

# B CELL STIMULATORY FACTOR 1 (INTERLEUKIN 4) IS A POTENT COSTIMULANT FOR NORMAL RESTING T LYMPHOCYTES

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B cell stimulatory factor 1 (BSF-1)<sup>1</sup> is a 20 kD murine T cell-derived glycoprotein that was first described as a costimulant of B cell entry into S phase in response to anti-Ig antibodies (1). BSF-1 also induces resting B cells to increase their expression of class II major histocompatibility complex (MHC) molecules (2, 3) and markedly promotes the secretion of IgG1 and IgE by lipopolysaccharide (LPS)-stimulated B cells (4–7). BSF-1 has been purified to homogeneity by affinity chromatography and high pressure liquid chromatography (8, 9, and Ohara, Maloy, Coligan, Zoon, and Paul; manuscript submitted for publication) and cDNA clones for murine BSF-1 (10, 11) and for its human analogue (12) have been obtained. A monoclonal anti-BSF-1 antibody that inhibits its biological activities has been derived (13). Recently, BSF-1 has been shown to cause proliferation of long-term T cell lines, such as HT-2 cells (9, 14, 15) and to enhance the capacity of interleukin 3 to stimulate the growth of mast cell lines (14). These results indicate that the range of action of BSF-1 is not limited to B cells. The designation interleukin-4 (IL-4) has been proposed for BSF-1 (10).

In this manuscript, we show that BSF-1 together with phorbol myristate acetate (PMA) stimulates resting T cells to enter the S phase of the cell cycle and to proliferate. Its action does not require accessory cells and is density independent. Virtually all normal T cells respond to BSF-1 plus PMA as judged by increase in cell volume. BSF-1 is required early in the T cell response, suggesting that it acts on resting T cells in a manner analogous to its action on resting B cells (16–18). However, BSF-1 is also necessary in the later phase (24–48 h) of the response of T cells to BSF-1 plus PMA. BSF-1 may prove to be of considerable importance in the physiologic activation of T cells, particularly in recruitment of T cell division in the environment of activated BSF-1-producing T cells, and possibly as an autocrine stimulatory factor.

## Materials and Methods

*Animals.* Virus-free BALB/c and DBA/2 female mice, 8–12 wk of age, were obtained from Biological Testing Branch of the National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD.

<sup>1</sup> *Abbreviations used in this paper:* BSF-1, B cell stimulatory factor 1; PI, propidium iodine; PKC, protein kinase C; PMA, phorbol myristate acetate.

**Culture Medium.** RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% FCS (Biofluids, Rockville, MD), L-glutamine (2 mM), 2-ME (0.05 mM), penicillin (50  $\mu\text{g}/\text{ml}$ ), and streptomycin (50  $\mu\text{g}/\text{ml}$ ) was used for culturing cells.

**Reagents and Monoclonal Antibodies.** A23187, phorbol-12-myristate-13-acetate (PMA), and propidium iodide (PI) were purchased from Sigma Chemical Company, St. Louis, MO. Con A and Percoll were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Recombinant mouse  $\beta$  interleukin 1 (IL-1) was from Hoffman-LaRoche, Nutley, NJ. Purified mouse interleukin 3 (IL-3) was kindly provided by Dr. James Ihle, NCI-Frederick Cancer Research Facility (19). Recombinant human interleukin 2 (IL-2) was from Cetus Corporation, Emeryville, CA. Recombinant mouse IFN- $\gamma$  was generously provided by Genentech, Inc., South San Francisco, CA. Monoclonal rat IgG1 anti-BSF-1 antibody 11B11 (13) and monoclonal rat IgG2a anti-IL-2 antibody S4B6.1 (14) were used as diluted ascitic fluids. Monoclonal rat IgG2a antidinitrophenyl (DNP) antibody (50C1), prepared from cells obtained from Dr. Joseph Davie, Washington University, St. Louis, MO served as a control ascitic fluid for 11B11 and S4B6.1 (20). Goat anti-mouse IgM heavy chain-specific antibody used in panning were prepared and purified in our laboratory (21).

**Preparation of T Cells.** Purified T cells were prepared from mesenteric lymph nodes by two nylon wool column passages and subsequent cytolysis with an anti-Ia monoclonal antibody (M5114; American Type Culture Collection, Rockville, MD) (22) plus low-tox-M rabbit complement (Cederlane Laboratories, Hornby, Ontario, Canada). To obtain purified L3T4<sup>+</sup> or Lyt-2<sup>+</sup> cells, monoclonal anti-Lyt-2 antibody (23) or anti-L3T4 antibody (24) plus MAR 18.5 mouse anti-rat  $\kappa$  antibody (25) was added to the antibody-complement cocktail. In some cases, the cells were further purified by depletion of IgM-bearing cells by an anti-IgM panning method (26). Small dense resting T cells were then separated by a modified discontinuous Percoll gradient procedure (27). Purified T cells that were included in the 66–70% band were considered to be small resting T cells. In selected cases, cells were incubated with a mixture of fluoresceinated anti-L3T4 (GK1.5) and anti-Lyt-2 (53.6.7). The 20% most intensely fluorescent cells were then obtained by electronic cell sorting using an EPICS V (Coulter Electronics, Hialeah, FL).

**Preparation of BSF-1.** BSF-1 was purified from supernatant fluids (SN) of EL-4 cells that had been induced with PMA. The SN was first passed over an anti-BSF-1 affinity column and the bound material was eluted with 0.1% trifluoroacetic acid. The eluate was applied to a C18 reverse-phase high performance liquid chromatography column. BSF-1 obtained in this manner contained one major protein of 20,000  $M_r$  on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Its biological activities were inhibited by monoclonal anti-BSF-1 antibody.

