Human Eosinophil Hematopoiesis Studied in vitro by Means of Murine Eosinophil Differentiation Factor (IL5): Production of Functionally Active Eosinophils From Normal Human Bone Marrow

By Elaine J. Clutterbuck and Colin J. Sanderson

The production of human eosinophils in vitro from normal bone marrow by using murine eosinophil differentiation factor (mEDF/interleukin 5) is described. Eosinophil production was selective and first detectable after 14 days and reached a peak between 21 and 35 days when they were the predominant cell type (41% to 89%). Until day 14, all the eosinophils were typical myelocytes, developing thereafter into metamyelocytes and mature cells. All cell types had characteristic light- and electron-microscopic features, apart from the absence of granules with crystalline cores. The eosinophils produced were readily recovered, and both immature myelocytes and mature cells were functionally active in an antibody-dependent, cell-mediated cytotoxicity assay. mEDF added into the assay enhanced the cytotoxicity but to a lower degree than previously reported for peripheral blood eosinophils, which suggests that they may be partially activated. The possibility that eosinophils could be deactivated was tested by removing mEDF from the culture medium. The eosinophils retained viability and functional activity, however, and showed no increased ability to be activated by mEDF for up to six days after removing the mEDF. The liquid culture of human bone marrow was shown to be an alternative assay for eosinophil differentiation factors to colony formation.

MATERIALS AND METHODS

Preparation of mEDF. mEDF was prepared from conditioned medium (CM) of the T cell hybrid NIMP-TH1. In some experiments mEDF-CM was partially purified (mEDF-PP) by 45% to 80% ammonium sulphate precipitation and by binding to lentil lectin (LcA-Sepharose, Pharmacia, Uppsala, Sweden) and eluting with 0.1 mol/L λ-methyl mannoside. No difference was found between mEDF-CM and mEDF-PP.

Human bone marrow cells. Human bone marrow was obtained from normal patients (peripheral blood eosinophil count less than 3%) who were donating marrow for allotransplantation at The Royal Free Hospital and Hammersmith Hospital, London, in accordance with a protocol approved by their local ethics committees. We are very grateful to Drs E. Gordon-Smith, M. Brenner, and H. Hislop for making this material available. After centrifugation over Ficoll-Paque (1.077 g/mL, Pharmacia), mononuclear cells were recovered from the interface and resuspended at 10^6 cells/mL in RPMI 1640 containing 15% fetal calf serum (FCS).

Liquid culture system. A quantity of 10^6 marrow mononuclear cells was cultured with diluted stimuli in 96-well round-bottomed microplates (Nunc, Roskilde, Denmark) at 37°C in humidified 5% CO2 in air. At seven- to 14-day intervals, 50 μL of the supernatant was replaced with fresh medium containing 5 μL of the appropriate stimulus. Unless stated otherwise in the text, when present in the culture medium mEDF was used at a final dilution of 1:10. After the culture period indicated in the text, the cells were resuspended and counted with a Coulter Counter (Coulter Electronics, Hialeah, FL) and differential cell counts performed on cyt centrifuge preparations. These were stained with 0.1% Luxol fast blue in urea-saturated 70% ethanol and Harris’ acidified hematoxylin, May-Grünwald-Giemsa, or Congo red and Harris’ acidified hematoxylin.

Cell viability was assessed by trypan blue exclusion.

For functional assays the cells were washed in fresh culture medium without mEDF and resuspended at a concentration of 1.63 x 10^6 cells/mL in HEPES-buffered RPMI 1640 containing 5% FCS, and 80-μL aliquots (containing 1.3 x 10^5 cells) were taken as effector cells.

Murine cultures were performed by using femoral bone marrow from Mecocentoides cortis-parasitised BALB/c NIMR mice. The peroxidase (EPO) assay was performed on both the human and murine cultures as described by Strath et al.

Colony-forming assay. A quantity of 8 x 10^4 mononuclear cells was incubated in medium containing diluted stimuli and 0.33% Bacto-Agar (Difco, Detroit) in 0.4-mL aliquots in leukocyte migration plates (Sterilin, Ltd, Teddington, England) at 37°C in 5% CO2.
in air for seven to 21 days. The cultures were methanol-fixed and stained with either Luxol fast blue and Harris’ acidified hematoxylin or Congro red and toluidine blue and all colonies (groups of more than 40 cells) counted.

