B CELL STIMULATORY FACTOR 1 (BSF-1) PREPARES RESTING B CELLS TO ENTER S PHASE IN RESPONSE TO ANTI-IgM AND LIPOPOLYSACCHARIDE

By EVELYN M. RABIN,* JAMES J. MOND,‡ JUNICHI OHARA,* and WILLIAM E. PAUL*

*From the *Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892; and the ‡Department of Medicine, Uniformed Services University of Health Sciences, Bethesda, Maryland 20814*

B cell stimulatory factor 1 (BSF-1)[1] is a 20,000 M_r T cell product that acts as a costimulant with anti-Ig antibodies to cause entry of B cells into the S phase of the cell cycle (1). This factor, initially designated B cell growth factor (BCGF or BCGF-I) (2) has recently been shown to act on small, resting B cells. It causes increases in cell volume (3, 4), marked enhancement in the expression of class II MHC molecules (5, 6), and preparation for a more prompt entry into S phase in response to subsequent culture with anti-IgM and BSF-1 (3, 7). Recently, BSF-1 has also been shown to induce switching in Ig class expression in LPS-stimulated B cells, which leads to the secretion of IgG1 by these cells (8–10).

The multiple outcomes of BSF-1 action on B cells and the indication that the resting and/or early G_1 phase B cell is a principal target of BSF-1 activity make important a careful examination of the requirements for BSF-1 stimulation of resting B cells and of the stimulants to which BSF-1-prepared B cells respond. In this study, we describe the requirements for BSF-1 action on resting B cells and show that anti-Lyb-2.1 mAb mimics these preparative effects. We show that BSF-1 by itself causes some DNA synthesis by B cell blasts prepared by stimulating B cells with anti-IgM and BSF-1. Our results indicate that BSF-1 has a principal role as an activation factor for resting B cells, but that it may function throughout the G_1 phase of the cell cycle.

Materials and Methods

Mice. Specific, pathogen-free female BALB/c, DBA/2, and (C57BL/6 × DBA/2)F_1 [BDF_1] mice were obtained from Charles River Breeding Laboratories, Wilmington, MA, and were used at 8–10 wk of age.

Culture Medium. RPMI 1640 medium (Biofluids Inc., Rockville, MD) supplemented with 10% FCS (Reheis, Kankakee, IL), penicillin (50 μg/ml), streptomycin (50 μg/ml), L-

J. J. Mond was supported, in part, by National Institutes of Health grant R01 A121328-01, Office of Naval Research grant N00014-WR24108, National Science Foundation grant PCM-8217009, and Armed Forces Radiobiology Research Institute grant 840026. This investigation is part of a dissertation submitted by E. M. Rabin in partial fulfillment of the requirement for the degree of Doctor of Philosophy, Department of Zoology, University of Maryland, College Park, Maryland 20742.

[1] *Abbreviations used in this paper:* BSF-1, B cell stimulatory factor 1; PDGF, platelet-derived growth factor; RP-HPLC, reverse-phase HPLC; SN, supernatant; TFA, trifluoroacetic acid.

glutamine (2 mM), and 2-ME (0.05 mM) was used in these experiments and is referred to as medium.

Antibodies and Mitogen. Goat anti-mouse IgM heavy chain-specific antibody, prepared and purified as described (11), was generously provided by Drs. W. Tsang and J. Mizuguchi, Laboratory of Immunology, NIAID. This antibody was also conjugated to CnBr-activated Sepharose 4B (10 mg antibody per gram of Sepharose) and was used in culture at 0.2–1.0 μg antibody per 0.2 ml. Hybridoma supernatants containing Bet-2, a rat anti-mouse IgM heavy chain-specific mAb, were prepared as described (12).

Monoclonal rat IgG1 anti-BSF-1 antibody, 11B11 (13), was used as diluted ascitic fluid. Ascitic fluid of a rat IgG2a anti-DNP antibody, 50C1, prepared from cells originally obtained from Dr. Joseph Davie (Washington University School of Medicine, St. Louis, MO), was used as a control for 11B11 (14). Purified anti-Lyb 2.1 mAb was prepared as described by Subbarao and Mosier (15). LPS W extracted from *Escherichia coli* 0111:B4 was obtained from Difco Laboratories, Inc. (Detroit, MI).

Preparation of B Cells. Enriched populations of B cells were obtained from spleen cells from which T cells had been eliminated by the method of Leibson et al. (16). Small, dense B cells were separated by the modified (3) Percoll gradient centrifugation procedure of DeFranco et al. (17). Cells forming a band between 66 and 70% Percoll and having a density of 1.081–1.086 g/ml were considered to be resting B cells. Cells banding between 50 and 60% Percoll were considered to be in vivo–generated blasts.

Preparation of B Cell Blasts. Small, dense B cells prepared as described above were stimulated with anti-IgM (5 μg/ml) plus BSF-1 (10 U/ml) for 20 h. Percoll density-gradient centrifugation was used to isolate blasts from nonactivated cells. Blasts formed a band between 50 and 60% Percoll.

Preparation of BSF-1. BSF-1 was partially purified from supernatants (SN) of EL-4 cells by a reverse-phase HPLC (RP-HPLC) technique, as described previously (18). Briefly, a large batch of serum-free SN from EL-4 cells that had been induced with PMA was adsorbed to trimethylsilylated-controlled pore glass beads (Sepralyte; Analytichem International, Inc., Harbor City, CA). The eluted material was applied to a C18 column (μ Bondapak, Waters Associates, Milford, MA) and a gradient of acetonitrile (40–60%) in 0.1% trifluoroacetic acid (TFA) was used for elution. BSF-1 is generally recovered in the 45–47% acetonitrile fraction. This material is enriched ~2,600-fold in comparison with its activity in serum-free SN of induced EL-4 cells (18). In some experiments, we used BSF-1 purified essentially to homogeneity. SN fluids from PMA-induced EL-4 cells were passed over an anti-BSF-1 column, the bound material was eluted with 0.1% TFA, and the eluate was applied to a C18 RP-HPLC column. The material obtained contained one principal protein of 20 M_r on SDS-PAGE (J. Ohara et al., manuscript in preparation). All of the biological activities of both BSF-1 preparations are inhibited by anti-BSF-1 mAbs (3, 10, 13); we have not observed any differences in the stimulatory activities of these preparations. A unit of BSF-1 is defined as the concentration required to cause half-maximal stimulation of [^{3}H]thymidine uptake by resting B cells in the anti-IgM costimulation assay (1).

