Effects of Recombinant Human Interleukin-3 on CD34-Enriched Normal Hematopoietic Progenitors and on Myeloblastic Leukemia Cells


Induction of proliferation and differentiation in response to recombinant human interleukin-3 (hIL-3) was studied in liquid and semisolid cultures of umbilical cord blood and bone marrow cells that were fractionated by “panning” with anti-My10 antibody according to expression of CD34 antigen. Cells from enriched fractions (70% to 90% CD34+) were found to proliferate strongly in response to hIL-3. Phenotypic analysis and morphologic characterization of the proliferating cells demonstrated a rapid decrease in CD34+ cells and an exponential increase in the number of cells belonging to the neutrophilic, eosinophilic, monocyte/macrophage, and thrombocytic lineages. When combined with recombinant human erythropoietin, burst colonies and cells expressing glycophorin-A were detected, thereby demonstrating the effects of hIL-3 on erythroid progenitors. Further, the development of mixed-erythroid colonies indicated that multipotential cells within CD34-enriched fractions responded to hIL-3. In addition, we examined the effect of hIL-3 on the proliferation of primary acute myeloblastic leukemia cells in liquid culture. We found that hIL-3 was able to induce cell proliferation in a proportion of the cases tested. Heterogeneity of the responses to hIL-3 was in part related to French-American-British classification but could not be correlated with CD34 antigen expression by the leukemic cells. These results indicate that, although the effects of hIL-3 on proliferation and differentiation of cells obtained from normal hematopoietic specimens were primarily borne by CD34+ cells, expression of the CD34 molecule per se is an insufficient condition to determine a growth response to this lymphokine.

A gibbon ape interleukin-3 (IL-3) cDNA probe that displays homology with the mIL-3 gene has been used to isolate the human counterpart from a genomic library. The predicted amino acid sequence of the human product has been shown to be 29% homologous with mIL-3. In parallel, a cDNA clone obtained by hybridization with mIL-3 cDNA that encodes human multilineage CSF (hMulti-CSF) has also been described. From the human T-cell library we have recently isolated cDNA clones encoding human IL-3 and whose predicted expression product (hIL-3) differs by one amino acid at position 27 from the sequence published by Yang et al.

In this report we describe the biologic effects of hIL-3 on cell fractions from normal umbilical cord blood and bone marrow. Cells were fractionated by “panning” according to their expression of CD34 surface membrane antigen, a 115-Kd molecule that is defined by monoclonal anti-My10 antibody and is present on human hematopoietic progenitor cells. This approach was taken to characterize the direct effects of hIL-3 in normal progenitors, thereby minimizing bystander activities mediated by accessory cells.

Finally, on the basis of observations that a number of murine myeloid leukemias require mIL-3 for growth in vitro and that human hematopoietic growth factors have been shown to induce proliferation of acute myeloblastic leukemia cells, we examined the effect of hIL-3 on the proliferative responses of primary myeloid leukemia cells in relation to their expression of CD34 antigen. Here we demonstrated that cells from normal hematopoietic specimens enriched for expression of CD34 antigen were strongly induced to proliferate and initiate multilineage myeloid differentiation in response to hIL-3, both in semisolid and liquid cultures. These effects were found to be primarily borne by cells of the CD34-enriched fractions.

Cells obtained from acute myeloblastic leukemia patients displayed a considerable heterogeneity in their proliferative...
EFFECTS OF IL-3 ON CD34+ PROGENITOR CELLS

MATERIAL AND METHODS

Collection and preparation of human cells. Bone marrow was obtained from 2 to 10^6 cells per tissue culture grade 35-mm Petri dish (Corning) in a volume of 1 mL. Isotope-modified Dulbecco's medium (10% MEM) containing 30% FBS, 10^-4 mol/L 2-mercaptoethanol, 0.9% methylcellulose (Methocult MC, 4,000 mPa·s, Fluka, Switzerland), 1 IU recombinant human Epo (hEpo) (Kirin-Amgen/Amersham, UK; specific activity, >70,000 IU/mg), 0.5% BSA neutralized with NaHCO3, and L-glutamine and antibiotics as previously described. After 14 days of incubation at 37°C and 5% CO2 in a moist atmosphere, colonies consisting of more than 50 cells were examined, classified, and counted by using phase-contrast microscopy. Classification of the colonies was confirmed through cytologic examination.

