Bryostatin 1 Modulates the Proliferation and Lineage Commitment of Human Myeloid Progenitor Cells Exposed to Recombinant Interleukin-3 and Recombinant Granulocyte-Macrophage Colony-Stimulating Factor

By Fei Li, Steven Grant, George R. Pettit, and Carl W. McCrady

The activity of protein kinase C (PK-C) has been implicated in the regulation of the growth and differentiation of both normal and neoplastic hematopoietic cells. We have examined the effects of the PK-C-activating agents phorbol 12,13-dibutyrate (PDBu), mezerein, and bryostatin 1 on the proliferation and lineage commitment of CD34+ human myeloid progenitor cells stimulated by recombinant interleukin-3 (rIL-3) and/or recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF). Although each of the PK-C activators administered alone induced no colony formation, coadministration of these agents with plateau concentrations of each cytokine (eg, 50 ng/mL) increased the number of day 14 granulocyte-macrophage colony-forming units by 100% to 150%. The number of pure and mixed neutrophil and macrophage colonies was substantially enhanced in the presence of PK-C activators, whereas the percentage and, in most cases, the absolute number of eosinophilic colonies was significantly reduced. The inhibition of eosinophilic colony formation was not overcome by the addition of rIL-5. Although addition of bryostatin 1 24 hours before rIL-3 abrogated the increase in total colony formation observed with simultaneous administration of factors, the inhibition of eosinophilic colonies and the increase in neutrophil/macrophage colonies persisted under these conditions. The addition of bryostatin 1 for up to 144 hours after rIL-3 continued to potentiate total colony formation, whereas the inhibition of eosinophilic commitment was lost after 120 hours. Together, these results suggest that pharmacologic interventions at the level of PK-C may regulate both the proliferation as well as the lineage commitment of human hematopoietic progenitors exposed to rGM-CSF and rIL-3.

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SEVERAL LINES of evidence suggest that the action of hematopoietic colony stimulating factors (CSFs) may be influenced by protein kinase C (PK-C) activity. For example, the PK-C–stimulating phorbol ester, TPA (12-O-tetradecanoylphorbol-13-acetate), is capable of serving as a source of colony-stimulating activity for murine and human myeloid hematopoietic progenitors in vitro1,2 and promotes the survival and proliferation of interleukin-3 (IL-3)-dependent cell lines.3 Agents with PK-C–inhibitory activity have also been found to inhibit in vitro colony formation by human bone marrow cells exposed to granulocyte-macrophage CSF (GM-CSF) and IL-3.4 In addition, PK-C activity has been implicated in regulating the growth and differentiation of human and murine leukemic cell lines.5,6

Although phorbol esters have been most commonly used for the direct manipulation of PK-C, their tumor-promoting activity makes them unsuitable for clinical use. Recently, a new class of PK-C–activating compounds, the bryostatins, has been identified.7,8 The bryostatins represent a group of macrocyclic lactones extracted and chemically purified from the marine bryozoan, Bugula neritina.10 Although PK-C–activating bryostatin analogs, such as bryostatin 1, mimic many phorbol ester effects, they block those phorbol ester–mediated events that they do not induce, including tumor promotion in mouse skin11,12 and induction of differentiation in some HL-60 sublines.13 The bryostatins have been shown to exert antiproliferative effects toward diverse neoplastic cell lines, and against primary cultures of human leukemic myeloblasts.14-16 The potential clinical use of bryostatin 1 as an antineoplastic agent is currently being evaluated in a phase I trial.

Recently, May et al17 showed that bryostatin 1 supported the in vitro growth of multipotent human hematopoietic progenitors, although additional studies by these investigators suggested that indirect, accessory cell–mediated effects contributed to this phenomenon.18 Subsequently, our group reported that bryostatin 1 or phorbol 12,13-dibutyrate (PDBu) significantly increased the formation of committed myeloid progenitors (day 14 granulocyte-macrophage colony-forming units [CFU-GM]) by human bone marrow mononuclear cells in response to recombinant GM-CSF (rGM-CSF).19 Together, these findings suggest an important role for PK-C activation in the regulation of human hematopoiesis, possibly at the level of growth factor release and/or responsiveness.