Cell Culture. For the 24 h preculture, cells were incubated at 10^6 cells per ml per well in 24-well Costar plates (3524; Costar, Cambridge, MA) in medium alone or in medium to which BSF-1 or anti-Lyb-2.1 had been added. After this preincubation, cells were washed twice in HBSS, resuspended in medium, and counted. For the secondary culture, 10^5 viable cells were plated per 0.2 ml per well in flat-bottomed microtiter plates (3596; Costar).

In some experiments, cells were cultured at 10^5 cells per well (0.2 ml) in microtiter plates for both the preculture and the secondary culture periods. 11B11 was added to the well at the end of the preculture period. This had the same effect as washing in the above procedure; it eliminated BSF-1 activity in the secondary culture. Stimulants were also added in small volumes (10 μl/well) at this time.

DNA synthesis was determined by measuring [^{3}H]thymidine incorporation (1 μCi per well, 6.7 Ci/mmol; ICN, Irvine, CA) after a 6 h labeling period. Cultures were harvested onto glass fiber filters using a PHD automated harvester (Cambridge Technology, Inc.,

TABLE I

Preculture with BSF-1 Prepares B Cells to Synthesize DNA in Response to Various Stimuli

Secondary culture conditions*	Preculture conditions					
	BALB/c		DBA/2		BDF1	
	Medium‡	BSF-1	Medium	BSF-1	Medium	BSF-1
Medium	671§	1,031	771	1,358	1,388	2,447
BSF-1 + anti-IgM (5 μg/ml) or + Bet-2‖	4,030	25,798	2,608	15,207‖	4,856	29,866‖
Anti-IgM (50 μg/ml)	2,548	22,223	ND	ND	ND	ND
Anti-IgM beads	4,941	22,863	17,22	57,153	13,456	66,529
LPS (50 μg/ml)	20,287	20,976	25,760	51,946	38,772	99,043

* Cells were washed twice after the preincubation period and cultured at 10^5 viable cells per 0.2 m per well for 34 h.

‡ Spleen cells from BALB/c, DBA/2, or BDF1 mice were treated with anti-Thy-1.2, anti-Lyt-1, and anti-Lyt-2 mAbs followed by mouse anti-rat κ mAb and C. Cells separated by Percoll density gradient centrifugation and banding between 66 and 70% Percoll were cultured at 10^6 cells/ml/well for 24 h in medium or in BSF-1 (10 U/ml).

§ [^{3}H]Thymidine uptake (cpm) was measured at 28–34 h of secondary culture period.

‖ Bet-2, rat anti-mouse IgM mAb.

Cambridge, MA). Filters wee assayed for radioactivity in a model LS3801 scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Measurement of numbers of cells in the S + G_2 and M phase of the cell cycle was carried out as previously described (17, 21, 22).

Results

BSF-1 Prepares Cells to Respond to Many Stimuli. We have previously shown that pretreatment of small resting B cells with purified BSF-1 prepares them to respond more promptly to anti-IgM (5 μg/ml) and BSF-1 than B cells precultured in medium alone. We wished to determine whether preculture with BSF-1 would prepare B cells to respond more promptly to other stimuli. Small, dense B cells recovered from Percoll gradient separation were cultured at 10^6 cells per ml per well for 24 h in medium alone or in BSF-1 (10 U/ml). After the preculture period, cells were washed twice and counted. 10^5 viable cells, as determined by trypan blue exclusion, were incubated for the secondary culture period in microtiter wells in medium alone or in various B cell mitogens. The stimulants used in secondary cultures were BSF-1 (10 U/ml) plus anti-IgM (5 μg/ml), anti-IgM (50 μg/ml), anti-IgM coupled to Sepharose beads, and LPS (50 μg/ml) (Table I). Cells were labeled with [^{3}H]thymidine for a 6 h period, beginning 28 h after initiation of the secondary culture, and they were harvested at 34 h. Cells precultured in BSF-1 responded much more strikingly at 28–34 h to anti-IgM plus BSF-1, to anti-IgM (50 μg/ml), and to anti-IgM beads than did cells precultured in medium. Preculture with BSF-1 thus speeds the pace of their subsequent response to all anti-IgM-based stimulants. Furthermore, previous work had shown that anti-IgM (50 μg/ml) caused very meager responses at cell densities of 10^5 per well or less. Thus, preculture with BSF-1 also allows responses to anti-IgM at lower cell densities. BSF-1 also prepared B cells from DBA/2 and BDF_1 mice to respond more promptly to LPS. However, LPS causes substantial responses at 28–34 h in B cells pretreated with medium alone, so that the effect

TABLE II
BSF-1 Preparation for Entry into S Phase: Analysis by Measurement of Cellular DNA

Pretreatment (24 h)	Net percent cells in S plus G_2 and M in response to anti-IgM and BSF-1	
	36 h	48 h
RPMI	4	9
BSF-1	12	21

Resting B cells (5×10^5/ml) were cultured for 24 h with RPMI or BSF-1 (purified by affinity chromatography and RP-HPLC; 10 U/ml). Cells were washed and recultured for 36 or 48 h with anti-IgM (50 μg/ml) and BSF-1 (10 U/ml) at 5×10^5 cells/ml. Colcemid (25 ng/ml) was added at 20 h of the second culture. Cells in S + G_2 and M were determined by staining with propidium iodide. Values recorded are (% cells in S + G_2 and M) − (% cells in S + G_2 which were observed in secondary cultures in RPMI alone [5–6%]).

of BSF-1 is mainly seen as an enhancement above these levels. In the experiment shown in Table I and in most experiments we observed no preparation of BALB/c B cells for response to LPS; in other experiments, we saw modest preparation of such cells for LPS response.

