In Vivo Induction of Terminal Differentiation of Malignant Myelopoietic Progenitor Cells by CSF-Inducing Biological Response Modifiers

By Erich Schlick and Francis W. Ruscetti

We have investigated the mechanisms by which colony-stimulating factor (CSF)-inducing biological response modifiers (BRM) may have beneficial effects on tumor-bearing hosts undergoing anti-tumor therapy. First, we have documented that treatment of mice with the chemically defined BRM maleic anhydride divinyl ether copolymer (MVE-2), which induces CSF secretion by macrophages (Mφ) and bone marrow cells (BMC), significantly increased growth and differentiation of normal myelopoietic cells and counteracted the myelosuppressive effects of cyclophosphamide (CY). Second, we established that MVE-2 may exert CSF-mediated antitumor effects on certain leukemic tumor cells. Serum from mice pretreated in vivo with MVE-2, which contained CSF, induced terminal differentiation of cloned tumor cells from the CSF responsive WEHI-3B D⁻ subline in vitro, but not from the WEHI-3B D⁺ subline, which is unresponsive to CSF. In vivo experiments showed that treatment of mice bearing the WEHI-3B D⁺ tumor first with CY and three days later with the CSF inducer MVE-2, significantly increased their survival time and rendered 20% to 50% of the tumor-bearing mice disease free. No such effects were obtained in mice bearing the WEHI-3B D⁻ tumor. Thus, the induction of CSF or other differentiation factors by some BRMs may result in therapeutic effects against certain leukemias based on at least two distinct mechanisms: In addition to their restorative effects on normal bone marrow functions, CSF-inducing BRMs may also prevent further leukemogenesis by induction of terminal differentiation of leukemic cells.

Several reports indicate that some immunotherapeutic agents can stimulate in vivo normal myelopoiesis, presumably mediated by CSFs, in addition to having an immunomodulating effect on Mφ and natural killer (NK)–cell activities. Since major treatment modalities for various cancers include chemotherapy and/or radiotherapy, which often result in severe suppression of hematopoiesis and immune functions, treatment with selected biologic response modifiers (BRMs) might be advantageous to the host, since it can result in cytoreductive tumor therapy, (1) by augmenting the host’s natural immunity (Mφ and/or NK cytotoxicity) against tumor cells, (2) by restoring the host’s suppressed hematopoietic functions, and (3) possibly by inducing terminal differentiation of certain leukemic tumor cells. Studies of several murine myeloid leukemia cell lines, especially of the WEHI-3B myelomonocytic leukemia, have shown that these undifferentiated tumor cells could be induced in vitro to differentiate into macrophages (Mφ) and/or granulocytes, which then exhibit properties similar to those of their normal counterparts. Exposure to a variety of chemical agents is able to induce such differentiation in these myeloid leukemia cell lines, but analogous differentiation could also be induced by biologic materials, such as endotoxin serum and certain conditioned media. Biochemical analysis of these biologic materials indicates that physiologic regulatory molecules such as colony-stimulating factors (CSF), which control in vitro growth and differentiation of normal granulocytes and/or Mφ, can induce terminal differentiation of myelomonocytic leukemias. Of special interest have been the findings that only two of three different subclasses of CSF are able to induce terminal differentiation. GM-CSF, controlling normal granulocyte formation, exhibited high differentiation-inducing activity. GM-CSF, controlling normal granulocyte and Mφ formation, was found to have only weak differentiation-inducing activity, whereas M-CSF, controlling normal Mφ formation, had no detectable effect on differentiation. Since previous studies have indicated that MVE-2 (maleic anhydride divinyl ether copolymer), a chemically defined BRM, might induce increased in vivo secretion of CSFs by Mφ and BMC, in addition to activating Mφ and NK cell cytotoxicity, we were therefore interested in testing the ability of MVE-2 to modulate the host’s myeloid progenitor and immune functions in vivo before and after antineoplastic treatment with cyclophosphamide (CY) and to define the potential of MVE-2 for differentiation of WEHI-3B leukemic cells.

