Activation and Growth of Murine Tumor-specific T-Cells Which Have in Vivo Activity with Bryostatin 1

Todd M. Tuttle, Thomas H. Inge, Kevin P. Bethke, Carl W. McCrady, George R. Pettit, and Harry D. Bear

ABSTRACT

We examined the ability of bryostatin 1 (Bryo), a novel protein kinase C activator, plus ionomycin (Io), a calcium ionophore, to activate T-cells with specific antitumor activity. Lymphocytes from the draining lymph nodes (DLN) of MCA-105 tumor-bearing host mice were stimulated with Bryo/Io, either fresh or after in vitro stimulation with autologous tumor, and then were incubated in interleukin-2 at 20 units/ml. Lymphocytes sensitized with tumor cells in vitro and then stimulated with Bryo/Io exhibited significant expansion (12-fold) after a total of 3 weeks in culture and moderate cytolytic activity (40% at an effector:tumor cell ratio of 80:1) and were exclusively CD8* T-cells. DLN cells activated immediately with Bryo/Io, without tumor antigen sensitization in vitro, displayed marked growth (130-fold expansion) over 3 weeks in culture, had weak cytolytic activity (8% at an effector:tumor cell ratio of 80:1), and were a mixed population of CD8* and CD4* cells. Despite the differences in phenotypes and in cytotoxicity, both groups of DLN cells were highly effective in vivo against MCA-105 pulmonary metastases. Bryo/Io-activated DLN cells from MCA-105 tumor-bearing hosts had no therapeutic efficacy against B16 melanoma or MCA-203 sarcoma metastases. Lymph node cells from normal mice and non-draining lymph node cells from tumor-bearing hosts could be expanded with Bryo/Io to a degree similar to that of DLN cells but had no antitumor activity. Phenotypic analyses and in vitro and in vivo depletion studies demonstrate that CD8* cells mediated tumor regression.

INTRODUCTION

Effective adoptive immunotherapy in humans will require large numbers of activated T-cells from cancer patients' own lymphoid cells. In contrast to LAK cells, T-cells require lower, less toxic doses of interleukin-2 in vivo. The activation and growth of tumor-reactive T-cells require periodic stimulation of the T-cell receptor with autologous tumor cells (1, 2). In the clinical setting, however, autologous tumor cells are not always available for such periodic T-cell stimulation. Monoclonal antibodies to T-cell surface structures such as CD3 or pharmacological activation of T-cell signal transduction pathways offer alternative strategies for activating T-cells for growth. The interaction between specific antigen and the T-cell receptor stimulates phospholipase C, which catalyzes the hydrolysis of phosphatidylinositol biphosphate to inositol triphosphate and diacylglycerol (3-5). Inositol triphosphate releases calcium from intracellular stores, thereby increasing intracellular calcium concentration. Diacylglycerol activates the enzyme PKC, which catalyzes the phosphorylation of numerous protein substrates. These two second messengers trigger two parallel pathways that act in concert to stimulate T-cell activation and proliferation.

Phorbol esters and the calcium ionophores mimic the intracellular signals mediated by diacylglycerol and inositol triphosphate, respectively (6). The combination of a PKC activator and a calcium ionophore stimulates each arm of this bifurcated pathway and may activate T-cells without repeated antigen stimulation. The clinical usefulness of the phorbol esters, however, is limited by their known tumor-promoting activity.

Bryostatin 1, a macrocyclic lactone isolated from the marine invertebrate Bugula neritina, can also activate PKC in vitro (7). Bryostatin 1 has many immunomodulating properties which include stimulating production of IL-2 and IFN-γ in human peripheral blood lymphocytes (8), induction of IL-2 receptor on human T-lymphocytes (9), and inhibition of natural killer and LAK cells (10). Moreover, unlike the phorbol esters, bryostatin 1 has no tumor-promoting activity and has potent anti-neoplastic properties (10, 11).

In this study, we examine the in vitro immunomodulating properties of bryostatin 1 on TBH lymphocytes and the ability of lymphocytes activated by bryostatin 1 to mediate in vivo tumor regression. The results indicate that bryostatin 1 plus a calcium ionophore can substitute for specific antigen and lead to the generation of large numbers of T-cells with specific antitumor activity.

