In Vivo Control of Differentiation of Myeloid Leukemic Cells by Recombinant Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin 3

By Joseph Lotem and Leo Sachs

The normal myeloid hematopoietic regulatory proteins include one class of proteins that induces viability and multiplication of normal myeloid precursor cells to form colonies (colony-stimulating factors [CSF]) and interleukin 3 [IL-3], macrophage and granulocyte inducing proteins, type 7 (MGI-1) and another class (called MGI-2) that induces differentiation of normal myeloid precursors without inducing cell multiplication. Different clones of myeloid leukemic cells can differ in their response to these regulatory proteins. One type of leukemic clone can be differentiated in vitro to mature cells by incubating with the growth-inducing proteins granulocyte-macrophage (GM) CSF or IL-3, and another type of clone can be differentiated in vitro to mature cells by the differentiation-inducing protein MGI-2. We have now studied the ability of different myeloid regulatory proteins to induce the in vivo differentiation of these different types of mouse myeloid leukemic clones in normal and cyclophosphamide-treated mice. The results show that in both types of mice (a) the in vitro GM-CSF- and IL-3-sensitive leukemic cells were induced to differentiate to mature cells in vivo in mice injected with pure recombinant GM-CSF and IL-3 but not with G-CSF, M-CSF, or MGI-2; (b) the in vitro MGI-2-sensitive leukemic cells differentiated in vivo by injection of MGI-2 and also, presumably indirectly, by GM-CSF and IL-3 but not by M-CSF or G-CSF; (c) in vivo induced differentiation of the leukemic cells was associated with a 20- to 60-fold decrease in the number of blast cells; and (d) all the injected myeloid regulatory proteins stimulated the normal myelopoietic system. Different normal myeloid regulatory proteins can thus induce in vivo terminal differentiation of leukemic cells, and it is suggested that these proteins can have a therapeutic potential for myeloid leukemia in addition to their therapeutic potential in stimulating normal hematopoiesis.

Material and Methods

Cells and cell culture in vitro. Three growth factor-independent clones of mouse myeloid leukemic cells were used in the present experiments. Clones 3 and 11 originated from a spontaneous myeloid leukemia in an SL mouse, and clone 7-M12 was from a myeloid leukemia obtained after x-irradiation in a SJL/J mouse.

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Clone 3 and 11 cells are inducible to differentiate to mature cells in vitro by MGI-2 and to immature cells by G-CSF but are not differentiated in vitro by GM-CSF, IL-3, or M-CSF. Clone 7-M12 cells are differentiated in vitro only by GM-CSF or IL-3. The leukemic cells were cultured at 37°C in Dulbecco's modified Eagle's medium (EM; H-21, Gibco, Grand Island, NY) and 10% heat-inactivated (56°C, 30 minutes) horse serum (GIBCO). They multiplied in culture in suspension as myeloblasts to promyelocytes with population doubling times of 20 and 16 hours for clones 3 and 7-M12, respectively. Normal bone marrow cells for assay of CSF or IL-3 (– MGI-1) were obtained from the femurs of 2- to 3-month-old normal SJL/J mice.