Electron microscopy. Cells cultured in the liquid assay in the presence of optimal concentrations of mEDF were fixed with 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.25, followed by 1% osmium tetroxide in the same buffer and then 1% aqueous uranyl acetate. After dehydrating with absolute ethanol they were embedded in araldite and post-stained with uranyl acetate and Reynold’s lead citrate.

Antibody-dependent, cell-mediated cytotoxicity assay. The ADCC assay previously described by Vadás et al. was used with minor modifications. Briefly, 40 μL (4 × 10⁶) 51Cr-labeled P815 mastocytoma cells were incubated in V-bottomed radioimmunoassay vials (Sarstedt, Leicester, England) with 24 μL rabbit anti-P815 antiserum, 16 μL stimulus (culture medium or mEDF), and 80 μL (1.3 × 10⁶) effector cells. After three hours at 37°C, 80 μL of the supernatant was removed and ¹⁰⁷Cr release determined. The percent cytotoxicity was calculated according to the following formula: 

\[
\text{Cytotoxicity} = \frac{\text{Test}(\text{cpm}) - \text{Control}(\text{cpm})}{\text{Control}(\text{cpm})} \times 100
\]

with absolute ethanol they were embedded in araldite and post-stained with uranyl acetate and Reynold’s lead citrate.

Statistics. Results were expressed as means ± 1 SD of three or four replicates unless stated otherwise in the text and compared by Student’s paired t test. Fifty percent end points were determined by linear regression using log-transformed data.

RESULTS

mEDF selectively stimulates the growth of eosinophils in liquid culture. Table 1 summarizes the complete data from 12 patients. In the absence of mEDF, total cell numbers diminish progressively over the first 21 days, mainly because of the loss of cells of the neutrophil and lymphocyte lineages.

<table>
<thead>
<tr>
<th>Days in Culture</th>
<th>0 (n = 8)</th>
<th>14 (n = 6)</th>
<th>21 (n = 12)</th>
<th>35 (n = 3)</th>
<th>49 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>5.9 ± 1.6</td>
<td>3.3 ± 3.9</td>
<td>0.4 ± 0.5</td>
<td>0.1 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>M</td>
<td>5.9 ± 1.6</td>
<td>2.9 ± 2.3</td>
<td>0.6 ± 1.0</td>
<td>0.1 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>L</td>
<td>0.2 ± 0.3</td>
<td>0.7 ± 0.3</td>
<td>0.9 ± 0.8</td>
<td>0.6 ± 0.6</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>E</td>
<td>3.9 ± 1.5</td>
<td>2.0 ± 1.0</td>
<td>1.1 ± 0.8</td>
<td>0.7 ± 0.2</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>T</td>
<td>3.9 ± 1.5</td>
<td>2.1 ± 0.9</td>
<td>1.1 ± 1.1</td>
<td>1.0 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

Table 1. Differential Cell Counts on Culturing Normal Human Bone Marrow in the Presence and Absence of mEDF

Results are expressed as means ± 1 SD (× 10⁶ cells/mL) for n replicates. P represents the significance of the difference between counts in the absence and presence of mEDF.

Abbreviations: N, neutrophils; M, macrophages; L, lymphocytes/macrophages; E, eosinophils; T, total cells; -, in the absence of mEDF; +, in the presence of mEDF-CM.

Cells remaining viable in culture thereafter are monocyte-macrophages and lymphocytes.

Culture in the presence of mEDF causes a selective eosinophil proliferation, with no effect on the other cell lineages. Eosinophil production was first detectable after 14 days (P < .01) and reached a peak between days 21 and 35 depending on the individual marrow (Fig 1), although the mean maximal eosinophil count occurred on day 21. The
Fig 2. Electron micrographs of eosinophils induced by mEDF: (A) myelocyte, and (B) mature cell, and their granules (C) and (D), respectively. The bar represents 1 μm.

