Regulation of Human Eosinophil Precursor Production by Cytokines: A Comparison of Recombinant Human Interleukin-1 (rhIL-1), rhIL-3, rhIL-5, rhIL-6, and rh Granulocyte-Macrophage Colony-Stimulating Factor

By Elaine J. Clutterbuck and Colin J. Sanderson

The effect of a panel of recombinant human (rh) cytokines on the generation of human eosinophil precursors was assessed using a two-step culture technique. Normal human bone marrow was preincubated with different cytokine combinations in liquid culture before assessment of the number of eosinophil progenitors, which give rise to eosinophil colony-forming units (CFU-Eo) on secondary semi-solid culture with either interleukin-5 (IL-5), IL-3, or granulocyte-macrophage colony-stimulating factor. rhIL-3 or rhGM-CSF, but not rhIL-5, increased the number of CFU-Eo. CFU-Eo production by rhIL-3 or rhGM-CSF was maximal after 7 days' preincubation. Neither rhIL-1 or rhIL-6 acted on eosinophil precursors, either alone or in combination with rhIL-5, rhIL-3, or rhGM-CSF. A similar spectrum of activity of the cytokines was demonstrated whether rhIL-5, rhIL-3, or rhGM-CSF was used in the secondary cultures as the eosinophil CSF. However, rhIL-3 induced relatively more rhIL-5-responsive CFU-Eo than rhIL-3-responsive CFU-Eo, suggesting that rhIL-3 is pushing progenitors into an rhIL-5-responsive compartment.

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Survival of hematopoietic progenitor cells in vitro is dependent on the presence of a family of glycoprotein peptide molecules. Six members of this family of cytokines have been characterized: erythropoietin (EPO), granulocyte-colony-stimulating factor (G-CSF), macrophage-CSF (M-CSF/CSF-1), granulocyte-macrophage CSF (GM-CSF), interleukin-3 (IL-3/multi-CSF), and interleukin-5 (IL-5/EDF).

The type of hematopoietic cell produced depends on the nature of the CSF present in the cultures. EPO, G-CSF, M-CSF, and IL-5 primarily stimulate single cell lineages, whereas IL-3 and GM-CSF act on a broader spectrum of target cells. There is considerable overlap, with several different cytokines active on an individual lineage. Synergism between the growth factors also appears to play an important role in the regulation of hematopoiesis in vitro.1,2 In addition, other cytokines synergize with the CSFs, although they are unable to support hematopoiesis alone (eg, IL-1, IL-6, IL-4, and IL-4').

Most of the available information concerns the control of the granulocyte and macrophage lineages, and apart from a single report on murine cultures,13 little is known about the eosinophil lineage. The development of eosinophilia is particularly interesting because it occurs only in response to a limited spectrum of immune responses, and also because it is also frequently specific. This specificity (occurring in the absence of increases in other cell lineages) suggests a mechanism of control that is different from the other lineages. In a previous study, we showed that while recombinant human (rh) IL-3, rhGM-CSF, and rhIL-5 all induce human eosinophil production in vitro, only rhIL-5 was specific for the eosinophil lineage.14 Surprisingly, although rhIL-5 stimulated the production of more eosinophils than rhIL-3 or rhGM-CSF in liquid cultures, it stimulated fewer colonies in semi-solid cultures. Thus, it appears that rhIL-5 acts on a smaller population of precursors but is capable of a greater proliferative effect.

In this report an analysis of the production of eosinophil precursors in a two-step culture system has been performed. Normal human bone marrow mononuclear cells were incubated first in liquid cultures. The number of colony-forming cells (CFU-Eo) were then assayed in semi-solid cultures.

Materials and Methods

Cytokines. rhIL-5 (1.4 x 10^7 U/mL) was purified from the culture supernatant of COS 7 cells transfected with the rhIL-5 gene as previously described.13 One unit of activity was defined as the amount required to stimulate half-maximal human eosinophil production after 21 days in liquid culture. rhGM-CSF (3.4 x 10^7 chronic myelocytic leukemia cell [CML] assay u/mg protein, 10 ng/mL) was kindly provided by Dr. J.F. DeLamarter (Biogen SA, Cambridge, MA). rhIL-6 (1 x 10^7 B-cell assay dilution u/mg protein, 335 ng protein/mL), and rhIL-3 (COS supernatant) were a kind gift of Dr. G.G. Wong (Genetics Institute, Boston, MA). rhIL-1a (>10^7 thymocyte mitogenesis u/mg protein, 100 ng/mL) was a gift from Dr. S. Gillis (Immunex Corp, Seattle, WA).