Myeloid cell growth and differentiation-inducing proteins. Myeloid differentiation-inducing protein (MGI-2) devoid of any growth inducing (MGI-1 = CSF or IL-3) activity was prepared from conditioned medium of Krebs' II ascites tumor cells. Twenty milliliters of 100-fold-concentrated, serum-free Knebs' tumor cell conditioned medium was dialyzed against Tnis-HCl (10 mmol/L, equilibrated in the same buffer. The unbound material contained va; mouse M-CSF was from the conditioned medium of L929 experiments and showed a specific activity of 300 milliliters of 100-fold-concentrated, serum-free Knebs' tumor cell conditioned medium of Krebs' II ascites tumor cells. Twenty five milliliters of 100-fold-concentrated, serum-free Krebs' II ascites tumor cell conditioned medium was dialyzed against Tris-HCl (10 mmol/L, pH 7.0) and loaded on a 1.6 x 10-cm diethyl aminoethyl (DEAE)-Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden) column equilibrated in the same buffer. The unbound material contained only MGI-2 activity active on myeloid leukemia clone 3 and 11 cells and did not contain any CSF or IL-3 activity. This material is called MGI-2B to distinguish it from the MGI-2 activity that binds to DEAE-Sephacel at pH 7 and is eluted by a NaCl gradient at a concentration of 0.05 mol/L NaCl. This MGI-2 activity, called MGI-2A, was separated from residual GM-CSF activity by using a phenyl-Sepharose column (1.6 x 10 cm) to which GM-CSF did not bind, and MGI-2A was eluted by a gradient of 1 mol/L ammonium sulfate to water (Shabo, Lotem, and Sachs, to be published). Both MGI-2B and MGI-2A were used in the present experiments and showed a specific activity of 300 and 600 U/mg protein, respectively.

Pure recombinant human G-CSF13 was kindly provided by Dr Lawrence Souza of Amgen, Thousand Oaks, CA. This human G-CSF is active on human as well as on mouse myeloid cells. Pure recombinant mouse GM-CSF28 was kindly provided by Drs Ken-Ichi Arai and Atsuchi Miyajima of the DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA, and pure recombinant mouse IL-328 was kindly provided by Dr Pierre Vassali of the Department of Pathology, Faculty of Medicine, University of Geneva; mouse M-CSF was from the conditioned medium of L929 fibroblasts. The amount of lipopolysaccharide (LPS) in these CSF, IL-3, and MGI-2 preparations was assayed with the limulus amebocyte lysate (Sigma Chemical Co, St Louis) gelation test.24 Assays for growth-inducing (MGI-1 = CSF or IL-3) and differentiation-inducing (MGI-2) activity. Growth-inducing activity (CSF or IL-3) was assayed in 35-mm Petri dishes by seeding 5 x 10⁴ normal nucleated bone marrow cells in 0.8 mL of 0.33% agar on top of a 2.5-mL harder agar base (0.5%) that contained different numbers of the material to be assayed. Both layers contained EM without serum, the chambers filled with 0.2 mL cell suspension containing 3 x 10⁴ clone 3 or 1 x 10⁴ clone 7-M12 leukemic cells (due to different growth rates) and implanted into the peritoneal cavity of 3-month-old normal C3H/HeJ mice that are resistant to bacterial LPS or into CD-1 mice that were injected 24 hours earlier with 200 mg/kg cyclophosphamide (ICN Pharmaceuticals, Inc, Plainview, NY). Two chambers were implanted, and at least three experiments were carried out for each point. Chambers were removed for analysis of their cellular content after ten days as described.26 Cell types were determined on May-Grünwald-Giemsa-stained smears by counting at least 300 cells. Differentiated cells were scored as intermediate stages of differentiation (myelocytes, metamyelocytes, and monocyte-like cells) and mature cells (mature granulocytes and macrophages). The reproducibility of the number of cells per chamber and the percentage of different cell types in different experiments was up to ±25% of the mean values.

Effect of injected myeloid regulatory proteins on normal myelo-poiesis in the spleen and peritoneal cell content. The different proteins were injected in a volume of 0.2 mL (diluted in phosphate-buffered saline [PBS]) into the peritoneal cavity of CD-1 or C3H/HeJ mice implanted with diffusion chambers or into normal C3H/HeJ mice. Generally, four injections (one injection per day) were carried out on days 4, 6, 7, and 8 after implantation of chambers, and the spleens and peritoneal cells were analyzed two days after the last injection (day 10). Control mice were injected with 0.2 mL PBS at the aforementioned schedule. The spleens were removed and cut into small pieces and their cells teased out. The total number of cells per spleen was determined, and 2 x 10⁶ cells were seeded in agar containing 5 ng/mL recombinant GM-CSF to determine the number of colony-forming cells per spleen. The peritoneal cells from the same mice were collected by flushing the peritoneal cavity with 5 mL EM containing 10% horse serum and 6 U/mL heparin (Novo, Copenhagen). The total number of peritoneal cells was determined and the different cell types analyzed on May-Grünwald-Giemsa-stained smears.

