Regulation of Myelopoiesis In Vitro: Partial Replacement of Colony-Stimulating Factors by Tumor-Promoting Phorbol Esters


Tumor-promoting phorbol esters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or phorbol-12,13-didecanoate (PDD) stimulate freshly isolated murine bone marrow cells in vitro to form colonies of myeloid cells in semisolid medium without added colony-stimulating factors (CSF), while phorbol and its nonpromoting derivatives do not induce marrow colony formation. TPA-stimulated colonies are composed primarily of monocyte-macrophage cells with admixed granulocytes in some colonies, and cell dose–response curves for colony formation suggest clonal origin of the colonies. The cells forming colonies in the presence of TPA alone appear to be a subset of marrow granulocyte-macrophage colony-forming cells (GM-CFC) characterized by larger mean cell size (determined by velocity sedimentation), and TPA-stimulated colony formation is inhibited by dexamethasone and prostaglandin E,
5 agents that also inhibit GM-CFC colony formation stimulated by CSF. The active phorbol esters can apparently replace exogenous CSF in agar cultures of murine bone marrow and may do so by either (1) directly stimulating GM-CFC, (2) inducing endogenous CSF secretion, or (3) increasing GM-CFC’s responsiveness to CSF. We were unable to demonstrate induction of CSF secretion by marrow cells in liquid culture in the presence of TPA. We did find that TPA synergistically enhanced colony formation stimulated by suboptimal concentrations of CSF from two sources, and this effect of TPA was concentration dependent. PDD also enhanced colony formation stimulated by suboptimal CSF concentrations, while phorbol had no effect. In long-term flask cultures of normal murine marrow, TPA caused rapid, concentration-dependent, terminal differentiation (to macrophages), and decline in the progenitor cell compartment. Our data indicate that tumor-promoting phorbol esters may alter the function of cell surface receptors for CSF on GM-CFC, causing stimulation of proliferation and differentiation in the absence of CSF or at very low concentrations of CSF. These compounds should be important in the study of hematopoietic regulation.

AMONG normal bone marrow cells, there are single cells that produce clonal colonies of differentiated granulocytes and/or macrophages in semisolid medium cultures.1,2 These granulocyte-macrophage progenitor cells, designated granulocyte-macrophage colony-forming cells (GM-CFC) are morphologically unidentifiable and relatively rare (< 0.5% of marrow cells), but can be detected and enumerated in colony-forming assays such as the soft agar system. The proliferation of GM-CFC and differentiation of their progeny require the presence of specific glycoprotein colony-stimulating factors (CSF) as well as serum-containing culture medium.3,4 In most species, CSF-producing cells are present in marrow cell populations, and endogenous colony formation is observed when high cell concentrations are cultured. Mouse bone marrow, however, does not seem to contain cells producing sufficient CSF to stimulate endogenous colony formation by GM-CFC, and colony formation occurs only in the presence of exogenously supplied CSF.

We recently reported that tumor-promoting phorbol esters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) but not their nonpromoting analogs, stimulate mouse bone marrow cells to form myeloid colonies in agar cultures without added CSF.5 Our initial attempts to define the mechanism of action of the phorbol esters form the basis of this report.

MATERIALS AND METHODS

Cells

Bone marrow cells from B6D2F1, mice (Cumberland View Farms, Inc.) were obtained by perfusing the femoral marrow cavity with cold McCoy’s 5A medium and pipetting repeatedly to obtain a single cell suspension. Cell counts were performed using standard hemocytometer technique or an electronic cell counter (Coulter Electronics, Inc., Hialeah, Fla.) and viability determined by Trypan blue dye exclusion.

