The Effects of Tumor-Promoting Phorbol Esters on Human Granulopoiesis In Vitro

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In order to determine whether the tumor-promoting phorbol esters are capable of inducing normal human committed granulocytic-monocytic progenitor cells (CFUc) to proliferate and differentiate in the absence of granulocyte-monocyte colony-stimulating activity (CSA), we studied the effects of these compounds on human granulopoiesis in vitro. We found that when light-density human marrow cells or peripheral blood leukocytes were depleted of adherent cells and then incubated in semisolid tissue culture medium under conditions optimal for CFUc growth, phorbol myristate acetate (PMA) and its congeners produced no measurable stimulatory effect on the proliferation of CFUc in the absence of added CSA. Likewise, when light-density marrow cells that had not been depleted of adherent cells were plated in the cultures, no stimulation of CFUc colony growth resulted from the addition of PMA. However, when light-density peripheral blood leukocytes were used as a target source of CFUc without first subjecting them to adherence separation, enhanced proliferation of CFUc was noted in cultures that contained PMA. To investigate the possibility that CSA production by monocytes in these cultures in response to activation by PMA might account for the enhanced colony formation that we observed, we incubated isolated peripheral blood monocytes in short-term liquid suspension cultures and found that in the presence of PMA, large quantities of CSA were secreted into the surrounding medium. Finally, we noted that when marrow cell suspensions were suboptimally stimulated by low concentrations of CSA added to the cultures, the effects of PMA on CFUc proliferation were unpredictable, enhancing colony formation in some cases and inhibiting it in others. Our data indicate that although the tumor-promoting phorbol esters do not appear capable of directly stimulating the proliferation or differentiation of human CFUc in the absence of CSA, they may do so indirectly by causing auxiliary cells such as monocytes to secrete CSA.

The TUMOR-PROMOTING phorbol esters, of which phorbol myristate acetate (PMA) has been the most widely studied to date, are a group of plant diterpenes that produce an extraordinary array of biologic effects in many classes of mammalian cells, both in vivo and in tissue culture. In recent years, these compounds have provoked considerable interest in experimental hematology, not only because they have been found to induce some myeloid leukemic cells to differentiate along the monocyte-macrophage pathway of development, but also because they seem to be capable of modulating the in vitro proliferation and differentiation of normal erythroid and granulocytic-monocytic progenitor cells (CFUc) as well. Of particular interest to our laboratory have been a growing number of reports that imply that PMA and other biologically active phorbol esters may bias the differentiation of committed granulocytic-monocytic progenitor cells (colony-forming units in culture, CFUc) towards monocyte and macrophage production and may in addition either amplify the trophic effects of specific regulatory glycoproteins known as granulocyte/monocyte colony-stimulating factors (GM-CSFs)—or collectively referred to as colony-stimulating activity (CSA)—upon CFUc in tissue culture or substitute for these growth factors, which heretofore have been considered to be absolutely requisite for both the proliferation and differentiation of CFUc in vitro.

Several studies now indicate that CFUc present in cell suspensions of normal murine marrow can be induced by PMA to form colonies of mature granulocytes, monocytes, and macrophages in semisolid tissue culture medium in the absence of added CSA; however, it remains a point of controversy whether the trophic effect of PMA under these conditions is exerted directly upon the CFUc or is instead aimed towards auxiliary marrow cells that become stimulated to produce CSA in culture. In the human system, although it seems clear that the biologically active phorbol esters can stimulate the growth of small clusters of monocytes and macrophages in the absence of added CSA, the evidence that PMA induces CFUc to proliferate and differentiate into large colonies containing granulocytes, monocytes, and macrophages is more restricted, and in general, the effects of PMA on human granulopoiesis in vitro are less well defined.

The experiments reported here were designed specifically to determine whether the tumor-promoting phorbol esters are capable of inducing human CFUc to give rise to full-sized colonies of mature phagocytes in...
the complete absence of CSA. Our data indicate that although these agents do not directly induce human CFUc to proliferate, they may do so indirectly by stimulating auxiliary cells such as monocytes and macrophages to elaborate CSA.

