Bryostatin-1 and IL-2 Synergize to Induce IFN-γ Expression in Human Peripheral Blood T Cells: Implications for Cancer Immunotherapy[1]

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Bryostatin-1 (Bryo-1), a protein kinase C modulator with antineoplastic activity, may exert some of its antitumor activity through activation of the immune response. Studies in tumor-bearing hosts have indicated that the T cell response, particularly IFN-γ production, is impaired. To evaluate whether Bryo-1 plus IL-2 may affect the activation pattern of T cells, we investigated the expression of IFN-γ mRNA and protein in human primary T cells. Northern blot analysis and ELISAs demonstrated that Bryo-1 and IL-2 synergized to induce both IFN-γ mRNA and protein expression. This synergistic induction was seen within 3 h of treatment and with as little as 10 U/ml IL-2 and 1.0 ng/ml Bryo-1. In vitro transcription assays revealed that Bryo-1 plus IL-2 induced transcriptional activation of the IFN-γ gene. Furthermore, mRNA stability studies indicated that this treatment also enhanced the IFN-γ mRNA half-life. Both $CD4^+$ and $CD8^+$ T cells responded to the treatment with IFN-γ expression. The induction of the IFN-γ expression was decreased by a specific p38 mitogen-activated protein kinase inhibitor, but not by a protein kinase C inhibitor. Our results demonstrate for the first time that Bryo-1 in combination with IL-2 control IFN-γ gene expression at both the transcriptional and post-transcriptional levels through a p38 mitogen-activated protein kinase-dependent process. Given the pivotal role that IFN-γ plays in the orchestration of an effective Th1 type of response, our results suggest that Bryo-1 plus IL-2 may be a valuable combined therapy for cancer treatment. *The Journal of Immunology,* **2001, 167: 4828–4837.**

Bryostatin-1 (Bryo-1),[3] a macrocyclic lactone derived from the marine bryozoan, *Bugula neritina* (1), exhibits a unique pattern of biological effects (2). Bryo-1 is a potent ligand and modulator of the phorbol ester receptor protein kinase C (PKC) (3, 4) and can mimic certain effects of phorbol esters in some biological systems (5). However, several other properties of Bryo-1 are distinct from those of the phorbol esters (6); of critical importance and unlike the phorbol esters, this compound lacks tumor-promoting capabilities and actually counteracts tumor promotion induced by phorbol esters (7). Bryo-1 has received considerable attention in the past few years because of its potent and broad antineoplastic activity both in vitro and in vivo. Bryo acts as a cytostatic, cytotoxic, and/or differentiating agent on several types of tumors. Application of Bryo either to freshly isolated cells from patients with acute or chronic myeloid and lymphocytic leukemia (8, 9) or to myeloid and lymphoid human cell lines, promotes their terminal differentiation and halts their growth (10, 11). Bryo-1 also inhibits the in vitro growth of a variety of murine and human tumor cell lines, including breast (12), lung (13), and melanoma (14), and significant antitumor activity has been observed in preclinical models for melanoma, sarcoma, and B cell lymphoma (14, 15). In addition to its direct antitumor activity, Bryo-1 may also inhibit tumor growth in vivo by indirect mechanisms related to its ability to stimulate host immune response. It has been reported that Bryo-1 enhances human polymorphonuclear leukocyte cytotoxicity, oxidative burst, and degranulation (4, 16). Recently, our group reported that Bryo-1 is a potent activator of human monocytes (17). In that report we demonstrated that subnanomolar concentrations of Bryo-1 induce the production and secretion of IL-l, IL-6, IL-8, and TNF-α, which are proinflammatory cytokines endowed with antitumor and immunomodulatory properties (17). Bryo-1 also induces the proliferation and activation of B and T cells (18–20) and triggers tumor-specific T cells that can traffic and mediate tumor regression (21, 22). Furthermore, Bryo-1 induces IL-2Rα and IL-2Rγ chains expression on human PBMC leading to an enhanced response to IL-2 (17, 19). Based on its direct antitumor activity and on its broad immunomodulatory effects, Bryo-1 is being clinically developed. Moreover, initial clinical studies have demonstrated that Bryo-1 is a novel anticancer agent with biochemical and immunomodulatory activities in cancer patients (19, 23).

IFN-γ, a potent immunomodulatory cytokine produced by T cells and NK cells, influences all aspects of the immune system, including the development, activation, and maturation of monocytes/macrophages, T cells, NK cells, and B cells (24). It is widely recognized that IL-12 and IFN-γ are key to the development of Th1 cells, and current studies indicate that type 1 immune responses are strongly correlated with antitumor immune activity (25–30). These reports clearly underscore the importance of IFN-γ in the immune response against tumors. The present study was designed to investigate: 1) whether treatment of human T lymphocytes Bryo-1

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[3] Abbreviations used in this paper: Bryo-1, bryostatin-1; Act-D, actinomycin D; BI, bisindolylmaleimide; CHX, cycloheximide; DEX, dexamethasone; IC_{50}, half-maximal inhibitory concentration; MAPK, mitogen-activated protein kinase; PKC, protein kinase C.

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plus IL-2 induce IFN-γ gene expression, and, if so, 2) to determine the molecular mechanisms regulating the Bryo-1- and IL-2-induced IFN-γ gene expression. Our results demonstrate for the first time that, through mechanisms dependent on p38 mitogen-activated protein kinase (MAPK) and independent of PKC, the antineoplastic agent Bryo-1 synergizes with IL-2 to induce IFN-γ gene expression in freshly isolated human peripheral blood T cells as well as in purified $CD4^+$ and $CD8^+$ T cells. Herein we also demonstrate that both transcriptional and post-transcriptional levels of regulation control IFN-γ gene expression by this combined treatment. Lastly, we show that mRNA expression induced by Bryo-1 and IL-2 leads to productive secretion of IFN-γ protein.

Materials and Methods

T cell isolation, culture condition, and stimulation

Peripheral blood leukocytes were obtained from normal healthy volunteers by leukapheresis using a Fenwall CS-3000 blood cell separator (Fenwell Laboratories, Deerfield, IL). Lymphocytes were separated by density gradient centrifugation on lymphocyte separation medium (Organon Teknika, Durham, NC) and then purified in suspension from the unfractionated mononuclear leukocyte preparation by countercurrent centrifugal elutriation in a Beckman JE-6 elutriation chamber and rotor system (Beckman Instruments, Palo Alto, CA) as described previously (31). Total T, $CD4^+$, and $CD8^+$ T cells were purified with magnetic bits from the elutriated lymphocyte fraction according to the manufacturer's specifications (MACS; Miltenyi Biotec, Auburn, CA). For any given experiment, only cell populations with a purity of 95–96% for total T cells or 95–97% for $CD4^+$or $CD8^+$ cells were used in the experiments described below. Cell purity was assessed by flow cytometry using mAbs against CD3, CD4, CD8, CD14, CD16, and CD19 (BD Biosciences, San Jose, CA). Viability, as determined by the trypan blue exclusion test, was >99%. T cells were cultured in RPMI 1640 (BioWhittaker, Walkersville, MD), supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 20 mM HEPES (Life Technologies, Gaithersburg, MD), and 10% heat-inactivated FBS (HyClone, Logan, UT). T lymphocytes were cultured at the indicated time points in a 100-mm^2 tissue culture plates (Corning Glass Works, Corning, NY) at 2×10^6 cell/ml in medium alone or medium supplemented with either Bryo-1, highly purified IL-2, or a combination of both biologicals. The specific activity of IL-2 was 18×10^6 IU/mg; Chiron units are used throughout the manuscript, 1 Chiron unit = 6 IU; LPS content, < 0.06 IU/ml. IL-2 was kindly provided by Cetus (Emeryville, CA) (32). Clinical grade Bryo-1 was a gift from A. Fallavollita, Jr. (Cancer Therapy Evaluation Program, Division of Cancer Treatment, Diagnosis, and Centers, National Cancer Institute, National Institutes of Health, Bethesda, MD). Bryo-1 conversion factor is equal to 1.0 ng/ml = 0.904 nmol/l. Dexamethasone (DEX) was purchased from Sigma (St. Louis, MO). Bisindolylmaleimide (BI) and SB203580 were purchased from Calbiochem (San Diego, CA). Cell-free supernatants were collected at the designated time points, and cytokine secretion (IFN-γ, IL-4, IL-12, IL-13, IL-18) was evaluated using an ELISA according to the manufacturer's instructions (BD PharMingen, San Diego, CA).