One unit of BSF-1 is defined as the amount of BSF-1 required for half-maximal stimulation of [<sup>3</sup>H]thymidine uptake by resting B cells stimulated with 5  $\mu\text{g}/\text{ml}$  of goat anti-IgM antibody (1).

**Cell Culture.** Functional assays were carried out in 96-well flat-bottom Costar plates, in triplicate, with 200  $\mu\text{l}$  final volume. The plates were incubated at 37°C in a humidified 6% CO<sub>2</sub> atmosphere. DNA synthesis was determined by [<sup>3</sup>H]thymidine uptake (1  $\mu\text{Ci}/\text{well}$ , 6.7 Ci/nmol; [ICN, Irvine, CA]) after a 16-h labeling. For time-course experiments, a 4-h labeling time was used. Cells were harvested onto glass filter paper and [<sup>3</sup>H]thymidine incorporation was determined by liquid scintillation spectrometry.

**Cell Cycle Analysis.** Cells were cultured at a concentration of  $2.5 \times 10^5$  cells/ml in 24-well Costar plates. Colcemid (25 ng/ml) was added 20 h after the initiation of culture. Cultures were terminated at 48 h, and viable cell number was determined. Cells ( $10^6$ ) were pelleted and the pellet was resuspended with hypotonic PI (0.1% sodium citrate, 0.1% Triton X (vol/vol) and 0.05 mg/ml PI made up in double distilled water). DNA content was determined by measuring PI fluorescence (28, 29) using a FACS analyzer (Becton-Dickinson Immunocytometry Systems, Mountain View, CA).

**Volume Analysis.** Cell volumes were measured with a Coulter Counter (Model ZB1) and Coulter Channelizer L (C-1000; Coulter Electronics).  $4 \times 10^4$  cells were counted to obtain histograms.

**Determination of  $[Ca^{2+}]_i$ .** Intracellular free calcium concentration ( $[Ca^{2+}]_i$ ) in T cells

in response to BSF-1 and/or PMA was determined as previously described using the fluorescent calcium-binding dye Quin 2 (30).

## Results

*BSF-1 Acts on Highly Purified T Cells.* To investigate the activity of BSF-1 on T cells, mesenteric lymph node cells were purified by an extensive procedure involving two rounds of passage over nylon wool, treatment with anti-Ia antibodies and complement, and depletion of IgM-bearing cells by panning on anti-IgM-coated dishes. The resultant cells were then centrifuged on a discontinuous Percoll density gradient to obtain small cells. These were then stained with a mixture of fluoresceinated anti-L3T4 and Lyt-2 antibodies. Fluorescent cells were obtained by electronic cell sorting.

These highly purified small T cells showed a striking response to BSF-1 plus PMA, yielding incorporation of [<sup>3</sup>H]thymidine comparable to that observed in response to PMA plus the calcium ionophore A23187 (Table IA). Neither BSF-1 nor PMA alone caused any detectable DNA synthesis. It should be noted that many conventional T cell stimulants, such as Con A, fail to stimulate T cells purified to this degree. Indeed, purified T cells developed very meager responses to Con A plus PMA (Table IA).

The possibility that the costimulatory activity of the purified BSF-1 preparation might be due to contamination with IL-2 was addressed by examining the inhibitory capacity of monoclonal anti-BSF-1 and anti-IL-2 antibodies. Small BALB/c lymph node T cells were purified by passage over nylon wool treatment with anti-Ia antibodies and complement and centrifugation on a discontinuous Percoll density gradient. The response of these cells to BSF-1 plus PMA was completely inhibited by the monoclonal anti-BSF-1 antibody 11B11 but was not affected either by the monoclonal anti-IL-2 antibody S4B6.1 or by the monoclonal anti-DNP antibody 50C1 (Table IB). These results indicate that the costimulatory activity of purified BSF-1 was a property of BSF-1 itself. In other experiments, PC 61 (31), a monoclonal rat IgG1 anti-IL-2 receptor antibody failed to inhibit responses of T cells to purified BSF-1 and PMA (data not shown). These results provided further support for the conclusion that it was BSF-1 itself, and not a putative IL-2 contaminant, which was responsible for the stimulation. Furthermore, PC 61 provides a class-matched control for 11B11, which is also a rat IgG1.

Although the T cells used in these experiments were very extensively purified, it is possible that the small number of B cells that might remain contributed to this response, possibly by becoming efficient accessory cells as a result of stimulation by BSF-1. We reasoned that if B cells were essential for the T cell responses to BSF-1 plus PMA, the addition of resting B cells to a highly purified T cell population might cause striking enhancement of its response. As shown in Table IC, addition of purified B cells in amounts far in excess of the number of B cells that might possibly remain in the T cell population had no effect on the response to BSF-1 plus PMA. These experiments provide strong evidence that the BSF-1 effect is mediated by direct action on T cells.