Cytologic cell characterization. After collection, cells were cytocentrifuged for seven minutes at 400 rpm in a Cytospin-2 (Shandon Southern Products, Ltd, Aylesbury, UK) containing 50% FBS. Microscope slides were then stained with May-Grünwald-Giemsa, mounted, and subsequently observed by using an oil-immersion objective.

Cytoscourometric cell surface phenotyping. Indirect immunofluorescence was performed according to standard techniques with a panel of murine monoclonal antibodies detected by fluorescein-conjugated sheep F(ab')2, anti-mouse Ig (Bio-Art, Meudon, France). Double-color fluorescence was carried out by sequential incubation of the cells with anti-My7, fluorescein-conjugated antimouse Ig (Bio-Art, Meudon, France), and anti-Leu-M3-PE (CD14), anti-Leu-M1 (CD15), anti-Leu-M16 and anti-Leu-M3-PE (CD14), anti-Leu-M1 (CD15), anti-Leu-16 and anti-Leu-16-PE (CD20), anti-HLA-DR-PE (all obtained from Becton Dickinson) My7 (CD13) (Coulter Immunology, Hialeah, FL), P2 (anti-glycoprotein IIb-IIa, CD41) (Immunotech, Marseille, France), and CS4 (anti-glycoprotein A (GPA)) (kindly provided by Dr G. Mouchiroud, Université C. Bernard, Lyon, France).

Fluorescence analysis was performed either with a FACScan or with a FACS 440 (Becton Dickinson, Sunnyvale, CA), each equipped with an argon laser tuned at 488 nm and respectively emitting 15 and 400 mW. Detection sensitivity of the FACScan is several times higher than that of the FACS 440 (500 v 2,000 sites/cell, Becton Dickinson). Gating was set according to forward and 90-degree light scatter parameters to exclude erythrocytes, platelets, and subcellular particles from acquisition data.

Cells recovered after fractionation by panning were again saturated with anti-My10 antibody before FACScan analysis to allow optimal detection of CD34 antigen.
Recombinant human lymphokines. hIL-3, the cDNA of which was cloned from a human T-cell library as previously indicated, was used as supernatant from mammalian COS7 cells transfected with a pcD expression vector containing the hIL-3 cDNA sequence. Unless otherwise stated, experiments were performed by using a 1% vol/vol final concentration of COS7 hIL-3 transfection supernatant, which was consistently found to induce maximal proliferative responses. Mock supernatant was derived from COS7 cells transfected with an unrelated cDNA clone inserted into the expression vector and was used as a negative control. hIL-2 was obtained through the courtesy of Dr. R. Kastelein (DNAX Research Institute, Palo Alto, CA). Biologic activity was determined on murine CTLL cells and expressed in reference units according to Biological Response Modifiers Program standards (National Cancer Institute, Frederick, MD).

RESULTS

Recovery and characterization of cells fractionated according to expression of CD34 antigen. Physical fractionation of normal bone marrow or umbilical cord blood into “panned” (adherent) and nonadherent cells by the use of anti-MylO antibody resulted in high enrichment of CD34+ cells in panned fractions. The outcome of representative fractionation runs is summarized in Table 1 and Fig 1.

In the first experiment, CD34 antigen expression of unfractinated bone marrow was found to be 3.7% (Table 1, Fig 1A). Cell recovery of the anti-MylO panned fraction was 1.7% of the total light-density mononuclear cells. FACScan analysis further showed that 93.5% of the cells in the panned (CD34-enriched) fraction expressed CD34 antigen (Table 1, Fig 1B) whereas nonsignificant numbers of CD3+ and CD14+ cells were detected (Table 1), thus indicating the absence of relatively mature lymphoid or myeloid cells.