Activity of eosinophils in the ADCC assay. To establish the optimal conditions for the assay, cytotoxic activity at different cell ratios and different antibody dilutions in the presence of mEDF were carried out. Figure 3B shows that an eosinophil preparation (74% eosinophil myelocytes) gave significant cytotoxicity at an E:T ratio of 3.2:1, whereas the control culture (containing 8% myelocytes) gave significant cytotoxicity at a ratio of only 32:1. No significant cytotoxicity was obtained in the absence of antibody (Fig 3C), and this antiserum gave optimal levels of killing at a dilution of 1:53 or higher. A similar response curve was obtained when the eosinophils were assayed in the absence of mEDF (data not shown), which indicated that there was no increased effect at subthreshold levels of antibody. Subsequent experiments were performed at an E:T ratio of 32:1 and an antibody dilution of 1:53.

Marrow cultures with mEDF for 21 to 39 days (containing from 75% to 87% eosinophils) caused from 18% to 40% cytotoxicity (Fig 3A). The same marrows cultured in medium alone contained 0% to 7% eosinophils, and all but one caused less than 4% cytotoxicity in the three-hour assay. (The one exception contained 7% eosinophils and caused 10% cytotoxicity.) The significance of the difference between eosinophil and control samples was <.001. The remaining cells in both the stimulated and control cultures were lym-
phocytes and macrophages. Thus, although the cell preparations were not pure, most of the cytotoxic activity could be attributed to the eosinophils and very little to the contaminating lymphocytes and macrophages.

The effect of time in culture on the cytotoxic activity of the eosinophils is shown in Fig 4. Marrow cultured with mEDF contained between 37% and 91% eosinophils (43% to 99% myelocytes until day 28, 66% to 100% metamyelocytes or mature cells after day 35). Although there was considerable variation between different marrows, both mature cells and immature myelocytes caused the same degree of cytotoxicity. Cytotoxic activity progressively declined, however, when preparations were cultured for longer than 40 days. In three experiments mature eosinophils cultured for 53 days, although more than 90% viable and having normal light microscopic appearances were no more active than control preparations cultured in the absence of mEDF, which caused less than 8% cytotoxicity throughout.

mEDF present during the cytotoxic assay acted as an activating agent in 25 of 29 experiments and enhanced cytotoxicity by a mean of 5% (range, 0% to 13%; P < .001; data not shown). Experiments were carried out to test the possibility that cells could be deactivated (returned to a resting state) by incubation in the absence of mEDF. At each time point all the eosinophil preparations were washed once and cells resuspended in fresh medium with or without mEDF so that there was very little possibility of mEDF remaining in the mEDF-deprived wells, and all cells were subjected to the same number of medium changes. mEDF deprivation for up to six or seven days had little effect on eosinophil cytotoxic activity (Fig 5). Figure 5B shows an apparent increased activating effect of adding mEDF to the cultures after six to nine days’ deprivation, but this effect was relatively small. Furthermore, eosinophil numbers were maintained for relatively prolonged periods in the absence of mEDF: 70% of the initial number at seven days in experiment 5A, 61% at six days, and 25% at nine days in experiment 5B.

Liquid culture as an assay for EDFs. Counting eosinophil numbers was most sensitive between days 21 and 35 (data not shown), but day 21 was chosen as the optimal time point to assay for mEDF because the cultures then only required a single medium change. Of 22 marrow samples assayed, mEDF induced significant eosinophil production in all of them, with two broad groups of responders: the majority with a high eosinophil production (4 to 9 × 10⁸/mL) and three with a tenfold-lower eosinophil count (0.4 to 0.9 × 10⁸/mL) (Fig 6A). Although the numbers are small, there appears to be no relationship between induced eosinophil production and either initial bone marrow eosinophil count, donor age, sex, or HLA type. This system was a reproducible assay of mEDF activity: it gave only a fourfold variation of 50% end points (Table 2) (disregarding the three marrows where eosinophil production did not reach a plateau) and was capable of detecting mEDF activity at dilutions down to 1:3 × 10⁴ (Fig 6A) when eosinophil counts were two to five times the background.

Fig 3. Percent cytotoxicity in the ADCC assay of marrow cultured in the absence (●), and presence (○) of mEDF-CM. (A) Data for seven marrows cultured for 23 to 39 days. (B) Effect of E:T ratio (antibody dilution, 1:13). (C) Antibody dilution on marrow cultured for 25 days. Data are means ± 1 SD.

Fig 4. Effect of time in culture on percent cytotoxicity of marrow cultured in the absence (filled symbols) and presence (open symbols) of mEDF-CM. Each symbol represents an individual marrow. Data are means ± 1 SD.

