ABSTRACT

The effect of cyclic adenosine 3':5'-monophosphate, cyclic guanosine 3':5'-monophosphate, and prostaglandins E₁ and F₂α on the proliferation of normal and abnormal human myeloid progenitor cells was examined. In systems relatively free from endogenous colony-stimulating cells, cyclic adenosine 3':5'-monophosphate was found to be a dose-dependent inhibitor of normal granulocyte-macrophage progenitor (CFU-C) proliferation. Cyclic guanosine 3':5'-monophosphate increased the number of colonies observed in most experiments, although no clear-cut dose-response relationship was observed. Other compounds tested [prostaglandin F₂α, prostaglandin E₁ (PGE₁), carbachol, isoproterenol] affected normal CFU-C's in a manner consistent with their ability to increase either intracellular cyclic adenosine 3':5'-monophosphate or cyclic guanosine 3':5'-monophosphate. PGE₁ had effects on both CFU-C proliferation and colony-stimulating factor elaboration. The conditions of exposure to PGE₁ dramatically affected whether a stimulatory or inhibitory effect was noted. Abnormal peripheral blood granulocyte-macrophage colony-forming units from patients with chronic myelogenous leukemia responded to cyclic nucleotides in a manner similar to that of normal CFU-C's but showed defective inhibition by PGE₁ in the presence of colony-stimulating factor. Peripheral blood blast colony-forming units from patients with acute leukemia also responded to cyclic nucleotides and PGE₁ in a manner similar to that of normal CFU-C's, with one exception. Peripheral blood monoblast colony-forming units showed increased rather than decreased proliferation in the presence of PGE₁ at concentrations of 10⁻⁷ to 10⁻⁵ M. These data support the contention that cyclic nucleotides can alter in vitro proliferation of human committed myeloid progenitors. They also indicate a possible pivotal role for PGE₁ in regulation of normal myelopoiesis and abnormalities of response to PGE₁ in myeloid progenitors in certain types of hematological cancer.

INTRODUCTION

In recent years, the development of in vitro techniques for the growth of normal hemopoietic progenitors (6, 21, 30) has allowed investigation of potential regulators of normal hemopoiesis (7). The proliferation of normal granulocyte-macrophage progenitors (CFU-C) in these systems is absolutely dependent on the presence of a specific glycoprotein termed CSF (22). CSF remains the most likely candidate identified to date as an in vivo humoral regulator of normal granulopoiesis. Once stimulated by CSF, however, the proliferation of normal CFU-C's may be modulated by several naturally occurring compounds and hormones (12, 13). The proliferation of committed erythroid progenitors in vitro may also be modified by such compounds (2).

Studies by others (2, 5, 12, 13, 18, 28, 29, 32) have indicated that hemopoietic stem cell and committed progenitor proliferation may be subject to alteration in these systems by cyclic nucleotides or by hormones and drugs which cause changes in the intracellular concentration of cyclic nucleotides. It has been suggested that such compounds might modulate, in vivo, the ongoing proliferation of normal granulocyte progenitors. Many of these studies in the past were performed in murine systems (5, 18, 28) or failed to take into account possible effects of the cyclic nucleotides on elaboration of CSF (32). We have reexamined the question of the effect of cyclic nucleotides on normal human CFU-C proliferation in systems relatively free from CSF-elaborating cells, and we have extended these studies to assess the effects of cyclic nucleotides on the in vitro proliferation of hemopoietic progenitor cells from patients with hematological cancers.