The response of T cells to BSF-1 plus PMA was dependent on the BSF-1 concentration used. Maximal responses were obtained with 10–30 U/ml (Fig. 1)

TABLE I  
T Cells Synthesize DNA in Response to BSF-1 + PMA

A. Highly Purified T Cells		[ <sup>3</sup> H]Thymidine uptake (cpm)	
Stimulant		-PMA	+PMA (10 ng/ml)
RPMI		396	396
BSF-1 (30 U/ml)		463	108,113
A23187 (100 ng/ml)		ND	98,465
Con A (3 μg/ml)		396	3,331

B. Inhibition by Anti-BSF-1		[ <sup>3</sup> H]Thymidine uptake (cpm) in response to:		
Monoclonal antibody	Dilution	[ <sup>3</sup> H]Thymidine uptake (cpm) in response to:		
		Medium	BSF-1 (100 U/ml) + PMA	BSF-1 (1,000 U/ml) + PMA
None		280	54,100	172,200
Anti-BSF-1	1:1,000	180	200	2,700
Anti-BSF-1	1:300	210	210	550
Anti-IL-2	1:1,000	240	53,100	166,500
Anti-IL-2	1:300	190	55,900	194,600
Anti-DNP	1:1,000	180	70,500	154,300
Anti-DNP	1:300	330	69,700	146,700

C. Adding B Cells Does Not Increase the Response		[ <sup>3</sup> H]Thymidine uptake (cpm)			
Stimulant	B cells:	[ <sup>3</sup> H]Thymidine uptake (cpm)			
		0	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>4</sup>
RPMI		379	373	331	872
BSF-1 (30 U/ml)		604	361	342	1,310
PMA (10 ng/ml)		702	259	211	510
PMA + BSF-1		28,064	31,929	30,926	25,128

(A) Highly purified T cells respond to BSF-1 plus PMA. BALB/c lymph node cells were purified by two passes over nylon wool, lysis with anti-Ia + C, and panning to deplete IgM<sup>+</sup> cells. The remaining cells were then subjected to Percoll gradient centrifugation; the small resting T cells banding at the interface between 66–70% Percoll were retained. These cells were then stained with fluoresceinated anti-L3T4 and anti-Lyt-2 and fluorescent cells obtained by electronic cell sorting. Sorted cells were cultured in 96-well flat-bottom plates at  $2.5 \times 10^4$  cells/well. [<sup>3</sup>H]Thymidine was added at 48 h and the cells were harvested at 64 h of culture.

(B) Small T cells were prepared from mesenteric lymph nodes by passage over nylon wool, cytolysis with anti-Ia and C, and Percoll density gradient centrifugation. T cells were incubated at  $2.5 \times 10^4$  cells/well with medium or BSF-1 plus PMA (1 ng/ml) in the presence of ascitic fluid containing the monoclonal rat antibodies 11B11 (anti-BSF-1), S4B6.1 (anti-IL-2), or 50C.1 (anti-DNP). [<sup>3</sup>H]Thymidine was added at 48 h, and the cells were harvested at 64 h of culture.

(C) Resting BALB/c spleen cells were treated with anti-Thy-1.2, anti-Lyt-1, and anti-Lyt-2 monoclonal antibodies followed by MAR 18.5 and complement. Dense B cells were obtained by Percoll density gradient centrifugation. Resting mesenteric lymph node T cells were prepared by two passes over nylon wool, cytolysis with anti-Ia and C, panning with anti-IgM, and Percoll density gradient centrifugation. T cells were incubated at  $2.5 \times 10^4$  cells/well with added B cells, at various densities from 0 to  $10^4$  cells/well. [<sup>3</sup>H]Thymidine was added at 48 h and the cells were harvested at 64 h of culture.

indicating that T cell responses to BSF-1 require somewhat higher concentrations than do B cell responses, where costimulation with anti-IgM is half-maximal at 1 U/ml (8). Essentially equivalent T cell responses are obtained with PMA concentrations of 1 ng/ml and 10 ng/ml (data not shown).

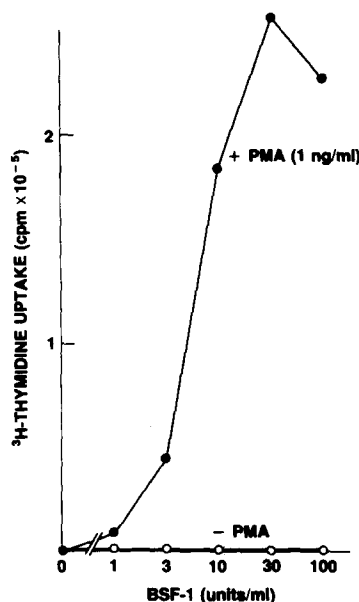


FIGURE 1. BSF-1 concentration/response curve. BALB/c mesenteric lymph node T cells were prepared by two rounds of passage over nylon wool, complement-mediated lysis to remove Ia-bearing cells, and Percoll density gradient centrifugation to obtain small, dense T cells. Cells were then cultured with various concentrations of BSF-1 at  $5 \times 10^4$  cells/well in 96-well flat-bottom plates. [ $^3\text{H}$ ]Thymidine was added at 48 h and cells were harvested at 64 h.

*BSF-1 and PMA Stimulates Both L3T4<sup>+</sup> and Lyt-2<sup>+</sup> Cells in a Density-independent Manner.* To gain further insight regarding the possible role of cellular interactions in the response of T cells to BSF-1 and PMA, we examined the density dependence of this response. Purified small T cells were cultured at densities from  $7.8 \times 10^2$  cells/well to  $2.5 \times 10^4$  cells/well. Over this range the T cell response to BSF-1 plus PMA was essentially density independent (Fig. 2). The slope of the line relating the log of [ $^3\text{H}$ ]thymidine incorporation to the log of the number of cells cultured was  $0.96 \pm 0.12$ , with a correlation coefficient ( $r^2$ ) of 0.97. Even at the lowest density ( $7.8 \times 10^2$  cells/well), BSF-1 plus PMA caused a significant stimulation of T cells over the response to medium, BSF-1 alone, or PMA alone. This result strongly suggests that the T cell response to BSF-1 plus PMA does not depend on accessory cells, since the interaction of two or more cell types should be expected to yield a line with a log cell number/log response slope  $>1.0$ . Indeed, the T cells used in this experiment had been purified to an extent that the actual number of non-T cells in populations cultured at the lowest density ( $7.8 \times 10^2$  cells/well) was very small, probably  $<20$ .