**MATERIALS AND METHODS**

**Collection of Marrow and Peripheral Blood**

Bone marrow samples (3 to 6 mL) were obtained from normal volunteers or from patients undergoing diagnostic marrow aspiration. In all cases, marrow specimens were determined to be free of malignancy. Venous blood (50 to 100 mL) was collected from healthy volunteers. Marrow and blood were obtained immediately before each experiment and anticoagulated with preservative-free heparin. All marrow and blood samples were collected following guidelines approved by the Institutional Review Board for Human Experimentation of University Hospital.

**Fractionation of Hemic Cell Suspensions**

In all experiments, marrow and blood were diluted with an equal volume of Hanks' balanced salt solution (HBSS) containing 50 IU/mL penicillin G and 50 μg/mL streptomycin, and bouyant nucleated cells were isolated by centrifugation of the diluted sample on density gradients of sodium metrizoate-Ficoll. In certain cases, adherent cells were then removed by incubation of the bouyant cells in columns containing nylon fiber. At the end of the separation procedures, the cells were washed twice in HBSS and finally resuspended in HBSS containing 10% fetal bovine serum (HBSS 10% FBS).

Monocytes were isolated from the peripheral blood of healthy volunteers by adherence separation using a modification of the technique described by Shaw et al. Adherent cells prepared in this manner were resuspended in McCoy's 5A medium containing 50 IU/mL penicillin G, 50 μg/mL streptomycin, and 15% fetal bovine serum (McCoy's 15% FBS). Viability of these cells after separation consistently exceeded 91% as assessed by exclusion of trypan blue and the percentage of monocytes as determined by acridine orange staining exceeded 87%.

**Culture of Granulocytic-Monocytic Progenitor Cells**

Granulocytic-monocytic progenitor cells were assayed in biphasic agar cultures using a modification of the method of Pike and Robinson, which we have previously described. Marrow cells or peripheral blood cells, separated as outlined above, were resuspended in supplemented Eagle's medium containing 0.3% agar at concentrations of 1 x 10⁶ marrow cells per milliliter or 2 x 10⁶ peripheral blood cells per milliliter. These cell suspensions were then distributed in 1 mL volumes into 33-mm culture plates onto previously prepared 1-mL underlayers containing the same medium with 0.5% agar, in which a source of CSA or additives to be tested for their effects on CFUc proliferation had been suspended. Maximal stimulation of CFUc proliferation (or 100% CSA activity) was defined for each experiment as the number of colonies of phagocytes that developed in three to six control cultures maximally stimulated by feeder layers containing 10⁶ peripheral blood leukocytes obtained from healthy volunteers. Cultures were incubated for 7-13 days at 37°C in a humid incubator containing 5% CO₂. Colonies were defined as globular aggregates containing fewer than 40 cells. In our laboratory, we have previously established that the colonies which we identify in semisolid tissue culture medium on the basis of their gross morphology, using an inverted microscope, are composed of either neutrophilic granulocytes, eosinophilic granulocytes, monocytes and macrophages, or mixtures of these types of phagocytes, by fixing large numbers of these cultures with formalin, mounting them on glass slides, and staining them with hematoxylin-eosin, luxol fast blue, or α-naphthyl butyrate for the identification of granulocytes, eosinophils, and monocytes, respectively.

**Sources of Colony-Stimulating Activity**

In most experiments, feeder layers containing human leukocytes served as a source of CSA. Venous blood was collected from healthy volunteers, anticoagulated with preservative-free heparin, and allowed to sediment at room temperature. The leukocyte-rich plasma was separated, and cells obtained in this manner were added to the underlayers of cultures at a concentration of 10⁶ cells per milliliter. In other experiments, CSA-rich lymphocyte-conditioned medium prepared as previously described was used.