MATERIALS AND METHODS

Culture of Normal Human Bone Marrow Granulocyte-Macrophage Progenitor Cells (CFU-C). Normal human bone marrow was collected in preservative-free heparin, diluted 1:1 with Hanks' balanced salt solution, and subjected to Ficoll-Hypaque sedimentation (3). Light-density cells were collected and washed 3 times in McCoy's Medium 5A plus 10% FCS. Adherent bone marrow cells were removed by incubation in tissue culture flasks in McCoy's Medium 5A with 10% prescreened FCS (Flow Laboratories) for 1 hr. Bone marrow specimens contained a mean of 6 ± 2.3% (S.E.) monocytes before adherence and 4 ± 2% (S.E.) monocytes after adherence as determined by α-naphthylesterase staining. Peripheral blood specimens contained 2.6 ± 1% (S.E.) monocytes after adherence as determined by α-naphthylesterase staining. Adherent peripheral blood and bone marrow cells were 90% or greater monocyte-macrophages as determined by α-naphthylesterase staining. Nonadherent cells were plated in triplicate at 1 to 2 x 10⁵/ml (marrow) or 5 x 10⁵/ml (peripheral blood) in 0.3% agar and supplemented McCoy's Medium 5A + 15% FCS as described by Pike (30). CSF was provided by (a) 2 lots of pooled media conditioned by Ficoll-Hypaque-separated peripheral blood mononuclear cells stimulated with pokeweed mitogen or (b) WBC feeders in 0.5% agar as described by Pike (30). Cultures were incubated at 37°C in 5.0% CO₂ for 10 days, and aggregates of greater than 40 cells were scored with an inverted microscope. Bone marrow and peripheral blood specimens subject to adherence displayed no spon-
taneous colony formation. The composition of the colonies was assessed by picking individual colonies with a fine capillary pipet, preparing cytocentrifuge preparations, and staining with Wright-Giemsa or α-naphthyl- or chloroacetate esterase. Control bone marrow cultures in these studies grew 114 ± 71 (S.D.) granulocyte-macrophage colonies/2 × 10⁵ cells.

Culture of Peripheral Blood Granulocyte-Macrophage Colony-forming Units from Patients with CML. Peripheral blood from patients with CML was collected in preservative-free heparin and subjected to Ficoll-Hypaque separation. Light-density cells were washed 3 times in McCoy’s Medium 5A + 10% FCS. The washed cells were then plated in triplicate without CSF at 5 × 10⁵/ml, or after adherence in tissue culture plates, with CSF at 1 to 2 × 10⁵/ml. Cultures were incubated and scored as described above.

Peripheral Blood Blast Colony-forming Assay. Peripheral blood from 5 untreated patients with acute nonlymphocytic leukemia was cultured by a modification of the technique described by Minden et al. (24). Peripheral blood was collected in preservative-free heparin and subjected to Ficoll-Hypaque separation. SRBC-rosetting cells were then removed on Ficoll-Hypaque. The nonrosetting fraction contained less than 0.1% SRBC rosette-positive cells. The non-SRBC-rosetting fraction was plated in triplicate at 4 × 10⁵/ml in 0.8% methylcellulose in α-medium + 15% FCS and 10% PHA-lymphocyte conditioned medium. Aggregates of greater than 20 cells were scored after 5 to 7 days of incubation at 37° in 7.5% CO₂. The myeloid or monocytic nature of the colony-forming cells was established by esterase staining of pooled cytopsin preparations of colonies picked with a fine capillary pipet and by their lack of SRBC rosette-forming capacity.

Preparation of Conditioned Media. Pokeweed mitogen-conditioned medium was prepared by incubating Ficoll-Hypaque-separated mononuclear cells (1 × 10⁶/ml normal peripheral blood) in McCoy’s Medium 5A + 10% FCS + 2% pokeweed mitogen (Grand Island Biological Co., Grand Island, N. Y.) for 1 week. The conditioned medium was then centrifuged (500 × g, 10 min), Millipore filtered, and stored at −20° until used. All studies were conducted at a final concentration of 10%, using one of 2 batches of pokeweed mitogen-conditioned medium, and gave identical results. PHA-leukocyte-conditioned medium was prepared by incubating Ficoll-Hypaque-separated bone marrow cells in McCoy’s Medium 5A + 10% FCS in a 35-mm Falcon tissue culture dish for 1 hr. Nonadherent cells were washed 3 times in McCoy’s Medium 5A + 10% FCS and subjected to Ficoll-Hypaque separation. SRBC-rosetting cells were then removed on Ficoll-Hypaque. The nonrosetting fraction contained less than 0.1% SRBC rosette-forming capacity.

