited by IL-3. No inhibition was detected by other neutrophil-stimulating factors, such as G-CSF or other cytokines, that do not stimulate neutrophil function, such as IL-4, IL-5, and IL-6. The reciprocal inhibition of IL-3 and GM-CSF binding found on eosinophils but not on neutrophils was also seen with eosinophils and neutrophils purified from the same donor and with incubation times of 4 hr instead of 16 hr (data not shown).

Because the experiments showing partial reciprocal inhibition of IL-3 and GM-CSF binding to eosinophils used a single concentration of heterologous ligand and the fact that

FIG. 3. Competition for specific ^{125}I -IL-3 and ^{125}I -GM-CSF binding to human eosinophils and neutrophils. Radioligands were added to 60 pM, and the concentration of competitors was 10 nM. Total specific binding to eosinophils was 1074 ± 49 cpm (18,972 cpm was added) for IL-3 and 199 ± 11 cpm (2652 cpm was added) for GM-CSF. GM-CSF binding to neutrophils was 1905 ± 55 cpm (9244 cpm was added).

levels of this inhibition varied between different individuals, the possibility that complete inhibition could be achieved by using optimal concentrations of heterologous competitor was examined by carrying out quantitative inhibition experiments. We found that ^{125}I -IL-3 binding to eosinophils could be inhibited by increasing concentrations of GM-CSF, although this inhibition did not reach the levels obtained with unlabeled IL-3 up to a concentration of 100 nM (equivalent to 1500-fold excess) (Fig. 4). The converse was also observed, with increasing concentrations of unlabeled IL-3 inhibiting the binding of ^{125}I -GM-CSF. In this case, the heterologous inhibition was greater although not complete.

DISCUSSION

The results presented here show that IL-3 and GM-CSF specifically interact on the surface of eosinophils partially inhibiting the binding of each other. These findings suggest a possible mechanism for the overlapping activities of IL-3 and GM-CSF in eosinophilopoiesis and the stimulation of function of mature eosinophils. These results also show that eosino-

phils behave differently to neutrophils, on which no inhibition of GM-CSF binding by IL-3 or other cytokines was observed.

The binding of IL-3 and GM-CSF to eosinophils is consistent with the ability of these growth factors to stimulate the function of these cells and suggests that IL-3 and GM-CSF receptors are expressed on the eosinophil lineage throughout differentiation. In contrast, we found that, although IL-3 stimulates the neutrophil myeloid series (31), no detectable receptors were found on mature neutrophils, a result that correlates with the lack of stimulation of neutrophil function by IL-3 (31). Neutrophils, however, bound GM-CSF, in agreement with previous reports (32, 33).

IL-3 and GM-CSF bound to a small number of high-affinity receptors (300–1000) on eosinophils, with the K_d of these interactions within the range of concentrations required to stimulate their function. Interestingly, IL-3 bound to eosinophils with a K_d approximately 10 times that of GM-CSF, a result that correlates with the requirement for higher concentrations of IL-3 than GM-CSF to stimulate eosinophil function. The apparent K_d of GM-CSF binding to eosinophils (40–60 pM) was similar to that of neutrophils (70 pM).

FIG. 4. Reciprocal competition for specific binding of ^{125}I -IL-3 (Left) and ^{125}I -GM-CSF (Right) to human eosinophils. ^{125}I -labeled cytokine concentrations were 60–80 pM. Each point represents the mean $\pm$ SD of determinations from four (^{125}I -IL-3) or three (^{125}I -GM-CSF) experiments, each containing two replicates per determination. The values obtained with IL-3 and GM-CSF at 100 nM were significantly different from each other in experiments with both ^{125}I -IL-3 ($P < 0.02$) and ^{125}I -GM-CSF ($P < 0.001$). The eosinophil purity in these experiments ranged from 96% to 99%.

The binding of radiolabeled IL-3 to eosinophils was found to be inhibited not only by unlabeled IL-3 but also by GM-CSF, and the converse was also seen. This was surprising in view of the demonstration of specific receptors for these factors in the mouse system in experiments carried out at 4°C, although inhibition of binding occurred at 37°C, a phenomenon termed down-modulation (34). It is unlikely that the reciprocal inhibition of IL-3 and GM-CSF binding to eosinophils shown here is due to down-modulation, since these experiments were carried out at 4°C in the presence of sodium azide. More importantly, acid elution experiments with ¹²⁵I-GM-CSF demonstrated that after 16 hr at 4°C 87% of the radioactivity was dissociable from the eosinophil surface (see *Results*).

Another difference between the binding of IL-3 to human and to mouse cells is the finding that there were no detectable IL-3 receptors on human neutrophils; however, mouse IL-3 binding to mouse neutrophils has been observed (35). It remains to be seen whether IL-3 binding results in the functional activation of mouse neutrophils.

These results also illustrate heterogeneity between primary human cell types in their binding to IL-3 and GM-CSF. Thus, although IL-3 and GM-CSF interact on the surface of eosinophils (this communication) and monocytes (36, 37), this is not the case in neutrophils. In addition, while eosinophils show a single class of high-affinity receptors for IL-3 and for GM-CSF, monocytes express high-affinity as well as low-affinity receptors for each cytokine (37).

The precise mechanism of the IL-3 and GM-CSF reciprocal inhibition on eosinophils is not yet understood. It is not clear, for example, why IL-3, which has an apparent K_d higher than that of GM-CSF on eosinophils, appears to be the better competitor at low concentrations. Similarly, it remains to be elucidated whether the IL-3 and GM-CSF reciprocal inhibition is due to a common binding protein or to a specific association of receptors for these cytokines on the eosinophil membrane. A precedent for the former can be found in a binding protein of 70,000–74,000 Da present in the GH3 pituitary cells capable of binding activin, inhibin, and transforming growth factor β (38). Alternatively, IL-3 and GM-CSF may have only unique receptors that become associated on the cell membrane.

Both IL-3 and GM-CSF have been shown to stimulate similar numbers of eosinophil colonies (31) and the same types of functions on mature eosinophils including ADCC (31), superoxide production (5), and phagocytosis (5). The reciprocal inhibition of binding of IL-3 and GM-CSF reported here may provide a basis for the common biological effects of IL-3 and GM-CSF on human eosinophils.

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