Interleukin-4 Has Basophilic and Eosinophilic Cell Growth-Promoting Activity on Cord Blood Cells

By C. Favre, S. Saeland, C. Caux, V. Duvert, and J.E. De Vries

Effects of human recombinant interleukin-4 (IL-4) on cord blood cells depleted of T cells and monocytes were tested in colony assays and liquid cultures. IL-4 did not induce colony formation in semisolid medium, but enhanced generation of basophil colonies induced by conditioned medium (CM) of the bladder carcinoma cell line 5637. In liquid cultures, variable degrees of basophil growth were observed in the presence of IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, and 5637 CM, or even with IL-4 alone, but the highest number of basophils were obtained when IL-4 was used in combination with IL-3 or 5637 CM. Progressive basophil growth was observed during 3 to 4 weeks of culturing, whereas the numbers of basophils remained stationary for another 3 weeks. Interestingly, cord blood cell cultures performed with IL-3 contained variable percentages of eosinophils that were further enhanced in the presence of combinations of IL-3 and IL-4. These latter cultures contained approximately 50% eosinophils and 50% basophils. Kinetic studies indicated that basophils were present 7 days after onset of the cultures, whereas eosinophils did not appear before day 13. In contrast to the pronounced effects of IL-4 and 5637 CM on basophil development, relatively low numbers of eosinophils were observed under these culture conditions. Our results indicate that eosinophil and basophil development are regulated by different sets of factors, and that IL-4 has an enhancing effect of both cell lineages in association with the appropriate factors.

INTERLEUKIN-4 (IL-4) has pleiotropic biologic activities. Among other activities, murine IL-4 has been shown to induce optimal proliferation of mouse mast cell lines when added with IL-3.2,3 Furthermore, freshly isolated mouse peritoneal mast cells have been shown to proliferate in response to combinations of IL-3 and IL-4, but not to IL-4 alone.4

In rodents, mast cells can be classified in two types according to their proteoglycan content. The mucosal mast cell or E-mast cell (E-MC) contains chondroitin sulfate E, whereas the connective tissue type mast cell, or H-mast cell (H-MC) synthesizes heparin.5 Thus far, it has been difficult to make this distinction in humans. Recently, E-MC have been identified in normal human colon6 and in human gastric mucosa,7 whereas H-MC were found to be present in human lung tissue.8 Both types of mast cells are closely related, but their relationship to circulating basophils in human and rodent systems is still unclear. In rodent systems, it has been suggested that basophils and mast cells have a common bone marrow progenitor cell that gives rise to precursors specific for each lineage.9

Human basophilic cells can be generated in vitro from cord blood,10 fetal liver,11 or bone marrow12 using T-cell conditioned medium (CM) or purified proteins isolated from these CM. Cells obtained in these cultures display features of basophilic cells rather than mast cells. Similar results have been obtained using purified recombinant lymphokines.13 Basophilic cells were selectively generated in cord blood cell cultures performed with recombinant human IL-3. This basophil growth-promoting effect of IL-3 was enhanced by IL-4, but no mast-cell development was observed under these conditions.13

To determine the role of IL-4 on the growth and development of basophilic cells, cord blood cell depleted of monocytes and T cells were cultured in the presence of IL-4 alone or with IL-4 in combination with other hematopoietic factors. IL-4 alone has no significant colony-inducing effect in semisolid medium, but can enhance the number and size of the basophil colonies induced by 5637 CM. In liquid cultures, basophil growth and some eosinophil growth was observed with IL-4 alone, but the highest number of basophils was obtained when IL-4 was used in combination with 5637 CM or IL-3. Interestingly, in cord blood cell cultures performed with IL-3, IL-4 was found not only to enhance the growth of basophils but also of eosinophils.