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Reagents. The following agents were tested for their effects on normal and abnormal colony-forming unit proliferation: cAMP, Sigma Chemical Co., St. Louis, Mo.; cGMP, Sigma; carbamylcholine, Sigma; isoproterenol, Sigma; PGE₁; and PGF₂α. Prostaglandins were kindly provided by Dr. John Pike (Upjohn Company, Kalamazoo, Mich.). Stock solutions of isoproterenol were prepared in 10⁻⁶ M ascorbic acid. Stock solutions of prostaglandins were prepared in ethanol at 5 × 10⁻³ m, and ethanol controls were used when appropriate. For certain studies, bone marrow cells were exposed for 2 hr to cyclic nucleotides or PGE₁, either without adherence or after adherence. The cells were then washed and plated on WBC feeders to assess the effects of preincubation with these compounds on CFU-C proliferation.

Patients. Normal human bone marrow was obtained from hematologically normal patients during routine orthopedic and cardiothoracic surgical procedures. The peripheral blood of 3 patients with Philadelphia chromosome-positive CML was cultured on 3 separate occasions. All 3 patients had a WBC of greater than 30,000/ml and had been off all therapy for greater than 7 days. None were considered to be in blast crisis or accelerated phase. The peripheral blood of 4 untreated patients with acute myelogenous leukemia and peripheral WBC of greater than 40,000/ml with greater than 60% blasts were studied. One untreated patient with acute monocytic leukemia with greater than 90% circulating blasts was also studied.

Assessment of Cyclic Nucleotide Effects on Non-S-Phase CFU-C’s. To determine whether the effect of cyclic nucleotides and PGE₁ on normal bone marrow CFU-C’s was limited to colony-forming units currently in S phase, light-density, nonadherent bone marrow cells were incubated for 1 hr with 10⁻³ M hydroxyurea, washed, and plated with PGE₁, or cyclic nucleotides. This procedure resulted in a reduction of colony numbers of 30%.

RESULTS

The effect of addition of cAMP, cGMP, PGE₁, or PGF₂α granulocyte-macrophage colony growth from normal, adherent cell-depleted cultures of human bone marrow is shown in Chart 1. All results represent the mean ± S.E. of determinations from 6 separate experiments. In general, the results are similar to the effects of these compounds on the growth of murine CFU-C’s (18, 27, 28). cAMP was found to be a dose-dependent inhibitor of CFU-C proliferation. cGMP enhanced colony growth, although the magnitude of response and concentration at which maximal enhancement occurred was not dose dependent. This variability in response is reflected in the large standard errors in Chart 1. The trend, however, to enhance proliferation with cGMP was consistent, beginning at concentrations greater than 10⁻⁸ M.

PGE₁ was found to be a dose-dependent inhibitor of normal CFU-C proliferation consistent with its known ability to raise intracellular levels of cAMP (20). The magnitude of this inhibition even at very high levels of PGE₁ was much less than that noted by others in murine systems (18). At concentrations of 10⁻⁵ M PGE₁, only 55% inhibition was noted, while nearly complete inhibition of murine CFU-C growth was noted by others at this concentration (18).

When the effect of PGE₁ on normal CFU-C proliferation was assessed using marrow containing adherent cells, or marrow
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These results indicate that cyclic nucleotides and compounds causing intracellular elevations of cyclic nucleotides can modulate normal human CFU-C proliferation. The results obtained with cAMP and cGMP appear opposite in nature, similar to results noted in other cell systems in which cAMP is found to be an inhibitor of proliferation, and cGMP has no effect on or is a mild enhancer of proliferation (11, 27, 28).

