Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSFp-2)

(Ig production)

Toshio Hirano, Tetsuya Taga, Naoko Nakano, Kiyosi Yasukawa, Shinichiro Kashiwamura, Kazuo Shimizu, Koichi Nakajima, Kwang H. Pyun, and Tadamitsu Kishimoto

Division of Cellular Immunology, Institute for Molecular and Cellular Biology, Osaka University, 1-3. Yamadaoka, Suita, Osaka 565, Japan

ABSTRACT Human B-cell differentiation factor (BCDF) was purified to homogeneity by sequential filtration and chromatography of culture supernatants from TCL-Na1 cells on an AcA34 gel column and then on a Mono P column with fast protein liquid chromatography and reversed-phase HPLC. A 5300-fold enrichment in specific activity of BCDF with about 25% recovery was attained. The homogeneity of purified BCDF was evidenced by the following: (*i*) the specific activity was 1.7×10^7 units/mg of protein, (*ii*) only two bands, M_r 19,000 and 21,000, were identified by NaDodSO$_4$/PAGE under reduced as well as nonreduced conditions, and (*iii*) BCDF activity was recovered from the gel after NaDodSO$_4$/PAGE in the fractions corresponding to protein bands of M_r 19,000 or 21,000. Purified BCDF induced Ig secretion in Epstein–Barr virus-transformed cell lines; as little as 3 pM gave 50% of the maximum reaction achieved by 30–80 pM BCDF. Purified BCDF induced Ig production in activated B cells without any effect on cell growth. Purified BCDF did not show any activity of interleukin 1 or 2, B-cell stimulatory factor (BSF)p-1, B-cell growth factor II (BCGF-II), or interferon. Since BCDF was isolated and characterized as described, we propose that the BCDF that induces the final differentiation of B cells into high-rate Ig-secreting cells be designated BSFp-2.

Involvement of B-cell-specific growth and differentiation factors in the T-cell-dependent activation of B cells into antibody-secreting cells has been demonstrated (1, 2). However, their number, molecular or biochemical characteristics, and modes of action are still controversial and a definitive resolution of the controversy will require the chemical isolation and molecular cloning of the various B-cell stimulatory factors (BSFs).

Previous studies have demonstrated that factors inducing Ig secretion in Epstein–Barr virus (EBV)-transformed B-cell lines are present in culture supernatants from a human T-cell hybrid clone (3) or mitogen-stimulated T cells (4–7). This activity was called T-cell replacing factor (TRF) (4, 5), B-cell differentiation factor (BCDF) (3, 6), or BCDF-II (7). Several experimental results indicated that BCDF acted on B cells in the final maturation to antibody-secreting cells and seemed to be the human equivalent of murine TRF [originally reported by Schimpl and Wecker (8)]; (*i*) BCDF induced IgG or IgM secretion in EBV-transformed human B-cell lines without any effect on cell growth (4, 5, 9), (*ii*) BCDF induced an increase in biosynthesis of secretory-type heavy chains of Ig as well as their mRNA (10), and (*iii*) BCDF induced IgM and IgG secretion in *Staphylococcus aureus* Cowan I (SAC)-activated normal B cells (3, 11, 12).

We have established a human T-cell leukemia virus-transformed T-cell line, TCL-Na1, that secretes a relatively

large amount of BCDF (13). Culture supernatants from TCL-Na1 cells were used as a source of BCDF and in this report we describe the purification of human BCDF to homogeneity. As little as 1 pM purified BCDF induces Ig secretion in activated B cells without inducing any cell growth.

MATERIALS AND METHODS

Preparation of Supernatants from TCL-Na1 Cells. TCL-Na1 cells were maintained in spinner culture bottles as described (13). Serum-free culture supernatant fluids were obtained by culturing TCL-Na1 cells at a density of 1×10^6/ml in RPMI 1640 medium in a spinner culture bottle for 2 days and centrifuging successively at 1500 rpm for 5 min, 7000 rpm for 20 min, and $100,000 \times g$ for 120 min.

