Release of Granulocyte-Specific Colony-Stimulating Activity by Human Bone Marrow Exposed to Phorbol Esters

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Granulocyte-macrophage colony growth depends on the presence of colony-stimulating activity (CSA). Phorbol esters induce concentration-dependent colony formation in the absence of exogenous CSA. We questioned whether phorbol esters mimicked the action of CSA by directly stimulating colony growth, or whether phorbol esters acted indirectly by inducing marrow cells to release CSA. First, after incubating human bone marrow cells with phorbol 12,13-dibutyrate (PDB) for 3 days, we separated PDB from the protein peak of the conditioned medium by Sephadex G-10 gel filtration and tested this peak for the presence of CSA. When diluted 1:10 in the agar colony assay, this material induced 133 ± 15 colonies/10^6 bone marrow cells. Second, to determine whether bone marrow cells required the continued presence of PDB in order to release CSA, PDB was removed from bone marrow cells by washing, and these cells were reincubated in fresh medium in the absence of PDB. CSA was found in the medium of these cultures; its release was maximal after reincubation of bone marrow cells with 5 x 10^{-8} M PDB for 3 days, followed by incubation for 3 days in the absence of PDB. This CSA stimulated granulopoiesis out of proportion to monocytopoiesis, with 85% ± 17% of the colonies being granulocytic (as indicated by histochemical staining for chloroacetate esterase), and 12% ± 3% being monocytic (as indicated by nonspecific esterase). Inhibitors of monocYTE colony formation, including PGE_2, were not present in the medium that contained this CSA. These studies demonstrate that normal human bone marrow cells exposed to PDB release CSA and that this CSA selectively stimulates granulopoiesis in vitro.

The growth of bone marrow precursor cells into granulocyte-macrophage colonies in vitro is dependent on a growth factor, which, in its unpurified form, is called colony-stimulating activity (CSA). Most human sources of CSA consist of growth medium conditioned by cultured cells. Significant amounts of CSA are released from placental cells, monocytes, T cells, endothelial cells, and cell lines of pancreatic and lung neoplasms. The action of CSA appears to be specific. It has been purified into a related group of glycoproteins called colony-stimulating factors (CSF) and these factors fully account for the ability of CSA to induce colonies.

The release of CSA appears to be controlled by humoral interactions between cells and by cell-cell contact. Monocytes elaborate a factor that increases CSA release by T cells, endothelial cells, and fibroblasts. CSA is also released by mitogen-stimulated T cells and by endothelial cells exposed to endotoxin. Cell-cell interactions may be important regulators of CSA release, particularly in the bone marrow, where CSA concentrations are low. Although studies of human bone marrow have been lacking, studies of murine bone marrow have found that substances that stimulate or inhibit the action of CSA are produced during different phases of marrow growth in vitro and that CSA is both released and consumed by adherent bone marrow cells.

Recent studies indicate that the phorbol ester, 12-0-tetradecanoylphorbol-13-acetate (TPA), can mimic the action of CSA in a concentration-dependent manner. The authors of these studies postulate that TPA has a direct effect on the differentiation of normal colony-forming cells for granulocytes/macrophages (CFC-GM) in marrow, similar to its ability to directly promote macrophage differentiation of human myeloid leukemic cell lines and leukemic cells freshly obtained from most patients with ANLL. However, it is unclear whether the action of TPA is directly on the CFC-GM or whether TPA also acts indirectly by inducing the release of CSA from the other bone marrow cells present in culture. CSA has not been detected in cultures of normal blood cells incubated with TPA, but has been detected in cultures of TPA-treated leukemic cell lines. Moreover, when CFC-GM were cocultured with either TPA-treated murine bone marrow-derived macrophages or fresh human leukemia cells, colonies were formed in the absence of exogenous CSA. However, in these experiments, the detection of CSA has been hampered by the use of TPA, which is lipophilic and cannot effectively be removed from cells. Thus, some of the putative CSA could have been due to residual TPA. This problem can be avoided by the use of the more hydrophilic phorbol ester, phorbol-12,13-dibutyrate.
(PDB), which can be removed from cell cultures. Using this compound, we investigated whether phorbol esters induce colony formation through the release of CSA from normal human bone marrow cells.