To determine whether the preparative effects of BSF-1 were limited to a small subpopulation of B cells or were a feature of a major fraction of these cells, we measured the percentage of BSF-1-pretreated B cells that entered the S phase of the cell cycle upon subsequent stimulation with anti-IgM (50 μg/ml) and BSF-1. Resting B cells (5×10^5/ml) were cultured for 24 h in BSF-1, purified by affinity chromatography and RP-HPLC, or in medium. The cells were washed and recultured as 5×10^5/ml in anti-IgM and BSF-1. Colcemid (25 ng/ml) was added at 20 h of the second culture and the fraction of the cells entering S phase was determined at 36 and 48 h. A net stimulation of 12 and 21% of the cells to enter S phase at 36 and 48 h, respectively, was observed in B cell precultured with BSF-1 (Table II). As observed previously, B cells precultured with BSF-1 appeared to respond ~12 h faster to anti-IgM and BSF-1 than did B cells cultured in medium alone (3) (compare RPMI and BSF-1 preculture groups in Table II). These results indicate that BSF-1 acts upon a substantial fraction of resting B cells for preparation to enter S phase.

Low Concentrations of BSF-1 Are Effective in Preparing B Cells. DBA/2 B cells precultured with as little as 0.1 U/ml of BSF-1 exhibited enhanced responses to LPS at concentrations from 1 to 100 μg/ml, to Bet-2 plus BSF-1, and to anti-IgM beads (Fig. 1). The degree of preparation increased as preculture concentrations of BSF-1 were increased to 100 U/ml.

BSF-1 Must Be Present for an Extended Time during the Preculture Period. To determine the duration of contact between BSF-1 and B cells necessary for preparation to occur, we varied the times BSF-1 was present in the preculture period. This was done in two ways. BSF-1 was added to cells at the initiation of the preculture, and 11B11, the anti-BSF-1 antibody, was added at various times to block further BSF-1 activity. At the same times, control groups received a control antibody, 50C1 (Fig. 2 *A*); 11B11 was added to control cells at the end

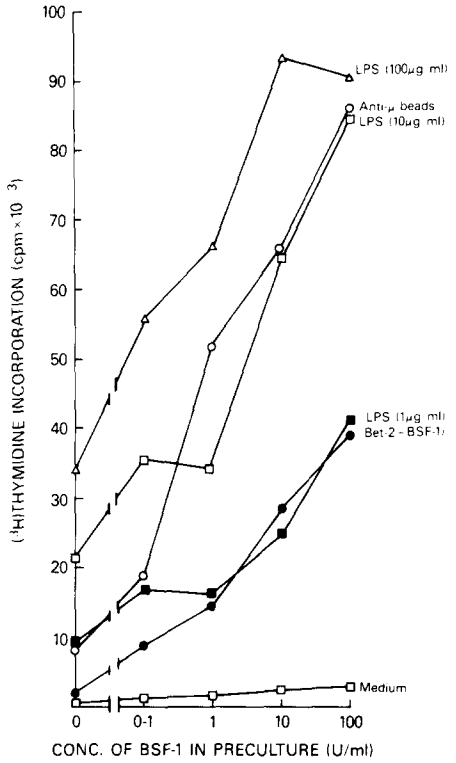

FIGURE 1. Effect of preculture in various concentrations of BSF-1 on subsequent response to secondary stimuli. Small, dense B cells were prepared from spleens of DBA/2 mice using complement-mediated lysis to remove T cells and Percoll gradient centrifugation to obtain dense cells. Cells banding betweeni 66 and 70% Percoll were cultured for 24 h in medium or concentrations of BSF-1 from 0.1–100 U/ml. At 24 h, after two washes, 10^5 viable cells per well were cultured for an additional 28 h in medium alone, anti-IgM beads, Bet 2 + BSF-1, and LPS (1 μg/ml, 10 μg/ml, or 100 μg/ml). Cells were labeled with [^{3}H]thymidine for the last 6 h of culture.

of the preculture period. Alternatively, cells were cultured in medium, and BSF-1 was added at various times after initiation of culture and 11B11 was added at the end of the 24 h preincubation (Fig. 2 *B*). In both of these experiments, we took advantage of the fact that BSF-1 prepared cells to respond to anti-IgM (50 μg/ml) and that responses to anti-IgM (50 μg/ml) can be obtained in the presence of 11B11 (E. Rabin, J. Ohara, and W. E. Paul, manuscript in preparation).

B cells precultured in BSF-1 for 24 h responded much more promptly to anti-IgM than did cells pretreated with medium. The addition of 11B11 at the outset of the preculture completely inhibited preparation (Fig. 2 *A*). Addition of 11B11 at any time thereafter led to partial inhibition. Inhibitions of 50% were obtained by adding 11B11 midway through the preculture period (i.e., at ~12 h). Similarly, delaying the addition of BSF-1 diminished the preparative effect (Fig. 2 *B*). 50% diminution was observed when BSF-1 was not added until 12 h of the 24 h preculture. Both results emphasize that BSF-1 effects require extended time for full induction; however, partial induction can be achieved by the presence of BSF-1 for as little as 4 h.

