Phorbol Myristate Acetate Stimulates Macrophage Differentiation and Replication and Alters Granulopoiesis and Leukemogenesis in Long-Term Bone Marrow Cultures

By Joel S. Greenberger, Peter E. Newburger, and Maryann Sakakeeny

The effects of the tumor-promoter phorbol myristate acetate (PMA) on normal hemopoiesis and Friend leukemia virus (FLV) granulocytic leukemogenesis in long-term bone marrow cultures were examined. FLV-anemia-inducing strain (FLV-A) infected, Rauscher R-MuLV clone M52R infected, or uninfected control NIH Swiss mouse marrow cultures were treated weekly with PMA or 4-0-methyl-PMA at 2.0 ng/ml or 200.0 ng/ml. Addition of PMA to control uninfected or R-MuLV-infected cultures decreased production of nonadherent granulocytic cells and granulocyte-macrophage progenitor cells (GM-CFU-c), and increased the numbers of adherent macrophages. Addition of PMA to FLV-A-infected cultures did not inhibit generation of granulocytic leukemia cell lines even though the numbers of adherent adipocytes were decreased and adherent macrophages were increased. PMA treatment of freshly explanted whole bone marrow but not purified nonadherent GM-progenitor cells from long-term bone marrow cultures stimulated GM-CFU-c and cluster formation in the absence of added colony-stimulating factor (CSF). The sensitivity of purified GM-progenitor cells to L929 or WEHI-3 CSF was not altered by PMA; however, PMA treatment of bone marrow macrophages or peritoneal exudate macrophages stimulated detectable GM-CFU-c and cluster formation by purified GM-progenitor cells under conditions where equal numbers of untreated macrophages failed to be stimulatory. Thus, several PMA effects on hemopoietic stem cells in vitro are mediated through indirect action on adherent stromal cells including macrophages.

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promoting effect of PMA on FLV-A or R-MuLV (M52R) granulocytic leukemogenesis in vitro.

MATERIALS AND METHODS

**Mice**

NIH Swiss female mice were obtained from the animal colonies of the National Institutes of Health and housed 10 per cage.

**Long-Term Bone Marrow Cultures**

The methods for establishment of long-term marrow cultures have been reported previously. Briefly, the system described by Dexter was modified to facilitate study of hematopoietic stem cells of a single age during a longer period of continuous proliferation in vitro. Cultures were established from the contents of both a femur and tibia of adult 30-33-g mice. Cultures contained 10.0 ml Fischer’s medium supplemented with 25% fetal calf serum (Gibco, Grand Island, N.Y.) and 10^{-7} M hydrocortisone sodium hemisuccinate (Solucortef, UpJohn). Cultures were established and maintained in 40 cm² Corning plastic flasks, incubated at 33°C, 7% CO₂, and were depopulated by weekly removal of all medium with all nonadherent cells. Fresh medium with hydrocortisone was added weekly. These conditions facilitate longer maintenance of the critical adherent cell component due to decreased fat accumulation compared to that observed with 25% horse serum. The adherent stromal compartment is required for prolonged hematopoiesis; however, fat accumulation by the cells is not essential.

**Hemopoietic Colony-Forming Cells**

The methods for assay of granulocyte-macrophage progenitor cells (CFU-c) have been previously described. Briefly, nonadherent cells from long-term marrow cultures or fresh marrow were suspended in 1.0 ml of 0.3% agar (Difco) and McCoy’s 5A supplemented medium with 10% fetal calf serum (Gibco). As a source of colony-stimulating factor (CSF), conditioned medium from WEHI-3 (clone c54) was dialyzed and concentrated five-fold by Amicon filtration and added in a 10% volume. 1.929 cell conditioned medium was similarly prepared. Groups of cultures were also plated in the absence of an exogenous source of CSF. Cultures were incubated at 37°C, 7% CO₂, and scored on day 7. CFU-c were scored as colonies containing ≥ 50 cells, and GM-clusters as those of 10-49 cells.

**Establishment of Leukemogenic Cell Lines**

Nonadherent cells from long-term marrow cultures were centrifuged at 1000 g for 10 min to a cell pellet and re suspended at 10^6 or 10^8 in a 4.0-ml Petri dish containing McCoy’s 5A supplemented medium, 10% fetal calf serum, and 10% WEHI-3 SCM. Cultures were passaged by 1:2 split every 3 days for 7 wk, 1:5 split for 2 wk, then 1:100. Cultures sustaining passage for 100 days were cloned in 0.8% methylcellulose according to previous procedures and subclonal lines established. Cell lines were designated permanent if generated subclonal lines grew under these conditions for over 70 days. DCM - dialyzed conditioned medium.

**Chemicals**

Phorbol myristate acetate (PMA) and 4-0-methyl PMA were obtained from Consolidated Midlands Chemical Co., Brewster, N.Y. These were stored in DMSO at 2 mg/ml and thawed freshly each week. PMA and 4-0-methyl PMA were diluted to an initial 20 μg/ml concentration then to the desired concentration in Fischer’s medium and added as a 0.1-ml volume to each culture at the time of weekly feeding to give a final concentration of 2.0 or 200.0 ng/ml.