Colony Assay

Marrow cells were cultured in McCoy’s 5A (modified) medium (Grand Island Biological Company [GIBCO], Grand Island, N. Y.) supplemented with sodium pyruvate, essential and nonessential amino acids, vitamins, and 15% fetal calf serum (GIBCO), and containing 0.3% Bacto-agar (Difco Laboratories, Detroit, Mich.). This mixture was added, in 1.0-mL aliquots, to 35-mm plastic Petri dishes (Lux, M. A. Bioproducts, Walkersville, Md.) containing colony-stimulating factors and/or phorbol derivatives and/or other test substances. After 7 days incubation at 37°C in a humidified atmosphere of 7.5% CO2 in air, discrete cellular aggregates of ≥ 50 cells were scored as colonies using a dissecting microscope (Olympus Optical Co., Ltd., N. Y.) at 25× magnification. For morphological analysis of colony cells, sequential individual colonies were picked.
out of the agar with a micropipettor, transferred to glass slides, stained with aceto-orcein, and examined microscopically at 100–400× magnification. Colonies were scored as pure macrophage or pure neutrophil if >95% of the colony cells were of one type, and mixed colonies otherwise.

**Colony-Stimulating Factors**

Colony-stimulating factors were obtained from media conditioned by cells of the WEHI-3 myelomonocytic cell line or the L-cell line. The cell lines were individually cultured in flasks at 37°C, 5% CO₂, in McCoy’s 5 A medium supplemented with glutamine and 2% fetal calf serum (FCS). Medium was harvested once or twice weekly and pooled. Batches of conditioned medium were dialyzed for 3 days at 4°C against glass distilled water and concentrated by ultrafiltration or lyophilization and resuspension. Alternatively, some batches were desalted by Sephadex G-50 gel filtration and lyophilized, a method that gave equivalent results. All batches were assayed for activity and stored at −20°C. Except where specified otherwise, CSF was added at concentrations previously demonstrated to maximally stimulate colony formation ("plateau" levels).

**Liquid Marrow Cultures**

Freshly isolated marrow cells at 10⁶/ml were cultured in flasks in supplemented McCoy’s 5A with 15% FCS at 37°C, 5% CO₂ for 1–5 days. Some of the liquid marrow cultures also contained 10⁻¹ M TPA or equivalent solvent (DMSO) addition. In addition, some flasks contained the identical McCoy’s medium and TPA but no cells. After the appropriate culture period, the supernatant medium was harvested, either dialyzed against distilled water as above or held at 4°C for 3 days, and stored at −70°C. The media were assayed simultaneously for colony-stimulating activity by adding 0.1 ml to 35-mm plates and overlaying with 10 ml of fresh medium containing 10 freshly isolated marrow cells in agar medium for incubation and subsequent colony counts.

**Defined Reagents**

Phorbol, 12-0-tetradecanoyl-phorbol-13-acetate (TPA), and phorbol-12,13-didecanoate (PDD) (Consolidated Midland Corp., Brewster, N. Y.) were stored at −70°C in 102 M solutions in dimethylsulfoxide (DMSO). They were diluted just before use in the McCoy’s 5A culture medium (without agar) and added to the plates with precautions against excessive light exposure. Dexmethasone, prostaglandin E₁, and prostaglandin F₂ (Sigma Chemical Co., St. Louis, Mo.) were stored at −70°C in 10⁻¹ M solutions in absolute ethanol and diluted with culture medium just before use.

**Velocity Sedimentation**

Freshly isolated marrow cells were fractionated by velocity sedimentation at unit gravity at 4°C, using the Statup apparatus (O. H. Johns Scientific, Toronto, Canada), as described by others. Recovery of viable cells in replicate experiments ranged from 68% to 95%. Equal numbers of cells from each fraction were cultured in the agar colony assay in the presence of CSF from WEHI-3-conditioned medium or 10⁻¹ M TPA, and the total number of colony-forming cells per fraction calculated.

**Long-Term Marrow Culture**

Long-term marrow culture in flasks was carried out as described. Briefly, the contents of a single mouse femur or two tibias were flushed into 25 sq cm plastic flasks (Corning) containing 10 ml of Fischer’s medium (GIBCO) supplemented with glutamine, antibiotics, and 20% prescreened horse serum (GIBCO). The flasks were sealed after gassing with 5% CO₂ in air and incubated at 33°C. Weekly feedings were carried out by removal of half the medium and nonadherent cells and replacement with fresh medium. At the third feeding, all the medium and suspended cells were replaced with 10 ml of fresh medium containing 10² freshly isolated marrow cells. Weekly feedings, as before, then allowed assay of the removed nonadherent cells for cell number, differential count, and number of GM-CFC.