Double-color fluorescence analysis performed on the panned fraction from a second bone marrow sample demonstrated that the CD34+ cells simultaneously expressed HLA-DR (Fig 1C). In addition, the CD34+ cells were found to lack significant expression of the lymphoid markers CD5 and CD20 or the monocyte/macrophage marker CD14, as evaluated by double-color fluorescence analysis using anti-MylO and a cocktail of PE-conjugated antibodies (CD5, CD20, and CD14) (Fig 1D).
**EFFECTS OF IL-3 ON CD34+ PROGENITOR CELLS**

### Table 2. Induction of Hematopoietic Colonies by CD34-Enriched Cells From Cord Blood and Marrow

<table>
<thead>
<tr>
<th>Sample</th>
<th>Stimulation</th>
<th>CFU-GM</th>
<th>CFU-G</th>
<th>CFU-M</th>
<th>CFU-E0</th>
<th>BFU-e</th>
<th>CFU-mix</th>
<th>Total</th>
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<td>1</td>
<td>0</td>
<td>6</td>
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<tr>
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<td>0</td>
<td>9</td>
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<td>2</td>
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<td>35</td>
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*Day 14 colonies/2 x 10^3 cells plated. The number of colonies represents the average counts of two replicate plates. hEpo (1IU/ml), added at day 0.
†COS7 transfection supernatants (1% vol/vol).
§Supernatant from activated human T-cell clone as a positive control (5% vol/vol).

In the second experiment illustrated in Table 1, the anti-My10 panned fraction obtained from cord blood yielded 1.2% of the total light-density population and consisted of 74.5% CD34+ cells as detected by FACSscan analysis. In addition, 14.2% of the cells were found to express CD20 antigen.

These results indicate that our fractionation method resulted in high enrichment of CD34+ cells from both bone marrow and cord blood.

**Effects of hIL-3 on induction of hematopoietic colonies from CD34-fractionated cells.** Culturing CD34-enriched cells from bone marrow or cord blood in the presence of hIL-3 resulted in the formation of well-developed hematopoietic colonies, thereby demonstrating a capacity to trigger clonal proliferation of progenitor cells. As shown in Table 2, the colonies induced in the presence of COS7 hIL-3 (1% vol/vol) could be categorized by morphologic and cytologic criteria into granulocyte-macrophage (CFU-GM), neutrophilic granulocyte (CFU-G), eosinophil (CFU-E0), monocyte/macrophage (CFU-M), erythroid bursts (BFU-e), and mixed (CFU-Mix) consisting of erythroid and nonerythroid elements. The development of significant numbers of large, well-hemoglobinized, mixed colonies indicated that multipotential myeloid hematopoietic progenitors were included among target cells of hIL-3.

Mock COS7 transfection supernatant, tested under the same conditions, was unable to promote any significant colony formation (Table 2). Supernatants from the activated human helper T-cell clone 2F1 established in our laboratory (Dr D. Blanchard), which promotes multilineage colony development, served as positive controls in these experiments. In T-cell clone 2F1 mRNA transcripts for IL-3 and GM-CSF have been detected (Dr R. De Waal Malefijt, personal communication, 1988).

The overall frequency of colony-forming cells detected in response to saturating concentrations of COS7 hIL-3 transfection supernatant was found (Table 2) to be lower than that of 2F1 T-cell clone supernatants (144/256 = 0.56 and 160/213 = 0.75).

Finally, COS7 hIL-3 transfection supernatant, even when tested at concentrations up to 5% vol/vol, was not observed to induce any significant colony formation by CD34-depleted marrow or cord blood cell fractions (not shown).

**Effects of hIL-3 on induction of proliferation of CD34-fractionated cells in liquid culture.** hIL-3 was demonstrated to induce "H-TdR incorporation by CD34-enriched bone marrow or cord blood cells in a dose-dependent fashion (Fig 2). Maximum responses were obtained approximately at a 1% vol/vol final concentration of COS7 hIL-3 transfection supernatant. In addition, no inhibitory effects were observed when concentrations were increased up to 10% vol/vol (not shown). Mock transfection supernatants tested under the same conditions were unable to induce any significant "H-TdR uptake (Fig 2). Viability of the cells cultured in medium alone or in the presence of mock transfection supernatant and as judged by trypan blue exclusion was estimated to be on the order of 20% at the time of "H-TdR addition.