IL-3, otherwise known as multi-CSF, is a polypeptide growth factor that acts on early hematopoietic progenitors, as well as on their more committed progeny.20,21 IL-3 and GM-CSF share a high-affinity receptor on hematopoietic cells, which may account for certain overlapping biologic effects.22-24 Although the mechanisms responsible for signal transduction by these growth factors are not known, it is noteworthy that IL-3 has been shown to stimulate PK-C cytosol to membrane redistribution in FDC-P1 cells.25 Furthermore, the ability of phorbol esters to support the proliferation of IL-3–dependent murine cells lines provides additional evidence that some of the actions of IL-3 may be

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related to PK-C activation.\textsuperscript{27,28} In addition to their effects on cell proliferation, both IL-3 and GM-CSF have been implicated in regulating the lineage commitment of hematopoietic progenitors. For example, both IL-3 and GM-CSF, either alone or in conjunction with IL-5, are known to be potent stimulators of eosinophilic differentiation.\textsuperscript{29-33} The present studies were undertaken to characterize the effect of PK-C-activating agents, including bryostatin 1, PDBu, and the weak tumor promoting diterpene, mezerein,\textsuperscript{34} on cytokine-induced proliferation and lineage commitment of human hematopoietic progenitors. Our results suggest that PK-C activators significantly augment the proliferative response of human myeloid progenitors to IL-3 and GM-CSF, and also play an important role in regulating the lineage specificity of this response.

\section*{MATERIALS AND METHODS}

\textbf{Reagents.} All tissue culture reagents were obtained from Gibco (Grand Island, NY), unless otherwise stated. Bryostatin 1 was extracted and purified from \textit{Bugula neritina} as previously described,\textsuperscript{12} PDBu and mezerein were obtained from L.C. Services (Woburn, MA). Bryostatin 1, PDBu, and mezerein were dissolved in dimethyl sulfoxide (DMSO) at 100 \textmu M and diluted in complete medium just before use. Recombinant human IL-3 (rIL-3) was provided by Dr Jay Stoudemire (Genetics Institute, Cambridge, MA). rGM-CSF was a gift from Dr Paul Trotta, Schering Corp, Bloomfield, NJ). rIL-5 was purchased from Amgen (Thousand Oaks, CA). Conditioned medium from the cell line 5637 was harvested from confluent cultures, filtered, and stored at \textdegree C.\textsuperscript{35}

\textbf{Cell separation.} Bone marrow aspirates were obtained with informed consent from the posterior iliac crests of healthy volunteers. These studies have been approved by the Human Investigations Committee of the Medical College of Virginia. The cells were passed through 26-gauge needles to disperse clumps and diluted 1:5 in McCoy's 5a medium containing preservative-free heparin. The mononuclear cell fraction was recovered from a Ficoll-Hypaque gradient, washed three times, and resuspended in fresh medium with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). After incubation in culture flasks at 37\textdegree C in air with 5% CO\textsubscript{2} for either 90 minutes or overnight, the nonadherent mononuclear cell population was then recovered and incubated with anti-CD34 monoclonal antibody (MoAb; Becton Dickinson Laboratory Sciences Co, Corning, NY). The CD34+ cell fraction was recovered from a Ficoll-Hypaque gradient, washed three times, and resuspended in fresh medium with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). After incubation in culture flasks at 37\textdegree C in air with 5% CO\textsubscript{2} for either 90 minutes or overnight, the nonadherent mononuclear cell population was then recovered and incubated with anti-CD34 monoclonal antibody (MoAb; Becton Dickinson Immunocytochemistry Systems, Mountain View, CA) at 4\textdegree C for 60 minutes. The cells were subsequently washed twice in medium with 0.2% bovine serum albumin and incubated with goat antimouse IgG-coated M-450 immunomagnetic beads (Dynal Inc, Great Neck, NY) using a bead:total cell ratio of 1:35. The bead/cell mixture was gently vortexed and centrifuged at 400g for 10 minutes to obtain optimal rosette formation. The cells were further incubated at 4\textdegree C with gentle agitation for 30 minutes. Rosette formation was inspected in a phase-contrast microscope and rosettes and free particles were magnetically removed. The CD34+ cell fraction was washed twice and resuspended at appropriate cell density for plating.

\textbf{CFU-GM assay.} CD34+ myeloid progenitor cells were cloned in soft agar as previously described.\textsuperscript{21} Briefly, cells were plated in a double-layer soft agar system in 22-mm 12-well plates (Corning Laboratory Sciences Co, Corning, NY). The bottom layer consisted of 0.5 mL McCoy's 5a medium supplemented with sodium pyruvate, essential and nonessential amino acids, MEM vitamins, serine, asparagaine, glutamine, penicillin, streptomycin, 0.5% bacto agar (Difco, Detroit, MI), and 20% FBS. The top layer contained 4,000 CD34+ cells in 0.5 mL supplemented McCoy's 5a medium with 20% FBS, and 0.3% agar. After the agar was allowed to gel, 0.1 mL of medium containing experimental agents was added to each well. We have previously shown that when factors are added in this manner, colony formation is equivalent to that obtained when factors are incorporated into the bottom layer or incorporated into both the top and bottom layers.\textsuperscript{21} The plates were then placed in a 37\textdegree C, 5% O\textsubscript{2}, 5% CO\textsubscript{2}, fully humidified incubator. After 14 days, colonies consisting of groups of 50 or more cells with granuloctytic or macrophage-like appearance were scored using an inverted microscope. The effects of scheduling on hematopoietic cell growth were assessed by exposing cells to bryostatin 1 for intervals ranging from 0 to 144 hours before, or after, incubation with rIL-3.

