

# PURIFICATION TO HOMOGENEITY OF B CELL STIMULATING FACTOR

## A Molecule That Stimulates Proliferation of Multiple Lymphokine-dependent Cell Lines

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The differentiation of resting B lymphocytes to Ig secretion involves several sequential steps regulated by antigen, T lymphocytes, and macrophages. The requirement for T cells may in some cases be replaced by lymphokines secreted upon T cell activation (1, 2). A number of distinct T cell-derived lymphokines have been described (3-9) that directly regulate the growth and maturation of B cells. These include IL-2 (3, 4), IFN- $\gamma$  (5, 6), and at least two additional, yet less well-characterized molecules, B cell stimulating factor, (also known as B cell growth factor, BCGF or BSF-1)<sup>1</sup> (7, 8), and B cell differentiation factor (BCGF-II or BCDF) (9). BSF-1 was originally (7) described as a factor that stimulated proliferation of B cells in conjunction with a submitogenic concentration of anti-Ig. Recent studies (10, 11) have shown that semipurified BSF-1 acts on resting B cells, facilitating their entry into S phase upon subsequent interaction with anti-Ig. These studies (11) indicate that BSF-1 might actually be a differentiation factor, and they further suggest that BSF-1 may not have growth factor activity.

We have purified BSF-1 to homogeneity from culture supernatants of mitogen-activated EL4 thymoma cells. We used the proliferation of highly purified splenic B cells in the presence of anti-IgM as an assay in our purification procedure. The purification was also monitored for additional lymphokines using the factor-dependent cell lines CTLL-2 and FDC-P2. Interestingly, fractions containing BSF-1 always contained stimulatory activity for both IL-2- and IL-3-dependent cell lines, even though these fractions were devoid of IL-2 and IL-3 protein. *N*-terminal amino acid sequencing of homogeneous BSF-1 revealed a unique protein sequence completely different from that of murine IL-2 or IL-3. Based on these results, we conclude that BSF-1 is both a growth and differentiation factor that may have biologic effects beyond the B lymphocyte compartment.

### Materials and Methods

*Mice.* Female C57BL/6J mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and were used at 8-12 wk of age.

<sup>1</sup> *Abbreviations used in this paper:* BSF-1, B cell-stimulating factor; DIEA-Ac, *N,N*-diisopropylethylamine; TFA, trifluoroacetic acid; TMS-silica, trimethylsilyl-silica.

*Preparation of B Lymphocytes.* Mice were pretreated with intraperitoneal injections of T24 rat anti-mouse Thy-1 mAb (12). 3 d later, the spleens were removed and treated in vitro with a cocktail of T24, GK1.5 rat anti-mouse L3T4 mAb, rabbit anti-mouse thymocyte serum (liver- and bone marrow-absorbed) and rabbit complement (Pel-Freeze Biologicals, Rogers, AR). The T cell-depleted spleen cells were then passed over Sephadex G10 to remove adherent cells, and the B lymphocytes were positively selected by panning on petri dishes coated with goat anti-mouse IgM (Cooper Biomedical, Inc., Malvern, PA). Purified B cells contained undetectable levels of T cells and >98% B cells as judged by flow cytometer analysis (Epics-C; Coulter Electronics, Inc., Hialeah, FL) using T24 and anti-L3T4 rat mAb, followed by FITC-conjugated rabbit anti-rat Ig (Becton Dickinson Immunocytometry Systems, Mountain View, CA) to detect T cells, and FITC-conjugated rabbit anti-mouse IgM to detect B cells. In addition, such a B cell population was found to be completely unresponsive to the T cell mitogen, Con A, but retained full responsiveness to the B cell mitogen LPS.

*Cell Lines.* EL4 thymoma cells were maintained in RPMI-1640 supplemented with 5% FCS, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin and 2 mM glutamine. For the production of lymphokine-containing supernatants, EL4 cells were stimulated in serum-free medium with 1% PHA (PHA-M; Difco Laboratories, Detroit MI) and 10 ng/ml PMA (Sigma Chemical Co., St. Louis, MO). Cell-free supernatant was collected by centrifugation after 24 h.