**RESULTS**

As previously reported, mouse bone marrow forms colonies of macrophages with or without granulocytes in the presence of low concentrations (10⁻⁷ to 10⁻¹ M) of active tumor-promoting phorbol esters. The phorbol ester-stimulated colonies are rather uniform, discrete aggregates of 50–1000 cells which, by morphological and functional criteria, are mainly mature macrophages with lesser numbers of monocytes, neutrophils, and immature cells (Table 1). Figure 1 shows a linear relationship between numbers of cells plated and colonies formed in the presence of two active phorbol esters, TPA, and phorbol-12,13-didecanoate (PDD), suggesting single cell (clonal) origin of the colonies. No colonies developed in the presence of the nonpromoting parent compound, phorbol. It is also apparent that the efficiency of marrow colony formation at this previously determined optimal concentration of phorbol ester (10⁻¹ M) is lower than that seen with L-cell-derived CSF (LC-CSF).

These observations suggested that the target cell for the TPA effect might be either totally different from the LC-CSF responsive GM-CFC or a subpopulation thereof. To test these possibilities, marrow was cultured in dishes containing a plateau level of LC-CSF with or without 10⁻¹ M TPA. In multiple experiments, the addition of the TPA altered the colony morphology slightly ("tighter" aggregates), but did not increase the number of colonies seen with plateau

**Table 1. Properties of Cells From TPA-Stimulated Colonies**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Percent ± SE</th>
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<tbody>
<tr>
<td>Morphology</td>
<td></td>
</tr>
<tr>
<td>Macrophage</td>
<td>81.0 ± 4.0</td>
</tr>
<tr>
<td>Monocyte</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>12.0 ± 1.0</td>
</tr>
<tr>
<td>Immature</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td>Esterase positive</td>
<td>86.0 ± 5.0</td>
</tr>
<tr>
<td>Adherence to plastic (1 hr, 37°C):</td>
<td>60%</td>
</tr>
<tr>
<td>Phagocytosis (latex particles, 25°C):</td>
<td>75% 1 hr</td>
</tr>
<tr>
<td></td>
<td>90% 18 hr</td>
</tr>
</tbody>
</table>

*Mouse marrow cells in supplemented McCoy’s 5A with 15% FCS, 10⁻¹ M TPA and 0.8% methylcellulose were incubated for 7 days at 37°C, 5% CO₂. Colony cells were collected by dilution and aspiration. See Stuart and Hamilton.*
levels of LC-CSF alone (data not shown). The latter was consistent with the possibility that the active phorbol esters alone were causing colony formation by a subpopulation of GM-CFC, rather than by a different class of marrow cells.

Further support for this possibility was obtained in velocity sedimentation experiments. Fresh mouse bone marrow was fractionated by velocity sedimentation at unit gravity\(^1\) and each fraction assayed for colony formation in the presence of CSF or TPA. Figure 2 shows a representative experiment. We discovered that TPA-responsive colony-forming cells were found in more rapidly sedimenting fractions than the majority of the CSF-stimulated colony-forming cells (GM-CFC). Most interestingly, in the more rapidly sedimenting fractions (6–10 mm/hr), there was a virtual one-to-one correspondence between TPA-responsive colony-forming cells and CSF-responsive GM-CFC, while the slowest sedimenting fractions (3–4.5 mm/hr) contained CSF-responsive GM-CFC but no detectable TPA-responsive colony-forming cells. Intermediate fractions (4.5–6 mm/hr) contained both types of cells but fewer TPA-responsive cells. The cells forming colonies in the presence of TPA may, therefore, be a subset of GM-CFC characterized by larger mean cell size.