MATERIALS AND METHODS

Mice. Virus-free female C57Bl/6 mice, 8–12 weeks old, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The animals were caged in groups of five or fewer, fed laboratory chow, and given water ad libitum.

Tumors. The MCA-105 and 203 sarcomas are methylcholanthrene-induced tumors of C57Bl/6 origin and were kindly provided by Dr. Michael Lotze (National Cancer Institute, Bethesda, MD) and Dr. Alfred Chang (Ann Arbor, MI), respectively. After thawing from storage, tumor was transplanted by s.c. inoculation in syngeneic mice. Single-cell suspensions were prepared from solid tumors by mincing into small fragments followed by digestion with constant stirring in 40 ml of RPMI 1640 containing 4 mg of deoxyribonuclease, 40 mg of collagenase, and 100 units of hyaluronidase (Sigma Chemical Co., St. Louis, MO) for 3 h at room temperature. For establishing solid tumors, C57Bl/6 mice were inoculated in one hind footpad with 5 x 10^6 MCA-105 or 203 sarcoma cells in 0.05 ml HBSS (Biofluids, Rockville, MD). The B16 murine melanoma was obtained from Dr. Kimber White (The Medical College of Virginia) and grown in CM as an adherent monoculture. The B16 cells were composed of RPMI 1640 with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM l-glutamine, 100 units/ml peni-
cillin, 100 μg/ml streptomycin, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and 5 × 10⁻³ M 2-mercaptoethanol (Sigma).

Lymphoid Cell Suspensions. Ipsilateral popliteal lymph nodes draining the footpad (draining lymph nodes) were harvested 10 days after tumor injection. Single cell suspensions were prepared by pressing the nodes through a 100-mesh screen with the blunt end of a 3-cm plastic syringe plunger in HBSS under sterile conditions. Cells from the axillary lymph nodes and spleens of normal mice and the mesenteric lymph nodes of tumor-bearing mice were similarly prepared. The cell suspensions were filtered through no. 100 nylon mesh (Nytex; TETKO, Elmsford, NY), washed twice, and suspended in CM before culture.

Reagents. Bryostatin 1 was prepared from B. neritina as previously described (12). The calcium ionophore ionomycin was purchased from Calbiochem (San Diego, CA). Bryostatin 1 and ionomycin were kept as 10⁻² M stock solutions in dimethyl sulfoxide at -20°C. Human rIL-2 was kindly supplied by Hoffmann-La Roche (Nutley, NJ).

Stimulation of Lymphoid Cells. Fresh lymphoid cells were stimulated in one of the following regimen.

(a) In each well of a 24-well plate, 5 × 10⁵ lymphocytes were cultured with 1 × 10⁶ irradiated (2000 rads) MCA-105 tumor cells and 20 units/ml rIL-2 in CM. Lymphocytes were fed with fresh medium and rIL-2 every 3 days, restimulated with autologous tumor cells every 7 days, and cultured for a total of 3 weeks (DLN + AT). Essentially, this is the classical in vitro sensitization protocol described by Shu and Chang (1).

(b) Lymphocytes were stimulated with MCA-105 autologous tumor cells, and 20 units/ml rIL-2 for 7 days, then treated with Bryo/Io (see below), and cultured for an additional 2 weeks in CM with 20 units/ml rIL-2 (DLN + AT + B/I).

(c) Fresh lymphocytes were stimulated with Bryo/Io, without prior in vitro autologous tumor sensitization, and cultured in CM with 20 units/ml rIL-2. After 14 days in culture, the cells were restimulated with Bryo/Io and cultured for an additional 7 days (DLN + B/I).

Bryo/Io Treatment. Lymphoid cells at 1 × 10⁶ cells/ml were incubated with 5 nM Bryo, 1 mM Io, and 20 units/ml IL-2 for 7 days, then treated with Bryo/Io (see below), and cultured for an additional 2 weeks in CM with 20 units/ml rIL-2 (DLN + AT + B/I).

Statistics. For cytotoxicity assays, SE were always less than 10% and are not shown. Significance of treatment effects in vivo was assessed with the Wilcoxon rank-sum test (15).

