In Vivo Administration of Bryostatin 1, a Protein Kinase C Activator, Decreases Murine Resistance to Salmonella typhimurium

Andrew S. Kraft, Victor Adler, Patti Hall, George R. Pettit, William H. Benjamin, Jr., and David E. Briles

ABSTRACT

Bryostatin 1, a potent activator of protein kinase C, has antitumor activity against murine lymphoma, leukemia, and melanoma. In vitro, this compound stimulates the release of γ-interferon, interleukins, and hematopoietic growth factors from accessory cells and activates both T- and B-cells. Bryostatin 1 is also able to stimulate neutrophils to undergo oxidative burst and degranulation. Because of the ability of this compound to stimulate the immune system, cause release of immune mediators, and activate neutrophils, we have examined its effect on bacterial infection by using the gram-negative bacterium Salmonella typhimurium in mice. We find that animals given injections i.v. of S. typhimurium have a shortened life span if they are also given injections i.p. of nonlethal doses of bryostatin 1. There is a dose-response relationship with 100 μg/kg bryostatin 1 having a greater effect on survival than 40 μg/kg. Below 40 μg/kg there are no effects on survival. Analysis of the first 4 h of Salmonella infection demonstrates that bryostatin 1 does not affect the blood clearance of the bacterium. However, by day 2 of infection greater numbers of bacteria are found in the livers and spleens of mice given injections of bryostatin 1. By day 5,10-fold more S. typhimurium bacteria are found in the livers and spleens of mice receiving 40 μg/kg of bryostatin 1. To determine whether bryostatin 1 was affecting growth or causing the death of bacteria, we used a Salmonella carrying a plasmid which has a temperature-sensitive origin of replication and is unable to replicate when the bacteria are in mice. This experiment demonstrates that bryostatin 1 represses bacterial killing but does not affect bacterial growth. Bryostatin 1 given i.p. stimulates a transient syndrome of weight loss and diarrhea from which the mice recover and regain weight, suggesting that bryostatin 1 may release a number of important humoral mediators in vivo. The weight loss is exacerbated by Salmonella infection with mice receiving bryostatin 1 and S. typhimurium, in that they lose approximately 33% of body weight prior to death. Thus, at doses used to treat murine tumors, bryostatin 1 treatment does not affect the clearance of S. typhimurium from the blood but does decrease the killing of bacteria in the liver and spleen, leading to early animal death. Such potential effects of bryostatin 1 on the outcome of bacterial infections should be evaluated in ongoing human trials of this agent.

INTRODUCTION

Bryostatin 1, a naturally occurring macrocyclic lactone isolated from the marine animal Bugula neritina (a bryozoan) (1), is a potential anticancer agent. Studies in mice demonstrated that bryostatin 1 injection prolonged survival of those mice given injections of either P388 leukemia (2), B16 melanoma (3), or M5076 ovarian sarcoma (2). Bryostatin 1 injection i.p. decreased the number of pulmonary metastases from i.v. injected B16 melanoma cells. (3). One possible mechanism underlying the antiproliferative activity of bryostatin 1 may be its ability to differentiate specific tumor types. Application of bryostatin 1 to either freshly isolated cells from patients with acute and chronic myelogenous (4, 5) or leukemia cell lines HL-60 (6) or U937 (7) induces macrophage-like differentiation and halts the growth of those cells. Not only the myeloid lineage but also B-cells from chronic lymphatic leukemia are induced to differentiate by this compound (8).

In contrast to these tumor cells, adding bryostatin 1 to cells from patients with chronic myelomyelogenous leukemia does not induce differentiation but inhibits the growth of these cells (9). This inhibition may be caused by the bryostatin-stimulated release of tumor necrosis factor which is cytotoxic to these leukemic cells. Another possible mechanism of antitumor action of bryostatin 1 is through its stimulation of the immune system. Bryostatin 1 stimulates IL-2 receptor expression on CD4+ and CD8+ lymphocytes, and greatly enhances the efficiency of recombinant interleukin 2 in triggering the development of in vivo primed cytotoxic T-lymphocytes (10). Bryostatin 1 may activate the immune system indirectly by stimulating an increased release of IL-2 or γ-interferon from T-cells and macrophages (11). However, bryostatin 1 was found to block IL-2-induced lymphocyte activated killer cell activation, suggesting that bryostatin 1 might also inhibit certain segments of the immune system (12).