If TPA-responsive colony-forming cells are in fact GM-CFC, it is reasonable to expect that TPA-

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Fig. 1. Colony formation (≥ 50 cells) by normal murine bone marrow cells in the presence of "plateau" concentrations of LC-CSF (○), or 10\(^{-7}\) M concentrations of TPA (□), PDD (△), or phorbol (▽). Shown are results of triplicate 7-day cultures (mean ± SE) at varying cell concentrations from 1.5 x 10\(^3\) to 200 x 10\(^3\) per 1.1 ml culture. No colonies developed in cultures containing appropriate concentrations of solvent (DMSO).

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Fig. 2. Fractionation of normal murine bone marrow by velocity sedimentation at unit gravity and enumeration of total colony-forming cells (CFC) in each fraction in the presence of WEHI-3 CSF (△) or 10\(^{-7}\) M TPA (□). Shown are mean values of triplicate cultures (2.5 x 10\(^6\) cells/plate) for each fraction.
stimulated colony formation should be inhibited by agents that inhibit GM-CFC-derived colony formation by a direct action on GM-CFC. Such agents include corticosteroids\(^1\) and prostaglandins of the E series.\(^2\) When we cultured fresh marrow cells in the presence of \(5 \times 10^{-8} M\) TPA and graded concentrations of dexamethasone, prostaglandin E\(_1\) (PGE\(_1\)), or prostaglandin F\(_{2\alpha}\) (PGF\(_{2\alpha}\)), we observed marked concentration-dependent inhibition of colony formation by dexamethasone and PGE\(_1\), but virtually no effect with PGF\(_{2\alpha}\) (Fig. 3). We tentatively concluded that at least some murine marrow GM-CFC are capable of colony formation in the presence of tumor-promoting phorbol esters without added CSF\(_1\).

A possible explanation of our observations is that the active phorbol esters might act on marrow cells to induce the production or secretion of glycoprotein CSF, which in turn stimulates the GM-CFC. We attempted to test this hypothesis by exposing marrow cells in liquid culture to appropriate concentrations of TPA for varying periods of incubation. The cell-free supernatants were then collected and assayed for colony-stimulating activity (CSA) in the soft agar system using fresh marrow cells as targets (Table 2). Supernatants were also assayed after dialysis against distilled water in what proved to be futile attempts to remove the low molecular weight TPA from the media. We found in repeated experiments that supernatants from liquid marrow cultures containing TPA had detectable CSA, most of which persisted after dialysis. However, identically incubated samples of cell-free medium with TPA also contained CSA that persisted after dialysis, and no significant consistent quantitative differences were found between incubated cell-containing and cell-free TPA media (Table 2). We concluded that the CSA detected in the TPA-containing liquid marrow cultures was due to residual TPA alone (which could not be completely removed by dialysis from the serum-containing medium) and not due to factors produced by the marrow cells. To conclude otherwise, one would have to postulate that TPA induces CSF production by marrow cells in agar and methylcellulose cultures, but not in liquid cultures. These data do not exclude the possibility that TPA stimulates CSF production as its major mode of

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**Table 2. Colony-Stimulating Activity in Supernatants From Liquid Marrow Cultures**

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>Supernatant Treatment</th>
<th>Colonies per Plate (as % of Control ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium + BM cells</td>
<td>—</td>
<td>0±1</td>
</tr>
<tr>
<td>TPA-medium§ + BM cells</td>
<td>—</td>
<td>25.0±4.0, 32.0±4.0, 30.0±4.0, 30.0±1.0</td>
</tr>
<tr>
<td>TPA-medium</td>
<td>—</td>
<td>31.0±2.0, 21.0±2.0, 34.0±2.0, 22.0±2.0</td>
</tr>
</tbody>
</table>

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*Marrow cells were suspended in supplemented McCoy's medium with 15% FCS at \(10^6\) cells/ml and incubated for 1-5 days at 37°C, 5% CO\(_2\), and high humidity. Supernatants were harvested after centrifugation and frozen (-70°C) for later assay for colony stimulating activity.

†Colony-stimulating activity: Number of colonies per 10\(^6\) fresh bone marrow cells in agar cultures with 10% test supernatant (mean ± SE of triplicate cultures).

