Bryostatin 1 Induces Differentiation of B-Chronic Lymphocytic Leukemia Cells

By Hans G. Drexler, Suzanne M. Gignac, Richard A. Jones, Colin S. Scott, George R. Pettit, and A. Victor Hoffbrand

Peripheral blood cells from nine patients with B-chronic lymphocytic leukemia (B-CLL) were treated in vitro with bryostatin 1 (a macrocyclic lactone derived from a marine invertebrate). Like the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA), bryostatin 1 activates protein kinase C (PKC), which plays a central role in the phosphatidylinositol signal transduction pathway. The effects of bryostatin 1 alone and in combination with TPA or with the calcium mobilizing ionophore A23187 were assessed by morphological appearance, cell adherence and aggregation, RNA and DNA synthesis, and immunoglobulin (Ig) production. While eight of nine B-CLL cultures remained proliferatively inert, bryostatin 1 could effectively trigger activation and differentiation of B-CLL cells in all cases as inferred by the induction of morphological changes, RNA synthesis, and monotypic Ig production. Addition of calcium ionophore A23187 to bryostatin 1-exposed cells resulted in significantly increased values for RNA synthesis and Ig production and in the acquisition of plasmacytoid morphology. Bryostatin 1 and the dual signal of bryostatin 1 plus A23187 mimicked the stimulatory action of TPA and the combination of TPA plus A23187, respectively. Overall, bryostatin 1 was less active than equivalent concentrations of TPA. This lesser efficacy may, however, reflect a quantitative rather than qualitative difference. Bryostatin 1 partially antagonized TPA-mediated effects on B-CLL cells suggesting different modes of action by the two activators. These studies indicate that bryostatin 1 has effective differentiation-inducing properties on B-CLL cells that can be accentuated by a calcium ionophore.

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In contrast to phorbol esters, the bryostatins lack tumor promoting properties and may in fact be antipromoters.20 Two mechanisms that may partially explain the different action of these two agents are that the bryostatins induce accelerated degradation of PKC entailing a shorter duration of action, and they activate a second target in addition to the typical high affinity phorbol ester binding site. This lesser efficacy may, however, reflect a quantitative rather than qualitative difference. Bryostatin 1 partially antagonized TPA-mediated effects on B-CLL cells suggesting different modes of action by the two activators. These studies indicate that bryostatin 1 has effective differentiation-inducing properties on B-CLL cells that can be accentuated by a calcium ionophore.

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the production and release of lymphokines, for example interleukin-2 (IL-2) and IL-3.27,28

B-chronic lymphocytic leukemia (B-CLL) is characterized by the accumulation of small, resting, immunophenotypically distinct B cells with a low spontaneous proliferation rate. B-CLL cells are generally unresponsive to antigen and to conventional mitogens including anti-immunoglobulin (Ig), pokeweed mitogen, and lipopolysaccharide. They also fail to respond to growth factors such as IL-2 and IL-6 even though receptors for at least one of these cytokines (IL-2) are demonstrable.29 Unresponsiveness to normal regulatory or maturation signals may represent one mechanism by which these cells accumulate in excessive numbers.

We have previously shown that failure to respond to these growth and differentiation signals is not a consequence of a basic defect in a final common pathway of activation or of cellular differentiation mechanisms.30 Thus, activation and terminal differentiation can be induced by second messenger analogs acting on key enzymes in the signal transduction pathway located distal to initial signal elements.3,31

The above described observations provided the rationale for the current study in which we analyzed the response of B-CLL cells to bryostatin 1 and to compare its effect with that seen after treatment with the phorbol ester TPA. The results demonstrate that bryostatin 1 is an effective inducer for the current study in which we analyzed the response of B-CLL cells as assessed by changes in morphology, RNA synthesis, and Ig production.

MATERIALS AND METHODS

Cells. Leukemic cells were isolated from heparinized peripheral blood of patients with B-CLL by density gradient centrifugation on Ficoll-Hypaque (1.077 g/mL, Lymphoprep; Nyegaard, Oslo, Norway). The harvested cells were immediately immunophenotyped and stored (see below). The clinical and laboratory data of the patients studied are detailed in Table 1.

