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Immunology

Human B-cell differentiation factor defined by an anti-peptide antibody and its possible role in autoantibody production

(B-cell stimulatory factor 2/NH_2-terminal sequence/cardiac myxoma/autoimmunity)

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ABSTRACT **The partial amino acid sequence of the NH_2 terminus of a factor named human B-cell differentiation factor or B-cell stimulatory factor 2 (BSF-2) has been determined. Antibodies raised against the synthetic peptide corresponding to residues 1–13 of the NH_2-terminal sequence specifically react with BSF-2 generated by a T-cell line and by phytohemagglutinin-stimulated normal T cells. Furthermore, the anti-peptide antibodies react with a BSF-2-like factor produced by cardiac myxoma as well as uterine cervical carcinoma cells. The results show that BSF-2 functions *in vivo* as well and suggest that the constitutive production of BSF-2 may be involved in autoantibody production, since patients with cardiac myxoma and uterine carcinoma showed autoantibody production.**

Upon stimulation with the appropriate antigen and in the presence of a variety of lymphokines and monokines produced by T cells and monocytes (1–3), B cells begin to proliferate and differentiate into antibody-producing cells. Human B-cell differentiation factor or B-cell stimulatory factor 2 (BSF-2) has been reported to act in the late stages of B-cell differentiation (4–7) leading to the biosynthesis of secretory type immunoglobulin (Ig) (8). BSF-2 has been purified to homogeneity and is capable of inducing Ig production in Epstein–Barr virus-transformed B-cell lines at pM concentrations without any effect on cell growth (9). The same study with the purified BSF-2 also demonstrated that BSF-2 could induce Ig secretion in *Staphylococcus aureus* Cowan I-activated B cells, which could be augmented by the addition of interleukin 2 (IL-2). No activities of B-cell stimulatory factor 1 (BSF-1), B-cell growth factor 2, interleukin 1 (IL-1), and IL-2 were detected in the purified preparation of BSF-2 (9).

Production of autoantibodies may be caused by the unregulated expression of genes encoding lymphokines as well as monokines affecting B-cell growth and/or differentiation as suggested in the MRL *lpr* (10) and Motheaten (11) mice. Patients with cardiac myxoma frequently show several kinds of connective tissue disease-like symptoms (autoimmune), such as presence of fever, arthralgia, hypergammaglobulinemia, elevated erythrocyte sedimentation rate, Raynaud phenomenon, and autoantibodies (12). The above pathologies usually disappear upon the surgical removal of the tumor. Cardiac myxoma cells when cultured *in vitro* continue to produce BSF-2-like molecules for a period of 1 month, suggesting that the tumor or its products may be responsible for the autoimmune-like syndrome (M.S., unpublished results).

In this communication, we report the partial amino acid sequence at the NH_2 terminus of BSF-2 and show that the heterologous anti-peptide antibodies raised on the basis of the NH_2-terminal sequence of BSF-2 specifically absorb the BSF-2 activity generated by T cells and by cardiac myxoma cells. The results show that BSF-2 is a molecule distinct from other known lymphokines and monokines; furthermore, our observations with cardiac myxoma suggest that the constitutive production of BSF-2 *in vivo* may be crucial for the generation of autoantibody production.

MATERIALS AND METHODS

Purification of BSF-2. BSF-2 was purified from 40 liters of fetal calf serum-free culture supernatants of TCL-Nal cells (13) by HPLC using a TSK-2000SW (600 × 21.5 mm, Toyo Soda), a Mono P (Pharmacia), and a Synchropak RP-P (C_{18}, 250 × 4.1 mm, Synchrom, Linden, IN) as described (9). Of the two species of BSF-2 (M_r, 19,000 and 21,000), the M_r 21,000 species was further purified by preparative NaDodSO$_4$/PAGE (14) followed by HPLC using a Synchropak RP-P. BSF-2 was eluted from polyacrylamide gel by incubating the gel fraction in 0.01 M NH_4HCO_3/0.05% NaDodSO$_4$ at 37°C overnight.