\textbf{Colony characterization.} Colony types were characterized using a previously described in situ triple-stain technique.\textsuperscript{26} Briefly, cells were fixed by adding 1 mL of CAM fixative to the wells for 10 minutes, which were then washed three times with distilled water. The cells were then stained for the presence of specific and nonspecific esterase using commercially available kits (Sigma, St Louis, MO). The plates were submerged in water, and the agar disks floated to the surface and transferred to glass slides. After drying, the slides were stained with 0.1% Luxol fast blue solution (0.1 g Luxol fast blue in 100 mL of 70% ethanol saturated with urea) for 60 minutes at room temperature. The triple-stained slides were then counter-stained with Hematoxylin solution Gill No. 3 (Sigma).

\section*{RESULTS}

\textbf{Effects of PK-C-activating agents on rGM-CSF or rIL-3-induced colony formation by CD34+ cells.} The effect of varying concentrations of bryostatin 1, PDBu, and mezerein on rGM-CSF–induced and rIL-3–induced colony formation is shown by the data in Fig 1. None of the PK-C activators supported colony formation when administered alone. However, when 1 to 100 nmol/L bryostatin 1 was combined with concentrations of rGM-CSF or rIL-3 that had been determined to yield maximal colony numbers when administered alone (eg, 50 ng/mL), colony formation increased by more than 100% in each case \textit{(P < .01)}. The mean number of colonies formed in the combined presence of rGM-CSF or rIL-3 and 10 nmol/L bryostatin 1 (eg, 152 ± 28, n = 7, and 116 ± 16, n = 13, respectively) was equivalent to that observed in the presence of 10% 5637 conditioned medium (135 ± 8, n = 26) or the combination of rIL-3 and rGM-CSF (112 ± 15, n = 5).

In contrast, higher concentrations of PDBu (eg, 50 nmol/L) reduced rIL-3 and rGM-CSF colony formation (Fig 1), and eliminated growth entirely at concentrations ≥ 100 nmol/L (data not shown). However, lower concentrations of PDBu (eg, 25 nmol/L) increased colony formation \textit{(P < .01)} in response to either rIL-3 (114 ± 23, n = 6) or rGM-CSF (104 ± 25, n = 5) to levels equivalent to those obtained with 10% 5637 conditioned medium (135 ± 8, n = 26). Similar results were obtained with the PK-C–activating agent, mezerein, although optimal stimulatory as well as inhibitory concentrations were lower than those observed in the case of PDBu.

In view of the ability of bryostatin 1 to block those phorbol ester-mediated effects that it does not share,\textsuperscript{14,15} separate studies were performed to determine whether
Bryostatin 1 could reverse the inhibitory effects of high concentrations of PDBu. Coadministration of 50 nmol/L PDBu with 50 ng/mL of either rGM-CSF or rIL-3 resulted in a 90% reduction in colony formation. However, when 100 nmol/L bryostatin 1 was added to the combination, the inhibitory effects of PDBu were abrogated and colony formation was equivalent to that induced by the growth factor and bryostatin 1. For example, in a representative experiment, the combination of 50 nmol/L PDBu and rGM-CSF or rIL-3 gave rise to 8 ± 3 and 7 ± 2 colonies, respectively (mean ± SE, n = 3). These values increased to 156 ± 16 and 122 ± 17 when bryostatin 1 was present. However, 100 nmol/L bryostatin 1 was not able to reverse the inhibition of colony formation observed when cells were preincubated 24 hours with 50 nmol/L PDBu and rIL-3 (data not shown).