CTLL-2 an IL-2-dependent murine T cell line (13) was maintained in Click's medium (Atlick Associates, River Falls, WI) containing 10% FCS, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, and 100 U/ml of IL-2 from a supernatant of rat spleen cells cultured 24 h with Con A (Pharmacia Fine Chemicals, Piscataway, NJ).

FDC-P2, a factor-dependent murine cell line derived from long-term bone marrow cultures (14) was maintained in RPMI-1640 containing 20% horse serum, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, 50  $\mu$ M 2-ME, and 10% WEHI-3 cell line-conditioned medium.

32D, an IL-3-dependent murine hemopoietic cell line (15) was kindly provided by Dr. James Watson (Auckland Medical School, Auckland, New Zealand) and was maintained in Click's medium containing 10% WEHI-3 cell line-conditioned medium, 10% FCS, and antibiotics.

*Cellular Assays.* BSF-1 was assayed by its ability to stimulate the proliferation of purified B cells in the presence of a submitogenic concentration of goat anti-mouse IgM (7). B cells were cultured ( $10^5$  cells/culture) in 200  $\mu$ l containing 3–5  $\mu$ g/ml of affinity-purified goat anti-mouse IgM (Cooper Biomedical, Inc.) and serial dilutions of test sample. After 72 h, cultures received 2.0  $\mu$ Ci of [ $^3$ H]thymidine (75 Ci/mmol, New England Nuclear, Boston, MA) for 6 h, were harvested onto glass fiber filters, and incorporation of radioactivity was measured.

All proliferation assays using factor-dependent cell lines were performed using  $2 \times 10^3$  cells/culture in 100  $\mu$ l of Click's medium containing 10% FCS, antibiotics, and test samples. After 24 h, cultures received 2.0  $\mu$ Ci of [ $^3$ H]thymidine (75 Ci/mmol) for 6 h, were harvested onto glass fiber filters, and incorporation of radioactivity was measured. For all proliferation assays, 1 U of activity was defined as the amount of lymphokine that induced 50% of maximal proliferation in 100  $\mu$ l cultures. For example, if a sample induced 50% of maximal proliferation at a dilution of 1:20, then 1 U was said to be contained in one-twentieth of 100  $\mu$ l, or 5  $\mu$ l, and the sample said to contain 200 U/ml.

*Lymphokines.* A mouse IL-2 cDNA was cloned from a library prepared from LBRM-33 lymphoma cell mRNA by Immunex Corporation and was expressed in yeast using the yeast  $\alpha$  factor promoter and leader sequences to direct synthesis and secretion (16). rIL-2 was purified to homogeneity as previously described (17), and had a specific activity of  $10^9$  U/mg.

A mouse IL-3 cDNA was also cloned from the above-detailed library by Immunex Corporation, expressed in yeast, and resultant rIL-3 was purified to homogeneity (18). Purified murine rIL-3 had sp act of  $1.1 \times 10^{10}$  U/mg.

*BSF-1 Purification.* BSF-1 was purified from 71.5 liters of supernatant from PHA and

PMA-stimulated EL4 thymoma cells. BSF-1 activity was extracted from crude supernatants by adsorption to trimethylsilyl-silica (C-1, Sepalyte; Analytichem International, Harbor City, CA) as previously described (19), with modifications. Crude supernatant was acidified with 0.1% trifluoroacetic acid (TFA), and trimethylsilyl (TMS)-silica added (10 g/liter). After stirring 1.5 h, the supernatant was decanted. The TMS-silica was poured into a column, washed with 20% acetonitrile with 0.1% TFA, and the BSF-1 was eluted with 75% acetonitrile and 0.1% TFA. Acetonitrile was removed by rotary evaporation, and the aqueous phase was adjusted to 5 mM sodium citrate, 50 mM NaCl, pH 5.5.