MATERIALS AND METHODS

Cord blood cell preparations. Umbilical cord blood samples were collected according to institutional guidelines. Blood was collected in vessels containing preservative-free grade 1 sodium-heparin (Sigma, St Louis, MO) at a final concentration of 20 IU/mL. Light density mononuclear cell fractions were isolated by Ficoll-Hypaque (Lymphoprep, Nyegaard, Oslo, Norway) gradient separation at a density of 1.077 g/mL. Adherent cells were depleted by first incubating the cells at 10^6 cells/mL in tissue culture flasks overnight. Fifty-milliliter cell suspension in RPMI medium supplemented with 1% tissue culture grade bovine serum albumin (BSA) (Boehringer Mannheim, Mannheim, West Germany), 0.2% NaHCO3, 25 mmol/L Hepes, 100 U/mL penicillin, and 100 µg/mL streptomycin were added for each 150-cm² culture flask (Corning, NY). Subsequently, nonadherent cells were further depleted of T cells by rosetting with sheep erythrocytes according to standard procedures. Cell samples were checked for contaminating T cells and monocytes by immunofluorescence with monoclonal antibodies (MoAbs) directed against the appropriate cell surface antigens. Labeled cells were analyzed on a FACS IV (Becton Dickinson, Sunnyvale, CA). Contamination by T cells as judged by staining with the anti-CD3 MoAb IOT-3 (SPV-T3b, Immunotech, Marseille, France) was lower than 5%, whereas less than 2% monocytes were present according to staining with antimonocyte MoAb Leu M3 (Becton Dickinson).

Cell cultures. Liquid cultures of cord blood cells depleted of monocytes and T cells were established at 10^6 cells/mL in RPMI-
10% FCS (Flow 97156, Irvine, Scotland) supplemented with recombinant factors or 5637 CM. Cell cultures were split in two weekly by using fresh medium supplemented with the appropriate factors. Large-scale cultures were performed by using this procedure, but the cultures were set up with the total number of cells obtained from one umbilical cord sample (usually about 30 10^6 cells) in a 150-cm² flask in the presence of 5637 CM 5% and 100 U/mL IL-4.

**Cytologic characterizations.** Aliquots of cell cultures were taken for cytologic characterization weekly. 2 x 10^5 cells were centrifuged onto a microscope slide in a cytospin-2 (Shandon, Astmoor, UK) in medium containing 50% FCS at 400 rpm for 7 minutes. Slides were fixed in ethanol/acetic acid (3:1) for 10 minutes for staining with alcian blue or Biébrich scarlet. May-Grünewald-Giemsa staining was performed on air-dried cells. Slides were stained with 0.5% alcian blue (8GX, Sigma A 3137) in 0.3% acetic acid for 1 hour, then rinsed with distilled water and counterstained with 0.1% safranin 0 (Sigma S 8884) in 0.1% acetic acid. Eosinophil characterization was performed by staining the cells with Mayer's haematoxilin solution (MHS-1, Sigma) for 5 minutes, followed by washing with distilled water and incubation for 1 hour with 1 mg/mL Biébrich scarlet solution in 0.1 mol/L Tris buffer (stock solution: 50 mg/mL Biébrich scarlet Sigma B6008 in DMSO).

**Colonies assays.** Colony assays were performed by plating 10^5 cord blood cells depleted of monocytes and T cells per tissue culture grade 35-mm Petri dish (Corning, NY) in a volume of 1 mL Iscove's Modified Dulbecco's Medium (IMDM) containing 30% FCS, 10^-4 mol/L 2-mercaptoethanol, 0.9% methylcellulose (Methocel MC 4000 mP, Sigma, Switzerland), 1 IU purified human erythropoietin (hEpo, Terry Fox, Vancouver, Canada), 0.5% BSA (Boehringer Mannheim) neutralized with NaHCO₃, 1 mmol/L glutamine, and antibiotics. After 14 days of incubation at 37°C and 5% CO₂, in a serum-free medium, all factors were used at saturating concentrations.