Determination of NH_2-Terminal Amino Acid Sequence. Ten micrograms (≈500 pmol) or 3 μg (150 pmol) of purified BSF-2 (M_r, 21,000) was employed for the determination of amino acid sequence from the NH_2 terminus using a gas–(liquid–solid) phase protein sequencer (Applied Biosystems model 470A) essentially as described by Hewick *et al.* (15). The phenylthiohydantoin amino acid derivatives were separated by reverse-phase HPLC using ODS-120 T column (C_{18} 250 × 4.6 mm, Toyo Soda) as described (16).

Preparation of Antisera Against Synthetic Peptide. The peptide corresponding to residues 1–13 of the NH_2-terminal sequence of BSF-2 was synthesized by the conventional method of solid-phase synthesis using a Vega coupler 250 peptide synthesizer. The peptide was purified successively by reverse-phase HPLC on a C_{18} column (YMC S-15, 250 × 2.5 mm) with a linear gradient of 10–20% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid and water and by gel filtration on Sephadex G-10 with water. The entire sequence of the synthetic peptide was confirmed by Edman degradation with a 470A protein sequencer, and the amino acid analysis ($Ala_{1.9}$, $Asp_{1.9}$, $Glu_{1.0}$, $Gly_{1.0}$, $Lys_{1.0}$, $Pro_{2.6}$, $Ser_{0.9}$, and $Val_{1.9}$) was obtained. The peptide was introduced with thiol groups and conjugated to maleimide-modified ovalbumin (17) at a peptide-to-carrier protein molar ratio of 6:1. Peptide-ovalbumin (700 μg) in complete

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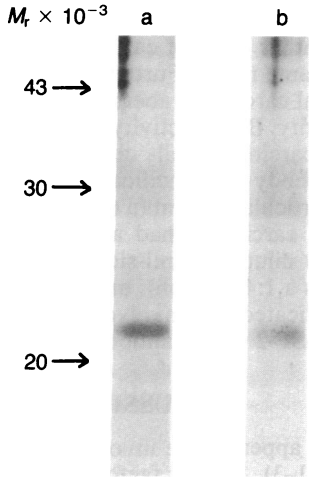
Abbreviations: BSF-1 and -2, B-cell stimulatory factor 1 and 2, respectively; Ig, immunoglobulin; HTLV, human T-cell leukemia virus; IL-1, -2, and -3, interleukin 1, 2, and 3, respectively; PHA, phytohemagglutinin.



H_2N-Pro-Val-Pro-Pro-Gly-Glu-Asp-
Ser-Lys-Asp-Val-Ala-Ala-Pro

FIG. 1. NaDodSO$_4$/PAGE of purified BSF-2 and its NH_2-terminal amino acid sequence. (*Upper*) Purified BSF-2 was analyzed by NaDodSO$_4$/PAGE (12) under reducing (lane a) and nonreducing (lane b) conditions and silver stained. Approximately 13 μg of purified BSF-2 with M_r 21,000 was obtained. The amount of protein was determined by comparison with a protein standard (soybean trypsin inhibitor) after silver staining. (*Lower*) The initial yield of the first amino acid (proline) was about 80%, and the NH_2-terminal 14 residues of the protein were eventually sequenced. The sequence analysis was repeated twice.

Freund's adjuvant was used to immunize rabbits, followed by additional booster injections with antigen in incomplete Freund's adjuvant at 3-week intervals for 3 months. All five rabbits produced antibodies that bound the peptide when tested by ELISA. Anti-peptide and anti-ovalbumin antibodies were purified with peptide-coupled and ovalbumin-coupled affinity columns, respectively, and then conjugated to CNBr-activated Sepharose CL-4B (Pharmacia).

