The antileukemic activity of murine recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) and a combination of rGM-CSF and recombinant interleukin-3 (rIL-3) was examined by using a murine model of spontaneous B-cell leukemia (BCL1) in BALB/c mice. All untreated mice inoculated with 2 × 10^7 BCL1 cells developed leukemia within 4 weeks, with extreme lymphocytosis and a massive increase in both spleen weight and cell number while the number of myeloid progenitors (CFU-C) per spleen was decreased. In contrast, rGM-CSF–or rGM-CSF– and rIL-3–treated recipients did not show any evidence of leukemia or splenomegaly at 4 weeks and showed a significant increase in CFU-C per spleen. Hematologic parameters in the peripheral blood of untreated mice showed anemia and thrombocytopenia. Significant elevations in these parameters were recorded in mice treated with either protocol of CSF. Treatment of recipient mice with either rGM-CSF or rGM-CSF and rIL-3 prolonged their median survival from 6 weeks in untreated controls (range, 5 to 9 weeks) up to the time they were killed at 105 days. Adoptive transfer of spleen cells obtained from mice treated with rGM-CSF, mice treated with a combination of rGM-CSF and rIL-3, and untreated controls, into normal secondary recipients indicated improved survival in recipients inoculated with rGM-CSF. These data indicate that CSFs may inhibit in vivo expansion of leukemic cells of lymphoid origin.

The in vitro proliferation and differentiation of hematopoietic precursors are regulated by the interactions of a group of specific glycoproteins, the colony-stimulating factors (CSFs). Four major different classes of murine CSFs have been defined as controlling murine granulocyte-macrophage populations: GM-CSF,1 multi-CSF (interleukin-3 [IL-3]),2 G-CSF,3 and M-CSF.4 Each has been purified to homogeneity and its range of in vitro biologic actions characterized.

Recent studies have indicated that CSFs affect mature leukocytes as well as hematopoietic progenitors. Human recombinant (r)GM-CSF5,6 and murine highly purified CSFs7 enhance antibody-dependent cell-mediated cytoxicity of neutrophils toward leukemic cells. Murine rGM-CSF, produced in Escherichia coli and native GM-CSF, was found to stimulate granulocyte and/ or macrophage colony formation by adult and fetal mouse progenitor cells. They also stimulate eosinophil colony formation by adult marrow cells and megakaryocyte as well as erythroid and mixed-erythroid colony formation.8

The use of rGM-CSF and rIL-3 for enhancement of normal hematopoiesis is well established in several experimental systems.9-13 The present work was designed to investigate a new potential use of rGM-CSF or a combination of rGM-CSF and rIL-3 for suppression of the development of leukemia through amplification of a natural defense mechanism against tumors by using an animal model of human lymphoid leukemia/lymphoma, the murine B-cell leukemia (BCL1).14,15 Our observations suggest that CSFs may provide an effective biologic tool for the control of leukemia in vivo and that similar approaches should be explored as an adjunct to chemotherapy for tumor eradication in humans.

**MATERIALS AND METHODS**

*Mice.* Inbred male BALB/c mice 6 to 8 weeks old were used throughout this study. All mice were kept in conventional animal facilities.

rGM-CSF and rIL-3. The murine rIL-3 and rGM-CSF used in these studies were a generous gift from Dr J.J. Mermod (Glaxo, Geneva). The production and purification of these CSFs synthesized by E coli have been described previously.16,17

The original preparations of rIL-3 (16 μg/mL) and rGM-CSF (100 μg/mL) were diluted in pyrogen-free saline to produce dilutions containing 1.25 ng/mL and 1.5 ng/mL, respectively. The stock solutions of rIL-3 and rGM-CSF contained <0.7 ng/mL and <0.14 ng/mL, respectively, of endotoxin as determined by a limulus amebocyte lysate assay. The materials were diluted 1:12,800 (rIL-3) and 1:64,000 (rGM-CSF) in pyrogen-free saline.