MATERIALS AND METHODS

Chemicals

Powdered PDB (LC Services Corp., Woburn, MA) was reconstituted at $10^{-3}$ M in acetonitrile, stored at $-70^\circ$C, and diluted with culture medium immediately before use. The final concentration of acetonitrile in culture medium was $<0.1\%$ and had no detectable effect on cell growth. $[^3H]PDB$ (New England Nuclear, Boston, MA), with an activity of $0.2$ mCi/cc at a concentration of $3.1 \times 10^{-3}$ M, was stored at $-20^\circ$C and diluted with culture medium immediately before use. Phytohemagglutinin (PHA, HA-15, Burroughs-Wellcome, Greenville, NC) was reconstituted in distilled water and stored at $-70^\circ$C. Indomethacin (Sigma Chemicals, St. Louis, MO) was reconstituted at $10^{-3}$ M in sterile water and stored at $-20^\circ$C until use.

Colonies-Forming Cell Assay

After obtaining informed consent, 8 ml of bone marrow was aspirated from the posterior iliac crest of normal volunteers into 1 ml of preservative-free heparin (Grand Island Biological Co., GIBCO), Grand Island, NY, 250 U/ml. These studies were performed after approval of the University of Pennsylvania Committee on Studies Involving Human Beings. The cell preparation previously described was modified slightly. Cells were diluted 1:2 in supplemented Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, Inc., MacLean, VA) containing 20% FCS, 1% penicillin/streptomycin (10,000 U/ml), 0.4 mg/ml L-asparagine, 0.25 mg/ml DEAE-Dextran, and 1.0% NaHCO$_3$ (Sigma); and 50 \mu g/ml gentamycin (Schering Corp., Kenilworth, NJ). The mononuclear cells were separated by centrifugation at 1,000 g over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) at 22°C for 30 min. The interface cells were washed twice and the adherent cells removed by incubation in 75 sq cm plastic culture flasks (Corning Glass Works, Corning, NY) at 37°C in 10% CO$_2$ for 1 hr. Single-layer agar cultures of the nonadherent marrow cells at 10$^5$ cells/ml were used to detect colony-stimulating activity present in various samples of conditioned medium according to the method of Met-calf as modified by Abraham. Briefly, cells were added at a 1:20 dilution to an equal mixture of double-strength supplemented DMEM and 0.6% bacto agar (Difco Laboratories, Detroit, MI). One-milliliter aliquots were pipetted into 35 sq cm plastic petri dishes (Lux Scientific Group, Newbury Park, CA), to which was added 0.1 ml of conditioned medium as a source of colony-stimulating activity (CSA). The mixture was incubated for 7–14 days in a fully humidified 10% CO$_2$ atmosphere at 37°C. Cytocentrifuge preparations (Shandon Southern Instruments Co., Sewickley, PA) were made from cell suspensions for each bone marrow sample. After incubation, duplicate agar cultures were scored for clusters (10–49 cells) and colonies (≥50 cells) using a Nikon dissecting microscope at 40×. Results are presented as the sum of clusters and colonies per 10$^5$ cells, and the total is referred to as colonies. Each agar culture was then fixed and stained.