**Histochemical and Hematologic Staining**

The methods for detection of myeloperoxidase, alkaline phosphatase, esterase-M (ASD-chloroacetate substrate) lysozyme, and superoxide by nitro blue tetrazolium (NBT) dye reduction have been described. Nonadherent cells from long-term marrow cultures or fresh marrow were tested in each of these methods. A method for NBT staining of cells in 0.3% agar by addition of reagents directly to the cultures has been previously described.

**Viruses and Virus Assays**

Friend leukemia virus anemia-inducing strain (FLV-A) and polycythemia-inducing strain (FLV-P) were generously provided by Dr. Charlotte Friend, Mt. Sinai Hospital, New York, N.Y. Virus stocks were prepared according to previously published methods. These were standardized to 10^9 polymerase induction units (PIU) per milliliter and stored at –85°C. A clonal strain of Rauscher murine leukemia virus designated M52R has previously been described. This virus causes only lymphatic leukemia after a mean latent period of 30 wk. Virus was titered on NIH/3T3 cells at 37°C and stored at –85°C. Virus in long-term marrow cultures was quantitated by an assay for virion-associated RNA-dependent DNA polymerase activity according to published procedures.

Virus was added to cultures at the time of initiation by addition of 0.1 ml containing 10^9 PIU to the contents of an adult mouse femur and tibia in 2.0 ml medium. These cells were then incubated in shallow monolayer for 2 hr with subsequent addition of 8.0 ml medium.

**RESULTS**

The effects of weekly addition of PMA or the relatively inactive tumor-promoting analogue, 4-0-methyl-PMA, to long-term marrow cultures were first evaluated. As shown in Fig. 1, uninfected control NIH Swiss long-term marrow cultures generated over 10^5 nonadherent cells per week through week 18. There was gradual decrease in cell number over subsequent weeks but morphologically mature granulocytes were generated to week 40. These results confirm and extend previous observations on NIH Swiss mouse marrow cultures that are intermediate in longevity between the extremely long-lived cultures derived from AkR/J, C58/J, and C57Ks/J strains and the relatively short-duration cultures derived from C3H/HeJ mice.
During the first 3–4 wk of culture, adherent cell islands were established and few nonadherent cells were generated. For this reason, PMA was not added until day 28. Following weekly addition of 2.0 ng/ml PMA (starting from week 4) to NIH Swiss marrow cultures, there was an initial increase in number of nonadherent cells removed between weeks 4 and 7 (Fig. 1). This was followed by a gradual but continued decrease in nonadherent cell numbers. No granulocytic cells were detected by week 30, and there were decreased numbers compared to uninfected cultures as early as week 16 (Fig. 1). PMA-treated cultures demonstrated increased numbers of adherent cells morphologically resembling macrophages and loss of the adipocyte colonies detected in the adherent cell compartment between weeks 12 and 20.

To determine whether a higher dose of PMA would accelerate this inhibition of hematopoiesis, other cultures were treated with 200.0 ng/ml PMA weekly starting from week 4. As shown in Fig. 1, cultures treated with 200 ng/ml demonstrated an increased number of nonadherent cells removed between weeks 4 and 6, with a subsequent rapid weekly decrease in total nonadherent cells removed to week 16. Nonadherent cells removed at week 16 demonstrated >90% macrophage morphology. Thus, PMA treatment at 2.0 ng/ml produced a decrease in the total number of nonadherent cells generated in long-term NIH Swiss mouse bone marrow cultures and shortened the duration of detectable hematopoiesis.

To determine whether the PMA effects described above were associated with tumor-promoting activity of the compound, other cultures were treated by weekly addition of 2.0 ng/ml of the compound 4-0-methyl-PMA. As shown in Table 1, 3.5 × 10⁵ cells/culture were removed at week 12 from 4-0-methyl-PMA-treated cultures compared to 0.8 × 10⁵ cells recovered from PMA-treated cultures. There were also higher numbers of nonadherent cells generated at week 16 in 4-0-methyl-PMA-treated cultures. The number of biologically detectable GM-CFU-c was significantly higher at weeks 8, 12, and 16 in 4-0-methyl-PMA-treated cultures compared to PMA-treated cultures (Table 1), and larger numbers of 10–49-cell GM clusters were formed by nonadherent cells removed at weeks 12–16 from 4-0-methyl-PMA compared to PMA-treated cultures. The GM colonies and clusters were grown in 0.3% agar in L-929-CSF. Cells removed at week 8 from PMA or 4-0-methyl-PMA-treated cultures failed to generate CFU-e, BFU-e, or CFU-GM-B-lymphocyte colonies, and control cultures generated only low numbers of BFU-e. Both PMA and 4-0-methyl-PMA-treated cultures generated cells over shorter duration and with less biologic activity than uninfected control cultures (Table 1). These data demonstrated that weekly addition of phorbol esters to long-term marrow cultures produced...
a decrease in biologically responsive as well as total numbers of nonadherent cells generated compared to control cultures. Furthermore, the more effective tumor promoter, PMA, was also more effective in this system compared to 4-0-methyl-PMA.

