The Antineoplastic Bryostatins Affect Human Basophils and Mast Cells Differently

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Bryostatins, macrocyclic lactones from the marine bryozoan Bugula neritina, are potent antineoplastic agents and multipotential stimulators of immune cells. We have examined the effects of bryostatins on mediator release from human basophilic leukocytes and human tissue mast cells. Bryostatins 1, 2, and 5 (10 to 3,000 nmol/L) induced histamine secretion from purified and unpurified peripheral blood basophils, whereas they caused no release of peptide-leukotriene C4 from these cells. The rate of histamine release caused by bryostatin 1 was slower than that caused by anti-IgE (t1/2 = 38.2 ± 4.7 minutes vs 8.9 ± 0.2 minutes; P < .01), whereas the temperature dependence was similar (optimum release at 37°C, approximately 30% less at 30°C, and no release at 22°C or 4°C). The addition of increasing concentrations of extracellular Ca2+ to the medium caused histamine release in the presence of bryostatins. Subeffective concentrations of bryostatins and anti-IgE produced a synergistic effect on histamine release from basophils. Staurosporine, chelerythrine, and calphostin C (0.1 to 10 mmol/L), which are protein kinase C inhibitors, inhibited the histamine secretion activated by bryostatin 1 and tetradeacyanophorbol-acetate (TPA). Preincubation with granulocyte-monocyte colony-stimulating factor (GM-CSF; 1 and 5 mmol/L) and interleukin-3 (IL-3; 10 ng/mL) potentiated the activation of human basophils induced by bryostatin 1. Neither bryostatin 1 nor bryostatin 2 induced the release of histamine from mast cells isolated from human lung or skin tissues. However, brief (10 minutes) preincubation with bryostatin 1 (3 to 300 nmol/L) potentely inhibited the histamine secretion induced by anti-IgE from skin or lung mast cells. Bryostatin 1 was a more potent (by approximately 30 times) inhibitor of IgE-mediated histamine release than was TPA. The heterogeneous effects exerted by bryostatins on human basophils and mast cells can be of interest for those designing therapeutic trials using these agents. © 1995 by The American Society of Hematology.

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MATERIALS AND METHODS

Reagents. The following reagents were purchased: 60% HClO4 (Baker Chemical Co, Deventer, The Netherlands); calphostin C, chelerythrine, N,N'-bis-(2-ethanesulfonic acid)-piperazine, staurosporine, synthetic LTC4, O-tetradeacyanophorbol 13-acetate (TPA) (Sigma Chemical Co, St Louis, MO); dextran T 70, Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden); (3H)-LTC4 (39.3 Ci/mmol; New England Nuclear, Boston, MA). Rabbit antihuman-Fc,
antibody was a gift from Dr. T. Ishizaka (La Jolla Institute for Allergy and Immunology, La Jolla, CA). The antipeptide-leukotriene C4 was a gift from Dr. L.M. Lichtenstein (The Johns Hopkins University, Baltimore, MD). Bryostatins I, 2, and 5 were isolated as previously described.  

Buffers. The Pipes buffer used in these experiments was composed of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, pH 7.35. The mixture referred to as P2CG contains, in addition to P, 2 mM CaCl2, and 1 g/L dextrose; pH was titrated to 7.4 with sodium bicarbonate.

Preparation of peripheral blood leukocytes containing basophils and basophil purification. The study protocol was approved by the Ethics Committee for Human Research of the University of Naples Federico II School of Medicine (Naples, Italy). Informed consent was obtained from normal subjects, aged 19 to 58 years (mean age, 36.5 ± 1.9 years), and venous blood was drawn into a final concentration of 0.008 mol/L EDTA and 1.1% dextran T70. Erythrocytes were allowed to settle for 90 minutes at 22°C. The leukocyte-rich upper layer was drawn off, pelleted (200 g for 30 minutes), and counted in a Spier-Levy eosinophil counter.  

Mast cells were stained with Alcian blue and counts were between 3 × 106 and 20 × 106, and purities ranged between 75% to 90%, as assessed by basophil staining with Alcian blue and counting in a Spier-Levy eosinophil counter.

Isolation of human lung and skin mast cells. Human lung tissue was obtained from patients undergoing thoracotomy and lung resection, mostly for lung cancer. The study protocol was approved by the Ethics Committee for Human Research of the University of Naples Federico II School of Medicine. Macroscopically normal lung tissue was dissected free from pleura, bronchi, and blood vessels, minced into 5- to 10-mm fragments, and dispersed into single-cell suspension as previously described.  