Preparation of Conditioned Medium

Conditioned medium from bone marrow cells pretreated with PDB was prepared in the following manner. Bone marrow cells were separated by centrifugation over Ficoll-Hypaque at 550 g for 30 min at 22°C. Interface cells were washed twice and incubated at 10$^6$ cells/ml in 25 sq cm flasks in a humidified 5% CO$_2$ incubator at 37°C in culture medium, which consisted of modified McCoy's 5A medium supplemented with 12.5% heat-inactivated bovine serum, 12.5% horse serum (GIBCO), and the following GIBCO supplements: 1% MEM essential amino acids, 1% nonessential amino acids, 1% vitamins, 1% 2 mM glutamine, 1% penicillin/streptomycin (10,000 U/ml), 1% pyruvate, and 0.075% NaHCO$_3$, and 10 mM HEPES buffer (from Sigma). PDB was added at the start of incubation to one container of each duplicate culture. Unless otherwise stated, 5 $\times$ $10^{-4}$ M PDB was used. After incubation periods of 1 hr to 7 days, the supernatant conditioned medium was removed, centrifuged at 550 g for 10 min, passed through a 0.45 \mu M filter (Gelman, Ann Arbor, MI), and stored at $-70^\circ$C until use. The cells that were adherent to the culture flask and the nonadherent cells were washed 4 times with 20 ml culture medium with a 15-min incubation between washes to facilitate removal of PDB from cells. The cells were then reincubated in fresh medium for 3 days in 5% CO$_2$ at 37°C, in the absence of PDB, and the supernatant was collected and stored as described above. Conditioned medium (CM) collected after the second incubation from bone marrow cells preincubated with PDB will be referred to as PDB-CM and that from cells incubated only in the absence of PDB will be referred to as control-CM. In some experiments, indomethacin at $10^{-2}$–$10^{-4}$ M was added throughout the culture.

Conditioned medium from peripheral blood leukocytes exposed to 1% PHA (PHA-CM) was prepared according to the method of Aye. Briefly, following informed consent, 200 cc of peripheral blood was drawn from a normal volunteer into 50-ml syringes containing 250 U of heparin and allowed to sediment at unit gravity in a 2:1 mixture of dextran 60 (Abbott Laboratories, Chicago, IL) for 90 min. The supernatant cells were removed and washed twice by centrifugation at 500 g for 10 min. The cells were resuspended at 10$^6$ cells/cc in Roswell Park Memorial Institute (RPMI) medium 1640 (Flow Labs) supplemented with 20 mM l-glutamine, 1% vitamins, 1% penicillin/streptomycin (10,000 U/ml), 15% bovine calf serum and 1% PHA, and cultured in 5% CO$_2$ at 37°C for 7 days. The supernatant was collected following centrifugation and the conditioned medium was filtered through a 0.45 \mu M membrane (Nalgene, Rochester, NY) and stored at $-70^\circ$C until use.

Removal of $[^3H]PDB$ by Washing of Bone Marrow Cells

In the preincubation experiments, PDB was washed off of cells between the first and second incubations. To determine the effectiveness of the washing procedure, Ficoll-Hypaque-separated bone marrow cells were incubated in 5 $\times$ $10^{-4}$ M $[^3H]PDB$ for 3 days in 5% CO$_2$ at 37°C. They were then centrifuged at 500 g for 10 min and resuspended in twice the initial volume. At each wash, triplicate 0.1-ml samples of the cell suspension were diluted into 10 ml of Aquasol (New England Nuclear) and counted in a Beta counter (Beckman, LS 7000). A total of five washes were performed. The mean counts were then converted to residual PDB molarity.

Removal of $[^3H]PDB$ by Gel Filtration

In certain experiments, conditioned medium was harvested from bone marrow cells following 3 days of incubation with 5 $\times$ $10^{-4}$ M $[^3H]PDB$. In order to assay for colony-stimulating activity, it was necessary to remove $[^3H]PDB$ from the medium. Ten-milliliter samples were concentrated fivefold over an Amicon YM-10 ultrafiltration membrane (Amicon, Bedford, MA). The sample was separated over a 40 x 1 cm Sephadex G-10 column (Pharmacia). Column flow rates had been optimized by separation of $[^3H]PDB$.
from fresh medium and from control conditioned medium. The fractions were sampled for OD at 280 nm and counts/minute (cpm). The protein peak (OD 280 nm) was pooled and assayed for colony-stimulating activity. Subsequently, protein peaks were collected from conditioned medium taken from bone marrow cells cultured in medium containing cold PDB, PHA, or no stimulant using the identical column.