Preparation of B Cells and the Induction of Ig Production in Normal B Cells. Tonsilar B lymphocytes were obtained from patients at tonsillectomy as described (13). B cells (1×10^6/ml) were cultured with SAC (0.0025%, vol/vol) for 3 days and low-density blast cells were separated by a discontinuous Percoll gradient centrifugation (30%, 40%, 50%, and 60% Percoll in phosphate-buffered saline at $1280 \times g$ for 12 min at 4°C). Blast B cells were recovered in the fractions between 40% and 50% and cultured at a cell density of 5×10^5/ml in 200 μl of culture medium consisting of RPMI 1640 containing 10% fetal calf serum and 50 μM 2-mercaptoethanol for 3 days. The concentration of IgG in culture supernatants was determined by an ELISA (14). Recombinant interleukin (IL) 2 was prepared as described (12) and was kindly provided by J. Hamuro (Ajinomoto Co., Ltd.).

Assay for BCDF Activity. Four thousand SKW6-CL4 (9) or 6×10^3 CESS (4) cells were cultured in 200 μl of culture medium in the presence of 1:3 dilutions of the samples to be tested. After 3 days of culture, the concentrations of IgM for SKW6-CL4 cells and IgG for CESS cells were determined by ELISA. The BCDF activity that induced 50% of the maximum response of IgM production in 1×10^4 SKW6-CL4 cells was defined as 1 unit/ml. Unless otherwise mentioned, BCDF activity was determined by using SKW6-CL4 cells.

Assay for BSFp-1, BCGF-II, IL-1, IL-2, and Interferon Activities. To determine BSFp-1 activity, 5×10^4 purified B cells were cultured in the presence of the F(ab')$_2$ fragment of affinity-purified goat anti-human IgM (Cappel Laboratories, Cochranville, PA; 5 μg/ml) and exposed to [^{3}H]thymidine (specific activity, 15.1 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) during the last 16 hr of a 72-hr culture (13). Phytohemagglutinin (PHA) supernatant is the preparation used previously (13). To detect BCGF-II activity, the *in vivo* BCL$_1$ cell line (kindly provided by E. S. Vitetta, University

Abbreviations: BSF, B-cell stimulatory factor; BCDF, B-cell differentiation factor; SAC, *Staphylococcus aureus* Cowan I; BCGF-II, B-cell growth factor II; IL, interleukin; FPLC, fast protein liquid chromatography; PHA, phytohemagglutinin.

of Texas) was employed as described (13). For the IL-1 assay, 2×10^5 thymocytes of 6-week-old BALB/c mice were cultured in 0.2 ml of culture medium containing PHA at 1 μg/ml and then exposed to [^{3}H]thymidine as described above. IL-2 activity was assayed as described (15). Interferon activity was assessed with WISH cells and vesicular stomatitis virus (16).

Gel Filtration. Culture supernatant was concentrated by using an Amicon ultrafiltration unit with a YM-10 membrane and then applied to an Ultrogel AcA34 (LKB) column (2.5 × 90 cm) previously equilibrated with phosphate-buffered saline and eluted at a flow rate of 20 ml/hr.

Chromatofocusing. Active fractions from the AcA34 column were pooled, dialyzed against 25 mM piperazine·HCl buffer (pH 6.3), and then applied to a Mono P column (Pharmacia) previously equilibrated with the same buffer and eluted with Polybuffer 74 (pH 4.5) using a fast protein liquid chromatography (FPLC) system (Pharmacia).

Reversed-Phase HPLC. Reversed-phase HPLC was carried out by using a Waters HPLC system with Synchropak RP-P (250 × 4.1 mm) (SynChrom, Linden, IN), RP-304 (250 × 4.6 mm) (Bio-Rad), and Pro-RPC HR 5/10 (Pharmacia) columns. Pooled fractions from the Mono P column were adjusted to a concentration of 0.1% (vol/vol) trifluoroacetic acid and applied to a reversed-phase column equilibrated with water containing 0.1% trifluoroacetic acid and eluted with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. For the Pro-RPC HR 5/10 column, a flow rate of 0.2 ml/min and a linear gradient (from 0% to 45% for 10 min and from 45% to 65% for 100 min) of acetonitrile were employed. In the case of the RP-304 column, a flow rate of 0.5 ml/min and a linear gradient (from 0% to 50% for 25 min and from 50% to 80% for 60 min) of acetonitrile were employed. For the Synchropak RP-P column, a flow rate of 1 ml/min and a linear gradient (from 0% to 50% for 25 min and 50% to 70% for 30 min) were employed. Ten microliters of each fraction was mixed with 490 μl of RPMI 1640 medium containing 10% fetal calf serum, dialyzed against RPMI 1640, and assessed for BCDF activity.