Effect of PK-C activators on the lineage commitment of day 14 CFU-GM stimulated by rGM-CSF and/or rIL-3. To determine whether PK-C activators alter the response of committed myeloid progenitors to rIL-3 and rGM-CSF in a lineage-specific manner, characterization of day 14 colonies was performed using cytochemical techniques (Table 1). When cells were cultured in the presence of 50 ng/mL rGM-CSF alone, 43% of the colonies were purely eosinophilic in nature and a total of 52% contained cosinophilic components. When cells were cultured in the combined presence of 100 nmol/L bryostatin 1 and 50 ng/mL rGM-CSF, a twofold increase was observed in the total number of colonies (compared with rGM-CSF alone), but the absolute number of cosinophilic colonies remained constant (21 ± 3 vs. 20 ± 2). Significant increases were noted in the number of pure macrophage (29 ± 6 vs. 11 ± 2), pure neutrophil (11 ± 4 vs. 3 ± 1), and mixed neutrophil-macrophage (31 ± 9 vs. 8 ± 3) colonies (P < .05 in each case). Consequently, the increase in growth of these noneosinophilic colony subtypes was responsible for the potentiation of total colony formation induced by bryostatin 1 in conjunction with rGM-CSF.

The response of cells to rGM-CSF and either 12.5 nmol/L mezerein or 25 nmol/L PDBu was similar to that of cells exposed to rGM-CSF and 100 nmol/L bryostatin 1, in that significant increases were noted in both the absolute number and percentage of macrophage, neutrophil, and mixed neutrophil-macrophage colonies, compared with cells exposed to rGM-CSF alone. However, in contrast to combinations containing bryostatin 1, which were associated with a reduction in the percentage but not the absolute number of eosinophilic colonies, coadministration of PDBu or mezerein with rGM-CSF significantly reduced the absolute number of pure eosinophilic colonies (eg, from 20 ± 2 to 7 ± 3 and 12 ± 3, respectively; P < .05).

When cells were exposed to 100 nmol/L bryostatin 1 in conjunction with 50 ng/mL rIL-3 (Table 1), the increase in the total number of colonies was similar to that observed when bryostatin 1 was combined with rGM-CSF. In addition, coadministration of bryostatin 1 with rIL-3 significantly enhanced the absolute number (and percentage) of pure macrophage (42 ± 13 vs. 7 ± 1), pure neutrophil (18 ± 4 vs. 2 ± 2), and mixed neutrophil-macrophage (36 ± 6 vs. 4 ± 1) colonies (P < .05 in each case). Collectively, these increases were responsible for the potentiation of total colony formation when bryostatin 1 was combined with rIL-3. However, in contrast to the results of studies using rGM-CSF, coadministration of bryostatin 1 with rIL-3 produced a significant decrease in the absolute number (in addition to the relative percentage) of pure eosinophilic colonies compared with rIL-3 alone (12 ± 2 v. 27 ± 4; P < .05). Finally, coadministration of rIL-3 with either 25 nmol/L PDBu or 12.5 nmol/L mezerein yielded results that were identical to those observed with 100 nmol/L bryostatin 1. Specifically, significant increases in the number of pure and mixed neutrophil-macrophage colonies were noted, whereas both the absolute number and relative percentage
Values represent the means ± SE for n separate experiments. The Student's t-test was applied for comparisons between samples. Figures in parentheses correspond to the percentage of each colony subtype relative to the total number of colonies. Values for mixed Eo/N and Eo/N/M colonies were minimal for all conditions and are excluded for the sake of clarity.

Abbreviations: Eo, eosinophil; M, monocyte/macrophage; N, neutrophil.

*Significantly greater than values for growth factor(s) alone (P < .05).
†Significantly less than values for growth factor(s) alone (P < .05).

of eosinophilic colonies substantially declined with the addition of PDBu or mezerein (P < .05 in each instance).

The effect of bryostatin 1 on the lineage specificity of colonies derived from CD34+ cells cultured in the presence of both rIL-3 and rGM-CSF is shown by the data shown in Table 1. As previously reported, exposure of enriched progenitor cells to both rGM-CSF and rIL-3 produced additive effects on colony formation. Coadministration of these cytokines resulted in a significant increase in the absolute number, but not in the percentage, of pure eosinophilic colonies compared with cells exposed to either factor alone (54 ± 3 v 27 ± 4 or 20 ± 2; P < .02). Significant increases were also noted in the number of mixed macrophage/eosinophil colonies. In contrast, only small increases were noted in the number of noneosinophilic colonies formed under these conditions. Consequently, enhanced colony formation induced by the combination of rIL-3 and rGM-CSF resulted primarily from the increase in pure and mixed eosinophil colonies. Although the addition of 100 nmol/L bryostatin 1 to the combination of rIL-3 and rGM-CSF produced no change in the total number of colonies, it did result in a significant reduction in both the absolute number (9 ± 3 v 54 ± 3; P < .01) and relative percentage (7% v 48%) of eosinophilic colonies, with a corresponding increase in the relative percentage and absolute number of pure macrophage and mixed neutrophil-macrophage colonies.