The TMS-silica-purified BSF-1 was fractionated on carboxymethyl cellulose (CM52; Whatman, Inc., Clifton, NJ). BSF-1 bound and was eluted with a linear NaCl gradient. The fractions containing BSF-1 were pooled, dialyzed against 20 mM Tris, pH 9.0, and fractionated on quarternary aminoethyl cellulose (QA52, Whatman, Inc.) to which BSF-1 bound, and was eluted with a linear NaCl gradient.

Reversed-phase HPLC fractionation of partially purified BSF-1 preparations was performed on a 4.6 × 250 mm Vydac 218TP (C<sub>18</sub>) column (The Separations Group, Hesperia, CA) using a Beckman Model 344 solvent delivery system. For purification step HPLC I (Table I), the column was equilibrated with 0.1% TFA in water at a flow rate of 0.8 ml/min. Fractions containing BSF-1 activity from the QA52 column were pooled, adjusted to pH 2 with TFA, and injected onto the column. The column was washed for 10 additional minutes with 0.1% TFA and then brought to 10% acetonitrile (containing 0.1% TFA) over 2 min. After an additional 8 min of equilibration at 10% acetonitrile, a linear gradient from 10 to 70% acetonitrile in 0.1% TFA was run over 60 min (1% per minute), and 1-min fractions were collected.

For HPLC II, active fractions from HPLC I were pooled and concentrated in vacuo to 250  $\mu$ l, and injected onto the same column, equilibrated with 50 mM acetic acid adjusted to pH 4.50 with *N,N*-diisopropylethylamine (DIEA-Ac), at a flow rate of 0.7 ml/min. After a 5 min wash with DIEA-Ac, the column was brought to 5% *n*-propanol over 2 min. After an additional 8 min of washing, a linear gradient from 5 to 40% *n*-propanol in DIEA-Ac over 70 min (0.5%/min) was run, and 1 min fractions were collected.

*N-terminal Protein Sequencing.* Amino-terminal amino acid sequencing was performed on an Applied Biosystems Model 470A protein sequencer (Applied Biosystems Inc., Foster City, CA). Homogeneous BSF-1 from HPLC II fractions 61 and 62 (Fig. 1) were concentrated in vacuo to a final volume of 30  $\mu$ l and then spotted onto a conditioned sequencer filter. Sequencing and PTH amino acid analysis was performed as described previously (20).

*SDS-PAGE.* Fractions from the purification steps were monitored by SDS-PAGE and subsequent silver staining, as described previously (21).

## Results

*Initial Purification.* BSF-1 was purified from serum-free supernatants of PHA- and PMA-stimulated EL4 cells as described in Materials and Methods and Table I. BSF-1 was extracted from crude supernatants by adsorption to TMS-silica. BSF-1 was eluted in TFA/acetonitrile, and further fractionated by a combination of cation- (CM52) and anion- (QA52) exchange chromatography (Table I).

*HPLC.* Biologically active fractions eluted from the QA52 column were pooled and fractionated by HPLC on a C18 column as described in Materials and Methods. After one fractionation using a TFA/acetonitrile buffer system (HPLC I), an intense band at 18.4 kD was detected by silver staining on SDS-PAGE gels (Fig. 1, lane 1, SM), which correlated with BSF-1 biological activity. BSF-1 biological activity was eluted from the HPLC column in 42% acetonitrile.

The biologically active fractions from HPLC I were further fractionated using the same HPLC column equilibrated in a different buffer system (see Materials and Methods). As shown in Fig. 1, this fractionation yielded homogeneous BSF-

TABLE I  
Purification of BSF-1

Step	Total activity	Specific activity	Yield
	$U \times 10^{-6}$	$U/\mu g$	%
Crude	21.4	3	100
TMS-silica	19.7	100	92
CM52	13.7	2,500	64
QA52	5.6	26,000	26
HPLC I	5.1	127,100	24
HPLC II	1.6	328,000	8

BSF-1 was purified from 71.5 liters of crude EL4 supernatant as described in Materials and Methods. Units of BSF-1 activity were determined using purified B cells. Protein was measured using the Bradford protein assay (BioRad Laboratories, Richmond, CA).