Fig 5. Effect of incubating cultured eosinophils in the absence of mEDF before the ADCC assay. (A) Day 32 marrow (80% eosinophils, 92% to 100% myelocytes). (B) A different marrow at day 47 (83% eosinophils, 100% mature cells). ○, mEDF-CM absent; , mEDF-CM present in the assay. Data are means ± 1 SEM. (a) The eosinophil count (cell viability) was 5.5 × 10⁸/ml (99%), 5.9 × 10⁸/ml (77%), and 3.9 × 10⁸/ml (80%), after 0, 4, and 7 days of EDF deprivation. (b) The eosinophil count fell from 6.4 × 10⁸/ml (99% viable) to 3.9 × 10⁸/ml (98% viable) after six days and 1.6 × 10⁸/ml (97% viable) after nine days.

Fig 6. Effect of incubating cultured eosinophils in the absence of mEDF before the ADCC assay. (A) Day 32 marrow (80% eosinophils, 92% to 100% myelocytes). (B) A different marrow at day 47 (83% eosinophils, 100% mature cells). ○, mEDF-CM absent; , mEDF-CM present in the assay. Data are means ± 1 SEM. (a) The eosinophil count (cell viability) was 5.5 × 10⁸/ml (99%), 5.9 × 10⁸/ml (77%), and 3.9 × 10⁸/ml (80%), after 0, 4, and 7 days of EDF deprivation. (b) The eosinophil count fell from 6.4 × 10⁸/ml (99% viable) to 3.9 × 10⁸/ml (98% viable) after six days and 1.6 × 10⁸/ml (97% viable) after nine days.
The peroxidase assay detects neutrophil myeloperoxidase, which is not specifically inhibited by potassium cyanide. However, peroxidase activity does assess eosinophil numbers after day 21 when, in the absence of neutrophil growth factors, neutrophil counts are low (data not presented). As summarized in Table 2, although this assay was three- to 14-fold more sensitive than differential counting, the 50% end points were more variable (tenfold). Compared with marrow from parasitized mice, normal human bone marrow was two- to 30-fold less sensitive at detecting mEDF when using this assay (Table 2).

mEDF induced selective eosinophil colony production maximally at day 14 (data not presented). The dose-response curves of eosinophil colony production by mEDF-CM are shown in Fig 6B, the lowest detectable mEDF dilution varying from 1:10⁶ to 1:10⁵, and Table 2 shows the 50-fold variation in 50% end points.

**DISCUSSION**

Few lymphokines show cross-species activity between mouse and human; however, where they exist, they have provided a means for studying activities when the homologous factor has not been available. In this paper mEDF has been used to study human eosinophil hematopoiesis (because this source of mEDF is known to be free of other lymphokines⁴ and produces selective eosinophil colony production).⁵ Other factors known to induce eosinophil differentiation are granulocyte-macrophage colony-stimulating factor (GM-CSF)⁶ and interleukin 3,⁷ but their activity is not specific to the eosinophil lineage, so eosinophils are produced along with large numbers of other cell types.

It is shown that functionally active human eosinophils can be produced, which opens up the possibility of studying eosinophil differentiation and activation under controlled conditions. The time required for maximal eosinophil production in the liquid culture system (21 to 35 days) is similar to that previously reported for maximal colony formation,¹²,¹³ and eosinophils of more than 90% viability were maintained in culture for up to 7 weeks.

The morphology and maturation of eosinophils produced in liquid culture by mEDF resembled that seen in the bone marrow of normal subjects. Eosinophil maturation in colonies has been studied by Zucker-Franklin et al who used peripheral blood lymphocytes stimulated by white cell underlayers.¹⁴ They found that promyelocytes were present on day 9, with myelocytes and metamyelocytes being most prevalent on days 15 and 21. Our colony results were similar, but the stage of maturation in the liquid culture system was delayed by about 1 week. The main difference between cultured mature eosinophils and circulating eosinophils was the