In view of previous studies showing that coadministration of IL-5 with IL-3 primarily promotes the growth of eosinophilic progenitors, experiments were performed to determine whether bryostatin 1 could block this process (Table 2). Coadministration of IL-5 and rIL-3 resulted in approximately 90% eosinophilic colonies. Coadministration of bryostatin 1 with rIL-5 alone did not significantly reduce the number of eosinophilic colonies, but did permit a limited number of noneosinophilic colonies to form. The addition of bryostatin 1 to the combination of rIL-5 and rIL-3 significantly reduced (P < .01) the absolute number, and percentage, of eosinophilic colonies when compared with rIL-3 or rIL-5 alone or in combination. Substantial increases in the absolute number (and percentage) of pure and mixed neutrophil and macrophage colonies were also noted when cells were exposed to bryostatin 1 in conjunction with rIL-3 and rIL-5.

### Table 1. Effect of PK-C Activators on the Growth and Lineage Commitment of Myeloid Progenitors Exposed to rGM-CSF and/or rIL-3

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>Eo</th>
<th>Eo/M</th>
<th>M</th>
<th>N</th>
<th>N/M</th>
<th>Total n</th>
</tr>
</thead>
<tbody>
<tr>
<td>rGM-CSF</td>
<td>20 ± 2</td>
<td>4 ± 1</td>
<td>11 ± 2</td>
<td>3 ± 1</td>
<td>8 ± 3</td>
<td>46 ± 7</td>
</tr>
<tr>
<td>rGM-CSF + bryostatin 1</td>
<td>21 ± 3</td>
<td>8 ± 1</td>
<td>29 ± 6</td>
<td>11 ± 4</td>
<td>31 ± 9</td>
<td>99 ± 16</td>
</tr>
<tr>
<td>rGM-CSF + PDBu</td>
<td>7 ± 3</td>
<td>2 ± 1</td>
<td>48 ± 18</td>
<td>14 ± 6</td>
<td>25 ± 9</td>
<td>94 ± 16</td>
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<tr>
<td>rGM-CSF + mezerein</td>
<td>12 ± 3</td>
<td>2 ± 1</td>
<td>36 ± 13</td>
<td>17 ± 6</td>
<td>20 ± 8</td>
<td>86 ± 16</td>
</tr>
<tr>
<td>rIL-3</td>
<td>27 ± 4</td>
<td>6 ± 1</td>
<td>7 ± 1</td>
<td>2 ± 2</td>
<td>4 ± 1</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>rIL-3 + bryostatin 1</td>
<td>12 ± 2</td>
<td>5 ± 1</td>
<td>42 ± 13</td>
<td>18 ± 4</td>
<td>36 ± 6</td>
<td>110 ± 13</td>
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<td>rIL-3 + PDBu</td>
<td>7 ± 3</td>
<td>2 ± 1</td>
<td>44 ± 13</td>
<td>7 ± 3</td>
<td>20 ± 11</td>
<td>80 ± 18</td>
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<tr>
<td>rIL-3 + mezerein</td>
<td>3 ± 1</td>
<td>1 ± 0</td>
<td>69 ± 37</td>
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<td>145 ± 27</td>
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<tr>
<td>rGM-CSF + rIL-3</td>
<td>54 ± 3</td>
<td>16 ± 2</td>
<td>12 ± 3</td>
<td>12 ± 5</td>
<td>18 ± 5</td>
<td>112 ± 15</td>
</tr>
<tr>
<td>rGM-CSF + rIL-3 + bryostatin 1</td>
<td>9 ± 3</td>
<td>6 ± 2</td>
<td>60 ± 21</td>
<td>40 ± 4</td>
<td>49 ± 11</td>
<td>132 ± 13</td>
</tr>
</tbody>
</table>

Cells were plated in the presence of rGM-CSF (50 ng/mL) and/or rIL-3 (50 ng/mL) in conjunction with bryostatin 1 (100 nmol/L), PDBu (25 nmol/L), or mezerein (12.5 nmol/L). After 14 days of incubation, colonies were scored and characterized cytochemically as described in the text. Values represent the means ± SE for n separate experiments. Figures in parentheses correspond to the percentage of each colony subtype relative to the total number of colonies. Values for mixed Eo/N and Eo/N/M colonies were minimal for all conditions and are excluded for the sake of clarity.