**Lymphokines and conditioned media.** Supernatants of Cos7 cells transfected with the cDNAs of IL-3 and G-CSF were used as sources of recombinant human IL-3 and G-CSF. Mock transfection supernatants were used as controls (all kindly provided by Dr N. Arakawa, DNAX Research Institute, Palo Alto, CA). Human IL-4 [13] and GM-CSF [14] purified from supernatant of transfected CHO cell supernatant were provided by Dr T.L. Nagabhushan (Schering Research, Bloomfield, NJ). CM of the bladder carcinoma cell line 5637 (ATCC HTB9) was collected after culturing the cells in RPMI 1640 supplemented with 10% FCS, antibiotics. After 14 days of incubation at 37°C and 5% CO₂ in a moist atmosphere, colonies were counted using an inverted phase-contrast microscope.

**RESULTS**

**Effects of IL-4 on colony formation induced by 5637 CM.** Effect of IL-4 on colony formation in semisolid medium by cord blood cells depleted of T cells and monocytes was studied. In Fig 1, it is shown that the supernatant of the bladder carcinoma cell line 5637, which contains various hematopoietic growth factors [15] and was used as a positive control, induced the generation of mainly G and GM colonies, and to a lesser extent M colonies, as well as erythroblast lineage colonies (BFUe and CFUe). The small number of colonies obtained with IL-4 alone did not differ significantly from those observed in medium only. Interestingly, IL-4 enhanced both the number and size of colonies induced by 5637 CM, whereas these effects were not observed for GM and M colonies. Cytologic characterization of individual G and GM colonies obtained in the presence of combinations of IL-4 plus 5637 CM by May-Grünewald-Giemsa staining indicated that those colonies contained mainly basophils at different stages of maturation. Collectively, these data demonstrate that IL-4 has a potentiating effect on the generation of basophil colonies induced by 5637 CM.

**Effect of IL-4 on basophil growth in liquid cultures.** To investigate whether basophils could be generated in liquid cultures, cord blood cells depleted of T cells and monocytes were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics in the presence of hematopoietic growth factors. The cultures were split twice and fresh medium and growth factors were added twice per week. Aliquots for cytologic analysis were taken weekly. Results of one representative experiment out of three is shown in Table 1, and Fig 2 shows a more detailed cytologic analysis. In medium alone, the number of viable cells declined slowly and after 3 weeks no further viable cells were observed. Variable cell growth was observed in cultures performed with IL-4, G-CSF, GM-CSF, IL-3, or 5637 CM. In all instances, cell proliferation induced by the individual factors was enhanced in the presence of IL-4 (Table 1). However, in some samples, increased cell numbers were not observed before 2 weeks' culture. At the onset of the culture, no more than 5% reactivity with the anti-CD3 MoAb SPV-T3b (see Materials and Methods). In the presence of IL-4, which has T-cell growth-promoting activity [16], this percentage of T cells increased to a maximum of 10%, but no T cells could be detected after 10 days of culture (data not shown).

Large numbers of blast cells were observed in cultures containing actively proliferating cells. The highest number of blast cells were obtained with G-CSF alone or G-CSF with...
IL-4 Promotes Eosinophil and Basophil Growth

Appearance of these cells always preceded the emergence of the more mature granulocytes, which likely derived from immature cells present within the blast cell population.

In the presence of GM-CSF alone or in combinations of GM-CSF with IL-4, large numbers of adherent monocytes were generated. Since only nonadherent cells were counted, the total cell number in these cultures was considerably underestimated. IL-4 alone did not induce the appearance of monocytes, but it enhanced the number of nonadherent monocytes induced by GM-CSF (Fig 2). Microscopic examination showed that the number of adherent monocytes were also enhanced in these cultures (not shown).

Basophils developed in cultures performed with IL-4, GM-CSF, G-CSF, IL-3, and 5637 CM, with IL-3 being the most potent in inducing basophil growth. IL-4 considerably enhanced the number of basophils in cultures performed with each of the other factors. The strongest basophil proliferation was obtained with the combinations of IL-4 with 5637 CM or IL-3 (Table 1, Fig 2).