Isolated cells were incubated in RPMI 1640 medium (GIBCO, Uxbridge, Middlesex, UK) supplemented with l-glutamine (2 mmol/L) (GIBCO), penicillin (100 IU/mL) (GIBCO), streptomycin (100 μg/mL) (GIBCO), and 10% (vol/vol) heat-inactivated (45 minutes at 56°C) fetal calf serum (Sera-Lab, Crowley Down, Sussex, UK).

Immunophenotyping. Surface antigen expression was examined by direct and indirect immunofluorescence staining in suspension using the microtiter plate system. Intracytoplasmic and nuclear antigens were demonstrated on cytopsin slide preparations. Positivity was analyzed under an epi-immunofluorescence microscope (Nikon Labophot, Telford, Shropshire, UK) equipped with appropriate filters for double-staining.

In short, cells were first incubated with the specific murine anti-human monoclonal antibodies (MoAbs) (Table 2). Labeling of the cells was visualized with fluorescein isothiocyanate (FITC)- or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse F(ab')2 isotype-specific antisera (Southern Biotechnobodies, Birmingham, AL). Surface Ig expression was determined by double-staining using combinations of directly conjugated goat anti-human heavy chain- (FITC-IgG and TRITC-IgM) and light-chain- (FITC-lambda and TRITC-kappa) specific antisera (Southern Biotechnology). Intracytoplasmic Ig was examined on acetone-fixed cytopsin slides. The composite immunophenotypes of the samples studied are listed in Table 2.

The MoAbs were provided by Drs G. Janossy, London (RF-11); J. Armitage, London (UCHB-1); A. Freedman, Boston (B5, B7); T.A. Waldmann, Bethesda, MD (Tac); and W. Knapp, Vienna, Austria (VIM-13). OKT-10, Ki-67, anti-BrdU, and FMC-7 MoAbs; R. Armitage, London (UCHB-1); A. Freedman, Boston (B5, B7); T.A. Waldmann, Bethesda, MD (Tac); and W. Knapp, Vienna, Austria (VIM-13). OKT-10, Ki-67, anti-BrdU, and FMC-7 MoAbs were purchased from Ortho (High Wycombe, Bucks, UK), Dako (High Wycombe), Becton Dickinson (Cowley, Oxford, UK), and Sera-Lab (Cowley Down, Sussex, UK), respectively.

Cells were stained for 30 minutes with Ki-67 or with 5* 14C thymidine. Mild alkaline denaturation with NaOH allowed for double-staining.

Table 1. Clinical Characteristics of Patients Studied

<table>
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<tr>
<th>Patient No.</th>
<th>Diagnosis*</th>
<th>Age/sex</th>
<th>WBC (x 10^3/L)</th>
<th>Treatment</th>
<th>Percent Poly PhD</th>
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<tr>
<td>1</td>
<td>B-CLL</td>
<td>70/M</td>
<td>42</td>
<td>Chi, P</td>
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<tr>
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<td>95</td>
<td>None</td>
<td>4</td>
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<tr>
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<td>90</td>
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<tr>
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<td>460</td>
<td>Chi</td>
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<td>76/F</td>
<td>55</td>
<td>CHOP</td>
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Table 2. Immunophenotypes of B-CLL Cells Studied

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<tr>
<td>IgM</td>
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<tr>
<td>Kappa</td>
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<tr>
<td>Cytoplasmic</td>
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<tr>
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<tr>
<td>RFT-1</td>
<td>2 97 97 12 95 80 95 98 93</td>
</tr>
<tr>
<td>RFT-12</td>
<td>96 88 96 80 93 97 91 94 95</td>
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<tr>
<td>RFT-9</td>
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<td>RFB-7</td>
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<td>RFD-2</td>
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<td>B-cell activation markers</td>
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<td>B5</td>
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<tr>
<td>B7</td>
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<td>OKT-10</td>
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<tr>
<td>Tac</td>
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</table>

Abbreviations: Chi, chlorambucil; C, cyclophosphamide; H, Adriamycin (doxorubicin); Adria Laboratories, Columbus, (OH); O, vincristine; P, prednisolone.

