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Bryostatin 1 activates splenic lymphocytes and induces sustained depletion of splenocyte protein kinase C activity *in vivo* after a single intravenous administration

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Bryostatin 1 activates and subsequently down-regulates protein kinase C (PKC) *in vitro* and has potential use as an immunomodulator and as an anti-cancer agent. Despite extensive examination of its activities *in vitro* and anti-tumor effects *in vivo*, previous studies have failed to document that bryostatin 1 modulates total cellular PKC activity in tumor or normal tissues when administered *in vivo*. After a single bolus injection of bryostatin 1 (1.0 μg) in normal C57Bl/6 mice, blood was drawn at various intervals and assayed for bryostatin 1 levels. In addition, spleens from bryostatin-treated mice were harvested 10 min to 10 days after treatment, weighed and analyzed for cell numbers, PKC activity and cell surface phenotypes. Bryostatin 1 levels in plasma rose rapidly, reaching peak levels of 56.5 nM less than 1 min after injection, and then declined to undetectable levels by 1 h. A similar pattern was observed when bryostatin 1 was incubated with leukemia cells *in vitro*, raising the possibility that the rapid fall in plasma levels results from intracellular uptake and binding. Bryostatin 1 induced marked depletion of total splenocyte PKC activity (as much as 69% relative to control values) at 24–96 h after drug administration, but not at earlier times (i.e. 1 h). A single injection of bryostatin 1 also induced expression of the T cell activation marker CD69, leading to positivity in 53% of cells at 3–24 h versus 11% in control mice, and resulted in marked splenomegaly, associated with increased numbers of nucleated cells at 48–96 h. Together, these studies demonstrate that despite rapid disappearance of the drug from plasma, a single i.v. dose of bryostatin 1 exhibits significant and sustained effects on normal murine spleen cells, including early lymphocyte activation, prolonged depletion of PKC activity, spenocyte proliferation and splenomegaly. These findings may have implications for attempts to understand the *in vivo* effects of bryostatin 1 in normal host tissues.

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Introduction

Bryostatin 1 is a macrocyclic lactone isolated from the marine bryozoan *Bugula neritina*,[1] and has been shown to exhibit antitumor and immunomodulatory properties in preclinical studies.[2] Like the phorbol esters, bryostatin 1 is a potent activator of the calcium- and phospholipid-dependent serine-threonine kinase, protein kinase C (PKC).[3] The basis for the unique pattern of activity of bryostatin 1 remains unclear, but may result from isoform-specific effects[4] or from its capacity to induce extensive PKC down-regulation.[5] Unlike the phorbol esters, however, bryostatin 1 is not a tumor promoter; in fact, it can block certain phorbol-mediated actions that it does not itself possess.[6] In view of its promising preclinical activity, several phase I trials of bryostatin 1 have recently been conducted in humans[7–9] and others are currently underway.

Bryostatin 1 has been found to modify the response of normal and malignant cells to ionizing radiation and chemotherapeutic agents, and to potentiate host immune mechanisms. For example, Sredni *et al.* reported that when combined with the ogranotellurium compound AS101, bryostatin 1 accelerated the recovery of murine bone barrow and spleen cell counts following radiation or chemotherapy.[10] Recently, we have shown that bryostatin 1 can be employed to expand T lymphocytes with anti-tumor activity *ex vivo*,[11,12] to enhance the radioprotective effect of granulocyte macrophage colony stimulating factor *in vivo*[13] and to potentiate ara-C-induced apoptosis in human leukemia cells *in vitro*.[14] However, the results of an earlier report

suggest that bryostatin 1 administered *in vivo* does not significantly alter PKC activity in normal murine tissues, including the spleen, or in tumor cells.[15] It should be noted that the latter study involved only short-term (i.e. 1 h) assessment of PKC activity in target tissues, even though *in vitro* data suggested that PKC depletion may be delayed and more prolonged. Furthermore, our interest in the use of bryostatin 1 *in vivo* to potentiate chemotherapy effects on cancer cells made it essential that the kinetics and duration of any effects on PKC be delineated in detail. The present studies were therefore undertaken to test the hypothesis that a single bolus injection of bryostatin 1 could in fact exert measurable effects (i.e. as determined by total PKC activity and/or T cell activation markers) in normal splenic cells when monitored over more prolonged intervals.