Effect of IL-4 on eosinophils. In addition to basophils, relatively small numbers of eosinophils were obtained in cultures performed with IL-4, GM-CSF, G-CSF, and 5637 CM (Table 1), whereas IL-3 induced considerable, but variable, numbers of eosinophils. In the experiment shown in Table 1, cultures with IL-3 contained 11% eosinophils at day 20, whereas in the example shown in Fig 2 this was 32% after 24 days of culturing. Interestingly, IL-4 enhanced the generation of eosinophils in the presence of IL-3. After 3 weeks, cultures performed with IL-3 plus IL-4 contained approximately 50% basophils and 50% eosinophils. The eosinophils always emerged later in these cultures than the basophils (Table 1), indicating that these cell types are generated with different kinetics. In contrast, low percentages of eosinophils were detected in cultures performed with IL-4 plus 5637 CM, and no eosinophil colony formation was noticed in the presence of IL-4 plus 5637 CM (Fig 1).

Cytologic analysis. To distinguish eosinophils and basophils more precisely, cytologic characterization was also performed with Biebrich scarlet and alcian blue, which specifically stain eosinophilic granules and basophilic/mast cell granules, respectively. Photomicrographs of cells cultured with combinations of IL-3 plus IL-4 for 3 weeks showed that cells stained either with alcian blue or Biebrich scarlet (Fig 3). These results confirmed data obtained after May-Grünwald-Giemsa staining and indicate that these cultures consisted predominantly of basophils and eosinophils.

Basophilic cells generated in the different cultures shown here were immature with a kidney shaped nucleus (Fig 3). They contained small granules that stained with alcian blue, confirming their basophilic nature. No cells were found that stained with safranin O (data not shown), indicating that mast cells were not present in these cultures.

A small number of neutrophils was observed only in cultures performed in the presence of G-CSF (data not shown).

Large-scale basophil cultures. Large-scale cord blood cell cultures were prepared by adding the total number of cells obtained from one sample after depletion of monocytes and T cells. Culture conditions allowing the recovery of high numbers of basophils were used, ie, combinations of 5637 CM (5%) and IL-4 (100 U/mL) in RPMI-1640 supplemented with 10% FCS and antibiotics. These cultures were split in two and fresh medium supplemented with growth factors was added weekly. Results of three of these cultures are summarized in Table 2. After approximately 3 weeks of culturing, a large increase in cell number (up to 10-fold) was obtained; about 30% of these cells were alcian blue positive basophils. Progressive cell proliferation was observed during the first 3 weeks, then the total number of cells and the number of basophils remained stable for 3 additional weeks and decreased thereafter (not shown). These data indicate that these culture conditions allow the generation of relatively large numbers of basophils suitable for functional studies.

Table 1. Long-Term Culture of Cord Blood Cells

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 13</th>
<th>Day 20</th>
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<tbody>
<tr>
<td></td>
<td>Total Cell No.</td>
<td>Total Cell No.</td>
<td>% Basophils</td>
</tr>
<tr>
<td>Medium</td>
<td>5</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>IL-4</td>
<td>5</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>IL-3</td>
<td>5</td>
<td>9</td>
<td>14</td>
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<tr>
<td>IL-3 + IL-4</td>
<td>5</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>GM</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>GM + IL-4</td>
<td>5</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>G</td>
<td>5</td>
<td>5</td>
<td>29</td>
</tr>
<tr>
<td>G + IL-4</td>
<td>5</td>
<td>10</td>
<td>12</td>
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<tr>
<td>5637</td>
<td>5</td>
<td>3</td>
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<tr>
<td>5637 + IL-4</td>
<td>5</td>
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Abbreviation: ND, not done.

IL-4 (Fig 2). Appearance of these cells always preceded the emergence of the more mature granulocytes, which likely derived from immature cells present within the blast cell population.

In the presence of GM-CSF alone or in combinations of GM-CSF with IL-4, large numbers of adherent monocytes were generated. Since only nonadherent cells were counted, the total cell number in these cultures was considerably underestimated. IL-4 alone did not induce the appearance of monocytes, but it enhanced the number of nonadherent monocytes induced by GM-CSF (Fig 2). Microscopic examination showed that the number of adherent monocytes were also enhanced in these cultures (not shown).