*Anticancer according to the proposals by Melo et al.22; CLL (<11% polyphocytes); CLL/PL (11% to 55%); PLL (>55%).

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anti-BrdU MoAbs; this was followed by 30-minute incubation with FITC-conjugated goat anti-mouse antibodies. Cells were analyzed by immunofluorescence microscopy.

The Ki-67 nuclear antigen is expressed on all proliferating cells, preferentially during the late G1, S, G2, and M phases of the cell cycle, whereas cells in the G0 phase consistently lack the antigen.34 Uptake of BrdU, a thymidine analog, after a certain cell cycle, whereas cells in the G0 phase consistently lack the DNA during the late G1, S, G2, and M phases of the cell cycle.

In a dose-dependent fashion, bryostatin 1 induced RNA synthesis and Ig production with maximal response at 10^{-4} mol/L bryostatin 1.

RESULTS

Dose-response and time course kinetics of bryostatin 1 induction. Initial studies were undertaken to determine the range of concentrations at which bryostatin 1 was effective. B-CLL cells were cultured with graded doses of bryostatin 1. At 24-hour intervals RNA synthesis and Ig production were examined by ^3H-uridine uptake and ELISA, respectively. A representative experiment is illustrated in Fig 1. In a dose-dependent fashion, bryostatin 1 induced RNA synthesis and Ig production with maximal response at 10^{-4} mol/L bryostatin 1.

Fig 1. Dose-response curve of B-CLL cells to treatment with bryostatin 1 in one representative patient. ^3H-uridine incorporation (solid line) at its maximum at day 3 and Ig production (broken line) after six days of culture were used as parameters of response. Three other patients showed similar data. Morphological examination equally identified the range of 10^{-7} mol/L to 10^{-9} mol/L as the most effective concentrations.
Analysis of time course kinetics showed the peak of RNA synthesis to occur at the second or third day of in vitro treatment. A representative example is given in Fig 2. An increase in Ig production was seen after 24 hours, and this continued steadily in the following days.

Treatment of one case with TPA showed similar dose-response curves and time course kinetics as compared with those found after induction with bryostatin 1. However, as pointed out later, TPA was more effective at inducing \(^3\)H-uridine incorporation and Ig production.

*Morphologic examination.* As early as 24 hours after initiation of the bryostatin 1-treated cultures, compact cell aggregates could be observed with the inverted microscope. At days 2 and 3, these clumps became large, densely packed cell clusters with surrounding single cells, which under higher magnification showed cytoplasmic extensions and pseudopodia (Fig 3B). The most pronounced clustering of cells was detected in cultures treated with \(10^{-7}\) mol/L or \(10^{-8}\) mol/L bryostatin 1.

It has been documented that lymphocyte activation is accompanied by increased adhesiveness and this adhesion of lymphocytes to one another is a leukocyte function antigen (LFA-1)-dependent process. This antigen-independent aggregation of a single cell type (homotypic adhesion) is known to be inducible by phorbol esters, ie, through direct stimulation of PKC. Light microscopic examination of B-CLL cells treated with bryostatin 1 showed the acquisition of the following morphologic features beginning after 24 hours of incubation: enlargement of the cytoplasm, which remained clear; bizarre cellular forms and shapes in the majority of cells; cytoplasmic extensions and development of pseudopodia ("stretching") in most cells (Fig 4B).

*DNA synthesis.* To determine if bryostatin 1 induced proliferation, B-CLL cells were cultured with graded doses of the agent. The cultures were incubated for one to six days and labeled with \(^3\)H-thymidine. Except for one of the nine patients the proliferative response to bryostatin 1 in the B-CLL cells after one to six days was negligible, exceeding the control values by not more than two to fivefold. These results were confirmed by immunostaining with the MoAbs Ki-67 and anti-BrdU as these markers were positive in less than 0.1% of the control and treated cells. No mitotic figures (in <0.01% of the cells) were seen on cytopsin microscopic slides stained with May-Gruenwald-Giemsa stain.

