Recombinant Gibbon Interleukin 3 Supports Formation of Human Multilineage Colonies and Blast Cell Colonies in Culture: Comparison With Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor

By Anne G. Leary, Yu-Chung Yang, Steven C. Clark, Judith C. Gasson, David W. Golde, and Makio Ogawa

The genetic sequences encoding the gibbon and human interleukin 3 (IL 3) proteins were molecularly cloned. The amino acid sequence of the mature gibbon IL 3 protein proved to share 93% homology with the corresponding human protein. We examined the effects of biosynthetic (recombinant) gibbon IL 3 on the proliferation and differentiation of an enriched population of human hematopoietic progenitors and compared the results with the effects of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF). Gibbon IL 3 supported the formation of various types of single lineage as well as multilineage colonies by My-10 bone marrow cells in the presence of human erythropoietin (Ep). In contrast, recombinant human GM-CSF supported the formation of single-lineage colonies and only a small number of multilineage colonies. Both IL 3 and GM-CSF had significant erythroid burst-promoting activity (BPA). Delayed addition of gibbon IL 3 to low serum culture of My-10 marrow cells supported the formation of blast cell colonies with variable but high replating capability. Human GM-CSF was less effective than IL 3 in support of multipotential blast cell colonies. These results are analogous to the effects of murine IL 3 and GM-CSF on murine progenitors and support the notion that the primary factor for multipotential progenitors is IL 3.

\[ \text{Equation} \]

**HEMATOPOIETIC CELL** proliferation and differentiation in semisolid culture is supported by protein/glycoprotein hormones called colony-stimulating factors (CSFs). CSFs are produced by a variety of tissues and include CSF-1, a macrophage CSF (GM-CSF), granulocyte-macrophage CSF (GM-CSF), granulocyte CSF (G-CSF) and interleukin 3 (IL 3). These CSFs have been purified, and the genes encoding the proteins have been molecularly cloned. Whereas CSF-1 and G-CSF act primarily on mononuclear progenitors, murine IL 3 and GM-CSF have been reported to support proliferation of multipotential progenitors. Murine IL 3 was purified by Ihle et al in 1982 and subsequently was shown to support formation of multilineage colonies and blast cell colonies in culture. Recently, the genes for gibbon and human IL 3 were identified using expression cloning techniques, and the gibbon IL 3 has been expressed in Cos-1 monkey cells. Comparison of the amino acid sequences of the human and gibbon proteins revealed that they are 93% homologous. This extensive homology suggests that gibbon and human IL 3 may be functionally similar.

Using granulocyte-macrophage colony formation in agar as an assay, murine GM-CSF was initially purified from medium conditioned by mouse lung tissue. Subsequent studies carried out by Metcalf et al and in our laboratory suggested that it is capable of supporting proliferation of multipotential progenitors. Emerson et al reported that human GM-CSF supports proliferation of multipotential progenitors. In this report, we examined the effects of gibbon recombinant IL 3 on hematopoietic colony formation by highly enriched human bone marrow cells and compared the results with the effects of human recombinant GM-CSF in culture.

**MATERIALS AND METHODS**

Recombinant factors. The recombinant gibbon IL 3 used in this study was obtained by transfecting monkey Cos-1 cells with gibbon IL 3 cDNA clone, pCSF-MLA. Five micrograms of pCSF-MLA DNA was used to transfec a 100-mm dish of Cos-1 cells by diethylaminoethanol (DEAE) Dextran-mediated DNA transfection with the addition of 0.1 mmol/L chloroquine. Conditioned media (CM) from transfected Cos-1 cells were harvested 72 hours after transfection. The concentration of IL 3 was estimated to be 100 nmol/L according to the bioassay developed by Griffin et al.

Human GM-CSF was purified as previously described from serum-free medium conditioned by monkey Cos-1 cells transfected with human GM-CSF cDNA clone, p91203(B). The purified GM-CSF appeared as a single protein band of 22,000 mol wt on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the same mol wt as the purified natural GM-CSF. Protein concentration was estimated by amino acid analysis.