**Histochemical Staining**

The technique of fixing and staining the semisolid agar cultures was the method of Salmon et al., as modified by Abrahm et al. Briefly, the agar culture dishes were cooled to 4°C for 15 min, rinsed with 1 ml of cold Hanks’ balanced salt solution without calcium and magnesium (GIBCO) for 10 min, and fixed in 4% buffered formol calcium for 10 min at 4°C. The agar was transferred to a 3 × 2 inch glass slide and spread with a cellulose acetate membrane (Schleicher and Schuell, Keene, NH) and allowed to dry. Slides were stained for nonspecific esterase (NSE), a monocyte marker, and then for chloroacetate esterase (CAE), a granulocyte marker, as previously described. CAE appeared as blue granules in the cytoplasm; NSE as brown cells. Cluster and colony cell type were scored as CAE-positive when >90% of cells contained blue granules and as NSE-positive if >90% of cells were brown; otherwise, the cell type was scored as mixed. The number of cells per aggregate was counted following fixation by randomly picking 25 aggregates of one slide from each PHA-CM and PDB-CM stimulated culture in 8 separate experiments.

**Prostaglandin Radioimmunoassay**

Samples of conditioned medium were kindly assayed for the presence of PGE, and PGE2 during incubation with PDB, PDB was separated from this stimulation was due to the [3H]PDB was present in the pooled fractions of the G-10 gel filtration (Fig. 1). In medium containing conditioned medium from BM cells exposed to 5 × 10^-8 M PDB 136 ± 15 colonies compared to fresh medium containing 5 × 10^-8 M PDB may be explained by the sharp dose-response curve of the PDB effect. At 5 × 10^-8 M PDB, near maximal effects were seen during the liquid cultures. However, the CSA assay performed using a 10% v/v aliquot of medium exposed the target cells to only 5 × 10^-9 M PDB, which can induce colony formation but is only twice the concentration at which no colony stimulation occurs directly (data not presented). Thus, small differences in dilution and differences in bioavailable, unbound PDB in fresh medium compared to control-conditioned medium in which PDB was added could explain the differences in colony stimulation.

In contrast, the postfiltration protein fraction of medium that had been incubated with bone marrow cells in the presence of 5 × 10^-8 M PDB for 3 days was added or to the release of CSA from bone marrow cells during incubation with PDB, PDB was separated from the protein fraction of the culture medium by Sephadex G-10 gel filtration (Fig. 1). In medium containing 5 × 10^-8 M [3H]PDB, approximately 1% of the [3H]PDB was present in the pooled fractions of the protein peak (OD 280 nm), and 99% was present as a broad peak readily separated from the protein peak.

When added to marrow cultures at a concentration of 10% (v/v), fresh culture medium containing 5 × 10^-8 M PDB induced 43 ± 8 colonies/10^5 bone marrow cells, whereas the protein fraction induced 5 ± 3 colonies (Table 1). The reduced number of colonies observed in prefiltration samples of control conditioned medium supplemented with 5 × 10^-8 M PDB (11 ± 2 colonies) compared to fresh medium containing 5 × 10^-8 M PDB was determined by measuring cpm for each 0.75-cc fraction. Recovery of [3H]PDB was greater than 95%.

**RESULTS**

**Identification of CSA That Is Distinct From PDB**

PDB at concentrations from 10^-9 to 5 × 10^-8 M directly stimulated colony growth of cultured mononuclear nonadherent human bone marrow cells plated at 10^5 cells/cc in the absence of exogenous CSA. Colony induction was maximal (61 ± 14 colonies) at a final PDB concentration of 5 × 10^-9 M. The supernatant medium from cultures of PDB-treated bone marrow cells was also able to stimulate colony formation. To determine whether this stimulation was due to PDB itself or to the release of CSA from bone marrow cells during incubation with PDB, PDB was separated from the protein fraction of the culture medium by Sephadex G-10 gel filtration (Fig. 1). In medium containing 5 × 10^-8 M [3H]PDB, approximately 1% of the [3H]PDB was present in the pooled fractions of the protein peak (OD 280 nm), and 99% was present as a broad peak readily separated from the protein peak.