Effects of PMA on Myeloid Differentiation

The morphology of nonadherent cells removed from PMA-treated long-term bone marrow cultures was next evaluated. Cultures exposed to PMA, 2.0 ng/ml, demonstrated a relative increase in the number of nonadherent mononuclear cells to as high as 31% by week 12. In cultures treated with 200 ng/ml PMA, the increase in relative numbers of nonadherent mononuclear cells was detected earlier and to a higher 88% level. Treatment with 4-0-methyl-PMA also increased the percent of nonadherent mononuclear cells; however, to a level less than that detected with PMA (Table 2).

The nonadherent cells removed from each of 8 PMA-treated cultures were carefully examined for biologic, histochemical, and physiologic properties of mononuclear phagocytes. Nonadherent cells from each 12-wk-old culture treated with 2.0 ng/ml PMA were transferred to 4.0-ml plastic Petri dishes containing glass coverslips in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and allowed to adhere at 37°C, 7% CO₂, for 2, 4, or 24 hr. Coverslips were then tested for histochemically detectable esterase-M (ASD chloroacetate substrate specific) found in mature granulocytes but not macrophages38 and nonspecific esterase (alpha-naphthol butyrate) found in mononuclear phagocytes, lysozyme, NBT dye reduction, myeloperoxidase, and phagocytosis of added Candida albicans. Between 33% and 78% of cells adhered to glass coverslips and showed the characteristic surface spreading of macrophages. Histochemically detectable nonspecific esterase and lysozyme were present in 51%–58% and 63%–75% of cells, respectively, and 53%–58% were positive for NBT dye reduction. Fifteen to 25% of cells phagocytized C. albicans. There was no histochemically detectable myeloperoxidase. In contrast, nonadherent cells from control 12-wk cultures not exposed to PMA showed predominantly polymorphonuclear leukocyte morphology, and <10% of cells adhered to plastic coverslips within 24 hr. There were <20% cells positive for nonspecific esterase, and 20%–27% of cells stained positively for myeloperoxidase. Lysozyme, NBT dye reduction, esterase-M, and phagocytosis of C. albicans were detected in 17%–35% of nonadherent cells. The results indicated that nonadherent cells from PMA-treated cultures were predominantly functionally active mononuclear phagocytes.

Effects of PMA on GM-CFU-c Generated in Long-Term Marrow Cultures

The biologic properties of CFU-c detected in nonadherent cells removed from each PMA-treated group of cultures were next tested. As shown in Table 3, 35 7-day CFU-c from the 4-wk nonadherent cell population of control cultures were removed from agar culture and examined for morphology. Of these 35, 21 were pure macrophage, 6 were mixed granulocyte-macrophage, and 8 were pure granulocyte. These results confirm and extend previous reports of the effects of L-929-CSF on NIH Swiss long-term marrow culture derived CFU-c.31 The proportions of

<table>
<thead>
<tr>
<th>Virus* Added</th>
<th>Chemical Added</th>
<th>Number of Cultures</th>
<th>Differential Cell Count (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>8</td>
<td>41</td>
</tr>
<tr>
<td>PMA (2.0 ng/ml)</td>
<td>10</td>
<td>9</td>
<td>32</td>
</tr>
<tr>
<td>(200.0 ng/ml)</td>
<td>10</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>4-O-methyl PMA</td>
<td>8</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>FLV-A</td>
<td>None</td>
<td>10</td>
<td>45</td>
</tr>
<tr>
<td>R-MuLV</td>
<td>None</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>FLV-A</td>
<td>PMA (2.0 ng/ml)</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>R-MuLV</td>
<td>PMA (2.0 ng/ml)</td>
<td>25</td>
<td>11</td>
</tr>
</tbody>
</table>
pure G, pure M, and mixed GM-CFU-c were maintained to weeks 8 and 12. In contrast, cultures treated with PMA from week 4 demonstrated a significant increase in the relative proportion of pure macrophage CFU-c at weeks 8 and 12 (Table 3). Cultures treated with 200 ng/ml PMA or 4-O-methyl-PMA (2.0 ng/ml) also demonstrated increased relative proportions of pure macrophage CFU-c. Thus, PMA treatment increased both the number of mononuclear cells in the nonadherent cell population and the likelihood of macrophage differentiation of GM-CFU-c in those cell harvests.