**Monoclonal antibodies.** Rat antimeast Lyt-2, L3T4, and Thy 1 (IgG2b) were obtained from Drs S. Cobbold and H. Waldmann (Department of Pathology, Cambridge University School of Medicine, UK). Normal rat IgG served as a negative control. Rabbit antimeast asialo-GM1 was purchased from Wako Chemicals (Neuss, West Germany). Affinity-purified IgG served as a control. The M1/70 rat antimeast Mac-1 monoclonal antibody (MoAb)18 was kindly provided by Dr I. Zan-Bar, Tel Aviv University, Israel. Fluorescent rabbit antimeast IgG and fluorescent goat antirabbit IgG were purchased from Bio-Yeda Rehovot, Israel (Catalog nos. 3275-1 and 3271-1, respectively).

Immunofluorescent rabbit antimeast IgG was absorbed on normal mouse spleen cells at a 1:3 ratio by volume for 30 minutes on ice.

**BCL1.** BCL1, originally discovered by Slavin and Strober,4 was maintained in vivo by intravenous (IV) passage in BALB/c mice. Three weeks after tumor passage of 5 × 10^6 peripheral blood leukocytes (PBLs) from a tumor-bearing animal, the mice were bled, and their PBLs were used as a source of tumor cells. PBLs were washed twice with pyrogen-free saline and resuspended in saline. Varying numbers of viable (trypan blue exclusion test) nucleated...
cells in 0.2 mL saline were injected into the tail vein of BALB/c mice (ten mice per group). Immediately following inoculation with BCL<sub>i</sub> cells, the mice were injected IV with 0.3 ng rGM-CSF per mouse or a combination of rGM-CSF (0.3 ng/mouse) and rIL-3 (0.25 ng/mouse). Following the first injection, the mice received intraperitoneal injections of equal doses of CSFs twice daily 5 days each week for 3 weeks. Control mice were injected into 0.2 mL pyrogen-free saline.

**Evaluation of leukemogenesis.** The appearance of leukemia was monitored by weekly PBL counts and evaluation of splenomegaly. Peripheral blood obtained from the retroorbital veins of tumor-bearing mice was collected in heparinized (44.7 µL) capillaries and counted by a Coulter counter (model S+). Peripheral blood obtained from the retroorbital veins of tumor-bearing mice was collected in heparinized capillaries and then dispersed into single cells by repeated aspiration through a 22-gauge needle. The cells were washed in phosphate-buffered saline (PBS), counted, and then plated in agar for assaying myeloid precursor cells. Spleens were removed and weighed, and spleen cell suspensions were prepared in IMDM containing 15% FCS as described previously.<sup>12</sup> The cells were washed in PBS, counted, and then plated in agar for assaying hematopoietic precursor cells. Nucleated cells were cultured from the spleens (1.5 x 10<sup>7</sup>/mL) and BM (1.5 x 10<sup>4</sup>/mL) of individual mice of each group in medium composed of 0.3% agar, IMDM, 15% FCS, and 5% pokeweed mitogen-spleen-conditioned medium and incubated in humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C. Colonies of more than 40 cells were counted on day 7 by using an inverted microscope. The counted number of CFU-C was multiplied by the number of spleen or marrow cells flushed from the organ to obtain the total CFU-C per hematopoietic organ. Results are expressed as means ± SE of two experiments, each performed in duplicate.

**Immunofluorescence assays.** Spleen cells (2 x 10<sup>9</sup>) obtained from mice receiving rGM-CSF alone, mice receiving rGM-CSF and rIL-3, or untreated controls were assayed at 4 weeks following inoculation with BCL<sub>i</sub> cells. The spleen cells were incubated with primary antibodies at a final concentration of 100 µg/mL (Tly-1, L<sub>5</sub>T<sub>6</sub>, and Lyt-2) or at a final dilution of 1 µL/mL for asialo-GM<sub>1</sub>, MoAb for 30 minutes on ice. After one washing in PBS with azid (0.02%), the cells were fixed with 0.5% paraformaldehyde and kept in the dark. The proportion of positive fluorescence was determined by using a FACS 440 flowcytometer (FACS) (Becton Dickinson, Mountain View, CA).