Experimental procedures

Mice

Virus-free C57Bl/6 mice were obtained from Charles River (Wilmington, MA). Mice were used at 8–12 weeks of age and were caged in groups of six or fewer, with food and water *ad libitum*. All guidelines at the Medical College of Virginia, which are in conformity with AAALAC and US Department of Agriculture recommendations for the care and humane experimental use of animals, were followed.

Materials

Bryostatin 1 was isolated from the marine intertebrate *B. neritina* as originally described[1] and material for these studies was obtained from the National Cancer Institute (Bethesda, MD). Bryostatin 1 was reconstituted from powder by preparing a stock solution in DMSO at 10^{-4} M concentration for short-term storage; this was diluted in Hanks' balanced salt solution (HBSS) to 10^{-6} M working solution immediately prior to use. For i.v. injection, the final concentration was 2 μg/ml and 0.5 ml was injected as a bolus. This dose (1 μg/mouse) was based on previously published data on toxicity, anti-tumor and immunomodulatory effects,[2,15,16] as well as our own preliminary experiments. Recombinant human interleukin-2 (IL-2) was kindly provided by Chiron (Emeryville CA).

Treatment and cell preparation

Mice were treated with bryostatin 1 employing a staggered schedule so that all spleens could be harvested simultaneously for fluorescence activated cell sorting (FACS) and PKC assays. After euthanizing mice, spleens were rapidly removed and weighed. Splenocyte suspensions were prepared by pressing spleens through 100-mesh stainless steel screens with a syringe piston and washing with cold HBSS. Erythrocytes were lysed with Gey's solution, as previously described.[17] Single cell suspensions were placed on ice within 30 min of sacrifice. Nucleated cells were counted using a Coulter Counter (Coulter Electronics, Hialeah, FL) after lysis of erythrocytes.

PKC assays

Activity of the total cellular PKC was determined by an adaptation[18] of the method of Yasuda *et al.*,[19] using materials provided by Gibco/BRL (Gaithesburg, MD). Pelleted cells were homogenized in 0.01 M Tris, 5×10^{-4} M EDTA, 5×10^{-4} M EGTA, pH 7.5, containing 25 μg/ml protease inhibitors (aprotinin, leupeptin) and 0.5% Triton X-100. The homogenate was incubated on ice for 30 min, centrifuged for 2 min at 1200 *g* and maintained on ice pending assay. The homogenates were partially purified by passage over DEAE–cellulose with elution in homogenization buffer containing 0.01 M β-mercaptoethanol and 0.2 M NaCl. Enzyme fractions were normalized for total protein content and added directly to an assay reaction mixture containing mixed micelles of phosphatidylserine and PMA in suspension. The reaction was initiated by addition of 2.5×10^{-5} Ci/ml [γ-^{32}P]ATP, 2×10^{-5} M non-isotopic ATP and 5×10^{-5} M synthetic peptide substrate (acetylated myelin basic protein peptide; $AcMB_{4-14}$). After a 5 min incubation at 30°C, aliquots of the reaction mixture were transferred to nitrocellulose filters and the reaction was terminated by immersion of the discs in cold 1% (V/V) phosphoric acid. The discs were washed thoroughly and radioactivity was quantified by conventional liquid scintillation. Total cellular PKC activity was expressed as counts per minute (c.p.m.)/mg of cellular protein. Values for each condition were obtained from the average of duplicate determinations and control spleens (from untreated mice) were assayed in each experiment. Data were normalized based upon control values for each individual experiment and presented as a percentage of

control activity for all mice at each time point, representing the results of seven separate experiments.