Assay for BSF-2 Activity. SKW6-CL4 cells (9) were cultured (10^4 cells per 200 μl of culture medium) in the presence of 1:3 diluted test samples. After 3 days of culture, the concentration of IgM was determined by ELISA. The BSF-2 activity that induced 50% of the maximum response of IgM production in 1 × 10^4 SKW6-CL4 cells was defined as 1 unit/ml.

RESULTS

Determination at NH_2-Terminal Amino Acid Sequence of BSF-2. BSF-2 with M_r 21,000 was purified from culture supernatants of the human T-cell leukemia virus type I-(HTLV I) transformed T-cell line TCL-Nal (13), following essentially the same method described (9) (Fig. 1 *Upper*). The sequence of the first 14 NH_2-terminal amino acids was determined with a gas–(liquid–solid) phase protein sequencer (Fig. 1 *Lower*). This sequence was confirmed when BSF-2 was cleaved by lysylendopeptidase, and the amino acid sequences of two of the fragments are Pro-Val-Pro-Pro-Gly-Glu-Xaa-Xaa-Lys and Xaa-Val-Ala-Ala-Pro, which is in agreement with the sequence shown in Fig. 1 *Lower*.

Preparation of Antiserum to the Synthetic Peptide. To confirm that the amino acid sequence determined belongs to the BSF-2 molecule, antibodies were raised in rabbits against a synthetic peptide corresponding to the first 13 residues of the NH_2-terminal sequence. The peptide was conjugated to ovalbumin and used to immunize rabbits. The anti-peptide and anti-ovalbumin antibodies were affinity purified from the immune sera and coupled to activated Sepharose CL-4B. As shown in Fig. 2*A*, the anti-peptide but not anti-ovalbumin antibody bound purified ^{125}I-labeled BSF-2. In addition, BSF-2 activity generated by HTLV I-transformed T-cell line (Fig. 2*B*) as well as phytohemagglutinin (PHA)-stimulated normal T cells (Fig. 2*C*) were adsorbed and subsequently eluted from the anti-peptide immunoaffinity column but not from anti-ovalbumin column. Furthermore, IL-2 activity could not be adsorbed to the anti-peptide column (Fig. 2*C*).

Computer Analysis. Comparison of the amino acid sequence at NH_2 terminus of BSF-2 was made with those of more than 5000 proteins by utilizing the Protein Sequence