In the experiment shown in Fig. 2, *right*, purified T cells treated with anti-Lyt-2 and C (L3T4<sup>+</sup>) or with anti-L3T4 and C (Lyt-2<sup>+</sup>) were cultured at various densities with BSF-1 plus PMA. For both populations, the slope relating the log cell number and the log response approached 1. Lyt-2<sup>+</sup> cells responded approximately threefold more vigorously than L3T4<sup>+</sup> cells. It is very unlikely that the response of the L3T4<sup>+</sup> cells could be due to residual Lyt-2<sup>+</sup> T cells, because this

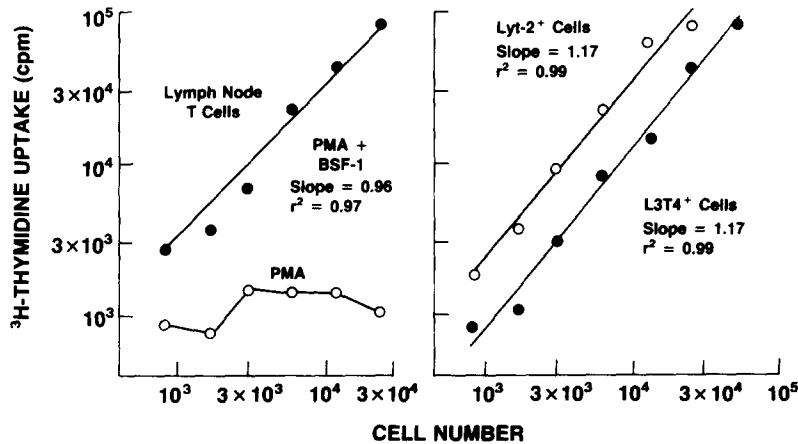


FIGURE 2. T cell responses to PMA + BSF-1 are density independent. Small dense BALB/c T cells (*left*), L3T4<sup>+</sup> and Lyt-2<sup>+</sup> DBA/2 T cells (*right*) were cultured with BSF-1 (30 U/ml) and PMA (1 ng/ml) at various densities for 64 h. [<sup>3</sup>H]Thymidine was added for the last 16 h of culture. Cell number per well and [<sup>3</sup>H]thymidine uptake (cpm) were plotted on a log/log scale. Slopes and correlation coefficients ( $r^2$ ) were determined by linear regression analysis. Controls for response to BSF-1 plus PMA were PMA alone (1 ng/ml), BSF-1 alone (30 U/ml), and medium only. The PMA alone curve for BALB/c T cells is shown for illustration; each of the other control responses were comparable.

would require a content of one-third Lyt-2<sup>+</sup> T cells in the L3T4<sup>+</sup> population. This is a proportion of Lyt-2<sup>+</sup> T cells that exceeds their frequency in the starting cell population, where it is 15–20%.

**BSF-1 Plus PMA Leads to T Cell Growth.** The effect of BSF-1 plus PMA on resting T cell populations is not limited to causing entry into S phase, but also causes actual proliferation of these cells. Small T cells were cultured with BSF-1 plus PMA for 5–6 d and the yield of living cells and [<sup>3</sup>H]thymidine incorporation was measured on each day. The number of viable cells recovered from cultures containing BSF-1 plus PMA fell over the first two days from  $2.5 \times 10^4$  (the number of cells seeded) to  $1.1 \times 10^4$  (Fig. 3). Thereafter, the yield of living cells increased with a doubling time of  $\sim 24$  h, reaching  $7.6 \times 10^4$  cells/well on day 5. The yield of living cells from cultures containing PMA plus A23187 was essentially the same as that from cultures containing BSF-1 plus PMA. Cultures containing medium alone showed progressive cell death over the duration of the culture, reaching  $0.4 \times 10^4$  cells/well on day 5. PMA accelerated the rate of cell loss. Strikingly, cultures treated with BSF-1 alone maintained good viability over the duration of the culture, falling from  $2.5 \times 10^4$  cells/well at the outset to  $1.5 \times 10^4$  at day 5. Since these cells incorporated essentially no thymidine over this period, we conclude that treatment of resting T cells with BSF-1 alone maintained their viability. In turn, this indicates that BSF-1 acts on resting T cells.

This viability-promoting activity of BSF-1 does not appear to be associated with any gross activation of the T cells. Over a 48-h period, T cells cultured with BSF-1 alone remain small. Fig. 4 shows results of a 24-h culture of T cells. Purified small T cells cultured in medium alone or in BSF-1 are a homogeneous population of small cells at 24 h (median cell volume,  $113$ – $120 \mu\text{m}^3$ ). PMA causes a small increase in the volume of these cells, but BSF-1 plus PMA causes a

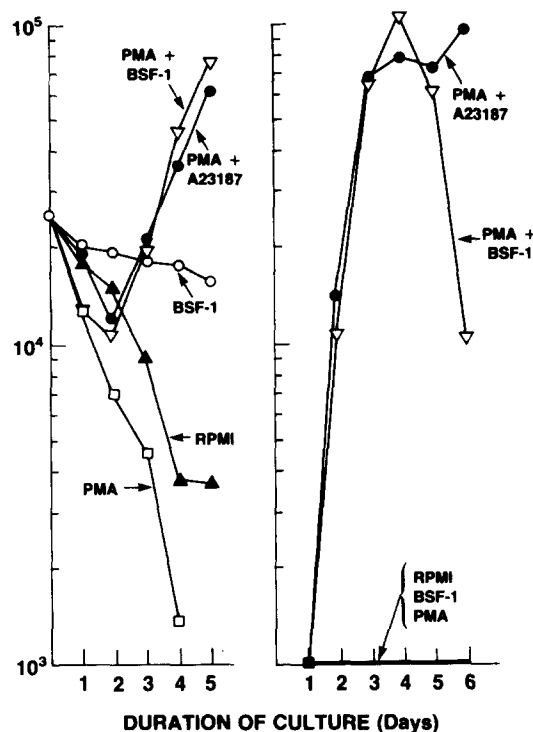


FIGURE 3. T cell responses to PMA + BSF-1 and to BSF-1. Dense BALB/c T cells were cultured at  $2.5 \times 10^4$  cells/well in medium alone (RPMI), BSF-1 (30 U/ml), PMA (1 ng/ml), PMA plus BSF-1, or PMA plus A23187 (100 ng/ml). Yield of viable cells (left) and uptake of [ $^3$ H]thymidine during a 4-h pulse (right) are shown.