Northern blot analysis

Human peripheral blood T cells were cultured in medium alone or supplemented with the indicated reagents. Total RNA was extracted by lysis with TRIzol (Life Technologies) and purified according to the manufacturer's specifications. Northern blot analysis was performed in accordance with the previously described protocol (17). Twenty micrograms of total RNA from each sample was electrophoresed under denaturing conditions, blotted onto Nytran membranes (Schleicher & Schuell, Keene, NH), and cross-linked by UV irradiation. Membranes were prehybridized at 42°C in Hybrisol (Oncor, Gaithersburg, MD) and hybridized overnight with 2×10^6 cpm/ml of ^{32}P-labeled probe. Membranes were then washed twice at 42°C for 15 min in 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0), 0.1% SDS and twice at 65°C for 20 min in 0.2× SSC, 0.1% SDS before being autoradiographed using Kodak Biomax-MR (Eastman Kodak, Rochester, NY) films and intensifying screens at −70°C. The human cDNA IFN-γ probe (a gift from Dr. G. Ricca (Rhone-Poulenc Rorer Biotechnology, King of Prussia, PA) and Dr. H. A. Young (Laboratory of Experimental Immunology, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD) and the murine-18S-DECAtemplate probe (Ambion, Austin, TX) were labeled by random priming using [α-^{32}P]dCTP (3000 Ci/mmol; Amersham, Arlington Heights, IL). For mRNA synthesis inhibition, actinomycin D (Act-D; Sigma) was dissolved in ethanol at 1 mg/ml and used at a final concentration of 5 μg/ml as indicated in the text. For protein synthesis inhibition experiments, cycloheximide (CHX; Sigma) was used at a final concentration of 10 μg/ml. An AlphaImager 2000 (alphaInnotech, San Leandro, CA) was used to analyze the band intensities of the autoradiographs of the Northern blots. The graphs were generated from the intensities of the distinct IFN-γ mRNA bands after being normalized to the relative abundance of 18S RNA present in each sample.

Nuclear run-on

Nuclear run-on experiments were performed as previously described (33). Briefly, nuclei were isolated from 5×10^7 cells/sample by lysing cells in 4 ml lysis buffer (10 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 10 mM NaCl, 150 mM sucrose, and 0.5% Nonidet P-40 (Sigma)) for 5 min on ice. Nuclei were spun at $167 \times g$ for 5 min at 4°C, and pellets were resuspended in lysis buffer without Nonidet P-40. Nuclei were pelleted again as described above and resuspended in 150 μl freezing buffer (50 mM Tris-HCl (pH 8.3), 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA). Run-on assays were performed by adding 150 μl 2× transcription buffer (20 mmol, Tris-HCl (pH 8.0), 300 mmol KCl, 10 mM $MgCl_2$, 200 mM sucrose 20% glycerol, 1 mmol dithiothreitol, and 0.5 mmol each of ATP, GTP, and CTP) and 100 μCi 800 Ci/mmol [α-^{32}P]UTP (New England Nuclear, Boston, MA) to 150 μl nuclei suspension. The samples were incubated at 29°C for 30 min. Thirty microliters of 200 mmol $CaCl_2$ and 30 μl 1 U/ml RNase-free DNase 1 (Promega, Madison, WI) were added to each reaction and further incubated for 10 min at 29°C. Labeled transcripts were isolated using TRIzol (Life Technologies) and purified according to the manufacturer's specifications. Equal amounts of radioactivity (~2×10^6 cpm labeled RNA) were added in 2 ml Hybrizol (Oncor, Gaithersburg, MD) to Nytran membranes on which 500 ng denatured full-length human IFN-γ cDNA (1.0 kb, lacking most of the 3′-untranslated region) and chicken β-actin cDNA (1.8 kb, *Hin*dIII fragment; Oncor) were immobilized using a slot-blot apparatus (Life Technologies) and a UV cross-linker (Fisher Scientific, Pittsburgh, PA). Hybridization was conducted at 42°C for 48 h. Filters were washed twice at 42°C for 15 min with 2× SSC/0.1% SDS and twice at 65°C for 20 min with 0.2× SSC/0.1% SDS. Filters were then autoradiographed at −70°C. Data were normalized for the content of β-actin present in each sample using an AlphaImager 2000 (alphaInnotech).

Results

Bryo-1 and IL-2 synergistically induce IFN-γ mRNA expression in human T lymphocytes

To determine whether Bryo-1, IL-2, or the combination of these two agents induced IFN-γ mRNA expression in peripheral blood human T cells, T lymphocytes were cultured for 12 h in medium alone or in the presence of 1.0 ng/ml Bryo-1, 50 U/ml IL-2, or the combination of the two agents. Total RNA was extracted, and Northern blot analysis was performed. As shown in Fig. 1, no basal expression of IFN-γ mRNA was detected in the medium control. Stimulation with Bryo-1 or IL-2 failed to induce IFN-γ mRNA expression, whereas treatment of T cells with the combination of Bryo-1 and IL-2 led to a mayor induction of IFN-γ mRNA. Dose-response experiments were performed to determine the optimal concentration of Bryo-1 and IL-2 needed to induce maximal IFN-γ mRNA expression. T lymphocytes were cultured in medium, Bryo-1 (1.0 ng/ml), or IL-2 (50 U/ml) alone or in the presence of various combined concentrations of Bryo-1 and IL-2. After 12 h stimulation, total RNA was extracted and analyzed by Northern blot for IFN-γ mRNA expression. As shown in Fig. 2, Bryo-1 plus IL-2 induced IFN-γ mRNA in a dose-dependent manner. As little as 10 U/ml IL-2 in combination with 1.0 ng/ml Bryo-1 (Fig. 2, *lane 4*) was sufficient to induce a modest, but reproducible expression of IFN-γ mRNA. Subnanomolar concentrations of Bryo-1 in combination with 50 U/ml IL-2 induced a significant expression of the IFN-γ transcript (*lane 6*), while 1.0 ng/ml Bryo-1 plus 50 U/ml IL-2 were required for maximal expression of IFN-γ mRNA (*lane 5*). Therefore, a combined dose of 50 U/ml IL-2 and 1.0 ng/ml Bryo-1 was used in all subsequent experiments. Neither Bryo-1

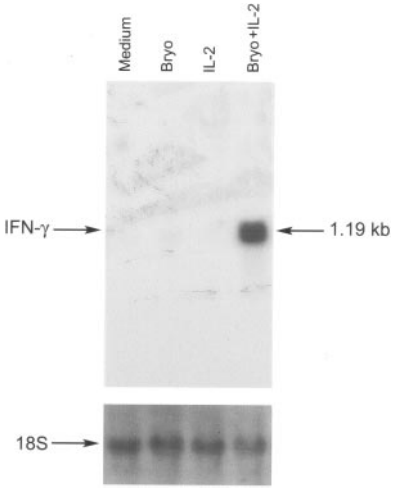

FIGURE 1. Bryo-1 plus IL-2 synergize to induce IFN-γ mRNA expression in human T lymphocytes. T lymphocytes were cultured for 12 h in the absence or the presence of 1 ng/ml Bryo-1, 50 U/ml IL-2, or a combination of 1 ng/ml Bryo-1 plus 50 U/ml IL-2. Total cellular RNA was extracted and analyzed by Northern blot for IFN-γ expression. The same membrane was rehybridized with 18S-DECAtemplate probe to confirm that equal amounts of RNA were loaded in each lane. Data shown are from one representative experiment of four performed.