‡Dialysis: distilled water, 3 days, 6 changes, 4°C.

§TPA-medium: supplemented McCoy's medium with 15% FCS and TPA \(10^{-7} M\).
action, but they do not strongly support such a mechanism.

Another possible mechanism of action suggested by our initial observations was that TPA and other active phorbol esters might increase the GM-CFC's responsiveness to CSF (which might be present in trace amounts in the serum-containing culture medium). In this manner, GM-CFC would be stimulated by concentrations of CSF that are normally insufficient to cause colony formation, and colonies would form in the apparent absence of CSF. Preliminary support for this possibility was obtained when we cultured marrow cells, stimulated by optimal concentrations of LC-CSF or TPA, in media containing graded concentrations of fetal calf serum (FCS) (Fig. 4). CSF-stimulated colony formation was dependent on the concentration of FCS up to 10%-15%; further increases in FCS concentration did not augment colony formation. Similar results were seen with other sources of CSF, such as WEHI-3 conditioned medium, mouse lung conditioned medium, and endotoxin mouse serum (data not shown). By contrast, the efficiency of TPA-stimulated colony formation was augmented by increasing FCS concentrations up to a maximum of 40%.

More detailed studies were then carried out to determine the effect of the phorbol esters on colony formation stimulated by suboptimal concentrations of CSF. Fresh marrow cells were cultured in agar and stimulated by the addition of 0.1 ml per plate of serial twofold dilutions of 5× concentrated LC-CSF, resulting in the classic sigmoid-shaped dose–response curve characteristic of CSF (Fig. 5). Identically prepared groups of culture plates containing the same LC-CSF dilutions plus graded concentrations of TPA were similarly cultured, and several observations were made. First, although $10^{-7} \, \text{M} \, \text{TPA}$ alone stimulated the formation of approximately 55 colonies per plate (10^5 cells plated), the addition of TPA to plates containing plateau concentrations of CSF resulted in no additional colonies compared to the CSF alone, confirming our previous observation. Second, at suboptimal concentrations of LC-CSF, the addition of $10^{-7} \, \text{M} \, \text{TPA}$ increased the number of colonies that developed, compared to that concentration of CSF alone. Third, the TPA augmentation of colony formation was a dose-dependent phenomenon; that is, the degree of augmentation of colony formation at any suboptimal CSF concentration increased with increasing concentrations of TPA up to a maximum of $10^{-7} \, \text{M} \, \text{TPA}$.

It is also important to note that the interaction of TPA and LC-CSF is not merely additive but, in fact, synergistic. Thus, $10^{-7} \, \text{M} \, \text{TPA}$ alone stimulated 55 ± 1 colonies and 0.1 ml of a 1:32 dilution of LC-CSF stimulated 7 ± 2 colonies, but the combination resulted in 117 ± 3 colonies (Fig. 5). Synergism is even more evident when $10^{-9} \, \text{M} \, \text{TPA}$ is added to the low concentrations of LC-CSF, since $10^{-9} \, \text{M} \, \text{TPA}$ alone stimulated only a few colonies.

TPA’s enhancement of colony formation at suboptimal CSF doses does not appear to be unique for LC-CSF. Similar results were obtained in an identical experiment in which CSF from WEHI-3-conditioned medium (WEHI-CSF) was used as the source of CSF (Fig. 6). Serial dilutions of WEHI-CSF gave a slightly different dose–response curve, as reported by others, with less of a plateau and activity detectable at higher dilutions. Nevertheless, we found the same concentration-dependent, synergistic enhancement of colony formation...
formation by TPA at suboptimal concentrations of WEHI-CSF.

In addition, we found that another active tumor-promoting phorbol ester, PDD, also showed enhancement of colony formation at low CSF doses. When LC-CSF (Fig. 7) or WEHI-CSF (Fig. 8) were assayed in serial dilutions for stimulation of colony formation, the addition of $10^{-7} M$ PDD enhanced colony formation at suboptimal CSF concentrations almost as much as $10^{-7} M$ TPA. In both experiments, $10^{-7} M$ phorbol, the nonpromoting parent alcohol, had no effect on CSF-stimulated colony formation.