**Methods for colony assay.** Bone marrow cells were aspirated from the posterior iliac crest of healthy adult volunteers and placed in 16-mL Falcon tissue culture tubes containing 400 U of preservative-free heparin (Invenex Laboratories, Melrose Park, IL). The donors were advised of the risks of marrow aspiration and gave informed consent. These studies were approved by the Institutional Review Board for Human Research. Mononuclear cells were isolated by use of Ficoll-Paque (Pharmacia, Piscataway, NJ), and the nonadherent fraction was separated by overnight adherence to plastic. Samples were further enriched for colony-forming cells by positive indirect immune adherence using human progenitor cell antibody (HPCA) (Becton Dickinson, Mountain View, CA) which is the commercial preparation of monoclonal anti-My-10 antibody, and the “panning” technique of Engleman et al. Details of this procedure have been reported previously.

Panned My-10 bone marrow cells were cultured in 35-mm Lux suspension culture dishes (Miles Laboratories, Naperville, IL) using a modification of the technique of Iscove et al. One milliliter of...
culture consisted of 2,000 My-10 bone marrow cells, α-medium
(Flow Laboratories, Rockville, MD), 1.2% of 1,500 centipoise
methylcellulose (Fisher Scientific, Norcross, GA), 30% fetal calf
serum (FCS) (Flow Laboratories), 1% deionized bovine serum albumin (BSA) (Sigma Chemical, St Louis), 5 x 10^-5 mol/L
mercaptoethanol, 2 U/mL human urine erythropoietin (Ep) with
a specific activity of 370 U/mg protein (kindly provided by Dr
Makoto Kawakita, Kumamoto University School of Medicine,
Japan) and variable concentrations of IL 3 and GM-CSF. Dishes
were incubated at 37°C in a humidified atmosphere of 5% CO2 in
air. On days 14 through 16 of incubation, the types and sizes of
the colonies were estimated in situ. Abbreviations for colony types were:
GM, granulocyte and/or macrophage colonies; Meg, megakaryo-
cyte colonies; Eo/Baso, eosinophil and/or basophil colonies; E,
erthyroid bursts; and E-mix, mixed colonies containing erythroid
cells. When cytologic confirmation was required, colonies were
individually lifted from methylcellulose by an Eppendorf micropipette
and slides were prepared using a cytospin centrifuge (Shandon
Southern Instruments, Sewickley, PA). Slides were stained with
May-Grunwald-Giemsa (Gallard Schlesinger Chemical, Carle
Placc, NY) for differential counting.

Blast cell colony formation. The factors were also tested for
their capacity to support blast cell colony formation using a tech-
nique we developed recently. In brief, 1 x 10^6 panned My-10 bone
marrow cells were plated in methylcellulose culture containing:
α-medium; 2% FCS; 1% crystallized, deionized BSA; 5 x 10^-5
mol/L mercaptoethanol; and 300 μg/mL fully iron saturated human
transferrin (98% pure) 160 μg/mL soybean lecithin 96 g/mL
cholesterol, and 10^-7 mol/L sodium selenite (Sigma). On day 14 of
incubation, 0.1 mL of a 1:50 dilution of IL 3 or 10 or 1 nmol/L
GM-CSF was layered over the culture, and the dishes were exam-
ined daily for the appearance of blast cell colonies between days 21
and 32.

When identified, blast cell colonies were replated in culture for
confirmation of their ability to form secondary colonies. For these
experiments, blast cell colonies were individually lifted from the
culture and suspended in 100 μL α-medium. The samples were
gently pipetted and then added to 0.9 mL methylcellulose medium
containing 30% FCS, 1% deionized BSA, 2 U/mL Ep, and 30% C5MJ CM. Cultures were incubated at 37°C and 5% CO2 in air.
When colonies appeared to have matured (generally after 12 to 16
days of culture), individual colonies were prepared for differential
counting as described above.