RESULTS

Bryo/Io Effectively Activate Lymphocytes for Expansion. DLN cells stimulated with autologous tumor cells alone, autologous tumor cells followed by Bryo/Io, or Bryo/Io alone were counted after 3 weeks in culture. Fig. 1 shows the means and SD of cumulative expansion over 3 weeks for three separate experiments. DLN cells stimulated every 7 days with MCA-105 tumor cells expanded an average of 15-fold. Lymphocytes stimulated with autologous tumor cells only on day 0 and then treated with Bryo/Io grew 12-fold over the same time period.

![Fig. 1. Cumulative 3-week expansion of DLN cells. Lymphocytes obtained from the popliteal DLNs ipsilateral to a progressively growing footpad tumor were stimulated in three different regimens: DLN + AT, DLN + AT + B/I, and DLN + B/I. After 3 weeks in culture with 20 units/ml IL-2, the lymphocytes were counted, and the fold expansion was determined. Columns, pooled means for three separate experiments; bars, SD.](https://cancerres.aacrjournals.org)
Expansion of Antitumor T-Cells Using Bryostatin

Figure 2: Fluorescence-activated cell sorter analysis of antigen- and bryostatin-expanded cells from MCA-105 DLNs. Ordinate, relative numbers of cells; 10,000 cells were analyzed in each frame.

Figure 3. In vitro cytotoxicity of expanded cells. After 3 weeks in culture, a 4-h 

**Cr release assay was performed against MCA-105 target cells. Results are representative of 3 separate experiments. Treatment groups are defined in "Materials and Methods."**

In Vitro Cytotoxicity of Cultured Cells. The DLN cells used for cytotoxicity assays were stimulated in the same manner as the cells shown in Fig. 1. DLN cells serially stimulated with autologous tumor cells demonstrated significant cytotoxicity against MCA-105 targets (Fig. 3), and DLN cells initially cultured with autologous tumor and then restimulated with Bryo/Io had slightly less cytolytic activity. However, fresh DLN cells activated with Bryo/Io, without prior autologous tumor sensitization in vitro, displayed only low levels of killing. Freshly harvested DLN cells were not cytolytic (6% lysis at an effector:target cell ratio of 80:1) before expansion, a finding consistent with the results previously reported by others (16). Similar results were obtained in two separate experiments. Cells from the lymph nodes of normal mice could be expanded with Bryo/Io in a similar fashion but had no cytotoxicity against MCA-105 targets (Fig. 3).

Therapeutic Efficacy of Bryostatin-expanded Lymphocytes. In an i.v. lung metastasis model, all untreated mice had more than 250 MCA-105 pulmonary nodules (Table 1). In contrast, there were no lung metastases in mice that received lymphocytes obtained from DLNs and were stimulated with irradiated MCA-105 tumor cells every 7 days. Similarly, lymphocytes sensitized to autologous tumor cells in vitro and then expanded with Bryo/Io completely eradicated experimental metastases. Furthermore, DLN cells activated directly with Bryo/Io, without antigen sensitization in vitro, also eliminated metastases, despite their limited in vitro cytotoxic activity. This finding with Bryo/Io-stimulated DLN cells was reproduced in four separate experiments. Although lymph node cells from normal mice and non-draining lymph node cells from tumor-bearing mice could be expanded just as well as tumor-draining lymph node cells using Bryo/Io, they did not reduce pulmonary metastases.

Mice treated with IL-2 alone, without the adoptive transfer of cultured cells, had 250 lung metastases (Table 2). Freshly harvested DLN cells given along with IL-2 had no in vivo antitumor activity. However, DLN cells acquired therapeutic activity after in vitro stimulation with bryostatin 1/ionomycin and culture in IL-2.

Specificity of in Vivo Antitumor Activity. While lymphocytes obtained from MCA-105 TBH reduced MCA-105 pulmonary metastases after expansion with Bryo/Io, these same cells had no effect on B16 melanoma or MCA-203 metastases (Table 3).

