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B-cell growth factor (B-cell growth factor I or B-cell-stimulating factor, provisional 1) is a differentiation factor for resting B cells and may not induce cell growth

(lymphokine/anti-Ig)

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ABSTRACT **B-cell growth factor I [BCGF I or B-cell-stimulating factor, provisional 1 (BSFp1)] has been defined as a T-cell-derived lymphokine that acts as a co-stimulator of polyclonal B-cell growth in B cells cultured with anti-μ, anti-δ, or anti-Ig. Based on a number of studies it has been suggested that anti-Ig induces cell enlargement, entry into the G_1 phase of the cell cycle, and expression of receptors for BSFp1. BSFp1 then induces entry of the cells into S phase. By adding BSFp1 prior to anti-Ig, we have found evidence that BSFp1 renders cells susceptible to anti-Ig-mediated entry of cells into G_2/S phase. In contrast, if cells are first treated with anti-Ig, washed, and then cultured with BSFp1, they do not enter S phase. Taken together, these results suggest that BSFp1 acts on the resting B cells *not* as a growth factor but rather as a lymphokine that prepares cells for anti-Ig-mediated activation. Taken together with previous reports that BSFp1 induces increased expression of Ia antigens on resting B cells, these studies suggest that BSFp1 may be a differentiation factor rather than a growth factor and that it acts on resting B cells.**

T cells and macrophages secrete cytokines that effect B-cell growth and differentiation (reviewed in ref. 1). One such B-cell growth factor (BCGF) is derived from supernatants (SN) of several T-cell lines, hybridomas or clones, and is termed BCGF I [or B-cell-stimulating factor, provisional 1 (BSFp1)] (2, 3). When cultured in the presence of both anti-Ig and BSFp1, resting G_0 B cells proceed through G_1 and enter the G_2/S phase of the cell cycle. It has been suggested that BSFp1 acts during the first 12 hr of coculture with anti-μ (1). B-cell growth is affected by three other cytokines: (*i*) interleukin 1 (IL-1), a macrophage-derived factor that enhances entry of B cells into S phase after their treatment with anti-μ and BSFp1 (4, 5); (*ii*) BCGF II, a T-cell-derived late-acting BCGF that does not costimulate with anti-μ (3, 6); and (*iii*) interleukin 2 (IL-2), a T-cell-derived factor that acts on B-cell blasts (5, 7, 8).

Recent studies by Noelle *et al.* (9) and Roehm *et al.* (10) have demonstrated that the BSFp1-containing SN (9, 10) or highly purified BSFp1 (9) induces a selective increase in the expression of Ia antigens on small, resting B cells. Such treated B cells do not enter the cell cycle (9). These results indicate that BSFp1 can function as a differentiation factor and that receptors for BSFp1 are present on resting B cells. Hence, the results are incompatible with models in which BSFp1 receptors are only expressed *after* the cells have been induced by anti-μ to enlarge and enter G_1 (1, 5).

We considered the possibility that BSFp1 is not a growth factor but rather induces B cells to become susceptible to stimulation by ligands. To test this hypothesis, highly purified B cells were cultured with sources of BSFp1 or anti-δ-Sepharose. Ater 24 hr, cells were washed and recultured for an additional 48 hr with the opposite reagent. Cells were then stained with acridine orange (AO) and analyzed by flow cytometry for their status in the cell cycle.

MATERIALS AND METHODS

Animals. Female BDF_1 mice (from the University of Texas Health Science Center breeding colony) 8–12 weeks of age, were used for all experiments.

Preparation of B Cells. Spleen cells were stained with biotin-conjugated, monoclonal anti-Thy-1.2 (HO-13.4) (11) and fluoresceinated avidin (Vector Laboratories, Burlingame, CA). Small B cells were sorted based on their negative surface fluorescence and low forward light scatter on a FACS III (Becton Dickinson). The sorted cells were always >99% Thy-1.2$^-$ and represented a homogeneous population of small cells that were 90–95% surface IgD$^+$.