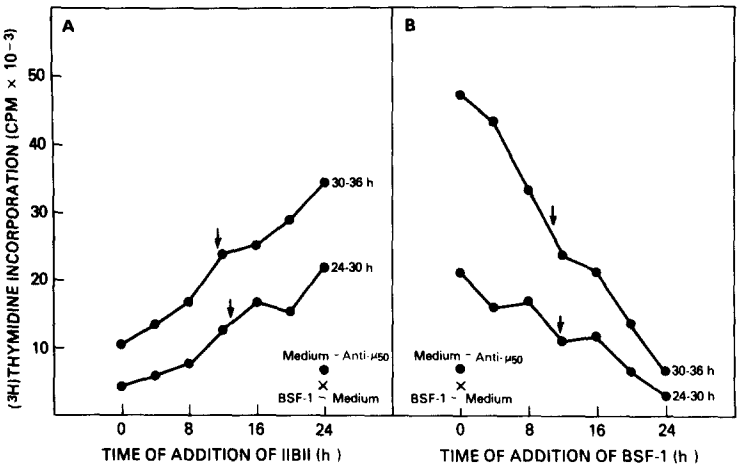

FIGURE 2. Requirement for extended exposure to BSF-1 for BSF-1-induced preparation for response to anti-IgM (50 μg/ml). (*A*) Small, dense B cells (ρ = 1.081–1.086 g/ml) were cultured at 10^5 per well in medium or in BSF-1 (10 U/ml). An anti-BSF-1 mAb, 11B11, was added to the cultures either at time 0 or at increasingly later time intervals. Replicate cultures received 50C1, an anti-DNP antibody. Both were added at a final dilution of 1:5,000 of ascitic fluid. At 24 h, all wells that had received 50C1 throughout the preculture received 11B11 to eliminate BSF-1 activity in the secondary cultures. (*B*) Other cells were precultured in medium, and BSF-1 or medium was added to the cultures at increasing time intervals during preculture. The final concentration of BSF-1 was 10 U/ml. At 24 h, cells received 11B11 (1:5,000). (*A* and *B*) At 24 h, medium or anti-IgM was added to cultures. Final concentration of anti-IgM in the secondary culture was 50 μg/ml. Cells were cultured for an additional 24 or 30 h, labeled for 6 h with [^{3}H]thymidine, and harvested. Control cultures of cells precultured in medium and stimulated with anti-IgM in secondary culture, and of cells precultured in BSF-1 then cultured in medium alone are also shown. Such cultures were labeled at 30 h and harvested at 36 h of culture. Arrow designates points of half-maximal stimulation.

BSF-1 Does Not Mediate Its Preparative Effect by Increased B Cell Viability. We considered the possibility that BSF-1 might mediate its function simply by promoting B cell viability rather than by preparing the cells to respond more promptly to subsequent stimuli. We tested this possibility by preculturing B cells for 24 h in RPMI, BSF-1 (10 U/ml), or EL-4 SN. Numbers of viable cells at the end of the preculture period were essentially the same in each group (Table III). A fixed number of viable cells were then cultured with RPMI or anti-IgM (50 μg/ml) for 28 h and the yield of viable cells was measured. The yield of viable cells was virtually identical whether precultured in RPMI, BSF-1, or EL-4 SN after secondary culture in anti-IgM (Table III). Nonetheless, B cells precultured with either BSF-1 or EL-4 SN took up substantially more [^{3}H]thymidine in response to anti-IgM than B cells precultured in RPMI alone. This, together with our previous finding that BSF-1 pretreatment markedly speeds the rate at which cells enter S phase in response to anti-IgM (3), leads us to conclude that the BSF-1 preparatory effect cannot be explained simply by enhanced B cell viability.

Anti-Lyb-2.1 Mimics BSF-1 in Preparing B Cells to Respond to Secondary Stimulants. Lyb-2 is an alloantigen expressed on primitive and mature cells of the B lineage (21). Anti-Lyb-2 mAbs have been reported to stimulate B cell proliferation by themselves and to synergize with anti-IgM to heighten DNA synthesis

TABLE III

BSF-1 Does Not Mediate Its Effects by Increasing B Cell Viability

A	Preculture* conditions	Yield of viable cells
	RPMI	5.4×10^6
	BSF-1 (10 U/ml)	5.8×10^6
	EL-4 SN (1:100)	6.2×10^6

B	Preculture condition	Secondary culture condition‡	Yield of viable cells after secondary culture	[^{3}H]Thymidine uptake§
	RPMI	RPMI	4.4×10^5	179
		Anti-IgM	5.0×10^5	186
	BSF-1	RPMI	6.2×10^5	181
		Anti-IgM	5.6×10^5	4,246
	EL-4 Sn	RPMI	5.4×10^5	171
		Anti-IgM	5.6×10^5	4,293

Purified splenic B cells from BALB/c mice were separated by Percoll density gradient centrifugation. Cells banding between 66 and 70% Percoll and pelleting through 70% Percoll were cultured at 10^6 cells/ml for 22 h in RPMI, BSF-1 (10 U/ml), or EL-4 SN (1:100). At the end of the preculture period, numbers of viable cells were counted. Viable cells were then cultured at 10^6 cells/ml in RPMI or anti-IgM (50 μg/ml). The number of viable cells at 28 h of secondary culture were determined. Cells were also cultured at 10^5 cells/0.2 ml/culture well, tritiated thymidine was added at 28 h, and cells were harvested at 36 h.

* 8×10^6 cells; 22 h.

‡ 10^6 cells; 28 h.

§ 10^5 cells/well; 28–36 h.

responses. Subbarao and Mosier (22) and Yakura et al. (23) have suggested that anti-Lyb-2 may react with the receptor for BSF-1. We wished to determine whether the similar modes of action of anti-Lyb-2 and BSF-1 extended to roles as preparatory agents, allowing a prompter entry into S phase on subsequent stimulation.

DBA/2 B cells were used in this experiment since they possess the Lyb-2.1 alloantigen. DBA/2 B cells were precultured for 24 h with medium, BSF-1, or anti-Lyb-2.1. Precultured cells were washed and cultured with a series of secondary stimulants. Both anti-Lyb-2.1 and BSF-1 prepared DBA/2 B cells to respond to all the stimulants tested, including LPS (Table IV). BALB/c cells, which lack the Lyb-2.1 alloantigen, were cultured in parallel with DBA/2 cells in this experiment, and they were not prepared by anti-Lyb-2.1 to respond to secondary stimulants such as anti-IgM plus BSF-1 (data not shown).

The Effect of BSF-1 on Blast Cells. BSF-1 has been clearly shown to activate resting B cells, as judged by volume increases, induction of class II MHC molecules, and preparation to enter S phase more promptly. We now wished to determine whether BSF-1 also caused entry into S phase of B cell blasts prepared by stimulating resting B cells with anti-IgM (5 μg/ml) and BSF-1. Blasts isolated by Percoll density gradient centrifugation responded very well to Bet-2 and to goat anti-IgM. They showed meager responses to BSF-1 used at low concentrations (≤10 U/ml); when BSF-1 concentrations were raised to 100 U/ml a clearer

TABLE IV

Anti-Lyb 2.1 Prepares B Cells to Respond to Several Stimulants

Preculture conditions‡	[^{3}H]thymidine uptake (cpm)*				
	Secondary culture conditions§				
	Medium	BSF-1 + anti-IgM5	anti-IgM50	anti-Lyb-2 + IgM5	LPS
Medium	1,435	2,413	1,948	8,807	22,877
BSF-1	2,584	13,571	20,249	40,666	64,331
anti-Lyb-2.1	7,575	27,479	23,739	59,246	136,163

* [^{3}H]thymidine uptake was measured at 22–28 h of the secondary culture period.