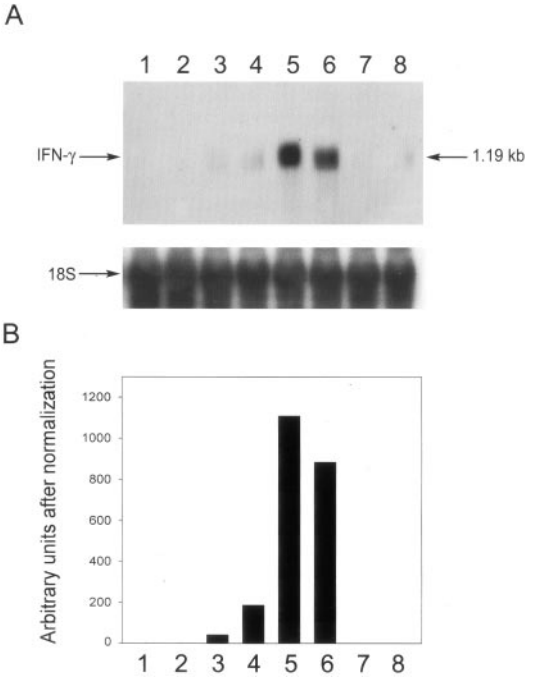

FIGURE 2. IFN-γ mRNA expression is induced in a dose-dependent manner by Bryo-1 and IL-2 treatment. T lymphocytes were cultured for 12 h in the absence or the presence of varying concentrations of Bryo-1 and IL-2 as follows: medium (*lane 1*), 1 ng/ml Bryo-1 (*lane 2*), 1 ng/ml Bryo-1 plus 2 U/ml IL-2 (*lane 3*), 1 ng/ml Bryo-1 plus 10 U/ml IL-2 (*lane 4*), 1 ng/ml Bryo-1 plus 50 U/ml IL-2 (*lane 5*), 0.1 ng/ml Bryo-1 plus 50 U/ml IL-2 (*lane 6*), 0.01 ng/ml Bryo-1 plus 50 U/ml IL-2 (*lane 7*), and polyethylene glycol plus 50 U/ml IL-2 (*lane 8*). Total cellular RNA was extracted and analyzed by Northern blot for IFN-γ expression. The same membrane was rehybridized with 18S-DECAtemplate probe to confirm that equal amounts of RNA were loaded in each lane. Data shown are from one representative experiment of two performed. *A*, Northern blot analysis for IFN-γ mRNA expression. *B*, Quantitative analysis of normalized levels of IFN-γ mRNA expression. As described in *Materials and Methods*, the band intensities were normalized to the 18S RNA control, and the graph was generated with the relative values obtained after normalization.

alone (*lane 2*) nor the combination of 50 U/ml IL-2 plus polyethylene glycol, the diluent for Bryo-1 (*lane 8*), induced the expression of IFN-γ mRNA.

To establish the kinetics of IFN-γ mRNA induction by the combination of Bryo-1 and IL-2, T cells were incubated in medium alone or in the presence of 1.0 ng/ml Bryo-1 plus 50 U/ml IL-2 for the indicated lengths of time. Total RNA was extracted, and Northern blot analysis was performed to detect IFN-γ mRNA expression. As shown in Fig. 3, an early induction of IFN-γ mRNA was detected within 3 h after Bryo-1 plus IL-2 stimulation. IFN-γ message expression was further increased at 6 h and reached maximal levels by 12 h. A small decrease in IFN-γ mRNA amounts was noted by 24 h, the last time point examined.

Mechanisms controlling IFN-γ gene expression by Bryo-1 plus IL-2 treatment

To investigate whether the induced expression of IFN-γ by Bryo-1 and IL-2 involved the activation of IFN-γ gene transcription, nuclear run-on experiments were performed. Human T cells were incubated in medium alone or supplemented with 1.0 ng/ml Bryo-1 plus 50 U/ml IL-2. The nuclei were isolated at 4 and 6 h after treatment, and nuclear run-on assays were performed. As shown in Fig. 4, the IFN-γ gene was not transcriptionally active in medium-treated control cells. On the other hand, Bryo-1- plus IL-2-treated cells displayed a mayor induction of IFN-γ gene transcription, which was further augmented at 6 h posttreatment. These results indicate that the induction of IFN-γ mRNA in Bryo-1- plus IL-2-treated cells was associated at least in part with the transcriptional activation of this gene. Next, experiments were performed to determine whether the combined Bryo-1 and IL-2 treatment influenced the stability of IFN-γ mRNA. Inasmuch as IFN-γ mRNA is not constitutively present in human T lymphocytes, we compared the half-life of Bryo-1- plus IL-2-induced IFN-γ mRNA with that of cells treated with Bryo-1 plus the potent T cell activator PMA. T cells were incubated for 12 h in medium alone or supplemented with 1.0 ng/ml Bryo-1 plus 50 U/ml IL-2 or 1.0 ng/ml Bryo-1 plus 10 ng/ml PMA. After the 12-h incubation period, Act-D was added to the cultures for the indicated lengths of time to block further RNA transcription. Northern blot analysis revealed that IFN-γ mRNA decayed with different kinetics in Bryo-1- plus PMA-treated samples compared with Bryo-1- plus IL-2-treated cells (Fig. 5). The level of IFN-γ mRNA in Bryo-1- plus PMA-treated cells decreased by 50% ($t_{1/2}$) after 55 min, and IFN-γ mRNA became almost undetectable after 4 h of Act-D treatment. On the other hand, Bryo-1- plus IL-2-treated cells displayed an enhanced IFN-γ mRNA stability, resulting in a $t_{1/2}$ of 3 h and 20 min. Furthermore, almost 40% of the IFN-γ mRNA was still present in the Bryo-1- plus IL-2-treated cells after 6 h. Taken together, these results demonstrate that treatment with Bryo-1 and IL-2 induced IFN-γ gene expression in human peripheral blood T cells through a dual mechanism involving transcriptional and post-transcriptional levels of regulation.