Cell Culture Conditions. Sorted B cells were cultured as described (12). In addition, small Thy-1.2$^-$ cells were cultured at 50,000 per well for 24 hr (phase 1) with either PK 7.1 SN, BSFp1, or anti-δ-Sepharose. Cells were then centrifuged at 300 rpm for 3 min to remove the anti-δ-Sepharose or at 1000 rpm for 10 min to remove SN from the cells. The cells were then washed once at 1000 rpm/10 min and replated at 50,000 per well under the same culture conditions, but with different combinations of ligand or SN (or both) (phase 2). After 48 hr of additional culture, the cells were washed and the cell cycle status was determined by AO analysis.

Lymphokines. Two sources of lymphokines were used: (*i*) the SN of Con A-pulsed PK 7.1 cells (13), which contains BCGF I (BSFp1) and BCGF II (14) but lacks interferon-γ (IFN-γ) and IL-2 (13), and (*ii*) a preparation of BSFp1 purified by HPLC.

Preparation of BSFp1. Partially purified BSFp1 was prepared by a technique to be described in detail elsewhere (J. Ohara, S. Lahet, J. Inman, and W. E. Paul, personal communication) and was the generous gift of J. Ohara. Briefly, EL-4 cells were induced with phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) in serum-free culture medium. The cell-free SN was harvested after 48 hr and incubated with trimethylsilyl-controlled pore glass beads (Sepralyte; Analytichem International, Harbor City, CA). The beads were

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Abbreviations: AO, acridine orange; BCGF, B-cell growth factor; BSFp1, B-cell-stimulating factor, provisional 1; IFN-γ, interferon-γ; IL-1, interleukin 1; IL-2, interleukin 2; PMA, phorbol 12-myristate 13-acetate; SN, supernatant(s).

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washed with increasing concentrations of acetonitrile. Material from the 50% acetonitrile wash was applied to a reversed-phase C_{18} HPLC column and eluted with a 10–60% gradient of acetonitrile in 0.1% trifluoroacetic acid. The samples eluting at 46–48% acetonitrile contained the peak of BSFp1 activity. This material was lyophilized, reconstituted with water, and stored at −70°C at 3×10^6 units/ml.

Cell Cycle Analysis. Cell cycle analysis was performed by using the metachromatic nucleic acid dye AO, as described by Darzynkiewicz *et al.* (15). Briefly, cells were permeabilized in a low-pH detergent buffer, stained with AO, and subjected to cytofluorometric analysis. The green (515–575 nm) and red (600–650 nm) fluorescence emissions were monitored independently by separate photomultipliers. Doublets were distinguished from diploid DNA-containing cells by a nonlinear relationship of green pulse peak vs. green pulse area. Cells that were dead prior to permeabilization were eliminated from the analysis by their characteristic low content of DNA (15). Each cytogram was constructed from the analysis of 5000–10,000 cells.

RESULTS AND DISCUSSION

As seen in Table 1, when small resting B cells (>98% of which were surface Ig^+ and >95% were in G_0) were cultured for 48 hr with either PK 7.1 SN or purified BSFp1, only 3% of the cells entered the cell cycle. In contrast, when cells were cultured with anti-δ-Sepharose, ≈21% of the B cells entered the G_1 phase of the cell cycle but few (10%) proceeded further. When cells were cultured with both anti-δ-Sepharose and either PK 7.1 SN or purified BSFp1, 68–70% of the cells entered all phases of the cell cycle and 18% of the cells were in the G_2/S phase after 48 hr. In general, these results confirm previous reports (1, 5) that both anti-Ig and BSFp1 are required for the entry of cells into G_2/S. They also suggest that the active lymphokine in the PK 7.1 SN is BSFp1. It should be noted that anti-δ-Sepharose and anti-μ-Sepharose (data not shown), but not normal Ig-Sepharose, had the same effect.

