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Antineoplastic bryostatins are multipotential stimulators of human hematopoietic progenitor cells

(differentiation/proliferation/colony-forming unit/recombinant human granulocyte–macrophage colony-stimulating factor/neutrophil activation)

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ABSTRACT **The bryostatins are macrocyclic lactones, extracted from the marine bryozoan *Bugula neritina*, and have been reported to be potent antineoplastic agents. Results described here demonstrate that the bryostatins may also be useful as stimulators of normal human hematopoietic cells since they can (*i*) directly stimulate bone marrow progenitor cells to form colonies *in vitro* and (*ii*) functionally activate neutrophils. Structure–activity studies with bryostatin congeners indicate that these stimulatory properties may be dependent on the chain length and the unsaturated nature of the acylated group at carbons 20 and 7 of the bryostatin molecule. These stimulatory properties demonstrate that the naturally occurring bryostatins can mimic many of the biological effects of multipotential granulocyte–macrophage colony-stimulating factor. Thus, the coupling of antineoplastic activity with stimulatory growth properties for normal hematopoietic cells makes this agent an excellent probe to dissect the mechanism(s) of normal hematopoiesis. In addition, bryostatin may represent a clinically attractive agent useful for treating bone marrow failure states.**

The bryostatins are a group of macrocyclic lactones, which are extracted and chemically purified from the marine animal *Bugula neritina* of the phylum bryozoa (1, 2). These agents represent a group of congeners with potent antineoplastic properties discovered during initial screening (1, 2). Since the administration of antineoplastic agents can be limited severely by their inhibitory effects on normal bone marrow progenitor cells, we tested whether the bryostatins would inhibit hematopoietic progenitors. Surprisingly, we found that bryostatins can stimulate normal human bone marrow cells to form colonies *in vitro*. Since polypeptide colony-stimulating factors (CSFs) are the known physiological stimulators of normal hematopoiesis, we compared these results with bryostatin to those for the multipotential recombinant human granulocyte–macrophage colony-stimulating factor (rHGM-CSF) (3–5).

The results indicate that like rHGM-CSF, bryostatins 1, 3, 8, and 9, but not bryostatin 13 (Fig. 1), are highly active and are potent stimulators of normal bone marrow progenitor cell growth. However, bryostatin 1, but not bryostatin 13, was found to rapidly activate mature neutrophils unlike that for rHGM-CSF. The results indicate that bryostatins can mimic many of the effects of the multipotential rHGM-CSF (3, 4).

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EXPERIMENTAL PROCEDURES

Materials. rHGM-CSF was a kind gift from Genetics Institute, Boston, MA. Phorbol 12-myristate 13-acetate was purchased from Sigma. Fetal calf serum was purchased from Hyclone. Bryostatins were purified by one of us (G.R.P.) as described (1, 2). All other reagents used were from commercial sources.

Colony Forming Assays. Granulocyte–macrophage colony-forming unit (CFU-GM), erythroid burst-forming unit (BFU-E), and erythroid colony-forming unit (CFU-E) colonies were enumerated from light-density, adherence-depleted normal human bone marrow cells. For CFU-GM colonies, cells were suspended in supplemented McCoy's 5A medium containing 0.3% agar and plated in 35-mm Petri dishes as described (6). Assays for both early (BFU-E) and late (CFU-E) progenitors were enumerated as described in the plasma-clot method containing optimal concentrations of erythropoietin (7).

Clone Transfer Experiment. Assays were performed essentially as described (3). Briefly, cultures of normal human bone marrow were initiated in medium containing 10 nM bryostatin 1. Following 4 days of growth, clones of 4–8 cells were carefully removed using a glass pipette to avoid contamination by surrounding cells and transferred to secondary plates containing either rHGM-CSF (50 ng/ml) or bryostatin 1 (10 nM) as the source of CSF (3). After an additional 10 days of culture, the number of clones that had proliferated to form a colony of 40 cells or more were determined.

Neutrophil Chemiluminescence Assay. Normal human peripheral blood neutrophils were highly purified on a Ficoll density gradient and suspended in Dulbecco's phosphate-buffered saline supplemented with Ca^{2+} and Mg^{2+}. First, substances to be tested were incubated with the neutrophils for 15 min. At this point 10 μM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was added to each sample. The generation of chemiluminescence was measured in a luminometer at 1.25-min intervals for up to 20 min (8).