Bryo-1 and IL-2 synergistically induce IFN-γ secretion in human T lymphocytes

To evaluate whether the induced expression of IFN-γ mRNA led to protein secretion, supernatants from stimulated human T lymphocytes were assayed for the presence of IFN-γ. T cells were cultured for 24 h in the absence or presence of 1.0 ng/ml Bryo-1 plus 50 U/ml IL-2, and their supernatants were analyzed by ELISA. As shown in Table I, medium-treated T lymphocytes have

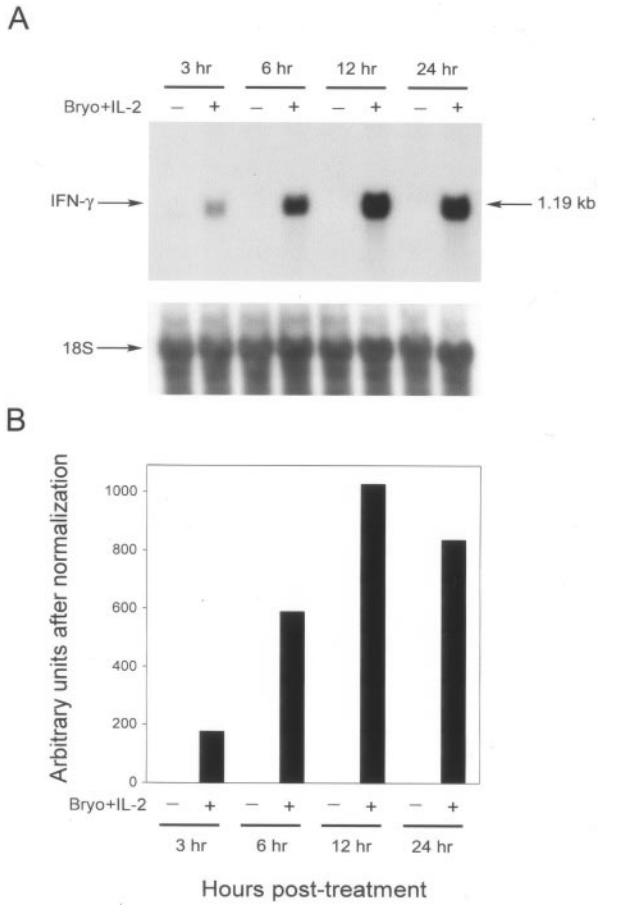

FIGURE 3. Kinetics of Bryo-1 plus IL-2-induced IFN-γ mRNA expression. T lymphocytes were stimulated in the absence or the presence of 1 ng/ml Bryo-1 plus 50 U/ml IL-2 for the indicated times. Total cellular RNA was isolated, and Northern blot analysis for IFN-γ mRNA expression was performed. The same filter was subsequently probed with 18S-DECAtemplate probe to ensure that comparable amounts of RNA were loaded in each lane. Data shown are from one representative experiment of two performed. *A*, Northern blot analysis for IFN-γ mRNA expression. *B*, Quantitative analysis of normalized levels of IFN-γ mRNA expression. As explained in *Materials and Methods*, the band intensities were normalized to the 18S RNA control, and the graph was generated with the relative values obtained after normalization.

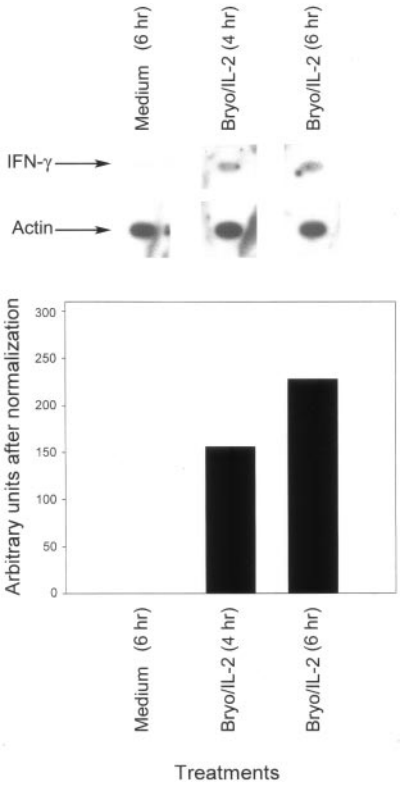

FIGURE 4. Bryo-1 plus IL-2 induce IFN-γ gene transcription in human T lymphocytes. T lymphocytes (5×10^7 cells/point) were treated with medium alone or with 1 ng/ml Bryo-1 plus 50 U/ml IL-2. Nuclei were isolated at the indicated time points, and the rate of transcription of IFN-γ gene was then assessed by nuclear run-on analysis as described in *Materials and Methods*. Data presented are from one of two similar experiments. The graph was generated as described in *Materials and Methods* with the relative values obtained after normalization of the band intensities to the respective amounts of β-actin.

no basal production of IFN-γ. Treatment with Bryo-1 or IL-2 alone also failed to induce IFN-γ protein expression, while the combined treatment of Bryo-1 plus IL-2 induced a significant secretion of IFN-γ. These results indicate that Bryo-1- plus IL-2-induced IFN-γ mRNA expression in human T lymphocytes is associated with the expression of IFN-γ protein in culture supernatants. Overall these results provide the first evidence indicating that Bryo-1 and IL-2 act synergistically to induce IFN-γ gene expression and secretion in human T cells

Protein synthesis is not required for IFN-γ mRNA induction by Bryo-1 plus IL-2

To determine whether active protein synthesis was necessary for the Bryo-1 plus IL-2 induction of IFN-γ mRNA, T cells were incubated for 4 h in the absence or the presence of 1.0 ng/ml Bryo-1 plus 50 U/ml IL-2 and in the absence or the presence of the protein synthesis inhibitor CHX. As shown in Fig. 6, the addition of CHX to Bryo-1- and IL-2-treated T cells did not decrease the induced IFN-γ mRNA expression. Interestingly, addition of CHX to Bryo-1- and IL-2-treated T cells caused a major increase in the induction (>2-fold) of IFN-γ mRNA expression over the level seen in Bryo-1- plus IL-2-treated samples. These results suggest that the Bryo-1 plus IL-2 induction of IFN-γ mRNA is not dependent on de novo protein synthesis.

Induction of IFN-γ gene expression by Bryo-1 plus IL-2 is blocked by DEX, but not by PKC, inhibition

Inhibitors are powerful tools that may help to dissect the biochemical mechanisms responsible for gene regulation. DEX has been shown to block both NF-κB nuclear translocation (34, 35) and AP-1/CREB interaction with IFN-γ promoter (36, 37). These two nuclear transcriptional complexes are known to play a major role in IFN-γ transcription. To investigate whether DEX affects Bryo-1- plus IL-2-mediated IFN-γ gene expression, human peripheral blood T cells were cultured with DEX in the presence or the absence of Bryo-1 and IL-2 for 4 h. As shown in Fig. 7*A*, DEX significantly inhibited IFN-γ gene expression induced by the combined treatment. These results demonstrate that IFN-γ mRNA expression induced by Bryo-1 and IL-2 was highly sensitive to DEX treatment and suggest that IFN-γ induction by this combined treatment may be dependent on NF-κB and/or AP-1/CREB transcriptional regulatory factors.