<table>
<thead>
<tr>
<th>Table 1 Therapeutic efficacy of MCA-105 DLN cells</th>
</tr>
</thead>
</table>

C57Bl/6 mice were given injections of 5 x 10⁵ MCA-105 tumor cells in the tail vein. Three days later, the mice received i.v. injections of 5 x 10⁶ cultured cells. Fourteen days after tumor injection, mice were sacrificed, and pulmonary nodules were counted. Lungs with metastatic foci too numerous to count were assigned an arbitrary value of 250.

<table>
<thead>
<tr>
<th>Cells transferred</th>
<th>IL-2</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>−</td>
<td>250+ (0)</td>
<td>250+ (0)</td>
</tr>
<tr>
<td>DLN + AT</td>
<td>+</td>
<td>0 (0)</td>
<td>ND</td>
</tr>
<tr>
<td>DLN + AT + B/I</td>
<td>+</td>
<td>0 (0)</td>
<td>ND</td>
</tr>
<tr>
<td>DLN + B/I</td>
<td>+</td>
<td>0 (0)</td>
<td>10 (10)</td>
</tr>
<tr>
<td>NLN + B/I</td>
<td>+</td>
<td>250+ (0)</td>
<td>ND</td>
</tr>
<tr>
<td>TBH Mes LN + B/I</td>
<td>+</td>
<td>ND</td>
<td>250+ (0)</td>
</tr>
</tbody>
</table>

* DLN + AT, DLN cells from MCA-105-bearing mice stimulated weekly with autologous tumor; DLN + AT + B/I, DLN cells stimulated with autologous tumor for 7 days, then expanded with Bryo/Io; DLN + B/I, DLN cells expanded with Bryo/Io alone. TBH Mes LN + B/I, mesenteric lymph nodes from mice with 10-day footpad tumors expanded with Bryo/Io; NLN + B/I, lymphocytes from non-tumor-bearing mice expanded with Bryo/Io.

† IL-2 given as 7500 units i.p. twice a day for 3 days on day of adoptive transfer.

‡ Mean for at least 5 animals/group. Parentheses, SD.

§ Significantly different from untreated groups (P < 0.05, two-sided values; Wilcoxon rank-sum test).

ND, not determined.
Table 2 Draining lymph nodes contain pre-effector cells which possess potent in vivo antitumor activity after stimulation in vitro

<table>
<thead>
<tr>
<th>Cells transferreda</th>
<th>IL-2b</th>
<th>MCA-105 pulmonary metastasesc</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>250+</td>
<td>(0)</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>250+ (0)</td>
</tr>
<tr>
<td>Fresh DLN</td>
<td>250+</td>
<td>(0)</td>
</tr>
<tr>
<td>DLN + B/I</td>
<td>0</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

- Fresh DLN, freshly harvested DLN cells from day 10 MCA-105 tumor-bearing mice; DLN + B/I, DLN cells expanded with Bryo/Io alone.
- IL-2 given as 7500 units i.p. twice a day for 3 days starting on day of adoptive transfer.
- Mean for at least 5 animals/group. Parentheses, SD.

Table 3 Specificity of in vivo antitumor activity of bryostatin 1-expanded DLN cells

C57Bl/6 mice were given injections of MCA-105, MCA-203, or B16 tumor cells in the tail vein. Three days later, the mice received i.v. injections of 5 x 10^6 cultured cells. Fourteen days after tumor injection, the mice were sacrificed and pulmonary nodules were counted. Lungs with metastatic foci too numerous to count were assigned an arbitrary value of 250.

<table>
<thead>
<tr>
<th>Cells transferreda</th>
<th>IL-2</th>
<th>Pulmonary metastasesb,c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>250+ (0)</td>
</tr>
<tr>
<td>MCA-105 DLN + B/I</td>
<td>250+</td>
<td>(0)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>250+ (0)</td>
</tr>
<tr>
<td>MCA-105 DLN + B/I</td>
<td>250+</td>
<td>(0)</td>
</tr>
</tbody>
</table>

- DLN + B/I, DLN cells from MCA-105- or MCA-203-bearing mice expanded with Bryo/Io alone. IL-2 was given as 7500 units i.p. twice a day for 3 days, starting on the day of adoptive transfer.
- Mice were given injections of 5 x 10^6 MCA-105 or MCA-203 tumor cells or 4 x 10^5 B16 tumor cells in the tail vein.
- Means and SD (parentheses) for at least 5 animals/group.
- Significantly different from untreated groups (P < 0.05, two-sided values; Wilcoxon rank-sum test).