MATERIALS AND METHODS

Male BALB/c mice were obtained from the Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, NCI-FCTR, NIH, Frederick, Md. The animals were 6 to 8 weeks of age before they were used for experiments. Maleic anhydride divinyl ether copolymer (MVE-2) was the generous gift of Dr. R. Corrano (Adria Laboratories, Columbus, OH). Lipo polysaccharide from S. typhimurium (LPS) was purchased from Difco Laboratories (Detroit, Mich). The alkylating agent cyclophosphamide (CY) was obtained from Sigma Chemical (St. Louis). The media and BRM preparations were tested for endotoxin contamination with the Limulus lysate assay and found to contain less than 0.125 ng/mL.

Tumor cells. WEHI-3B leukemia cells were originally obtained from mineral oil–injected BALB/c mice in 1968. This leukemia cell line grows in liquid culture as a population of undifferentiated blast cells and promyelocytes. The leukemic cells form large colonies in agar medium that are composed of similar cells. Their plating efficiency varies from 30% to 70%. The WEHI-3B D⁺ and WEHI-3B D⁻ sublines were a generous gift from Dr. Peter Ralph, Sloan-Kettering Institute, New York. Cells of the WEHI-3B D⁺ subline are responsive to induction of differentiation, whereas cells of the WEHI-3B D⁻ subline are unresponsive to the induction of differentiation. The present studies were performed using...
WEHI-3B D+ and D- cells grown in liquid suspension culture in RPMI medium with 10% FCS and containing 0.5-2.0 x 10^6 cells/mL. Both cell lines have a doubling time of about 20 hours in vitro and of about 24 hours in vivo. WEHI-3B D+ and D- cells are equally sensitive to the in vitro cytoxic effects of an active metabolite of CY, 4-sulfonfthiocyclophosphamide [4-SET-CY; Asta Z7557] (donated by Dr P. Hilgard from Asta Werke, Bielefeld, West Germany). The respective ED50 (concentration inducing 50% of the maximal effect) for 4-SET-CY-induced growth inhibition were 3.9 μg/mL (WEHI-3B D+ cells) and 5.0 μg/mL (WEHI-3B D- cells) for 1 h incubation with 4-SET-CY (data not shown).

Agar cultures of WEHI-3B D+ and D- cells were prepared using cloned cells grown in suspension culture in complete RPMI medium (RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum [Gibco, Grand Island, NY]), 292 μg/mL L-glutamine (Gibco) and 100 μg/mL gentamycin (Sigma). All semisolid agar cultures were prepared as 1 mL cultures in 35 mm Petri dishes using an equal volume mixture of double strength Dulbecco's modified Eagle's medium and 0.6% agar, supplemented with 20% FCS, 292 μg/mL L-glutamine (Gibco), and 100 μg/mL gentamycin (Sigma). The WEHI-3B cells were added to the liquid agar medium and 1 mL volumes containing 300 cells were pipetted into each culture dish containing 0.1 mL of serially diluted serum from mice pretreated 3 hours before with 5 μg/mouse of LPS (postendotoxin serum) or 0.1 mL of serially diluted sera from MVE-2-treated mice. Serially diluted conditioned media (CM) from macrophages (Mφ) or bone marrow cells (BMC) from MVE-2-treated mice were tested likewise. The cultures were allowed to gel and then incubated for 7 days at 37 °C and 8% CO2 in a fully humidified atmosphere. Colonies (>50 cells) were counted after 7 days of incubation and were classified after Wright staining in situ as undifferentiated if they consisted of a tight aggregate of cells with no outlying cells. Differentiated colonies were composed of loosely dispersed differentiating granulocytes/Mφ around a central aggregate. Cells were terminally differentiated, when they failed to form new tumor colonies after being replated in subsequent agar cultures without addition of growth and/or differentiation factors.

**Macrophages.** Resident peritoneal macrophages (Mφ) harvested by peritoneal lavage, were seeded into 16 mm wells (Costar, Cambridge, Mass) in 1 mL of complete medium. The cultures were incubated for 2 hours and the adherent Mφ-monolayers (>95% Mφ as determined by esterase and Wright staining) were washed twice before testing in experiments. The assay for measuring the macrophage-mediated cytoxicity has been previously described. Briefly, to measure in vivo activation, monolayers of 8 x 10^4 peritoneal Mφ, harvested at different times after in vivo treatment of the mice with MVE-2, were overlaid with 8 x 10^6 MBL-2 tumor cells in complete medium and 10 ng/mL of LPS were added. All cultures were incubated for an additional 48 hours at 37 °C and 5% CO2 and viable MBL-2 cells were then determined by trypan blue dye exclusion. The percent inhibition of MBL-2 cell growth due to Mφ activation was calculated by:

\[
\text{% inhibition} = \frac{\text{mean of MBL cells from drug treated Mφ}}{\text{mean of MBL-2 cells from nontreated Mφ}} \times 100
\]