considerably more striking increase; such T cells have a median volume of  $196 \mu\text{m}^3$ . What is particularly interesting is that the entire size histogram of the T cell population treated with BSF-1 plus PMA is displaced in comparison to that of the T cell population treated with PMA alone, implying that virtually all resting T cells are responsive to costimulation by these agents.

Further demonstration that BSF-1 plus PMA acts on a significant proportion of T cells was obtained by measuring the percent of T cells in the S, G<sub>2</sub>, and M phases of the cell cycle at 48 h after stimulation with BSF-1 plus PMA (Table II). At this time, 58% of the surviving cells are in S, G<sub>2</sub>, or M, emphasizing that the action of BSF-1 plus PMA is not confined to a small subpopulation of T cells.

*BSF-1 Is Required Both in the Early and Late Phases of the Initial Response of T Cells.* BSF-1 has been shown to act on resting B cells to enhance expression of class II MHC molecules (2, 3) and to prepare these cells to enter S phase more promptly in response to high concentrations of anti-Ig antibodies or to LPS (16–18). We therefore tested the time requirements of BSF-1 and of PMA in the response of resting T cells. T cells were cultured for 24 h in medium, BSF-1, PMA, or BSF-1 plus PMA. To the cells precultured in medium for 24 h, medium, PMA, BSF-1, or BSF-1 plus PMA were added. These cells failed to respond to PMA or to BSF-1. They did respond to PMA plus BSF-1 (Fig. 5), but only at 48 h of secondary culture, indicating that the initial cohort of cells responding to

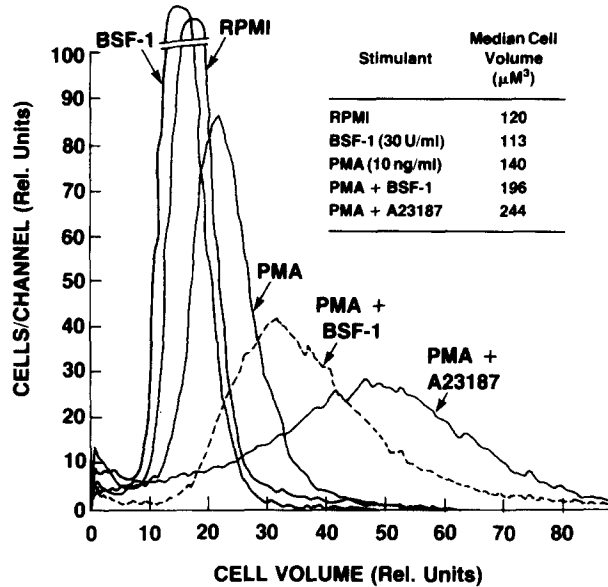


FIGURE 4. BSF-1 plus PMA causes T cell enlargement. Dense BALB/c T cells were cultured for 24 h at  $2.5 \times 10^5$  cells/ml/well in medium (RPMI), BSF-1 (30 U/ml), PMA (10 ng/ml), BSF-1 plus PMA, or PMA plus A23187 (100 ng/ml). Cell volume was measured at the termination of culture.

TABLE II  
Cells in S, G<sub>2</sub>, and M Phases at 48 h in Response to BSF-1 + PMA

Stimulant	Cells in S, G <sub>2</sub> and M (%)
RPMI	4.0
BSF-1 (30 U/ml)	7.2
PMA (10 ng/ml)	10.9
BSF-1 + PMA	57.9
A23187 (100 ng/ml) + PMA	63.2

Small resting T cells were prepared by two rounds of passage over nylon wool, treatment with anti-Ia plus C, and Percoll density centrifugation. These cells were cultured at  $2.5 \times 10^5$  cells/ml for 48 h with various stimulants. Colcemid was added 20 h after initiation of assay. Cells were pelleted at  $10^6$  cells/tube and resuspended in hypotonic PI. Suspended nuclei were analyzed for DNA content after 24 h at 4°C.

BSF-1 plus PMA does not enter S phase until after 24 h. By contrast, cells precultured in BSF-1 plus PMA and then cultured with the same stimulants during the secondary culture showed a striking response at 24 h and an even greater response at 48 h. We then asked whether preculture in either BSF-1 or in PMA would prepare cells to respond at 24 h of secondary culture in BSF-1 plus PMA. As shown in Fig. 5, neither BSF-1 or PMA preculture accelerated the response of these cells to PMA plus BSF-1, indicating that both agents were required from the outset of the culture. Thus, BSF-1 is required early in the first round of T cell proliferation to PMA plus BSF-1.