RESULTS

In vitro differentiation of myeloid leukemic cells by different myeloid regulatory proteins. The in vitro induction of differentiation on clone 3 and 7-M12 myeloid leukemic cells was determined by incubating the cells with pure recombinant proteins GM-CSF, G-CSF, or IL-3 and also natural M-CSF or MGI-2. The synthesis of lysozyme and morphological maturation were determined after four and ten days, respectively. The results (Table I) indicate that clone 3 can be induced to differentiate in vitro to mature cells by MGI-2A or MGI-2B and partially (up to 10% of the cells in intermediate stages of differentiation) by G-CSF. This clone did not show any differentiation with GM-CSF, IL-3, or M-CSF. In contrast, clone 7-M12 cells differentiated in vitro with GM-CSF and IL-3 (Table I).

Contamination with bacterial LPS can interfere with interpretation of the experiments in vitro and in vivo. Both clones 3 and 7-M12 did not show a significant degree of in vitro differentiation with high concentrations (1 to 10 μg/mL) of LPS (Table I). Results in vitro with another clone of myeloid leukemic cells (clone 11) that can be differentiated to mature cells by MGI-2A and MGI-2B and to intermediate stages by G-CSF and that is very sensitive to induction of
IN VIVO DIFFERENTIATION IN MYELOID LEUKEMIA

**Table 1. In Vitro Differentiation of Myeloid Leukemic Cells by Myeloid Regulatory Proteins**

<table>
<thead>
<tr>
<th>Clone No.</th>
<th>Material Added</th>
<th>Concentration (per mL)</th>
<th>Lysozyme (µg equivalent/5 x 10⁶ Cells)</th>
<th>Differentiation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intermediate Stages</td>
<td>Cell Types (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Granulocytes</td>
<td>Macrophages</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>—</td>
<td>0.2 ± 0.1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td>r GM-CSF</td>
<td>2-200 ng</td>
<td>0.2 ± 0.1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td>r IL-3</td>
<td>2-200 ng</td>
<td>0.2 ± 0.1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td>r G-CSF</td>
<td>20 ng</td>
<td>0.2 ± 0.1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td></td>
<td>M-CSF</td>
<td>20-400 U</td>
<td>0.2 ± 0.1</td>
<td>10 ± 2</td>
</tr>
<tr>
<td></td>
<td>MGI-2A</td>
<td>1 U</td>
<td>2.5 ± 0.8</td>
<td>28 ± 5</td>
</tr>
<tr>
<td></td>
<td>MGI-2B</td>
<td>1 U</td>
<td>2.2 ± 0.6</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>7-M12</td>
<td>None</td>
<td>—</td>
<td>0.1 ± 0.1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td></td>
<td>r GM-CSF</td>
<td>2 ng</td>
<td>6.8 ± 3.2</td>
<td>57 ± 5</td>
</tr>
<tr>
<td></td>
<td>r IL-3</td>
<td>5 ng</td>
<td>17.6 ± 6.0</td>
<td>50 ± 8</td>
</tr>
<tr>
<td></td>
<td>r G-CSF</td>
<td>20 ng</td>
<td>24.5 ± 6.2</td>
<td>42 ± 10</td>
</tr>
<tr>
<td></td>
<td>M-CSF</td>
<td>20-400 U</td>
<td>6.5 ± 2.1</td>
<td>39 ± 4</td>
</tr>
<tr>
<td></td>
<td>MGI-2A</td>
<td>1 U</td>
<td>18.6 ± 3.6</td>
<td>41 ± 11</td>
</tr>
<tr>
<td></td>
<td>MGI-2B</td>
<td>1 U</td>
<td>0.1 ± 0.1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>1-10 µg</td>
<td>0.6 ± 0.1</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

*Cells were assayed for lysozyme after four days and for analysis of morphological differentiation after ten days as described in Materials and Methods. CSF or IL-3 = MGI-1.
Abbreviation: r, recombinant; U, units.