NaDodSO$_4$/PAGE. Fractions obtained from the reversed-phase column were analyzed by electrophoresis on a 1% NaDodSO$_4$/12% polyacrylamide gel (17) and proteins were determined by silver staining using Daiichikagaku silver stain (Daiichikagaku, Tokyo). To measure BCDF activity, after electrophoresis, the gel was cut into 1-mm slices, and each sliced gel was minced, put into a tube, mixed with phosphate-buffered saline containing 10% fetal calf serum, and shaken at 4°C overnight. Eluted samples were dialyzed against RPMI 1640 and filtered through a 0.45-μm membrane, and BCDF activity was determined.

RESULTS

Purification of BCDF. BCDF activity was eluted from an Ultrogel AcA34 column in the fractions corresponding to M_r 30,000–35,000 (Fig. 1A). The active fractions (67–75) were pooled, and dialyzed against piperazine·HCl buffer (25 mM, pH 6.3), and applied to a Mono P column, which was eluted with Polybuffer 74 (pH 4.5). As shown in Fig. 1B, BCDF activity was found in the fractions corresponding to a pI of 5.0 to 5.1. The active fractions (fractions 18 and 19) were pooled and divided into four aliquots. One aliquot was then applied to a Pro-RPC (C_{1+8}) column, one to a RP-304 (C_4) column, and one to a Synchropak-RP-P (C_{18}) column. The representative chromatographic profile from the Synchropak RP-P column is shown in Fig. 1C. BCDF activity, eluted with 55% acetonitrile, had a minor peak of protein after the elution of several major peaks of protein.

Purified BCDF preparations obtained from different kinds of reversed-phase columns were analyzed by NaDod-
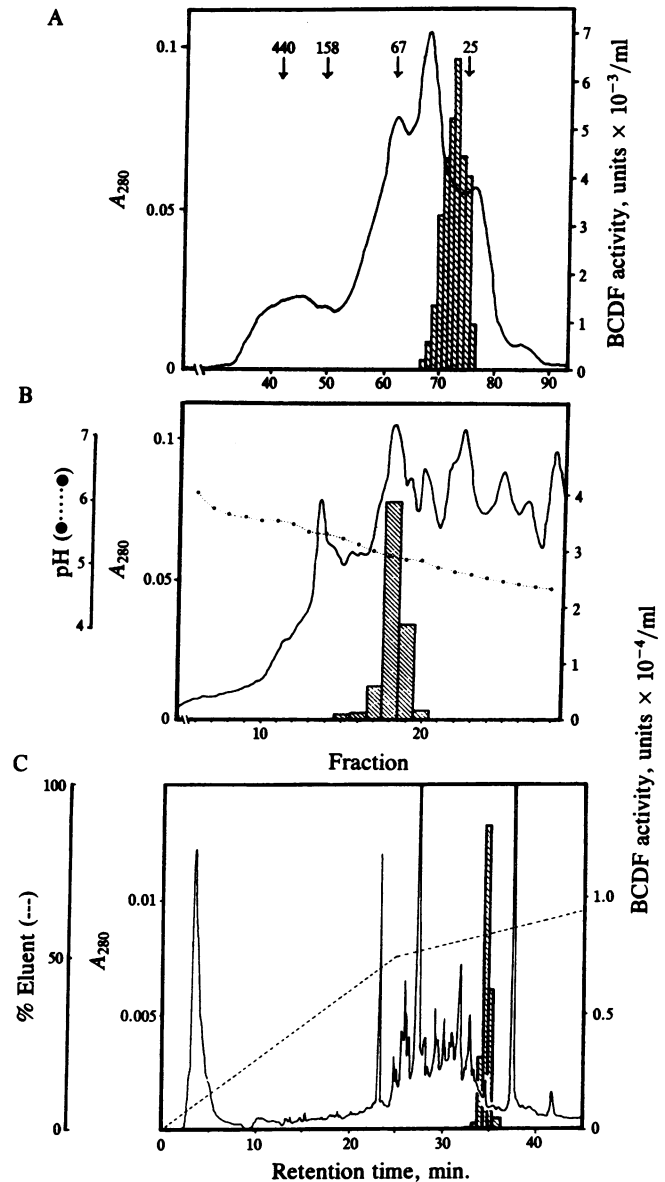

FIG. 1. Purification of BCDF. (A) Serum-free culture supernatant (5 liters) of TCL-Na1 cells was concentrated to 10 ml, applied to an Ultrogel AcA34 column, and eluted at a flow rate of 20 ml/hr. Chymotrypsinogen A (M_r 25,000; 25), bovine serum albumin (M_r 67,000; 67), aldolase (M_r 158,000; 158), and ferritin (M_r 440,000; 440) (Pharmacia) were used as standard marker proteins. (B) Active fractions from the AcA34 column (67–75) were pooled and chromatofocused on a Mono P column with a pH interval between 4.5–5.5 using an FPLC system. (C) Active fractions from a Mono P column (18 and 19) were pooled and one-fourth of the sample was applied to a Synchropak RP-P column and eluted with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing BCDF activity are indicated by hatching.