Mast cells yields with this technique ranged from 3 × 106 to 8 × 106, and purities between 75% to 90%, as assessed by basophil staining with Alcian blue and counting in a Spier-Levy eosinophil counter.

RESULTS

Activation of human basophils induced by bryostatins. In a first group of experiments, we assessed the effects of increasing concentrations of bryostatins 1, 2, and 5 on histamine release from purified peripheral blood basophils (75% to 90% pure) obtained from normal donors. In a series of six experiments, bryostatin 1 (10 to 1,000 nmoL/L), bryostatin 2 (10 to 3,000 nmoL/L), and bryostatin 5 (10 to 3,000 nmoL/L) concentrations dependedently caused histamine release from basophils. As previously reported, TPA (15 to 500 nmoL/L) induced the release of histamine from basophils (Fig 1). Bryostatin 1 was a more potent activator than was TPA, while bryostatins 2 and 5 were much less potent than the other two stimuli. The maximal histamine release induced by bryostatin 1 and TPA was similar (approximately 60%).
while bryostatins 2 and 5 were much less effective (maximal response, approximately 20%). Similar results were obtained in a series of eight experiments in which unpurified (approximately 1% pure) basophils were used (data not shown).

**Characteristics of bryostatin-induced histamine release.**

We next compared the kinetics of histamine release induced by bryostatins, TPA, and anti-IgE from basophils. The time course of histamine release induced by bryostatins 1 and 2 was slow, reaching a maximum at 40 to 60 minutes after the addition of the stimulus. As previously shown, anti-IgE–induced histamine release was significantly faster, being complete after 15 minutes (Fig 2). Bryostatin 5–induced histamine release was extremely slow, reaching its plateau after 90 minutes. The t½ of the release reaction induced by TPA (35.6 ± 2.5 minutes) was similar to that caused by bryostatin 1 (38.2 ± 4.7 minutes) and bryostatin 2 (45.1 ± 6.1 minutes), longer than that caused by anti-IgE (8.9 ± 3.2 minutes; P < .05), and faster than that of bryostatin 5–induced histamine release (47.3 ± 4.2 minutes; P < .05; n = 3).

We studied the histamine-releasing activity of bryostatin 1 on basophils incubated at 37°C, 30°C, 22°C, and 4°C. Figure 3 shows that the bryostatin 1–induced response was abolished by lowering the temperature of the incubation buffer from 37°C to 22°C or 4°C and decreased approximately 30% at 30°C. Similar results were obtained in two other experiments.

**Effect of extracellular Ca²⁺ on the histamine release from basophils by bryostatin.**

We also investigated the effect of the presence of increasing concentrations of extracellular Ca²⁺ (0.1 to 5 mmol/L) on the histamine-releasing activity of bryostatin 1 and bryostatin 2 (Fig 4). The presence of increasing concentrations of extracellular Ca²⁺ increased the percent histamine release induced by bryostatin 1 and bryostatin 2. All subsequent experiments were conducted with physiologic concentrations of extracellular Ca²⁺ (2 mmol/L).

**Effect of bryostatin 1 on LTC₄ release.**

The immunologic activation of human basophils leads to de novo synthesis and release of LTC₄ through the 5-lipoxygenase pathway in addition to the secretion of preformed histamine. In a series of four experiments, we compared the effects of various concentrations of bryostatin 1, TPA, and anti-IgE on the release of LTC₄ from basophils. As previously demonstrated with TPA, optimal concentrations of bryostatin 1 (30 to 1,000 nmol/mL) did not induce the release of LTC₄ from peripheral blood basophils (Table 1). In a control experiment,
anti-IgE (3 x 10^{-2} to 1 \mu g/mL) stimulated the release of \( \text{LTC}_4 \) from these cells. Synergy between bryostatin 1 and anti-IgE. Anti-IgE, which induces histamine release by crosslinking cell surface IgE, was tested for synergy with bryostatin 1 and TPA. Table 2 shows that the presence of bryostatin 1 potentiated the anti-IgE–induced release of histamine from basophils. A potentiation of release was detected at each condition studied in all three experiments. As previously reported, TPA also potentiated anti-IgE–induced histamine release from basophils. Similar results were obtained with bryostatin 2 (data not shown).

