

RECEPTORS FOR B CELL STIMULATORY FACTOR 2 Quantitation, Specificity, Distribution, and Regulation of Their Expression

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Several factors are involved in the regulation of growth and differentiation of B cells (reviewed in 1). In fact, B cell stimulatory factor 2 (BSF-2),¹ which has recently been cloned (2), is a distinct cytokine of 21 kD that acts on activated normal human B cells as well as EBV-transformed B-lymphoblastoid cell lines to induce immunoglobulin secretion (3, 4). The study with the antipeptide antibody specific to BSF-2 demonstrated that several tumor cells, including cardiac myxomas, produce BSF-2, and patients with such tumors show hypergammaglobulinemia and autoantibody production (5). From these earlier investigations it was suggested that BSF-2 has an important role in the regulation of antibody production.

Recent reports have indicated identity between BSF-2 and other cytokines known as interferon β_2 (IFN- β_2) and hybridoma plasmacytoma growth factor (HPGF) (6, 7). Another identical molecule is also reported as a 26 kD protein (8) that is expressed in human fibroblast by poly (IC) in the presence of cycloheximide (9) or (IL-1) (10). These facts suggest that BSF-2 has an important role not only in the immunoglobulin production of B cells but also in the regulation of growth and differentiation of several other types of cells. The recombinant BSF-2 had no detectable antiviral activity² (11), and it has been proposed that it be called IL-6 (11).

However, no information is available as to the presence of specific BSF-2 receptors (BSF-2-R) mediating the physiological effects. The availability of highly purified BSF-2 produced in *Escherichia coli* by recombinant DNA techniques enabled us to study the presence and the properties of BSF-2-R. We report here in this study the number and distribution of BSF-2-R. Our findings indicate that BSF-2-R are widely distributed in several tissues and cell lines. In contrast to BSF-1-R, which are found on resting B cells (12), our observations indicate that normal B cells express BSF-2-R only after activation.

¹ *Abbreviations used in this paper:* AET, S-(2-aminoethyl)isothiuronium bromide; BSF-2, B cell stimulatory factor 2; G-CSF, granulocyte colony-stimulating factor; MNC, mononuclear cell; SAC, *Staphylococcus aureus* Cowan I.

² Hirano, T., T. Matsuda, K. Hosoi, A. Okano, H. Matsui, and T. Kishimoto. B cell stimulatory factor 2 (BSF-2/IFN- β_2) does not belong to the family of interferons. Manuscript submitted for publication.

Materials and Methods

Lymphokines. Recombinant BSF-2 was prepared by expressing a cDNA for BSF-2 (2) in *E. coli*, followed by further purification. Specific activity was determined as 3.6×10^9 U/g by using the BSF-2-responsive human B lymphoblastoid cell line, SKW6-CL4 (4). Human IFN- β and IFN- γ were kind gifts from Toray, Co., Ltd. (Tokyo, Japan) and possessed specific activities of 1.3×10^{11} and 5.9×10^9 U/g, respectively. Recombinant human IL-1 β and IL-2 were supplied by Otsuka pharmaceutical Co., Ltd. (Tokushima, Japan), at specific activities of 2.0×10^{10} and 5.0×10^{10} U/g, respectively. Recombinant human granulocyte colony-stimulating factor (G-CSF) with a specific activity of $2.5\text{--}10 \times 10^{10}$ U/g was generously provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan).

Iodination of BSF-2. ^{125}I -BSF-2 was prepared as described by Bolton and Hunter (13) with some modifications. 5 μg of recombinant BSF-2 dissolved in 10 μl of 0.1 M borate buffer, pH 8.5, was added to 500 μCi of dry Bolton and Hunter reagent (2,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) and the reaction mixture was agitated for 15 min on ice. The reaction was stopped by adding 0.5 ml of 0.2 M glycine in 0.1 M borate buffer, pH 8.5, with further mixing for 5 min on ice. ^{125}I -labeled BSF-2 was separated from ^{125}I -labeled products of low molecular mass using a gel filtration column (PD-10; Pharmacia Fine Chemicals, Piscataway, NJ). ^{125}I -BSF-2 was diluted with RPMI 1640, 25 mM Hepes, pH 7.2, 3 mg/ml BSA, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 U/ml penicillin (binding medium), filter sterilized, and stored at 4°C. Purity and molecular mass of ^{125}I -BSF-2 was examined by SDS-PAGE on a slab gel (10–20% continuous gradient of acrylamide) followed by autoradiography.

Binding Assay. Usual binding assay was performed as follows: cells for assay were washed twice and incubated in binding medium for at least 10 min at 37°C. 10^6 cells were mixed with ^{125}I -BSF-2 with or without a 200-fold excess of unlabeled BSF-2 in a final volume of 70 μl of binding medium, and incubated on ice for 150 min with occasional agitation every 10–15 min. At the end of incubation, the reaction mixture was layered on a 300- μl cushion of FCS in a 400- μl polypropylene tube and centrifuged at 9,000 rpm for 90 s. The tube was cut just above the cell pellet, and the cell-associated radioactivity was measured in a Beckman Gamma 9000 (Beckman Instruments, Fullerton, CA). The specific binding was calculated after subtracting the count of the samples with 200-fold excess unlabeled BSF-2 (nonspecific bound count).

Determination of Specific Radioactivity of ^{125}I -BSF-2. Maximal binding capacity was measured according to a described method (14). Briefly, varying numbers of EBV-transformed B-LCL, CESS cells were mixed with 9,300 cpm of ^{125}I -BSF-2 with or without a 200-fold excess of unlabeled BSF-2 in a final volume of 70 μl of binding medium. Reaction mixtures were incubated on ice for 150 min with frequent agitation every 10–15 min. Specific binding was calculated as indicated above and data were plotted as reciprocal radioactivity specifically bound versus reciprocal cell number. From the ordinate intercept of a regression line of the plots, maximal binding capacity at infinite cell number was determined.

Self-displacement analysis was performed according to Calvo et al. (14). 10^6 CESS cells were mixed with 9,300 cpm of ^{125}I -BSF-2 and increasing amounts of unlabeled BSF-2 or ^{125}I -BSF-2 were added in a total volume of 70 μl of binding medium. At the end of 150 min of incubation on ice, the cells were separated by centrifugation on FCS, and cell-associated radioactivity was measured. Data were transformed into a bound/free ratio and plotted against \log_{10} (amount of unlabeled BSF-2 [in nanograms]) or \log_{10} (amount of ^{125}I -BSF-2 [counts per minute]). Two regression lines were drawn and the specific radioactivity was determined as the amount of radioactivity divided by the amount of unlabeled BSF-2 added to obtain the same bound/free ratio.