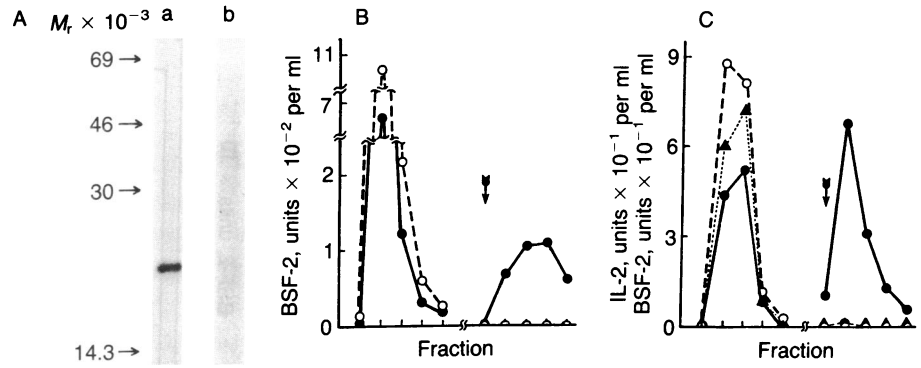


FIG. 2. Immunoaffinity chromatography of BSF-2 with anti-peptide antibody. (*A*) Reverse-phase HPLC-purified BSF-2 (7600 units, ≈456 ng) was labeled with ^{125}I by the lactoperoxidase by using Enzymobead (Bio-Rad) according to the manufacturer's instructions, and half of this was mixed with 400 μl of affinity-purified anti-peptide antibody conjugated to Sepharose CL-4B (0.6 mg of protein per 1 ml of gel) (lane a), and the other half was mixed with affinity-purified anti-ovalbumin antibody conjugated to Sepharose CL-4B (lane b) and rotated at 4°C overnight. The gel was extensively washed with PBS (0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2) containing 0.1% of bovine serum albumin, and bound proteins were eluted by boiling in the presence of 2-mercaptoethanol (5% vol/vol) and a sample buffer for NaDodSO$_4$/PAGE. The eluted proteins were separated by NaDodSO$_4$/12% polyacrylamide gel electrophoresis (13) and autoradiographed. BSF-2 was partially purified from culture supernatants of TCL-Nal cells by gel filtration and isoelectric focusing (*B*), the fraction of PHA-conditioned tonsillar cell supernatant corresponding to the M_r 15,000–30,000 by gel filtration (*C*) was applied to the anti-peptide (●, ▲) or an anti-ovalbumin (○) column, both of which were equilibrated with PBS containing 10% (vol/vol) fetal calf serum. After extensive washing with PBS containing 10% (vol/vol) fetal calf serum, bound proteins were eluted with 3 M KSCN (indicated by an arrow). BSF-2 (●, ○) and IL-2 (▲) activities of each fraction were assayed employing a BSF-2-responsive B-lymphoblastoid cell line, SKW6-CL4, and an IL-2-dependent T-cell line, MTH.41.16, respectively, as described (8).

Database,¶ Genetic Sequence Data Bank,|| and EMBL Nucleotide Sequence Data Library.** This analysis showed that BSF-2 is distinct from other known proteins including human IL-1, IL-2, interferons, colony-stimulating factor type 1, granulocyte–macrophage colony-stimulating factor, and BSF-1 and including murine IL-1, IL-2, granulocyte–macrophage colony-stimulating factor, IL-3, and BSF-1.

Characterization of BSF-2-Like Molecule Produced by Cardiac Myxoma and Uterine Cervical Carcinoma Cells. It was found that cardiac myxoma cells when cultured *in vitro* produce a BSF-2-like molecule, and this seemed to be involved in the autoimmune-like condition frequently observed in the patients. One of these patients, a 50-year-old woman, had anti-nuclear factor (serum titer was a 1:10 dilution), anti-DNA antibodies (29 unit/ml), rheumatoid factor (serum titer was a 1:640 dilution), elevated level of immune complexes (26 μg/ml), arthritis, and Raynaud phenomenon. The tumor was aseptically removed during autopsy. As shown in Fig. 3*A*, the culture supernatant of the tumor cells contained a high level of BSF-2 activity (≈1300 units/ml). This was more than 100-fold higher than BSF-2 levels in culture supernatants of PHA-stimulated tonsillar lymphocytes. To examine whether the anti-peptide antibody reacted with this BSF-2-like molecule, culture supernatants of myxoma cells were applied to the anti-peptide immunoaffinity column. Most of the BSF-2 activity was adsorbed and could be recovered after elution with 3 M KSCN, indicating that the BSF-2-like molecule produced by cardiac myxoma cells is identical or closely related to the T-cell-derived BSF-2 (Fig. 3*B*). Five other cases of cardiac myxoma also showed essentially the same result. Furthermore, it was shown that a uterine cervical carcinoma aberrantly produced molecules with BSF-2 activity. BSF-2 activity in the culture supernatant of the cervical carcinoma cells could be absorbed with the anti-peptide antibody and specifically eluted with 3 M KSCN (Fig. 4). This particular patient (a 65-year-old woman) with a uterine cervical carcinoma had anti-nuclear factor (serum titer was a 1:20 dilution), anti-sicca syndrome-A antibody (serum titer was a 1:4 dilution), and Raynaud phenomenon. All results implicated the involvement of the unregulated production of BSF-2 in autoantibody production.