Table 4 Effect of in vitro depletion of CD4+ and CD8+ cells with monoclonal antibodies and complement prior to adoptive immunotherapy

C57Bl/6 mice were given injections of 2 x 10^6 MCA tumor cells in the tail vein. Three days later, the mice received i.v. injections of 5 x 10^5 cultured cells treated with complement alone or monoclonal antibody and complement. Fourteen days after tumor injection, the mice were sacrificed and pulmonary nodules were counted. Lungs with metastatic foci too numerous to count were assigned an arbitrary value of 250.

<table>
<thead>
<tr>
<th>Cells transferreda</th>
<th>In vitro treatmentb</th>
<th>MCA-105 metastasesc</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>144 (44)</td>
</tr>
<tr>
<td>DLN + B/I, C'</td>
<td></td>
<td>0 (0)</td>
</tr>
<tr>
<td>DLN + B/I a-CD4 + C'</td>
<td></td>
<td>1 (1)</td>
</tr>
<tr>
<td>DLN + B/I a-CD8 + C'</td>
<td></td>
<td>62 (20)</td>
</tr>
</tbody>
</table>

- DLN + B/I, DLN cells from MCA-105-bearing mice expanded with Bryo/Io alone. IL-2 was given as 7500 units i.p. twice a day for 3 days starting on day of adoptive transfer.
- Depletion of CD4 and CD8 subsets resulted in 69% and 30% yield of cells, respectively, compared to C' alone.
- Means and SD (parentheses) of at least 5 animals/group.
- Significantly different from untreated group (P < 0.05, two-sided values; Wilcoxon rank-sum test).
- Significantly different from C' alone group (P < 0.05, two-sided values; Wilcoxon rank-sum test).

Discussion

Although the activation and growth of antigen-specific T-cells require intermittent stimulation with a specific antigen, autologous tumor cells are not always available from human cancer patients. Moreover, even when they are present, cancer cells may not be good immunogens in vitro, either because they bear weak or sparse antigenic determinants or because they release immunosuppressive factors (17–19). Therefore, alternative methods of activating T-cells in vitro may broaden the applicability of adoptive immunotherapy. Several investigators have reported that monoclonal antibody against the TcR/CD3 complex can be used to activate and expand either murine or human T-cells with antitumor activity (20–24). Pharmacological manipulation of the T-cell activation pathways offers another option which may further overcome T-cell unresponsiveness. However, it is probably necessary to use a source of lymphocytes with a high frequency of antitumor clones if either anti-receptor antibodies or pharmacological agents are to be used. Tumor-infiltrating lymphocytes or draining lymph nodes represent potential sources of such cells (24–29).

A novel aspect of the studies reported here is the utilization of a short-term pulse of bryostatin 1 and ionomycin. We have previously shown that the short-term manipulation of PKC and cytosolic calcium levels is sufficient for the proliferation of T-cells (30). This brief exposure to bryostatin 1 avoids the down-regulatory effects of chronic PKC activation as well as the toxicity observed with long-term exposure to calcium ionophores such as ionomycin.

The results of the present studies demonstrate that bryostatin 1 combined with ionomycin and low-dose IL-2 can activate and expand T-lymphocytes with antitumor activity. DLN cells sensitized in vitro with tumor antigen and then expanded with Bryo/Io displayed significant growth in the absence of repeated experiments 1 and 2, respectively). In experiment 2, the reciprocal specificity of Bryo/Io-expanded cells from MCA-203 DLNs also was shown; these cells had potent antitumor activity against MCA-203 metastases but no effect on MCA-105 experimental metastases (Table 3, experiment 2).

Phenotype of Effector Cells. Results of the in vitro and in vivo depletion experiments are shown in Tables 4 and 5, respectively. Although CD8-depleted cells had some in vivo effect, these lymphocytes no longer completely eliminated experimental metastases, and in one experiment (Table 5, experiment 2) elimination of CD8+ cells completely abrogated the therapeutic effect. In all experiments, the number of metastases in animals in which CD8+ effector cells were depleted was significantly greater than for the positive control groups (C' alone in vitro or HBSS in vivo). Conversely, Bryo/Io-activated DLN cells consistently and significantly reduced pulmonary metastases when CD4+ cells were depleted.