**Effect of PMA on Granulocyte-Macrophage Colony Formation by Fresh Marrow GM-CFU-c**

The above experiments demonstrated that weekly addition of PMA to long-term marrow cultures was associated with detection of increased numbers of macrophages in the adherent cell compartment and increased numbers of pure macrophage CFU-c in the nonadherent cell compartment. These effects were associated with decreased longevity of hemopoiesis in the cultures. The effects of PMA on fresh bone marrow were next tested. As shown in Table 4, fresh marrow suspended in 0.3% agar supplemented with 10% L-929-CSF at 0.5 x 10^5, 1.0 x 10^6, or 2.0 x 10^6 cells/ml generated ≥50 cell colonies and 10-49-cell clusters in an increasing number proportional to cell plating density. In contrast, transfer of cells to agar cultures in the absence of added CSF produced spontaneous cluster and colony formation only at the higher densities. These latter results reflect the presence of adequate numbers of CSF-producing macrophages and other adherent cells at high plating densities. Treatment of cells with PMA in each of 2 doses of PMA or 4-O-methyl-PMA at the time of

**Table 3. Effect of PMA or 4-O-Methyl-PMA on Morphology of GM-CFU-c Produced in NIH Swiss Long-Term Marrow Cultures**

<table>
<thead>
<tr>
<th>Chemical (Dose)*</th>
<th>Total Colonies Removed</th>
<th>Total Colonies Removed</th>
<th>Total Colonies Removed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 4</td>
<td>Week 8</td>
<td>Week 12</td>
</tr>
<tr>
<td>None</td>
<td>G M GM</td>
<td>G M GM</td>
<td>G M GM</td>
</tr>
<tr>
<td>PMA (2.0 ng/ml)</td>
<td>35 8 21 6</td>
<td>50 6 39 5</td>
<td>52 3 41 8</td>
</tr>
<tr>
<td>NT</td>
<td>24 0 21 3</td>
<td>20 0 20 0</td>
<td>20 0 20 0</td>
</tr>
<tr>
<td>PMA (200.0 ng/ml)</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>4-O-methyl-PMA (2.0 ng/ml)</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

* Cultures established as described in Materials and Methods were treated with weekly PMA or 4-O-methyl-PMA starting from day 28. Cells were removed weekly and transferred to 0.3% agar cultures supplemented with 10% fetal calf serum and L-929-CSF (10%). Colonies of ≥50 cells were scored at day 7 following incubation in 7% CO2, 37°C. At this time, individual colonies were removed by Pasteur pipette under an inverted microscope for microscopic scoring at day 7 following incubation as G. and those 'anUloCies (Unitron), smeared onto glass coverslips, and stained with Wrights/Giemsa. At least 20 cells per colony were visualized by light microscope, oil immersion 1000x, and categorized as granulocyte or macrophage. Colonies with all cells scored as macrophage are designated M, those of pure granulocytes as G, and those mixed colonies as GM. NT, not tested.

**Table 4. Effect of PMA on Spontaneous GM-CFU-c and GM-Cluster Formation by Fresh NIH Swiss Mouse Marrow and Nonadherent Cells From Long-Term Marrow Cultures**

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>Additive† (mg/ml)</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBM</td>
<td>None</td>
<td>Colonies</td>
<td>Clusters</td>
<td>Colonies</td>
<td>Clusters</td>
<td>Colonies</td>
<td>Clusters</td>
</tr>
<tr>
<td>PMA (2.0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6 ± 1</td>
<td>2 ± 1</td>
<td>1 ± 0.2</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>PMA (200.0)</td>
<td>1 ± 0.1</td>
<td>0</td>
<td>2 ± 1</td>
<td>11 ± 3</td>
<td>133 ± 13</td>
<td>6 ± 1</td>
<td></td>
</tr>
<tr>
<td>4-O-methyl-PMA (2.0)</td>
<td>0</td>
<td>3 ± 1</td>
<td>2</td>
<td>18 ± 2</td>
<td>3 ± 0.1</td>
<td>69 ± 6</td>
<td></td>
</tr>
<tr>
<td>10% CSF</td>
<td>113 ± 8</td>
<td>152 ± 6</td>
<td>167 ± 13</td>
<td>215 ± 17</td>
<td>203 ± 21</td>
<td>315 ± 29</td>
<td></td>
</tr>
<tr>
<td>FBM</td>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PMA (2.0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PMA (200.0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10% CSF</td>
<td>159 ± 10</td>
<td>217 ± 24</td>
<td>219 ± 8</td>
<td>301 ± 9</td>
<td>251 ± 17</td>
<td>341 ± 14</td>
<td></td>
</tr>
</tbody>
</table>

* Colonies of ≥50 cells and clusters of 10-49 cells were scored at day 7. Results are expressed as the mean ± SEM for at least 4 plates at each point.
† Fresh bone marrow (FBM) was removed from adult NIH Swiss mouse femurs and single cell suspensions prepared as described by drawing cells through successively smaller gauge needles to a 30-gauge needle in 5.0 ml McCoy's 5A medium. Cells were transferred to 0.3% agar cultures in the indicated final plating densities. Nonadherent cells were removed from long-term NIH Swiss mouse cultures at time of the eighth weekly medium change. These were transferred to a cell pellet and plated at the indicated densities alone or in the presence of 10% L-929-CSF.
‡ Each chemical was added as a 0.1-ml (tenfold) concentrate to the fresh agar culture at the time of plating the cells.
transfer to agar cultures, resulted in spontaneous cluster formation and increased colony formation at lower plating densities. In particular, clusters were detected at 0.5 × 10⁴ cells/ml, a density where none were detected in control cultures. These results suggested three possible effects of PMA: induction of spontaneous colony and cluster formation by direct PMA action on the GM-CFU-c; indirect action of PMA through induction of CSF from other cells present in the fresh bone marrow; or indirect action of PMA through sensitizing the GM-CFU-c to respond to a lower level of CSF.