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**Table 1. Colony Induction by Conditioned Medium Before and After Gel Filtration**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Prefiltration (CFC-GM/10^5 BM Cells)</th>
<th>Postfiltration (CFC-GM/10^5 BM Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>McCoy's 5A</td>
<td>4 ± 2</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>McCoy's 5A plus 5 × 10^-8 M PDB</td>
<td>43 ± 8</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Conditioned medium from control BM cells</td>
<td>12 ± 3</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>Conditioned medium from control BM cells plus 5 × 10^-8 M PDB</td>
<td>11 ± 2</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>Conditioned medium from BM cells exposed to 5 × 10^-8 M PDB</td>
<td>136 ± 15</td>
<td>133 ± 15</td>
</tr>
<tr>
<td>Conditioned medium from BM cells exposed to 1% PHA</td>
<td>154 ± 18</td>
<td>164 ± 25</td>
</tr>
</tbody>
</table>

*Mean ± SE of colonies scored at 14 days of agar incubation in 7 experiments.

BM, bone marrow.
induced 133 ± 15 colonies. This number of colonies was similar to the number of colonies induced by a standard source of colony-stimulating activity, PHA-conditioned medium (164 ± 25 colonies). Thus, conditioned medium by incubation with PDB-treated bone marrow cells contains a colony-stimulating activity (CSA) that is present in the protein peak and is distinct from PDB.

CSA Release by Bone Marrow Cells Preincubated With PDB

To determine whether PDB could be removed from cells in culture, bone marrow cells were incubated for 3 days in medium containing \(5 \times 10^{-8} \text{M} \)^{[3H]}PDB, after which the cells were washed 5 times in 2 volumes of fresh culture medium (Fig. 2). Following 4 washes, less than \(4 \times 10^{-11} \text{M} \)^{[3H]}PDB remained in the cell suspension, indicating that PDB could effectively be removed from cell cultures. This enabled us to assess whether bone marrow cells preincubated with PDB would release CSA during subsequent periods of incubation. We observed that CSA was released into the medium when bone marrow cells were preincubated with PDB for 1-7 days and subsequently incubated for 3 additional days (Fig. 3). CSA release was maximal in cultures preincubated with PDB for 2 days, with no further increment after up to 7 days of PDB exposure.

To determine the time course of CSA release by bone marrow cells preincubated with PDB, cells were grown in the presence of \(5 \times 10^{-8} \text{M} \) PDB for 5 days, and, after washing to remove PDB, they were incubated for varying periods of time. CSA production was maximal 3 days after washing. In subsequent experiments, both the preincubation with PDB and the subsequent incubation in its absence were for 3 days, and the resulting conditioned medium was referred to as PDB-CM. The level of CSA in PDB-CM depended on the initial PDB concentration (Fig. 4), with maximal CSA levels occurring in cultures preincubated with PDB at concentrations between \(5 \times 10^{-8} \) and \(10^{-6} \text{M} \).

Cellular Composition of PDB-Treated Marrow Cultures

The bone marrow cells responsible for generating CSA were examined morphologically. The PDB-treated bone marrow cultures displayed a marked shift toward mononuclear phagocyte differentiation. Of the nonadherent cells, 74.6% ± 4.6% were large mononuclear cells that stained for nonspecific esterase (NSE), a mononuclear phagocyte marker, whereas 15.2% ± 3.2% were positive for chloroacetate esterase (CAE), a granulocyte marker. This compared to 18.0% ± 3.2%
NSE-positive cells and 73.8% ± 3.3% CAE-positive cells in control cultures. In addition to the relative shift toward mononuclear phagocytes in the nonadherent cell fraction, there was an absolute increase in mononuclear phagocytes adherent to the culture dish. When expressed as the mean of 5 cell counts/200× field, PDB induced an increase from 3 ± 0.5 cells in control cultures to 22 ± 3.5 in PDB-treated cultures. However, recovery by exposure to cold versene and scraping was incomplete, and an absolute count could not be determined. Thus, the release of CSA by PDB-treated bone marrow cultures was associated with an increase in mononuclear phagocytes present in these cultures.