BSF-1 is also required in the latter phases of the T cell response to BSF-1 plus



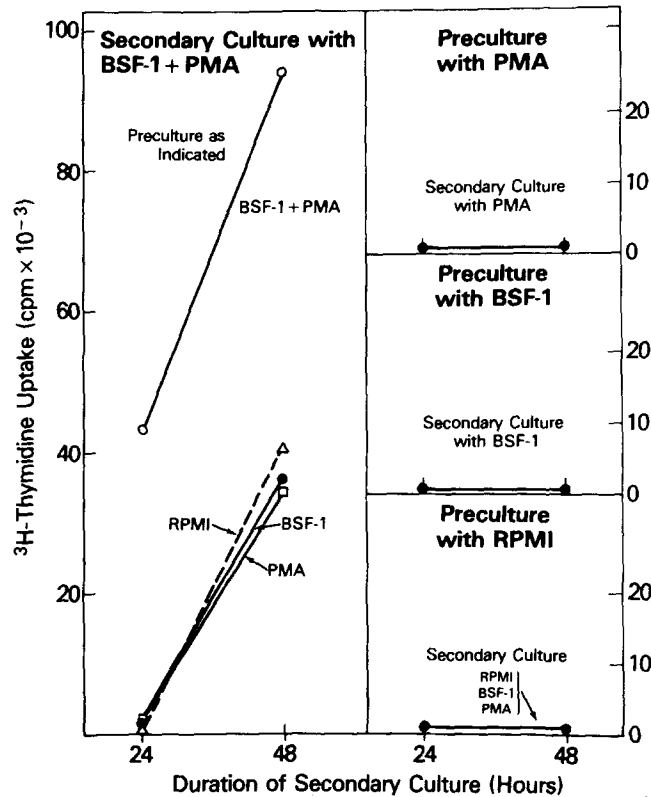


FIGURE 5. Both BSF-1 and PMA are required early in culture. Dense BALB/c T cells were precultured for 24 h at  $2.5 \times 10^4$  cells/well, in medium (four sets of replicates), BSF-1 (30 U/ml) (two sets of replicates), PMA (1 ng/ml) (two sets of replicates) or BSF-1 plus PMA. Medium, BSF-1, PMA, or BSF-1 plus PMA was added to cells precultured in medium; medium or PMA was added to cells precultured in BSF-1; medium or BSF-1 was added to cells precultured in PMA; medium was added to cells precultured in BSF-1 plus PMA. In each of these cultures, the additive(s) present in the preculture was also present in the secondary culture because the cells were not washed. Cells were pulsed for 4 h with [ $^3$ H]thymidine before harvest at 24 or 48 h of secondary culture.

PMA. Removal of BSF-1 from cultures after 24 h inhibits entry into S phase. T cells were precultured for 24 h with medium alone or with PMA plus BSF-1. After 24 h, these cells were washed and recultured for 24 h with medium, PMA, BSF-1, or PMA plus BSF-1. These cells were then incubated with [ $^3$ H]thymidine for 16 h before harvest. Cells pretreated for 24 h with PMA plus BSF-1 do not take up [ $^3$ H]thymidine in secondary culture with medium alone, or with PMA (Table III). By contrast, they show striking responses to BSF-1 plus PMA and to BSF-1 alone. This result indicates the need for BSF-1 at times beyond the first 24 h of culture. The fact that PMA did not have to be added in the second culture does not establish a lack of requirement for PMA at that point in the response, since PMA is poorly removed by washing. Nonetheless, because prolonged PMA treatment leads to a depletion of cellular protein kinase C in many systems (32), it is quite possible that PMA, even if present, exerts relatively little action after 24 h. Cells pretreated in medium alone make a modest response to BSF-1 plus PMA in secondary culture, but this is far less than that of cells

TABLE III  
*BSF-1 Is Required Late in Initial Response to BSF-1 + PMA*

Secondary culture (24-h + 16-h pulse)	<sup>3</sup> H]Thymidine uptake after 24-h preculture	
	Medium	BSF-1 + PMA
	<i>cpm</i>	
RPMI	2,161	1,205
PMA (10 ng/ml)	628	1,324
BSF-1 (30 U/ml)	4,846	80,684
BSF-1 + PMA	25,184	146,690

Resting T cells were precultured at  $2.5 \times 10^5$  cells/well in 24-well Costar plates in either culture medium alone or BSF-1 (30 U/ml) plus PMA (10 ng/ml) for 24 h. The cells were then washed two times. Viable cells were recultured, with various stimuli, at  $2.5 \times 10^4$  cells/well for 24 h and labeled for an additional 16 h with [<sup>3</sup>H]thymidine.

TABLE IV  
*Other Lymphokines Do Not Replace PMA as a Costimulant  
 with BSF-1*

Stimulant	<sup>3</sup> H]Thymidine incorporation	
	Without BSF-1	With BSF-1 (30 U/ml)
	<i>cpm</i>	
Medium	448	1,264
PMA (1 ng/ml)	524	53,713
IL-1 (10 U/ml)	186	760
IL-1 + PMA	312	56,761
IL-2 (30 U/ml)	690	2,681
IL-2 + PMA	84,837	155,463
IL-3 (30 U/ml)	279	894
IL-3 + PMA	262	64,690
IFN- $\gamma$ (100 U/ml)	223	938
IFN- $\gamma$ + PMA	411	62,894

Dense BALB/c T cells were cultured either in culture medium or in BSF-1 (30 U/ml) at  $5 \times 10^4$  cells/well with various stimuli. [<sup>3</sup>H]Thymidine uptake was measured at between 48 and 64 h of culture.

precultured in PMA plus BSF-1, indicating that the response measured in the latter case is not simply a response commencing at the onset of the secondary culture. These results suggest that BSF-1 is required both in the early and later portions of the G<sub>1</sub> phase of the first cell cycle in the response of resting T cells to BSF-1 and PMA.