In the four experiments averaged in Table 2, the cells were initially cultured with either PK 7.1 SN, anti-δ-Sepharose, or both (phase 1); after 24 hr, the ligands and SN were washed away and the cells were recultured for an additional 48 hr with the reagents listed in Table 2 (phase 2). In all experiments, >90% of the cells were recovered after each treatment and their viability was >90%. If cells were first cultured with anti-δ-Sepharose, washed, and then cultured with PK 7.1 SN (line 1), they did not enter the G_2/S phase of the cell cycle. In contrast, if they were first cultured with PK 7.1 SN and then cultured with anti-δ-Sepharose, 23% entered G_2/S (line 2). Finally, the addition of *both* anti-δ-Sepharose and PK 7.1 SN to the primary and secondary phases of the cell culture gave results similar to those obtained when only PK 7.1 SN was present in the primary cultures and only anti-δ-Sepharose was present in the secondary cultures—i.e., the same percentage of cells was in the G_2/S phase (line 3). None of the control treatments (lines 5–10) caused >5% of the cells to enter G_2/S, as compared with no treatment at all (line 4). Furthermore, as shown in line 11, the addition of anti-δ to phase 1 cultures did not prevent the cells from responding to PK 7.1 SN since they did respond if both anti-δ and BSFp1 were present in phase 2 cultures.

One potential problem in the failure of anti-δ-Sepharose to activate the resting cells in the "wash out" experiments is that the surface Ig^+ cells could bind to the Sepharose and be removed during centrifugation. To avoid this problem, soluble fragments of anti-Ig antibodies were used in place of the anti-δ-Sepharose. $F(ab')_2$ anti-Igs have been reported to be particularly potent activators of resting B cells (16). The results of these experiments (Table 3) were similar except that a larger percentage of the cells entered G_2/S. Thus, either some of the cells had been removed with the Sepharose or soluble $F(ab')_2$ anti-Ig is a more potent activator of B cells. Regardless, the results shown in Tables 2 and 3 strongly suggest that PK 7.1 SN acts on small resting B cells to prepare them for anti-Ig-mediated entry into G_2/S. Conversely, culturing cells with anti-δ-Sepharose or $F(ab')_2$ anti-Ig did not render them susceptible to growth-promoting activities of the PK 7.1 SN. Similar results were obtained by using a highly purified preparation of BSFp1 (Table 4).

In the earlier studies using murine B cells, cells were not washed between the addition of anti-μ and BSFp1 (1–3). The interpretation of the results was that the anti-μ acted first to induce the expression of BSFp1 receptors and BSFp1 subsequently acted as the growth factor. The present studies, however, suggest that BSFp1 acts as a differentiation factor

Table 1. Effect of anti-δ and PK 7.1 SN on entry of B cells into the cell cycle

Addition	% cells			
	G_0	G_{1A}	G_{1B}	S + G_2
None	98.6	0.7	0.3	6.4
PK 7.1 SN	95.2	3.6	0.6	0.6
BSFp1	95.7	3.1	0.5	0.7
Anti-δ	67.6	22.0	5.4	5.0
+ PK 7.1 SN	29.9	25:3	27.3	17.5
+ BSFp1	30.3	19.7	31.5	18.5

B cells were prepared by negative sorting of spleen cells from $(C57BL/6 \times DBA_2)F_1$ (BDF_1) mice on the FACS III (Becton Dickinson) using Biotin-conjugated monoclonal anti-Thy-1.2 (HO-13.4) and fluoresceinated avidin. Sorted B cells were cultured as described (12). Optimal amounts of anti-δ-Sepharose (10 μg/ml), BSFp1 (2.5 units/ml), or PK 7.1 SN [2.5% (vol/vol)] were added at the initiation of culture. Forty-eight hours later, anti-δ-Sepharose and PK 7.1 were removed and cell cycle analysis was performed on a Systems 50H Ortho cytofluorograph by using the metachromatic nucleic acid dye AO, as described by Darzynkiewicz *et al.* (15). Each cytogram was constructed from the analysis of 5000–10,000 cells. Fifteen experiments were done with PK 7.1 SN and three were done with BSFp1. The data shown are from a representative experiment.