**Bone marrow cells.** Sterile single-cell suspensions from femoral bone marrow were prepared as previously described. The number of viable nucleated cells in the femur of each mouse was counted in a hemocytometer. To determine the number of granulocyte-Mφ committed stem cells (GM-CFU-C), a previously described in vitro soft agar culture technique was used. Briefly, 10^6 unfractionated bone marrow cells (BMC) were suspended in 2 mL of 0.3% agar, supplemented with 15% horse serum (Gibco), 15% fetal calf serum (Gibco), and 100 μg/mL of gentamycin/mL (Sigma), and were plated in 35 mm Petri dishes (Falcon, Oxnard, Calif). As CSF source, 0.2 mL supernatant from WEHI-3B cells was used. The cultures were incubated at 37 °C in a fully humidified atmosphere of 7% CO2 in air. Colonies (>40 cells) were scored and classified on the basis of their morphology at days 7 and 10 of the culture period.

**CSF assay.** Cell-free supernatants from in vitro- or in vivo-activated Mφ and mononuclear BMC were harvested after 48 hours of subsequent in vitro culture (37 °C and 5% CO2) at a cell density of 10^6 Mφ/mL or 10^5 BMC/mL and kept at −20 °C until assayed. CSF concentrations were then determined using a bioassay as previously described. Briefly, serial dilutions of the supernatants or sera to be tested for CSF were added to each 35 mm Petri dish containing 10^6 nonadherent BMC from normal mice in 2 mL of 0.3% agar, supplemented with 15% horse serum, 15% fetal calf serum, and 100 μg/mL gentamycin. At the end of the culture period (10 days at 37 °C and 7% CO2), the colonies (>40 cells) were counted and classified on the basis of their morphology. No colony formation was noted in the absence of either the standard CSF (supernatant from the WEHI-3B cell line), a supernatant from Mφ or bone marrow cells, or serum. CSF activity is expressed as U/mL. The Student's t test was used for statistical analysis.

**RESULTS**

The results of the experiments on the effects of in vivo treatment with MVE-2 on CSF secretion and myeloid progenitor cells in normal and CY-treated mice are summarized in Table 1. MVE-2 at a dose of 25 mg/kg induced an increase in CSF secretion by BMC and Mφ and in serum CSF levels which was followed by significant augmentation in granulocyte-Mφ progenitor cells and in nucleated BMC per femur (Table 1, group 2 v 1 and Fig 1). Optimal effects could be found 3 days after treatment with MVE-2 at 25 mg/kg and lower or higher doses of MVE-2 were less effective. MVE-2, when given 3 days after the bone marrow suppressive agent CY (150 mg/kg), also induced increased secretion of CSF by Mφ and BMC, stimulated growth and differentiation of myelomonocytic progenitor cells, and thus restored CY-caused myelosuppression (Table 1, group 6 v 5). MVE-2, given 1 day after CY, however, failed to enhance CSF secretion by either Mφ or BMC, or to counteract the myelosuppressive effects of CY (Table 1, group 4 v 3).