¶National Biomedical Research Foundation (1985) Protein Sequence Database, Protein Identification Resource (Natl. Biomed. Res. Found., Washington, DC 20007) Release No. 6.

||National Institutes of Health (1985) Genetic Sequence Databank: GenBank (Research Systems Div., Bolt, Beranek, and Newman, Inc., Boston), Tape Release 38.0.

**European Molecular Biology Laboratory (1985) EMBL Nucleotide Sequence Data Library (Eur. Mol. Biol. Lab., Postfach 10.2209, 6900 Heidelberg, F.R.G.), Tape Release No. 6.

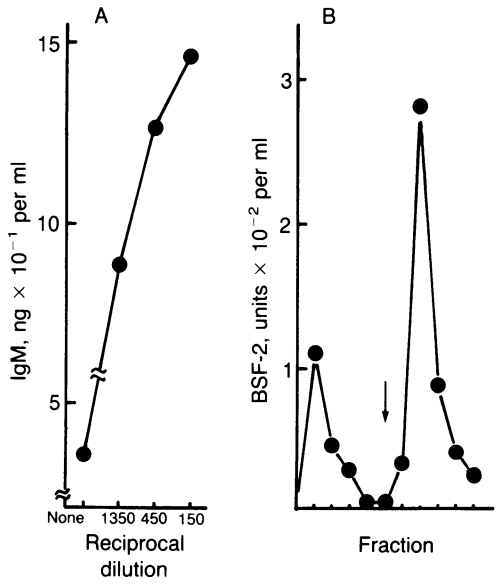


FIG. 3. BSF-2 activity of the culture supernatant of cardiac myxoma cells. Cardiac myxoma cells were aseptically obtained during autopsy and cultured in RPMI 1640 containing 10% (vol/vol) fetal calf serum at the cell concentration of 1×10^5 cells per ml with a weekly change of medium for a month. The culture supernatant was dialyzed against RPMI 1640, and the BSF-2 activity was determined (*A*). The culture supernatant of cardiac myxoma cells was applied to the anti-peptide column and bound proteins were eluted with 3 M KSCN as described in Fig. 2. BSF-2 activity of each fraction was determined (*B*).

DISCUSSION

Various factors appear to be involved in B-cell growth and differentiation (1–3). Several factors that have already been identified are IL-1 (18, 19), IL-2 (20), γ-interferon (21), and BSF-1 (22–24). To clarify the mechanism of B-cell maturation, it is essential to isolate each factor and to define its function(s) and its relationship to other known factors. We have demonstrated (7–9) that BSF-2 is involved in the final maturation of activated B cells. Functional studies have suggested that BSF-2 is a molecule distinct from other known lymphokines. In this study, we report the identification of BSF-2 as a distinct molecule, based on functional and biochemical analyses.

Human BSF-2 (M_r, 21,000) derived from an HTLV I-transformed T-cell line, TCL-Nal, was purified, and its partial NH_2-terminal amino acid sequence was determined. The antibody against the synthetic peptide corresponding to residues 1–13 in the NH_2-terminal sequence adsorbed ^{125}I-labeled purified BSF-2 (M_r, 21,000) as well as BSF-2 activity, whereas the anti-ovalbumin control antibody did not. These data indicated that the determined amino acid sequence was actually that of BSF-2. In addition, the anti-peptide antibody adsorbed BSF-2 activity derived from PHA-stimulated tonsillar lymphocytes, indicating that BSF-2 molecules generated by the HTLV I-transformed T-cell line and by normal T lymphocytes are homologous. In addition, the specificity of the anti-peptide antibody was further demonstrated by the fact that the anti-peptide antibody did not react with IL-2.