As shown in Fig 5A, treatment (10 minutes at 37°C) with a subeffective concentration of bryostatin 1 (100 nmol/L) resulted in a marked potentiation of histamine release from basophils caused by a wide range of concentrations of anti-IgE. As shown in Fig 5B, isoeffect analysis of the interaction between anti-IgE and bryostatin 1 confirmed that the CI values for this interaction were consistently less than 1.0 over a broad range of anti-IgE concentrations, indicating a high degree of synergy.

Effects of staurosporine, chelerythrine, and calphostin C. We next examined the effects of staurosporine and two more specific PKC inhibitors, chelerythrine and calphostin C\(^{57,44}\) on bryostatin 1−, TPA−, and anti-IgE–induced histamine release from basophils. Pretreatment (15 minutes) of basophils with staurosporine, chelerythrine, and calphostin C (0.1 to 10 nmol/L) inhibited bryostatin 1- and TPA-induced histamine release in a concentration-dependent fashion (Fig 6). These experiments were performed in the light because calphostin C is known to be light-activated.\(^{45}\) The concentrations (mean ± SEM) required to reduce histamine release by 50% (IC\(_{50}\)) were 1.5 ± 0.7 nmol/L and 3.6 ± 0.7 nmol/L (staurosporine), 6.8 ± 4.4 nmol/L and 6.6 ± 4.1 nmol/L (chelerythrine), and 2.2 ± 1.7 nmol/L and 9.3 ± 2.1 nmol/L (calphostin C) when cells were activated by bryostatin 1 and TPA, respectively. As previously reported, staurosporine,\(^{46}\) chelerythrine, and calphostin C\(^{44}\) had some inhibitory effect on anti-IgE–induced histamine release from basophils. However, the potency of these drugs was much reduced; indeed, the IC\(_{50}\) for anti-IgE (4.5 ± 0.9 nmol/L, 5.1 ± 2.8 nmol/L, and 5.1 ± 2.9 nmol/L for staurosporine, chelerythrine, and calphostin C, respectively) was greater than in bryostatin 1-induced (1.3 ± 0.6 nmol/L, 2.8 ± 1.9 nmol/L, and 0.15 ± 0.1 nmol/L; \( P < .05 \)) and TPA-induced
DISCUSSION

These studies show that three different bryostatins (1, 2, and 5) synthesized by the marine bryozoan Bugula neritina exert differential effects in human basophils and in tissue mast cells. The activating property of different bryostatins on basophils purified from peripheral blood of normal donors varies considerably. The activating property of bryostatin 1 on basophils can be greatly enhanced by preincubation with some (GM-CSF and IL-5) but not at all (IL-3) cytokines. There was a synergy between bryostatin 1 or 2 and anti-IgE in activating histamine release from human basophils. In

Interaction between GM-CSF, IL-3, IL-5, and bryostatin 1. The antineoplastic activities of bryostatins are mediated, at least in part, by the release of lymphokines. In addition, coadministration of bryostatin 1 increased the radioprotective capacity of GM-CSF. Human basophils possess surface receptors for various cytokines (GM-CSF, IL-3, IL-5, etc.), and preincubation of basophils with GM-CSF, IL-3, and IL-5 can modulate their activation. Therefore, in a series of four experiments with purified basophils, we examined the possible interaction between GM-CSF, IL-3, and IL-5 and bryostatin 1. Figure 7A shows that preincubation of basophils with GM-CSF (1 and 5 nmol/L) concentration dependently potentiated the release of histamine caused by bryostatin 1 (10 to 300 nmol/L). The priming effect of GM-CSF was observed even at concentrations of bryostatin 1 (10 nmol/L) that caused no histamine release. Similar results were obtained with IL-3 (10 ng/mL) but, interestingly, not with IL-5 (10 U/mL; Fig 7B). In these experiments, IL-3, IL-5, and GM-CSF alone did not activate purified basophils (Fig 7A and B).

Effect of bryostatins on human mast cells isolated from human lung and skin. Given the heterogeneity of human basophils and mast cells, we tested whether bryostatins could induce histamine release from human lung or skin mast cells and compared their effects with anti-IgE and calcium ionophore A23187. Figure 8 shows that a wide range of concentrations of bryostatins 1 and 2 (30 to 3,000 nmol/L) did not affect the secretion of histamine from either human lung (Fig 8A) or skin mast cells (Fig 8B). In contrast, both of these mast cell types responded to a challenge with optimal concentrations of anti-IgE or A23187 with a concentration-dependent release of histamine (Fig 8A and B). TPA alone did not activate human lung and skin mast cells (data not shown).