Neither IL-1, IL-3, or IFN- $\gamma$  synergizes with BSF-1 or with PMA in the stimulation of resting T cells, nor did these agents inhibit the response to BSF-1 plus PMA (Table IV). IL-2 also failed to synergize with BSF-1. However, IL-2 showed striking synergy with PMA, and this response was additive with the BSF-1 plus PMA response.

*BSF-1 Does Not Increase [Ca<sup>2+</sup>]<sub>i</sub> in Resting T Cells.* The fact that BSF-1 plus PMA causes responses comparable to those obtained with PMA plus the calcium

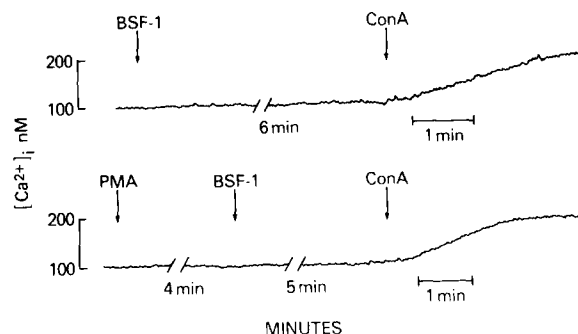


FIGURE 6. BSF-1 does not cause increased  $[Ca^{2+}]_i$  in T cells. Dense BALB/c T cells were loaded with Quin 2 methyl ester. Cells ( $6 \times 10^6$  in 3 ml) were stimulated with BSF-1 (30 U/ml) or PMA (10 ng/ml) followed by BSF-1. Con A (10  $\mu$ g/ml) was added 6 or 5 min after BSF-1.  $[Ca^{2+}]_i$  was calculated based on fluorescence at 492 nM.

ionophores A23187 (Table I and Fig. 3) or ionomycin (data not shown) naturally raises the possibility that BSF-1 functions in resting T cells by elevating  $[Ca^{2+}]_i$ . We measured  $[Ca^{2+}]_i$  in resting T cells treated with BSF-1 alone or with BSF-1 plus PMA and found no elevation (Fig. 6). By contrast, Con A increases  $[Ca^{2+}]_i$  in both untreated and PMA-treated cells, but shows poor synergy with PMA for T cell entry into S phase (Table I). This strongly suggests that BSF-1 costimulatory effects are not mediated in a manner entirely analogous to those of the calcium ionophore.

### Discussion

In this communication, we show that the T cell-derived lymphokine BSF-1 synergizes with PMA in the stimulation of resting normal T cells. This response can be obtained with highly purified populations of small T cells cultured at very low cell density (<1,000 cells/well). Furthermore, the slope relating the log of cell number cultured and the log of the response is  $\sim 1$ . Both results strongly suggest that the costimulatory activity of BSF-1 plus PMA does not depend upon an accessory cell. Thus, BSF-1 and PMA, acting together, are sufficient for the activation of resting T cells.

Essentially all T cells are responsive to BSF-1 plus PMA, as indicated by the finding that virtually all cells treated with this combination of stimulants have enlarged by 24 h. A substantial fraction of the stimulated cells enter S phase.  $Lyt-2^+$  cells incorporate more  $[^3H]$ thymidine in response to BSF-1 plus PMA than do  $L3T4^+$  cells, but we have not established that the fraction of  $Lyt-2^+$  T cells that enter S phase in response to BSF-1 plus PMA exceeds the fraction of  $L3T4^+$  cells that mount such a response.

T cell activation has been proposed to involve the elevation of  $[Ca^{2+}]_i$  and the stimulation of protein kinase C (PKC)-catalyzed protein phosphorylation (33, 34). PMA and calcium ionophore stimulation of T cells is in keeping with this concept, since PMA activates PKC (35, 36) and calcium ionophores elevate  $[Ca^{2+}]_i$ , but it is more difficult to use this model to explain how BSF-1 synergizes with PMA in stimulating T cells. Over short periods of time, we have observed no elevation of  $[Ca^{2+}]_i$  in response to BSF-1 alone or to BSF-1 plus PMA. By

contrast, T cells do show elevation of  $[Ca^{2+}]_i$  to Con A, despite the fact that their incorporation of  $[^3H]$ thymidine in response to Con A and PMA is much poorer than to BSF-1 plus PMA. We cannot fully exclude the possibility that BSF-1 might cause a very slow but prolonged increase in  $[Ca^{2+}]_i$ . However, BSF-1 also fails to cause increases in  $[Ca^{2+}]_i$  in B cells, and some BSF-1 effects in B cells can be obtained in calcium-deficient media (37). These results strongly suggest that elevation of  $[Ca^{2+}]_i$  is not required for T cell activation in response to BSF-1 plus PMA. The lack of requirement for  $[Ca^{2+}]_i$  elevation in lymphocyte activation is not without precedent, since the response of B cells to lipopolysaccharide (LPS) is not associated with increased  $[Ca^{2+}]_i$  or with increased inositol phospholipid metabolism (38, 39). However, an important difference here is that the LPS response in B cells also appears to be independent of PKC (Mizuguchi and Paul, unpublished observations), whereas BSF-1 effects on T cells depend upon PMA and, presumably, upon protein phosphorylation catalyzed by PKC. Thus, the capacity of BSF-1 plus PMA to stimulate efficient, accessory cell-independent activation of resting T cells raises questions about how accessory cells act in T cell stimulation and about the central role of  $[Ca^{2+}]_i$  elevation in the responses. One possible explanation for this result is that the response to PMA plus A23187 requires both an induction of sensitivity to lymphokine, such as IL-2 or BSF-1, and the stimulation of lymphokine production. In the response to BSF-1 plus PMA and to IL-2 plus PMA (discussed below), it is possible that only sensitivity to lymphokine need be induced, since BSF-1 or IL-2 is supplied exogenously. This leads to the conclusion that induction of sensitivity to lymphokine may be due to the action of PKC (or possibly through the action of another cellular target of PMA) without any requirement for increase in  $[Ca^{2+}]_i$ . An important issue is to identify the natural ligand(s) that replaces PMA in its synergy with BSF-1 and with IL-2. Although receptor crosslinking agents are obvious candidates, their role in replacing PMA has not been established.