The one exceptional case (patient no. 8) clearly displayed proliferative activity in cultures treated with bryostatin 1 as evidenced by the following parameters: 1% to 2% of the cells showed mitotic figures on cytopsin slides; \(^3\)H-thymidine uptake values increased to 200-fold relative to control values (20,000 cpm vs 100 cpm); 12% of the cells were Ki-67 positive; 4% of the cells were anti-BrdU positive.

*RNA synthesis.* \(^3\)H-uridine incorporation was used as an indicator of RNA synthesis and consequently as a parameter of cellular activation of bryostatin 1-treated B-CLL cells. A significant increase in \(^3\)H-uridine uptake was noted in all cases exposed to bryostatin 1 compared with control cultures, which began at day 1 and peaked at day 2 or 3 of treatment (Figs 2 and 5A). The mean percentage increases in \(^3\)H-uridine uptake of the population as a whole (cases 2 to 9) caused by the inducers were as follows: bryostatin 1 \(v\) medium (182% ± 94%); bryostatin 1 + A23187 \(v\) medium (587% ± 448%); bryostatin 1 + A23187 \(v\) bryostatin 1 (134% ± 107%).

*Immunoglobulin production.* The terminal stage of B-cell differentiation is antibody-forming cells. To evaluate whether bryostatin 1 could mediate terminal B-cell maturation, the Ig present in the culture wells was quantified using a specific ELISA. After 24 hours an increase in the values for Ig was observed in all patients treated with bryostatin 1 (Figs 2 and 5B). In all cases, the type of Ig produced (IgM vs IgG and kappa vs lambda light chain) was examined; the isotype of the analyzed Ig corresponded to that detected on the cell surface or in the cytoplasm by immunophenotypical staining.
BRYOSTATIN INDUCES B-CLL DIFFERENTIATION

Fig 3. Phase photomicrograph of B-CLL cells in culture (patient no. 5). Control and treated cells were photomicrographed through an inverted microscope with phase-contrast illumination after three days of culture. (A) Control: cells did not adhere or aggregate; (B) bryostatin 1: clustering in densely packed clumps and adherence of single cells with cytoplasmic extensions; (C) TPA: same as bryostatin 1, but larger, more numerous aggregates; (D) TPA + bryostatin: similar to TPA or bryostatin 1 alone; (E) TPA + A23187: smaller, less densely packed clumps, but more numerous than with the single inducers; (F) bryostatin 1 + A23187: similar to TPA + A23187. Concentrations of inducers used: TPA (10⁻⁴ mol/L); A23187 (7.5 × 10⁻⁷ mol/L); bryostatin 1 (10⁻⁴ mol/L). Original magnification x 25. Identical results were found in all cases.

(Table 2) and the type of Ig produced was exclusively monoclonal and monotypic. The mean percentage increases in IgM production of cases 3 to 9 together caused by the biomodulators were as follows: bryostatin 1 v medium (438% ± 543%); bryostatin 1 + A23187 v medium (699% ± 811%); bryostatin 1 + A23187 v bryostatin 1 (51% ± 41%).

Interaction with calcium ionophore. Previously, we showed that addition of the calcium ionophore A23187 to CLL cells cultured with TPA had an additive or even synergistic effect on induction of differentiation.⁷⁻⁸ We studied whether A23187 could likewise potentiate bryostatin 1-promoted activation and differentiation of CLL cells.

The results for ³H-uridine uptake (RNA synthesis) and Ig production (final maturation), which are summarized in Fig 5, indicate that A23187 clearly increased the effect of bryostatin 1 in an additive or synergistic fashion in all cases tested. No increase in ³H-thymidine incorporation could be
triggered by the treatment with both A23187 and bryostatin 1 compared with the application of either inducer alone. The cells stimulated with both reagents clustered; these aggregates were far more numerous, but smaller and less tightly knit than the bryostatin 1-induced clusters (Fig 3F). Morphologically, the cells resembled plasmacytoid cells being large, round or oval, with basophilic cytoplasm, eccentrically located nuclei, and clear, Hof-like perinuclear zones (Fig 4F).