### Table 2. Effect of Bryostatin 1 on the Growth and Lineage Commitment of Myeloid Progenitors Exposed to rIL-3 and/or rIL-5

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>Eo</th>
<th>Eo/M</th>
<th>M</th>
<th>N</th>
<th>N/M</th>
<th>Total</th>
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<tbody>
<tr>
<td>rIL-3</td>
<td>28 ± 3</td>
<td>11 ± 2</td>
<td>5 ± 1</td>
<td>0</td>
<td>2 ± 1</td>
<td>46 ± 3</td>
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<td>rIL-5</td>
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<td>0</td>
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<td>10 ± 1</td>
</tr>
<tr>
<td>rIL-3 + rIL-5</td>
<td>44 ± 3</td>
<td>8 ± 1</td>
<td>6 ± 2</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>59 ± 4</td>
</tr>
<tr>
<td>rIL-5 + bryostatin 1</td>
<td>7 ± 1</td>
<td>1 ± 1</td>
<td>4 ± 1</td>
<td>1 ± 1</td>
<td>2 ± 1</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>rIL-3 + rIL-5 + bryostatin 1</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>25 ± 3</td>
<td>1 ± 1</td>
<td>52 ± 13</td>
<td>90 ± 14</td>
</tr>
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</table>
Schedule dependence of bryostatin 1-mediated effects on rIL-3-stimulated colony formation. To determine the role of scheduling considerations might play in modulating the interaction between bryostatin 1 and rIL-3, factors were added to the plates separated by intervals of 0 to 144 hours (Table 3). When bryostatin 1 was added to the plates at any time up to and including 144 hours after rIL-3, a significant increase \((P < .01)\) in the number of colonies was observed when compared with cells exposed to rIL-3 alone. In addition, the total number of colonies formed when bryostatin 1 was administered after rIL-3 was equivalent to the values obtained when both agents were added together at the initiation of cultures. Similarly, a decrease in the fraction and absolute number of eosinophilic colonies, and a corresponding increase in the percentage of noneosinophilic colonies, was observed when bryostatin 1 was added within 120 hours after the addition of rIL-3. When the addition of bryostatin 1 was delayed 120 or 144 hours, the reduction in the absolute number of eosinophilic colonies was abrogated, suggesting that a subpopulation of hematopoietic progenitors exposed to rIL-3 had become committed to the eosinophilic lineage by that time.

A different response pattern was noted when bryostatin 1 was added before rIL-3. Under these conditions, the addition of rIL-3 to the cultures 4 hours after bryostatin 1 resulted in colony formation equivalent to that observed when these agents were added simultaneously (eg, 89 ± 9 vs 89 ± 3). However, when the interval between bryostatin 1 and rIL-3 administration was increased to 24 hours, colony formation was significantly reduced compared with cells simultaneously exposed to these agents (50 ± 7 v 89 ± 3; \(P < .01)\). Nevertheless, significant reductions in the number and percentage of eosinophilic colonies (\(P < .05\)), and increases in the numbers of pure and mixed neutrophil colonies persisted. Although a 72-hour delay in the addition of rIL-3 resulted in a further decline in colony formation to levels equivalent to those stimulated by rIL-3 alone, the percentage and absolute number of eosinophilic colonies remained significantly reduced compared with rIL-3-treated cells (\(P < .01\)). Together, these findings suggest that events occur shortly after administration of bryostatin 1 that require the presence of rIL-3 for optimal enhancement of progenitor-cell colony formation. In contrast, the inhibition of eosinophilic commitment was not abrogated by bryostatin 1 pretreatment. Thus, perturbations in the temporal coordination of signals induced by bryostatin 1 and rIL-3 appear to exert divergent effects on the proliferative capacity versus lineage commitment of hematopoietic progenitors.

DISCUSSION

The regulation of hematopoiesis by IL-3 and GM-CSF is a coordinated process involving not only stimulation of the proliferation of hematopoietic progenitors, but also determination of their lineage commitment. For example, the growth and differentiation of cells of the eosinophilic lineage are known to be regulated by the concerted actions of IL-3, GM-CSF, and IL-5.29-33 Eosinophilic colonies have been shown to account for a major fraction of IL-3–stimulated and GM-CSF–stimulated day 14 CFU-GM in both murine and human systems, and this component can be further increased by coadministration of IL-5.32,33,38 The available evidence indicates that IL-3 and GM-CSF induce proliferation and commitment of more primitive eosinophilic progenitors, whereas IL-5 acts to support terminal differentiation of cells committed to the eosinophilic lineage.32,33,38 Our observation that PK-C activators effectively blocked eosinophilic differentiation induced by rGM-CSF and rIL-3, but not rIL-5, suggests that PK-C–mediated events alter the differentiation program of more primitive hematopoietic progenitors that have not undergone irreversible lineage commitment events.