The cellular basis of the antitumor action of bryostatin 1 is based on the ability of this compound to activate protein kinase C (13). Bryostatin 1 binds to this enzyme, stimulating phosphorylation of proteins (14), causing the translocation of this enzyme to the membrane, and leading to the rapid degradation of this protein kinase (14). Although bryostatin 1 modulates protein kinase C in a similar fashion to the tumor-promoting phorbol esters (15, 16), these two compounds have different biological effects. Unlike phorbol esters, bryostatin 1 when injected into animals is not a tumor promoter (17); it does not induce the growth of GH4 cells (18); and it does not induce squamous differentiation in human tracheobronchial epithelial cells (19). Bryostatin 1 is a much weaker stimulant of nonadherent growth of mouse JB6 epidermal cells than phorbol esters (20). The differences in the biological effects of these two compounds may result from their slightly different effects on protein kinase C. Bryostatin 1 causes a much more rapid degradation of this enzyme than phorbol esters (14) and, unlike phorbol esters, stimulates the translocation of protein kinase C beta to the nucleus (21, 22).

The observation that bryostatin 1 stimulates colony growth of hematopoietic progenitors suggests that it could also be used as an adjuvant to cytotoxic chemotherapy that decreases normal bone marrow function. Using in vitro human bone marrow cultures, bryostatin 1 stimulates CFU-GM, CFU-erythroid, and burst-forming units-erythroid (23). The effects of bryostatin 1 may be secondary to the release of GM-CSF or IL-3 from T-cells or other accessory cells (24). Alternatively, bryostatin 1 may sensitize in vitro responses of progenitor cells to GM-CSF,
which is normally secreted from accessory cells (25). Bryostatin 1 also has stimulatory effects on mature cells in the hematopoietic system, and it stimulates oxidative burst and degranulation of mature neutrophils and monocytes (26).

Gram-negative sepsis is a frequent occurrence in patients undergoing intensive chemotherapy for hematopoietic or solid malignancies. Because bryostatin 1 activates neutrophils to undergo oxidative burst and degranulation, stimulates the release of γ-interferon, activates T-cell proliferation, and stimulates B-cell differentiation, we have evaluated its effect on the course of Gram-negative sepsis. S. typhimurium infection is a complex challenge to various arms of the immune system. The initial i.v. infection is cleared over the first 4 h by the reticuloendothelial system (27). The bacteria remaining find sanctuary in the liver and spleen in mononuclear cells in which they multiply, and the host is eventually overwhelmed by increasing blood-borne infection (28–30). The lty locus on chromosome I of the mouse plays a major role in the resistance of certain mouse strains to systemic S. typhimurium infection. Mice that are lty susceptible demonstrate slightly decreased killing of bacteria in the first few hours of infection, thus allowing a much greater growth of bacteria in the liver and spleen (31).

We have found that the injection of bryostatin 1 at nonlethal doses to C57BL/6 mice infected with S. typhimurium caused the more rapid death of these mice. This effect was dose dependent, with those mice receiving 100 µg/kg dying much faster than those receiving 40 µg/kg. The injection of bryostatin 1 did not cause any change in clearance of bacteria over the first 4 h, but it allowed an increase in the number of bacteria in the liver and the spleen. Using S. typhimurium infected with a temperature-sensitive plasmid we have been able to demonstrate that bryostatin 1 decreases the ability of mice to kill this bacterium. These data predict that, while decreasing tumor growth, treatment with bryostatin 1 may have adverse effects on the outcome of certain Gram-negative infections.

MATERIALS AND METHODS

C57BL/6 and LAF, mice were obtained between 6 and 14 weeks of age from The Jackson Laboratory, Bar Harbor, ME. All mice arrived with negative serology for mouse hepatitis virus and were used for experiments within 2 days. Bryostatin 1 was diluted to the appropriate concentration in Dulbecco's phosphate-buffered saline containing 10% DMSO. Where necessary, bryostatin 1 was diluted first with 100% DMSO, so that the final concentration of DMSO injected was never lower than 10%. Bryostatin 1 was injected i.p. in a 0.2-ml volume.

Mice were infected i.v. with indicated numbers of strain WB600 of S. typhimurium (32), and were sacrificed at varying days postinfection; the numbers of Salmonella in the spleen and liver were determined as previously described (31).