The proliferation kinetics of cells fractionated from bone marrow demonstrated that CD34-enriched cells responded rapidly to hIL-3 as indicated by "H-TdR uptake measured after a six-hour pulse on day 3 of culture (Fig 3). Under the experimental conditions used, incorporation of "H-TdR, reflecting accumulation of cells undergoing DNA synthesis, reached a maximum on day 9 where the uptake by CD34-
enriched cells was found to be approximately six times higher than that of the CD34-depleted fraction. After prolonged culturing a rapid decrease of the proliferative responses was observed due to unfavorable culture conditions (see later).

CD34-enriched cells obtained from cord blood also responded to hIL-3 by rapid and strong ^H-TdR uptake (Fig 4A). In contrast, these cells were unresponsive to hIL-2 and could not be achieved by concanavalin A (ConA). The proliferative response of cord blood total light-density unfractionated cells to hIL-3 (Fig 4B) was delayed and of lower magnitude than that of the CD34-enriched fraction, thus suggesting the presence of lower numbers of target cells. Finally, CD34-depleted cells were inducible by hIL-2 and ConA but did not respond to hIL-3 (Fig 4C). Only a marginal proliferative response to hIL-3 was observed in this experiment after prolonged culture. The addition of higher concentrations of COS7 hIL-3 (up to 5% vol/vol) did not enhance the proliferation of CD34-depleted fractions (not shown).

Cell counts performed in parallel on cord blood cultures also demonstrated that hIL-3 induced the proliferation of CD34-enriched cells (Fig 5), which indicated that ^H-TdR uptake reflected actual cell multiplication. Nevertheless, the experiment presented in Fig 5, which illustrates the cumulative generation of cells by maintaining optimal culture conditions by passaging the cells every five to seven days in the presence of hIL-3, indicated a progressive loss of the proliferative response to hIL-3 in long-term culture and reflected differentiation (see later). In contrast to the CD34-enriched population, cell numbers in CD34-depleted fractions rapidly declined despite the presence of hIL-3 (Fig 5).

Phenotype and morphology of CD34-enriched cells in liquid culture. We examined the changes induced by hIL-3 in the phenotype composition of liquid cultures of CD34-enriched cells from bone marrow or cord blood (Fig 6). Evaluation of the phenotypic variations was obtained by combining the percentages of cells staining with each antibody with total viable cell counts (Fig 6A and F). The results are expressed as absolute numbers of positive cells to avoid discrepancies associated with a loss of certain subpopulations due to culture conditions.

Culturing CD34-enriched fractions in the presence of COS7 hIL-3 transfection supernatant (1% vol/vol) resulted in an exponential increase in total number of cells, but a gradual decrease in the number of cells expressing CD34 antigen was observed (Fig 6B). In addition, the number of cells of the monocyte/macrophage and granulocytic lineages, as measured by expression of CD14, CD13, and CD15 antigens, was increased in all experiments either from marrow or cord blood (Fig 6C, D, and E).

In combination with hEpo, hIL-3 promoted the emergence of erythroid lineage elements as indicated by induction of
cells expressing GPA (Fig 6G). In the absence of hEpo, there was only a small detectable change in the number of cells expressing GPA, and control cultures containing hEpo but no hIL-3 resulted in a rapid loss of viability of the cells (not shown).

COS7 hIL-3 transfection supernatant was also found to induce an important increase in the number of cells expressing thrombocytic-associated CDw41 antigen (Fig 6H). Furthermore, the addition of hEpo at the beginning of the cultures was observed to potentiate this effect of hIL-3.

In contrast to these promoting effects, the number of CD20+ cells present in some CD34-enriched fractions rapidly declined upon culturing in the presence of hIL-3 (not shown).

Finally, we were unable to demonstrate CD10+ or CD3+ cells after culturing in hIL-3.

Results obtained by phenotypic analysis of CD34-enriched cells grown in hIL-3 were further compared with a morphologic evaluation of the cultures by May-Grünwald-Giemsa staining of cytospin preparations. Our observations indicated a progressive predominance and maturation of morphologically recognizable cells of the neutrophilic, eosinophilic, and monocytic/macrophage series, thus confirming the phenotypic analysis and colony data and further demonstrating that hIL-3 induced myeloid differentiation (not shown).