Pharmacokinetic studies

For studies of bryostatin 1 levels, mice were first anesthetized by inhalation of methoxyflurane (Pitman-Moore, Mundelein, IL) and then injected with 1.0 μg of bryostatin 1 i.v. At various times after infusion (30 s to 24 h), citrated blood was obtained from the orbital plexus and centrifuged at 400 *g* for 10 min to obtain plasma. Mice were re-anesthetized as needed to obtain blood samples and each sample was pooled from five mice. Plasma was assayed for levels of bryostatin 1 using a minor modification of a previously described platelet aggregation based assay, based on the aggregation lag-phase of ionophore-stimulated platelets.[20] Platelet aggregation was measured using a Chronolog whole blood lumi-aggregometer (Havertown, PA). Platelet-rich plasma (0.5 ml) was placed in an aggregometer cuvette equipped with a stirring bar. The platelet concentration was set to 30 000/μl by diluting platelet-rich plasma with platelet-poor plasma. Calcium ionophore (Sigma, St Louis, MO), final concentration 0.038 μM, was added to the platelet-rich plasma and allowed to incubate for 1 min at 37°C. Once a stable baseline was obtained, prewarmed test plasma or medium (0.4 ml) was then added to the calcium ionophore-stimulated platelets. Platelet aggregation was monitored electrically as an increase in impedance (ohms). Using this assay, lag phase time in minutes is inversely proportional to bryostatin 1 concentration and is sensitive to bryostatin 1 concentrations as low as 2 nM. A lag phase of 60 min or greater corresponds to the absence of detectable bryostatin 1. Bryostatin 1 levels were extrapolated from a standard curve created by adding known amounts of bryostatin 1 to normal mouse plasma.

FACS analysis of surface markers

For FACS analysis of lymphocyte surface markers, cells were stained with fluorescein-labeled monoclonal antibodies, as previously described,[21] and anlayzed on a Becton-Dickinson FACStar (Mountain View, CA). Control isotype matched antibody, anti-CD69, anti-CD3, anti-CD4, anti-CD8, anti-CD56 and anti-CD25 antibodies were obtained from PharMingen (San Diego, CA).

Statistical methods

Data on PKC levels, spleen weights and cell counts were tested for normality and homogeneity; for statistical analysis of PKC levels, a log transformation, which satisfied the normality assumption, was used. The statistical significance of treatment effects was determined by analysis of variance (ANOVA), and pairwise comparisons between experimental groups and to controls were made utilizing Dunnett's test for unpaired observations.[22]

Results

Immediately (30 s) after i.v. infusion, bryostatin 1 could be detected in plasma at 56.5 nM. Subsequently, bryostatin 1 levels rapidly declined and were undetectable (i.e. below 2 nM) by 60 min (see Figure 1). A possible explanation for the rapid disappearance of bryostatin 1 from plasma is provided by the data shown in Figure 2. In these studies, bryostatin 1 was added to cultures of HL-60 cells (2×10^5 cells/ml) at a concentration of 25 nM. At various intervals, aliquots of the cell suspension were removed, centrifuged and the supernatant

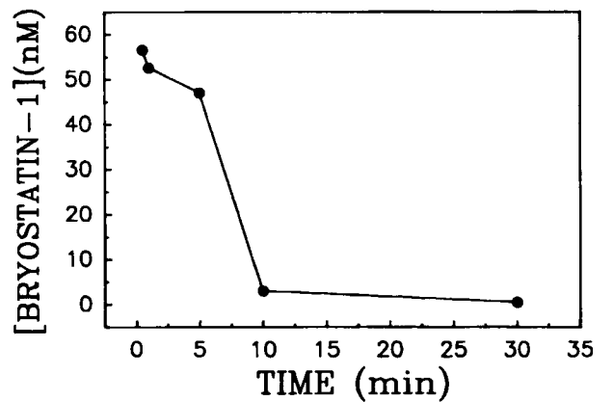

Figure 1. Pharmacokinetics of bryostatin 1 *in vivo*. At various times after a single infusion of bryostatin 1 (1.0 μg/mouse), plasma samples were obtained and assayed for bryostatin 1 as described in Methods. Each data point represents the concentration in a pooled sample of plasma from two mice obtained at the time shown. Samples obtained at 60 min, 120 min and 24 h exhibited lag phases of greater than 60 min (not shown), indicating the absence of detectable bryostatin 1. This experiment was performed twice, with identical results.