**Discussion**

Differentiation with LPS (sensitive to as little as 5 ng/mL) also did not show any differentiation with 200 ng/mL GM-CSF or IL-3 or with M-CSF. In addition, induction of differentiation in clone 11 by a high concentration of LPS (200 ng/mL) is completely blocked by adding 5 µg/mL polymyxin B, but this concentration of polymyxin B did not show any inhibition of the differentiation-associated properties induced in clones 3 and 11 by G-CSF, MGI-2A, and MGI-2B or in clone 7-M12 cells by GM-CSF or IL-3. In the absence of LPS, the amounts of LPS detected in the stock solutions of the proteins were less than 1 ng/mL with the recombinant proteins GM-CSF, IL-3, G-CSF, and our preparation of M-CSF and 10 ng/mL in the MGI-2A and MGI-2B preparations. These results indicate that the LPS content of the materials used was low and cannot account for the induction of differentiation by the myeloid regulatory proteins on clones 3 and 7-M12 in vitro. To avoid any possible effects of even these small amounts of LPS in vivo, however, the following in vivo experiments were carried out both in CD-1 mice and in LPS-resistant C3H/HeJ mice, and the amount of LPS in the materials injected was never higher than 2 ng per injection. Injections of LPS in vivo were also carried out to determine its effect on the in vivo differentiation of the leukemic cells.

**In vivo differentiation of myeloid leukemia cells in response to injected myeloid regulatory proteins.** We have previously shown that both clone 3 and 7-M12 cells differentiated in vivo to mature cells in normal mice in the presence of 10% horse serum or even a single antigen such as ovalbumin in the diffusion chambers. This in vivo differentiation was inhibited in both clones when the cells were inoculated into chambers in the absence of antigen (only in EM) or in cyclophosphamide-treated mice. The effect of the different injected materials was, therefore, tested under conditions where the basal level of differentiation was low, namely, in cyclophosphamide-treated CD-1 mice or in LPS-resistant C3H/HeJ mice without including antigen in the chambers. The following experiments gave similar results in both these strains of mice.

The results with clone 7-M12, which was inducible in vitro by GM-CSF and IL-3 (Table 1), indicate that four intraperitoneal injections of 1 µg pure recombinant GM-CSF (45 µg/kg/d) on days 4, 6, 7, and 8 after implantation of the chambers (a total of 4 µg per mouse) induced the in vivo differentiation of these leukemic cells to mature macrophages (Fig 1A and C, Table 2). Even a tenfold lower amount of GM-CSF showed a significant increase in the in vivo differentiation of clone 7-M12 (Fig 2), and similar results were also obtained with recombinant IL-3 (Fig 2, Table 2). These results show that clone 7-M12 leukemic cells are differentiated by GM-CSF and IL-3 both in vitro and in vivo. Injection of 1 µg IL-3 only once (on day 4) or twice (days 4 and 6) was less efficient than four injections of IL-3 (Fig 3). Four injections with 0.5 µg IL-3 per injection (Fig 2) gave a better differentiation than the same cumulative dose given in two injections of 1 µg per injection (Fig 3). This
Table 2. In Vivo Differentiation of Myeloid Leukemic Cells by Myeloid Regulatory Proteins