SO$_4$/PAGE in a nonreduced condition. Protein bands, M_r 19,000 and 21,000, were identified only in the fractions that showed BCDF activity (data not shown). In addition, a fraction from the Synchropak RP-P column with a peak BCDF activity was shown to contain only protein bands of M_r 19,000 and 21,000 by silver staining (Fig. 2A). In a reduced condition, both the M_r 19,000 and 21,000 proteins were also identified (Fig. 2B). To examine whether proteins of M_r 19,000 and 21,000 had BCDF activity after electrophoresis in a nonreduced condition, the gel was cut into strips 1 mm in width, protein was eluted from the gel, and BCDF activity of each fraction was examined. As shown in Fig. 2A, BCDF
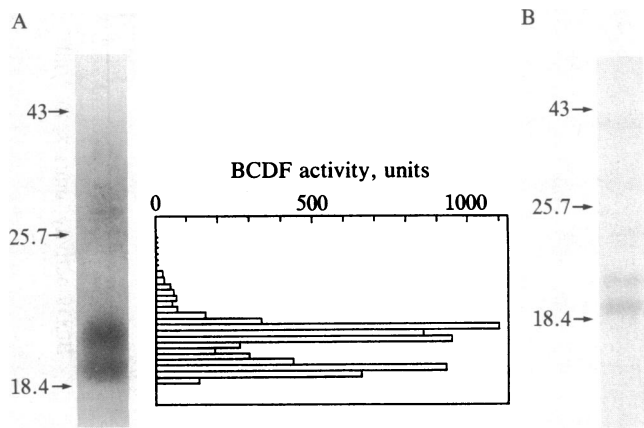
FIG. 2. NaDodSO$_4$/PAGE of reversed-phase HPLC-purified BCDF. (*A*) Reversed-phase HPLC (C$_{18}$)-purified BCDF was analyzed by NaDodSO$_4$/PAGE in a nonreduced condition. After electrophoresis, one lane was silver-stained and the other lane was cut into strips 1 mm in width, and these were minced, transferred to a tube, mixed with phosphate-buffered saline containing 10% fetal calf serum, shaken vigorously at 4°C overnight, dialyzed against RPMI 1640 medium, and assessed for BCDF activity. (*B*) A fraction from the Synchropak RP-P column with a peak BCDF activity in another series of purifications was analyzed by NaDodSO$_4$/PAGE in a reduced condition and silver-stained. Ovalbumin (M_r 43,000; 43), α-chymotrypsinogen (M_r 25,700; 25.7), and β-lactoglobulin (M_r 18,400; 18.4) (Bio-Rad) were used as standard marker proteins.

FIG. 3. Induction of Ig production by purified BCDF in EBV-transformed B-cell lines. Cells [4 × 10^3 SKW6-CL4 (*A*) or 6 × 10^3 CESS (*B*)] were cultured with various concentrations of BCDF for 3 days and the concentration of IgM (*A*) or IgG (*B*) was determined. Data represent means of triplicate cultures. Standard errors (of the means) were <5%.

activity was detected in the fractions corresponding to protein bands of M_r 19,000 and 21,000. All of these results indicate that both the M_r 19,000 and 21,000 protein bands represent BCDF and that BCDF is composed of a single polypeptide chain.

The representative data of overall purification of BCDF that was achieved using 5.7 liters of serum-free culture supernatant are shown in Table 1. The protein content of the Synchropak RP-P (C$_{18}$)-purified material was estimated by comparing the density of the silver-stained protein band with that of a silver-stained soybean trypsin inhibitor. A specific activity of 1.7 × 10^7 units/mg of protein and a final purification of 5300-fold were obtained. The overall recovery of BCDF activity was 25%. These data show that the concentration of BCDF at 1 unit/ml corresponds to approximately 3 pM.