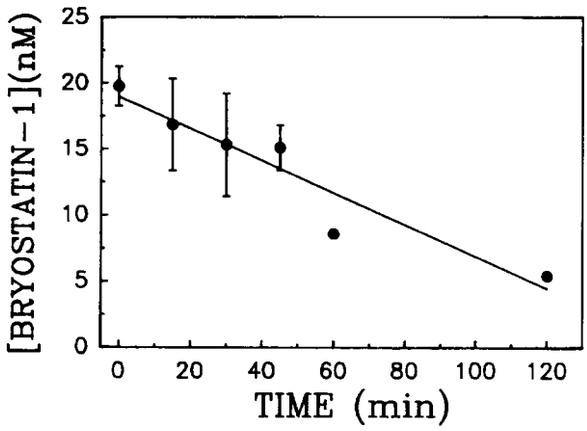

Figure 2. Pharmacokinetics of bryostatin 1 disappearance from culture medium after addition to HL-60 leukemia cells. Bryostatin 1 was added to cultures of HL-60 cells (2×10^5 cells/ml) at an initial concentration of 25 nM. Cells were pelleted and samples of the medium removed at various times as shown and assayed for bryostatin 1. Data shown are combined results from four separate experiments, with duplicate measurements for each sample; thus, each point represents the mean concentration ± SEM of a total of eight determinations. Samples obtained at 240 min exhibited lag phases of greater than 60 min (not shown), indicating the absence of detectable bryostatin 1.

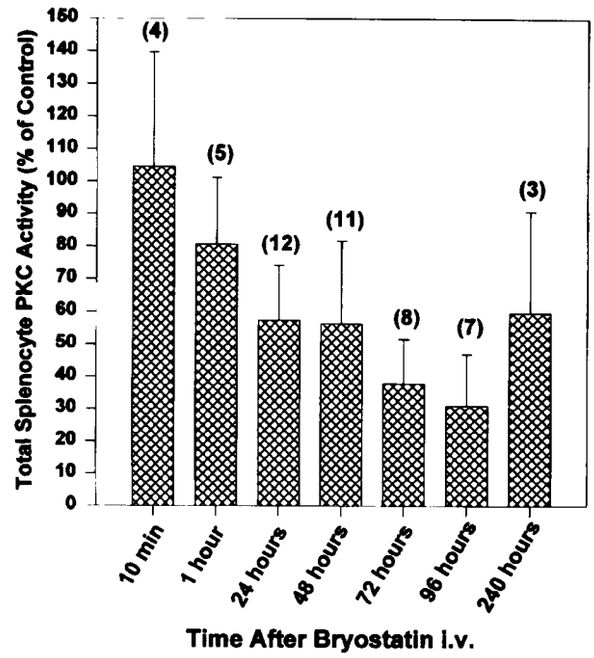

Figure 3. Total cellular PKC activity in splenocytes from mice treated with bryostatin 1. Spleens were harvested from mice at various times after infusion of 1 μg of bryostatin 1 i.v. Results from seven separate experiments were pooled and bars represent the mean percentage relative to controls for the same experiment (± SD). The number of spleens assayed at each time point is shown in parentheses above each bar. Overall, time after treatment effect was significant by ANOVA, with $p < 0.001$. Pairwise comparisons to the control spleens by Dunnett's tests resulted in $p < 0.05$ for 24, 48, 72, 96 and 240 h. Other groups (10 min and 1 h) were not statistically different from controls.

assayed for bryostatin 1 levels. Under these conditions, concentrations of bryostatin 1 in the medium declined to undetectable levels by 4 h. In these studies, levels of bryostatin 1 incubated in medium without cells remained unchanged (data not shown).

As depicted in Figure 3, bryostatin 1 did not modify total splenocyte PKC activity at intervals of 1 h or less following i.v. administration (1.0 μg). However, by 24 h, a statistically significant reduction in total splenocyte PKC activity was noted (i.e. to 57% of control values; $p < 0.05$). Moreover, significant reductions in PKC activity were noted at longer intervals and persisted for at least 96 h after a single i.v. injection of bryostatin 1 (i.e. 56% of control at 48 h, 38% of control at 72 h and 31% at 96 h; $p < 0.05$ in each case). Overall, ANOVA indicated that the effect of time after treatment was significant, with $p < 0.001$. In addition, marked reduction in PKC activity was observed as long as 10 days following bryostatin 1 injection, although these results were based on a limited number of animals.