Activation of PKC by Bryo-1 appears to play a significant role in cellular responses to this antineoplastic agent. To ascertain the role of PKC in the induction of IFN-γ mRNA by Bryo-1 and IL-2, T cells were treated with either medium or Bryo-1 plus IL-2 in the presence of increasing concentrations of the specific PKC inhibitor

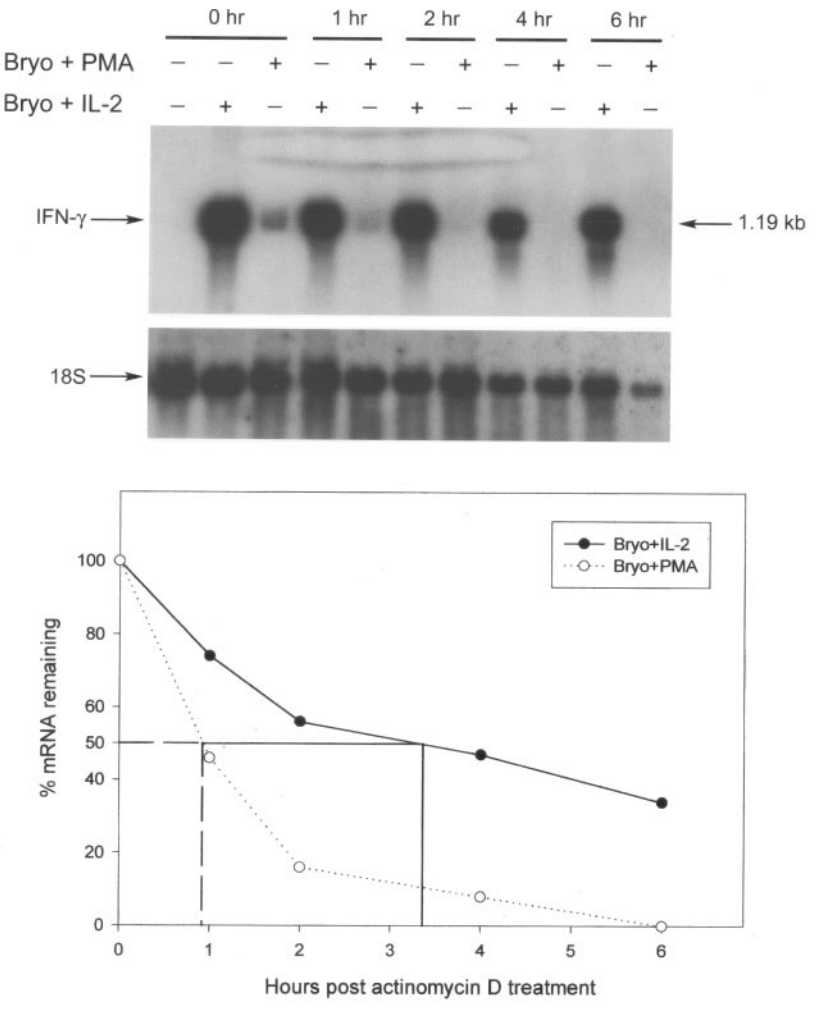

FIGURE 5. Bryo-1 plus IL-2 augment IFN-γ mRNA stability. T lymphocytes were incubated for 12 h in medium alone or in medium supplemented with either 1 ng/ml Bryo-1 plus 50 U/ml IL-2 or 1 ng/ml Bryo-1 plus 10 ng/ml PMA. After 12 h, cells were treated with 5 μg/ml Act-D, and their total cellular RNA was collected and analyzed by Northern blot for IFN-γ mRNA expression at the indicated time points. Data shown are from one representative experiment of two performed. The graph was generated as described in *Materials and Methods*, and data are presented as the relative amounts of IFN-γ mRNA remaining after adding Act-D and normalizing to the respective amounts of 18S RNA.

BI, and IFN-γ expression was evaluated. As shown in Fig. 7*B*, BI did not inhibit the Bryo-1 plus IL-2 induction of IFN-γ mRNA at either 0.01 μM, the half-maximal inhibitory concentration (IC_{50}), or 0.1 μM (38, 39). On the contrary, BI at doses 100 times the IC_{50} for PKC did inhibit IFN-γ induction by Bryo-1 plus IL-2. These findings indicate that the Bryo-1- and IL-2-induced IFN-γ gene expression occurs by PKC-independent mechanisms.

The p38 MAPK inhibitor decreases IFN-γ expression induced by Bryo-1 plus IL-2

p38 MAPK has recently been reported to be involved in the ability of T cells to express IFN-γ (40, 41). To ascertain the role of p38 MAPK in the present system, we investigated the effects of SB203580, a p38 MAPK-specific inhibitor (42), on the expression of IFN-γ mRNA in human lymphocytes treated with Bryo plus IL-2. Purified human peripheral blood T cells were pretreated with SB203580 at the indicated doses for 20 min, and then cells were activated with Bryo-1 plus IL-2 for 6 h. Total RNA was extracted, and Northern blot analysis was performed. As depicted in Fig. 8, 0.1 μM SB203580 inhibited Bryo-1- plus IL-2-induced IFN-γ mRNA expression. Further reduction of Bryo-1- plus IL-2-induced IFN-γ expression was observed when cells were treated with 1 μM SB203580. We next tested whether SB203580 also decreased IFN-γ production by Bryo-1- plus IL-2-activated T cells. Lymphocytes were cultured with Bryo-1 plus IL-2 in the presence or the absence of increasing concentrations of SB203580, and IFN-γ levels were measured by ELISA in the supernatants after 24 h of culture. As shown in Fig. 9, SB203580 inhibited the production of IFN-γ by activated T cells in a dose-dependent manner. The inhibitory effects of SB203580 observed in these experiments (0.1 μM SB203580 decreased IFN-γ production by ~50%) occurred with a potency similar to that previously reported to block p38 MAPK activity in several cellular systems (40–43). Overall, these results suggest that in human primary lymphocytes the induction of IFN-γ expression by Bryo-1 plus IL-2 is at least partially controlled by activation of p38 MAPK.

Table I. *Bryo-1 synergizes with IL-2 to induce IFN-γ secretion in human T lymphocytes*[a]

Treatment	IFN-γ (pg/ml)		
	Donor 1	Donor 2	Donor 3
Medium	0	0	0
Bryo-1 (1 ng/ml)	0	0	0
IL-2 (50 U/ml)	0	0	0
Bryo-1 (1 ng/ml) + IL-2 (50 U/ml)	658	581	572

[a] Highly purified T lymphocytes from three normal donors were cultured for 24 h in medium alone or supplemented as indicated above. Cell-free supernatants were tested by ELISA for the presence of IFN-γ.

Treatment with Bryo-1 and IL-2 induces IFN-γ mRNA expression in human $CD4^+$ and $CD8^+$ T lymphocytes

To determine the pattern of IFN-γ expression in the different subset of T cells, highly purified $CD4^+$ or $CD8^+$ T cells were cultured for 12 h in medium alone or in the presence of 1.0 ng/ml Bryo-1

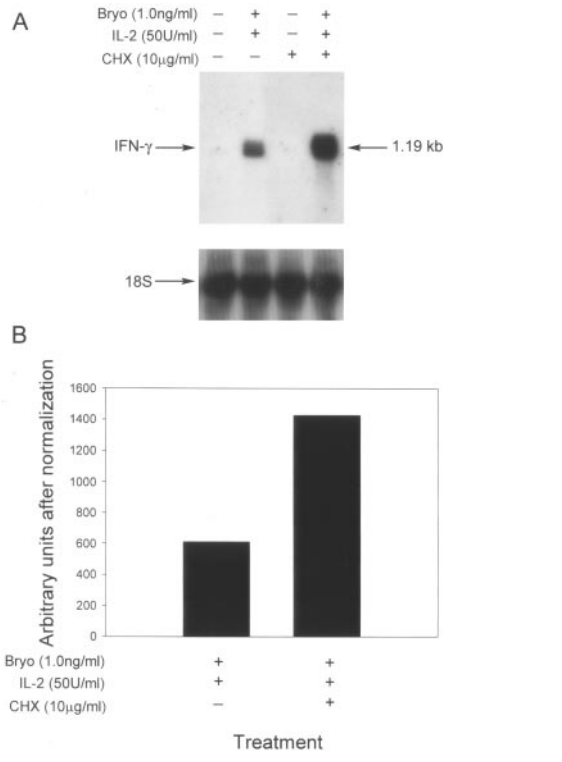

FIGURE 6. De novo protein synthesis is not required for the Bryo-1- plus IL-2-induced expression of IFN-γ mRNA. T lymphocytes were incubated for 4 h in the absence or presence of 1 ng/ml of Bryo-1 plus 50 U/ml of IL-2 and in the absence or presence of 10 μg/ml CHX. *A*, Total cellular RNA was extracted and analyzed by Northern blot for IFN-γ mRNA expression. Data shown are from one of two similar experiments. *B*, Quantitative analysis of normalized levels of IFN-γ mRNA expression. As stated in *Materials and Methods*, the band intensities were normalized to the 18S RNA control, and the graph was generated with the relative values obtained after normalization.

plus 50 U/ml IL-2. Total RNA was extracted, and Northern blot analysis was performed. As shown in Fig. 10, no basal expression of IFN-γ mRNA was detected in medium-treated cells, whereas Bryo-1- plus IL-2-treated $CD4^+$ or $CD8^+$ T lymphocytes displayed comparable levels of IFN-γ mRNA expression.