**Addition of Phorbol Esters to the Cultures**

For most experiments, the tumor-promoting phorbol ester, phorbol-12-myristate-13-acetate (PMA, Sigma Chemical Co, St Louis, Mo) or its inert parent compound phorbol (PHR, P-L Biochemicals, Inc, Milwaukee, Wis) were added directly to the underlayers of bilayer cultures. Other related compounds obtained from P-L Biochemicals, Inc, that were used included the tumor-promoting ester phorbol 12,13-didecanoate (PDD) and the inert esters phorbol 12-monomyristate (PM) and 4α-phorbol 12,13-didecanoate (4α-PDD). These agents were all dissolved in dimethylsulfoxide (DMSO, Fisher Scientific Co, Fair Lawn, NJ) at 1 mg/mL and stored at -20°C; subsequent dilutions were made in HBSS. Diluted samples were sterilized using 0.45 μm Millex-HA filters (Millipore Corporation, Bedford, Mass).

**Measurement of Membrane Depolarization**

Depolarization of the plasma membrane of monocytes in response to phorbol esters was measured using the cationic lipophilic fluorescent cyanine dye 3,3-dipropylthiodicarbocyanine [di-S-C₃(5)] using a modification of the method described by Horne and Simons. Monocytes prepared as described above were resuspended at 10⁶cells per milliliter in 0.15 mol/L sodium chloride (NaCl) at 37°C and placed into the cuvette of a Perkin-Elmer (Oak Brook, Ill) MPF-2A fluorescence spectrophotometer equipped with a stirring apparatus. The sample was excited by light at a wavelength of 620 nm, and fluorescence emitted by the sample was monitored at 670 nm. The di-S-C₃(5), 2 x 10⁻⁶ mol/L, was then added to the sample and allowed to equilibrate until a plateau in fluorescence was achieved. Phorbol esters or appropriate controls were then added to the stirring cell suspension, and the fluorescence was monitored for 15 minutes. The results were reported as relative fluorescence, which represents a ratio (F/FO) of the fluorescence measured after addition of the membrane perturbant (F) to the fluorescence of the sample previously recorded after equilibration with di-S-C₃(5) (FO).

**Statistical Methods**

Standard error of the mean was used as estimate of variance, and means were compared by using Student's t test.

**RESULTS**

Because the biologic activity of the tumor-promoting phorbol esters may decay in storage and because these compounds have been observed to be sensitive to inactivation after exposure to light, efforts were made
to assay periodically the stock solutions of the two tumor-promoting esters PMA and PDD that we used throughout this study. Solutions of PMA and PDD that were dissolved in DMSO and stored in the dark at −20 °C were assayed by Dr Robert Clark in the Section of Infectious Diseases of University Hospital for their capacity to stimulate iodination of human serum albumin in the presence of human neutrophils, an activity that depends upon perturbation of the plasma membrane of neutrophils by biologically active phorbol esters.3 Using this method, the stock solutions of PMA and PDD that we used were shown to retain measurable biologic activity in concentrations as low as 1 ng/mL during the course of this study. In addition, because the capacity of PMA to exert a demonstrable stimulatory effect on the membrane of monocytes was critical for the experiments in this report, we measured the ability of this compound to depolarize the membrane of isolated monocytes in liquid suspension cultures. As shown in Figure 1, we observed that PMA produced a predictable wave of membrane depolarization after its addition to freshly isolated monocytes, whereas no such effect was observed when the biologically inert parent compound PHR was added to the cells.

In order to determine whether PMA induced isolated monocytes to release CSA, suspensions of these cells were incubated for 24 hours in McCoy's 15% FBS containing serial concentrations of PMA or DMSO. At the end of the culture period, the cell-free supernatant media were harvested, dialyzed, and assayed for CSA. Controls included PMA incubated in McCoy's 15% FBS without cells, and medium conditioned by 2 × 10⁶ monocytes per milliliter with no additional additives, medium incubated with a concentration of DMSO equivalent to that present in each of the cultures containing PMA or PHR, and medium conditioned by monocytes incubated in the presence of DMSO. Each bar represents the mean ± SEM of three cultures from one representative experiment that was repeated three times with similar results.

As shown in Figure 2, cell suspensions highly enriched for monocytes were stimulated by PMA to secrete abundant quantities of CSA. No CSA was produced by monocytes incubated alone, and the cell-free media containing PMA exerted no stimulatory effect when tested for CSA activity in the CFUc bioassay.