In an attempt to separate the first from the other two explanations, nonadherent cells from long-term bone marrow cultures were enriched for GM-CFU-c by Ficoll-Hypaque density centrifugation. The nonadherent cells from 8-wk NIH Swiss long-term bone marrow cultures have been shown to have ~1% macrophages, fibroblasts, and other adherent cells with a 5–10-fold increase in relative numbers of GM-CFU-c as compared with fresh bone marrow. This purity is only reliably observed when the long-term marrow cultures are maintained from weeks 4 to 8 in fetal calf serum. Nonadherent cells from 8-wk cultures were harvested, pelleted by centrifugation, and resuspended in 0.3% agar at each of the cell densities indicated in Table 4. As shown in Table 4, there were no detectable spontaneous colonies or clusters forming at plating densities up to 2.0 × 10⁵ cells/ml. Addition of PMA (2.0 or 200.0 ng/ml) failed to induce spontaneous cluster or colony formation. There was no effect of 2.0 ng/ml PMA on shifting or altering the shape of the dose–response curve of 10⁴ NIH Swiss purified GM-CFU-c to increasing doses of L-cell or WEHI-3 CSF over a range of 0.1%–20% CSF (data not shown). These results indicated that the mechanism by which PMA increased colony and cluster formation in fresh marrow was indirect and mediated through some cell population that was absent from GM-CFU-c-enriched nonadherent cells from long-term bone marrow cultures, but present in fresh whole marrow. Alternatively, the transition of myeloid progenitor cells to macrophages, including cells capable of forming macrophage clusters in semisolid medium, might not require cell division or a humoral factor released by PMA action on adherent cells. If this were the case, then the failure to detect macrophage clusters in agar by PMA-treated purified populations of GM-CFU-c would indicate the absence of a specific subpopulation of multipotential stem cells that respond to PMA by differentiating to macrophages.

**PMA-Treated Macrophages Induce GM-Cluster Formation by Purified GM-Progenitor Cells**

To demonstrate that PMA was acting through adherent cells in fresh marrow to stimulate GM-CFU-c and cluster formation, nonadherent cells from long-term bone marrow cultures (responder cells) were plated at each of several densities with addition of increasing numbers of peritoneal exudate macrophages (stimulator cells) harvested according to published procedures. As shown in Table 5, addition

<table>
<thead>
<tr>
<th>Macrophages Added (x 10⁵)</th>
<th>Phorbol (µg/ml)</th>
<th>Colonies</th>
<th>Clusters</th>
<th>Colonies</th>
<th>Clusters</th>
<th>Colonies</th>
<th>Clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 x 10⁵</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6 ± 1</td>
<td>73 ± 11</td>
<td>21 ± 6</td>
</tr>
<tr>
<td>5 x 10⁴ PMA (2.0)</td>
<td>0</td>
<td>2 ± 0.1</td>
<td>13 ± 1</td>
<td>27 ± 5</td>
<td>163 ± 18</td>
<td>48 ± 13</td>
<td>216 ± 37</td>
</tr>
<tr>
<td>5 x 10⁴ 4-O-methyl-PMA</td>
<td>0</td>
<td>3 ± 0.3</td>
<td>83 ± 5</td>
<td>83 ± 5</td>
<td>27 ± 8</td>
<td>189 ± 14</td>
<td></td>
</tr>
<tr>
<td>1 x 10⁴</td>
<td>—</td>
<td>0</td>
<td>13 ± 2</td>
<td>15 ± 2</td>
<td>97 ± 11</td>
<td>53 ± 11</td>
<td>211 ± 31</td>
</tr>
<tr>
<td>1 x 10⁴ PMA (2.0)</td>
<td>0</td>
<td>7 ± 0.8</td>
<td>28 ± 3</td>
<td>47 ± 4</td>
<td>218 ± 52</td>
<td>76 ± 8</td>
<td>227 ± 15</td>
</tr>
<tr>
<td>1 x 10⁴ 4-O-methyl-PMA</td>
<td>0</td>
<td>2 ± 0.2</td>
<td>26 ± 2</td>
<td>19 ± 3</td>
<td>187 ± 15</td>
<td>61 ± 15</td>
<td>219 ± 43</td>
</tr>
<tr>
<td>2 x 10⁴</td>
<td>—</td>
<td>3 ± 0.1</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>2 x 10⁴ PMA (2.0)</td>
<td>0</td>
<td>31 ± 5</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>2 x 10⁴ 4-O-methyl-PMA</td>
<td>0</td>
<td>18 ± 2</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
</tbody>
</table>

*Nonadherent cells from 8-wk NIH Swiss long-term marrow cultures were harvested and plated at the densities indicated and according to the legend to Table 4. Results are the mean ± SEM of at least 4 plates at each point.