Neutrophil Cytotoxicity Assay. Neutrophils used were purified as described above. Neutrophils were incubated at a density of 3×10^6 cells per ml first with various concentrations of bryostatin 1 (0.1 μM to 0.1 nM), rHGM-CSF (50 ng/ml), or in medium alone for 2 hr. Following two washes, the cells were added to the ^{51}Cr-labeled K562 target cells at effector/target cell ratios of 30:1, 15:1, or 7.5:1 as indicated. Control plates containing ^{51}Cr-labeled K562 cells and either

Abbreviations: CSF, colony-stimulating factor; rHGM-CSF, recombinant human granulocyte–macrophage colony-stimulating factor; CFU-GM, granulocyte–macrophage colony-forming unit; BFU-E, erythroid burst-forming unit; CFU-E, erythroid colony-forming unit.

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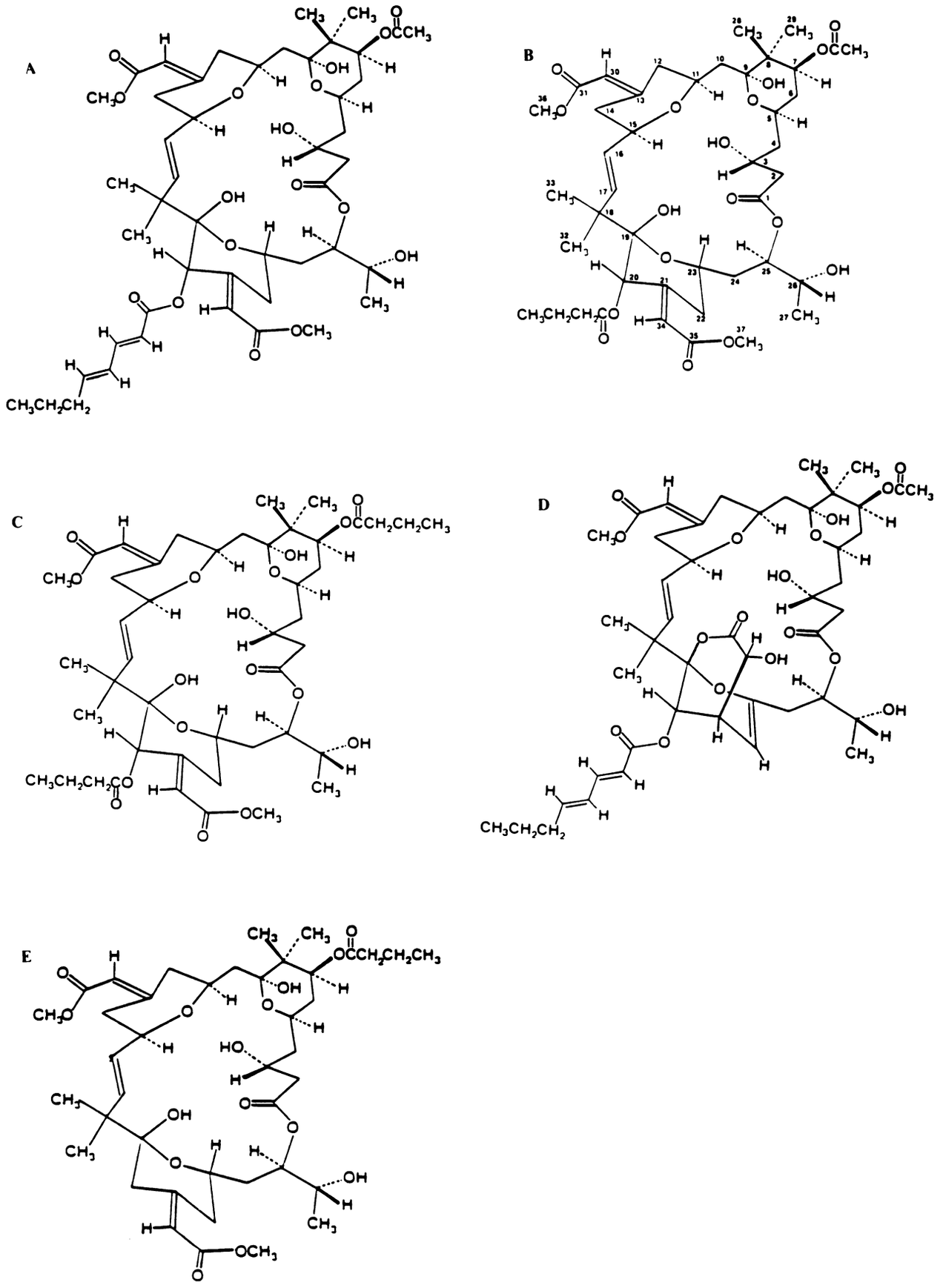