Human Cell Lines. SK-MG-4 and SK-N-MC were kindly provided by Dr. R. Ueda, Aichi Cancer Institute, Nagoya, Japan. Daudi, HSB, CEM, Jurkat, and U937 were provided by Dr. P. Ralph, Memorial Sloan-Kettering Cancer Center, New York. Reh and KM-3 were provided by K. Kikuchi, Sapporo Medical College, Sapporo, Japan. HL60 and U373 were obtained from the American Type Culture Collection, Rockville, MD. BL41 and BL41/45 were from Dr. G. Klein (Karolinska Institute). Louckes and EBNA2-

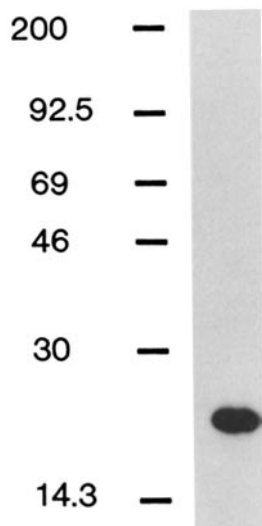


FIGURE 1. Autoradiograph of ^{125}I -BSF-2 analyzed by SDS-PAGE. Recombinant BSF-2 was iodinated and an aliquot was applied on polyacrylamide gel with a continuous gradient of 10–20% acrylamide. The molecular mass (kD) of protein standards are also indicated.

transfected Louckes cells were from Dr. E. Kieff (University of Chicago, Chicago, IL). EBV-transformed B cell lines SKW6-CL4, LCL13, and LCL14 were described elsewhere (2, 15). All human cell lines except U373 were maintained in RPMI 1640, 10% FCS, 5×10^{-5} M 2-ME, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin. U373 was cultured in MEM, 10% FCS, 100 $\mu\text{g}/\text{ml}$ sodium pyruvate, and nonessential amino acid mixture (Gibco, Grand Island, NY). All were cultured at 37°C , in humidified 5% CO_2 in air.

Normal Human T and B Cells. Human T and B cells were prepared as follows. Tonsillar cells were dispersed on a plastic dish and mononuclear cells (MNC) were isolated by centrifugation on Ficoll-paque (Pharmacia Fine Chemicals). T cells were prepared from MNC by two sequential rosettings with *S*-(2-aminoethyl)isothiuronium bromide (AET)-treated SRBC. B cells were isolated from MNC by AET-treated SRBC-rosetting twice, and rosette-negative cells were further treated with anti-CD2 (kindly provided by Dr. E. L. Clark) and rabbit complement to eliminate possible contaminating T cells. The purity of T and B cells were examined by FACS analysis. T and B cells were cultured in the same medium described above for most of the cell lines at a density of $2 \times 10^6/\text{ml}$. PWM (Sigma Chemical Co., St. Louis, MO), 2.5 $\mu\text{g}/\text{ml}$ final concentration; PHA (Sigma Chemical Co.), 0.1% wt/vol, final concentration; and *Staphylococcus aureus* Cowan I (SAC) from Bethesda Research Laboratories, Gaithersburg, MD, 0.003% (vol/vol), final concentration, were used as stimulants. Size fractionation of B and T cells was performed by Percoll (Pharmacia Fine Chemicals) discontinuous gradient centrifugation. Large cells were separated from the interface between 40 and 50% Percoll, and small cells were from the interface between 55 and 70% after centrifugation at 3,500 rpm for 25 min at 4°C .

Results

Kinetics of Binding of ^{125}I -BSF-2. Recombinant BSF-2 was radiolabeled with ^{125}I as described in Materials and Methods, and radioiodinated BSF-2 was analyzed by SDS-PAGE. Autoradiography showed only one major band, with an M_r of 21,000 (Fig. 1). This preparation was used for the analysis of BSF-2-R.

The EBV-transformed B-LCL, CESS, which is known to be responsive to BSF-2 (16), was used for the initial binding studies. As shown in Fig. 2, the binding of BSF-2 at 0°C achieved a steady-state level within 150 min. Maximum binding at 37°C was about one-half that observed at 0°C . The addition of 0.02% sodium azide could keep the binding stable at 37°C , but could not increase the maximum

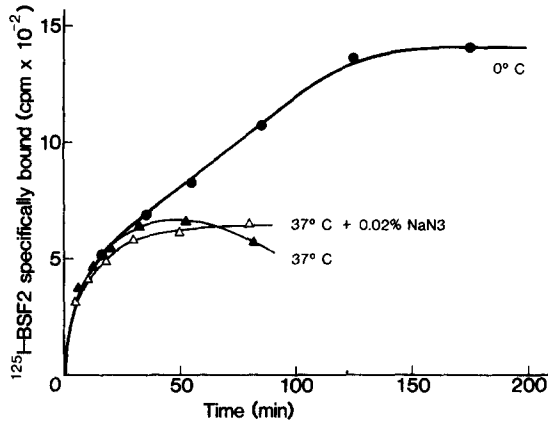


FIGURE 2. Kinetics of ^{125}I -BSF-2 binding to CESS cells. 10^6 CESS cells in $70\ \mu\text{l}$ binding medium with $11,000\ \text{cpm}$ ^{125}I -BSF-2 in the presence or absence of 200-fold excess unlabeled BSF-2 were incubated for the indicated times at 0°C or 37°C with or without 0.02% sodium azide. Data represent the mean specific binding of duplicate samples with the deviation usually $<5\%$.