Table 5 Effect of in vivo depletion of CD4+ and CD8+ cells with in vivo mAb treatment

C57Bl/6 mice were given injections of 2 x 10^6 or 4 x 10^6 MCA tumor cells (experiments 1 and 2, respectively) in the tail vein. Three days later, the mice received i.v. injections of 5 x 10^5 cultured cells. Within 1 h of adoptive transfer, mice were injected i.v. with 0.2 ml mAb ascites diluted to 1.0 ml in HBSS. Fourteen days after tumor injection, the mice were sacrificed and pulmonary nodules were counted. Lungs with metastatic foci too numerous to count were assigned an arbitrary value of 250.

<table>
<thead>
<tr>
<th>Cells transferreda</th>
<th>In vivo mAb</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>144 (44)</td>
<td>250+ (0)</td>
</tr>
<tr>
<td>DLN + B/I, HBSS</td>
<td></td>
<td>0 (0)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>DLN + B/I a-CD4</td>
<td></td>
<td>3 (2)</td>
<td>67 (23)</td>
</tr>
<tr>
<td>DLN + B/I a-CD8</td>
<td></td>
<td>49 (13)</td>
<td>250+ (0)</td>
</tr>
</tbody>
</table>

- DLN + B/I, DLN cells from MCA-105-bearing mice expanded with Bryo/Io alone. IL-2 was given as 7500 units i.p. twice a day for 3 days starting on day of adoptive transfer.
- Means and SD of at least 5 mice/group.
- Significantly different from untreated group (P < 0.05, two-sided values; Wilcoxon rank-sum test).
- Significantly different from HBSS alone group (P < 0.05, two-sided values; Wilcoxon rank-sum test).
stimulation with autologous tumor. These cells were all CD8+ T-cells and were capable of eradicating MCA-105 pulmonary metastases. In contrast, DLN cells stimulated with autologous tumor on the day of harvest from the animal and cultured in IL-2 alone, without periodic restimulation with either antigen or Bryo/Io, did not grow in culture.

Fresh lymphocytes from TBH activated with Bryo/Io without in vitro tumor stimulation demonstrated more than 8-fold more growth than bryostatin 1-expanded antigen-sensitized DLN cells or cells stimulated only with tumor cells. In addition, unlike lymphocytes exposed to tumor antigen in vitro, DLN cells stimulated with Bryo/Io contained a mixed population of CD8+ and CD4+ cells. It is not clear at this time why tumor-stimulated DLN cells don't grow as well as fresh DLN cells after bryostatin 1 stimulation. One explanation may be that during the 7-day culture with tumor cells, "accessory cells" important for T-cell growth after Bryo/Io stimulation are lost. Moreover, the phenotypic analysis indicates that immediate activation with Bryo/Io expands CD4+ helper cells, which may provide additional lymphokines which increase T-cell proliferation. Alternatively, stimulation with tumor cells, but not Bryo/Io, may activate suppressor cells, which may limit the subsequent in vitro growth of T-cells. Our initial prediction was that pharmacological activation of fresh T-cells, regardless of the source, would induce the overgrowth of irrelevant clones of T-cells and therefore would be less effective than using such agents after in vitro selection of tumor-specific cells by stimulation with tumor cells. Indeed, the cytotoxicity data reported here supported that hypothesis; DLN cells activated immediately with Bryo/Io had minimal in vitro cytotoxicity against autologous tumor cells compared to either AT-stimulated cells or AT-stimulated cells expanded with Bryo/Io. Nevertheless, without prior sensitization to MCA-105 tumor cells in vitro, Bryo/Io-activated DLN cells induced the regression of MCA-105 pulmonary metastases but had no antitumor effect on B16 melanoma or MCA-203 metastases. These data suggest that in vivo sensitization of draining lymph node T-cells may provide an enriched population of tumor-specific pre-effector cells that can be expanded in vitro without the necessity for further antigen stimulation. We and others have shown that lymph nodes draining the site of tumor growth are a better source of tumor-sensitized T-cells for adoptive immunotherapy than non-draining nodes or splenocytes (28, 29, 31). Moreover, we have recently shown that the same method of T-cell expansion also yielded T-cell which were effective in vivo in a different tumor model (13).