The effects of short-term (2-hr) exposure of marrow cells to cGMP, cAMP, and PGE1 are shown in Chart 3A. In these experiments, normal bone marrow cells were preincubated for a period of 2 hr with either cyclic nucleotide or PGE1, washed, and plated on WBC feeders. With 2 hr of exposure, the cAMP and cGMP curves were very similar to those seen with more continuous exposure. The retention of this pattern of exposure with only 2 hr of incubation suggests strongly that the action of these compounds is to alter the response of the CFU-C to some early event in the culture.

The effect of PGE1 with short-term exposure at first appeared paradoxical; enhancement rather than inhibition of proliferation was noted (Chart 3A). However, when the adherent bone marrow cells were removed from the specimen by incubation in tissue culture flasks prior to exposure to PGE1, enhancement of proliferation was greatly attenuated (Chart 3B). Removal of adherent bone marrow cells had no effect on the response to cGMP or cAMP (Chart 3B). This finding would be consistent with an effect of PGE1 on CFU-C growth through increased elaboration of CSF by endogenous, adherent bone marrow cells. In contrast, the effects of short-term exposure to cyclic nucleotides appear to be primarily on the colony-forming cell itself, since removal of the adherent cells did not change the pattern of response.

This latter hypothesis is supported by the effects of PGE1 on elaboration of CSF by adherent bone marrow cells shown in Chart 4. When adherent bone marrow cells were allowed to condition media in the presence of variable concentrations of

plated over WBC feeders, PGE1 was still found to be a dose-dependent inhibitor of similar potency (data not shown). The effect of PGE1 with protracted exposure appeared to be an inhibitory one, even in the presence of CSF-producing cells.

The response of normal human CFU-C's to PGE2a, which is known in some cell systems to elevate intracellular levels of cGMP (10), was similar to that seen with cGMP itself. Enhancement of proliferation was noted over the entire concentration range from $10^{-8}$ to $10^{-5}$ M. The variability of response reflected in the cGMP curve is similarly reflected in the response of the colony-forming units to PGE2a.

Incubation of the marrow cells with hydroxyurea for 1 hr failed to change the pattern of response to these agents, indicating that their ability to modulate human CFU-C proliferation was not restricted to cells currently in the process of synthesizing DNA (data not shown).

When carbamylcholine, which may increase intracellular concentrations of cGMP (9), and isoproterenol, which may increase intracellular concentrations of cAMP (20), were substituted for the cyclic nucleotides, the expected patterns of response remained unchanged (Chart 2). These results represent the mean ± S.E. of triplicate determinations from 6 experiments. These results strongly suggest that the observed alterations in proliferation noted with the prostaglandins and cyclic nucleotides were due to increases in the intracellular concentration of cyclic nucleotides.

Chart 1. The effect of the addition of cAMP, cGMP, PGE1, or PGF2a on normal bone marrow granulocyte-macrophage colony growth. Normal bone marrow was depleted of endogenous CSF-producing (adherent) cells and cultured with the cyclic nucleotide or prostaglandin. Similar effects on CFU-C proliferation were seen when PGE1 was cultured with bone marrow cells without removal of adherent cells or when bone marrow or peripheral blood was cultured on WBC feeders. Concentrations are in log10 units. Points, mean of 6 experiments; bars, S.E.; CFU's, colony-forming units.

Chart 2. The effect of addition of carbamylcholine or isoproterenol on normal granulocyte-macrophage colony growth. Concentrations are in log10 units. CFU's, colony-forming units; Bars, S.E.
PGE₁ for 1 week, a dramatic increase in CSF elaboration occurred over the same concentration range in which the growth enhancement with short-term exposure to PGE₁ was noted. These findings support the contention of others (16, 17, 19) in murine systems that PGE₁ may serve both to inhibit CFU-C proliferation and to enhance CSF release. These findings also indicate that the time course of exposure to the prosta
glandin is critical in determining which of these opposing influences on CFU-C proliferation predominates in culture. It is of note that we were unable to duplicate these actions of PGE₁ on elaboration of CSF by adherent peripheral blood cells using the cell concentrations in these studies.