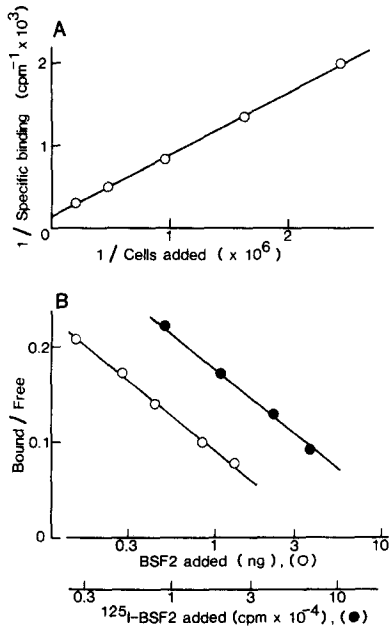


FIGURE 3. Determination of the maximal binding capacity and specific radioactivity of ^{125}I -BSF-2. (A) $9,300\ \text{cpm}$ of ^{125}I -BSF-2 was incubated for 150 min with five different concentrations of CESS cells (up to 5×10^6) in the presence or absence of 200-fold excess amount of unlabeled BSF-2. Data (averages of duplicates) are plotted as reciprocal specific binding versus reciprocal cells added. The ordinate intercept of the regression line means the reciprocal counts that would bind at infinite number of cells (maximal binding capacity). (B) Self-displacement analysis is performed as described in Materials and Methods. $9,300\ \text{cpm}$ of ^{125}I -BSF-2 and increasing amounts of unlabeled BSF-2 (up to $1.3\ \text{ng}$) or ^{125}I -BSF-2 (up to $80,000\ \text{cpm}$) were incubated with 10^6 CESS cells. The mean specific binding of duplicates is plotted as a bound/free ratio (corrected for maximal binding capacity). The specific radioactivity is determined as mentioned in the text.

binding. In addition, the level of the nonspecific binding of ^{125}I -BSF-2 at 37°C in the presence of 200-fold excess unlabeled BSF-2 was twofold as high as that at 0°C (data not shown). Therefore, the standard experimental condition of 150 min incubation at 0°C , was used through the study.

Determination of Specific Radioactivity of ^{125}I -BSF-2. To determine how much of the total radiolabeled BSF-2 was bindable to CESS, we used a graphic method presented previously (14). As briefly described in Materials and Methods, increasing numbers of CESS cells were incubated with a constant amount of ^{125}I -BSF-2 in a final volume of $70\ \mu\text{l}$. Nonspecific binding of ^{125}I -BSF-2 was measured by the addition of 200-fold excess of unlabeled BSF-2. Fig. 3A shows the regression line of the plot of reciprocal specific binding versus reciprocal cell number. From its ordinate intercept, the maximal binding capacity at the infinite cell number

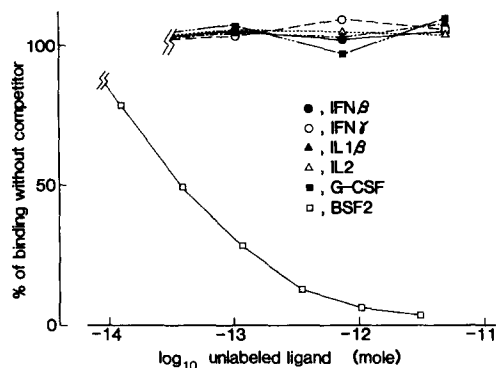


FIGURE 4. Competition for the binding of ^{125}I -BSF-2 by unlabeled BSF-2 and other lymphokines to CESS cells. 10^6 CESS cells and 5.7×10^{-15} mol of ^{125}I -BSF-2 are incubated in $70 \mu\text{l}$ binding medium on ice for 150 min with indicated amount of lymphokines. Data are mean of duplicate samples.

was calculated to be 82.6% of the total radioactivity. This means only 17.4% of the initial BSF-2 lost its binding ability during radioiodination.

The specific radioactivity of ^{125}I -BSF-2 was determined by self-displacement analysis (14). Details are described in Materials and Methods. Fig. 3B shows two regression lines of bound/free ratio versus additional amount of unlabeled BSF-2 or additional ^{125}I -BSF-2 to a constant set of initial ^{125}I -BSF-2 and CESS cells. The identical slope of the two lines indicates that there was no significant difference in binding affinity between unlabeled BSF-2 and ^{125}I -BSF-2 to its receptors. The results indicate that 2.89×10^4 cpm of ^{125}I -BSF-2 was equivalent to 0.386 ng of BSF-2 in the meaning of the induction of the same bound/free ratio of 0.15, thus the calculated specific radioactivity (corrected for the maximal binding capacity) of ^{125}I -BSF-2 was 6.16×10^{13} cpm/g.

Specificity of ^{125}I -BSF-2 Binding on CESS Cells. The ability of unlabeled BSF-2 and other cytokines to compete with ^{125}I -BSF-2 for binding sites on CESS cells is shown in Fig. 4. Unlabeled BSF-2 inhibited the binding of ^{125}I -BSF-2 in a dose-dependent manner. As much as 96.5% of the total radioactivity of ^{125}I -BSF-2 bound to CESS without competitor was inhibited by 560-fold excess unlabeled BSF-2. No significant competition was observed with any other cytokines, such as IFN- β , IFN- γ , IL-1 β , IL-2, and G-CSF. Previous studies had shown sequence homology between BSF-2, G-CSF (2), and IFN- β (17). However, large excess of G-CSF or IFN- β could not inhibit the binding of ^{125}I -BSF-2 to CESS, indicating that BSF-2, IFN- β , and G-CSF are using different receptors.

Scatchard Plot Analysis of Binding of ^{125}I -BSF-2. The specific binding of ^{125}I -BSF-2 to CESS cells as a function of the concentration of ^{125}I -BSF-2 is shown in Fig. 5A. ^{125}I -BSF-2 bound to CESS in a saturable manner. Analysis of the binding data by the method of Scatchard (18) revealed only one linear regression line, indicating that there was a single set of binding sites on CESS (Fig. 5B). The negative inverse of the regression coefficient gave a dissociation constant of 3.4×10^{-10} M and the abscissa intercept provided a number of 2,700 binding sites per cell.

BSF-2-R on Human Cell Lines. Scatchard analyses were carried out on various human cell lines and are summarized in Table I. All the analyzed EBV-transformed B cell lines had BSF-2-R with an affinity of 2.0 – 4.0×10^{-10} M. The number of receptors on B-LCL was in the range of 200–2,700 per cell. In contrast, none of the Burkitt's lymphoma cell lines were found to express any

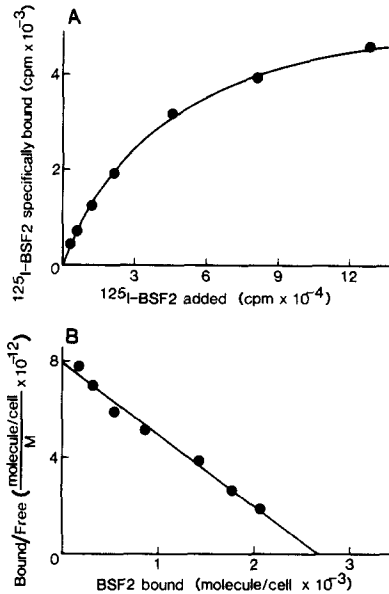


FIGURE 5. (A) Specific binding of ^{125}I -BSF-2 to EBV-transformed B cell line CESS. (B) Scatchard plot of the same data. The average of the duplicate is shown.

detectable number of receptors. The infection of EBV into EBV⁻ Burkitt's cells (BL41) or the transfection of EBNA2 into Burkitt's cells (Louckes) (19) did not induce any expression of the BSF-2-R. The plasma cell lines ARH-77 and U266 displayed 120 and 11,000 receptors per cell, respectively, with affinities of 1.5×10^{-10} and 2.5×10^{-10} M. Four T cell lines tested had no BSF-2-R.