Differential production of IL-4 and IL-13 by Bryo-1- plus IL-2-activated T cells

The differential roles of Th1- and Th2-type responses in tumor-bearing hosts have been described, and several reports suggest a correlation between generation of a predominant Th1-type response and antitumor activity (25–30). The presence of IL-12 and IFN-γ promotes a Th1 response, whereas IL-4 and IL-13 are associated with a Th2 response (44–46). To evaluate whether Bryo-1 plus IL-2 also affect the expression of type 2 cytokines, we investigated the profile of IL-4 and IL-13 expression. Primary human lymphocytes were cultured with medium alone or medium supplemented with Bryo-1 plus IL-2, and IL-4 and IL-13 levels were measured by ELISA in the supernatants after 24 h of culture. As shown in Table II, medium-treated cells did not produce either IL-4 or IL-13. Bryo-1- plus IL-2-treated lymphocytes produced either very low levels of IL-4 or no IL-4 at all. On the other hand, IL-13 production was observed in all cells treated with the combination of Bryo-1 plus IL-2. Functional IL-13R-α has not been directly demonstrated in human T cells, and IL-13, unlike IL-4, does not induce proliferation of mitogen-activated T cells (47). Taken together these results suggest that Bryo-1 plus IL-2 may induce a pattern of cytokine that is skewed toward a Th1-type response, i.e., high IFN-γ/low IL-4.

Discussion

Bryo-1 has been shown to have a potent and broad in vitro and in vivo antineoplastic activity. In addition to its direct antitumor activity, recent studies suggest that Bryo-1's antitumor effects may be partly due to its immunomodulatory activity. IL-2 has also been recognized for its immunoregulatory functions and for its antitumor activity in both animal models and clinical trials (48). Presently, little information exists regarding the mechanisms controlling the effects of Bryo-1 plus IL-2 on human T lymphocytes. The data presented in this study demonstrate for the first time that Bryo-1 can synergize with IL-2 to induce significant IFN-γ gene expression in freshly isolated human peripheral blood T cells. This work also provides the first look at the molecular mechanisms involved in the Bryo-1- and IL-2-induced expression of IFN-γ mRNA. In agreement with previous reports (49) we have shown that Bryo-1 alone does not induce IFN-γ in human lymphocytes. On the other hand, we have demonstrated that as little as 10 U/ml IL-2 and 1.0 ng/ml Bryo-1 are sufficient to induce IFN-γ mRNA expression. Bryo-1 plus IL-2 synergized not only to induce IFN-γ mRNA expression, but also to induce high levels of IFN-γ protein production similar to those obtained with the potent IFN-γ inducer IL-12 (50). Induction of IFN-γ gene expression occurred as early as 3 h posttreatment and was further augmented up to 12 h. The rapid induction of IFN-γ mRNA in primary cultured T lymphocytes suggests a direct effect of Bryo-1 and IL-2 on the expression of this gene rather than a secondary effect mediated by another Bryo-1- and IL-2-inducible gene. This conclusion is further supported by the absence of IL-12 and IL-18 in the supernatant of lymphocytes treated with Bryo-1 plus IL-2 (data not shown). Our group has reported that in human peripheral blood monocytes Bryo-1 is capable of up-regulating the expression of the IL-2R γ-chain while not affecting the constitutive expression of IL-2R β-chain or inducing the expression of IL-2R α-chain (17). In an attempt to determine whether Bryo-1 was synergizing with IL-2 through the induction of the IL-2R, we examined the surface expression of the three chains of the IL-2R complex on Bryo-1-treated T lymphocytes. No significant increase in the expression of any of the IL-2R chains at 3 and 6 h post-treatment was observed (data not shown). In agreement with a previous report (20), we noticed that Bryo-1 induces IL-2R α-chain only after 24 h of treatment. These data demonstrate that the synergistic mechanism(s) operating in the early induction of IFN-γ mRNA by Bryo-1 and IL-2 does not involve up-regulation of the IL-2R complex. To better understand the nature of Bryo-1 and IL-2 synergism, we are currently undertaking efforts to further characterize the Bryo-1 signal transduction cascade leading to IFN-γ gene regulation.

To further dissect the molecular mechanisms responsible for the Bryo-1- plus IL-2-induced IFN-γ mRNA expression, nuclear run-on experiments were performed. These assays showed that the IFN-γ gene was not transcriptionally active in medium-treated T cells and that Bryo-1 plus IL-2 treatment induced transcriptional activation of the gene. The above-mentioned results from both the kinetics and run-on experiments provide the first evidence that this combined treatment can exert transcriptional control of the IFN-γ gene and that this effect contributes at least in part to the expression of IFN-γ mRNA in treated human T lymphocytes. It has been reported that PKC activation or increased cAMP levels have a stabilizing effect on IFN-γ mRNA expression (51). In an attempt to determine whether message stabilization was one of the mechanisms involved in the Bryo-1- and IL-2-induced IFN-γ gene expression, experiments with the transcription inhibitor Act-D were