To evaluate bacterial killing versus growth, mice were infected with a strain of TML S. typhimurium carrying the nonreplicating temperature-sensitive plasmid pHSG422. The presence of Salmonella carrying this plasmid was detected by plating the Salmonella on kanamycin plates. This plasmid fails to replicate at body temperature; therefore, mice used in this study were maintained at 30°C to prevent the plasmid from replicating as the Salmonella pass through the extremities (30, 31).

RESULTS

Prior to examining the effects of bryostatin 1 on mice infected with S. typhimurium, the effects of bryostatin 1 alone on mice were first investigated. Ten C57BL/6 mice were given injections daily i.p. of 100 µg/kg of bryostatin 1. These animals experienced a syndrome of diarrhea and weight loss; however, after daily injections no deaths from bryostatin 1 had occurred. Other mice were given injections i.v. of 10³ S. typhimurium and i.p. of bryostatin 1 at 12 and 24 h after bacterial injection and then at 24-h intervals until 72 h postinfection. Because the complete blood clearance of bacteria occurs during the first 4 h after infection, the injection of bryostatin 1 at 12 h allowed examination of the effect of this compound on the chronic phase of this disease. By day 3, all of the control mice infected with S. typhimurium were alive, but none of the mice receiving 100 µg/kg and only 70% of the mice receiving 80 µg/kg were alive (Fig. 1). By day 5 none of the mice receiving 80 or 60 µg/kg were alive. On day 6, the mice given injections of Salmonella and 40 µg/kg of bryostatin, all of which remained alive, were sacrificed. Their livers and spleens were removed, sterilely homogenized, and bacterial levels were determined by plating out dilutions of this homogenate. The animals given injections of 40 µg/kg dose of bryostatin 1 had over 10-fold more bacteria in their liver and spleen; animals given injections of bryostatin had an average of 8.230 ± 0.100 (log₁₀ mean ± SE) (n = 3) and those given injections of vehicle alone had an average of 7.183 ± 0.119 (n = 6) bacteria in the liver and spleen. These values were significantly different at the P < 0.001 level.

All subsequent studies of the effect of bryostatin on level of S. typhimurium in the liver and spleen of chronically infected mice, a 40-µg/kg dose of bryostatin was used. The effect of varying Salmonella dosages was next examined. When mice given daily bryostatin 1 were given injections of 10² or 10³ Salmonella, the same 10-fold enhancement by bryostatin 1 in the numbers of Salmonella in the livers and spleens was observed (Table 1). In another set of experiments mice were given injections of 10⁶ S. typhimurium and received bryostatin 1 at 12, 24, and 48 h. Because of the large number of bacteria injected, mice were sacrificed at 72 h. In these mice there was no difference in the number of bacteria in the liver and spleen between the bryostatin 1-treated and untreated animals. Thus, the effects of bryostatin are seen only with low doses of Salmonella, which allows for prolonged animal survival. The increase in the level of bacteria in the liver and spleen is dependent...
on the concentration of bryostatin 1 injected. Lower dosages of bryostatin 1 (20, 4, and 0.4 µg) did not affect the level of bacteria in the liver and spleen (Table 2).

To study whether the effects of bryostatin 1 were seen soon after infection and treatment, we gave animals injections of S. typhimurium and injected bryostatin at 12 and 36 h. The animals were sacrificed on day 2, and the number of bacteria in the livers and spleens were counted. Mice treated with bryostatin 1 had an average of 5.569 ± 0.136 (log_{10} ± SE) (n = 5), while control animals had an average of 4.976 ± 0.081 (n = 5) bacteria in their livers and spleens (P < 0.01). Therefore, following only 2 doses of bryostatin 1, it was possible to measure the difference in bacterial levels caused by bryostatin 1 treatment.

The studies described above focused on the chronic tissue phase of Salmonella bacterial infection. To evaluate the effects of 40 µg/kg bryostatin on the blood clearance of Salmonella, bryostatin 1 at 40 µg/kg was given approximately 1 h before injecting 10⁶ bacteria i.v. Mice were bled at 5 min, 30 min, 2 h, and 4 h, and the blood levels of the bacteria were determined. Bryostatin 1 injection had no effect on the level of clearance (Table 3). In other experiments, bryostatin 1 was injected either simultaneously with bacteria or 3 h after the bacterial injection with no change in the clearance rate.