BSF-2-R levels equivalent to CESS cells were detectable on histiocytic leukemia cell line U937 and promyelocytic leukemia HL60, whereas the receptor number on astrocytoma U373 and glioblastoma SK-MG-4 was in the low detectable range. Several other hematopoietic cell lines, such as a non-T, non-B line, KM3 and Reh, did not express any receptors.

BSF-2-R on Normal Lymphocytes. Normal human T and B cells were purified from tonsillar mononuclear cells and examined for the expression of BSF-2-R. Fig. 6 shows specific binding of ^{125}I -BSF-2 to human T cells and its Scatchard analysis. The results showed that resting T cells expressed BSF-2-R with a homogeneous affinity. The binding sites per cell were around 300 and the affinity was 1.4×10^{-10} M. Upon stimulation of T cells for 3 d with 0.1% PHA or 0.003% (vol/vol) SAC + 2.5 $\mu\text{g}/\text{ml}$ PWM, the level of binding sites remained constant or were slightly decreased. Activated large T cells displayed fewer receptors than the smaller ones. These results, summarized in Table II, are in contrast to the fact that four human T cell lines examined had no significant number of BSF-2-R (Table I).

In a marked contrast, freshly prepared resting human B cells displayed no detectable BSF-2-R in the absence of stimulation (tonsils 1, 2, and 3 in Table II, lower half). The level of receptor number increased upon activation of B cells with 0.003% SAC for 1, 2, or 3 d (tonsils 3, 4, and 6). The result obtained with B cells from the tonsil of individual 3 showed that B cells without any detectable BSF-2-R before stimulation were induced to express the receptors after 3 d of culture with SAC, but not with medium alone. Another result with tonsillar B

TABLE I
 Characteristics of BSF-2-R on Human Cell Lines

Cell line	Cell type	Sites per cell	K_d
			$\times 10^{-10} M$
CESS	EBV-B	2,700	3.4
SKW6-CL4	EBV-B	210	2.0
LCL13	EBV-B	310	4.0
LCL14	EBV-B	410	3.9
BL29	Burkitt's	NS*	—
BL36	Burkitt's	NS	—
BL41	Burkitt's	NS	—
BL41/95	Burkitt's (EBV-infected)	NS	—
Daudi	Burkitt's	NS	—
Raji	Burkitt's	NS	—
Louckes	Burkitt's	NS	—
Louckes/EBNA2	Burkitt's (EBNA2-transfected)	NS	—
ARH-77	Plasma cell	120	1.5
U266	Plasma cell	11,000	2.5
GEM	T	NS	—
HSB	T	NS	—
Jurkat	T	NS	—
OM1	T	NS	—
KM3	Non-T non-B	NS	—
Reh	Non-T non-B	NS	—
U937	Histiocytoma	2,800	3.3
HL60	Promyelocytic leukemia	3,600	6.4
U373	Astrocytoma	170	1.3
T24	Bladder carcinoma	NS	—
SK-MG-4	Glioblastoma	150	3.9
SK-N-MC	Neuroblastoma	NS	—

Cells were analyzed by usual binding assay (see Materials and Methods) and a Scatchard plot. At least four different doses of ^{125}I -BSF-2 were used. The averages of duplicate determinations were taken for analysis. BL41/95 (27) is an EBV-infected cell line originated from a Burkitt's lymphoma cell line, BL41.

* Not significant (<30).

cells from individual 4 demonstrated that activated larger B cells on day 1 of stimulation with SAC expressed ~600 binding sites per cell with a K_d of $4.2 \times 10^{-10} M$, whereas smaller B cells displayed barely detectable number of receptors (120 receptors), that was similar to the number before stimulation (see also Fig. 7). The result with the tonsil of individual 5 also demonstrated that freshly prepared large B cells which could be considered in vivo-activated cells, expressed a small but substantial number of BSF-2-R. Freshly prepared small resting B cells devoid of BSF-2-R (tonsil 6) were shown to express BSF-2-R after activation for 2 d with SAC, compared to unstimulated controls. The above results indicate that BSF-2-R are expressed constitutively on resting T cells, but are inducible on B cells.

FACS Analysis of Normal T Cells and B Cells. To know the purity of T cells and B cells prepared from tonsillar mononuclear cells, cells were stained with fluorescein-conjugated anti-CD3 or anti-CD20 and examined by FACS. The T

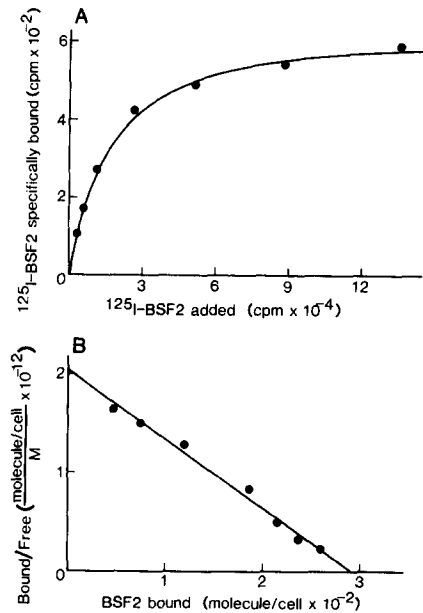


FIGURE 6. (A) Specific binding of ^{125}I -BSF-2 to normal human T cells. (B) A Scatchard plot of the data in A. Data represent the mean of the duplicate.

cell fraction used in these studies contained >96% T cells and the B cell fraction consisted practically of 100% B cells (data not shown). To demonstrate that BSF-2-R were expressed mainly on the final maturation stage of B cells, large and small B cell fractions were stained with anti-Ba and anti-IgD antibodies (20). As shown in Fig. 8, a large B cell fraction, which expressed BSF-2-R, consisted mainly of $\text{Ba}^{++}/\text{IgD}^-$ or Ba^-/IgD^- cells, while large portion of smaller B cell fraction was IgD^+ .