Effect of bryostatin 1 on IgE-mediated histamine release from human mast cells. It has been previously shown that prolonged pretreatment (18 hours) of human mast cells with the PKC activator TPA inhibits the subsequent response to anti-IgE. We compared the effect of brief preincubation (10 minutes) with low concentrations of bryostatin 1 (0.1 to 300 nmol/L) and TPA (0.1 to 300 nmol/L) on the secretion of histamine from skin mast cells induced by anti-IgE. Figure 9 shows that both TPA and bryostatin 1 caused a concentration-dependent inhibition of IgE-mediated response of skin mast cells. Interestingly, bryostatin 1 was a much more potent inhibitor (approximately 30-fold) than TPA (IC50 = 6.3 ± 1.2 nmol/L v 200 ± 11.1 nmol/L; P < .001). Similar results were obtained in three experiments with human lung mast cells (data not shown). Brief (10-minute) incubation of human lung and skin mast cells with bryostatin 1 or TPA did not affect A23187-induced histamine release (data not shown).

We also evaluated the kinetics of the inhibition of IgE-mediated histamine release by bryostatin 1 (100 nmol/L) in human lung and skin mast cells. In the preincubation time range between 10 and 120 minutes, we found no significant differences in the inhibiting effect of bryostatin 1 in human lung and skin mast cells (n = 3; data not shown).
contrast, bryostatins were unable to activate mediator release from human lung and skin mast cells; however, low concentrations of bryostatin 1 caused a rapid and potent inhibition of IgE-mediated histamine release from these cells.

Bryostatins activated human basophils presumably by a noncytotoxic mechanism, as cell viability was not affected and bryostatin's releasing activity from basophils was temperature-dependent. Indeed, bryostatin releasing activity was blocked by incubation at a low temperature (22°C and 4°C) and was markedly reduced by lowering the temperature from 37°C to 30°C. A noncytotoxic mechanism is also suggested by the lack of cytotoxicity of similar concentrations of bryostatins in systems using other immune cells (such as neutrophils, lymphocytes, etc.).

The kinetics and Ca²⁺ dependence of basophil activation induced by bryostatins differed markedly from anti-IgE-induced release, but are similar to TPA-induced secretion of histamine from basophils. The kinetics of basophil activation by different bryostatins vary to some extent, with bryostatin 5 being the slowest activator of human basophils.

Bryostatins act as incomplete secretagogues on human basophils (no release of LTC₄), as previously shown for TPA. As similar results were obtained with highly purified and unpurified basophils, it is likely that bryostatins activate directly peripheral blood basophil. However, the possibility cannot be excluded that bryostatins, which induce the release of specific granules from human neutrophils, might induce the secretion of histamine from basophils through the activation of neutrophils or other contaminating cells.

It is not unfeasible that the mechanism of activation of basophils could be mediated by interaction of bryostatins with PKC. In this context, appropriate concentrations of staurosporine and two more specific PKC inhibitors, chelerythrine and calphostin C, inhibited mediator release caused by bryostatin 1 and TPA. Although direct measurements of PKC activation by bryostatins were not made, basophils activated by anti-IgE appeared less sensitive to the modulation of staurosporine.

A potentially relevant finding of this study is the synergy between bryostatin 1 and immunologic stimuli or cytokines. We found that pretreatment with bryostatins 1 and 2 synergistically potentiated the immunologic release of histamine induced by anti-IgE; even low concentrations of bryostatins 1 and 2 that alone caused little or no release of histamine markedly potentiated the releasing activity of anti-IgE on basophils. Another interesting interaction occurs between cytokines and bryostatins. Preincubation of cells with such
cortisol as GM-CSF and IL-3 potentiated the activating properties of bryostatins. The specificity of this observation is suggested by the finding that IL-5 did not possess a similar priming effect. This observation may be relevant to those conditions in which cytokines (e.g., GM-CSF, IL-3, IL-5, etc.) and, possibly, bryostatins could be used alone in combination in the treatment of some patients with tumors. Furthermore, the interaction between GM-CSF, IL-3, and bryostatin 1 may be particularly relevant, because in several systems bryostatin 1 promotes some of the biologic effects of GM-CSF.