**Colony Cell Type Induced by PDB-Conditioned Medium**

The types of colonies induced by the CSA present in PDB-CM at 14 days were determined by staining for NSE and CAE. 85% ± 17% were granulocytic (CAE positive), 12% ± 3% were monocytic (NSE positive), and 3% ± 2% were mixed. This relative distribution differed markedly from the colonies induced by PHA-CM. When tested over a range of CM concentrations from 2% to 20% (v/v), PHA-CM induced equivalent numbers of granulocyte and monocyte colonies at each concentration tested, whereas PDB-CM induced predominantly granulocyte colonies over the same concentration range (Fig. 5). At low concentrations of CM, both the size and total number of colonies were greater with PHA-CM than with PDB-CM. However, at 20% CM, colony size was equivalent using either PDB-CM or PHA-CM. The same relative distribution of colony types observed at 14 days was obtained after only 7 days of incubation, although the absolute number of monocyte colonies at 7 days was approximately one-third that present at 14 days. Moreover, there was no further increase in the number of monocyte colonies induced with PDB-CM at 21 days. Thus, although the absolute number and size of colonies formed was a function of time and of CM concentration, the relative numbers of granulocyte and monocyte colonies induced by PHA-CM and PDB-CM were constant.

**Absence of a Soluble Inhibitor in PDB-CM**

The fact that PDB-CM appeared to lack the ability to stimulate a significant number of monocyte colonies, may have been due either to the presence of a soluble inhibitor of monocyte colony formation or to a decreased amount of monocyte colony-stimulating material. Prostaglandin E₁ (PGE₁) is a known inhibitor of monocyte colony formation. We were unable to detect PGE₁ by radioimmunoassay (PGE₁ =0.5 µg/ml) in fresh medium, PDB-CM, or PHA-CM. To explore the potential role of PGE₁ further, PGE₁ production was inhibited by indomethacin, which was added throughout the culture period (Fig. 6). The presence of indomethacin had no significant effect on the proportion of monocyte colonies relative to granulocyte colonies over a concentration range of 10⁻³—10⁻⁴ M. At high concentrations, indomethacin decreased the total number of colonies induced.

To test for the presence of other soluble inhibitors to monocyte colony formation that might have been present in PDB-CM, mixtures of PDB-CM and PHA-CM were assayed for their ability to induce monocyte colonies. This was compared to the monocyte colony formation as induced by PHA-CM alone (Fig. 7). PDB-CM did not inhibit monocyte colony formation as...
induced by PHA-CM. Instead, the activities appeared additive. Because neither PGE\(_1\) nor a soluble inhibitor were found, we concluded that PDB-CM contains less monocyte colony-stimulating activity than PHA-CM.

**DISCUSSION**

These experiments indicate that bone marrow cells exposed to the phorbol ester, PDB, release colony-stimulating activity (CSA) into the culture medium. The CSA produced under these conditions is present in the protein fraction of the medium. It primarily stimulates granulopoiesis, with little effect on monocytopoiesis.

Previous studies have shown that phorbol esters, when cultured directly with either human or murine CFC-GM, induce the formation of monocyte colonies.\(^{15-17}\) This direct effect may be mediated by accessory cells, as TPA-treated cultures of partially purified CFC-GM fail to form colonies in the absence of exogenous CSA.\(^{25,26}\) This observation is strengthened by our studies, which indicate that phorbol esters have an indirect effect on marrow CFC-GM through the induction of CSA release from marrow cells. Once primed by PDB, bone marrow cells continue to release CSA when grown in the absence of PDB.