1 protein as detected by silver-stained SDS-PAGE, and as confirmed by protein sequencing. The peak of BSF-1 biological activity eluted at 32% *n*-propanol. Although acceptable yields of protein were recovered at this step, some loss of biological activity was observed, which possibly was due to denaturation of the BSF-1 molecule in the propanol solvent. The observed sp act of this BSF-1 was  $3.28 \times 10^8$  U/mg.

*Protein Sequencing.* Fractions 61 and 62 (Fig. 1) from the HPLC II-purified preparation were subjected to amino-terminal protein sequencing. Only one sequence was obtained, consistent with the preparation of BSF-1 having been purified to homogeneity. The yields for each cycle of amino-terminal sequencing are shown in Table II. The initial yield, 56%, is based upon isoleucine in cycle two, due to typically poor yields for histidine residues (cycle one). The absolute yield of 67.4 pmol isoleucine in cycle two is consistent with the expected yield (50–70%) from the 120 pmol (2.2  $\mu g$ ) of BSF-1 applied to the sequencer filter. The first 20 residues, His-Ile-His-Gly-Cys-Asp-Lys-Asn-His-Leu-Arg-Glu-Ile-Ile-Gly-Ile-Leu-Asn-Glu-Val, were found to be a unique sequence when compared to previously published reports (22). The assignment of Cys to position 5 is tentative, since no signal was observed for cycle 5 and the protein was not modified before sequencing.

*Biological Activity.* All fractions throughout the purification were simultaneously monitored for BSF-1, IL-2 (CTLL proliferation), and IL-3 (FDC-P2 proliferation) activities. The majority of IL-2, as measured by CTLL proliferation, separated from BSF-1 on the CM52 column. However, subsequent to that step, all CTLL activity eluted precisely with the BSF-1 activity. EL4 supernatants contained no IL-3 protein, and all FDC-P2 activity eluted with BSF-1. The CTLL and FDC-P2 cell line response assays, using HPLC-purified BSF-1 fractions, are shown in Fig. 2. It is clear that purified BSF-1 has the capacity to score positively in both conventional IL-2 and IL-3 assays.

As seen in Fig. 3, several factor-dependent cell lines were compared for their responses to IL-2, IL-3, and BSF-1. CTLL, FDC-P2, and 32D all respond to BSF-1, but to a lesser extent (a lower maximum incorporation and a shallower slope) than to either IL-2 or IL-3.