**Sera from MVE-2-treated mice induce differentiation in WEHI-3B D+ leukemia cells.** Next, we investigated whether MVE-2-induced CSF would cause in vitro differentiation of WEHI-3B D+ cells, as compared to WEHI-3B D- cells. Incubation of WEHI-3B D+ cells in agar cultures with sera derived from MVE-2-treated mice (14 hours after injection) caused a significant increase in differentiation into granulocytes/Mφ of the leukemia cells (up to a serum dilution of 1/64) as compared to sera from PBS-treated animals (Table 2). The differentiation induced in these WEHI-3B D+ cells was in about two thirds of the cases terminal as shown by the failure of these differentiated cells to form new tumor colonies when replated in subsequent agar cultures without any growth and/or differentiation factors (data not shown). Sera from MVE-2-treated mice (harvested 14 hours after injection) also significantly inhibited (up to a serum dilution of 1/32) the growth of WEHI-3B D+ cells in liquid suspension cultures (Fig 2). The effects of sera from mice harvested at different times after treatment with
MVE-2 on terminal differentiation of WEHI-3B D⁺ cells paralleled their stimulatory effects on normal myelopoiesis at all times (Fig 1). Incubation of WEHI-3B D⁺ cells in agar or liquid suspension cultures with serial dilutions of conditioned media from MΦ and BMC of MVE-2–treated mice gave similar results with respect to terminal differentiation (Table 3) and growth inhibition (data not shown) as sera from the same animals. The effects of CM from MΦ or BMC harvested at different times after treatment with MVE-2 on terminal differentiation of WEHI-3B D⁺ cells paralleled their stimulatory effects on normal myelopoiesis at all times (data not shown). Postendotoxin serum served as a positive internal control. Cell lines of the WEHI-3B D⁺ subline were unresponsive to sera from PBS or MVE-2–treated mice with respect to terminal differentiation (Fig 2). Conditioned media from BMC or MΦ of MVE-2–treated mice also failed to induce either differentiation (Table 3) or growth inhibition of WEHI-3B D⁺ cells (data not shown). Likewise, WEHI-3B D⁺ cells failed to respond to postendotoxin serum (data not shown). Heating of the sera and CM of MΦ and BMC from MVE-2–treated mice (56 °C and 30 min) did not change their stimulatory effect on normal myelopoiesis or their differentiation-inducing activity on WEHI-3B D⁺ cells (data not shown), suggesting that CSF inhibitors were not present in these sera or CMs. We also incubated WEHI-3B D⁺ and D⁻ cells in agar and in liquid suspension cultures directly with MVE-2, at concentrations ranging from 1 to 100 μg/mL to rule out any direct cytotoxic effects of the agent. MVE-2 at these concentrations had no significant growth inhibitory effect and did not induce differentiation of the leukemic cells (data not shown).

### Table 1. Effect of MVE-2 on CSF Secretion and Bone Marrow Cellularity in Normal and Cyclophosphamide-Pretreated Mice

<table>
<thead>
<tr>
<th>Group *</th>
<th>Cyclophosphamide (150 mg/kg IP) Treatment at</th>
<th>Treatment at Day 3 With MVE-2 (25 mg/kg IP)</th>
<th>GM-CFU-C/Femur (% of Control)</th>
<th>BMC/Femur (x 10⁵)</th>
<th>CSF Secretion†</th>
<th>U/10⁶ MΦ</th>
<th>U/10⁷ BMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>—</td>
<td>100</td>
<td>9.9</td>
<td>70</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>+</td>
<td>142‡</td>
<td>13.7‡</td>
<td>165‡</td>
<td>87‡</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>d 4</td>
<td>—</td>
<td>26‡</td>
<td>3.6‡</td>
<td>81</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>d 4</td>
<td>+</td>
<td>42‡</td>
<td>4.4‡</td>
<td>90</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>d 6</td>
<td>—</td>
<td>63‡</td>
<td>7.3‡</td>
<td>108</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>d 6</td>
<td>+</td>
<td>114§</td>
<td>11.9§</td>
<td>198</td>
<td>96</td>
<td></td>
</tr>
</tbody>
</table>

*BALB/c mice were pretreated with CY at the days indicated. They received additionally a single injection of PBS or MVE-2 at day 3. The mice were killed at day 0 for determination of bone marrow cellularity and CSF secretion by MΦ and BMC. The results shown here are the mean of a typical experiment (5 mice per group); SD was less than 15% of the mean. The experiment was repeated 2 times with comparable results.

†CSF secretion by in vivo activated MΦ and BMC was determined after 2 days of subsequent in vitro culture of 10⁵ MΦ/mL or 10⁷ BMC/mL in complete medium without any stimulus.

‡P < 0.01 as compared to group 1.

§P < 0.01 as compared to group 5.

### Fig 1. BALB/c mice received a single IP injection of either PBS (open symbols) or 25 mg/kg of MVE-2 (closed symbols). The mice were killed at the times indicated and their sera were harvested for determination of CSF concentrations and for induction of differentiation of WEHI-3B D⁺ cells. The results are expressed as units of CSF/mL serum or % differentiated WEHI-3B D⁺ colonies (at a serum dilution of 1/8) and are the mean values of a typical experiment (SD was less than 20%). The experiment was repeated two times and yielded similar results. Incubation of WEHI-3B D⁺ cells with sera from PBS– or MVE-2–treated mice resulted in up to 5% differentiated WEHI-3B D⁺ colonies (data not shown).