**Detection of dormant BCL<sub>i</sub> cells.** Spleen cells from mice treated by various modalities from each group were inoculated IV into normal secondary BALB/c recipients (the number of recipient mice is indicated in Results). Each mouse was inoculated with 1 x 10<sup>4</sup> spleen cells. Leukemia-related mortality was recorded as evidence by various modalities from each group were inoculated IV into normal secondary BALB/c recipients (the number of recipient mice is indicated in Results). Each mouse was inoculated with 1 x 10<sup>4</sup> spleen cells. Leukemia-related mortality was recorded as evidence.

### RESULTS

**Development of leukemia following inoculation with low numbers of BCL<sub>i</sub> cells.** Leukemia developed in all BALB/c mice inoculated with as few as ten BCL<sub>i</sub> cells. The kinetics of leukemia development is manifested in the peripheral blood was clearly dose dependent. The pattern of leukemia development was uniform in all groups receiving BCL<sub>i</sub> inoculation and ranged from ten to 10<sup>5</sup> cells/mouse (Fig 1A). As shown in Fig 1B, all recipients of ten to 10<sup>5</sup> cells died of typical leukemia within 63 days.

**The effect of rGM-CSF and a combination of rGM-CSF and rIL-3 on the development of leukemia following inoculation with BCL<sub>i</sub> cells.** All recipients of 2 x 10<sup>5</sup> BCL<sub>i</sub> cells developed leukemia at 4 weeks following inoculation (Table 1). In the two independent experiments that are represented in Table 1, none of the mice treated with rGM-CSF or a combination of rGM-CSF and rIL-3 developed leukemia, whereas all recipients inoculated with a similar number of BCL<sub>i</sub> cells developed leukemia within the observation period of 4 weeks. The mean number of PBLs in each of these groups is detailed in Fig 2, which represents one of two experiments with identical results.

**The effect of rGM-CSF and a combination of rGM-CSF and rIL-3 on spleen and BM of mice inoculated with BCL<sub>i</sub> cells.** At 4 weeks, four of ten mice of each group of two independent experiments and their controls were killed. The weight and cell numbers of the spleens of each group obtained from one representative experiment are shown in Table 2. A massive increase in both the spleen weight and cell number occurred in all untreated recipients of BCL<sub>i</sub>.
cells. In contrast, recipients of BCL1 cells treated with either rGM-CSF or a combination of rGM-CSF and rIL-3 did not show any evidence of splenomegaly by weight or cell number (Table 2).

A significant decrease in the spleen content of CFU-C was observed in recipients of BCL1 cells. There was a significant increase \( (P < .05) \) in the number of CFU-C per spleen in mice treated with either one of the two rCSFs protocols as compared with untreated recipients of BCL1 cells in one of two experiments (Table 2).

The effect of rGM-CSF and a combination of rGM-CSF and rIL-3 on hematologic parameters in the peripheral blood of BALB/c mice inoculated with BCL1 cells. Hematologic parameters in the peripheral blood were studied in three groups of BALB/c mice inoculated with \( 2 \times 10^5 \) BCL1 cells with or without additional rCSFs. As shown in Table 3, untreated mice inoculated with \( 2 \times 10^5 \) BCL1 cells developed a significant anemia within 4 weeks (reduced Hb level \( (P < .05) \), reduced RBC count \( \times 10^{12} /L \) \( (P < .05) \), and reduced Hct \( (P < .025) \), as well as thrombocytopenia \( (P < .0005) \) (Table 3).

Significant elevations of Hb concentration \( (P < .05) \), RBC count \( (P < .05) \), Hct \( (P < .025) \), as well as platelet counts \( (P < .0005) \) in comparison with untreated leukemia recipients were recorded in mice treated with rGM-CSF alone or a combination of rGM-CSF and rIL-3 (Table 3). Interestingly, platelet counts also seemed to respond to rGM-CSF and a combination of rGM-CSF and rIL-3. Overall, the data suggest a delayed onset of leukemia, improved survival, and improvement in several hematologic parameters of leukemic mice treated with rGM-CSF or a combination of rGM-CSF and rIL-3.