†Peritoneal exudate macrophages were harvested from adult NIH Swiss mice according to published procedures. 24 hr after intraperitoneal inoculation of 2.0 ml casein. Cells were washed in ice-cold McCoy's 5A medium and differential cell counts performed. Polymorphonuclear leukocytes were removed by Ficoll-Hypaque density centrifugation and the resultant macrophage-enriched fraction tested by differential cell count. This was >95% pure. Macrophages were added to each culture at the indicated numbers at the time of plating responder cells.

‡Each chemical was added freshly to agar cultures at the time of plating as described in the legend to Table 4. NT, not tested.
of 5 × 10⁴ peritoneal exudate macrophages to 5.0 × 10⁴ responder cells failed to induce spontaneous colony or cluster formation; however, colonies and clusters were detected with a higher responder cell population, 10⁵. In contrast, 5.0 × 10⁴ PMA-treated peritoneal exudate macrophages did produce detectable colony and cluster formation by 5.0 × 10⁴ responder cells. With 2.0 × 10⁴ responder cells, greater numbers of colonies and clusters were detected at a given macrophage cell number in the presence of PMA compared to that number cocultivated without PMA.

Increasing the number of macrophages to 10⁵ produced more colonies and clusters by a given number of responder cells, and this effect was also increased by addition of PMA (Table 5). Addition of 4-O-methyl-PMA produced a lower but detectable increase over control in colonies and clusters compared to PMA. Spontaneous cluster formation by macrophages alone was seen at the high plating density, 2.0 × 10⁵, and was increased by treatment of the macrophages with PMA (Table 5; Fig. 2). These data indicate that PMA stimulation of colony and cluster formation by bone marrow CFU-c in the absence of added CSF was only detected in the presence of adherent cells including macrophages. PMA was not completely removed from our treated macrophages before cocultivation with purified GM-CFU-c. Therefore, these experiments do not rule out the possibility that PMA was altered by macrophages and macrophage cluster-forming cells in the stimulator-cell fraction and then induced purified responding cells to form clusters in agar.

**PMA Fails to Simulate Growth of the DCM-Dependent Acute Promyelocytic Leukemia Cell Line FLVA-161V Clone 20**

The above data indicated that the PMA effect on stimulation of colony formation by GM-CFU-c was indirect and required other cells in addition to purified GM-CFU-c. To further explore the possibility of an additional direct effect of PMA on the GM-CFU-c target cell, PMA was tested for its ability to stimulate proliferation of the DCM-dependent clonal acute promyelocytic leukemia cell line FLVA-161V clone 20. 3 This cell line has been passaged in suspension culture for over 2 yr in the presence of 10% WEHI-3 DCM, upon which its growth is dependent. 3 As shown in Table 6, cells plated at 10⁴ cells per Petri dish in 10% WEHI-3 DCM demonstrated growth over a period of 4 wk. Transfer of cells to McCoy's 5A supplemented medium in the absence of DCM resulted in cell death. Cells were next transferred to medium supplemented with 10% L929 cell DCM, PMA 2.0 ng/ml, 200 ng/ml, or each of the combinations listed in Table 6. Only WEHI-3 DCM stimulated growth of FLVA-161V clone 20 cells. Exposure of triplicate groups of cultures of FLVA-161V clone 20 cells in no PMA, 2.0 ng/ml, or 200 ng/ml PMA, respectively, to several concentrations of WEHI-3 DCM from 10% to 0.1% was carried out. There was no detectable difference in the minimum required DCM concentration (1.0%) between groups. There was no detectable differentiation of FLVA-161V clone 20 cells to either mature polymorphonuclear leukocytes
or macrophages following exposure to PMA. Rather, cells grown in PMA-supplemented medium in the absence of WEHI-3 DCM disintegrated over the subsequent 10 days.

**PMA Effects on Murine Leukemia Virus-Infected Long-Term Marrow Cultures**

Addition of FLV-A to long-term marrow cultures accelerated generation of increased numbers of nonadherent cells compared to uninfected controls (Fig. 3). Similarly, addition of R-MuLV to other cultures produced an increase over control in total cells generated. Nonadherent cells were generated in virus-infected cultures for periods in excess of 50 wk, while control cultures had markedly run down by week 40 (Fig. 3). Nonadherent cells removed from each of 4 FLV-A-infected cultures at week 6 generated permanent granulocytic leukemia cell lines. In marked contrast, nonadherent cells similarly removed from R-MuLV-infected cultures at weeks 4, 5, 6, or 7 failed to generate permanent cell lines. Furthermore, nonadherent cells removed at week 20 from FLV-A- or R-MuLV-infected cultures failed to generate permanent leukemia cell lines. These data confirm and extend our prior studies and indicate that permanent granulocytic cell lines are induced from the early harvests of cultures infected by FLV-A but not R-MuLV.

To determine whether addition of PMA to R-MuLV-infected cultures would act as a tumor promoter and stimulate generation of permanent cell lines, the following experiment was designed. Groups of 16 cultures were infected with FLV-A or R-MuLV clone M52R. Results are presented for total nonadherent cells removed as described in the legend to Fig. 1 for (a) uninfected controls, or cultures infected with (e) FLV-A or (a) R-MuLV (M52R). Asterisk indicates number of culture harvests that established a secondary suspension culture line that was cloned in methylcellulose and sustained 100 days passage in vitro over the number of cultures tested at that time point.