Table 2. Effect of anti-δ and PK 7.1 SN on entry of B cells into G_2/S

Sample	Addition		% cells in G_2/S
	Phase 1	Phase 2	
Experimental			
1	Anti-δ	PK 7.1	3.5 ± 3.2
2	PK 7.1	Anti-δ	23.4 ± 6.5
3	PK 7.1 + anti-δ	PK 7.1 + anti-δ	27.0 ± 8.5
Control			
4	—	—	1.3 ± 0.9
5	Anti-δ	—	2.3 ± 1.8
6	—	Anti-δ	5.8 ± 0.2*
7	Anti-δ	Anti-δ	3.7 ± 0.8*
8	PK 7.1	—	3.8 ± 2.1
9	—	PK 7.1	0.8 ± 0.2
10	PK 7.1	PK 7.1	4.0 ± 1.6
11	Anti-δ	Anti-δ + PK 7.1	19.6 ± 6.7

Sorted $Thy.1^-$ small cells were cultured for 24 hr (phase 1) and then removed by gentle pipetting and centrifugation at low speed to separate the Sepharose beads from the cell suspension. Recovered cells (92%) were at least 90% viable following removal of the Sepharose. The cells were then washed once and replated for 48 hr (phase 2). Cell cycle analysis was performed as described in the legend to Table 1. Values are given as mean ± SEM. Data are from four experiments, except as noted.
*Data are from two experiments.

Table 3. Effect of $F(ab')_2$ anti-Ig and PK 7.1 SN on entry of B cells into G_2/S

Addition		% cells in
Phase 1	Phase 2	G_2/S
—	—	0.9 ± 0.5
Anti-Ig	PK 7.1	10.2 ± 1.7
PK 7.1	Anti-Ig	41.2 ± 1.6
PK 7.1 + anti-Ig	PK 7.1 + anti-Ig	47.0 ± 0.8

See legend to Table 2. $F(ab')_2$ anti-Ig fragments were used. Values are given as mean ± SEM. Data are from two experiments.

that induces resting B cells to become responsive to anti-δ (or anti-Ig). The present results do not exclude the possibility that BSFp1, once bound to its receptors, plays a role in the maintenance of cell growth as suggested by others (1). Furthermore, in the continuous presence of anti-Ig, BSFp1 may play a role in cell growth.

Experiments similar to ours have been reported using B cells from human tonsil (14), but in these experiments anti-μ-activated B cells responded to human BCGF (17) *after* anti-μ had been removed. The differences between these results and those reported here may be due to the source of B cells used. Thus, cells from human tonsil may be memory B cells that have already received an activation signal by BSFp1 *in vivo* and do not require BSFp1 *in vitro*. It is also possible that BSFp1 does not act on memory B cells.

The mechanism by which SN-containing BSFp1 induces B cells to become receptive to the growth-promoting activities of ligands such as anti-δ or anti-Ig is not known. Thus, it is unclear whether the BSFp1-mediated increase in Ia antigen levels or another effect of the BSFp1—e.g., membrane depolarization or phospholipid hydrolysis—increases the sensitivity of the cells to ligands such as anti-Ig. The mechanism(s) by which anti-Ig promotes growth is also unclear. One possibility suggested by several studies is that there are interactions between surface Ig and surface Ia (18, 19) and that these interactions promote cell growth. Indeed, the activation signal may be transmitted by surface Ia. Since BSFp1 causes an increase in levels of surface Ia antigens (9, 10), surface Ig–surface Ia interactions may be facilitated when cells are cultured with anti-μ or anti-δ. In support of this concept is the finding that some anti-Ia-reactive T-cell clones (20–24) or anti-Ia antibodies (25) induce B-cell proliferation. Presumably, continued replication of such stimulated B cells would require growth-promoting lymphokines. Candidates for such lymphokines include BCGF II, IL-1, and IL-2.

One can speculate on the physiological role of BSFp1. Activated T cells (stimulated by antigen presented on macrophages) would secrete BSFp1, which would induce an increase in the expression of Ia antigens on neighboring B cells. (These B cells could also be bound to the macrophage surface by native antigen.) The B cells that bind antigen specifically via surface Ig would then process it and present it to T cells in the context of the elevated levels of surface Ia. Thus, cognate T-cell help for such B cells would be facilitated.

Table 4. Effect of anti-δ and BSFp1 on entry of B cells into G_2/S

Addition		% cells in
Phase 1	Phase 2	G_2/S
—	—	2.1 ± 2.0
Anti-δ	BSFp1*	3.3 ± 1.3
BSFp1	Anti-δ	23.9 ± 6.4
BSFp1 + anti-δ	BSFp1 + anti-δ	24.3 ± 4.9

See legend to Table 2. Values are given as mean ± SEM. Data are from two experiments.
*0.5 unit (0.5 μl) per well.

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