FIG. 1. Chemical structures of the bryostatins. (*A*) Bryostatin 1. (*B*) Bryostatin 9. (*C*) Bryostatin 8. (*D*) Bryostatin 3. (*E*) Bryostatin 13.

bryostatin or medium alone were evaluated to determine the spontaneous release of isotope. The plates containing the cells were centrifuged at 250 × *g* for 2 min and then incubated for 3 hr at 37°C. One hundred microliters of the supernatant was harvested from each well, and the amount of radioactivity present was determined in a γ scintillation counter. The maximum release of ^{51}Cr was determined by assaying supernatant obtained from wells in which the cells were completely lysed with 6 M HCl.

RESULTS

Direct Stimulatory Effect of Bryostatin on CFU-GM. Bryostatins 1, 3, 8, and 9 were found to mediate a dose-dependent stimulation of CFU-GM with the maximal effective concentration between 1 and 100 nM (Fig. 2). Colony-stimulating activity was observed with a concentration as low as 0.01 nM for bryostatins 1 and 8, which are the most potent of the four active congeners tested (Fig. 2). While the amplitude of granulocyte–macrophage-colony response was similar for bryostatin 3 at the higher concentrations (i.e., 10–100 nM), this agent was clearly less-potent than bryostatin 1 or 8 (Fig. 2). Bryostatin 9 was only a weak stimulator of normal colony growth, whereas bryostatin 13 was completely inactive (Fig. 2). Analysis of colony morphology revealed that bryostatin 1, like rHGM-CSF, stimulated primarily mixed granulocyte–macrophage and pure granulocyte colonies at 7 and 14 days of culture (Fig. 3).

The effect of bryostatin 1 on transferred hematopoietic clones was investigated to determine whether bryostatin stimulated colony formation directly. The light-density fraction of human bone marrow was depleted of adherent cells and cultured in methylcellulose medium containing 1 nM bryostatin. After 4 days, when initiated clones of 4–10 cells were detected, they were removed carefully to avoid contamination with surrounding cells, and individually transferred to a second plate containing either 1 nM bryostatin, rHGM-CSF, or no added growth-stimulating factor (Fig. 3). Further growth was not observed in colonies transferred to plates containing no added CSF. The clones from primary

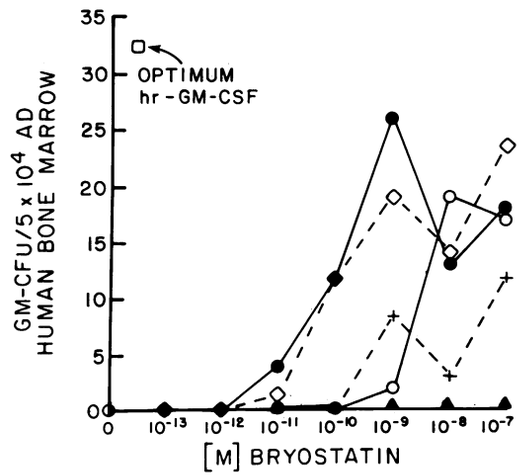

FIG. 2. Effect of bryostatins 1, 3, 8, 9, and 13 on granulocyte–monocyte colony growth *in vitro*. Colonies were enumerated following incubation of bone marrow with cells with rHGM-CSF at 50 ng/ml (Genetics Institute) or bryostatin 1 (●), bryostatin 3 (○), bryostatin 8 (■), bryostatin 9 (+), or bryostatin 13 (▲), at the concentrations indicated. After 14 days of culture at 37°C in a humidified atmosphere containing 7.5% CO_2/92.5% air, colonies of ≥50 cells were scored. Representative results from one of several experiments are reported as the mean colony number from quadruplicate samples. SEM is within ±10% of the mean number. AD, adherence depleted.