When splenocytes were monitored for evidence that bryostatin 1 induced T lymphocyte activation (as it does *in vitro*), no significant change was noted in the majority of T cell markers, including CD25 (data not shown). There was, however, a rapid and short-lived increase in the proportion of cells expressing CD69, an early marker of lymphocyte activation (Figure 4). The peak percentage of $CD69^+$ cells (as high as 53%) was observed 3–25 h after bryostatin 1 treatment (in three separate experiments) versus 11% positive in control mice. Beyond 24 h, CD69 expression rapidly declined, eventually returning to control levels. In addition, as shown in Figure 5, bryostatin 1 treatment induced a statistically significant increase in spleen weights 48–96 h after drug treatment ($p < 0.001$ overall; for pairwise comparisons versus controls, $p < 0.05$ at 48, 72 and 96 h). In a separate series of three additional experiments, involving a total of 18 mice per group, control mice and mice treated with bryostatin 1 ± IL-2 (7500 IU i.p. twice a day for six doses) were weighed and sacrificed 3 days after bryostatin 1 infusion. As shown in Figure 6, mean spleen weight for the bryostatin-treated groups was more than 200% that of controls. In contrast, mean total body weight was slightly less for the bryostatin-treated group than for controls (20.0 ± 0.38 versus

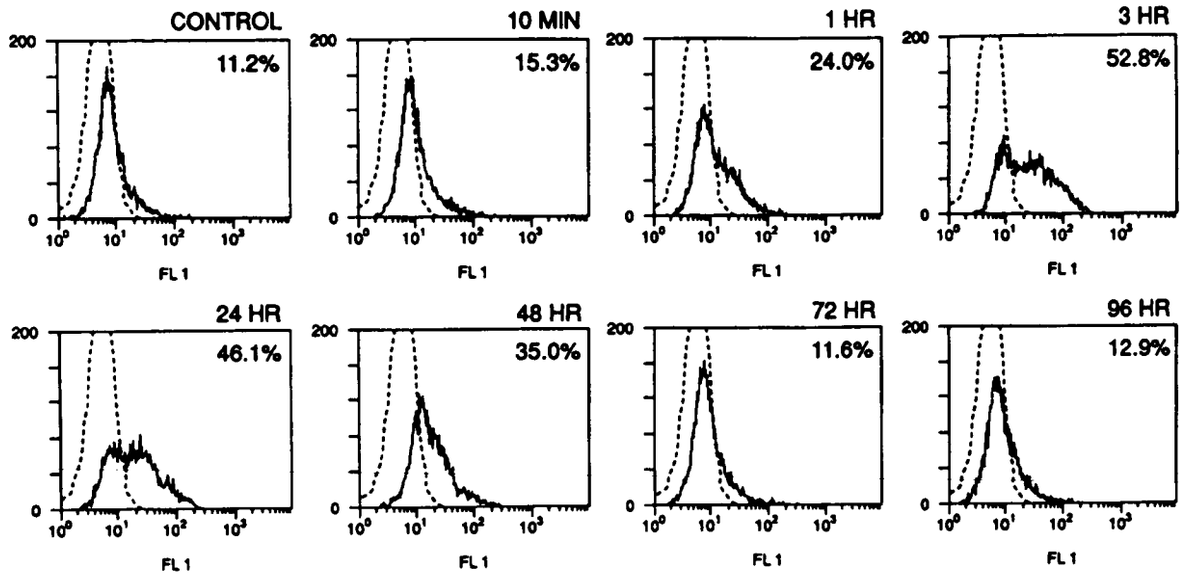

Figure 4. FACS analysis of splenocyte CD69 expression at various intervals after infusion of bryostatin 1. Spleens were harvested at times shown and analyzed as described in the text for expression of CD69 and other cell surface markers. Dotted curves correspond to cells stained with control isotype-matched antibody (10 000 events) and solid lines correspond to anti-CD69 antibody (5000 events). Results shown are representative of three separate experiments.