We began a series of studies to evaluate the response of abnormal myeloid progenitor cells to cAMP, cGMP, and PGE₁. We first examined the peripheral blood granulocyte-macro
dage colony-forming units from patients with CML. Patients with CML have a 100- to 1000-fold increase over normals in the concentration of granulocyte-macrophage colony-forming units in their peripheral blood (2, 14, 26). At times, the concentration of the colony-forming units in the peripheral blood of CML patients may exceed that seen in the bone marrow (25). The proliferation of these abnormal colony-forming units remains dependent on the presence of either adherent peripheral blood cells or added CSF (21, 26).

The results obtained when cAMP, cGMP, or PGE₁ were added to spontaneously proliferating (without added CSF, but with adherent cells) peripheral blood granulocyte-macrophage colony-forming units from CML patients are shown in Chart 5. Results are expressed as mean ± S.E. of 3 experiments. The response of these colony-forming units to all 3 compounds was similar under these conditions to that of normal CFU-C's.

When adherent peripheral blood cells were removed and the same source of CSF used in previous studies was added to CML peripheral blood cultures, PGE₁ was found to no longer inhibit the abnormal colony-forming unit proliferation (Chart 6). This effect occurred even at extremely high concentrations of PGE₁. These data suggest that CSF may be more easily able to overcome the inhibitory effect of PGE₁ on abnormal progenitor cells than their normal counterparts. The possibility that this pattern of response of CML colony-forming units was due to differences in the content of CSF-producing cells in the cultures or to differences between marrow and peripheral blood granulocyte-macrophage colony-forming units was considered. When included in the cultures, PGE₁ remained a dose-dependent inhibitor of normal marrow cells cultured without prior adherence, marrow cells cultured on WBC feeders, and adherent cell-depleted cultures of peripheral blood CFU-C's. As noted above, the content of α-naphthyl-esterase-positive cells in bone marrow and peripheral blood specimens did not differ after adherence. The lack of inhibitory effect of PGE₁ in the presence of CSF appeared to be a finding restricted to CML peripheral blood colony-forming units.

Finally, the effect of cAMP, cGMP, and PGE₁ on the proliferation of blast progenitors from the peripheral blood of 5 patients with acute nonlymphocytic leukemia was studied (Chart 7). These colonies contained only cells with appear-
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Chart 4. The effect of PGE₁ on elaboration of CSF by adherent bone marrow cells. Adherent bone marrow cells were incubated with medium in the presence of variable concentrations of PGE₁, dialyzed, and tested for CSF activity. CFU's, colony-forming units. Concentrations are in log₁₀ units.

ances morphologically and histochemically consistent with those of blasts, promyelocytes, or immature macrophages and monoblasts. The effect of all 3 compounds on the proliferation of these abnormal progenitors was very similar to those on normal progenitors, with one exception. This single exception was the response of the blast colony-forming units from a patient with acute monocytic leukemia to PGE₁. These cells showed marked enhancement of colony-forming unit proliferation in response to concentrations of 10⁻⁷ to 10⁻⁸ M PGE₁. Cells comprising the monoblast colonies morphologically and histochemically resembled immature macrophages and clearly differed from those seen in the blast colonies from the other 4 patients. These monoblast colony-forming units will be described in detail in a separate publication (31). The possible implications of this abnormal response to PGE₁ are discussed below.

DISCUSSION

The data presented here substantiate the ability of cyclic nucleotides to alter in vitro proliferation of normal human myeloid progenitors under conditions relatively free from possible effects on CSF elaboration. Similar effects were seen with prostaglandins and drugs known to cause intracellular elevations of cAMP and cGMP. In general, these studies support the contention that increases in the intracellular concentration of cGMP...
cyclic nucleotides can affect the proliferation of normal myeloid progenitor cells (18). These data are also consistent with data in other cell systems in which sustained elevations of cAMP inhibit proliferation and elevations of cGMP have no effect or enhance proliferation (11, 15). Suggested in vivo mechanisms by which modulation of ongoing hemopoiesis might occur include elaboration of PGE, by bone marrow macrophages (16) and secretion of catecholamines and cholinergic compounds by neural innervation of the bone marrow (8).