| Material Injected | No. of Cells per Chamber (x 10^4) | Cell Types (%) | | No. of Cells per Chamber (x 10^4) | Cell Types (%) |
|-------------------|-----------------------------------|----------------|---------------------------------|----------------|
| PBS               | 69.3 ± 8.1 | 15 ± 4 | 0 | 33.9 ± 8.1 | 19 ± 4 | 1 ± 1 |
| rGM-CSF (1 μg)    | 16.1 ± 2.1 | 70 ± 5 | 10 ± 2 | 1 ± 1 | 4.1 ± 1.3 | 61 ± 8 | 29 ± 7 |
| rIL-3 (1 μg)      | 70.6 ± 8.3 | 22 ± 5 | 2 ± 1 | 0 | 15.4 ± 6.1 | 67 ± 4 | 18 ± 4 |
| (5 μg)            | 14.5 ± 4.1 | 70 ± 6 | 11 ± 3 | 1 ± 1 | NT | NT | NT |
| rG-CSF (5 μg)     | 53.8 ± 8.6 | 19 ± 3 | 1 ± 1 | 0 | 38.5 ± 7.2 | 18 ± 1 | 1 ± 1 |
| M-CSF (10^4 U)    | 56.8 ± 9.5 | 18 ± 4 | 0 | 0 | 31.0 ± 4.5 | 24 ± 3 | 0 |
| MGI-2A (200 U)    | 17.5 ± 4.1 | 64 ± 4 | 14 ± 2 | 1 ± 1 | 39.5 ± 3.6 | 17 ± 3 | 0 |
| MGI-2B (200 U)    | 14.3 ± 3.2 | 68 ± 6 | 16 ± 3 | 2 ± 1 | 35.0 ± 5.4 | 21 ± 1 | 1 ± 1 |
| Ovalbumin (1 μg)  | 56.2 ± 6.5 | 21 ± 2 | 0 | 0 | 32.5 ± 3.6 | 16 ± 2 | 0 |
| (5 μg)            | 50.6 ± 4.8 | 23 ± 4 | 0 | 0 | 30.4 ± 4.8 | 18 ± 1 | 0 |
| LPS (0.1 μg)      | 55.5 ± 6.1 | 12 ± 3 | 1 ± 1 | 0 | 35.6 ± 3.5 | 22 ± 3 | 0 |
| (1 μg)            | 31.6 ± 4.5 | 19 ± 4 | 2 ± 1 | 0 | 26.9 ± 4.8 | 30 ± 5 | 1 ± 1 |

Clone 3 (3 x 10^6 cells) and clone 7-M12 (1 x 10^6 cells) in diffusion chambers containing EM were implanted into the peritoneal cavity of CD-1 mice pretreated with cyclophosphamide or into normal LPS-resistant C3H/HeJ mice as described in Materials and Methods. Results with both strains of mice were similar. Materials were injected intraperitoneally (one injection per day) in a volume of 0.2 mL (diluted with PBS) on days 4, 6, 7, and 8 after implantation of the chambers. CSF or IL-3 = MGI-1.

Abbreviation: NT, not tested; U, units.

indicates that the material injected has to be available to the leukemic cells in vivo for prolonged periods. In contrast to the effect of GM-CSF or IL-3, there was no induction of in vivo differentiation of clone 7-M12 leukemic cells in mice injected four times with 1 or even 5 μg pure recombinant G-CSF, with 10^6 units M-CSF, or with 200 units (0.33 to 0.66 mg protein) MGI-2A or MGI-2B (Table 2). These molecules also did not induce differentiation of clone 7-M12 cells in vitro (Table 1).

Similar experiments were carried out with leukemic clone 3, which differs from clone 7-M12 in its susceptibility to the induction of differentiation by the different myeloid regulatory proteins in vitro (Table 1). The results indicate that clone 3 cells that respond in vitro to MGI-2A or MGI-2B are also induced by these molecules to differentiate in vivo (Table 2). Clone 3 cells that did not differentiate in vitro with GM-CSF or IL-3 (Table 1), however, were induced to differentiate in vivo mainly to mature granulocytes by four injections of 1 μg GM-CSF (Fig 1B and D) or 5 μg IL-3 (Table 2). The results have also shown that four injections of LPS (0.1 to 1 μg per injection) into C3H/HeJ mice did not induce the in vivo differentiation of either clone 3 or 7-M12 leukemic cells (Table 2). This shows that the small amounts of LPS in the injected materials (less than 2 ng per injection) cannot explain the in vivo differentiation induced by the myeloid regulatory proteins injected. There was no differentiation in vivo by 1 to 5 μg G-CSF or by 10^6 units M-CSF (Table 2). These results indicate that the in vivo differentiation induced by GM-CSF or IL-3 on clone 3 leukemic cells may be induced indirectly, presumably by stimulating cells to produce MGI-2. In contrast to the results obtained after injection of GM-CSF, IL-3, and MGI-2, the injection of a
IN VIVO DIFFERENTIATION IN MYELOID LEUKEMIA