‡ Spleen cells from DBA/2 mice were prepared as described for Table I and cultured at 10^6 cells/ml/well for 24 h in medium, BSF-1 (10 U/ml), or anti-Lyb 2.1 antibody (50 μg/ml).

§ Cells were washed twice after the preincubation period and cultured at 10^5 viable cells per 0.2 ml per well for 28 h.

TABLE V

Response of B Cell Blasts Prepared with Anti-IgM and BSF-1

Stimulants*	Response (uptake of [^{3}H]thymidine [cpm])‡		
	Exp. 1	Exp. 2	Exp. 3
Medium	26,311	4,111	2,854
Bet-2	180,950	57,763	14,451
Anti-IgM (50 μg/ml)	204,830	141,520	93,700
BSF-1 (1 U/ml)	41,093	4,993	—
(10 U/ml)	56,937	12,966	4,338
(100 U/ml)	83,430	25,135	—
BSF-1 (1) + TEPC 183	40,184	—	—
BSF-1 (10) + TEPC 183	47,722	—	—
BSF-1 (100) + TEPC 183	78,066	—	—
TEPC 183 (10 μg/ml)	23,180	—	—

Resting B cells were stimulated with anti-IgM (5 μg/ml) plus BSF-1 (10 U/ml) for 20–24 h. Blasts banding between 50 and 60% Percoll were isolated after Percoll gradient centrifugation.

* Blasts were cultured at 10^5 per well in Bet-2, anti-IgM (50 μg/ml), BSF-1 with or without TEPC 183, or medium with or without TEPC 183. Bet-2 is a rat anti–mouse IgM mAb; TEPC-183 is a mouse IgM myeloma protein.

‡ [^{3}H]thymidine incorporation was measured at 22–28 h of the secondary culture period.

response was observed, although it was considerably less than that to Bet-2 or goat anti-IgM (Table V). Responses of B cell blasts to high concentrations of BSF-1 were not inhibited by the IgM myeloma protein TEPC-183 (10 μg/ml), suggesting that residual anti-IgM from the initial stimulation of the B cells was not required for this response.

We also prepared large B cells directly from the spleens of normal BALB/c mice by Percoll density gradient centrifugation. These B cell blasts responded well to LPS and to anti-IgM-beads, but failed to respond to BSF-1 (10 U/ml) alone (Table VI).

These experiments thus indicate that BSF-1 is relatively inefficient at stimulat-

TABLE VI
Large B Cells Fail to Enter S Phase to BSF-1 Alone

Stimulants*	[^{3}H]thymidine uptake (cpm)‡			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Medium	3,521	5,148	4,897	18,558
BSF-1	6,199	5,761	6,111	21,234
LPS	71,150	90,824	99,847	190,373
Anti-IgM beads	—	113,286	91,770	166,813

Spleen cells from BALB/c mice were depleted of T cells as described in Table I. Cells banding between 50 and 60% Percoll by density gradient centrifugation were cultured.

* Cells were cultured for 28 h at 10^5 per well in medium, BSF-1 (10 U/ml), LPS (50 μg/ml), or anti-IgM coupled to Sepharose beads.

‡ Cells were labeled with [^{3}H]thymidine for the last 6 h of culture.

ing entry into S phase of B cell blasts prepared with anti-IgM and BSF-1, and that it fails to stimulate large B cells found in mouse spleens.

There Is A Requirement for BSF-1 at Later Times in Responses to Anti-IgM. Although B cell blasts are not efficiently stimulated to enter S phase when cultured with BSF-1 alone, we did observe that isolated B cell blasts (Fig. 3 *B*) or a total B cell population (Fig. 3 *A*) prepared by culture with BSF-1 and anti-IgM or Bet-2 did respond better to Bet-2 plus BSF-1 than to Bet-2 alone. However, when these responses were analyzed carefully, we found that responses to Bet-2 plus BSF-1 early in secondary culture (i.e., 18–24 h or 22–28 h after initiation) were only modestly diminished (20–30%) by removing BSF-1, but responses later in secondary culture (40–46 h) were much more dependent on BSF-1; they were diminished by 74% by removal of BSF-1.

These results are in keeping with the finding that anti-BSF-1 antibody added at 20 h of primary culture inhibits responses to anti-IgM plus BSF-1 by only 41% when measured at 46–52 h from the initiation of culture, but by 60% when measured at 64–70 h (Fig. 4).

The late requirement for BSF-1 in responses to Bet-2 or to low concentrations of anti-IgM, taken together with the inefficiency of BSF-1 in causing blast cells to divide, suggests that BSF-1 may be required in the early stages of the second cell cycle of B cells responding to anti-IgM.

Discussion

BSF-1 is now recognized as an activation factor for resting B cells, based on its clearly established effects on preparation for DNA synthesis, induction of MHC class II molecules, and increases in cell volume (3–7). How BSF-1 causes these responses is not yet understood. We can gain some insight into the mechanisms involved in BSF-1 preparation by examining the types of responses affected by BSF-1 preparation and also by further characterizing the requirements for the preparation itself.

BSF-1 prepares BALB/c cells to respond to BSF-1 plus anti-IgM (5 μg/ml), anti-IgM (50 μg/ml), and anti-IgM beads, but only modestly, if at all, to LPS. However, the failure to regularly prepare BALB/c B cells for LPS responses is