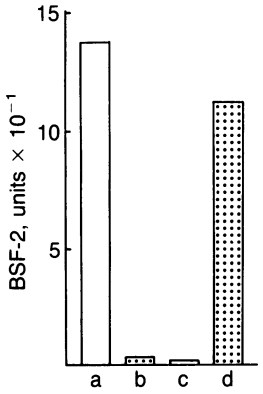


FIG. 4. Affinity chromatography of a molecule with BSF-2 activity generated by uterine cervical carcinoma cells. A uterine cervical carcinoma was obtained by a surgical operation, minced, and cultured in RPMI 1640 containing 10% (vol/vol) fetal calf serum at the cell concentration of 2×10^6 cells per ml for 2 days. Half of the culture supernatant containing 154 units of BSF-2 activity was applied to the anti-peptide column (bars b and d), and the other half was applied to the control anti-ovalbumin column (bars a and c). After washing the gels, the bound protein was eluted with 3 M KSCN. BSF-2 activities in the effluent (bars a and b) and eluate (bars c and d) were determined.

Computer analysis of the determined NH_2-terminal amino acid sequence demonstrated that human BSF-2 was distinct from other known proteins including (*i*) human IL-1, IL-2, interferons, colony-stimulating factor-1, and BSF-1 and (*ii*) murine IL-1, IL-2, granulocyte/macrophage colony-stimulating factor, IL-3, and BSF-1. In addition, purified BSF-2 did not show any IL-1, IL-2, interferon, BSF-1, or B-cell growth factor type 2 activity as reported (9). These facts indicate that BSF-2 is a distinct lymphokine acting on B cells.

It has been reported that BSF-2 induced biosynthesis of secretory-type Ig at the mRNA level in an Epstein–Barr virus-transformed B-lymphoblastoid cell line (8) and was synergistic with IL-2 in Ig production in *S. aureus* Cowan I-activated normal B cells (9). However, BSF-2 did not induce cell growth (9). In the murine system, Swain *et al.* (25) and Leibson *et al.* (26) reported a factor(s) that synergistically functions with IL-2 in Ig production, and γ-interferon and IL-2 have been reported to act synergistically in Ig production (27, 28). Since purified human BSF-2 has not shown any interferon activity and its partial amino acid sequence does not have any homology with that of interferon, it has become clear that BSF-2 is a distinct molecule acting on Ig production. On the basis of the above observations and other reports (3–9), we conclude that BSF-2 is a factor inducing the final differentiation of B cells to antibody-producing cells and may be the human equivalent to murine T-cell replacing factor originally proposed by Schimpl and Wecker (29).

Autoimmune conditions may involve an unregulated production of lymphokines and/or monokines affecting B-cell proliferation and/or differentiation as demonstrated in the MRL *lpr* (10) and the Motheaten (11) mice. It has been reported that T cells of MRL *lpr* mouse constitutively produce B-cell differentiation factor, and B cells of Motheaten mouse spontaneously produce B-cell maturation factor. For these mice, the constitutive production of such lymphokine(s) may be related to the genetically determined autoimmune diseases. In this report, we have shown that the antibody against the NH_2-terminal region of BSF-2 adsorbed BSF-2 activity generated by cardiac myxoma cells. In fact, patients with cardiac myxoma frequently have connective tissue disease-like symptoms, including the presence of hypergammaglobulinemia and autoantibodies (12). Furthermore, these symptoms disappear upon the surgical removal of the tumor, suggesting that the myxoma itself or its products are involved in the autoimmune condition of these patients. Our observations indicate that cardiac myxoma cells produce large amounts of a BSF-2-like molecule *in vitro*, and this BSF-2-like molecule could be one of the factors that induces or perpetuates the autoimmune symptoms in these patients. In addition, uterine cervical carcinoma cells obtained from a particular patient who had autoantibodies produced molecules with BSF-2 activity *in vitro*. One of the important findings in this report is that the BSF-2 activity derived from cardiac myxoma and uterine cervical carcinoma cells could be adsorbed by the anti-peptide antibodies, demonstrating that molecules with BSF-2 activity derived from these cells are identical or closely related to BSF-2 produced by T cells. This "experiment of nature" suggests that a constitutive production of BSF-2 *in vivo* may be involved in the induction of certain autoimmune diseases.

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