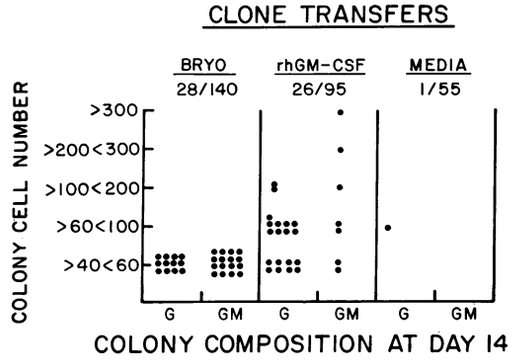

FIG. 3. Effect of bryostatin 1 (Bryo) on the growth of transferred clones. The number of cells in each of the colonies and their cellular morphology was determined. Each symbol (●) represents a colony. Its height in each column is proportional to the number of cells in that colony. The data directly above sections G of the column represent colonies composed entirely of granulocytic cells. The data above sections GM in the column represent colonies composed of both granulocytic and monocytic cells. No colonies in secondary plates were detected that were composed of macrophage–monocyte cells only. The numerator in the fraction at the top of each column represents the number of colonies that developed in the secondary plate, and the denominator represents the total number of clones transferred.

plates initiated with bryostatin produced full-size colonies only on the secondary plates to which bryostatin or rHGM-CSF had been added (Fig. 3). This indicates that the stimulatory effect of bryostatin 1 and rHGM-CSF is exerted directly. Further, an attempt to produce a conditioned medium from bone marrow cells in the presence of bryostatin was unsuccessful. Nonadherence-depleted human bone marrow, which was composed of a mixture of progenitor plus ancillary cells, was incubated in culture medium that contained a maximal stimulatory concentration of bryostatin (i.e., 1–10 nM) for 5 days. The supernatant was aspirated and tested for the presence of a GM-CSF after fractionation by passage over a Sephadex G-25 gel filtration column to remove the bryostatin (i.e., M_r, 884; refs. 1, 2, 9, 10). The resulting void volume, which contains the large molecular weight polypeptide growth factors, was evaluated for CSF activity. This supernatant was tested at both 10% and 50% (vol/vol) final concentrations in the culture plates and failed to stimulate GM-CFU growth (data not shown).

Stimulatory Effect of Bryostatin on BFU-E and CFU-E. Bryostatin 1 stimulated both early, BFU-E, and late, CFU-E, human erythroid progenitors grown in plasma clots (Fig. 4). The maximal stimulatory concentration was between 1 and 10 pM, with an inhibitory effect noted at concentrations above 1 nM.

Bryostatin Activates Purified Neutrophils. The ability of active bryostatins to stimulate the proliferation as well as the differentiation of normal hematopoietic bone marrow progenitor cells *in vitro* is consistent with the multipotential characteristics described for rHGM-CSF (3, 4, 11). In addition to the stimulatory effects on progenitor cells, rHGM-CSF was found to activate the functional properties associated with mature myeloid cells as well (11, 12). Accordingly, bryostatin was tested for its ability to activate neutrophil chemiluminescence and cytotoxic killing of K562 target cells. Bryostatin 1, but not bryostatin 13, was found to be a potent activator of neutrophil chemiluminescence, a measure of oxide radical formation (8). Activation follows a similar time course to that for the known activator phorbol 12-myristate 13-acetate (Fig. 5). Furthermore, activation of neutrophil cytotoxicity, as measured by killing of ^{51}Cr-labeled K562 tumor target cells (13), was also stimulated by bryostatin 1 (Table 1). Neutrophil activation was maximal between 10 and

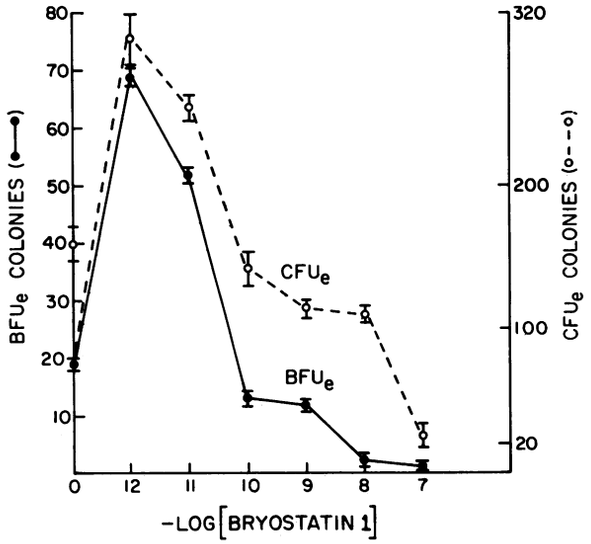

FIG. 4. Bryostatin 1 stimulates the growth of human erythroid progenitors. Bryostatin 1 was added to the culture medium as indicated. Results are reported as the mean colony number ±SEM. In the absence of erythropoietin, no colonies (either early or late) were seen at any of the concentrations of bryostatin used (data not shown).