### Table 2. Summary of Results of Dose Response of Individual Marrow Cultures

<table>
<thead>
<tr>
<th>mEDF</th>
<th>Experiment</th>
<th>50% End Point*</th>
<th>Eosinophil Count</th>
<th>Colony Assay</th>
<th>Peroxidase Assay (hEPO)</th>
<th>Ratio of mEPO/hEPO†</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEDF-PP</td>
<td>1</td>
<td>32</td>
<td>135</td>
<td>11.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20†</td>
<td>150</td>
<td>10.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>100</td>
<td>969</td>
<td>1.6</td>
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<tr>
<td>mEDF-CM</td>
<td>6</td>
<td>72</td>
<td>279</td>
<td>4.4</td>
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</tr>
<tr>
<td></td>
<td>9</td>
<td>135</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td>28†</td>
<td>2,156</td>
<td>3.1</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>11</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>12</td>
<td>31†</td>
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<td>18</td>
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<td>924</td>
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<td></td>
<td>21</td>
<td>62</td>
<td>1,722</td>
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<td></td>
<td>22</td>
<td>43</td>
<td>137</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Abbreviations: mEPO, murine EPO; hEPO, human EPO.

*Reciprocal of the dilution, calculated from results at a 1:10 dilution of mEDF where no plateau was reached (†).
†mEPO = 50% end point in the mouse peroxidase assay (mEDF-PP = 1.565; mEDF-CM = 1.236).
absence of crystalline cores in the granules. A similar observation was made by Zucker-Franklin et al.\textsuperscript{14} and Butterfield et al.\textsuperscript{12} in their studies of eosinophil colonies. Like these workers found, granules with cores of low electron density were not detected.

An array of different factors including mEDF have been shown to activate eosinophils.\textsuperscript{15} This term is usually used to describe an increase in activity in a short-term assay, but no clear idea of the mechanisms behind activation has emerged. It is not clear whether it is a part of the differentiation pathway or a separate, perhaps reversible, pathway. If activation were a final step in differentiation, only fully mature cells would exhibit fully functional activity.

Cultured eosinophils were as functional in an ADCC assay as peripheral blood eosinophils.\textsuperscript{3} Moreover, it appears that this functional activity is present to the same degree in both immature eosinophil myelocytes and mature cells produced in vitro. Mature cells, however, lost their activity after 7 weeks despite being more than 90% viable and having normal light-microscopic appearances. This may indicate that functional activity is of shorter duration than cell viability assessed by trypan blue exclusion or that other factors may be required at this stage to maintain functional activity.

The cytotoxic activity of peripheral blood eosinophils has been reported to depend on the presence of activating factors.\textsuperscript{3,4,11} In the present study, however, cultured eosinophils alone were cytotoxic, and the effect of the presence of mEDF was small, which suggests that the eosinophils were already partially activated. In an attempt to test whether activation was reversible, cultured eosinophils were deprived of mEDF. It was surprising to find that eosinophil numbers were well maintained for up to six days in the absence of mEDF with little loss of cytotoxic activity because previous work has shown that 50% of peripheral blood eosinophils survive only 36 hours in the absence of mEDF.\textsuperscript{16} It is possible that mEDF was not removed by washing but remained bound to glycosaminoglycans in the extracellular matrix as shown for GM-CSF.\textsuperscript{17} Nonetheless, the observation that cultured immature eosinophil myelocytes are as active as mature cells and that both cell types appeared to be partially activated suggest that activation is a separate pathway from differentiation.

Human hematopoietic growth factors are mainly assayed by their ability to induce colony formation. As in the mouse,\textsuperscript{7} a liquid culture system using either differential cell counting or peroxidase assay was shown to be a useful alternative. It is interesting to note that in the mouse the colony assay is very insensitive compared with liquid cultures.\textsuperscript{4,15} None of these assay systems, however, provides an ideal assay for eosinophil differentiation factors in humans because human bone marrow is not readily available and the culture periods are lengthy. Human bone marrow is less sensitive than murine bone marrow at detecting murine EDF. It remains to be seen whether human EDF is active on mouse cells because this would provide an assay system with several advantages over the human bone marrow systems.

ACKNOWLEDGMENT

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REFERENCES

8. Warren DJ, Sanderson CJ: Production of a T cell hybrid producing a lymphokine stimulating eosinophil differentiation. Immunology 54:615, 1985
Human eosinophil hematopoiesis studied in vitro by means of murine eosinophil differentiation factor (IL5): production of functionally active eosinophils from normal human bone marrow

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