**Table 6. Effect of PMA on Growth of DCM-Dependent AML Cell Line FLVA 16 IV Clone 20**

<table>
<thead>
<tr>
<th>Medium + 10% FCS Supplemented With</th>
<th>Cell Number per Culture (× 10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nothing</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>10% WEHI-3 DCM</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>10% L929 cell DCM</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>PMA, 2.0 ng/ml</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>PMA, 200 ng/ml</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>10% L929 cell</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>DCM + PMA</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>(2.0 ng/ml)</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>10% L929 cell DCM + PMA</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>(2.0 ng/ml)</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

* Results are presented as the mean ± SEM of total cells generated over 4 wk for at least 3 plates counted at each point. NT, not tested.

Fig. 3. Effect of FLV-A or R-MuLV (clone M52R) on hemopoiesis in long-term bone marrow cultures. Groups of 8 cultures established according to Materials and Methods were left uninfected or were infected at initiation with 10⁵ PIU of FLV-A or R-MuLV clone M52R. Asterisk indicates number of culture harvests that established a secondary suspension culture line that was cloned in methylcellulose and sustained 100 days passage in vitro over the number of cultures tested at that time point.
Fig. 4. Effect of PMA on FLV-A leukemogenesis in long-term marrow cultures. Groups of 8 cultures were left uninfected, infected at initiation with 10^6 PIU of FLV-A, or were treated with addition of PMA weekly at day 28 following initial infection with virus. Results are for the mean of total cells removed from at least 4 cultures at each point as described in the legend to Fig. 1. (c) Infected with FLV-A; (s) FLV-A-infected plus weekly addition of PMA (2.0 ng/ml); (c) FLV-A-infected plus weekly addition of PMA (200 ng/ml); (c) uninfected and untreated control. (*) Number of culture harvests generating permanent cell line/number tested.

Fig. 5. Effect of PMA on R-MuLV (MS2R) infection in long-term marrow cultures. Groups of 8 cultures, prepared as described in the legend to Fig. 1, were infected with 10^6 PIU of R-MuLV (MS2R) at initiation and were either left untreated or were treated with weekly addition of PMA starting from day 28 after infection. Results are for the mean total cells removed from 4 cultures at each point: (c) infected with R-MuLV (MS2R); (c), R-MuLV (MS2R) infected with weekly addition of PMA (2.0 ng/ml); (c), R-MuLV (MS2R) infected with weekly addition of PMA (200 ng/ml); or (c) infected and untreated control. (*) Number of culture harvests generating permanent cell line/number tested was % in each instance.

cultures with addition at that time of 10^6 WEHI-3 DCM plus 2.0 or 200.0 ng/ml PMA also did not result in generation of permanent cell lines.

Virus-infected cultures that were treated with PMA demonstrated a combination of effects distinct from cultures receiving one agent (Table 2). While there were increased numbers of immature granulocytic cells generated in FLV-A-infected cultures exposed to PMA at weeks 4, 8, and 12, the relative number (16%) was less than that found in virus-infected cultures alone (41%) at the same 12-wk time point. There were also increased numbers of mononuclear cells (20%) at week 12 in FLV-A-infected PMA-treated cultures compared to uninfected cultures (6%). FLV-A-infected cultures exposed to PMA generated ~90% pure macrophage GM-CFU-c after week 12, while cultures infected with FLVA but not exposed to PMA generated 20%–50% pure granulocytic and mixed GM-CFU-c, and 50%–80% pure macrophage CFU-c.

DISCUSSION

Tumor-promoting phorbol esters have been intensively investigated for the ability to modulate growth of mammalian cells. The growth of established cell lines in semisolid medium is altered by PMA.\(^1\) Reported effects of PMA on cell differentiation are also of great interest. PMA has been shown to inhibit differentiation of erythroleukemia cells,\(^6\) melanoma cell lines,\(^11\) an adipocyte cell line,\(^10\) neuroblastoma cells,\(^9\) and a clone of myeloid leukemia cells.\(^3\) Other studies have demonstrated that PMA can paradoxically stimulate differentiation of a distinct erythroleukemia clonal cell line,\(^8\) human or mouse myeloid leukemia cell lines,\(^31\) and melanoma cells.\(^44\) Biochemical effects of PMA include stimulation of respiratory burst activity in macrophages\(^30\) and polymorphonuclear leukocytes\(^47\) and increases in the synthesis of plasminogen activator and ornithine decarboxylase\(^30\) and enzymes associated with cellular differentiation.\(^32,53\)