The finding that lymphocytes obtained from TBH after activation with bryostatin 1 had potent in vivo antitumor activity, despite minimal in vitro cytotoxicity, is similar to the results recently reported with anti-CD3 mAb as a primary in vitro stimulus for TBH T-cells (24) and suggests that the mechanism of tumor regression may not be simply direct cytolysis by adoptively transferred cells. Phenotypic analysis of bryostatin-expanded lymphocytes demonstrates that TCR/CD3 expression was not reduced; therefore, lack of cytolytic activity was probably not merely a result of bryostatin-induced down-regulation of cell surface receptors (data not shown). Other investigators have recently reported that noncytolytic CD8+ T-cells from antigen-stimulated murine tumor-infiltrating lymphocyte cultures were effective in vivo (32). The in vivo efficacy of those tumor-infiltrating lymphocytes was found to correlate with the secretion of IFN-γ and/or tumor necrosis factor, and anti-IFN-γ mAb inhibited the in vivo effect. Others have demonstrated that bryostatin 1 induces IFN-γ and IL-2 production in human lymphocytes (8). Further studies in progress will determine whether tumor-specific CD8+ cells activated with bryostatin 1 mediate tumor regression through lymphokine production and/or recruitment of host effector cells.

The in vitro and in vivo depletion experiments and phenotypic analyses indicate that CD8+ T-cells are primarily responsible for tumor regression. The precise mechanism whereby bryostatin-expanded CD8+ cells exert their in vivo antitumor effect remains unclear. The low dose of IL-2 used for in vitro stimulation and for adoptive immunotherapy does not induce LAK activity, and a recent report indicates that bryostatin 1 actually inhibits the generation of LAK cells (10). The regression of MCA-105 pulmonary metastases does not appear to be mediated by the direct antitumor properties of bryostatin 1, because lymph node cells from normal mice and non-draining lymph node cells from tumor-bearing mice expanded with bryostatin had no antitumor activity. The DLN cells utilized in these studies were washed extensively after incubation in bryostatin 1, and the stimulating agents were further diluted as the rapidly growing cultures were split, thus minimizing the potential direct antitumor effect of bryostatin 1.

These studies demonstrate that T-lymphocytes expanded by direct pharmacological manipulations of second messenger systems are capable of in vivo antitumor immune function. Such pharmacological manipulations may provide effective means of expanding T-cells for adoptive immunotherapy by overcoming T-cell nonresponsiveness upstream of PKC activation and cytosolic calcium elevation.

The optimal method for growing T-cells with bryostatin 1 for adoptive immunotherapy has not yet been settled upon. Both antigen-sensitized and fresh DLN cells stimulated with Bryo/Io mediate tumor regression with relatively small numbers of adoptively transferred cells. DLN cells activated immediately with Bryo/Io yield larger numbers of cells than those stimulated first with specific antigen. If these cells are as effective, on a per-cell basis, as cells obtained by serial stimulation with specific antigen, then the strategy of immediate pharmacological stimulation would clearly be advantageous. Conversely, obtaining a greater yield of lymphocytes with much weaker antitumor reactivity would not be as effective for adoptive immunotherapy. In either case, this regimen would still provide an alternative method for T-cell activation when autologous tumor is not available and has a potential for the expansion of T-cells for adoptive immunotherapy.

REFERENCES


Activation and Growth of Murine Tumor-specific T-Cells Which Have *in Vivo* Activity with Bryostatin 1


Updated version  Access the most recent version of this article at: [http://cancerres.aacrjournals.org/content/52/3/548](http://cancerres.aacrjournals.org/content/52/3/548)

**E-mail alerts**  Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  To request permission to re-use all or part of this article, use this link [http://cancerres.aacrjournals.org/content/52/3/548](http://cancerres.aacrjournals.org/content/52/3/548). Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.