Human bone marrow. Human bone marrow was obtained from normal patients (peripheral blood eosinophil count less than 3%) donating marrow primarily for allotransplantation at The Royal Free Hospital and Hammersmith Hospital, London, UK. Marrow was collected in accordance with a protocol approved by the Ethics Committees of both hospitals. Mononuclear cells were obtained by centrifugation over Ficoll-Hypaque (1.077 g/mL) (Pharmacia Fine Chemicals, Uppsala, Sweden), and resuspended at 10^6 cells/mL in RPMI 1640 containing 15% fetal calf serum (FCS) and 10^-6 mol/L hydrocortisone (RPMI/FCS).

Bone marrow culture. Cells were preincubated in liquid culture in the manner previously described.14 Briefly, 10^5 mononuclear cells were cultured in 100-μL medium with 10 μL of medium or diluted cytokine (at 37°C in humidified 5% CO2 in air) for the culture period indicated in the text. All cells were recovered by repeated pipetting, washed in fresh medium, and counted with a Coulter Counter (Coulter Electronics, Luton, UK). Differential cell counts were performed on cytacentrifuge preparations stained with 0.1% Luelol.
Fast Blue in urea-saturated 70% ethanol, and Harris' acidified hematoxylin or May-Grünwald Giemsa.

Cells were then cultured as previously described19 in 0.4-mL duplicate semi-solid cultures in medium containing 0.33% Bacto-agar (Difco Laboratories, Detroit, MI), and either 40 μL medium (RPMI/FCS) or cytokine as specified in the text. Each semi-solid culture contained the number of cells present after the preincubation of 8 × 10^6 mononuclear marrow cells. After 14 days at 37°C in 5% CO₂ in air, whole cultures were methanol-fixed and stained with either Luxol Fast Blue and Harris' acidified hematoxylin or Congo Red and Toluidine Blue. The number of colonies (groups of more than 40 cells) and eosinophil clusters (groups of more than four cells, inclusive of colonies) in each culture was counted.

Statistics. The data were analyzed by analysis of variance and Student's paired t-test. To account for missing values, the sums of squares associated with each variable of interest was adjusted by computing the increase in the residual sum of squares (RSS) that resulted when a single term was excluded from the fitted model. The increase in RSS was used for testing significance. As a guide to the errors, 1 SD is indicated in the figures and tables.

RESULTS

Optimal cytokine dose. Preliminary titration experiments established for each cytokine the dilution at the end of the plateau of the dose-response curve that stimulated maximal eosinophil production. Subsequent experiments were performed using these concentrations, which were: rhIL-5, 70 u/mL; rhIL-3 1:1,000 final dilution; and rhGM-CSF 10 ng/mL. rhIL-1 and rhIL-6 do not stimulate eosinophil colony formation,24 but were used at a final dilution of 1:1,000 (0.1 ng/mL and 330 ng/mL, respectively), as higher concentrations were inhibitory on other cell lineages.

Kinetics of precursor formation. Marrow was preincubated with the different cytokines before secondary semi-solid culture with rhIL-5 for 14 days (Fig 1). In the absence of added cytokine there was a progressive decrease in CFU-Eo. Cultures containing rhIL-5, rhIL-1, or rhIL-6 were not significantly different from controls. However, cultures containing rhIL-3 or rhGM-CSF showed a marked increase in CFU-Eo between 5 and 7 days, rising to well above input numbers of CFU-Eo. Subsequent experiments were performed with a 7-day preincubation period.

After preincubation for 7 days, marrow cells were plated in semi-solid culture in halving dilution and cultured with rhIL-5 for 14 days. The number of colonies supported increased linearly with the number of cells plated (data not shown). This indicated that the action of rhIL-5 in the colony assay is probably independent of accessory cells and confirmed the validity of assessing colony numbers at a single number of plated cells.