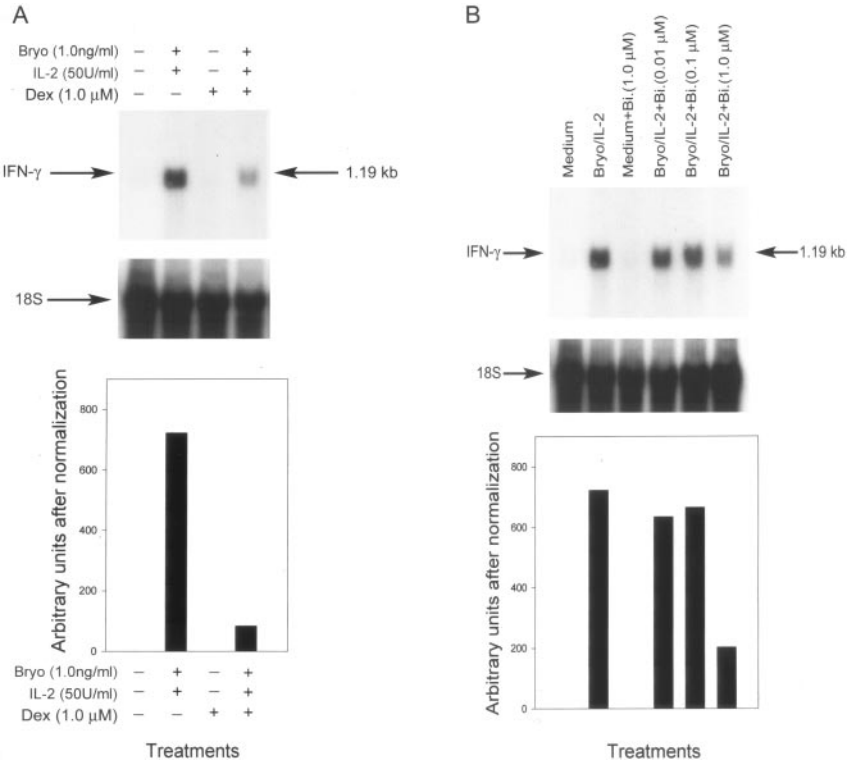

FIGURE 7. Induction of IFN-γ gene expression by Bryo-1 plus IL-2 is blocked by DEX, but not by the PKC inhibitor BI. *A*, T lymphocytes were incubated for 4 h in the absence or the presence of 1 ng/ml Bryo-1 plus 50 U/ml IL-2 and in the presence or the absence 1 μM DEX. *B*, T lymphocytes were preincubated in the presence or the absence of different concentrations of BI and then cultured for 4 h in the presence or the absence of 1 ng/ml Bryo-1 plus 50 U/ml IL-2. Total cellular RNA was extracted and analyzed by Northern blot for IFN-γ mRNA expression. Data shown are from one of two similar experiments. The graphs represent the quantitative analysis of normalized levels of IFN-γ mRNA expression. As explained in *Materials and Methods*, the band intensities were normalized to the 18S RNA control, and the graphs were generated with the relative values obtained after normalization.

performed. This study was complicated due to the fact that IFN-γ is not constitutively expressed in T lymphocytes; therefore, it was not possible to measure the IFN-γ mRNA half-life in the absence of stimulation. We observed that IFN-γ mRNA decayed with different kinetics in Bryo-1- plus IL-2-treated cells ($t_{1/2}$ = 3 h and 20 min) compared with that in Bryo- plus PMA-treated cells ($t_{1/2}$ = 55 min). These results suggest a potentially important role for post-transcriptional regulation of the IFN-γ mRNA. Importantly, the $t_{1/2}$ of IFN-γ induced by Bryo-1 plus IL-2 was much greater than the previously reported $t_{1/2}$ induced by IL-2 and PHA ($t_{1/2}$ = 1 h), IL-12 plus PHA ($t_{1/2}$ = 1 h and 15 min), or IL-2, IL-12, plus PHA ($t_{1/2}$ = 1 h and 55 min) (52). Rapid degradation of mRNAs encoding many oncogenes and cytokines is regulated in part by A+U-rich elements in their 3′-untranslated regions (53, 54). Proteins that bind to A+U-rich elements in the 3′-untranslated regions of these messages control their stability, and by doing so they also control the levels and timing of expression (53, 54). Furthermore, AU-rich sequences have been documented in the 3′-untranslated region of IFN-γ mRNA (55), strongly suggesting that IFN-γ gene expression is at least partly regulated through its mRNA stability. While at present we are unable to determine the natural half-life of the IFN-γ mRNA, our data suggest that post-transcriptional mechanisms are probably involved in the Bryo-1- plus IL-2-induced IFN-γ expression.

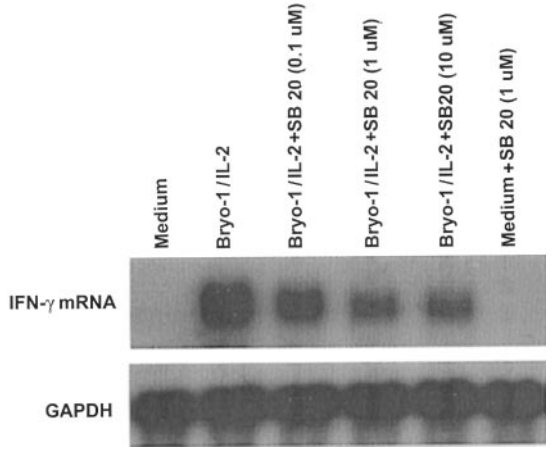

FIGURE 8. The p38 MAPK inhibitor decreases IFN-γ expression induced by Bryo-1 plus IL-2. T lymphocytes were preincubated for 20 min in the presence or the absence of increasing concentrations of SB203580 (SB20) and then cultured for 6 h in the presence or the absence of 1 ng/ml Bryo-1 plus 50 U/ml IL-2. Total cellular RNA was extracted and analyzed by Northern blot for IFN-γ mRNA expression. The same membrane was rehybridized with GAPDH probe to ensure that equal amounts of RNA were loaded in each lane. Data are from one representative experiment of two performed.

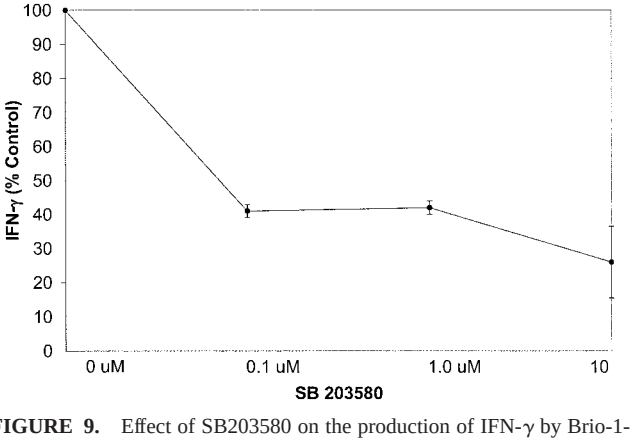

FIGURE 9. Effect of SB203580 on the production of IFN-γ by Brio-1-plus IL-2-activated lymphocytes. Cells were preincubated for 20 min in the presence or the absence of increasing concentrations of SB203580 and then cultured in the presence or the absence of 1 ng/ml Bryo-1 plus 50 U/ml IL-2. IFN-γ levels were measured by ELISA in the supernatants after 24 h of culture. Data are expressed as a percentage of the control response. Values are the mean ± SEM from three normal donors.

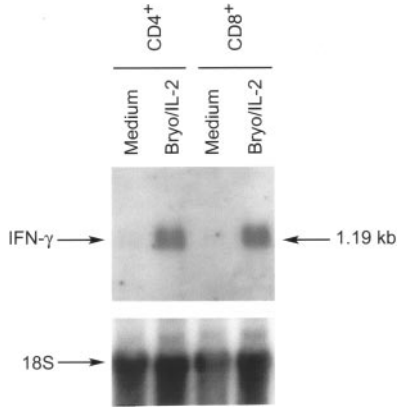

FIGURE 10. Bryo-1 plus IL-2 induce IFN-γ mRNA expression in human $CD4^+$ and $CD8^+$ T lymphocytes. Human peripheral blood $CD4^+$ and $CD8^+$ T lymphocytes were cultured for 12 h in the absence or the presence of 1 ng/ml Bryo-1 plus 50 U/ml IL-2. Total cellular RNA was extracted and analyzed by Northern blot for IFN-γ expression. The same membrane was rehybridized with 18S-DECAtemplate probe to ensure that equal amounts of RNA were loaded in each lane. Data shown are from one representative experiment of two performed.