Discussion

Cloning of the cDNA for BSF-2 (2) enabled us to obtain highly purified BSF-2 protein generated in *E. coli* by recombinant DNA technology and has made it possible to study receptors for BSF-2. We have found that an efficient radioiodination of BSF-2 without hindering its binding ability can be achieved with the use of the Bolton and Hunter reagent. In fact, 83% of the iodinated BSF-2 retained its binding ability, with a specific radioactivity of 6.16×10^{13} cpm/g (Fig. 3). This material has allowed us to analyze the binding properties of BSF-2 and to detect the presence of very low number of receptors per cell. In fact, the identical slope of the two regression lines obtained by the addition of excess ^{125}I -labeled or unlabeled BSF-2 (Fig. 3B) indicates that there was no significant difference in the binding affinity between labeled and unlabeled BSF-2 to its receptor. Native BSF-2 purified from a bladder carcinoma line, T24 (21), also gave a regression line parallel to those in Fig. 3B (data not shown). The molecular mass shown in Fig. 1 agrees well with the one of native BSF-2 shown by a previous study (5). Because the binding property of recombinant BSF-2 does not seem to be different from native BSF-2, it seems that glycosylation does not play a critical role in the binding of this cytokine to its receptor.

The kinetic study has demonstrated that binding of ^{125}I -BSF-2 to CESS cells

TABLE II
 Characteristics of BSF-2-R on Normal Human T Cells and B Cells

Cell type	Tissue (tonsil) from subject:	Cell size*	Stimulant	Culture duration	Sites per cell [‡]	K_d
				<i>d</i>		$\times 10^{-10} M$
T cells	1	—	—	0	290	1.4
	1	Large	0.1% PHA	3	220	—
	1	Small	0.1% PHA	3	310	1.6
	2	—	—	0	310	2.0
	2	Large	0.1% PHA	3	120	1.6
	2	Small	0.1% PHA	3	230	1.5
	3	—	—	0	350	1.4
	4	—	—	0	990	2.1
	4	—	PWM + SAC	3	270	2.8
B cells	1	—	—	0	NS	—
	2	—	—	0	NS	—
	3	—	—	0	NS	—
	3	—	Medium alone	3	NS	—2
	3	—	0.003% SAC	3	80	3.6
	4	—	—	0	70	4.5
	4	Large	0.003% SAC	1	570	4.2
	4	Small	0.003% SAC	1	120	5.0
	5	Large	—	0	87	4.2
	5	Small	—	0	NS	—
	6	Small	—	0	NS	—
	6	Unfractionated [§]	—	2	NS	—
	6	Unfractionated [§]	0.003% SAC	2	230	3.3

T cells and B cells were separated from tonsils of six individuals (numbered 1–6) and cultured with 0.1% PHA or 2.5 $\mu\text{g}/\text{ml}$ PWM + 0.003% SAC (T cells), or 0.003% SAC or medium alone (B cells) at an initial density of 2×10^6 cells/ml for the indicated duration. In some cases, cells were fractionated by size. Scatchard analyses were performed (see legend of Table I).

* —, unfractionated.

‡ NS, not significant (<30).

§ Freshly prepared small B cells with no detectable number of BSF-2-R were cultured in the absence of size-fractionation on the day of assay.

was saturated in 150 min at 0°C. In comparison, at 37°C the saturation was achieved in 60 min but the maximum level of binding was half that found at 0°C. This level was almost the same in the presence of 0.02% sodium azide at 37°C, although the maximum level was more stable than without sodium azide. Unlike other cytokines (22, 23), the binding property of BSF-2 to its receptor has a certain peculiarity, in that the level of binding at 0°C is higher than that at 37°C. In any case, as the nonspecific binding at 0°C was much lower than that at 37°C, the assay at 0°C provided a suitable condition for the measurement of BSF-2-R.

We have previously mentioned that the primary structure of BSF-2 has some homology at the NH₂-terminal region with G-CSF (2), and that the organization of the genomic genes for BSF-2 and G-CSF, both of which had five exons and four introns, are almost identical (24). Although it has been suggested that some

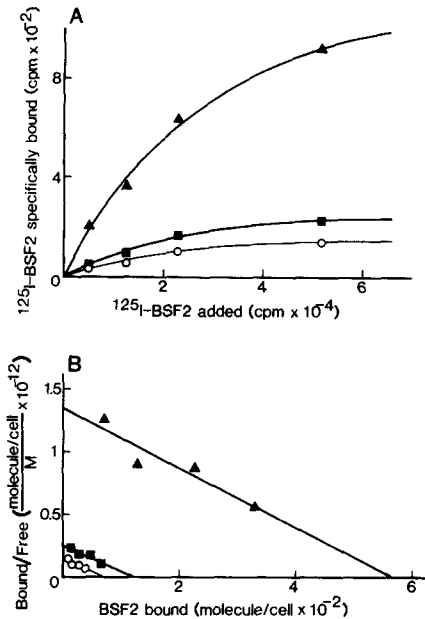


FIGURE 7. (A) Specific binding of ^{125}I -BSF-2 to normal B cells. B cells were purified from tonsillar mononuclear cells and cultured with 0.003% SAC for 1 d and fractionated into large (closed triangles) and small (closed squares) cells by centrifugation on discontinuous gradient of Percoll. Unstimulated nonfractionated B cells (open circles) are also used for binding assay. (B) Scatchard plots of the same data.