Induction of rhIL-5-responsive CFU-Eo. rhIL-5-responsive eosinophil precursors were consistently enhanced by rhIL-3 or rhGM-CSF (P < .05), whereas rhIL-5, rhIL-1, or rhIL-6 had no effect (Table 1 presents data from five experiments). There was considerable variation in the degree of this enhancement between different marrow donors (from three- to 57-fold).

Regardless of the cytokine present in the preincubation period, no eosinophil colonies developed on secondary semi-solid culture with medium alone (data not shown). This suggests that the washing procedure adequately removed the CSFs that had been present in the preincubation period.

rhIL-1 and rhIL-6 had a negligible effect on the induction of CFU-Eo. Neither rhIL-1 or rhIL-6 in combination with rhIL-3, rhIL-5, or rhGM-CSF in the preincubation period affected the number of CFU-Eo induced by the latter three cytokines when present alone. Table 1 shows data for rhIL-5-responsive CFU-Eo and Fig 2 for CFU-Eo detected by rhIL-3 in the secondary cultures. Although rhIL-1 appears to reduce the number of CFU-Eo induced by rhGM-CSF in the preincubation period (Fig 3), there was no reduction of other colony types in rhIL-1 preincubated cultures (data not shown), suggesting that the lack of effect on eosinophil colonies was not due to rhIL-1 toxicity.

Effect of different cytokines in the secondary cultures. The same spectrum of activity of the cytokines on eosinophil precursor generation was demonstrated regardless of which eosinophil CSF was used in the secondary cultures. Preincubation with rhIL-5, compared with controls, did not alter the number of CFU-Eo supported in the secondary cultures by rhIL-3 (24 v 22 CFU-Eo/culture, Table 2), and neither rhIL-1 or rhIL-6 altered the number of rhIL-3-responsive CFU-Eo (data not shown). In 4 of the 5 experiments (Table 2), preincubation with rhIL-3 significantly (P < .05) increased the number of rhIL-3-responsive CFU-Eo. However, overall this effect did not reach statistical significance (37 v 22 colonies, P < .2), because in one experiment there were a relatively large number of rhIL-3-responsive CFU-Eo in the control cultures, and preincubation with rhIL-3 did not alter their number.

rhGM-CSF was used in the secondary cultures in two experiments with similar results (data not shown).

rhIL-3 in the secondary cultures supported more CFU-Eo than rhIL-5. rhIL-3 supported three to four times as many
Table 1. Effect of Preincubation With Different Cytokines for Seven Days on Eosinophil Colony Production Supported on Subsequent Semi-solid Culture With rhlL-5

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Medium</th>
<th>rhlL-5</th>
<th>rhlL-1</th>
<th>rhlL-6</th>
<th>rhlL-3</th>
<th>rhlL-1 + rhlL-5</th>
<th>rhlL-1 + rhlL-6</th>
<th>rhlL-1 + rhlL-3</th>
<th>rhlL-1 + rhGM-CSF</th>
<th>rhlL-6 + rhlL-5</th>
<th>rhlL-6 + rhlL-3</th>
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<td>3 ± 4</td>
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<td>4 ± 1</td>
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<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>16 ± 2</td>
<td>15 ± 1</td>
<td>13 ± 1</td>
<td>9 ± 2</td>
<td>44 ± 8</td>
<td>57 ± 6</td>
<td>ND</td>
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<td>52 ± 0</td>
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<td>120 ± 27</td>
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<td>ND</td>
<td>ND</td>
</tr>
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<td>2 ± 2</td>
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<td>5 ± 2</td>
<td>5 ± 3</td>
<td>86 ± 2</td>
<td>18 ± 1</td>
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<td>61 ± 5</td>
<td>10 ± 2</td>
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<td>5 ± 0</td>
<td>13 ± 4</td>
<td>0 ± 1</td>
<td>1 ± 1</td>
<td>23 ± 4</td>
</tr>
</tbody>
</table>

P value  NS† NS‡ NS§ <.05 † <.05 ‡ NS§ NS§ NS§ NS§

The number of rhlL-5–responsive input CFU-Eo was determined in three of the experiments: 1, 5.5 ± 1.5; 2, 26 ± 4; 4, 32 ± 3.