The rapid induction of IFN-γ mRNA expression suggested a direct response independent of de novo protein synthesis. Indeed, we demonstrated that CHX treatment did not block the early induction of IFN-γ mRNA expression by Bryo-1 and IL-2. On the contrary, we observed a significant increase in IFN-γ mRNA expression in cells treated with CHX plus Bryo-1 and IL-2 compared with Bryo-1- and IL-2-treated lymphocytes. These results indicate that IFN-γ mRNA expression induced by Bryo-1 plus IL-2 might be partially controlled by a de novo synthesized repressor protein(s). This finding further confirms and extends a previous report showing that CHX superinduced IFN-γ expression in PHA-blasted peripheral mononuclear cells (52). It has been recently reported that the zinc finger transcription factor yin-yang-1 may act as a repressor of IFN-γ basal transcription (36). Future efforts in our laboratory will try to delineate the role of yin-yang-1 in IFN-γ gene expression induced by Bryo-1 and IL-2. Taken together our data from the kinetics and CHX experiments indicate that early induction of IFN-γ gene expression by Bryo-1 plus IL-2 does not require de novo protein synthesis, but it is probably down-modulated by a de novo synthesized repressor protein(s).

In an attempt to further explore the molecular mechanisms that led to the induction of IFN-γ gene expression by Bryo-1 and IL-2, the synthetic glucocorticoid DEX was used. We demonstrated that DEX produces a major inhibition of Bryo-1- plus IL-2-induced IFN-γ expression. DEX has been well characterized for its ability to block nuclear translocation of NF-κB factors (34, 35) and for interfering with binding of the transcriptional complex AP1/CREB-ATF to the IFN-γ promoter (37). NF-κB is a critical regulatory element present in the 5′-flanking region of the IFN-γ gene (56, 57), and we have recently demonstrated that Bryo-1 enhances the expression of NF-κB in human monocytic cells (C. S. Garcia et al., manuscript in preparation). Further work will be needed to investigate the roles of these transcriptional factors in the induction of IFN-γ expression by Bryo-1 plus IL-2.

Bryo-1 is known to be a potent ligand for PKC, and a large body of evidence suggests that many of its biological effects are mediated through the activation of PKC (3–5). However, Bryo-1 can antagonize several PKC-mediated effects (6, 7, 58), and recent work has indicated that Bryo-1 antitumor activity in a mouse melanoma model was exerted through a PKC-independent mechanism (59). In the present experimental model, the specific PKC inhibitor BI did not affect the Bryo-1 plus IL-2 induction of IFN-γ mRNA at either the IC_{50} of 0.01 μM or at 0.1 μM, a dose 10 times the IC_{50} (38, 39). In contrast, at concentrations 100 times the IC_{50} of BI for PKC, BI was able to block Bryo-1 and IL-2 induction of IFN-γ mRNA expression (Fig. 7*B*). It is noteworthy that this later concentration of BI not only inhibits PKC, but also inhibits other kinases such as cAMP-dependent protein kinase and phosphorylase kinase (38, 39). Overall, these findings indicate that the Bryo-1- and IL-2-induced IFN-γ gene expression occurs via PKC-independent mechanisms. Our data also suggest that other kinases, different from PKC, are key to the signal transduction cascade induced by Bryo-1 plus IL-2 leading to IFN-γ production. Since p38 MAPK has recently been involved in the ability of T cells to express IFN-γ (40, 41), we investigated the role of p38 MAPK in Bryo-1 plus IL-2 signaling. We clearly demonstrated that SB203580 inhibited the induction of IFN-γ expression in a dose-dependent manner. Although we did not measure p38 MAPK activity in our model, the efficacy and specificity of SB203580 have been extensively demonstrated in several cellular models (40–43). Taken together these results suggest that p38 MAPK activity is a fundamental component of the pathway leading to IFN-γ production by primary human lymphocytes activated with Bryo-1 plus IL-2. Future work in our laboratory will attempt to further define the signal transduction events induced by Bryo-1 in T lymphocytes.

In summary, we have demonstrated that at doses pharmacologically achievable, Bryo-1 plus IL-2 induce high levels of IFN-γ expression in primary human T cells without the need for pretreatment with PHA or other mitogenic activator(s). The present study also provides the first report dissecting the molecular mechanisms involved in the synergistic induction of IFN-γ by Bryo-1 plus IL-2. We demonstrated that in primary T lymphocytes, through a process dependent on p38 MAPK and independent of PKC activation, a dual mechanism involving transcriptional and post-transcriptional levels of regulation is responsible for the Bryo-1 plus IL-2 induction of IFN-γ gene expression and protein secretion. We

Table II. *Bryo-1 + IL-2 differentially regulate the production of IL-4 and IL-13 in human T lymphocytes*[a]

	Production (pg/ml)					
	Donor 1		Donor 2		Donor 3	
Treatment	IL-4[b]	IL-13[b]	IL-4	IL-13	IL-4	IL-13
Medium	ND[b]	ND	ND	ND	ND	ND
Bryo-1 (1 ng/ml) + IL-2 (50 U/ml)	ND	151	ND	305	ND	147

[a] Highly purified T lymphocytes from three normal donors were cultured for 24 h in medium alone or supplemented as indicated above. Cell-free supernatants were tested by ELISA for the presence of IL-4 and IL-13.
[b] ND, not detectable.

found that this combined treatment induces IFN-γ gene expression in both $CD4^+$ and $CD8^+$ T cells.

Studies in cancer patients and preclinical models have indicated that T cell responses to tumor cells are impaired. Of particular relevance, some reports have shown that in T cells from tumor-bearing animals IFN-γ production is deficient (60, 61). Several investigators have also suggested that T cells from tumor-bearing hosts have changed their cytokine production patterns from a Th1 to a Th2 pattern (25–30, 44, 45). These studies have indicated that IFN-γ and IL-12 tend to promote the development of T cells with a Th1-type pattern of cytokine expression while inhibiting the development of Th2 cells (25–30) Thus, it has been hypothesized that the failure to protect against tumor is not due to the lack of an immune response, but it is the result of the cytokine pattern deviation that impairs the proper development of an antitumor response. The present results suggest that Bryo-1 plus IL-2, by inducing a pattern of cytokine expression that is strongly skewed toward a Th1-type response (high IFN-γ/low IL-4), may play a crucial role in controlling the polarization of the immune response in a clinical therapeutic setting. Future efforts in our laboratory will try to determine whether Bryo-1 and IL-2 treatment can direct T cell differentiation toward Th1 and, if so, whether this combined treatment induces changes in the methylation status of the IFN-γ gene and/or induces the expression of the recently identified Th1 developmental factor T-bet (62).

The differential regulation of IL-4 and IL-13 by Bryo-1 plus IL-2 clearly emphasizes the complex nature of the cytokine network. IL-13 production is more ubiquitous than IL-4 production, and in agreement with our observation others have reported that human T cells that otherwise have a Th1 phenotype produce IL-13 (63–65). IL-13 shares approximately 30% homology with IL-4 and seems to have several overlapping biological activities with IL-4 (66). However, there are several functions of IL-4 that cannot be mimicked by IL-13. For instance, IL-13 transgene expression does not reverse the IgG1 deficiency observed in IL-4-deficient ($IL\text{-}4^{-/-}$) mice (46). Another major difference between IL-13 and IL-4 is the inability of the former to induce T cell proliferation, which is probably due to the lack of functional IL-13R on human T cells (47). Although it has been recently reported that IL-13 reverses the inhibitory effects of renal cell carcinoma on the functional differentiation of dendritic cells (67), at present the significance of Bryo-1- plus IL-2-induced IL-13 is not clear. Further studies are warranted to better understand the role of IL-13 in modulating the immune response against tumor.

Taking into account the well-characterized antineoplastic and immunomodulatory activity of both Bryo-1 and IL-2 and having shown in a murine model with B16-F10 melanoma cells that a combination of Bryo-1 and IL-2 had antitumor activity without significant toxicity (68), our group is currently conducting a National Cancer Institute-funded phase I clinical trial to evaluate the immune effects and toxicity of this combination in patients with cancer.

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