RESULTS

Colony formation supported by IL 3 and/or GM-CSF. First, we carried out titration of IL 3 for colony formation in culture. Two thousand My-10 marrow cells were plated in cultures containing different dilutions of IL 3 preparations and 2 U/mL Ep. As controls, cultures were also prepared with conditioned medium from nontransfected Cos-1 cells (mock) plus Ep, C5MJ CM plus Ep, or Ep alone. C5MJ supports a wide variety of colony types, including mixed colonies. The results are presented in Table 1. IL 3 preparations ranging from 1:50 dilutions to 1:5,000 dilutions supported formation of various types of single lineage as well as multilineage colonies. Although the number of colonies supported by three different dilutions of IL 3 were similar, the colonies were smaller in the 1:5,000 dilution of IL 3 than those grown in higher concentrations of IL 3. Most notably, the largest colonies in all categories were smaller in 1:5,000 dilutions than in 1:500 dilutions. Based on these observations, the 1:500 dilution was chosen as the standard concentra-
tion. The concentration of IL 3 in this dilution was estimated to be 200 pmol/L (described in the Materials and Methods section). The mock CM plus Ep or Ep alone supported only occasional GM colonies or clusters in the My-10 fraction of cells.

In the next experiment, My-10 marrow cells were plated in quadruplicate cultures in the presence of Ep, GM-CSF, and/or IL 3. The results are presented in Table 2. To confirm that 100 pmol/L GM-CSF is the optimal concentration for colony formation, marrow cells were cultured in five different concentrations of GM-CSF. As has been shown by Tomonaga et al, this concentration of GM-CSF was the optimal concentration for colony formation. A 1:500 dilution (~200 pmol/mL) of IL 3 and Ep supported formation of various types of colonies, including E-mix colonies. A representa-
tive mixed colony containing macrophages, eosinophils, and erythrocytes is shown in Fig 1. In contrast, GM-CSF rarely supported formation of E-mix colonies. As demonstrated by a number of investigators, GM-CSF in all concentrations augmented formation of erythroid bursts in culture. Mock CM and Ep failed to support formation of erythroid bursts.

Cytologic analysis of lineage expression. For determina-
tion of the precise lineages expressed in the colonies in culture, 2,000 My-10 marrow cells were plated in each dish with IL 3 plus Ep or GM-CSF plus Ep, and on days 14 through 16 of incubation, colonies in each group were individually analyzed for their cellular composition. The results are presented in Table 3. In two separate experiments, most colonies supported by GM-CSF were single-lineage colonies. A 100-fold increase in the concentration of GM-

### Table 1. Colony Formation From MY-10 marrow Cells Supported by IL 3 and Ep

<table>
<thead>
<tr>
<th>Factors</th>
<th>GM</th>
<th>EO/BASO</th>
<th>E</th>
<th>E-MIX</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5MJ CM</td>
<td>14</td>
<td>4</td>
<td>13</td>
<td>5</td>
<td>36</td>
</tr>
<tr>
<td>(50-700)*</td>
<td>(1,000-2,000)</td>
<td>(100-4,000)</td>
<td>(500-3,000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL 3 (1:50)</td>
<td>22</td>
<td>5</td>
<td>13</td>
<td>4</td>
<td>44</td>
</tr>
<tr>
<td>(50-700)</td>
<td>(100-1,000)</td>
<td>(200-2,500)</td>
<td>(1000-2,000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL 3 (1:500)</td>
<td>23</td>
<td>6</td>
<td>11</td>
<td>4</td>
<td>44</td>
</tr>
<tr>
<td>(50-2,000)</td>
<td>(300-3,000)</td>
<td>(200-6,000)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL 3 (1:5,000)</td>
<td>26</td>
<td>13</td>
<td>4</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>(30-300)</td>
<td>(100-1,000)</td>
<td>(300-1,000)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Average of colony numbers in duplicate cultures. Concentrations of IL 3 ranged from 1:50 to 1:5,000 dilutions, and Ep was constant at 2 U/mL.

*Numbers in brackets represent range of estimated size of colonies.
CSF showed no difference in the number and types of colonies. IL 3 plus EP supported formation of many types of multilineage colonies, including pentalineage colonies. In contrast, only a few mixed colonies were supported by GM-CSF plus Ep.