In previous studies, CSA has been found in the medium following the incubation with TPA of marrow-derived macrophages,\(^{14}\) leukemic cell lines HL-60 and U937,\(^{18,21}\) and fresh leukemic myeloblasts.\(^{20}\) However, these and other studies\(^{25,26}\) did not completely remove the TPA from the medium, which contained CSA, and some colony stimulation may have occurred through a direct TPA effect. Stuart et al.\(^{15}\) were also unable to separate the direct phorbol ester effect on CFC-GM from an indirect effect that depended on CSA production. They concluded that phorbol esters both stimulated CFC-GM directly and increased the efficiency of CFC-GM stimulation, possibly through altering the CSA receptor-mediated response. Thus, these and prior data are consistent with the observation that PDB causes marrow cells to release CSA. Our experimental design reduced the concentration of PDB to which the CFC-GM were exposed to \(5 \times 10^{-11}\) M, which is at least 2 logs below the level at which PDB itself stimulates colony formation. It is unlikely that residual PDB remaining after washing or gel filtration had a two log increase in specific activity to account for the colony stimulation in the absence of CSA.

In addition to stimulating CFC-GM through a direct action and through the release of CSA, phorbol esters have an inhibitory effect on CFC-GM growth when added to cultures containing other sources of CSA.\(^{17,26}\) The composite effect on proliferation is dependent on the concentration of phorbol esters and of CSA present in culture. This contrasts with the effect of phorbol esters on leukemic cells, where there is complete inhibition of proliferation\(^{18}\) and colony growth.\(^{27}\)

We have also found that phorbol esters have two opposing effects on the differentiation of human CFC-GM. Alone or in the presence of CSA, phorbol esters directly induce an increased level of monocyte colonies and a decreased level of granulocyte colonies.\(^{16,17}\) This action is similar to their ability to induce macrophage differentiation in HL-60 cells and myeloid leukemias.\(^{18,19}\) In contrast, the CSA released following PDB exposure was found to induce a predominance of granulocyte colonies. This effect was only observed after careful removal of PDB from the cultured medium so as to avoid contaminating the CSA assay by residual PDB. The predominance of granulocyte colonies was not due to the presence of inhibitors of monocyte colony formation, including PGE\(_1\). Rather, this CSA appears to specifically stimulate granulopoiesis.

Other sources of CSA have been reported to predominantly induce granulocyte colonies. The serum of endotoxin-treated mice contains granulocyte-specific CSA,\(^{28}\) and a DEAE-Sephadex fraction of WEHI-3 conditioned medium induces murine colonies that are 50%-80% granulocytic, 0%-16% monocytic, and 18%-30% mixed.\(^{29}\) Human placenta-conditioned medium induces colonies that are 50%-70% granulocytic after 14 days of culture.\(^{17,30}\) Other sources of CSA induce either monocyte colonies or a mixture of both monocyte and granulocyte colonies.\(^{1,2}\) Separation of murine and human CSA subclasses on the basis of size has been difficult. All appear to be glycoproteins that have similar physical properties,\(^{5,29}\) although they can be partially separated by DEAE-Sephadex\(^{29}\) and concan-
avalin A-Sepharose. Radioimmunoassays have been developed to a human monocyte CSA but not toward a human granulocyte CSA. Thus, it remains to be determined whether CSA subclasses can be separated and if they activate a selective population of colony-forming cells.

The mechanism by which PDB stimulates granulocyte-specific CSA remains unclear. Monocytes are a known source of granulocyte/macrophage CSA. They can regulate colony formation both by the production of CSA and by the release of compounds, such as monocyte-releasing activity, PGE, and acid ferritins, which modify either CSA release or colony formation. Because bone marrow cell cultures exposed to PDB contain large numbers of monocytes and macrophages, these cells may be responsible for the production of the granulocyte-selective CSA found in PDB-CM. However, phorbol esters are also known to be T cell mitogens, and the CSA we observed may have been of T cell origin. Alternatively, marrow stromal cells may have released CSA in direct response to phorbol esters or in response to mediators released by macrophages stimulated by PDB. Thus, the individual and collective contributions of PDB-treated bone marrow cells to the generation of granulocyte-selective CSA remains to be determined.

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