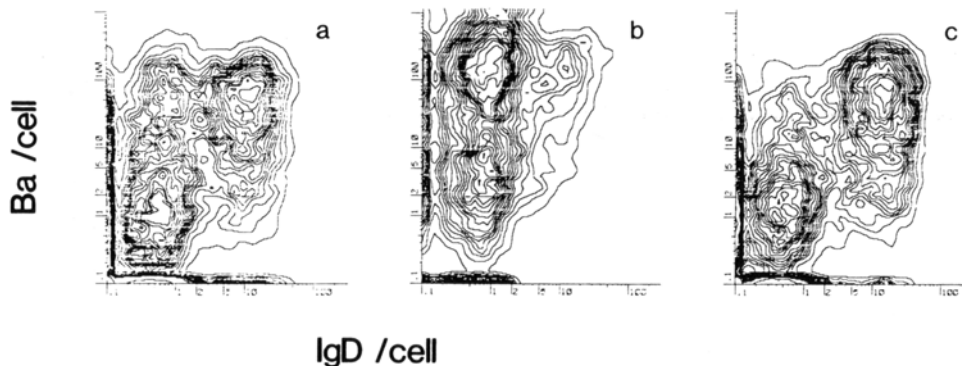


FIGURE 8. Two-color FACS analysis of tonsillar B cells stained with anti-Ba and anti-IgD. Freshly prepared tonsillar B cells (unfractionated, *a*; large, *b*; and small, *c*) were stained with FITC-anti-IgD and biotinylated anti-Ba, and developed with Texas Red-avidin. The expression of Ba and IgD on cells were analyzed by FACS 440.

similarity exists between BSF-2 and IFN- β in the COOH-terminal portion (17), the present study clearly demonstrates that BSF-2 receptors can only bind BSF-2, not other cytokines such as IFN- β and G-CSF.

The presence of high- and low-affinity receptors for IL-2 and several other growth factors has been demonstrated (25, 26). From the binding profile and the Scatchard analysis (Fig. 5), only a single class of BSF-2 receptors was detected on CESS cells numbering 2,700 and having a dissociation constant of 3.4×10^{-10} M. Several cell lines possessed BSF-2-R with similar dissociation constants in the range of 1.3 – 6.4×10^{-10} M (Table I). Because 1.2×10^{-9} M BSF-2 was used at the maximum concentrations in these binding studies, the existence of another class of receptors with much lower affinity could not be completely excluded.

The number of receptors was found to vary in the cell lines tested, ranging from 120 to 11,000. Considering the requirement of a very low amount ($\sim 10^{-14}$ mol) of BSF-2 for the induction of 50% maximum response of SKW6-CL4 (10,000 cells in 200 μ l medium), which expressed only 210 receptors, partial occupancy of low numbers of BSF-2-R expressed on EBV-transformed B cells may be sufficient to be functional. Therefore, the presence of a large number of receptors might not be necessary for BSF-2 signal transduction, as observed in IL-1 stimulation (27).

It is noteworthy that BSF-2-R were expressed on all EBV-transformed B cell lines examined here but not on any Burkitt's lines (Table I). Two possible explanations could be given: (a) EBV induces B cells to express BSF-2-R; or (b) EBV can cause the arrest of B cells at a specific stage of growth or differentiation in which BSF-2-R could be expressed. The results shown in Table I using two sets of two different variants of the Burkitt's lines, BL41 or Louckes, showed that these lines did not express detectable BSF-2-R even after EBV infection or EBNA2 transfection. EBV-infected BL41/95 has been reported to express lymphoblastoid cell associated antigens, whereas BL41 did not (28), and we also observed BL41/95 making clumps in culture, a typical characteristic of EBV-infected B cells. This also shows a marked contrast to the expression of a B cell-specific differentiation antigen, CD23 (Fc ϵ receptor), the expression of which could be inducible by the transfection of EBNA2 (19). This result implies that the former of the two possible explanations described above is probably not correct, but further study is required to draw any firm conclusions on the mechanism of the regulation of BSF-2-R expression.

Not only EBV-transformed B cell lines but also various other human cell lines expressed BSF-2-R. Among them, it should be noted that human plasma cell lines, ARH-77 and U266, expressed BSF-2-R, since several investigators reported that BSF-2 could function as a plasmacytoma growth factor in a murine system (7, 29). In a preliminary study, recombinant BSF-2 could induce the proliferation of myeloma cells freshly prepared from a patient with myeloma (our unpublished data). Besides B cell lines and plasma cell lines, the histiocytic line U937, the promyelocytic line HL60, the astrocytoma line U373, and the glioblastoma line SK-MG-4 displayed BSF-2-R. The finding that BSF-2-R are expressed on cell lines of different types is in agreement with the wide range of target cells for BSF-2 (4, 6, 30, 31). Further study should clarify the function of BSF-2 on those receptor-positive cells. It is interesting to note that in the four cell lines described above, including an astrocytoma and a glioblastoma line, BSF-2-R were expressed and BSF-2 itself was inducible after stimulation with IL-1 or TPA (24 and our unpublished data), whereas a neuroblastoma line neither expressed BSF-2-R nor produced BSF-2. These results may suggest the presence of an autocrine mechanism in BSF-2-induced differentiation or growth of various cells.

One of the interesting findings was the expression of BSF-2-R on resting T cells. The situation is similar with BSF-1 (IL-4), which can act on T cells to induce their proliferation (32). Fewer BSF-2-R were detected on large T cells compared to small-size T cell fractions (Table II). This might explain the lack in BSF-2-R on the T cell lines examined, as seen in Table I. The role of BSF-2 on

normal T cells is, as yet, unknown. But preliminary studies suggest that BSF-2 could induce IL-2-R expression on certain T cell lines (33).

Recent studies on receptors for cytokines such as IL-1, IL-2, BSF-1, and TNF (12, 34, 35, 36) demonstrated that cytokines do not have strict target cell specificity. The present studies show that BSF-2 is no exception. In such a situation, studies on the regulation of the expression of both cytokines and receptors are essential for the elucidation of the biological role of these molecules. Normal human B cells usually do not express a significant number of receptors for BSF-2 before activation (Table II). Upon activation with SAC they were observed to express 80–570 BSF-2 receptors per cell, with a K_d of $4-5 \times 10^{-10}$ M. This smaller number of the receptors may be due to a smaller proportion of receptor-positive cells among total B cells. Large B cells after 1 d stimulation with SAC displayed more receptors than smaller ones. As shown in Table II, even before stimulation *in vitro*, large B cells freshly prepared from a tonsil expressed 87 receptors per cell with a similar K_d to other cases, whereas smaller B cells did not. This means normal B cells can express BSF-2-R when they are activated *in vivo*. As reported previously (20), by using anti-Ba (a monoclonal antibody that recognizes activated B cells) and anti-IgD, tonsillar B cells were separated into four subpopulations with regard to their activation stage; Ba^-/IgD^+ , Ba^+/IgD^+ , Ba^{++}/IgD^- , and Ba^-/IgD^- . As shown in Fig. 8, more than half of the freshly prepared large B cells were Ba^{++}/IgD^- and most cells were IgD^- . This phenotype of B cell is known to be at the final maturation stage and ready to produce immunoglobulin (37). These results suggest that normal B cells become ready to respond to BSF-2 by expressing receptors only when they are activated. This is in contrast to the BSF-1-R, which are reported to exist on resting B cells (12, 38, 39). The difference between the mode of the expression of BSF-1- and BSF-2-R fits the functional difference between BSF-1 and BSF-2 on B cells; the former acts mainly on resting B cells to lead to proliferation in the presence of anti-IgM, and the latter acts on activated B cells to secrete immunoglobulin (4, 40). It is not necessary for normal B cells to have constitutive expression of the BSF-2-R, but only their transient expression might be sufficient to drive the cells to a final stage of differentiation.