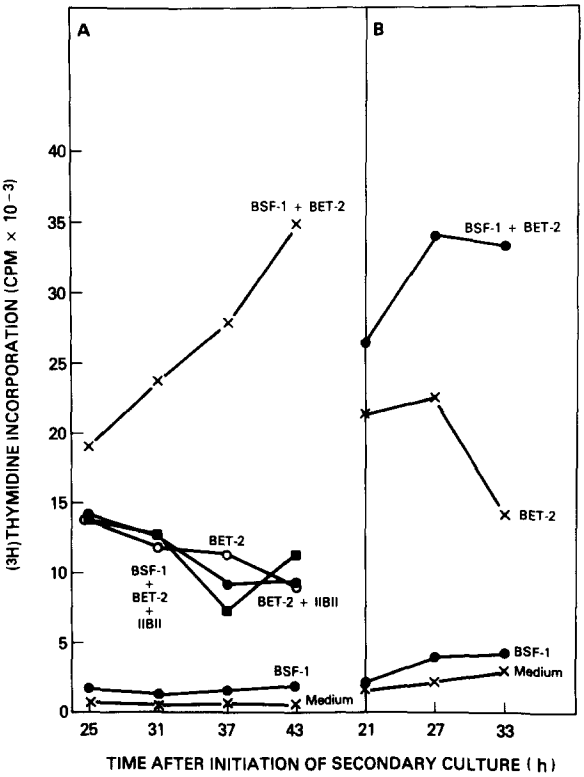

FIGURE 3. Late requirement for BSF-1 in the anti-IgM response. (*A*) Small, dense B cells were cultured for 24 h in BSF-1 plus Bet-2. At 24 h, cells were washed twice and cultured at 10^5 viable cells per well in BSF-1 (10 U/ml) plus Bet-2 (1:20); Bet-2; BSF-1 plus Bet-2 plus anti-BSF-1 mAb, 11B11 (1:5,000); Bet-2 plus 11B11; BSF-1; or medium alone. Cells were labeled with [^{3}H]thymidine beginning at 22 h, 28 h, 34 h, and 40 h, and harvested 6 h later. Time of harvest is indicated by the midpoint of the labelling period. (*B*) Resting B cells were stimulated with anti-IgM (5 μg/ml) plus BSF-1 (10 U/ml) for 20 h. Blasts banding between 50 and 60% Percoll were cultured at 10^5 per well in BSF-1 (10 U/ml) plus Bet-2 (1:20), Bet-2, BSF-1, or medium. Cells were labeled at 6-h intervals beginning at 18 h. After a 6 h labeling period in [^{3}H]thymidine, cells were harvested at 24 h, 30 h, and 36 h.

not general, since BSF-1 does prepare DBA/2 and BDF_1 B cells to respond to LPS.

Although the mechanism through which BSF-1 prepares B cells to respond to secondary stimulants is not clear, it does not appear to result from heightened initial signaling through membrane IgM molecules. Anti-IgM is known to cause rapid increases in intracellular free calcium $[Ca^{++}]_i$ and in concentration of inositol phosphates. Preculture of resting B cells with BSF-1 does not enhance these increases (J. Mizuguchi et al., manuscript in preparation), despite the heightened reactivity of the prepared B cells as measured by increased rate of entry into S phase. Moreover, Mizuguchi et al. (manuscript submitted for publication) have shown that BSF-1 does not, itself, lead to increased $[Ca^{++}]_i$ in resting B cells.

The preparative effects of BSF-1 require extended contact between the B cells and BSF-1 for full effect. Delaying the addition of BSF-1 for 4–8 h in a 24-h

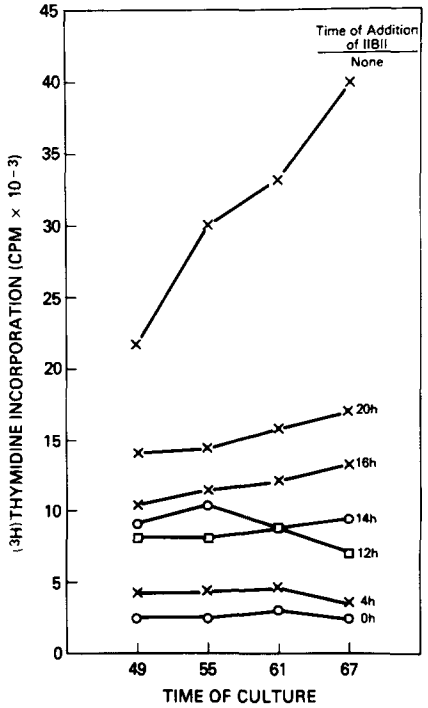

FIGURE 4. Inhibitory effect of anti-BSF-1 antibody on the response to anti-IgM plus BSF-1 at later periods of culture. Resting B cells were cultured at 10^5 per well in anti-IgM (5 μg/ml) plus BSF-1 (10 U/ml). The anti-BSF-1 mAb, 11B11, was added to cultures at 0, 4, 12, 14, 16, and 20 h for a final dilution of 1:5,000 of ascites fluid. 50C1, an anti-DNP mAb, was added to control cultures (data not shown). Cells were labeled with [^{3}H]thymidine at 46, 52, 58, and 64 h and harvested 6 h later.

preculture period diminishes the degree of preparation; similarly, removal of BSF-1 by adding anti-BSF-1 mAb 4–8 before the end of a 24-h preculture diminishes the subsequent response. On the other hand, the presence of BSF-1 for relatively short periods (4–8 h) does have a measurable effect. This suggests that BSF-1 can prepare cells to varying degrees for secondary responses and that maximal preparation requires extended contact between BSF-1 and B cells. We cannot fully exclude the possibility that individual B cells require BSF-1 for only a short time, but that only some B cells are in a BSF-1-responsive state during any particular period of the 24-h preculture. However, the most likely heterogeneity of cells with regard to stimulant sensitivity would be position in the cell cycle. Since essentially all the B cells used in these experiments are in G_0 or the early portion of the G_1 phase, it seems very unlikely that heterogeneity in responsiveness can explain the requirement for extended contact. Furthermore, other well studied factors, including IL-2, epidermal growth factor, and insulin require extended contact with their cellular targets to achieve maximal effects (24–26). Indeed, anti-IgM itself will only cause B cells to become committed to entry into S phase after 20–30 h of contact with B cells that have not been prepared by prior culture with BSF-1 (17).

The capacity of BSF-1 to prepare resting B cells to enter S phase in response

to subsequent stimuli is reminiscent of the effects of platelet-derived growth factor (PDGF) on quiescent fibroblast lines. Thus, PDGF does not, itself, cause growth-arrested BALB/c-3T3 cells to enter S phase but induces a state of competence, allowing them to divide in response to epidermal growth factor or to somatomedins (27). However, our finding that BSF-1 action on resting B cells requires extended contact, and the fact that BSF-1 does not cause increased $[Ca^{++}]_i$ in resting B cells, stand in contrast to the effects of PDGF on fibroblasts. PDGF induces a state of competence in BALB/c-3T3 cells within 30 min (27), and this induction is associated with an increase in $[Ca^{++}]_i$ (28). This suggests that the mode of action of BSF-1 on B cells may be quite different from that of PDGF on fibroblasts.