There are two possible explanations for the increase in S. typhimurium measured in the livers and spleens of bryostatin-treated mice: either bryostatin 1 is enhancing the ability of these bacteria to grow or it is repressing the ability of these animals to kill the bacteria. To evaluate these possibilities, we used S. typhimurium, TML, carrying a plasmid, pHSG422, which contains a temperature-sensitive origin of replication and antibiotic resistance genes (31). This plasmid shows virtually no replication at body temperature. Since the plasmid contains a kanamycin resistance gene, the number of plasmid containing Salmonella was measured by plating out the bacteria from the liver and spleen on plates containing this antibiotic. A decrease in plasmid number reflects killing of the Salmonella and thus the plasmid by the host. A decrease in the frequency of the plasmid per total Salmonella would be an indication of bacterial growth. We injected bacteria containing this plasmid into C57BL/6 mice susceptible to Salmonella (Ity) and LAF mice, which are resistant to Salmonella infection (Ity). Mice were given injections at 12 and 36 h of bryostatin 1 (40 µg/kg) and were killed at 2 days; the number of kanamycin-resistant and total bacteria from the liver and spleen were counted (Table 4). Bryostatin 1 treatment did not change the kanamycin-resistant:total organisms ratio but did increase the number of total and kanamycin-resistant colonies 3- to 4-fold. These data demonstrate that bryostatin 1 does not inhibit the growth of bacteria; thus, it must modify the ability of cells within the liver and spleen to kill these bacteria. As expected (31), more Salmonella growth and less killing was observed in Ity' than in Ity' mice.

One possible explanation of the effect of bryostatin 1 on bacterial killing might be the secondary effects of humoral factors, i.e., interferon, interleukins, or tumor necrosis factor, which are released after bryostatin 1 treatment on neutrophils and monocytes. Release of these humoral factors might be further stimulated by Salmonella infection. Bryostatin 1 treatment causes a syndrome of weight loss and diarrhea which might reflect the release of multiple humoral mediators. This syndrome is transient, and mice given injections for 7–10 days returned to starting weight (data not shown). We therefore evaluated the combined effects of bryostatin 1 (40 µg/kg) and Salmonella infection on animal weight. During the first 2 days after infection, the loss of weight found in these animals solely reflects bryostatin toxicity (Fig. 2). By days 3 and 4, however, the weight loss of the mice appears to be additive, combining the continued effects of bryostatin 1 and the effects of increasing bacterial load (Fig. 2). The weight loss induced by bryostatin 1 may reflect the release of multiple humoral mediators which prevent bacterial killing at early times, thus allowing for increased bacterial levels in tissue.

DISCUSSION

Because bryostatin 1 stimulates neutrophil degranulation and oxidative burst, activates the release of both interleukins and γ-interferon, and stimulates mitogenesis of both T- and B-cells, we hypothesized that this agent might have bactericidal activity against Gram-negative bacteria. Salmonella is a complex infection which first involves i.v. clearance, followed by multiplication of the remaining organisms in the reticuloendothelial system of the liver and spleen, and followed by overwhelming bacterial infection. The ability of mice to suppress this infection is at least partly mediated by a locus on chromosome 1, Ity. Animals that are Ity' allow the growth of these bacteria in the liver and spleen, while Ity' blocks this multiplication phase.

In contrast to our hypothesis, injection of 100 µg/kg bryostatin 1 shortened the life span of mice given injections of low doses (1000 CFU) of S. typhimurium. However, this bryostatin 1 effect is not apparent when high doses (10⁶) of bacteria are given because animals die of infection within the first 2 days.

To examine the ability of bryostatin 1 to regulate the growth of Salmonella in the liver and spleen, we used a dose of

<table>
<thead>
<tr>
<th>Time</th>
<th>Control (CFU)</th>
<th>Bryostatin 1 (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td>6.056 ± 0.031</td>
<td>6.072 ± 0.025</td>
</tr>
<tr>
<td>30 min</td>
<td>5.959 ± 0.017</td>
<td>5.999 ± 0.040</td>
</tr>
<tr>
<td>2 h</td>
<td>4.673 ± 0.115</td>
<td>4.336 ± 0.071</td>
</tr>
<tr>
<td>4 h</td>
<td>3.541 ± 0.089</td>
<td>3.217 ± 0.098</td>
</tr>
</tbody>
</table>

* Values shown are the log_{10} means ± SE for 5 mice in each group.