Unlike basophils, mast cells isolated from human lung or skin tissues did not respond to challenge with bryostatin 1, whereas they were normally responsive to anti-IgE or to the

Fig 7. (A) Effect of preincubation (60 minutes at 37°C) with GM-CSF (1 and 5 nmol/L) on histamine release from human basophils induced by increasing concentrations of bryostatin 1. Each point represents the mean ± SEM from duplicate determinations of four experiments. (B) Effect of preincubation (30 minutes at 37°C) with optimal concentrations of IL-3 (10 ng/mL) or IL-5 (10 U/mL) on histamine release from human basophils induced by increasing concentrations of bryostatin 1. Each point represents the mean ± SEM from duplicate determinations of four experiments. The different times of preincubation with cytokines were selected based on previous experience in our laboratory (University of Naples).

Fig 8. Effect of increasing concentrations of bryostatin 1, bryostatin 2, anti-IgE, and compound A23187 on histamine release from human lung (A) and skin (B) mast cells. Each point represents the mean ± SEM of three and four experiments for lung and skin mast cells, respectively. Error bars are not shown when graphically too small.
calcium ionophore A23187. These results agree with the observation that another PKC activator, TPA, is also unable to induce mediator release from human mast cells. Interestingly, low concentrations of bryostatin 1 rapidly and potently inhibited the IgE-mediated activation of human skin and lung mast cells. These results are compatible with the hypothesis that PKC plays a role in the release process of human mast cells induced by IgE crosslinking, because it has been shown that short-term treatment with bryostatin 1 causes rapid loss of PKC activity. \(^{53,54}\) Our results extend the observation by Massey et al\(^{15}\) in that it appears that even short-term (10 minutes) cell pretreatment with bryostatin 1 or TPA can cause inhibition of IgE-mediated mast cell activation. Therefore, it is conceivable that downregulation of PKC after brief incubation with bryostatin 1 causes a diminished mast cell response to anti-IgE. Direct PKC measurements cannot yet be performed because they require large amounts of highly purified mast cells that are not currently available from human tissues. The finding that bryostatin 1 was more potent than TPA as an inhibitor of IgE-mediated histamine release from skin mast cells is intriguing. In fact, it has been hypothesized that bryostatin 1 and TPA may act on different PKC isozymes,\(^{13}\) or that the two compounds may differ in their affinity to PKC.\(^{55}\)

Mast cells and basophils are commonly considered to play a major role in the pathogenesis of IgE-dependent immediate hypersensitivity.\(^{20,25}\) However, increasing evidence showing that human basophils and mast cells are sources of multifunctional cytokines\(^{24,25}\) implies that they play a more complex role in the immune regulation than originally thought. In particular, there is preliminary evidence that basophils and mast cells, through the release of chemical mediators and cytokines, might affect angiogenesis, tumor development, and tumor resistance.\(^{56,57}\) Bryostatin 1 has been shown to possess in vitro\(^{58}\) and in vivo\(^{59}\) antineoplastic activities, and recent findings suggest that bryostatins represent clinically attractive agents useful for treatment of various neoplasms.\(^{18}\) Previous findings have clearly demonstrated that bryostatin 1 causes substantial activation of T lymphocytes,\(^{17}\) B lymphocytes,\(^{14}\) neutrophils,\(^{5}\) and basophils.\(^{15,16}\)

The releasing activity of various bryostatins is similar but not identical, and bryostatin 1 appears to be more potent than the tumor promoter TPA. Previous studies have demonstrated that bryostatins bind to and activate PKC, but they differ from TPA in selectivity and in that they behave either as agonists or antagonists of PKC actions.\(^{9,16}\) The basis for the different selectivities of bryostatins and phorbol esters is not understood.\(^{2,16}\) Differences in the activating properties of different bryostatins have been already reported.\(^{20}\) Here we show that in activating human basophils, different bryostatins also differ in their potency and in some biochemical characteristics. Therefore, further studies of bryostatins as activators of human basophils should be profitable in the understanding of the mechanisms by which bryostatin-sensitive PKC is distinct from TPA-sensitive kinases in basophils. It is fascinating that in the human mast cells bryostatin 1 does not cause activation but, rather, inhibition of the IgE-mediated mediator release. This finding may indicate that downregulation of PKC can play an inhibitory role in mast cell activation induced by anti-IgE. This inhibitory effect of low concentrations of bryostatin 1 in human mast cells might be clinically relevant, because mast cells represent a major source of mediators.

The differential effects of bryostatins on human basophils and mast cells and their complex interaction with other immunologic stimuli or cytokines raise important issues that will need to be considered when designing clinical trials to evaluate the antineoplastic properties of these compounds.

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