Functional Properties of Purified BCDF. Purified BCDF at 100 pM did not cause a significant increase in the [^{3}H]thymidine uptake in either PHA–stimulated murine thymocytes or BCL$_1$ cells (data not shown). BCDF at 1 pM showed an IL-2 activity of <0.0003 unit/ml and an antiviral activity of <0.0014 unit/ml. Purified BCDF induced IgM secretion in SKW6-CL4 cells with as little as 1 pM and the maximum induction with 30–80 pM (Fig. 3*A*). Essentially the same results were obtained in the induction of IgG production in CESS cells as shown in Fig. 3*B*. It was previously shown that partially purified BCDF acted on leukemic B cells (6) or B
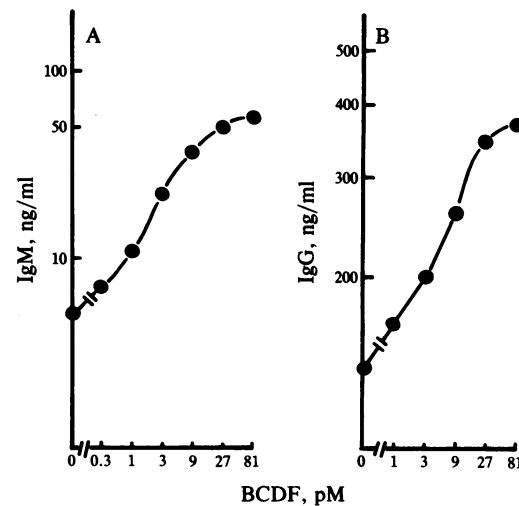
cells prestimulated with other kinds of BSFs (11) or IL-2 (12) and induced the final differentiation of B cells to high-rate Ig-secreting cells. To examine whether or not purified BCDF has these same functional properties, blast B cells were separated after 3 days' stimulation of tonsilar B cells with SAC. Blast B cells, 1 × 10^5, were cultured with various concentrations of BCDF and/or recombinant IL-2 for 3 days and the concentration of IgG in the culture supernatants as well as [^{3}H]thymidine uptake during the last 16 hr were determined. As shown in Table 2, BCDF alone induced a substantial degree of IgG production without any significant increase in [^{3}H]thymidine uptake. IL-2 alone also induced IgG production comparable to that with BCDF and in contrast to BCDF, induced a significant degree of [^{3}H]thymidine uptake. When B cells were cultured with IL-2 at 1 unit/ml and various concentrations of BCDF, IgG production was augmented but no enhancement of IL-2-induced [^{3}H]thymidine uptake was caused by the addition of BCDF. These data indicate that BCDF induces the final differentiation of B cells without any effect on [^{3}H]thymidine uptake. This was also shown by an experiment that showed no synergistic effect with anti-Ig on B-cell proliferation (Table 3).

DISCUSSION

Many BSFs with distinct functions, such as BSFp-1 (18, 19), BCGF-II (20), BCDF (6), BCDF-II (7), BCDFμ (21), BCDFγ (22), BGDF (13), B151TRF (23), BMF (24), BRMF (25), and EL-TRF (26) have been reported. In addition to these, IL-2

Table 1. Purification

Fraction	Total activity, U × 10^{-4}	Total protein, A_{280}	Specific activity, U/A_{280}	Purification, fold	Recovery, %
Crude	19	58.8	3.2 × 10^3	1	100
AcA34	31.8	5.25	6.0 × 10^4	19	167
Mono P	4.7	0.2	2.4 × 10^5	75	25
Synchropak RP-P (C$_{18}$)	4.7	(2.8 μg)*	(1.7 × 10^7 units/mg)	5300	25

The crude fraction was 5.7 liters serum-free culture supernatant. U, units.
*The amount of protein was determined after silver staining by comparison with a protein standard (soybean trypsin inhibitor).