Comparison with TPA-induced effects. In order to compare the responses triggered by bryostatin 1 and phorbol ester, all samples were also treated with the phorbol ester TPA and the following two combinations of reagents: TPA + A23187 and TPA + bryostatin 1. TPA and bryostatin 1 alone induced similar effects regarding adherence and clustering of the cells and morphological changes (Figs 3B and C and 4B and C). In all cases, however, bryostatin 1 was less effective than TPA at inducing 3H-uridine uptake and Ig production.

The additive or synergistic effects of the dual stimulation with PKC activators plus calcium ionophore were similar for the combinations TPA + A23187 and bryostatin 1 +
BRYOSTATIN INDUCES B-CLL DIFFERENTIATION

Fig 5. Response to treatment with bryostatin 1 or the double-stimulus of bryostatin 1 + A23187 in cells from different B-CLL patients as measured by RNA synthesis at day 3 (A) and Ig production after six days of incubation (B). Cells were treated under identical experimental conditions with 10⁻⁸ mol/L bryostatin 1 or bryostatin 1 + A23187 (7.5 × 10⁻⁸ mol/L). Not shown are the values for A23187-treatment as these are similar or identical to the control figures. Note the pronounced heterogeneity of the effects of the inducers in different patients. 1, Value below sensitivity of assay; 2, not done.

Modulation of TPA-induced differentiation. To assess if bryostatin 1 could have a potentiating or inhibiting effect on TPA-induced differentiation, B-CLL cells were cultured in the presence of both reagents. Little or no effect on the morphological features when compared with the results from either inducer alone (Figs 3D and 4D).

However, RNA synthesis and Ig production induced by TPA were significantly inhibited by the simultaneous presence of 10⁻⁷ mol/L bryostatin 1 (Fig 6). A mean reduction of 46% (±19%; range, 22% to 76%) of TPA-mediated ³H-uridine uptake was observed. Bryostatin 1 also caused a blockade of the TPA induction of Ig production: a mean decrease of 76% (±15%; range, 51% to 91%). 10⁻⁴ mol/L bryostatin 1 was less effective (Fig 6).

Heterogeneity of responses. While the extent of differentiation as evidenced by morphological appearance was similar in all cases, a striking heterogeneity between all CLL cultures was seen for the quantifiable parameters of ³H-uridine uptake and Ig production (Fig 5). No correlation between responsiveness to the inductive signals and clinical
DISCUSSION

In response to in vitro treatment with the phorbol ester TPA human B-CLL cells acquire distinct morphological and cytochemical changes, produce and secrete monotypic Ig, and express a variety of activation- and differentiation-associated surface antigens. The responses elicited by TPA are due to activation of PKC, which in a variety of other model systems causes different biological effects dependent on the type of target cell. While the many aspects of these classical PKC activators, phorbol esters, have been studied extensively, the bryostatins represent a newly discovered class of PKC modulators. A number of substantial differences between the effects mediated by either bryostatins or phorbol esters have been reported. In contrast to phorbol esters, bryostatins lack tumor-promoting activity, but demonstrate antineoplastic effects. Because these compounds displayed several immunomodulating properties, it has been suggested that bryostatins might possibly represent useful biological response modifiers. In this context, the present studies were designed to analyze the differentiation-inducing capacities of one of the bryostatins, namely bryostatin 1, and to test whether the two PKC activators, TPA and bryostatin 1, confer similar or different effects on B-CLL cells.

The results presented here demonstrate that the stimulatory and differentiation-inducing effects of bryostatin 1 and TPA on B-CLL cells were comparable, albeit TPA appeared...
to be more effective for the promotion of RNA synthesis and Ig production. Both biomodulators effectively induced differentiation while the cells remained proliferatively inert (except for one patient). As previously described for the two-signal treatment of TPA plus A23187, the combination of bryostatin 1 and this calcium ionophore also potentiated the differentiation of B-CLL cells compared with the use of either inducer alone. Not only did the double stimulus induce higher amounts of RNA and Ig than the single compounds, but also the morphological changes triggered were more compatible with differentiation toward plasmacytoid cells.