We next sought to determine whether the direct addition of PMA or PDD to CFUc cultures of human marrow cells would result in stimulation of the proliferation of colonies of phagocytes in the absence of added CSA. In these experiments, marrow cells were fractionated by centrifugation on sodium metrizoate-Ficoll gradients and then incubated in bilayer agar cultures in the presence of serial concentrations of several different phorbol esters. Shown in Figure 3 are the results of one of four identical experiments. Since granulocyte colonies have been observed to reach peak numbers in CFUc cultures earlier than colonies containing primarily monocytes and macrophages,32 in these experiments colony growth was enumerated on both day 7 and day 13. On both days, the number of colonies that proliferated in the cultures containing PMA or PHR was no different from that in the unstimulated control cultures. In all of these experiments, in addition to PMA and PHR, the phorbol esters PDD, 4αPDD, and PM were tested at all of the concentrations shown; no stimulatory or inhibitory effect of any of these compounds on CFUc proliferation was observed in the absence of added CSA.

In order to determine whether the PMA induced CFUc proliferation in the absence of added CSA but the effect was masked by background colony growth.
resulting from CSA produced by monocytes present in the marrow cell suspension, experiments similar to those depicted in Fig 3 were carried out before and after removal of monocytes by incubation of the marrow cells in columns containing nylon fiber. As shown in Fig 4, no colony growth was detected in cultures to which PMA was added after depletion of adherent cells.

Experiments similar to those described above were performed to determine the effect of PMA on the proliferation of CFUc present in the peripheral blood. As shown in Fig 5, when buoyant, nonadherent peripheral blood cells prepared by centrifugation on sodium metrizoate-Ficoll gradients and subsequent nylon fiber adherence were used as the source of CFUc, no stimulation of colony growth was evident in any one of seven identical experiments. However, when mononuclear cells that had not been depleted of monocytes were plated in culture, significant stimulation of CFUc colony growth, equal to that seen in control plates maximally stimulated by peripheral blood feeder layers, was noted in three of four experiments. Of interest is the observation that stimulation of CFUc proliferation was noted in the presence of concentrations of PMA as low as 10^{-9} \text{g/L} (Fig 6).

To determine whether PMA was capable of augmenting the proliferative response of CFUc to suboptimal concentrations of CSA as has previously been reported,\textsuperscript{9} we cultured buoyant, nonadherent marrow cells in the presence of serial concentrations of PMA and quantities of CSA-rich lymphocyte-conditioned medium inadequate to stimulate maximal colony growth. Although augmentation of CFUc proliferation by low concentrations of PMA was clearly noted in some of these cultures (Fig 7A), the degree of stimulation was highly variable and unpredictable from experiments.
Fig 6. Effect of PMA on the proliferation of CFUc in suspensions of light-density peripheral blood leukocytes not subjected to adherence separation. Blood cells prepared by sodium metrizoate-Ficoll centrifugation were cultured with PMA in plates containing no added CSA. Control cultures (hatched bars) were either maximally stimulated by leukocyte feeder layers or contained no added CSA (blank). Each bar represents the mean ± SEM of three cultures of one of three experiments.

Fig 7. Effect of PMA on CFUc growth in cultures submaximally stimulated by added CSA. In panel A, test cultures (white bars) all contained a uniform suboptimal concentration of lymphocyte-conditioned medium (1% LCM) with or without PMA or PHR. Controls (hatched bars) included cultures containing no added CSA (blank), or cultures maximally stimulated by leukocyte feeder layers or contained no added CSA (blank). Each bar represents the mean ± SEM of three cultures from a single experiment. In panel B, test cultures contained serial concentrations of CSA-rich, lymphocyte-conditioned media containing 10^{-4} g/L PMA, 10^{-4} g/L PHR, or neither (HBSS). Values shown represent the mean ± SEM of three cultures in one experiment.