**Blast cell colony formation.** In 8 experiments using 8 separate marrow samples, 67 blast cell colonies were identified in 50 culture dishes receiving delayed addition of IL 3. Thirty-one of 48 replated blast cell colonies yielded secondary colonies in the presence of C5MJ CM plus Ep. The size of blast cell colonies ranged from 21 cells to 41 cells/colony, and the percentage of replating efficiencies of the positive colonies ranged from 11% to 65%. As shown in Table 4, secondary colonies included single-lineage colonies such as neutrophil (n), macrophage (m), eosinophil (e), basophil (b), or erythrocyte (E) colonies as well as many bilineage and trilineage colonies. Occasional tetralineage secondary colonies revealed all four leukocyte lineages. Two concentrations of GM-CSF were tested for their ability to support blast cell colony formation. When IL 3 blast cell colonies were replated in C5MJ CM plus Ep, only two colonies produced secondary colonies revealing n, m, or nm lineages. When a tenfold higher concentration of GM-CSF was used, four of ten blast cell colonies produced secondary colonies, including eosinophil colonies. Most blast cell colonies supported by GM-CSF apparently do not possess significant proliferative capacity and probably consist of progenitors committed only to the n, m, and e pathways.

**DISCUSSION**

Despite extensive search by a number of investigators, the gene for human IL 3 eluded identification until recently. Using 3H-labeled thymidine uptake by blast cells from patients with chronic myelogenous leukemia as an assay in expression cloning, an IL 3 gene was identified from a cDNA library prepared from a gibbon lymphosarcoma cell line. This gibbon cDNA was used as a probe to identify and clone the gene for human IL 3. The nucleotide sequence of the coding region of gibbon IL 3 proved to share 96% homology with the human IL 3 sequence. From this observation it was deduced that the protein from the two species differ by 11 amino acids, 9 of which reside in the mature protein sequence. We found gibbon IL 3 to be active on purified human progenitors. The results presented in this article suggest that the function of gibbon IL 3 will be similar to human IL 3 since in the presence of Ep gibbon IL 3 supported formation of all types of single-lineage colonies, multilineage colonies, and multipotential blast cell colonies. A controversy exists regarding the targets of murine and human GM-CSF. Murine GM-CSF was originally consid-
Table 3. Cytologic Analysis of Lineages Expressed by Colonies Supported by IL 3 Plus Ep or GM-CSF Plus Ep

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lines</td>
<td>GM-CSF</td>
</tr>
<tr>
<td></td>
<td>1 nmol/L</td>
</tr>
<tr>
<td>+</td>
<td>18 16 18</td>
</tr>
<tr>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>+</td>
<td>2 2 2</td>
</tr>
<tr>
<td>+</td>
<td>4 5 5</td>
</tr>
<tr>
<td>+</td>
<td>2 3 2</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

Abbreviation of lineages are as recommended by a UCLA Workshop (30).

All colonies in a single dish containing 2,000 My-10 cells were individually analyzed.

Table 4. Summary of Blast Cell Colony Formation by IL 3 or GM-CSF

<table>
<thead>
<tr>
<th>Factor</th>
<th>Incidence of Blast Cell Colonies</th>
<th>Positive Colonies in CSMJ</th>
<th>Plating Efficiency</th>
<th>Lineages in Secondary Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL 3 *</td>
<td>67 colonies/51 dishes</td>
<td>31/48</td>
<td>11%-65%</td>
<td>n, m, e, b, E, nm, me</td>
</tr>
<tr>
<td>GM-CSF (100 pmol/L)</td>
<td>19 colonies/40 dishes</td>
<td>2/13</td>
<td>14%-20%</td>
<td>n, m, nm</td>
</tr>
<tr>
<td>GM-CSF (1 nmol/L)</td>
<td>10 colonies/20 dishes</td>
<td>4/10</td>
<td>18%-63%</td>
<td>n, m, e, nm</td>
</tr>
</tbody>
</table>

*Concentration of IL 3 in each dish was a 1:500 dilution, which is ~200 pmol/L.
than human GM-CSF in supporting the proliferation of multipotential progenitors. Whereas a number of multipotential blast cell colonies were identified in cultures receiving delayed addition of IL 3, fewer blast cell colonies were supported by GM-CSF and yielded secondary colonies. The secondary colonies were restricted to neutrophil, macrophage, and eosinophil lineages. These observations are analogous to the reported results of murine factors on multipotential progenitors.10 Our previous data suggested that the progenitors for murine blast cell colonies responding to murine GM-CSF represent only a subpopulation of those responding to murine IL 3.10 Human multipotential progenitors that respond to GM-CSF may also represent a small subpopulation of multipotential progenitors sensitive to IL 3.

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