Summary

B cell stimulatory factor 2 receptors (BSF-2-R) were studied using radioiodinated recombinant BSF-2 with a specific activity of 6.16×10^{13} cpm/g. Kinetic studies showed that binding of ^{125}I -BSF-2 to CESS cells reached maximum level within 150 min at $0^\circ C$. There was a single class of receptors with high affinity (K_d 3.4×10^{-10} M) on CESS, and the number of receptors was 2,700 per cell. Binding of ^{125}I -BSF-2 to CESS was competitively inhibited by unlabeled BSF-2 but not by IL-1, IL-2, IFN- β , IFN- γ , and G-CSF, indicating the presence of the receptors specific for BSF-2. EBV-transformed B lymphoblastoid cell lines (CESS, SKW6-CL4, LCL13, and LCL14) expressed BSF-2-R, whereas Burkitt's lines did not. EBV or EBNA2 did not induce the expression of the receptors on Burkitt's cells. The plasma cell lines (ARH-77 and U266) expressed BSF-2-R, fitting the function of BSF-2 as plasma cell growth factor. Several other cell lines, the histiocytic line U937, the promyelocytic line HL60, the astrocytoma line

U373 and the glioblastoma line SK-MG-4, in which BSF-2 was inducible with IL-1 or TPA, displayed BSF-2-R with K_d in the range of $1.3\text{--}6.4 \times 10^{-10}$ M, suggesting the autocrine mechanism in BSF-2 function. The four T cell lines (CEM, HSB, Jurkat, and OM1) did not express a detectable number of receptors, but normal resting T cells expressed 100–1,000 receptors per cell. BSF-2-R were not present on normal resting B cells but expressed on activated B cells with a K_d of $3.6\text{--}5.0 \times 10^{-10}$ M, fitting the function of BSF-2, which acts on B cells at the final maturation stage to induce immunoglobulin production.

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References

1. Kishimoto, T. 1985. Factors affecting B cell growth and differentiation. *Annu. Rev. Immunol.* 3:133.
2. Hirano, T., K. Yasukawa, H. Harada, T. Taga, Y. Watanabe, T. Matsuda, S. Kashiwamura, K. Nakajima, K. Koyama, A. Iwamatsu, S. Tsunasawa, F. Sakiyama, H. Matsui, Y. Takahara, T. Taniguchi, and T. Kishimoto. 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature (Lond.)* 324:73.
3. Kikutani, H., T. Taga, S. Akira, H. Kishi, Y. Miki, O. Saiki, Y. Yamamura, and T. Kishimoto. 1985. Effect of B cell differentiation factor (BCDF) on biosynthesis and secretion of immunoglobulin molecules in human B cell lines. *J. Immunol.* 134:990.
4. Hirano, T., T. Taga, N. Nakano, K. Yasukawa, S. Kashiwamura, K. Shimizu, K. Nakajima, K. H. Pyun, and T. Kishimoto. 1985. Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSFp-2). *Proc. Natl. Acad. Sci. USA* 82:5490.
5. Hirano, T., T. Taga, K. Yasukawa, K. Nakajima, N. Nakano, F. Takatsuki, M. Shimizu, A. Murashima, S. Tsunasawa, F. Sakiyama, and T. Kishimoto. 1987. Human B-cell differentiation factor defined by an anti-peptide antibody and its possible role in autoantibody production. *Proc. Natl. Acad. Sci. USA* 84:228.
6. Zilberstein, A., R. Ruggieri, J. H. Korn, and M. Revel. 1986. Structure and expression of cDNA and genes for human interferon-beta-2, a distinct species inducible by growth-stimulatory cytokines. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2529.
7. Van Damme, J., G. Opdenakker, R. J. Simpson, M. R. Rubira, S. Cayphas, A. Vink, A. Billiau, and J. Van Snick. 1987. Identification of the human 26-kD protein, interferon β 2 (IFN- β 2), as a B cell hybridoma/plasmacytoma growth factor induced by interleukin 1 and tumor necrosis factor. *J. Exp. Med.* 165:914.
8. Haegeman, G., J. Content, G. Volckaert, R. Derynck, J. Tavernier, and W. Fiers. 1986. Structural analysis of the sequence coding for an inducible 26-kDa protein in human fibroblasts. *Eur. J. Biochem.* 159:625.
9. Content, J., L. Dewit, D. Pierard, R. Derynck, E. De Clercq, and W. Fiers. 1982. Secretory proteins induced in human fibroblasts under conditions used for the production of interferon β . *Proc. Natl. Acad. Sci. USA* 79:2768.
10. Content, J., L. De Wit, P. Poupart, G. Opdenakker, J. Van Damme, and A. Billiau. 1985. Induction of a 26-kDa-protein mRNA in human cells treated with an interleukin-1-related, leukocyte-derived factor. *Eur. J. Biochem.* 152:253.
11. Poupart, P., P. Vandenabeele, S. Cayphas, J. Van Snick, G. Haegeman, V. Kruys, W.