Table 1 Effect of bryostatin 1 on liver and spleen bacterial level

<table>
<thead>
<tr>
<th>Dose (µg/kg)</th>
<th>Control 10³ CFU injected</th>
<th>Bryostatin 1 10³ CFU injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.392 ± 0.053*a</td>
<td>7.058 ± 0.142*</td>
</tr>
<tr>
<td>Bryostatin 1</td>
<td>7.513 ± 0.025*b</td>
<td>8.158 ± 0.081*b</td>
</tr>
</tbody>
</table>

* All mice were killed on day 5 following infection. Data shown are the log_{10} means of 4 mice ± SE.
* P < 0.001 versus control.

Table 2 Effect of varying bryostatin 1 doses on liver and spleen bacterial level

<table>
<thead>
<tr>
<th>Dose (µg/kg)</th>
<th>Liver/spleen (CFU)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.230 ± 0.300</td>
</tr>
<tr>
<td>40</td>
<td>8.260 ± 0.045*</td>
</tr>
<tr>
<td>8</td>
<td>7.484 ± 0.253</td>
</tr>
<tr>
<td>4</td>
<td>6.912 ± 0.329</td>
</tr>
<tr>
<td>0.4</td>
<td>7.400 ± 0.097</td>
</tr>
</tbody>
</table>

* Mice were killed on day 4 after injection of approximately 10⁶ Salmonella. Data shown are the log_{10} means of values from 5 mice ± SE.
* Different from no bryostatin 1 at P < 0.05.
The injection of bryostatin 1 i.p. does not affect the infection. Bryostatin 1 treatment did not modulate the Salmonella sensitivity phenotype of these animals. However, the numbers of Salmonella in both strains of mice in the liver and spleen were increased by bryostatin 1, demonstrating that this agent affects bacterial killing.

There are a number of possible explanations for this observation. Salmonella infection is associated with massive infiltration of neutrophils into the liver and spleen. To investigate whether bryostatin 1 treatment modifies this infiltration, we did microscopic examination of livers and spleens from animals receiving bryostatin 1 or Salmonella or both. Bryostatin 1 did not appear to decrease the neutrophil infiltration stimulated by these bacteria. Because bryostatin 1 activates both neutrophils and monocytes to undergo oxidative burst and degranulation in vitro, it remains possible that these cells in the liver are nonfunctional. The second possibility was that bryostatin 1 was stimulating the release of a humoral mediator which exacerbated the effects of Gram-negative infection. To examine this hypothesis, we chose to measure the effects of bryostatin 1 on tumor necrosis factor α levels in the blood. Using an enzyme-linked immunosorbent assay kit (Genezyme), we were unable to detect any change in tumor necrosis factor α levels at 1 and 24 h after bryostatin 1 injection, with or without Salmonella. Possibly this method was not sensitive enough to detect small changes in tumor necrosis factor α levels. Further studies will be necessary to clarify this result.

The observation that bryostatin 1 stimulates transient weight loss and diarrhea and that this weight loss is compounded by Salmonella infection suggests that although bryostatin 1 injection is nonlethal, this compound has profound effects on murine physiology. Recently, we have demonstrated that if mice are given injections of 5-fluorouracil, making them leukopenic, weight loss and diarrhea and that this weight loss is compounded by Salmonella infection. Possibly this method was not sensitive enough to detect small changes in tumor necrosis factor α levels. Further studies will be necessary to clarify this result.

Because both serine and tyrosine kinases have been implicated in the transformation and maintenance of cancer cells, these kinases are an appropriate target for anticancer drugs. Bryostatin 1 modulates the activity of the serine protein kinase, protein kinase C, stimulating phosphorylation of membrane and cytosolic proteins and leading to the eventual degradation of this kinase. While bryostatin 1 has antitumor activity in several murine models, including lymphomas, leukemias, and melanomas, our data suggest that bryostatin 1 may negatively affect the outcome of overwhelming bacterial infection. Therefore clinical trials should be closely monitored for this potential adverse effect.

REFERENCES

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