Since, in these last experiments, TPA seemed to be able to markedly enhance the action of low concentrations of CSF on marrow in agar culture, we were interested in its effect on marrow in long-term liquid flask culture of the type originally described by Dexter and colleagues. In this culture system, there are
Fig. 7. Dose–response curves for LC-CSF in the absence (○) or presence of TPA (□), PDD (●), or phorbol (△), all at $10^{-7} M$ concentration. Shown are mean ± SE of triplicate cultures ($5 \times 10^4$ cells/plate).

Fig. 8. Dose–response curves for WEHI-CSF in the absence (○) or presence of TPA (□), PDD (●), or phorbol (△), all at $10^{-7} M$ concentration. Shown are mean ± SE of triplicate cultures ($5 \times 10^4$ cells/plate).
prolonged granulopoiesis and maintenance of stem (CFU-s) and progenitor (GM-CFC) cell compartments despite virtual absence of detectable CSF in the culture medium.12

We established multiple flask cultures in the conventional manner with an initial inoculation of each flask with fresh mouse marrow. At the third weekly medium feeding, the flasks were totally depopulated of nonadherent cells and recharged with fresh medium containing 107 fresh marrow cells per flask. On the third weekly medium feeding after recharging, the flasks were randomly divided into a control (no drug) group and groups to which we added graded amounts (106 to 1010 M) of TPA or phorbol (106 M) (Table 3), and incubation was continued for another week. Although there was no significant difference in cell numbers per culture 1 wk later, the TPA flasks showed marked reduction in GM-CFC (assayed in a conventional manner with WEHI-CSF) and a shift toward macrophage differentiation at the higher TPA concentrations. Phorbol addition had no significant effect on the cultures.

**DISCUSSION**

In the two-step model of carcinogenesis, tumor promoters are agents which, while not themselves carcinogenic, can nevertheless complete a carcinogenic process by promoting tumor formation in tissues previously "initiated" by exposure to a subthreshold dose of a true carcinogen.19 The best studied tumor promoters are fatty acid diesters of the plant diterpene phorbol alcohol phorbol. Tumor-promoting phorbol esters have come under intense scrutiny recently because they exhibit a variety of dramatic effects on cultured cells in vitro. These include stimulation of DNA synthesis and cell proliferation,20 induction of enzyme production,24 changes in cell membrane properties,27 and either inhibition30 or induction31 of terminal differentiation.

It has been suggested by several authors that in some of these actions, the phorbol esters appear to mimic the action of natural growth regulators. For example, TPA can apparently reproduce a number of the biologic effects of epidermal growth factor (EGF) in vitro and apparently does so through changes in the cell surface receptor for EGF.27,28 In addition, TPA has been shown to have a synergistic enhancing effect on certain growth-promoting polypeptides (EGF, insulin, fibroblast-derived growth factor) in stimulating DNA synthesis by mouse and human fibroblasts in serum-free medium.23

We have reported previously that tumor-promoting phorbol esters can apparently mimic the growth-promoting action of CSF on at least some mouse marrow GM-CFC.2 The rank order of activity of the phorbol derivatives in marrow colony formation is the same as that of their activity in promoting skin tumors in vivo, implying that the same structure–activity relationships are important for both actions.5 In this report, we offer further evidence that the TPA-responsive colony-forming cells are in fact GM-CFC, probably a subset of GM-CFC characterized by a larger mean cell size, as determined by velocity sedimentation. The addition of TPA to agar cultures already maximally stimulated by CSF does not increase colony numbers, and TPA-stimulated colony formation is inhibited by agents that inhibit CSF-stimulated colony formation by a direct action on the GM-CFC (Fig. 3). The dexamethasone experiment alone is not clear-cut, since antiinflammatory steroids are antagonistic to active phorbol esters in other systems37,38 and its inhibitory action on TPA-stimulated colony formation might be attributed to some effect on the TPA rather than directly on the responding cell. However, E-type prostaglandins are not antagonists of active phorbol esters; in fact, TPA stimulates prostaglandin production in canine kidney cells, and PGE2 appears to play a positive role in TPA-induced DNA synthesis in mouse epidermis in vivo.39 Therefore, PGE inhibition of TPA-stimulated colony formation is more likely to represent a direct inhibitory action on the responding colony-forming cell.