### Table 2. Differentiation-Inducing Activity of Sera From MVE-2–Pretreated Mice on WEHI-3B Leukemia Cells

<table>
<thead>
<tr>
<th>Group*</th>
<th>Serum Dilution</th>
<th>WEHI-3B D⁺ Tumor Cells†</th>
<th>WEHI-3B D⁻ Tumor Cells†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1/4</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>1/8</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>1/16</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1/32</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1/64</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>MVE-2</td>
<td>1/4</td>
<td>8</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>1/8</td>
<td>5</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>1/16</td>
<td>2</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>1/32</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>1/64</td>
<td>3</td>
<td>14</td>
</tr>
</tbody>
</table>

*Groups of 20 mice were injected IP with either PBS or 25 mg/kg MVE-2. Serum was collected and pooled 14 hours after injection.

†300 WEHI-3B leukemia cells were plated in 1 mL 0.3% agar containing 0.1 mL serum at different dilutions. Each value represents the mean percentage of differentiated colonies of duplicate cultures; SD was less than 20% of the mean. The experiment was repeated 2 times with similar results.

‡300 WEHI-3B leukemia cells were plated in 1 mL of 0.3% agar containing 0.1 medium.
Combined treatment of WEHI-3B D⁺ or D⁻ tumor-bearing mice with CY and MVE-2. We then wanted to determine whether MVE-2 could be used in vivo, either alone or in combination with CY, for specific treatment of mice bearing WEHI-3B D⁻ cells.

Inoculation of BALB/c mice IP with 10⁵ WEHI-3B (D⁺ or D⁻) leukemic cells led to the development of ascitic tumors. The medium survival time (MST) of the tumor controls was about 19 to 20 days with no surviving animals, regardless of the subline inoculated (Figs 3 and 4). Subsequent incubation of WEHI-3B (D⁺ or D⁻) cells, harvested by peritoneal lavage from the tumor controls 8 days after tumor inoculation in agar cultures, resulted in a 3% to 5% spontaneous differentiation (Table 4).

Treatment of animals bearing the WEHI-3B D⁻ subline with MVE-2 (25 mg/kg), 3 and 6 days after tumor inoculation, increased the MST to day 27 (+35% above controls; Fig 3). MVE-2 also induced differentiation in 26% of the remaining peritoneal tumor cells (Table 4). A single IP injection of CY (150 mg/kg) 3 days after tumor inoculation, prolonged the MST to day 28.5 (+42% above controls; Fig 3) and induced differentiation in 14% of the remaining peritoneal tumor cells (Table 4). No surviving animals are found after treatment with either MVE-2 or CY alone. Combined treatment with CY (at day +3) and MVE-2 (at day +6), however, delayed MST to day 47.5 (+137% above controls), with 30% of the animals still disease free, when the experiment was terminated 90 days after tumor inoculation (Fig 3). The percent survivors after such combined CY/MVE-2 treatment varied in the different experiments between 20% and 50%. The combined CY/MVE-2 treatment also induced differentiation in 61% of the peritoneal tumor cells remaining at day 8 (Table 4).

MVE-2 injections (25 mg/kg) of animals bearing the WEHI-3B D⁻ subline on days 3 and 6 after tumor inoculation increased the MST of these animals to day 24 (+26% above controls), while a single IP injection of CY (150 mg/kg) on day 3 delayed their MST to day 28 (+47% above controls; Fig 4). Combined treatment of these mice with CY (at day +3) and MVE-2 (at day +6) prolonged MST to day 35, which was an increase of 84% above the MST of the controls (Fig 4). However, neither treatment with MVE-2 or CY alone nor combined treatment with CY/MVE-2 given at a 3-day interval resulted in any surviving animals (Fig 4). Furthermore, none of the treatment regimens used induced differentiation of the peritoneal WEHI-3B D⁻ tumor cells remaining at day 8 (Table 4).

We then decided to treat tumor-bearing animals with CY/MVE-2 at a one-day time interval (CY at day 3 and MVE-2 at day 4), which does not result in increased CSF levels in vivo (Table 1).