DISCUSSION

The primary focus of our study was to determine whether the tumor-promoting phorbol esters are capable of directly inducing human CFUc to proliferate and differentiate into colonies of granulocytes, monocytes, and macrophages in the absence of CSA. We found that even when PMA and PDD were added over a wide range of concentrations to cultures of bone marrow or peripheral blood cell suspensions that had first been depleted of adherent cells, no stimulation of CFUc proliferation, as assessed by the formation of colonies of mature phagocytes, could be detected. Likewise, when marrow cells that had not previously been subjected to adherence separation were plated in the cultures, no increase in CFUc growth resulted from the addition of PMA or PDD. However, when light-density peripheral blood leukocytes were used in similar experiments without prior removal of adherent cells, CFUc proliferation appeared to be enhanced in the presence of PMA. Since the addition of PMA to liquid suspension cultures of monocytes induced these cells to release substantial quantities of CSA into their surrounding medium, it is reasonable to assume that when hemic cell suspensions that contain relatively large numbers of monocytes are cultured in semisolid medium, the CFUc present in these cell preparations may be indirectly induced to develop into colonies of mature phagocytes because of stimulation of CSA production via the activation of monocytes. Since peripheral blood mononuclear cells prepared by sodium metrizoate-Ficoll centrifugation contain large numbers of monocytes, whereas light-density bone marrow cells fractionated by the same procedure have relatively few, the fact that we observed that PMA augmented the growth of CFUc in cultures of light-density peripheral blood mononuclear cells but not in cultures of marrow cells prepared in a similar manner strongly supports the notion that enhancement of CFUc proliferation by PMA in cultures that contain no added source of CSA is dependent upon the presence of a critical number of monocytes and macrophages.

Since the report of Lotem and Sachs, which observed that PMA stimulated murine bone marrow cells to produce clusters of macrophages, and that of Stuart and Hamilton, which showed that under appropriate conditions PMA induced murine CFUc to develop into large colonies containing granulocytes and monocytes in the absence of CSA added to the culture plates, similar reports have appeared from other labo-
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ratories, all of which have left little doubt that these compounds exert a potent trophic effect on murine CFUc in tissue culture and appear to induce their differentiation along the monocyte-macrophage pathway of development preferentially. Considerable controversy, however, generated primarily by these studies, has centered on the nature of the stimulation effected by PMA and its derivatives on progenitor cells within the CFUc compartment. In the murine system, these agents have been postulated to replace CSA in vitro, to augment the effects of CSA on CFUc, or to induce auxiliary cells present in the cultures to secrete CSA. Since the proliferation and differentiation of CFUc in all mammalian species have long been considered to be absolutely dependent upon CSA, the possibility that PMA might mimic the trophic effects of one or more GM-CSF in vitro suggested that this compound might prove to be a valuable tool with which to dissect the early subcellular events involved in the initiation of differentiation of granulocytic-monocytic progenitor cells. However, by using highly purified preparations of murine or human CFUc effectively devoid of monocytes and macrophages, two groups recently have independently provided convincing evidence that in the absence of CSA-producing cells, PMA is incapable of stimulating CFUc to form colonies in semisolid tissue culture medium. The data of these investigators, as do those of Greenberger et al, implicate the induction of CSA production by cells such as marrow macrophages as the most plausible mechanism for enhanced CFUc proliferation in PMA-stimulated cell cultures. We feel that the experiments we have cited in this report corroborate this notion and suggest that similar indirect stimulation by PMA is likely to be operable in human CFUc cultures.