7) or four times (days 4, 6, 7, and 8). --.
11-3 once (day 4), twice (days 4 and 6), three times (days 4, 6, and 7) or four times with different amounts of recombinant murine 11-3 or GM-CSF (abscissa shows the cumulative dose) as in Fig 1 and cell types in the chambers analyzed ten days after implantation. ● O, blasts; O--O, intermediate stages; △–△, mature macrophages.

Mice with chambers containing clone 7-Mi 2 cells were injected with pure recombinant 11-3 and GM-CSF. The leukemic cells were inoculated into the diffusion chambers at a low cell number, 1 or 3 × 10⁶ cells per chamber with clones 7-M12 and 3, respectively. As previously shown in mice treated with cyclophosphamide, when differentiation was inhibited, the cell number in the chambers increased continuously so that the final cell number per chamber was 200- to 300-fold higher than the initial input (Table 2, Fig 4). Under conditions where there was strong in vivo differentiation of the leukemic cells, the cells multiplied much less than when the cells showed little in vivo differentiation. This reduction in the cell number per chamber was also now induced, in a dose-dependent manner, when GM-CSF or IL-3 were injected in vivo to cyclophosphamide-treated mice implanted with chambers containing clone 7-M12 (Fig 4, Table 2) or clone 3 (Table 2) cells. This shows that the degree of in vivo differentiation of the myeloid leukemic cells is inversely associated with the final number of cells in the chambers. The in vivo induction of differentiation of leukemic cells can thus reduce the blast cell tumor load, and in the present experiments this reduction of the number of blast cells was by a factor of about 20 to 60 (Table 2).

Stimulation in vivo of normal myelopoiesis in the spleen and increase in the number of peritoneal macrophages by myeloid regulatory proteins. Two aspects of enhanced in vivo myelopoiesis by the myeloid regulatory proteins are the induced increase in the number of granulocyte and macrophage colony-forming cells in the spleen and in the number of peritoneal cells, mainly macrophages. We have now tested the effect of the different myeloid regulatory proteins on the aforementioned properties in cyclophosphamide-treated CD-1 mice carrying chambers with leukemic cells and in normal C3H/HeJ LPS-resistant mice. The results show that in both strains of mice all the myeloid regulatory proteins injected increased two- to fourfold the total number of peritoneal cells, mainly peritoneal macrophages, and increased four- to 14-fold the number of colony-forming cells in the spleen of normal C3H/HeJ mice (Table 3). Injection of up to 100 ng LPS into normal CD-1 mice or 1 μg LPS into C3H/HeJ mice did not induce these changes in the peritoneal cavity or in the spleens of the injected mice (Table 3). The results also show that the stimulatory effects of G-CSF and M-CSF on normal myelopoiesis did not result in the induction of in vivo differentiation of either type of leukemic clone tested (clones 3 or 7-M12) and the stimulatory effect of MGI-2A or MGI-2B on normal myelopoiesis did not result in induction of in vivo differentiation of clone 7-M12 cells (Table 2). This shows that the hematopoietic stimulatory effect of the myeloid regulatory proteins on normal hematopoiesis (Table 3) was not necessarily associated with the in vivo differentiation of the different classes of myeloid leukemic cells (Table 2).

DISCUSSION

Induction of differentiation of myeloid leukemic cells can have important implications in the treatment of leukemia.