The results in Table 5 show that such a treatment delayed the MST of animals bearing the WEHI-3B D⁺ or D⁻ subline to 32 or 33.5 days, respectively, and no survivors were found. Furthermore, combined treatment with CY on day 3 and
MVE-2 on day 4 induced a differentiation only in 17% of the peritoneal WEHI-3B D⁺ tumor cells remaining at day 8, which was not different from the CY control (14%; data not shown). No differentiation was found in the remaining WEHI-3B D⁻ tumor cells (data not shown).

The residual undifferentiated peritoneal WEHI-3B D⁺ tumor cells, after treatment with either MVE-2, CY, or CY combined with MVE-2, retained their sensitivity to subsequent in vitro induction of differentiation by postendotoxin serum when tested at day 8, whereas the residual peritoneal WEHI-3B D⁻ tumor cells remained insensitive to postendotoxin serum with regard to in vitro differentiation or growth inhibition, suggesting these subline are stable (data not shown).

**Susceptibility of WEHI-3B D⁺/D⁻ leukemic cells to cytotoxicity by Mφ or NK cells.** Since MVE-2 is reported to increase cytotoxicity by Mφ and NK-cells against various tumor targets²⁴ and treatment with MVE-2 alone or CY combined with MVE-2 was still effective in treating mice bearing the (CSF-insensitive) WEHI-3B D⁻ subline, we wanted to test whether both tumor sublines were susceptible to cytotoxic Mφ and/or NK cells.

Both sublines of WEHI-3B (D⁺/D⁻) were highly sensitive to Mφ cytotoxicity at E:T ratios of ≥5:1, whereas they were insensitive to NK-cell cytotoxicity at E:T ratios of ≤200:1 (Table 6). MBL-2 tumor cells were internal positive controls for cytolytic activity.

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**Table 4. In Vivo Induction of Differentiation of WEHI-3B Leukemia Cells by Combined Treatment with Cyclophosphamide and MVE-2**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment*</th>
<th>Percent Differentiated Colonies of WEHI-3B D⁺ Tumor Cells</th>
<th>Percent Differentiated Colonies of WEHI-3B D⁻ Tumor Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>MVE-2 (25 mg/kg); days 3 and 6</td>
<td>2</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>CY (150 mg/kg); day 3</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>CY (150 mg/kg) on day 3 and MVE-2 (25 mg/kg) on day 6</td>
<td>5</td>
<td>61</td>
</tr>
</tbody>
</table>

*BALB/c mice received on day 0 10⁸ WEHI-3B D⁻ or WEHI-3B D⁺ leukemia cells IP. Controls (C) were injected IP on day +3 and day +6 with PBS. Mice from group 2 received 25 mg/kg of MVE-2 IP on day +3 and day +6. Mice from group 3 and 4 were injected on day +3 with 150 mg/kg cyclophosphamide and on day +6 with either PBS or 25 mg/kg MVE-2. All mice were killed on day +8. Their peritoneal cavity was lavaged with 10 mL complete medium and the peritoneal fluid, containing peritoneal exudate cells and WEHI-3B leukemia cells, was harvested. The Mφ were separated by plastic adherence and the nonadherent WEHI-3B cells were used for determination of their differentiation status.

†300 leukemia cells were plated in 1 mL of 0.3% agar without addition of any sera. Each value represents the mean percentage of differentiated colonies of duplicate cultures, initiated from 5 mice per group; SD was less than 25% of the mean. The experiment was repeated two times with comparable results.
LEUKEMIC MYELOBLASTIC DIFFERENTIATION

Table 5. MST of WEHI-3B Tumor-Bearing Mice After Combined CY/MVE-2–Treatment Given at a One-Day Time Interval

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment*</th>
<th>WEHI-3B D+ Tumor Cells (days)</th>
<th>WEHI-3B D+ Tumor Cells (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>CY (150 mg/kg); day 3</td>
<td>28</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>CY (150 mg/kg) on day 3 and MVE-2 (25 mg/kg) on day 4</td>
<td>32</td>
<td>33.5</td>
</tr>
</tbody>
</table>

*BALB/c mice received on day 0 10³ WEHI-3B D+ or D- leukemic cells. Controls were injected IP on day +3 and day +4 with PBS. Mice from groups 2 and 3 were kept for median survival time and similar results.