A major unresolved issue is whether the observed shift in differentiation pattern induced by PK-C activators results from a change in the lineage commitment of individual cells, or, alternatively, involves recruitment of a separate population of committed neutrophil and monocyte-macrophage progenitors. In this regard, it should be noted that bryostatin 1 did not substantially alter the total number of

Table 3. Effect of Alternative Schedules of rIL-3 and Bryostatin 1 Administration on the Growth and Lineage Commitment of Myeloid Progenitors

<table>
<thead>
<tr>
<th>Time of Delay (h)</th>
<th>Bryostatin 1</th>
<th>rIL-3</th>
<th>Day 14 CFU-GM/4,000 CD34+ Cells Plated</th>
<th>Eos</th>
<th>Eos/M</th>
<th>M</th>
<th>N</th>
<th>N/M</th>
<th>Total</th>
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<tr>
<td></td>
<td>—</td>
<td>0</td>
<td>24 ± 3 (77)</td>
<td>5 ± 1 (16)</td>
<td>5 ± 2 (7)</td>
<td>2 ± 3 (7)</td>
<td>0</td>
<td>2 ± 3 (7)</td>
<td>31 ± 5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>13 ± 2 (15)</td>
<td>3 ± 1 (3)</td>
<td>41 ± 3 (46)</td>
<td>12 ± 3 (13)</td>
<td>20 ± 1 (22)</td>
<td>89 ± 3</td>
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<td></td>
<td>24</td>
<td>0</td>
<td>13 ± 2 (14)</td>
<td>3 ± 1 (3)</td>
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<td>17 ± 3 (18)</td>
<td>22 ± 1 (23)</td>
<td>94 ± 16</td>
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<td>39 ± 1 (46)</td>
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<td>11 ± 3 (48)</td>
<td>3 ± 1 (13)</td>
<td>2 ± 1 (9)</td>
<td>23 ± 3</td>
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Cells were plated in the presence of bryostatin 1 (100 nmol/L) before, in conjunction with, or after the addition of rIL-3 (50 ng/mL) as described in the text. After 14 days of incubation, colonies were scored and characterized cytochemically as described previously. Values represent the means ± SE for a representative experiment performed in triplicate: two additional experiments yielded essentially equivalent results. Figures in parentheses correspond to the percentage of each colony subtype relative to the total number of colonies. Values for mixed Eo/N and Eo/N/M colonies were minimal for all conditions and are excluded for the sake of clarity.

Abbreviations: Eo, eosinophil; M, monocyte/macrophage; N, neutrophil.
colonies formed in response to the combination of rIL-3 and rGM-CSF, but, instead, reduced the total number of eosinophilic colonies, while inducing an corresponding increase in noneosinophilic colonies. In scheduling studies, inhibition of eosinophilic differentiation did not occur if bryostatin 1 was added more than 120 hours after rIL-3, although enhanced colony formation persisted, suggesting that an irreversible lineage commitment step had occurred by that time. Together, these data are consistent with the view that bryostatin 1 and related agents alter the lineage commitment of a subpopulation of uncommitted myeloid progenitors.

Although other investigators have shown that bryostatin 1 or phorbol esters stimulate hematopoiesis through activation of accessory cells, it is unlikely that this mechanism, by itself, is responsible for the effects reported here. For example, the use of low-density, T-cell–depleted and adherent-cell–depleted, CD34+ bone marrow mononuclear cell populations substantially reduces the possibility that significant numbers of residual accessory cells remain in the cultures. Moreover, none of the PK-C–activating agents used in the study stimulated colony formation when administered alone. Although bryostatin 1 by itself has previously been reported to support the growth of a limited number of colonies derived from progenitor-cell–enriched populations, it is conceivable that differences in cell isolation and culture techniques (eg, the inclusion of crythropeotin in the culture by other investigators) may have contributed to colony formation. These considerations suggest that PK-C manipulations act directly to influence hematopoietic cell responsiveness to GM-CSF and IL-3, although the possibility of accessory cell influences cannot be ruled out. Single cell cloning assays may eventually address this issue.

Whereas previous studies related the hematopoietic stimulatory capacity of phorbol esters to their tumor promoting capabilities, the present findings suggest a discordance between the two effects. For example, whereas PDBu is a potent primary tumor promoter, mezerein is classified as a second-stage promoter in mouse skin tumor assay systems, and exhibits little primary tumor-promoting activity. In contrast to both of these agents, bryostatin 1 essentially lacks tumor-promoting activity. Nevertheless, each PK-C activator potentiates colony formation to an equivalent degree, and produced similar qualitative changes in colony composition, although the dose-response profile of these agents differed significantly. These findings suggest that the hematopoietic regulatory effects of these compounds are not directly related to tumor-promoting mechanisms.