Fig 2. Dose dependence of in vivo induction of differentiation of myeloid leukemic cells by pure recombinant IL-3 and GM-CSF. Mice with chambers containing clone 7-M12 cells were injected four times with different amounts of recombinant murine IL-3 or GM-CSF (abscissa shows the cumulative dose) as in Fig 1 and cell types in the chambers analyzed ten days after implantation. ● O, blasts; O--O, intermediate stages; △–△, mature macrophages.

Fig 3. Effect of number of injections of IL-3 on in vivo differentiation of myeloid leukemic cells. Mice with chambers containing clone 7-M12 cells were injected with 1 μg recombinant IL-3 once (day 4), twice (days 4 and 6), three times (days 4, 6, and 7) or four times (days 4, 6, 7, and 8). ● O, blasts; O--O, intermediate stages; △–△, mature macrophages.

Fig 4. Effect of pure recombinant GM-CSF or IL-3 on the final number of cells in the chambers in vivo. Clone 7-M12 leukemic cells (1 × 10⁶) were cultured in vivo, and the mice were injected four times with different amounts of recombinant GM-CSF or IL-3. The final cell number per chamber was determined on day 10.
We have demonstrated the potential of this concept by showing that mouse myeloid leukemic cells induced to undergo cell differentiation in vitro by a normal protein inducer of differentiation lose their leukemogenic potential when inoculated into mice and that injection of such a protein can inhibit the development of leukemia. Furthermore, the suppression of malignancy due to differentiation of malignant cells in vivo has been demonstrated with mouse teratocarcinoma cells injected into blastocysts and with mouse myeloid leukemic cells injected into midgestation embryos. The in vivo differentiation of malignant cells has been shown with human leukemia in diffusion chambers in mice and in the body of patients with myeloid leukemia in active disease and during remission. These studies have demonstrated that a suitable in vivo environment can support the differentiation of malignant into non-malignant cells in the body. The use of nonphysiological in vitro active differentiation-inducing materials such as low-dose cytosine arabinoside to treat human patients has also shown that this treatment can suppress malignancy and that this can be associated with induction of cell differentiation in vivo.

Our present experiments were carried out to analyze the ability of the different normal hematopoietic regulatory proteins to induce the in vivo differentiation of two classes of myeloid leukemic cells that differ in their in vitro susceptibility to these proteins. The injection of differentiation-inducing protein MGI-2, with no MGI-1 (CSF or IL-3) activity, has shown that only the in vitro MGI-2-inducible type of leukemic cells responded to this protein in vivo. This indicates that MGI-2 may have induced the in vivo differentiation of these leukemic cells directly. The results with pure recombinant GM-CSF and IL-3 have shown that both of these proteins induced in vivo differentiation of the two types of leukemic cells, although only one type could be induced to differentiate in vitro by GM-CSF and IL-3. This indicates that GM-CSF and IL-3 induced in vivo differentiation in a type of leukemic cell that was not inducible by these proteins in vitro, presumably indirectly by stimulating cells to produce MGI-2. Such an indirect in vivo effect on cells not responding in vitro has also been obtained in some cells with LPS and with interferon or tumor necrosis factor. The results, therefore, indicate that GM-CSF and IL-3 can induce in vivo differentiation of leukemic cells that are susceptible or even resistant to induction of differentiation by these proteins in vitro. Neither pure recombinant G-CSF nor M-CSF showed in vivo induction of differentiation with these leukemic cells. It is, however, possible that a much higher concentration of G-CSF or M-CSF may be required than that required for the other proteins. G-CSF may also be able to induce in vivo differentiation in another class of myeloid leukemic cells that can be differentiated to mature cells by G-CSF in vitro. The ability of G-CSF and M-CSF to stimulate normal hematopoiesis but not in vivo differentiation of the leukemic cells tested in the present experiments indicates that the effect of different myeloid regulatory proteins on normal hematopoiesis was not necessarily associated with their ability to induce in vivo differentiation of myeloid leukemic cells.