Table 6. Susceptibility of WEHI-3B D+/D- Leukemic Cells to Macrophage and NK-Cell Cytotoxicity

<table>
<thead>
<tr>
<th>MVE-2* (25 mg/kg IP)</th>
<th>Tumor Targets</th>
<th>% MΦ Cytotoxicity†</th>
<th>% NK Cell Cytotoxicity‡</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>10:1</td>
<td>5:1</td>
</tr>
<tr>
<td>–</td>
<td>WEHI-3B D+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>87%</td>
<td>36%</td>
</tr>
<tr>
<td>–</td>
<td>WEHI-3B D-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>82%</td>
<td>33%</td>
</tr>
<tr>
<td>–</td>
<td>MBL-2</td>
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<td>0</td>
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<tr>
<td>+</td>
<td></td>
<td>86%</td>
<td>39%</td>
</tr>
<tr>
<td>–</td>
<td>YAC-1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>79%</td>
<td>26%</td>
</tr>
</tbody>
</table>

*BALB/c mice received on day 0 10³ WEHI-3B D+ or D- leukemic cells. All mice were killed on day +3 for determination of peritoneal MΦ and spleen NK-cell cytotoxicities against different tumor targets. Each value represents the mean of two separate experiments; SD was less than 20% of the mean.

†MΦ cytotoxicity of peritoneal MΦ was determined at different E:T ratios.

‡NK cell cytotoxicity of splenic lymphocytes was determined at different E:T ratios in a 4 hour 51Cr-release assay.

§P < 0.001 relative to controls.

DISCUSSION

The data presented here show that a chemically defined BRM, MVE-2, may induce in vivo terminal differentiation of malignant myelopoietic progenitor cells, in addition to stimulating growth and differentiation of normal myelopoietic progenitors. These effects on normal or malignant myelopoietic cells, however, appear not to be caused directly by MVE-2, but rather to be mediated through CSFs. This is supported by the observation that MVE-2 has no direct stimulatory or inhibitory effects on normal bone marrow cells grown in soft agar with or without addition of CSF. Furthermore, MVE-2 failed to directly induce in vitro differentiation and/or growth inhibition of WEHI-3B D+ cells. On the other hand, sera or supernatants from BMC and MΦ of mice pretreated in vivo with MVE-2, which contained different CSFs (these data and reference 2), caused differentiation of malignant myelopoietic progenitor cells. A carry over of MVE-2 with serum to our in vitro cultures can be excluded, since pharmacokinetic studies on the distribution of MVE-2 after in vivo treatment showed that MVE-2 has a short serum half-life.

Although the CSF molecules in serum and supernatants of BMC and MΦ were not purified, the morphology of the bone marrow colonies induced by these fluids seem to point out that sera- and BMC-derived supernatants contained three types of CSF, namely M-CSF, GM-CSF, and G-CSF, as evidenced by the findings of pure MΦ colonies, mixed granulocyte-MΦ colonies, and pure granulocyte colonies. MΦ-derived supernatants contained only M-CSF and G-CSF, as evidenced by the outgrowth of pure MΦ and pure granulocyte colonies.

While it seems to be clear that CSFs are the major regulator of normal myelopoiesis, the problem of whether CSFs are also the major physiologic inducer of differentiation in myelomonocytic leukemias, such as WEHI-3B D+ cells, is not yet solved. There is experimental evidence that, besides CSFs, there exist factor(s), such as MGI-2, which is also referred to as MGI,34 D. factor,35 and GM-DF31, which seem to induce differentiation of myelomonocytic cells, while supposedly lacking growth regulatory effects on normal myelopoiesis. However, in conflict with this, others have suggested that GM-DF for myeloid leukemic cells might be identical with G-CSF, inducing growth and differentiation of normal and terminal differentiation of malignant myeloid cells.33 In our experiments, sera, MΦ, and BMC supernatants of MVE-2–pretreated mice contained the relevant subclasses of CSF (GM-CSF and G-CSF), which have been shown to induce in vitro differentiation of WEHI-3B D+ cells, while lacking such effects on WEHI-3B D- [these data and reference 17]. The fact that these activities on normal and malignant myeloid progenitors of sera and MΦ/BMC supernatants from MVE-2–pretreated mice, which were free of CSF inhibitors, showed a parallel time course would support the notion that relevant CSFs present in these fluids (GM-CSF and/or G-CSF) caused the terminal differentiation of the WEHI-3B D+ cells. This does not, however, exclude the possibility of the concomitant presence of a separate differentiating factor.