The ability of bryostatin 1 to reverse the inhibitory effects of high concentrations of PDBu toward GM-CSF–stimulated and IL-3–stimulated cultures is consistent with the results of previous studies in which bryostatin 1 was found to block other phorbol ester–mediated processes, such as tumor promotion in mouse skin and differentiation induction in human leukemia cells. However, the inhibition of colony formation could not be reversed by bryostatin 1 after 24 hours of pre-exposure to high concentrations of PDBu, suggesting that irreversible events precluding colony formation had occurred by that time. The basis for biologic response differences between bryostatin 1 and phorbol esters, in this and other systems, remains unknown.

After initial stimulation, continuous exposure to PK-C activating agents has been shown to induce degradation and persistent downregulation of PK-C in diverse cell types. For example, chronic stimulation by bryostatin 1 or phorbol esters has been shown to induce a 90% reduction in PK-C activity in the promyelocytic leukemic cell line HL-60. Consequently, the regulatory effects of PK-C-activating agents on hematopoietic cell development might stem from either PK-C stimulation or downregulation. In contrast to lymphoid systems, in which PK-C activation has been shown to “prime” cells to respond to subsequently administered growth-promoting cytokines, a 24-hour exposure of myeloid progenitors to bryostatin 1 before rIL-3 abrogated the potentiation of colony formation that occurred with simultaneous administration of agents. This suggests that a subpopulation of hematopoietic progenitors requires a coordinated exposure to rIL-3 in conjunction with PK-C activation to undergo clonal expansion. In contrast, prior exposure of cells to bryostatin 1 continued to result in inhibition of eosinophilic differentiation, indicating that alterations in lineage commitment might be related to PK-C downregulation.

The ability of PK-C–activating agents to promote the growth of IL-3–dependent cells has been taken as evidence that the growth promoting activity of IL-3 is mediated through PK-C stimulation. Although previous studies using murine hematopoietic cell lines have shown an association between IL-3 administration and PK-C–cytosol–membrane translocation, the inability to obtain sufficient quantities of human hematopoietic progenitors for biochemical determinations precludes making direct correlations between levels of PK-C activity and specific biologic responses in this system. Nevertheless, the finding that basal levels of rIL-3–induced colony formation was not reduced by conditions previously shown to induce PK-C downregulation (ie, 24-hour pre-exposure to bryostatin 1), and that PK-C–activating agents do not mimic IL-3 actions, suggest that the stimulation of human hematopoietic cell growth by IL-3 is not directly mediated by PK-C activation. Instead, they imply that PK-C activation exerts a regulatory influence on events induced by cytokines such as GM-CSF and IL-3. These observations suggest that the effects of PK-C activators on hematopoietic cell proliferation and differentiation involve separate and potentially dissociable events.

The observation that bryostatin 1 selectively inhibits eosinophilic development induced by IL-3, or by the combination of GM-CSF and IL-3, suggests a new role for PK-C in the regulation of hematopoietic cell lineage commitment. This finding takes on added significance in view of recent reports showing an undesirable eosinophilia resulting from the in vivo administration of rGM-CSF or rIL-3 in patients with various hematologic disorders.
colonic interventions by agents acting at the level of PK-C may thus be capable of blocking rIL-3–induced or rGM-CSF–induced eosinophilia in vivo, and potentially increasing the number of circulating neutrophils and macrophages in response to growth factor administration. The recent initiation of phase I trials of bryostatin 1 as a potential antineoplastic agent may make it possible to test these hypotheses directly in the near future.

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REFERENCES

1. Fibach E, Marks PA, Rifkin RA: Tumor promoters enhance myeloid and erythroid colony formation by normal mouse hemopoietic cells. Proc Natl Acad Sci USA 77:4152, 1980


27. Whetton AD, Monk PN, Consaydey SD, Huang SJ, Dexter TM, Downes CP: Interleukin-3 stimulates proliferation via protein kinase C activation without increasing inositol lipid turnover. Proc Natl Acad Sci USA 85:3284, 1988


41. Young S, Parker PJ, Ulrich A, Stabel S: Down-regulation of protein kinase C is due to an increased rate of degradation. Biochem J 244:775, 1987


Bryostatin 1 modulates the proliferation and lineage commitment of human myeloid progenitor cells exposed to recombinant interleukin-3 and recombinant granulocyte-macrophage colony-stimulating factor

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