Phenotype of the spleen cells of mice treated with rCSFs. The proportion of T lymphocytes in the spleen (Thy1-positive cells) tended to be reduced in recipients of BCL1 as compared with untreated controls (28% vs 38%, respectively); however, no consistent changes in the proportion of Thy1-positive cells were noted between the different groups of mice inoculated with BCL1 and treated with rCSFs (range, 24% to 32%). The proportion of Lyt-2-positive cells was highest (41%) in recipients of rGM-CSF as compared with untreated controls or mice inoculated with BCL1 without CSF (28% each) and similar to that observed in recipients of a combination of rGM-CSF and rIL-3 (Table 4). Likewise, recipients of rGM-CSF showed higher levels of Mac 1 cells and asialo-GM1-positive cells as compared with other groups of mice (Table 4).

The effect of rGM-CSF and a combination of rGM-CSF and rIL-3 on the survival of mice inoculated with BCL1 cells. Two independent sets of experiments were carried out to investigate the effects of rCSFs on the survival of BCL1-inoculated mice and yielded identical results; therefore, the data were combined. Each experimental group consisted of 20 BALB/c mice inoculated with \( 2 \times 10^5 \) BCL1 cells. Twelve untreated controls, 12 recipients of rGM-CSF plus rIL-3, and six recipients of rGM-CSF, were monitored for the onset of leukemia and survival. As shown in Fig 3, all untreated controls died of leukemia within 9 weeks (median, 6 weeks), whereas none of the mice treated with rCSFs showed evidence of disease at 15 weeks \( (P < .001) \).

Detection of dormant BCL1 cells in mice treated with rCSFs. Untreated controls (eight mice), mice treated with rGM-CSF plus rIL-3 (eight mice), and mice treated with rGM-CSF (four mice) were killed at 4 weeks, and \( 1 \times 10^6 \) spleen cells of each mouse were adoptively transferred into two normal syngeneic recipients for detection of leukemic death. As shown in Fig 4, adoptive recipients of spleen cells obtained from leukemic recipients treated with rGM-CSF or both rCSFs showed improved survival over controls. Interestingly, adoptive recipients of spleen cells obtained from mice treated with rGM-CSF alone showed superior survival (100% at 9 weeks) as compared with recipients of spleen cells obtained from mice treated with a combination of rGM-CSF and rIL-3 (47% at 9 weeks). The detrimental effects of rIL-3

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**Table 2. Effects of rCSFs on Dimensions of Spleens and Cell Contents of Femora of BALB/c Mice at 4 Weeks Following Inoculation With \( 2 \times 10^5 \) BCL1 Cells**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Proportion of Mice With Leukemia*</th>
<th>Weight (mg)</th>
<th>Total Cell No.</th>
<th>Total CFU-C/ Spleen</th>
<th>Total Cell No./Femur</th>
<th>Total CFU-C/ Femur</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls</td>
<td>0</td>
<td>135 ± 10</td>
<td>214 ± 20</td>
<td>25.3 ± 0.5</td>
<td>11.1 ± 1</td>
<td>9.2 ± 0.9</td>
</tr>
<tr>
<td>Untreated recipients of BCL1</td>
<td>4/4</td>
<td>1,500 ± 30</td>
<td>1,010 ± 170</td>
<td>1.8 ± 0.6</td>
<td>9 ± 0.4</td>
<td>5.7 ± 1.4</td>
</tr>
<tr>
<td>rGM-CSF-treated recipients of BCL1</td>
<td>0/4</td>
<td>170 ± 20</td>
<td>190 ± 40</td>
<td>11.8 ± 3.4</td>
<td>6 ± 2</td>
<td>9.3 ± 2.6</td>
</tr>
<tr>
<td>rGM-CSF + rIL-3-treated recipients of BCL1</td>
<td>0/4</td>
<td>140 ± 20</td>
<td>147 ± 20</td>
<td>10.1 ± 3.9</td>
<td>9 ± 1</td>
<td>6.5 ± 1.2</td>
</tr>
</tbody>
</table>