Abbreviation: NS, not significant at 5% level; ND, not done.

†Present in liquid culture.

‡Significance of the difference between the cytokine and medium alone (paired t-test).

§Significance of the difference between the presence and absence of rhlL-1 or rhlL-6 (paired t-test).

CFU-Eo as rhlL-5 from either control- or rhlL-5–preincubated cultures (Table 2). This was also seen for rhlL-1- or rhlL-6–preincubated marrow (data not shown). However, in cultures preincubated with rhlL-3 there was a marked increase in rhlL-5–responsive CFU-Eo, so that there was now at least as many rhlL-5–responsive CFU-Eo as rhlL-3–responsive CFU-Eo (41 v 37 CFU-Eo/culture, Table 2). Although not so extensively studied, preincubation with rhGM-CSF also enhanced rhlL-5–responsive CFU-Eo relatively more than rhlL-3–responsive CFU-Eo (Table 2).

Moreover, all of the colonies supported by rhlL-5 were small (containing 40 to 100 cells), whereas a significant proportion (27%, range 10% to 50%) of the colonies detected by rhlL-3 were composed of more than 100 eosinophils. RhGM-CSF in the secondary cultures also supported more CFU-Eo than rhlL-5 (although fewer than rhlL-3, P < .05), and 17% (range 3% to 44%) of the colonies contained more than 100 eosinophils.

rhlL-3, rhGM-CSF, and rhlL-5 increased the number of eosinophil cluster-forming precursors. Figure 3 demonstrates the effect of the cytokines on the number of eosinophil clusters present in the secondary cultures containing medium, rhlL-5, or rhlL-3. Compared with preincubation in medium alone, rhlL-1 and rhlL-6 did not affect cluster numbers. Clusters were modestly increased by preincubation with rhlL-5 (two- to eightfold, P < .02), and rhGM-CSF (two- to sixfold, P < .05) but more markedly by rhlL-3 (six- to 13-fold, P < .01). No synergism of either rhlL-1 or rhlL-6 on the enhancement of eosinophil clusters by rhlL-5, rhGM-CSF, or rhlL-3 was demonstrable (data not shown). rhlL-5 combined with rhlL-3 in the preincubation period did not significantly enhance cluster numbers compared with rhlL-3 alone.

The number of clusters supported by secondary cultures containing medium alone, rhlL-3, or rhlL-5 were not significantly different (Fig 3). Other combinations of cytokines in the secondary cultures also supported similar numbers of eosinophil clusters (rhGM-CSF, rhlL-5 with rhlL-3, or rhlL-5 with rhGM-CSF) (data not shown). This is sugges-
REGULATION OF HUMAN EOSINOPHIL PRECURSORS

The clear observation from these studies is that IL-5 is the cytokine of choice to induce the expansion of eosinophil precursors, and IL-3 acts on progenitors earlier in the hematopoietic pathway, giving rise to the larger colonies. The clear observation from these studies is that IL-3 and, to a lesser extent GM-CSF, induce a significant increase in CFU-Eo. Combinations of cytokines in the primary culture did not produce any spectacular effects, and there was no evidence of synergism with any of the combinations tested.

In particular there was no demonstrable effect of IL-1 or IL-6. This contrasts with the reported activity of IFN-γ and TNF-α in the generation of murine CFU-GM and blast cell colonies3 and the reported synergistic effect of IL-1 on the generation of CFU-Eo from murine marrow enriched for eosinophil precursors by treatment of the mice with 5-fluorouracil (5FU). It is possible that endogenously produced monocyte-derived IL-1 may have masked the action of exogenously derived cytokines in the generation of eosinophil colonies. However, an interesting observation was the relative increase in IL-5 responsive CFU-Eo compared with IL-3 responsive CFU-Eo induced by preincubation with IL-3. Previous work has shown that IL-3 consistently stimulates the production of more eosinophil colonies than IL-5,14 and similarly, cells from control primary cultures contained approximately four times as many IL-3 than IL-5 responsive CFU-Eo (Table 2). In contrast, after primary culture in IL-3, the number of IL-5 responsive CFU-Eo is similar to (or slightly higher than) the number responding to IL-3. One possible interpretation of these results is that IL-3 is not only increasing the number of CFU-Eo, but is pushing them toward the IL-5 responsive compartment. Although not so extensively studied, GM-CSF appears to have a similar, although less marked, action.