An alternative hypothesis offered by a number of groups is that PMA may enhance the stimulatory effect of CSA on murine CFUc by altering the plasma membrane of these progenitor cells in such a way as to render the cell hypersensitive to suboptimal concentrations of one or more GM-CSF in the surrounding environment. This proposal has recently been strengthened by the report of Guilbert et al, who have found that PMA mimics many of the effects of GM-CSF, and appears to be able to down-regulate the GM-CSF, receptor on macrophages but not to compete with 125I-CSF, for binding to the receptor. Taken together, these studies, all of which have investigated the effects of phorbol esters on CFUc present in normal murine bone marrow cell suspensions, point to the concept that the induction of enhanced responsiveness of at least some cells within the CFUc population may be another mechanism whereby PMA may augment murine CFUc growth. However, Abraham and Smiley and Griffin et al have reported an altogether different effect of PMA on human CFUc colony growth stimulated by CSA. These investigators have observed that CFUc colony formation induced by peripheral blood feeder layers or human placenta-conditioned medium appears to be predictably inhibited in the presence of PMA. Since we noted that CFUc growth in cultures containing submaximal concentrations of CSA-rich human lymphocyte-conditioned medium was clearly enhanced in some (Fig 7) but not all of the experiments that we carried out, we feel that if phorbol esters do have the capability of modulating the trophic effect of CSA on human CFUc, such interaction is likely to be exceedingly complex, perhaps involving either synergy between PMA and only one class of GM-CSF, as Guilbert et al have implied, or simultaneous inhibition of those progenitor cells within the CFUc compartment committed to granulocyte production and stimulation of other progenitors programmed for monocyte-macrophage development as has been postulated.

When we compared the total number of CFUc colonies that proliferated in cultures of light-density, nonadherent peripheral leukocytes (Fig 5) with the number that developed in similar cultures of leukocytes that had not first been depleted of monocytes (Fig 6), we found that substantially fewer colonies were present in the former. We have previously observed that nylon fiber cell chromatography, while a highly efficient technique for the depletion of monocytes and macrophages, typically results in the loss of about 45% of the total number of CFUc when suspensions of human marrow cells are fractionated using this procedure, and the discrepancy that we observed between the number of CFUc present in these two cell preparations may therefore be accounted for in part by trapping of CFUc in the nylon fiber columns we used. However, since the number of colonies that developed in “unstimulated” control cultures of light-density peripheral leukocytes was approximately 50% of that seen in cultures of these cells stimulated by feeder layers, it seems likely that the presence of the large number of monocytes in the overlayers contributed significantly to the enhancement of colony growth by augmenting the total amount of CSA generated in the cultures. Although we cannot with certainty rule out the possibility that proliferation of T lymphocytes might have accounted for some of the increased number of colonies we observed in cultures of peripheral leukocytes treated with PMA, this explanation seems inadequate in view of the fact that no increased colony formation occurred in the presence of PMA in cultures of leukocytes subjected to adherence separa-
tion, since this cell population is highly enriched for T lymphocytes. Because we noted an enhancement of CFUc proliferation both at high concentrations of PMA, which we found optimal in effecting the production of CSA by suspensions of monocytes (Fig 2), and at low concentrations, which we (Fig 7) and others have observed to enhance the proliferative response of CFUc to CSA, we feel it likely that the increased colony growth in these cultures that we observed over a wide range of concentrations of PMA may have resulted from more than one stimulatory effect directed by these compounds towards cells within the CFUc compartment.

Although others have noted profound suppression of CFUc proliferation by concentrations of PMA above 10–100 ng/mL in cultures stimulated by CSA-rich conditioned media and some have observed a more modest degree of inhibition of human CFUc growth in the presence of high concentrations of PMA in cultures stimulated by leukocyte feeder layers, we found no such inhibition in our experiments. Although the discrepancy between the data of these investigators and our own cannot be clearly explained at this time, our findings, as well as those of Griffin et al and Pegoraro et al suggest that when viable leukocyte feeder layers are used as the source of CSA, PMA-induced generation of CSA in the cultures may in part counteract the inhibitory effect of high concentrations of PMA on CFUc.

In summary, the data we have presented indicate that in the absence of monocytes and macrophages, human CFUc are not induced to proliferate or to differentiate by the tumor-promoting phorbol esters. Our experiments further show that PMA induces cell suspensions highly enriched for monocytes to elaborate significant quantities of CSA, which may result in secondary stimulation of CFUc growth. Finally, although we have found that in certain cases PMA appears to increase the trophic effect of CSA on human CFUc in vitro, the variability of this effect is likely to reflect a series of intricate interactions between the phorbol esters and progenitor cells within the CFUc compartment, the nature of which is only beginning to emerge.

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