*Mice featuring a WBC count \( \geq 20,000/\mu L \) and splenomegaly greater than the mean ± 2 SD of normal.
following the administration of rGM-CSF or a combination of rGM-CSF and rIL-3 had a marked effect against the BCL, hematopoietic system both rGM-CSF and a combination of rGM-CSF and rIL-3 indicate that in addition to their classic effects on the myelopoietic actions of combinations of rGM-CSF and rIL-3 previously reported by us3 and by others. Our present data as compared with either one of the rCSFs alone were significantly following the administration of rCSFs, as compared with data obtained from untreated controls (Table 3).

Table 3. Effects of rCSFs on Hematologic Parameters of BALB/c Mice at 4 Weeks Following Inoculation With 2 x 10^6 BCL1 Cells

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>RBC (x 10^9/L)</th>
<th>Hb (g/dL)</th>
<th>Hct</th>
<th>Platelets (x 10^9/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls</td>
<td>8.8 ± 0.4</td>
<td>14.8 ± 0.8</td>
<td>47.9 ± 3</td>
<td>705,000 ± 14,740</td>
</tr>
<tr>
<td>Untreated recipients of BCL1</td>
<td>6.4 ± 0.6*</td>
<td>11.2 ± 0.9*</td>
<td>34.9 ± 3</td>
<td>283,000 ± 7,910†</td>
</tr>
<tr>
<td>rGM-CSF-treated recipients of BCL1</td>
<td>9.3 ± 0.4</td>
<td>15.9 ± 0.1</td>
<td>49.3 ± 2.9</td>
<td>662,000 ± 24,537</td>
</tr>
<tr>
<td>rGM-CSF + rIL-3-treated recipients of BCL1</td>
<td>8.8 ± 0.15</td>
<td>15 ± 0.2</td>
<td>45.3 ± 0.9</td>
<td>555,000 ± 104,935</td>
</tr>
</tbody>
</table>

*Significantly different from normal control, P < .05.
†Significantly different from normal control, P < .025.
‡Significantly different from normal control, P < .0005.

Table 4. Effects of rCSFs on the Phenotype of Spleen Cells of BALB/c Mice at 4 Weeks Following Inoculation With 2 x 10^6 BCL1 Cells

<table>
<thead>
<tr>
<th>Source of Spleen Cells Tested</th>
<th>Cell Subsets (%)</th>
<th>Lyt-2</th>
<th>Mac-1</th>
<th>Asialo-GM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td>28</td>
<td>10</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>BCL1, untreated</td>
<td>28</td>
<td>10</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>BCL1, rGM-CSF treated</td>
<td>41</td>
<td>29</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>BCL1, rGM-CSF + rIL-3 treated</td>
<td>37</td>
<td>19</td>
<td>42</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.
*Net positive immunofluorescence.

on the survival of rGM-CSF-treated mice is significant (P < .001).