Table 1 Activated neutrophil cytotoxicity for K562 target cells

Addition	% specific lysis at effector/target ratio		
	30	15	7.5
Neutrophils Plus			
Bryostatin 1			
10^{-7} M	102.8	18.1	7.8
10^{-8} M	8.4	2.4	0
10^{-9} M	0.9	1.8	0
10^{-10} M	−0.8	1.0	0.9
Medium alone	0.9	1.0	−0.8
rHGM-CSF (50 ng/ml)	1.6	0.9	−0.7
Bryostatin alone			
10^{-7} M	1.5	0.3	−0.6
10^{-8} M	0.9	1.0	0.3

Assessment of neutrophil cytotoxicity induced by bryostatin 1. K562 cells were labeled with $Na^{51}Cr_2O_4$ and adjusted to a concentration of 10^5 cells per ml in RPMI 1640 medium. Activated neutrophil cytotoxicity was determined. The results are expressed as the percentage of specific isotope released at the indicated effector/target cell ratio calculated as follows: % specific lysis = [(mean cpm of sample − mean cpm spontaneously released)/(mean cpm maximal − mean cpm spontaneously released)] × 100. Maximum induced cpm released was 7364 ± 149. The maximum mean % specific lysis from triplicate determinations from a representative experiment is shown.

100 nM bryostatin 1, a concentration range that can promote myeloid hematopoietic growth (Fig. 2). Whereas rHGM-CSF has been reported to activate eosinophils and neutrophils (3, 4), we were unable to detect either the induction of chemiluminescence or of cytotoxicity in neutrophils when rHGM-CSF at 50 ng/ml, a maximal-stimulatory concentration for granulocyte–macrophage-colony formation (Fig. 2), was tested alone under these assay conditions (Fig. 5 and Table 1).

DISCUSSION

The ability of bryostatin to stimulate the proliferation and differentiation of normal human hematopoietic progenitor cells *in vitro* (Fig. 2) was consistent with similar effects observed when such cells were incubated with the multipotential recombinant growth factor rHGM-CSF (3, 4, 11). Both bryostatin and rHGM-CSF stimulated primarily mixed granulocyte–macrophage and pure granulocyte colonies (Fig. 3), reflecting equipotent differentiating properties.

Humoral growth factors, including rHGM-CSF (3), have been found to stimulate the growth of hematopoietic cells following the direct interaction with specific receptors on target cells (9, 14). This specific interaction serves to transduce the required intracellular growth signal(s). Likewise, bryostatin 1 exerts its growth stimulatory effect directly on hematopoietic progenitor cells (Fig. 3). Bryostatin was required continuously for granulocyte–macrophage-colony formation since the transferred clones initiated by bryostatin failed to grow in the absence of bryostatin (Fig. 3). These findings are in agreement with a report (3) that demonstrates that rHGM-CSF also acts directly on transferred clones and is required continuously to stimulate colony formation.

Furthermore, like the multipotential rHGM-CSF growth factor (15), bryostatin also was found to be a potent stimulator of erythroid colony formation (Fig. 4). The dose-dependent effect of bryostatin 1 was similar for both early and