**Effects of hIL-3 on induction of proliferation of myeloblastic leukemia cells in liquid culture.** Next we examined the proliferative capacity and CD34 antigen expression of cells from a panel of diagnostic specimens of myeloblastic leukemia that represented different stages of the FAB classification.

As illustrated in Table 3, cells from approximately half of the samples tested (n = 21) could be induced to proliferate in response to COS7 hIL-3 transfection supernatant (1% vol/vol). In addition, no correlation was found between spontaneous proliferation in the absence of exogenous growth factor and responsiveness to hIL-3.

FAB M1 cells (n = 5) were found to proliferate in response to hIL-3. The responsiveness was found to vary and did not correlate with expression of CD34 antigen since only the majority of the cells of patient 1 were CD34+. None of
the five FAB M4 specimens tested in our study responded significantly to hIL-3, although in two of five patients tested the cells expressed CD34 antigen (patients 11 and 13). Since proliferation of these M4 myelomonocytic cells could be induced by supernatant from ConA-activated peripheral blood lymphocytes (not shown), it could be excluded that the observed lack of proliferative response to hIL-3 was due to a general nonresponsiveness of the M4 cases to exogenous hematopoietic growth factors. Among the FAB M2 and FAB M5 specimens tested, examples of both hIL-3 responders and nonresponders were found independently of CD34 antigen expression. This is illustrated by patient 9 who had a high proportion of CD34+ cells that did not respond significantly to hIL-3 and by patient 17 who had less than 1% CD34 antigen expression but whose cells could be induced to rapid proliferation in the presence of hIL-3.

These results indicate that the heterogeneity of the proliferative responses of myeloblastic leukemia cells to hIL-3 is in part related to FAB classification but is not correlated with CD34 antigen expression.

**DISCUSSION**

By using CD34 antigen as a marker during fractionation of normal hematopoietic cells by panning with anti-Myel0 antibody, we have isolated CD34-enriched fractions consisting approximately of 70% to 90% CD34+ cells as detected by FACScan analysis. In addition, double-color fluorescence indicated that the CD34+ cells simultaneously expressed HLA-DR, which is in accordance with the literature.37 The use of this cell enrichment procedure enabled us to analyze the direct effects of hIL-3 on the proliferation and differentiation of normal hematopoietic progenitors, both in liquid and semisolid culture systems. The proliferative response to hIL-3 was found to be primarily borne by cells of the CD34-enriched fractions. This finding is consistent with the previously described progenitor nature of CD34-reactive cells.25-27 The growth-promoting effects of hIL3 on CD34+ cells was correlated with the emergence and differentiation of myeloid precursor cells. Cell expansion could be correlated with a gradual decrease in the number of cells expressing CD34 antigen, likely consequence of hIL-3–induced differentiation.

The observed increase in the number of CD13, CD14, and CD15-reactive cells in liquid cultures of CD34-enriched cells reflected the growth of myeloid colonies observed in semisolid medium. Since no significant numbers of CD34+ cells expressing CD14 or CD15 antigens could be detected in our enriched populations on fractionation, expansion of CD14+ and CD15+ cells on culturing in the presence of hIL-3 reflects the generation of cells that acquired these antigens. On the other hand, expression of CD13 antigen was already detected on a large proportion of CD34+ cells before culturing. Morphologic analysis of the proliferating cells indicated that, in addition to elements of the neutrophilic series as well as monocytes/macrophages and their precursors, hIL-3 led to the appearance of cells of the eosinophilic lineage. This observation is in agreement with the results of Lopez et al38 who described induction of eosinophil colonies from human bone marrow by gibbon IL-3 (gIL-3). Also, the finding that gIL-3 is an inducer of mature eosinophil functions38 may indicate that IL-3 plays a particular role in the development and functional activities of cells of the eosinophilic lineage.

hIL-3 was found to induce the appearance of BFU-e in semisolid and cells expressing GPA in liquid cultures of CD34-enriched cells in the presence of hEpo, thereby demonstrating the effects of hIL-3 on erythroid progenitor cells and confirming activities previously ascribed to gIL-322,38 and hMulti-CSF.29

The strong induction in the number of cells bearing CDw41 antigen, known to be expressed on megakaryoblasts/megakaryocytes,39-41 indicated that hIL-3 was able to induce CD34+ cells toward the thrombocytic lineage. This effect was observed to be further potentiated by hEpo. These results are in line with recent data of Lopez et al38 who demonstrated that gIL-3 induced the development of megakaryocytes in plasma cultures from nonadherent human bone marrow cells. Also, potentiating effects of hEpo on the formation of human megakaryocyte colonies have been described by Dessypriss et al.42

The CD20+ cells present in some CD34-enriched fractions were found to be of a relatively mature B-cell phenotype as judged by expression of surface Ig.