Our studies add to the current consideration of the role of Lyb-2 and of anti-Lyb-2.1 antibody in the stimulation of B cells. Both Yakura et al. (23) and Subbarao and Mosier (15) have reported that monoclonal anti-Lyb-2.1 stimulates DNA synthesis by resting B cells. Indeed, F(ab) fragments of anti-Lyb-2.1 have been reported to be stimulatory (15). Low concentrations of anti-Lyb-2.1 co-stimulate with anti-IgM, but not with crude preparations containing BSF-1. This has led to the suggestion that anti-Lyb-2.1 mimics BSF-1 activity. Yakura et al. have also observed that anti-Lyb-2.1 blocks absorption of BSF-1 by DBA/2 B cells; they have therefore proposed that Lyb-2 is the cellular receptor for BSF-1 (23). Our data indicate that anti-Lyb-2.1 mimics BSF-1 in that it prepares cells to respond more promptly to several stimulants, including anti-IgM plus BSF-1, anti-IgM (50 μg/ml), LPS, and anti-Lyb-2.1 plus anti-IgM. However, the finding that anti-Lyb-2.1 can stimulate B cell entry into S phase without costimulants, while BSF-1 alone fails to do so, calls for caution in concluding that anti-Lyb-2.1 is directed to the BSF-1 receptor. Progress in purifying BSF-1 should make possible the development of binding assays that will allow a more direct assessment of the nature of the BSF-1 receptor and its relationship to Lyb-2.

BSF-1 was initially described as a costimulant with anti-IgM antibodies of B cell entry into S phase. It was anticipated that BSF-1 acted on B cells in a manner analagous to the action of IL-2 on T cells (29). That is, it was expected that resting B cells would be unresponsive to BSF-1 and that activation with anti-IgM would induce a state of BSF-1 sensitivity. Addition of BSF-1 would then determine that the activated B cell would enter S phase. Our data and those of others have clearly shown that BSF-1 can act on resting B cells. Thus, at least part of the initial postulate of the mode of BSF-1 action must be rejected. To determine whether B cell blasts could be stimulated by BSF-1 to enter S phase, we prepared blasts by culture with BSF-1 and anti-IgM. These cells incorporated modest amounts of [^{3}H]thymidine when stimulated with relatively high concentrations of BSF-1; large B cells harvested directly from the spleens of mice failed to synthesize DNA when stimulated with BSF-1. This leads us to conclude that in the first round of cell division of B cells stimulated with BSF-1 and anti-IgM, BSF-1 exerts its major role as an activation factor, working on G_0 and early G_1 B cells. It appears to have only a minor role on late G_1 B cells; considering the high concentrations of BSF-1 required, the late G_1 effect may be of limited physiologic significance.

The observation that anti-BSF-1 mAb, added as late as 20 h of culture, has

inhibitory effects on responses to anti-IgM plus BSF-1 could nevertheless indicate an important requirement for BSF-1 late in the initial cell cycle. An alternative, and we believe more plausible, possibility is that this inhibition reflects a requirement for BSF-1 in the early G_1 phase of the second round of cell division stimulated by anti-IgM. This is supported by the finding that the requirement for BSF-1 is considerably more striking when [^{3}H]thymidine uptake is evaluated at 60–70 h of culture than at 46–52 h. Direct examination of the requirement of BSF-1 for the second round of cell division is now in progress.

Indeed, it is quite possible that the recently described IgG1-inducing activity of BSF-1 is due to its function on early G_1 cells in the first and subsequent cell cycles. Thus, it has been shown that B cells stimulated with LPS will secrete little or no IgG_1 unless BSF-1 is added to the culture. Moreover, the cells that are the antecedents of the IgG1-secreting cells lack membrane IgG_1 at the outset of the culture, indicating that Ig heavy chain switching occurs in response to the action of BSF-1 (30). We suggest that the action of BSF-1 in early G_1 phase may initiate, in properly stimulated cells, the induction of the intracellular signals required for switching. Whether the switching that occurs under the influence of BSF-1 is limited to the γ_1 H chain will await analysis of expression of other Ig classes and of costimulants other than LPS. Recent studies (31) indicate that BSF-1 also promotes the expression of IgE.

Summary

BSF-1 prepares resting BALB/c, DBA/2, and BDF_1 B cells to enter S phase more promptly in response to subsequent culture with anti-IgM–based stimulants. It prepares DBA/2 and BDF_1 B cells to respond to LPS, but its preparative effect for LPS responses of BALB/c B cells is both inconstant and meager. Preparation mediated by BSF-1 requires extended contact of B cells with the stimulant for full effect. Half-maximal preparation requires ~12 h of contact, as judged by delayed addition of BSF-1 or by inhibition of BSF-1 action with anti-BSF-1 mAbs. BSF-1 preparative action on resting DBA/2 B cells is mimicked by anti-Lyb-2.1 antibody. B cell blasts prepared by culture with BSF-1 and anti-IgM show modest responses to high concentrations of BSF-1; large B cells directly isolated from the spleen are not stimulated to enter S phase by BSF-1. These results lead us to conclude that BSF-1 functions principally as an activation factor for resting B cells.

We thank Robert Cunningham and Philip Noguchi, Center for Drugs and Biologics, for measurements of cellular DNA content by flow cytometry. We wish to thank Jane Hu Li for excellent technical assistance and Shirley Starnes for her assistance in the preparation of this manuscript.

Received for publication 23 April 1986.

References

1. Howard, M., J. Farrar, M. Hilfiker, B. Johnson, K. Takatsu, T. Hamaoka, and W. E. Paul. 1982. Identification of a T cell-derived B cell growth factor distinct from interleukin 2. *J. Exp. Med.* 155:914.