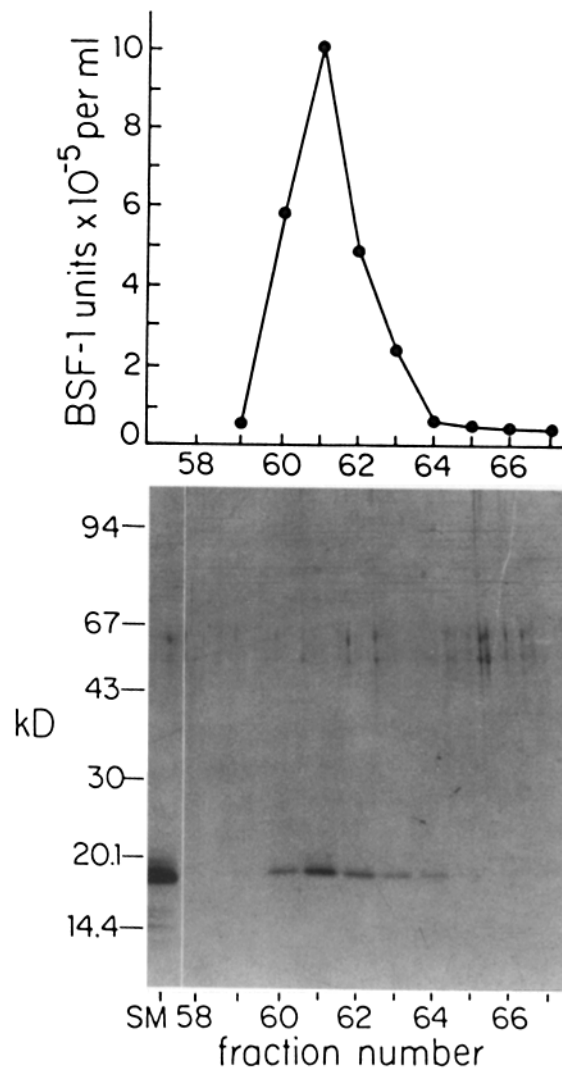


FIGURE 1. Purification of BSF-1 by HPLC. HPLC fractions from the final purification step (HPLC II) shown in Table I were assayed for BSF-1 activity (*top*) and analyzed by SDS-PAGE (*bottom*) as described in Materials and Methods. SM (starting material) in lane 1 is the pool of active fractions from HPLC I.

### Discussion

We have purified murine BSF-1 to homogeneity from supernatants of stimulated EL4 thymoma cells, and have determined its amino-terminal amino acid sequence. Homogeneous BSF-1 was found to have a molecular mass of 18.4 kD, with a sp act of at least  $3.28 \times 10^8$  U/mg. In addition, this lymphokine was found to stimulate the proliferation of both IL-2- and IL-3-dependent cell lines.

BSF-1 was originally described as B cell growth factor because it stimulated partially purified splenic B lymphocytes to proliferate in the presence of submitogenic concentrations of goat anti-mouse IgM (7). However, recent evidence

TABLE II  
Protein Sequence from Purified Murine BSF-1

Amino acid	Yield (pmol) from cycle number:																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Asn	—*	—	—	—	—	—	—	45.5	—	—	—	—	—	—	—	—	—	—	23.5	—
Ser	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Thr	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Gln	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Gly	—	—	—	23.4	—	—	—	—	—	—	—	—	—	—	17.3	—	—	—	—	—
His	34.6	—	26.2	—	—	—	—	—	19.6	—	—	—	—	—	—	—	—	—	—	—
Ala	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Asp	—	—	—	—	—	36.6	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Arg	—	—	—	—	—	—	—	—	—	—	24.8	—	—	—	—	—	—	—	—	—
Glu	—	—	—	—	—	—	—	—	—	—	—	22.7	—	—	—	—	—	—	17.1	—
Tyr	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Val	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	26.6
Pro	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Met	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ile	—	67.4	—	—	—	—	—	—	—	—	—	—	30.5	41.4	11.4	34.7	—	—	—	—
Leu	—	—	—	—	—	—	—	—	—	34.9	—	—	—	—	—	—	31.3	—	—	—
Phe	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Trp	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Lys	—	—	—	—	—	—	37.3	—	—	—	—	—	—	—	—	—	—	—	—	—
Cys	—	—	—	—	( ) <sup>‡</sup>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Sequence assigned to first 20 amino acids of BSF-1 is: His-Ile-His-Gly-Cys-Asp-Lys-Asn-His-Leu-Arg-Glu-Ile-Ile-Gly-Ile-Leu-Asn-Glu-Val.

\* Values <10 pmol are omitted for clarity.

<sup>‡</sup> Assigned as Cys due to lack of other signals; sulfhydryl groups were not modified before sequencing.

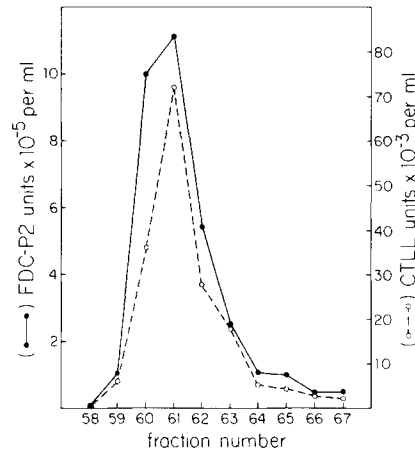


FIGURE 2. Assay of HPLC II fractions on CTLL and FDC-P2 cells. HPLC fractions from the final purification step (HPLC II) shown in Table I and Fig. 1 were assayed in the CTLL and FDC-P2 proliferation assays as described in Materials and Methods.