Therefore, in analogy to previously reported conclusions, the use of PKC activator plus calcium ionophore might convey a more physiological signal that is followed by adequate differentiative changes while TPA or bryostatin alone trigger maturation along a sideline away from the "normal" developmental pathway: TPA-treated B-CLL cells displayed, at least in part, hairy cell-like features. The additional signal mediated by the calcium ionophore might be required in order to initiate a differentiation process that resembles the one occurring under physiological conditions where the triggering of surface receptors then elicits activation of both second messengers, which subsequently transfer the signal from the cell membrane to various cellular sites.

The reason for the differing effectiveness of bryostatin 1 and TPA to mediate induction of RNA synthesis and Ig production are not known. Using another experimental system, Hess et al. speculated that in the light of the recent discovery of various PKC isoenzymes one or more PKC isoforms might be stimulated differentially by the two PKC agonists causing different, possibly even antagonistic effects. In fact, the results here show that bryostatin 1 can partially antagonize the differentiative response of B-CLL cells induced by TPA. Similar results have been reported by other investigators using T cells or leukemia cell lines. In this regard, an altered PKC substrate specificity has been proposed to explain differences and antagonism in biological responses seen in bryostatin 1- and TPA-treated cells. Recent studies have indicated that bryostatin 1 stimulates both cytoplasmic and nuclear PKC activity in the myeloid leukemia cell line HL-60, whereas TPA activates only the cytoplasmic form. Clearly, further biochemical studies are warranted to analyze in detail possible differences in the mode of action of bryostatin 1 and TPA.

Bryostatin 1 could also have effects on cellular systems other than PKC to account for its mechanism of action, e.g., the cAMP pathway. Further experiments have to be performed to invoke a specific mechanism to the exclusion of any others.

A striking patient-to-patient variation for the data on the induced RNA synthesis and Ig production was seen among our nine cases of B-CLL. Such pronounced heterogeneity in the response of CLL cells to phorbol ester and other stimulators has been documented previously. We could not establish any link between clinical and laboratory characteristics of the cells and the extent of their in vitro response to the inducers. Except for one patient, no proliferative activity could be induced by TPA or other biomodulators in our present or previous series of cases. Without apparent reasons, this single patient showed definite evidence for proliferation in the form of mitotic figures, positivity for the Ki-67 antigen, and incorporation of BrdU, which were studied at the single cell level. The fact that this case was exceptional raises the question of singling out individual patients whose cells were responsive to in vitro treatment while the majority of cases are not responsive, and of using these examples as representative models for B-CLL activation and differentiation.

In summary, the data presented here demonstrate that the PKC activator bryostatin 1 can induce differentiation of B-CLL cells paralleling the known effects elicited by the classical PKC stimulator TPA. The ability of the dual signals of bryostatin 1 plus the calcium ionophore A23187 to promote the final maturation of B-CLL cells into antibody-forming, plasmacytoid cells is similar to the capacity of TPA plus A23187 to mediate this response. Therefore, experimentally, phorbol esters can be substituted by bryostatins to dissect the various intracellular activation pathways and signal transduction components which might be stimulated by different isoenzymes of PKC; the understanding of heterogeneity in the PKC pathway is of great conceptual importance. Bryostatin 1, which itself lacks tumor-promoting activity, can also be antagonistic to the tumor-promoter TPA.

Finally, the partial integrity of the inositol phospholipid signal transduction pathway (downstream from PKC) seen in our study confirms that the defect, i.e., unresponsiveness to adequate inductive signals, most likely resides in a proximal component of the signaling system, possibly as a lack or dysfunction of signal receptors, blockade of the transducing machinery, or uncoupling of functional elements. Future attention will be focused on the role of G protein regulation of receptor signaling and its possible dysfunction in B-CLL cells.

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