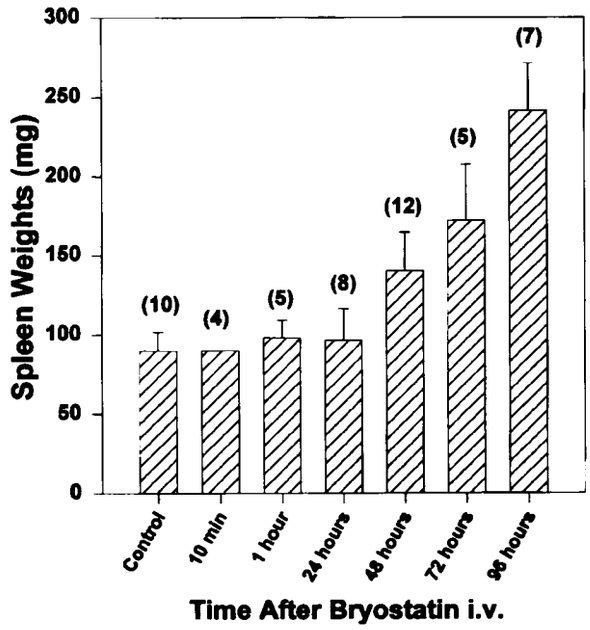

Figure 5. Spleen weights at various times after bryostatin 1 infusion. Spleens were harvested and weighed at times shown after infusion of 1 μg bryostatin 1 i.v. Each bar represents the mean (±SD) of spleen weights from six separate experiments. The number of mice in each group is shown in parentheses above the bar. By ANOVA, overall significance for treatment effect was $p < 0.001$. For pairwise comparisons using Dunnett's test, $p < 0.05$ for 48, 72 and 96 h versus controls. Spleens weighed at earlier time points were not significantly different from controls.

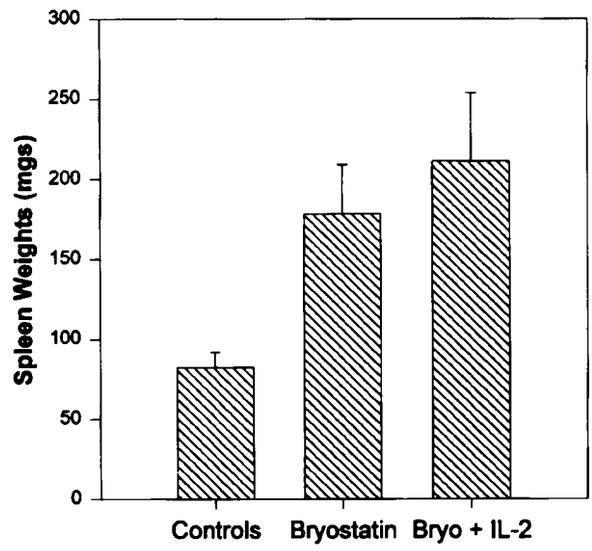

Figure 6. Spleen weights combined from three separate experiments in control mice, 3 days after iv. infusion of bryostatin 1 (1 μg) or after bryostatin 1 + IL-2 (7500 IU b.i.d. for 3 days). Each bar represent the mean for 18 mice (±SD). Both experimental groups were significalty different from the controls ($p < 0.05$) and spleen weights for bryostatin + IL-2 were significantly greater than for bryostatin 1 alone, analyzed by ANOVA.

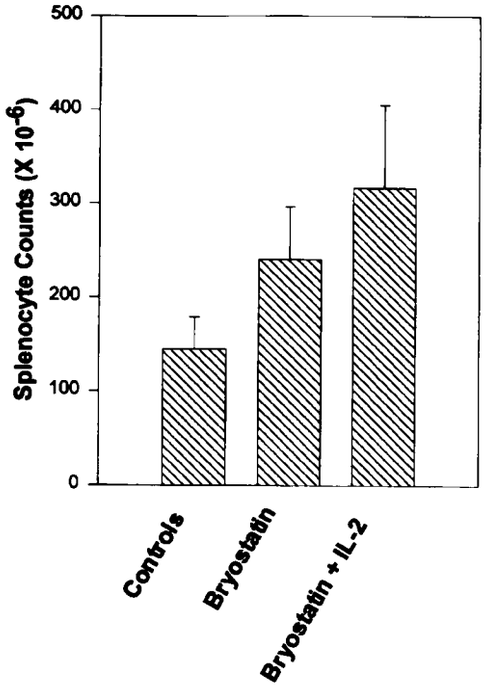

Figure 7. Nucleated spleen cell counts combined from three separate experiments, in control mice, 3 days after i.v. infusion of bryostain 1 (1 μg) or after bryostain 1 + IL-2 (7500 IU b.i.d. for 3 days). Each bar represents the mean for 18 mice (± SD). Both experimental groups were significantly different from the controls ($p < 0.05$) and cell numbers for bryostain + IL-2 were significantly greater than for bryostatin 1 alone, analyzed by ANOVA.