The observed therapeutic effects after treatment of mice bearing the WEHI-3B D+ or WEHI-3B D- subline with CY...
and/or MVE-2 could be due to either one or a combination of the following mechanisms: (1) direct cytotoxic effects of CY on either subline; (2) Mφ-mediated cytotoxicity on cells of either subline, and (3) CSF-mediated differentiation of the WEHI-3B D⁺, but not the WEHI-3B D⁻ subline. Since both sublines have the same origin, and were equally sensitive to Mφ-mediated cytotoxicity and CY toxicity, the differences in MST between both sublines, treated with MVE-2 alone or a combination of CY and MVE-2, are therefore most likely attributable to CSF-induced differentiation of WEHI-3B D⁺ cells. This hypothesis is supported by the finding of an increased percentage of differentiated WEHI-3B D⁺ cells after preceding in vivo therapy with MVE-2 alone or a combination of CY and MVE-2, given at an interval, which allows in vivo induction of CSF.

Treatment of either subline with MVE-2 alone was marginally effective, probably due to the fact that treatment commenced only on day 3, when the IP tumor burden already exceeded $6 \times 10^7$ tumor cells (data not shown). We therefore decided to dramatically reduce the tumor burden by CY to a stage of minimal disease before using MVE-2 as Mφ activator and differentiation-inducing agent. Since our studies have shown that an interval of about 3 days between CY and MVE-2 was optimal for CSF induction and reconstitution of CY-depressed myelopoiesis (these data and reference 36), and regrowth of CY-treated WEHI-3B D⁺ or D⁻ cells did not occur until about 4 days after CY (data not shown), we elected a 3-day interval for MVE-2 treatment after preceding high-dose CY. The combined CY/MVE-2 treatment given at a 3-day interval of WEHI-3B D⁻ tumor-bearing mice significantly increased their MST by about 84% above controls, most likely due to cytoreduction by CY and also more effective Mφ-mediated cytotoxicity at the minimal residual disease stage (ie, higher Mφ effectors to target cell ratio). The successful treatment, however, of mice bearing the WEHI-3B D⁺ subline with CY/MVE-2, when given at a 3-day interval (137% increase in MST and 20% to 50% cured mice), seems to be correlated not only with the reduced tumor burden (CY and Mφ-mediated), but also with the induction of differentiation in remaining tumor cells, which was in a high percentage of the cases terminal, thus preventing further leukemogenesis.33,37 This is supported by the findings that treatment of mice bearing WEHI-3B tumor cells with a combination of CY and MVE-2, given at a one-day interval, which does not allow the in vivo induction of CSF, resulted only in a rather small increase in MST with no surviving animals; also no differences were found between the WEHI-3B D⁺ and D⁻ sublines. Furthermore, MVE-2 given one day after CY failed to induce differentiation in the remaining WEHI-3B D⁺ cells. The finding that the residual undifferentiated WEHI-3B D⁺ tumor cells after treatment with CY/MVE-2 remain sensitive to differentiation with CSF seems to rule out the possibility of an immediate in vivo selection process for WEHI-3B D⁻ variants, which would preclude further cyclic treatment with CY/MVE-2.

Our studies thus show that combined treatment of WEHI-3B D⁺ tumor-bearing mice with CY and the BRM, MVE-2 depend on an optimal treatment schedule, which takes into account the time course of the cytoreductive effects of CY on the tumor cells, as well as the modification of the host's immune system. Furthermore, MVE-2 offers the distinct advantage of being a potent CSF inducer, which in turn has profound effects on CY-depressed myelopoiesis and terminal differentiation of certain myelomonocytic tumor cells. Even though it is unlikely that the results obtained with the differentiation-inducible murine WEHI-3B D⁺ leukemia can be extrapolated to the majority of primary murine or human myelomonocytic leukemias, our results and those of others33,38 clearly indicate that biologic response modification may occur in vivo leading to phenotypic changes of tumor cells, thus possibly eliminating the growth advantage of leukemic tumor cells relative to the normal myelomonocytic stem cell population39 and/or preventing further leukemogenesis.33,37

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In vivo induction of terminal differentiation of malignant myelopoietic progenitor cells by CSF-inducing biological response modifiers

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