The present studies were carried out to determine whether PMA would affect hematopoiesis in long-term bone marrow cultures and promote leukemogenesis by murine leukemia viruses. NIH Swiss mouse bone marrow cultures treated weekly with PMA and fresh bone marrow cells exposed to PMA demonstrated an increase in proliferation of adherent macrophages. There was an associated decrease in the numbers of nonadherent cells generated in long-term bone marrow cultures exposed to PMA. Increased numbers of pure macrophage CFU-c and mononuclear cells were detected in the nonadherent cell compartment, indicating either macrophage differentiation of nonadherent cells or release of macrophages from the adherent cell compartment.
To determine whether PMA demonstrated a direct effect on purified GM-CFU-c, nonadherent cells removed from long-term NIH Swiss marrow cultures were exposed to PMA immediately after transfer to 7-day GM-CFU-c assay. Under these conditions, no spontaneous colony or cluster formation was detected with purified GM-CFU-c, and no further colonies were detected in L929 or WEHI-3 CSF-stimulated cultures also exposed to PMA. Only following addition of macrophages or other marrow adherent cells was there a detectable PMA-induced increase in numbers of GM colonies and clusters. These results indicate that one effect of PMA in long-term marrow cultures as well as in fresh marrow is mediated through adherent cells including the macrophage. These results are consistent with data recently reported by Lotem and Sachs for fresh mouse marrow exposed to PMA. It is not known whether PMA only stimulates macrophage release of CSF or sensitizes the GM-CFU-c target cell to respond to a lower concentration of a CSF distinct from that produced by L929 or WEHI-3 cells. However, PMA did not induce GM-CFU colony and cluster formation by NIH Swiss mouse purified GM-progenitor cells in the absence of a source of CSF. Since significant mouse strain-specific differences are observed in long-term marrow cultures, it is possible that purified GM-CFU-c from other strains will show different responses to PMA in vitro. These studies are in progress.

The effects of RNA type C murine leukemia viruses (retroviruses) on hemopoiesis are a subject of much investigation. Viruses that naturally cause erythroid or lymphoid leukemia have been shown to induce granulocytic leukemia in long-term marrow cultures favoring granulocyte differentiation of pluripotent hemopoietic stem cells. In particular, 2 classes of viruses have been described with ability to produce either complete transformation generating permanent leukemic cell lines or dysmyelopoiesis associated with increased longevity of cultures, total cells generated, and dysplastic morphology of granulocytes.

Addition of PMA to FLV-A-infected cultures did not inhibit leukemogenesis. The earlier removal of cells capable of generating permanent cell lines in some FLV-A cultures treated with PMA should not necessarily be interpreted as a promoting effect of the phorbol esters in this setting and is more likely attributable to release of virally transformed cells from the adherent cell compartment. PMA did not stimulate R-MuLV-infected cultures to generate leukemia cell lines and could not replace the absolute growth requirements of FLV-A-transformed cell line FLVA161V clone 20 for WEHI-3 cell DCM. Furthermore, inhibitory effects of PMA on the duration of hemopoiesis in primary marrow cultures were still present in cultures infected with FLV-A or R-MuLV.

Whether the observed effects of PMA on bone marrow cultures are related to the tumor-promoting activity of the phorbol esters and whether PMA has similar biologic effects on hemopoiesis in vivo is unknown. In this regard there is evidence that PMA may not function as a tumor promoter for leukemogenesis in vivo. Effects of PMA on the cell phenotypes found in the present long-term marrow cultures might be altered or balanced in vivo by PMA effects on other cell phenotypes that are absent from the cultures, such as mature erythroid cells, lymphocytes, osteoblasts, and plasma cells. Whether PMA stimulated release of a macrophage CSF in the marrow cultures then causes production of inhibitors of normal granulopoiesis is not known; however, our present results would be consistent with such a mechanism. It is of interest that the effects of PMA on long-term marrow cultures were similar to those observed following infection with murine sarcoma viruses, in that both conditions indirectly depressed granulopoiesis through an action on the stromal component of the cultures. A common mechanism of action of murine retroviruses and PMA may involve alteration of cellular metabolic cooperation, in this case, between stromal and hemopoietic stem cells in the adherent cell compartment of the cultures. A balance between stromal and hemopoietic stem cells has been shown to be absolutely required for prolonged in vitro hemopoiesis. Further studies of the effects of phorbol esters on different populations of marrow cells may provide insight into the regulation of hemopoiesis and the physiologic events during viral leukemogenesis.

ACKNOWLEDGMENT

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NOTE ADDED IN PROOF

The present and previous cloned permanent promyelocytic leukemia cell lines and control short-term cultures of nonadherent cells removed from primary NIH Swiss mouse bone marrow cultures were initiated and maintained in McCoy's 5A medium supplemented with a 10% volume of WEHI-3 cell conditioned medium that had been dialyzed in Spectrapor* dialysis tubing (Fisher Scientific Corp., Pittsburgh, Pa., CAT #8667E, pore size 4.8 nm, which retains materials of -12,000 m.w.) against a 100-fold excess volume of distilled water for 48 hr and then concentrated five-fold by pressure using a model 402 filtration unit with a PM10 filter that retains materials of -10,000 m.w. (Amicon Corp., Lexington, Mass.). This concentrated WEHI-3 DCM (dialyzed conditioned medium) is indistinguishable from the preparation used as a source of WEHI-3 cell CSF.
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Phorbol myristate acetate stimulates macrophage differentiation and replication and alters granulopoiesis and leukemogenesis in long-term bone marrow cultures

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