(10, 11) suggests that semipurified BSF-1 is not a direct growth factor for small resting B cells, but rather primes them for subsequent entry into S phase on encounter with anti-IgM, indicating that BSF-1 is a differentiation factor. Consistent with this role of BSF-1 are the findings that partially purified BSF-1 induces the cell surface expression of Ia on B cells (23, 24), and the secretion of IgG1 by LPS-activated B cells (25–27). Roehm et al. (24) have also observed that partially purified BSF-1 augments the antigen-presenting function of B cells.

Our observation that BSF-1 directly stimulates proliferation of IL-2- and IL-

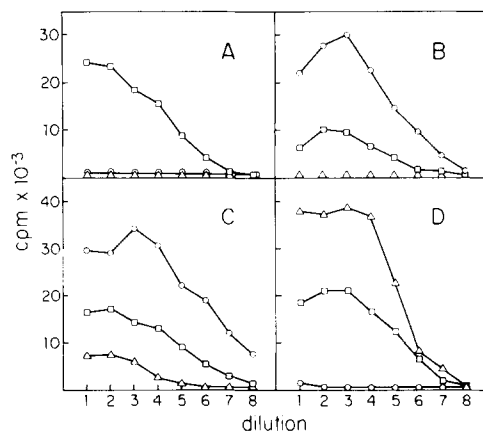


FIGURE 3. Response of B cells and factor-dependent cell lines to homogeneous lymphokines. Homogeneous BSF-1 ( $\square$ ), rIL-2 ( $\Delta$ ), and rIL-3 ( $\circ$ ) were tested for the stimulation of purified B cells (A), FDC-P2 (B), 32D (C), and CTLL (D) as described in Materials and Methods. Threefold dilutions of each lymphokine were tested starting at 500 U/ml and results reported in  $\text{cpm} \times 10^{-3}$ .

3-dependent cell lines indicates that BSF-1 is a growth factor as well as a differentiation factor. This should not be surprising, as similar results have been obtained in studies of other lymphokines. For example, IL-2 stimulates proliferation of T cells, however, it has recently become evident that IL-2 also elicits a variety of differentiated T cell functions (28–30). Moreover, granulocyte-macrophage-CSF (GM-CSF) has also been shown to activate differentiated macrophage (31) and granulocyte (32) effector functions, in addition to its traditional growth-stimulating activity on myeloid stem cells in the marrow.

Lymphokines that act on multiple lineages of cells are not without precedent. IL-2, once thought to act only on T lymphocytes, has recently (3, 4) been shown to drive proliferation of B cells as well. Additionally, several IL-3-dependent nonlymphoid cell lines have also been shown capable of responding to IL-2 (33, 34). Sanderson et al. (35) have reported that another B cell stimulating factor, BCGF-II, also acts as an eosinophil differentiation factor. One important caveat remains: although we have shown that BSF-1 stimulates non-B cell, factor-dependent cell lines to proliferate, it has yet to be determined whether this reflects a role of BSF-1 on normal cells, or whether those responses represent abnormal capabilities on the part of cells maintained *in vitro* for extended periods of time.

The ability of BSF-1 to act on IL-2- and IL-3-dependent cell lines suggests that BSF-1 has target cells beyond the B cell lineage, and may have significant functions other than the regulation of B cell growth and maturation.

### Summary

Murine B cell stimulating factor 1 (BSF-1) was purified to homogeneity from supernatants of a stimulated thymoma cell line. A protein of 18.4 kD with a unique *N*-terminal amino acid sequence was identified. BSF-1 had a sp act of at least  $3.28 \times 10^8$  U/mg. In addition to its B cell-stimulatory activity, BSF-1 also

stimulated the proliferation of several IL-2- and IL-3-dependent cell lines. We conclude that BSF-1 is both a growth factor and a differentiation factor. Finally, these results also suggest additional biologic properties of BSF-1 on lineages besides B lymphocytes.

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