22.1 ± 0.25 g, $p < 0.001$). The increase in spleen weights was paralleled by a marked increase in nucleated cells per spleen (Figure 7). Addition of IL-2 to the bryostatin 1 treatment resulted in modest further increases in both spleen cell counts and spleen weights, which were also statistically significant compared to bryostatin 1 alone or controls.

Discussion

The present data clearly indicate that a single i.v. bolus dose of bryostatin 1 exerts significant and sustained *in vivo* effects on murine splenocytes. Previous studies have either failed to demonstrate an effect of bryostatin 1 on normal or neoplastic tissues in intact animals or have not addressed this issue directly. For example, Schucter *et al.* reported that administration of bryostatin 1 reduced the number of pulmonary metastases in C57Bl/6 mice bearing the B16 melanoma, but were not able to document increased cell-mediated cytotoxicity of normal splenocytes exposed to this agent *in vitro*.[2] The results of other *in vivo* studies indicated that bryostatin 1 exerted anti-tumor activity against MO576 reticulum cell sarcoma, L10A B cell lymphoma, B16 melanoma and Waldenstrom's macroglobulinemia, and reduced resistance of mice to *Salmonella typhimurium*[16,23,24] However, in these studies the effects of bryostatin 1 on normal or tumor cell PKC activity was not examined. In a recent investigation, this issue was addressed through the performance of short-term (i.e. 1 h) assays of cytosolic and membrane PKC activity in several normal tissues (e.g. brain, liver, spleen) and in L10A lymphoma cells obtained from C57Bl/6 mice receiving an identical dose of bryostatin 1, and these did not reveal measurable changes.[15] In view of a precipitious decline in bryostatin 1 plasma levels, it was postulated that rapid clearance/degradation of bryostatin 1 accounted for its putative lack of *in vivo* activity. An early reduction in neutrophil cytosolic PKC activity was noted in that study, presumably reflecting enzyme translocation from cytosol to membrane. However, this decline should be distinguished from the decrease in *total* cellular activity described in the present communication. The latter phenomenon is analogous to that described in cells chronically exposed to certain PKC activators *in vitro* and presumably results from enzyme degradation.[25] More recently, in a phase 1 clinical trial of bryostatin 1 infused i.v. over 24 h, PKC activity in human peripheral blood showed neither a consistent pattern of PKC activation nor down-regulation during the time of treatment, but again, no data for later times were available.[26] Our findings (Figure 1), utilizing a bryostatin 1 bioassay which is over a log more sensitive than previously published methods,[15] confirm the rapid decline in plasma levels. However, the results of the *in vitro* studies shown in Figure 2 raise the possibility that mammalian cells may rapidly bind and/or incorporate bryostatin 1. Thus, the rapid disappearance of bryostatin 1 from plasma may reflect extensive uptake by circulating peripheral cells or tissues, rather than systemic degradation of the compound. Definitive resolution of this question awaits the availability of radiolabeled bryostatin 1 suitable for pharmacokinetic studies.

Relatively little information is currently available concerning the *in vivo* immunomodulatory activity of bryostatin 1, either in humans or in animals. Philip *et al.* have shown that plasma levels of interleukin-6 and tumor necrosis factor (TNF) rise in patients receiving bryostatin 1 by i.v. infusion.[8] More recently, Scheid *et al.* demonstrated that *in vitro* or *in vivo* exposure to bryostatin 1 increased