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DISCUSSION

We demonstrate that purified recombinant IL-4 alone is ineffective in inducing colony formation by light-density cord blood cells depleted of T cells and monocytes. However, IL-4 strongly enhanced the number and size of G colonies induced...
Development of basophils in liquid cultures of cord blood cells was also strongly induced by IL-3 (which is not produced by 5637 cell line; K.I. Arai, unpublished observation, 1987). This effect was also further enhanced by IL-4 (see Table 1). Additionally, these cultures contained varying numbers of eosinophils (Fig 2), indicating that IL-3 is able to promote the development of eosinophils from cord blood cells. These observations are in line with previous reports showing the appearance of eosinophil colonies from purified human marrow progenitors cultured in IL-3. IL-4 was also able to potentiate the IL-3-induced eosinophil development (Fig 2). The appearance of basophils in response to IL-3 in liquid cultures of cord blood cells and the potentiating effects of IL-4 were also described by Saito et al. However, in the latter study no eosinophils were described when combinations of IL-3 and IL-4 were used. Whether these differences are related to different culture conditions, ie, the presence of proliferating T cells that may inhibit eosinophil development, or to the higher IL-4 concentrations used here is not clear.

Monitoring the cultures at different time points showed that blast cells appeared first, followed by differentiated basophils, and to a lesser extent eosinophils, indicating that both culture systems are not completely comparable. However, cell proliferation induced by 5637 CM in liquid cultures was also strongly enhanced in the presence of IL-4. Cytologic characterization showed that a large proportion of the proliferating cells in these liquid cultures belonged to the basophilic lineage, indicating that IL-4 enhanced basophil growth induced by 5637 CM in both culture systems. Furthermore, it is shown that IL-4 also enhanced the proliferative responses of light density cord blood cells and basophil growth induced by G-CSF or GM-CSF. However, the highest proliferative responses and highest numbers of basophils were obtained with IL-4 in combination with 5637 CM or IL-3. Bladder carcinoma cell line 5637 has been shown to produce constitutively various hematopoietic growth factors including GM-CSF, G-CSF, IL-1α, and IL-6 (K.I. Arai et al, unpublished observations). Therefore, the potentiating effect of IL-4 and 5637 CM could be due to combined effects of IL-4 and one or more of these lymphokines.

Taken together, these results suggest that for optimal development of basophil and eosinophil cells, different combination of growth factors are required. Tanno et al also recently reported that the growth of these two types of cells is driven by different sets of T-cell-derived factors that could be separated by fractionation. The effect of IL-3 on the development of cells of both basophil and eosinophil lineages is likely to result from the stimulation of the common immature progenitor from which they derive. Whether the enhancement observed with IL-4 is due to a direct effect on this common progenitor cell or to induction of differentiation of a more mature, already committed precursor cell, remains to be determined.
Fig 3. Photomicrographs showing cytologic preparation of cell populations obtained after 24 days culture with IL-3 plus IL-4. (A) May-Grünwald-Giemsa stain. (B) Biebrich scarlet stain, specific for eosinophil granules. (C) Alcian blue stain, specific for basophil granules. Arrows indicate positive cells.
Morphology of the basophils obtained in our cultures was comparable with that of blood basophils and not with that of mast cells, probably due to insufficient culture conditions. Mast-cell development is induced by IL-3 and is dependent on the presence of an anchorage surface, which can be provided by fibroblast layers as demonstrated for murine IL-3-dependent bone marrow cells, or by an agar layer, as recently described for human bone marrow cell cultures.

Finally we demonstrated that cord blood cell cultures can be scaled up to provide a useful source of large numbers of basophils and eosinophils suitable for functional studies.

ACKNOWLEDGMENT

We thank Isabelle Durand for flow cytometric analysis; Dr J.P. Favier, Pr R.C. Rudigoz, and colleagues for providing umbilical cord blood samples; Dr A. Waitz for support; and Nicole Courbiere and Muriel Vatan for expert editorial assistance.

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