An alternate interpretation of the velocity sedimentation data (Fig. 2) can be offered. If the mode of action of TPA was primary induction of CSF secretion with secondary colony formation, one might suggest that only the more rapidly sedimenting fractions form colonies because they happen to contain CSF-producing cells. This would not, however, explain the consist-

| Table 3. Effects of TPA and Phorbol on 3-wk-old Long-Term Marrow Culture* |
|-----------------|-----------------|-----------------|-----------------|
| **Week** | **Group** | **Additive** | **Cells (x 10⁶)** | **Differential†** | **GM-CFC** |
| 3 | All (29) | — | 4.2 | 14 | 35 | 19 | 2 | 9 |
| 4 | A (9) | — | 3.3 | 10 | 33 | 57 | 15 | 6 |
| B (5) | TPA 10⁻⁸ M | 3.6 | 0 | 0 | 100 | 0 | 0 | 0 |
| C (5) | TPA 10⁻⁶ M | 3.2 | 0 | 31 | 49 | 0 | 0 | 0 |
| D (5) | TPA 10⁻⁴ M | 3.8 | 3 | 48 | 49 | 12 | 2 | 3 |
| E (5) | Phorbol 10⁻⁴ M | 3.6 | 4 | 37 | 59 | 15 | 8 | 3 |

*Long-term marrow cultures were established as described in Materials and Methods. At the third weekly feeding after recharging, the flasks were randomly assigned to receive no additive, phorbol or TPA at one of three concentrations. One week later, the cultures were assayed for total cells, differential count, and number of GM-CFC in the supernatant population.

†n = Number of flasks.

‡EG, early granulocyte (blasts, progranulocytes, myelocytes); LG, late granulocyte (metamyelocytes, band forms, polymorphonuclear cells); M/M, monocyte/macrophage.
tently lower cloning efficiency of unfractionated marrow in the presence of TPA, while the GM-CFC subset hypothesis is compatible with that observation. In addition, we have been unable to obtain convincing evidence to support induction by CSF secretion as the major action of TPA (Table 2).

In long-term flask cultures, TPA’s most pronounced effect seems to be induction of macrophage differentiation with adverse effects on maintenance of the progenitor cell compartment. In this effect, TPA again seems to mimic an effect of CSF, since it has been reported by others that the addition of exogenous CSF to long-term flask cultures promotes macrophage differentiation and loss of the stem and progenitor cell compartments. Alternatively, our observations may be explained by direct effects of the active phorbol esters on more primitive stem cells or by indirect effects mediated through other cells in the culture. For example, TPA may directly induce macrophage differentiation, with macrophage production of CSF secondarily causing a decline in the progenitor cell compartment. We are pursuing these intriguing preliminary observations.

If TPA and other active phorbol esters induce colony formation by mouse marrow GM-CFC in the apparent absence of CSF, the mechanism of this action becomes an important question. We have considered three hypotheses to explain this phorbol ester effect: (1) direct colony-stimulating ability; (2) induction of CSF secretion; and (3) alteration of GM-CFC responsiveness to CSF. Of course, these are not mutually exclusive hypotheses, and it is possible for the phorbol esters to have more than one effect that combines in stimulation of marrow colony formation. The first hypothesis is that TPA and its active analogs might be “artificial” colony-stimulating factors; that is, that they act directly on GM-CFC to induce cellular proliferation and differentiation, possibly by interaction with cellular receptors for CSF. This intriguing possibility is compatible with the data presented thus far, but it is a difficult hypothesis to prove.