The results obtained with short-term exposure to PGE, however, illustrate the problem of studying only part of an organ (bone marrow) in an isolated culture system. With short-term preincubation with this apparent inhibitor of CFU-C proliferation, normal bone marrow demonstrated an increase in colony proliferation. Thus, the same compound, under 2 different culture conditions, appeared to have opposing effects. The effects of short-term exposure to PGE, on the bone marrow cells appeared to be on the monocyte-macrophage population rather than on the progenitor cell itself. The attenuation of the observed increase in colony growth by removal of adherent bone marrow cells supports the contention that the observed enhancement was due in large part to some action on the adherent cell population. The effects of PGE, in preincubation studies were consistent with its effects in increasing the elaboration of CSF by adherent bone marrow cells. These data support the contention of Kurland et al. (16, 17, 19) that PGE, could play a central role in the regulation of normal granulopoiesis by monocyte-macrophages, acting both to inhibit the CFU-C and to stimulate CSF release from macrophages. Recent studies by Pelus et al. (29) also indicate that the inhibitory action of PGE, on the proliferation of murine CFU-C's may be specific for macrophage colony-forming units, with less effect on granulocyte or granulocyte-macrophage progenitors.

We tried to assess whether progenitors from patients with hematological cancers might show abnormalities in their in vitro patterns of response to cyclic nucleotides or PGE,1. The results obtained with blast colony-forming units suggest that these abnormal precursors retain their responsiveness to both cAMP and cGMP. The response of these progenitors to PGE, also appeared to be similar to that of normal CFU-C's. The only abnormality encountered in these studies of acute leukemia was in the response of blast colony-forming units from a patient with acute monocytic leukemia to PGE,. The similarity of the response of these cells to that of normal CFU-C's to short-term exposure to PGE, and to the effects of PGE, on adherent bone marrow cell elaboration of CSF is striking. It is tempting to speculate that these cells, possessing some properties of normal, mature monocyte-macrphages, react to PGE, by elaboration of a humoral stimulus to proliferation.

Finally, we studied the effect of the cyclic nucleotides and PGE, on the peripheral blood colony-forming unit of CML. These colony-forming units are known to be morphologically (resembling myeloblasts rather than lymphocytes) and physically distinct from the normal CFU-C population (26). Despite extensive in vitro and in vivo investigation, the factors allowing excessive myeloid proliferation in CML have remained obscure. Although progenitor cells in this disorder appear dependent on CSF for proliferation, they respond sluggishly to CSF (21, 23). Peripheral blood cells from these patients are also poor, rather than rich, sources of CSF themselves (14). It has been suggested that feedback inhibition by granulocyte-derived inhibitory factors is deficient in this disorder (4), but this finding has been disputed (1).

Our results indicate that in vitro inhibition of peripheral (CML) blood granulocyte-macrophage progenitors (by PGE,) is deficient in the presence of exogenous CSF. The fact that inhibition of these abnormal progenitors by PGE, appears similar to, or even more pronounced than, that of normal progenitors in the absence of exogenous CSF suggests that CSF may compete more effectively with PGE, in these cells than in normal cells. Studies in murine systems have shown that increasing concentrations of CSF can compete with the inhibitory effects of PGE, on normal CFU-C's (19). The disparity noted between the effects of cAMP and those of PGE, in CML is puzzling, however. Studies by Ziboh et al. (33) indicate that rat chloroleukemia cells can convert PGE, to PGF, at 8 times the rate of normal bone marrow cells. If such an accelerated rate of conversion also occurred with cells from patients with CML, this might provide an explanation for these observations. This matter is currently under investigation.

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Effects of Cyclic Nucleotides and Prostaglandins on Normal and Abnormal Human Myeloid Progenitor Proliferation

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