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Table 2. Effect of BCDF on IgG production and proliferation of SAC-stimulated B cells

Factor	Conc.	IgG, ng/ml	[³H]Thymidine uptake,* cpm
None		147 ± 31	2,137 ± 17
IL-2	0.1 unit/ml	314 ± 21	4,768 ± 86
	1 unit/ml	690 ± 42	13,959 ± 105
BCDF	0.1 pM	250 ± 28	2,394 ± 518
	1 pM	327 ± 24	2,579 ± 84
	10 pM	550 ± 42	2,912 ± 118
	100 pM	501 ± 30	3,041 ± 118
IL-2 (1 unit/ml)	0.1 pM	960 ± 14	15,316 ± 1,915
+ BCDF	1 pM	1120 ± 42	14,888 ± 2,400
	10 pM	1380 ± 84	16,238 ± 264
	100 pM	1590 ± 98	14,275 ± 1,012

Blast cells were separated from B cells stimulated with SAC for 3 days and 1×10^5 blast cells were cultured for 3 days.
*B cells were exposed to [³H]thymidine during the last 16 hr.

and immune interferon have also been considered as kinds of BSFs (27–29). BSFp-1 (1, 19) has been considered to act in the early activation process of B cells and to induce proliferation in the presence of anti-Ig. BCDF (6), BCDF-II (7), and EL-TRF (26) have been shown to function in the late stage of B-cell differentiation and to induce the final differentiation to antibody-forming cells. Several different kinds of BSFs, such as EL-BCGF-II, DL-BCGF-II, B151TRF, BRMF, and BGDF (13, 20, 23, 25), have been shown to have both growth and differentiation activities. Biochemical purification and/or molecular cloning of these BSFs are essential to solve the complexity and controversy with respect to BSFs; i.e., how many and what kinds of molecules are really involved in the activation process of normal B cells into antibody-producing cells. Several studies (30–32) have reported the partial purification of BSFs, but none of the BSFs have yet been purified to homogeneity in order to permit amino acid sequencing or gene cloning.

In this study, human BCDF produced by a human T-cell leukemia virus-transformed T-cell line, TCL-Na1, was purified to homogeneity. The purified BCDF exerted its biological function at a concentration of as little as 1 pM and did not contain any other lymphokine activity such as IL-1, IL-2, BSFp-1, BCGF-II, or interferon. NaDodSO₄/PAGE analysis showed that BCDF is composed of a single polypeptide chain with M_r either 19,000 or 21,000. Both species with different molecular weight seem to be BCDF molecule, because (*i*) fractions that contained either M_r 19,000 or 21,000 protein molecules had BCDF activity, (*ii*) BCDF activity was eluted from the fractions of NaDodSO₄/PAGE corresponding to M_r of either 19,000 or 21,000, (*iii*) BCDF, purified by Synchropak RP-P, was shown to contain only M_r 19,000 and 21,000 protein bands on NaDodSO₄/PAGE after silver-staining of the gels, (*iv*) purified BCDF exerted its biological function at a concentration of as little as 1 pM. We can only

Table 3. BCDF does not have a synergistic effect on anti-Ig-induced B-cell proliferation

Factor	[³H]Thymidine uptake,* cpm
None	1,215 ± 370
PHA-sup†	12,130 ± 1,724
BCDF	
1 pM	1,502 ± 490
10 pM	1,557 ± 224
100 pM	1,092 ± 290

*B cells (5×10^4) were cultured with the F(ab')₂ fragment of goat anti-human IgM (5 µg/ml) for 3 days.
†Ten microliters of the PHA supernatant preparation used previously (13) was used.

speculate that the presence of BCDF molecules with different molecular weights is the result of post-translational modification or the formation of breakdown products. A similar situation was previously observed with purified IL-2 (33). In the experiment reported in Table 1, 167% recovery of BCDF activity was obtained after gel filtration. This may be due to the depletion of inhibitory substances present in crude supernatants.

The purified BCDF induced a substantial amount of Ig production in SAC-stimulated blast B cells within 3 days at a concentration as little as 1 pM without any effect on [³H]thymidine uptake. If blast B cells were cultured with BCDF in the presence of IL-2, the IgG production was enhanced without any augmentation of proliferation, a situation in complete agreement with previous results (12). The data may be compared with those previously reported by several investigators who showed the synergy between IL-2 and a certain factor(s) in the induction of anti-sheep erythrocyte antibody response in murine spleen B cells (34, 35). BCDF does not have any growth activity and is reponsible for the final maturation of B cells into high-rate Ig-secreting cells. As discussed in several reports, three distinct signals for activation, proliferation, and differentiation are required for the maximum Ig induction in B cells. Anti-IgM and BSFp-1 may serve as the signals for activation and proliferation and IL-2 or BCGF(s) such as BCGF-II may be responsible for proliferation and Ig induction. The present study shows that BCDF is required for the final maturation of B cells and may be the human equivalent of the murine T-cell replacing factor originally reported by Schimpl and Wecker (8). We propose that our purified BCDF be designated BSFp-2.

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