A similar effect with IL-3 has also been reported with normal murine bone marrow.15 Preincubation of marrow with IL-3 (in either liquid or semi-solid cultures) increased the number of CFU-Eo compared with the numbers in the original cell inoculum. Furthermore, IL-3 induced the production of three times as many IL-5 responsive CFU-Eo as IL-3 responsive CFU-Eo.

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produced factor, but it should be noted that considerable differences exist in the literature between the effects of cytokines on the generation of progenitors in vitro.

For example, although IL-1 has been widely reported to have synergistic activity with many CSFs in murine cultures (IL-3, GM-CSF, and G-CSF), the effect on human marrow is less clear. No synergistic activity of IL-1 was demonstrable in two experiments designed to remove endogenously produced IL-1 with IL-3 on human CD34+ progenitor cells, or G-CSF on nonadherent human marrow cells plated at low cell concentrations, although IL-1 did induce a twofold increase of GM and erythroid progenitors produced by GM-CSF combined with erythropoietin. Similarly, although it has been reported that IL-6 directly induces human CD34+ progenitors, but it should be noted that considerable differences exist in the literature between the effects of cytokines on clusters supported the production of several different types of colonies from normal murine bone marrow. No colony-stimulating factor of IL-6 was demonstrated on either marrow from 5FU-treated mice or unfractionated marrow. However, IL-6 did synergize with IL-3 in the formation of blast cell colonies from both murine 5FU-treated marrow and human CD34+ progenitor cells. Further work with fractionated marrow will clarify the action of these cytokines on the eosinophil lineage.

There are a number of technical factors that influence the outcome of these types of experiments. Like most culture systems, different batches of FCS have a marked effect on the outcome of these types of experiments. Use FCS selected to give low background colonies. In the present experiments, a single batch has been used that was selected for its ability to support high production of eosinophils in murine culture systems, and also found to give satisfactory results with human marrow. The geometry of the culture vessel also has a significant effect. In our hands, round-bottom wells give considerably higher eosinophil production than flat-bottom wells, possibly reflecting the requirement for cell interactions in the proliferation and differentiation process. All these effects make comparisons between laboratories difficult. In addition, there is a significant difference between different marrows. We have attempted to minimize this latter effect by analyzing data from several experiments.

Eosinophil clusters were counted in all experiments. In general, effects of the cytokines on clusters supported the interpretation made on the basis of colony numbers. However, rhIL-5, which caused no increase in CFU-Eo, caused a significant increase in clusters. This is likely to be the result of proliferation of committed eosinophils and the production of cells only capable of few further cell divisions.

These and previous studies raise a number of questions concerning the biologic role of these phenomena. Firstly, it should be noted that this effect is more marked in murine cultures than in human cultures where very few eosinophil colonies are obtained in media that give rise to large numbers of eosinophils in the absence of agar. In both murine and human cultures, eosinophils are produced under conditions where cell interactions are encouraged. This may reflect a greater dependence on accessory cells by IL-5 than other cytokines.

Secondly, the roles of IL-1 and IL-6 are unclear. In general, positive effects with these factors have been obtained with bone marrow enriched for progenitor cells. It is possible that these effects are masked or replaced by accessory cells in unfractionated marrow.

Thirdly, it is difficult to explain the biologic specificity of eosinophilia if either of the multi-lineage factors IL-3 and GM-CSF are necessary for the generation of the eosinophil precursor. However, it should be recalled that mice undergoing a parasite-induced eosinophilia had detectable levels of circulating IL-5 but no detectable IL-3 or GM-CSF. It is possible that small amounts of IL-3 acting locally may provide an adequate production of precursors for the large amount of circulating IL-5 to produce a selective eosinophilia.

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