The results of our experiments were obtained by using material that contained little LPS; similar results were obtained in cyclophosphamide-treated CD-1 mice and in LPS-resistant C3H/HeJ mice, and an excess of LPS by itself did not induce the in vivo differentiation of the leukemic cells used in these experiments. This shows that the differential effects on the leukemic cells in vivo were not due to a possible indirect effect of small amounts of contaminating LPS. There are myeloid leukemic cells from patients with myeloid leukemia that still require a CSF or IL-3 for cell growth, so injection of one of these proteins may possibly stimulate the growth of these leukemic cells. However, the myeloid growth factor-independent clones of myeloid leukemic cells used in the present experiments also again require a CSF or IL-3 for growth when they are induced to differentiate. Despite this requirement of a myeloid growth factor for growth, the further induction of differentiation can change the balance between multiplying cells and nonmultiplying differentiated cells sufficiently to inhibit the development of leukemia. In view of these results and the inverse relationship between in vivo GM-CSF- and IL-3-induced differen-

Table 3. Stimulation of Normal Myelopoiesis in the Spleen and Number of Peritoneal Macrophages by Injection of Myeloid Regulatory Proteins

<table>
<thead>
<tr>
<th>Material Injected</th>
<th>Total No. of Cells</th>
<th>Total No. of Macrophages</th>
<th>No. of Peritoneal Cells (× 10⁴)</th>
<th>Total No. of Cells</th>
<th>Total No. of Macrophages</th>
<th>No. of Peritoneal Cells (× 10⁴)</th>
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<tr>
<td></td>
<td>CD-1</td>
<td>C3H/HeJ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>5.1 ± 1.3</td>
<td>3.4 ± 0.6</td>
<td></td>
<td>3.4 ± 0.5</td>
<td>2.3 ± 0.3</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>r GM-CSF</td>
<td>19.7 ± 2.3</td>
<td>16.3 ± 1.2</td>
<td></td>
<td>6.8 ± 0.4</td>
<td>5.7 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>r IL-3</td>
<td>16.0 ± 3.5</td>
<td>16.1 ± 2.3</td>
<td></td>
<td>5.1 ± 0.4</td>
<td>3.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>r G-CSF</td>
<td>12.6 ± 1.8</td>
<td>9.6 ± 2.1</td>
<td></td>
<td>6.0 ± 1.3</td>
<td>4.1 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>M-CSF</td>
<td>15.4 ± 4.3</td>
<td>10.9 ± 3.1</td>
<td></td>
<td>13.0 ± 4.3</td>
<td>9.9 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>MGI-2A</td>
<td>10.8 ± 1.6</td>
<td>7.8 ± 1.7</td>
<td></td>
<td>9.0 ± 1.0</td>
<td>6.3 ± 0.9</td>
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</tr>
<tr>
<td>MGI-2B</td>
<td>12.3 ± 3.2</td>
<td>8.6 ± 1.1</td>
<td></td>
<td>9.7 ± 1.5</td>
<td>6.6 ± 1.1</td>
<td></td>
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<tr>
<td>LPS</td>
<td>4.8 ± 1.6</td>
<td>3.9 ± 1.0</td>
<td></td>
<td>4.2 ± 0.5</td>
<td>3.0 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

Materials were injected (1 µg of all recombinant proteins) into chamber-bearing, cyclophosphamide-pretreated CD-1 mice or into normal untreated, LPS-resistant C3H/HeJ mice. The myeloid regulatory proteins and PBS were injected four times as in Table 2, and peritoneal and spleen cells were collected and analyzed as described in Materials and Methods. LPS was injected four times into CD-1 mice (100 ng per injection) or C3H/HeJ mice (1 µg per injection). CSF or IL-3 = MGI-1.
tiation of the leukemic cells and the number of blast cells shown in the present experiments, it can be suggested that in addition to their stimulatory effect on normal hematopoiesis, GM-CSF, IL-3, and MGI-2 can have a therapeutic potential for the treatment of myeloid leukemia.

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In vivo control of differentiation of myeloid leukemic cells by recombinant granulocyte-macrophage colony-stimulating factor and interleukin 3

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