In the course of this work, we also noted that PMA plus IL-2 caused resting T cells to become activated and to enter S phase. Like BSF-1, IL-2 is required during the first 24 h of the response (Hu-Li and Paul, unpublished observations) suggesting that it acts on  $G_0$  or, more likely, early  $G_1$  T cells. One possible mode of stimulation by this combination of agents would be the induction by PMA of a small number of IL-2 receptors and the upregulation of receptor number through the action of IL-2 (40). Such cells might then be stimulated to enter S phase by the binding of IL-2 to the heightened number of receptors present in late  $G_1$ .

BSF-1 has been shown to act directly on resting B cells (2, 3, 16–18). We show here that BSF-1 can act on resting T cells. Thus, the addition of BSF-1 to resting T cells causes striking increases in cell viability detectable by 2 d and persisting for periods of 5 d or more without any evidence of cell division. In addition, BSF-1 is required early in the costimulation of T cells; delaying the addition of BSF-1 by 24 h to cells cultured with PMA delays entry into S phase by 24 h. This strikingly parallels the action of BSF-1 on B cells, where delaying its addition to B cells cultured with anti-IgM delays their entry into S phase (18).

These results indicate that BSF-1 acts on resting T cells and thus raise the question of how BSF-1 may regulate T cell growth. Two major possibilities exist.

BSF-1 may function by acting on resting T cells, together with PMA, to induce IL-2 receptor expression and IL-2 production. This could be restated to say that BSF-1 plus PMA jointly act as a competence factor for resting ( $G_0$ ) T cells and that IL-2 acts as in late  $G_1$  to stimulate entry into S phase (i.e., as a progression factor). We have already proposed a similar model for the action of BSF-1 on B cells, where we suggest that BSF-1 and anti-IgM jointly act as a  $G_0$  and/or early  $G_1$  competence factor (18). Recently, BSF-1 has been shown to enhance the responses of some mast cell lines to IL-3 (14). Peschel, Paul, Ohara, and Green (manuscript submitted for publication) have shown that BSF-1 synergizes with recombinant erythropoietin and with IL-1 for the formation of megakaryocytic colonies in soft agar, with erythropoietin for the formation of erythroid colonies, and with granulocyte colony-stimulating factor for the formation of granulocytic colonies. Based on these observations, we suggest that BSF-1 may act as a competence factor (or a competence cofactor) for virtually all hematopoietic lineage cells.

A second possibility is that BSF-1 may act both as a lymphocyte activating factor and as a progression factor. According to this concept, rather than stimulating T cell responses by inducing IL-2 receptor expression and IL-2 production, BSF-1 causes resting T cells to enter S phase by acting with PMA to activate these cells and to render them sensitive to the growth stimulating activity of BSF-1 itself. BSF-1 would thus act as a cocompetence factor and as a progression factor. Indeed, it has been shown that many T cell lines fail to secrete IL-2 but do produce BSF-1 (41). The growth of one such line, D10, in response to antigen and antigen-presenting cells is inhibited by monoclonal antibodies to BSF-1, but not by antibody to the IL-2 receptor (42). Similarly, Fitch and colleagues (43) have described a cloned T cell line the growth of which was stimulated by antigen and antigen-presenting cells and did not require the addition of IL-2. Nevertheless, this line did not secrete detectable IL-2; IL-2 mRNA was not found by hybridization to dot blots, and antibody to the IL-2 receptor did not block its responses to antigen and antigen-presenting cells. These results suggest that lymphokines other than IL-2 can act as T cell progression factors. Indeed, finding that an anti-IL-2 antibody (S4B6.1) fails to inhibit responses to BSF-1 plus PMA suggests that IL-2 is not required for all growth in response to BSF-1 plus PMA.

In our opinion, understanding the mode of action of BSF-1 on T cells and relating it to that of IL-2 is a goal of considerable importance. Direct studies aimed at this issue are now in progress in our laboratory.

Finally, we note that IFN- $\gamma$  does not block the actions of BSF-1 plus PMA on resting T cells. IFN- $\gamma$  has been shown to be a powerful inhibitor of the action of BSF-1 on resting B cells, blocking both preparation to enter S phase (44) and induction of class II molecules (45). This result indicates that the inhibitory effect of IFN- $\gamma$  is cell type-specific and not general.

### Summary

Resting T cells proliferate in response to B cell stimulatory factor 1 (BSF-1; interleukin 4) plus phorbol myristate acetate (PMA). This response is obtained with highly purified T cells and is density independent, suggesting that accessory

cells are not required. Both L3T4<sup>+</sup> and Lyt-2<sup>+</sup> T cells respond to BSF-1 plus PMA.

Although BSF-1 alone does not cause T cell proliferation, it maintains the viability of small, dense T cells, indicating that it acts on resting T cells. Furthermore, BSF-1 is required early in the proliferative response of resting T cells to BSF-1 plus PMA, further supporting the concept that it acts on G<sub>0</sub> or early G<sub>1</sub> cells. However, BSF-1 is also needed late in the first round of division of T cells stimulated with BSF-1 plus PMA. Removing BSF-1 at 24 h of stimulation prevents entry into S phase. These results indicate that BSF-1 is involved in both the induction of competence and in the progression phases of T cell division.

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