interleukin-2 (IL-2)-induced proliferation in human peripheral blood mononuclear cells (PBMC).[9] Although CD25 expression was increased by *in vitro* exposure of human PBMC to bryostatin 1 + IL-2, no significant changes in PBMC lymphocyte markers, including CD25, were noted after *in vivo* treatment with bryostatin 1. The absence of any change in T cell subsets or CD25 expression *in vivo* is consistent with our findings in murine splenocytes, but Scheid *et al.* did not examine CD69 expression in their patients' cells nor did they monitor PKC activity in PBMC from patients receiving bryostatin 1. The results described herein indicate that, while bryostatin 1 does in fact disappear rapidly from plasma after infusion, it exerts a profound *in vivo* effect on normal murine splenocytes which can ony be detected considerably after the initial administration. The immunomodulatory actions of bryostatin 1 on T cell activation and proliferation have been temporally related to a rapid translocation of PKC to the cell membrane.[27,28] Moreover, the combination of bryostatin 1 and AS101 has been shown to induce splenocyte proliferation *in vivo* following irradiation,[10] indirectly suggesting a role for PKC activation in this setting. However, for technical reasons, it may not be feasible to harvest and prepare splenocytes rapidly enough to assess very early activation events, thereby explaining the failure to detect short-lived increases in membrane-associated PKC activity. Based upon what is known about the early events leading to lymphocyte activation,[27,28] the increased expression of CD69 at 3–24 h presumably results from early and transient activation and translocation of PKC, which would no longer be detectable by 10 min.

The protracted time course of the PKC inhibitory effect here was unanticipated and could be related to the pharmacokinetics of bryostatin 1 distribution or to prolonged binding of this compound to its intracellular target (i.e. PKC). With regard to the latter possibility, the results of the platelet-aggregation-based bryostatin 1 assay indicate that incubation of HL-60 cells with bryostatin 1 results in rapid and prolonged disappearance of this agent from the medium. A similar phenonomen might account for the rapid decline in plasma bryostatin 1 levels *in vivo*. Alternatively, the sustained *in vivo* effect of bryostatin 1 might stem from its action on peripheral blood cells which subsequently migrate to the spleen, or to elaboration of inflammatory cytokines (e.g. TNF or IL-6) by accessory cells, as previously reported.[8] A recent study in patients receiving bryostatin 1 by continuous infusion suggests that bryostatin 1 may in fact induce sequestrian of peripheral blood cells.[26] It is noteworthy that in the initial patients treated with 25 $\mu g/m^2$ bryostatin 1 as part of an ongoing phase Ib trial at our institution, prolonged down-regulation of total PKC activity has also been noted (unpublished observations), suggesting that this phenomenon is not species-specific.

Finally, whereas much attention has focused on the ability of bryostatin 1 to activate PKC, it is conceivable that down-regulation of PKC, or perhaps of specific PKC isoforms, might be responsible for at least some of this compound's actions. For example, bryostatin 1 has been shown to be highly effective in down-regulating PKCα in several systems.[5,28] Consequently, the long-term of effects of bryostatin 1 could stem from alterations in the pattern of expression of individual PKC isoforms, of which at least nine exist.[29] In this regard, it is noteworthy that inhibition down-regulation of PKC activity has been associated with potentiation of ara-C-induced apoptosis in human leukemia cells *in vitro*.[18] The present findings raise the possibility that a similar phenomenon might occur in the *in vivo* setting.

Conclusion

The purpose of this study was to characterize the pharmacokinetics and pharmacodynamics of bryostatin 1 after a single i.v. injection in mice. The present findings indicate for the first time that despite rapid disappearance from plasma after i.v. infusion, a single dose of bryostatin 1 caused rapid activation of splenic lymphocytes (53% $CD69^+$ at 3–24 h) and marked splenocyte proliferation. The expansion of spleen cell numbers and organ size (2- to 3-fold) was further increased by addition of modest doses of IL-2. Bryostatin 1 also induced late and sustained down-regulation of PKC activity *in vivo* (as much as 69% less than controls). Collectively, these observations indicate that bryostatin 1 exerts profound and sustained *in vivo* effects on normal murine lymphoid tissues, findings which may have significant implications for the clinical use of this compound as an anti-neoplastic and immunomodulatory agent. Studies designed to evaluate further the mechanisms by which bryostatin 1 exerts *in vivo* effects and to characterize additional immunologic and hematological consequences of *in vivo* bryostatin 1 administration are currently underway.

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