The present experiments were designed to investigate the effect of rGM-CSF and a combination of rGM-CSF and rIL-3 on tumor development and survival of BALB/c mice inoculated with low doses of BCL1, a highly tumorigenic and lethal leukemia of B-cell origin.14,15 Beneficial synergistic myelopoietic actions of combinations of rGM-CSF and rIL-3 as compared with either one of the rCSFs alone were previously reported by us13 and by others.20 Our present data indicate that in addition to their classic effects on the hematopoietic system both rGM-CSF and a combination of rGM-CSF and rIL-3 had a marked effect against the BCL1 disease. At 4 weeks following inoculation with BCL1, all untreated recipients developed overt leukemia, whereas none of the treated recipients developed any evidence of disease (Table 1 and Fig 2). All mice treated with rGM-CSF with or without rIL-3 showed long-term disease-free survival at 15 weeks (Fig 3). To test for the presence of clonogenic tumor cells in the spleens of treated recipients, 10^6 spleen cells were adoptively transferred to normal untreated secondary recipients. The advantages of the adoptive transfer system for detection of residual leukemia in treated recipients was previously described by Slavin et al21,22 and Weiss et al.23 Interestingly, while 93% of the recipients of 10^6 spleen cells obtained from untreated recipients of BCL1 cells died of leukemia within 9 weeks (median, 6 weeks, Fig 4), no tumorogenic BCL1 cells were documented by in vivo transfer experiments in the spleens of mice treated with rGM-CSF alone (>9 weeks). Fifty percent of mice spleens treated with a combination of rGM-CSF and rIL-3 showed evidence of disease at 9 weeks, whereas recipients of rGM-CSF alone showed no evidence of disease, thus suggesting that rIL-3 may have deleterious effects on the rGM-CSF-induced anti-BCL1 response. Documentation of leukemic-free recipients at >13 weeks following the original BCL1 inoculation (4 weeks in primary recipients and 9 weeks in secondary recipients) suggests that treatment with rGM-CSF may result in operational cure of BCL1.

The beneficial effects of rCSFs on parameters of BCL1 disease were also documented by monitoring spleen weight and cell contents as well as several basic hematologic parameters (Hb, RBC, and platelet counts), all of which improved significantly following the administration of rCSFs, as compared with data obtained from untreated controls (Table 3). The mechanisms of the antitumor effect observed following the administration of rCSFs, particularly rGM-CSF, are not

Fig 3. Survival of primary recipients of 2 x 10^6 BCL1 cells following the administration of rGM-CSF or a combination of rGM-CSF and rIL-3.

Fig 4. Survival of secondary recipients following the adoptive inoculation of 1 x 10^6 spleen cells obtained from BCL1-inoculated mice treated with rGM-CSF, a combination of rGM-CSF and rIL-3, and untreated controls.
well understood. Analysis of the phenotype of spleen cells of mice treated with CSFs as compared with mice inoculated with BCL, or untreated controls indicates a trend for an increase in the proportion of macrophages, cytotoxic T lymphocytes, and asialo-GM1-positive cells (Table 4). Potential antitumor effects may be exerted by GM-CSF-activated macrophages24 as well as by cytotoxic T lymphocytes23 or cells of the natural killer series (ie, “lymphokine-activated killer cells”). Amplification of natural antitumor effector mechanisms in the same tumor model system were recently described by Slavin et al21,22 and Weiss et al23 following allogeneic BM transplantation (BMT) or following the administration of rIL-2.25 Enhanced myelopoiesis of the normal hematopoietic progenitors as a result of rCSFs inoculation, which might compete with the proliferation of progenitors of BCL, cells, cannot be excluded. The marked potentially curative antileukemic effects of rGM-CSF rather than its potential enhancing effect on leukemic cells of lymphoid origins suggests that if similar effects will be observed in humans rGM-CSF may prove beneficial for the treatment of lymphoid malignancies in parallel with its conventional effect as a hematopoietic growth factor. This is in contrast to some concern about the use of rGM-CSF in nonlymphoid malignancies such as acute myeloid leukemia where rGM-CSF may potentially activate residual leukemic cells expressing rGM-CSF receptors and messenger RNA (F. Herrmann, personal communications).

The effects of rIL-3 have to be further investigated with care because it has been suggested that rIL-3 is capable of inducing proliferation of splenic lymphocytes.24 In contrast, the lack of proliferative effects of rGM-CSF on human B-lymphoid malignant cell lines was recently documented.27 Further evaluation of several rCSFs protocols in BCL, as well as in experimental models of BMT in leukemia are currently under study and have been encouraged by the dichotomy of the beneficial effects of rCSFs on normal hematopoiesis in parallel with marked antileukemic effects in vivo.

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Therapeutic potential of recombinant granulocyte-macrophage colony-stimulating factor and interleukin-3 in murine B-cell leukemia

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