The second hypothesis is that active phorbol esters induce differentiated marrow cells (presumably mature macrophages) to produce or secrete glycoprotein CSF. This mechanism is suggested by a previous report that TPA induces CSF production in a macrophage cell line; however, the data published do not exclude the other suggested mechanisms as explanations for the observed effects. Lotem and Sachs recently reported TPA-stimulated cluster formation by normal mouse marrow cells and suggested CSF induction as the mechanism, but they did not consider the possibility of direct stimulation. Our observations (Table 2) led us to conclude that CSF induction is not the major mechanism of action in TPA stimulation of myeloid colony formation.

The opposite conclusion was reached in a recent publication by Greenberger et al., who found that TPA-stimulated colony and cluster formation from freshly explanted bone marrow but not from GM-CFC-rich nonadherent cells from long-term bone marrow cultures (LTBMC). The authors attributed these different results to the absence (<1%) of macrophages in the LTBMC cells compared to their presence in fresh bone marrow and concluded that TPA acts through inducing CSF production by macrophages. However, the LTBMC cells were grown in the continuous presence of \(10^{-7}\) M hydrocortisone for 4-8 wk before assay in the agar system, and the cells were not washed before plating in agar. Since antiinflammatory steroids oppose many of the actions of phorbol esters, including myeloid colony formation (Fig. 3), the saturation of the LTBMC cells with hydrocortisone may account for the lack of response to TPA.

In this regard, it is notable that both Greenberger et al. and we (Table 3) have found that addition of TPA to LTBMC results in macrophage differentiation and loss of the progenitor cell compartment. However, the TPA effects were evident after 1 wk in our experiments using LTBMC without steroid additives; similar effects required 10 wk in Greenberger’s experiments with hydrocortisone-supplemented LTBMC. It may be that conditions of LTBMC (including steroid concentration) modify the effects of TPA.

We have considered a third hypothesis, that active phorbol esters increase the sensitivity of GM-CFC to CSF. Interestingly, this mechanism was suggested by Lotem and Sachs and considered by them and by us to be relevant to the mechanism of tumor promotion. This hypothesis was initially suggested to us by experiments that showed increasing efficiency of TPA-induced colony formation with increasing FCS concentrations up to 40% (Fig. 4).

Further support came from the demonstration that TPA synergistically enhances colony formation stimulated by suboptimal concentrations of various CSF preparations (Figs. 5-8). Similar results were reported very recently by Fibach, Marks, and Rifkind, who demonstrated stimulation of mouse myeloid colony formation by TPA in the absence of CSF and synergistic enhancement of myeloid (and erythroid) colony formation by TPA in the presence of suboptimal levels of CSF (or erythropoietin). These studies are consistent with the hypothesis that TPA and its active analogs may increase the GM-CFC’s responsiveness to CSF.

How could TPA produce such an effect? TPA has been shown to alter the function of cell surface recep-
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tors for EGF and its effects on epidermal cell growth presumably involve this action. TPA is also known to cause changes in cell membrane properties, and it may be that such changes alter certain cell surface receptors' numbers or affinity. CSF may well effect its action on GM-CFC by binding to cell surface receptors and thereby initiating signals for cellular proliferation. Alternately, if TPA alters the cell membrane of GM-CFC in a more general fashion, it might alter the function of CSF receptors. We postulate that such alteration results in either direct initiation of receptor-mediated signals or receptor-mediated actions occurring at lower than normal concentrations of CSF. Further studies are obviously indicated.

The importance of these observations is twofold. First, they indicate that the phorbol esters' effects on cellular proliferation may be related to their tumor-promoting properties. Since a cell must proliferate in order to express a carcinogenic event, the role of tumor promoters in carcinogenesis may be to influence cellular proliferation. Thus, further studies of phorbol esters and hematopoiesis may shed light on the cocarcinogenic action of these compounds. Second, the potent effects of these compounds on in vitro myelopoiesis suggest that they will be valuable tools in further study of the regulation of hematopoiesis. Such a conclusion was indicated by earlier work showing potent effects of phorbol esters on differentiation of mouse erythroblast or human promyelocytic cell lines. The demonstration of potent effects of phorbol esters on proliferation and differentiation of normal hematopoietic cells in primary culture adds to the potential importance of these compounds in experimental hematology and cell biology.

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