- Fiers, and J. Content. 1987. B cell growth modulating and differentiating activity of recombinant human 26-kD protein (BSF-2, HuIFN- β_2 , HPGF). *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1219.
12. Ohara, J., and W. E. Paul. 1987. Receptors for B-cell stimulatory factor-1 expressed on cells of haemopoietic lineage. *Nature (Lond.)*. 325:537.
 13. Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I -containing acylating agent: Application to the radio-immuno assay. *Biochem. J.* 133:529.
 14. Calvo, J. C., J. P. Padicella, and E. H. Charreau. 1983. Measurement of specific radioactivities in labelled hormones by self-displacement analysis. *Biochem. J.* 212:259.
 15. Teranishi, T., T. Hirano, N. Arima, and K. Onoue. 1982. Human helper T cell factor(s) (ThF): II. Induction of IgG production in B lymphoblastoid cell lines and identification of T cell-replacing factor (TRF)-like factor(s). *J. Immunol.* 128:1903.
 16. Muraguchi, A., T. Kishimoto, Y. Miki, T. Kuritani, T. Kaieda, K. Yoshizaki, and Y. Yamamura. 1981. T cell-replacing factor (TRF)-induced IgG secretion in a human B blastoid cell line and demonstration of acceptors for TRF. *J. Immunol.* 127:412.
 17. May, L. T., D. C. Helfgott, and P. B. Sehgal. 1986. Anti- β -interferon antibodies inhibit the increased expression of HLA-B7 mRNA in tumor necrosis factor-treated human fibroblasts: Structural studies of the β 2 interferon involved. *Proc. Natl. Acad. Sci. USA.* 83:8957.
 18. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* 51:660.
 19. Wang, F., C. D. Gregory, M. Rowe, A. B. Rickinson, D. Wang, M. Birkenbach, H. Kikutani, T. Kishimoto, and E. Kieff. 1987. Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23. *Proc. Natl. Acad. Sci. USA.* 84:3452.
 20. Kikutani, H., H. Nakamura, R. Sato, R. Kimura, K. Yamasaki, R. R. Hardy, and T. Kishimoto. 1986. Delineation and characterization of human B cell subpopulations at various stages of activation by using a B cell-specific monoclonal antibody. *J. Immunol.* 136:4027.
 21. Rowle, F. C., J. Shields, S. H. Smith, V. Iliescu, M. Merckenslager, P. C. L. Beverley, and R. E. Callard. 1986. B cell growth and differentiation induced by supernatants of transformed epithelial cell lines. *Eur. J. Immunol.* 16:1017.
 22. Robb, R. J., A. Munck, and K. Smith. 1981. T cell growth factor receptors: quantitation, specificity, and biological relevance. *J. Exp. Med.* 154:1455.
 23. Nicola, N. A., and D. Metocalf. 1985. Binding of ^{125}I -labeled granulocyte colony-stimulating factor to normal murine hemopoietic cells. *J. Cell. Physiol.* 124:313.
 24. Yasukawa, K., T. Hirano, Y. Watanabe, K. Muratani, T. Matsuda, S. Nakai, and T. Kishimoto. 1987. Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6) gene. *EMBO (Eur. Mol. Biol. Organ.) J.* In press.
 25. Robb, R., W. C. Greene, and C. M. Rusk. 1984. Low and high affinity cellular receptors for interleukin 2: Implications for the level of Tac antigen. *J. Exp. Med.* 160:1126.
 26. Walker, F., and A. W. Burgess. 1985. Specific binding of radioiodinated granulocyte-macrophage colony-stimulating factor to hemopoietic cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:933.
 27. Dower, S. K., S. R. Kronheim, C. J. March, P. J. Conlon, T. P. Hopp, S. Gillis, and D. L. Urdal. 1985. Detection and characterization of high affinity plasma membrane receptors for human interleukin 1. *J. Exp. Med.* 162:501.
 28. Torsteinsdottir, S., M. G. Masucci, B. Ehlin-Henriksson, C. Brautbar, H. Ben Bassat, G. Klein, and E. Klein. 1986. Differentiation dependent sensitivity of human B-cell

- derived lines to major histocompatibility complex-restricted T cell cytotoxicity. *Proc. Natl. Acad. Sci. USA.* 83:5620.
29. Van Snick, J., A. Vink, S. Cayphas, and C. Uyttenhove. 1987. Interleukin-HP1, a T cell-derived hybridoma growth factor that supports the in vitro growth of murine plasmacytomas. *J. Exp. Med.* 165:641.
 30. Kohase, M., D. H.-De Stefano, L. T. May, J. Vilcek, and P. B. Sehgal. 1986. Induction of β 2-interferon by tumor necrosis factor: A homeostatic mechanism in the control of cell proliferation. *Cell.* 45:659.
 31. Billiau, A. 1987. Interferon β 2 as a promotor of growth and differentiation of B cells. *Immunol. Today.* 8:84.
 32. Hu-Li, J., E. M. Shevach, J. Mizuguchi, J. Ohara, T. Mosmann, and W. E. Paul. 1987. B cell stimulatory factor 1 (interleukin 4) is a potent costimulant for normal resting T lymphocytes. *J. Exp. Med.* 165:157.
 33. Noma, T., T. Mizuta, A. Rosen, T. Hirano, T. Kishimoto, and T. Honjo. 1987. Enhancement of the interleukin 2 receptor expression on T cells by multiple B-lymphotropic lymphokines. *Immunol. Lett.* 15:249.
 34. Dower, S. K., and D. Urdal. 1987. The interleukin-1 receptor. *Immunol. Today.* 8:46.
 35. Waldman, T. A., C. K. Goldman, R. J. Robb, J. M. Depper, W. J. Leonard, S. O. Sharrow, K. F. Bongiovanni, S. J. Korsmeyer, and W. C. Greene. 1984. Expression of interleukin 2 receptors on activated human B cells. *J. Exp. Med.* 160:1450.
 36. Baglioni, C., S. McCandless, J. Tavernier, and W. Fiers. 1985. Binding of human tumor necrosis factor to high affinity receptors on HeLa and lymphoblastoid cells sensitive to growth inhibition. *J. Biol. Chem.* 260:13395.
 37. Kuritani, T., and M. D. Cooper. 1982. Human B cell differentiation (II): Pokeweed mitogen-responsive B cells belong to a surface immunoglobulin D-negative subpopulation. *J. Exp. Med.* 155:1561.
 38. Park, L. S., D. Friend, K. Grabstein, and D. L. Urdal. 1987. Characterization of the high-affinity cell-surface receptor for murine B-cell-stimulating factor 1. *Proc. Natl. Acad. Sci. USA.* 84:1669.
 39. Nakajima, K., T. Hirano, K. Koyama, and T. Kishimoto. 1987. Detection of receptors for murine B cell stimulatory factor 1 (BSF1): Presence of functional receptors on CBA/N splenic B cells. *J. Immunol.* 139:774.
 40. Howard, M. 1982. Identification of a T cell-derived B cell growth factor distinct from interleukin 2. *J. Exp. Med.* 155:914.