Expression of CD20 was rapidly lost in culture in the presence of hIL-3. In addition, no proliferation of CD3+ cells was detected in cultures of CD3-depleted cord blood cells. These findings indicate that hIL-3 is not mitogenic for cord blood lymphoid cells expressing these antigens.

Although it has been demonstrated43,44 that a proportion of CD34+ cells express CD10, we did not detect any CD10+ cells upon culturing CD34-enriched fractions in the presence of hIL-3.

Biologic activities of hIL-3 appear largely similar to those described for mIL-3.2 It may be noted, however, that we did not observe induction of basophils even after prolonged culture of CD34-enriched cells in the presence of hIL-3. Whether this reflects a divergence in the biologic activities of hIL-3 and mIL-3 or is due to lack of the relevant target cells after CD34 enrichment is currently under investigation.

When compared with CD34-enriched populations, CD34-depleted cell fractions from bone marrow and cord blood proliferated only marginally in response to hIL-3. It is possible that responsiveness of CD34-depleted fractions is related to low concentrations of residual CD34+ cells. On the other hand, such populations may contain cells responsive to hIL-3 that do not express detectable CD34 antigen. This notion is supported by our observations that in cultures of CD34-enriched cord blood cells hIL-3 induced the appearance of myeloid precursor cells that lacked detectable CD34 antigen expression but continued to proliferate in the presence of hIL-3.

hIL-3 was also capable of inducing growth in liquid culture of cells obtained from diagnostic specimens of acute myeloblastic leukemia, which is in line with observations of Delwel et al who described the effects of hMulti-CSF on leukemic progenitor cells.39 However, induction of proliferation of myeloid leukemia cells is not a specific property of hIL-3 since comparable data have previously been described
for other hematopoietic growth factors. We further observed a heterogeneity with respect to responsiveness of the leukemic cells to hIL-3, as has also been demonstrated for GM-CSF and G-CSF. Responsiveness to hIL-3 could not be associated with the degree of spontaneous proliferation in the absence of exogenous growth factor, but a partial correlation emerged between FAB classification and induction of proliferation by hIL-3. Thus, a high percentage of M1 specimens was shown to proliferate in response to hIL-3, whereas M4 cases were not significantly responsive. Recently, an extended survey has confirmed this trend and indicated responsiveness to hIL-3 in approximately 90% of the M1 cases tested (Mannoni et al, unpublished, 1988).

Among the M2 and M5 specimens tested in the present study, we found a heterogeneity with respect to the proliferative response to hIL-3. The finding that some M5 cases were responsive indicated that in the leukemic state growth-promoting effects of hIL-3 are not restricted to the most immature cells. Our data further demonstrated that the observed heterogeneity of responsiveness of myeloblastic leukemia cells to hIL-3 could not be related to CD34 antigen expression since CD34-negative cells from several specimens (patients 4, 17, and 19) were found to proliferate in response to hIL-3. The kinetics of the latter responses exclude that the observed $^3$H-TdR incorporation could be due to the selective outgrowth of CD34+ cells possibly present in low numbers within these populations. Furthermore, our results demonstrated the existence of myeloblastic leukemia specimens that express CD34 antigen on a high proportion of cells yet are unresponsive to hIL-3. This implies that the presence of the CD34 membrane structure is an insufficient condition to determine growth responsiveness to hIL-3 in myeloblastic leukemia. In this context, it is not known whether expression of CD34 antigen by leukemic cells parallels that of normal hematopoietic progenitors.

The definite characterization of the target cells for hIL-3 in relation to expression of the 115-kd molecule defined by CD34 will be greatly facilitated by detection of the receptor for hIL-3.

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