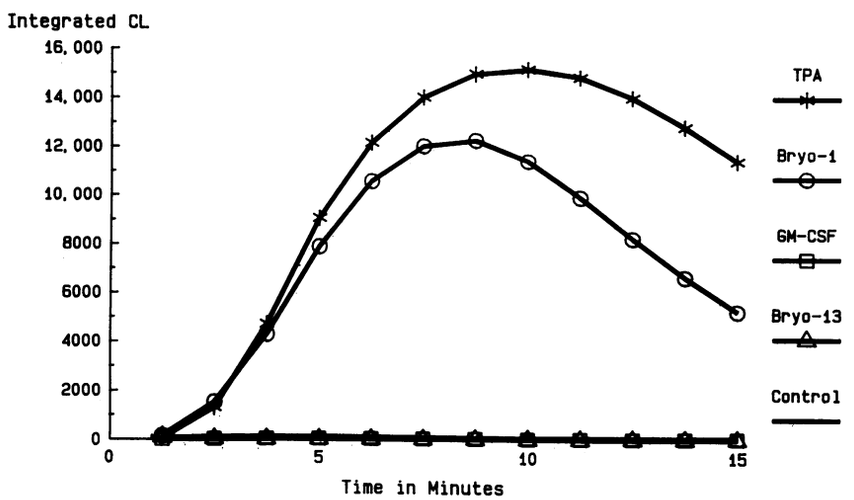

FIG. 5. Generation of chemiluminescence by neutrophils incubated with bryostatins 1 and 13 (Bryo-1 and -13, respectively). Bryostatin 1 or 13 was added to the cells at the indicated concentration. rHGM-CSF was added at 50 ng/ml, and phorbol 12-myristate 13-acetate (TPA) was added at 150 nM. The generated chemiluminesence was recorded as integrated chemiluminesence (CL) and expressed as mV·sec.

late human erythroid progenitors (i.e., BFU-E and CFU-E) grown in plasma clots (Fig. 3). However, a significant difference was noted between the maximal stimulatory concentration for erythroid progenitors (i.e., ≈1 pM) compared to that for nonerythroid progenitors (1–100 nM, Fig. 2). While the explanation for this concentration difference is not readily apparent, it may be related to the sensitivity of BFU-E and CFU-E to bryostatin 1 for erythroid colony formation *in vitro* and may serve to distinguish erythroid and nonerythroid progenitor cells.

In addition to the growth stimulatory properties of multipotential hematopoietic progenitor cells, rHGM-CSF was reported to activate the functional properties of mature neutrophils (4, 12). Bryostatin 1 but not bryostatin 13 was found to be a potent direct activator of neutrophils purified from peripheral blood (Fig. 5, Table 1, and ref. 16). Bryostatin 1 induced rapid neutrophil chemiluminescence, a measure of oxide radical formation (8), in a manner identical to that for the known activator phorbol 12-myristate 13-acetate (Fig. 5). Furthermore, bryostatin 1 also stimulated direct neutrophils cytotoxicity, as measured by killing of ^{51}Cr-labeled K562 tumor target cells (Table 1). In these same studies, a maximally active concentration of rHGM-CSF for colony growth (i.e., 50 ng/ml) was unable to directly activate neutrophils. This finding is consistent with a "priming" effect on neutrophil activation mediated by rHGM-CSF (12) and highlights one important difference between bryostatin 1 and rHGM-CSF.

When structure–activity relationships are examined, the results indicated that both the hematopoietic colony-stimulating activity as well as the functional activation of neutrophils demonstrated by bryostatin may be dependent upon acylation of carbon 20 (Figs. 1, 2, and 5). Thus, when the short-chain, saturated butyric acid was substituted in bryostatin 1 at carbon 20, the resulting bryostatin 9 was a much less potent stimulator of GM-CFU (Fig. 2). This decreased activity was restored when a substitution involving a longer acyl group at carbon 7 was made, as evidenced by bryostatin 8 (Fig. 1). However, when the acyl substitution on carbon 20 was completely removed, as for bryostatin 13, both colony-stimulating activity and neutrophil activation were lost (Figs. 2 and 5). Furthermore, the loss of activity does not result from a toxic effect of bryostatin 13 since when bryostatin 1 was added with bryostatin 13, the expected degree of CFU-GM colony formation obtained with bryostatin 1 alone was observed (data not shown).

Since the inactive bryostatin 13 congener still retains potent antineoplastic activity (17), this indicates that the stimulatory properties of bryostatin described here may be able to be dissociated from the intrinsic antineoplastic activity of this molecule based on the molecular substitutions at carbon 20.

Collectively our findings indicate that the bryostatins can mimic certain of the growth-promoting activities of rHGM-CSF but that this agent clearly has a broader spectrum of activities that includes antineoplastic and neutrophil activation. While the mechanism of action of rHGM-CSF and bryostatin on normal human hematopoietic progenitor cells *in vitro* is not known, the potential for the convergence of these pathways into a common growth-stimulatory effect for normal hematopoiesis exists. Bryostatin has been found to activate protein kinase C (15, 18, 19), a crucial intracellular enzyme thought to be intimately linked in controlling many growth regulatory properties of cells (20–22), including primary neutrophil activation (16). Thus, while speculative, certain action(s) of both bryostatin and rHGM-CSF may be mediated, at least in part, by activation of this enzyme.

The bryostatins will, therefore, be useful as probes to help dissect the mechanism(s) involved in normal hematopoiesis, in particular those regulated by the multipotential GM-CSF. These agents may also prove useful for managing clinical situations related to neoplastic bone marrow failure states.

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