2. Paul, W. E. 1984. Nomenclature of lymphokines which regulate B-lymphocytes. *Molec. Immunol.* 21:343.
3. Rabin, E. M., J. Ohara, and W. E. Paul. 1985. B cell stimulatory factor (BSF)-1 activates resting B cells. *Proc. Natl. Acad. Sci. USA.* 82:2953.
4. Thompson, C. B., M. E. Schaefer, F. D. Finkelman, I. Scher, J. Farrar, and J. J. Mond. 1985. T cell-derived B cell growth factor(s) can induce stimulation of both resting and activated B cells. *J. Immunol.* 134:369.
5. Roehm, N. W., J. Liebson, A. Zlotnick, J. Kappler, P. Marrack, and J. C. Cambier. 1984. Interleukin-induced increase in Ia expression by normal mouse B cells. *J. Exp. Med.* 160:679.
6. Noelle, R., P. H. Krammer, J. Ohara, J. W. Uhr, and E. S. Vitetta. 1984. Increased expression of Ia antigens on resting B cells: an additional role for B-cell growth factor. *Proc. Natl. Acad. Sci. USA.* 81:6149.
7. Oliver, K., R. J. Noelle, J. W. Uhr, P. H. Krammer, and E. S. Vitetta. 1985. B-cell growth factor (B-cell growth factor I or B-cell-stimulatory factor, provisional 1) is a differentiation factor for resting B cells and may not induce cell growth. *Proc. Natl. Acad. Sci. USA.* 82:2465.
8. Vitetta, E. S., K. Brooks, Y.-W. Chen, P. Isakson, S. Jones, J. Layton, G. C. Miskra, E. Pure, E. Weiss, C. Word, D. Yuan, P. Tucker, J. W. Uhr, and P. H. Krammer. 1984. T cell-derived lymphokines that induce IgM and IgG secretion in activated murine B cells. *Immunol. Rev.* 78:137.
9. Sideras, P., S. Bergstedt-Lindqvist, and E. Severinson. 1985. Partial biochemical characterization of IgG_1-inducing factor. *Eur. J. Immunol.* 15:593.
10. Vitetta, E. S., J. Ohara, C. Myers, J. Layton, P. H. Krammer, and W. E. Paul. 1985. Serological, biochemical, and functional identity of B cell–stimulatory factor 1 and B cell differentiation factor for IgG1. *J. Exp. Med.* 162:1726.
11. Sieckmann, D. G., R. Asofsky, D. E. Mosier, I. M. Zitron, and W. E. Paul. 1978. Activation of mouse lymphocytes by anti-immunoglobulin. I. Parameters of the proliferative response. *J. Exp. Med.* 147:814.
12. Kung, J. T., S. O. Sharrow, D. G. Sieckmann, R. Lieberman, and W. E. Paul. 1981. A mouse IgM allotypic determinant (Igh-6.5) recognized by a monoclonal rat antibody. *J. Immunol.* 127:873.
13. Ohara, J., and W. E. Paul. 1985. B cell stimulatory factor BSF-1: production of a monoclonal antibody and molecular characterization. *Nature (Lond.).* 315:333.
14. DerBalian, P., J. Slack, B. J. Clevinger, H. Bazin, and J. M. Davie. 1980. Subclass restriction of murine antibodies. III. Antigens that stimulate IgG_3 in mice stimulate IgG_{2c} in rats. *J. Exp. Med.* 152:209.
15. Subbarao, B. and D. E. Mosier. 1983. Induction of B lymphocyte proliferation by monoclonal anti-Lyb 2 antibody. *J. Immunol.* 130:2033.
16. Liebson, J. H., P. Marrack, and J. W. Kappler. 1981. B cell helper factors. I. Requirement for both interleukin 2 and another 40,000 mol wt factor. *J. Exp. Med.* 154:1681.
17. DeFranco, A., E. Raveche, R. Asofsky, and W. E. Paul. 1982. Frequency of B lymphocytes responsive to anti-immunoglobulin. *J. Exp. Med.* 155:1523.
18. Ohara, J., S. Lahet, J. Inman, and W. E. Paul. 1985. Partial purification of BSF-1 by HPLC. *J. Immunol.* 135:2518.
19. Krishan, A. 1975. Rapid flow cytofluorimetric analysis of mammalian cell cycle by propidium iodide staining. *J. Cell Biol.* 66:188.
20. Gazdar, A. 1980. DNA content analysis by flow cytometry and cytogenetic analysis in mycosis fungoides and Sezary syndrome. *J. Clin. Invest.* 65:1440.

21. Shen, F. W., M. Spanondis, and E. A. Boyse. 1977. Multiple alleles of the Lyb-2 locus. *Immunogenetics.* 5:481.
22. Subbarao, B. and D. E. Mosier. 1982. Lyb antigens and their role in B lymphocyte activation. *Immunol. Rev.* 69:81.
23. Yakura, H., I. Kawabata, F. W. Shen, and M. Katagiri. 1985. Lyb-2 as a possible B cell growth factor (BCGF)1 receptor. *Fed. Proc.* 44:1532.
24. Cantrell, D. A. and K. A. Smith. 1984. The interleukin-2 T-cell system: a new cell growth model. *Science (Wash. DC).* 224:1312.
25. Schechter, Y., L. Hernaeg, and P. Cuatrecasas. 1978. Epidermal growth factor: biological activity requires persistant occupation of high-affinity cell surface receptors. *Proc. Natl. Acad. Sci. USA.* 75:5788.
26. Straus, D. S. 1984. Growth-stimulatory actions of insulin *in vitro* and *in vivo. Endocr. Rev.* 5:356.
27. Singh, J. P., M. A. Chaikin, W. J. Pledger, C. D. Scher, and C. D. Stiles. 1983. Persistance of the mitogenic response to platelet-derived growth factor (competence) does not reflect a long-term interaction between the growth factor and the target cell. *J. Cell Biol.* 96:1497.
28. Berridge, M. J., J. P. Heslop, R. F. Irvine, and K. D. Brown. 1984. Inositol triphosphate formation and calcium mobilization in Swiss 3T3 cells in response to platelet-derived growth factor. *Biochem. J.* 222:195.
29. Cantrell, D. A. and K. A. Smith. 1983. Transient expression of interleukin 2 receptors. Consequences for T cell growth. *J. Exp. Med.* 158:1895.
30. Layton, J. E., E. S. Vitetta, J. W. Uhr, and P. H. Krammer. 1984. Clonal analysis of B cells induced to secrete IgG by T cell-derived lymphokine(s). *J. Exp. Med.* 160:1850.
31. Coffman, R. L., J. Ohara, M. W. Bond, J. Carty, E. Zlotnik, W. E. Paul. 1986. B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J. Immunol.* 136:4538.