PURIFICATION TO HOMOGENEITY OF A HIGH MOLECULAR WEIGHT HUMAN B CELL GROWTH FACTOR; DEMONSTRATION OF SPECIFIC BINDING TO ACTIVATED B CELLS; AND DEVELOPMENT OF A MONOCLONAL ANTIBODY TO THE FACTOR

By JULIAN L. AMBRUS, JR., CYNTHIA H. JURGENSEN, ERIC J. BROWN, AND ANTHONY S. FAUCI

From the Laboratory of Immunoregulation and the Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

A wide range of lymphokines have been described that enhance the proliferation of B lymphocytes, including interleukin 1 (IL-1)[1] (1), IL-2 (2, 3), interferon (IFN) (4), and several B cell growth factors (BCGF) or B cell stimulatory factors (BSF) (5–10). However, the precise molecular distinctions among these factors and the exact role that each plays in B cell function remains unclear. Many important questions regarding the biology and chemistry of these lymphokines are unanswered, in part because sufficient quantities of purified proteins have not been available.

Recently, substantial progress has been made in the purification and chemical and biologic characterizations of several lymphokines. IL-1 has been purified (11) and cloned (12), as have IL-2 (13–15) and various IFN (16–18). However, investigations related to BCGF have lagged behind those of other lymphokines because of several major problems. First, it has been difficult or impossible to completely separate IL-2 from BCGF when both are present in the same culture supernatant (19). Second, the currently available assay systems for BCGF, which depend on costimulation of normal B cells with antigen (20) or surface membrane immunoglobulin crosslinking agents such as *Staphylococcus aureus* Cowan I (SAC) (21), are time consuming and insensitive. Third, monoclonal antibodies have been invaluable in the purification of certain lymphokines. In the purification of IL-2, Smith et al. (13) found that a monoclonal antibody was necessary to detect IL-2 in column fractions because functional activity, as defined by the ability to enhance the proliferation of an IL-2-dependent cell line, was lost during purification. No monoclonal antibody has yet been developed to any human BCGF,

[1] *Abbreviations used in this paper:* BCGF, B cell growth factor; BSF, B cell stimulation factor; HMW, high molecular weight; HPLC, high performance liquid chromatography; IFN, interferon; IL-1 and IL-2, interleukin 1 and 2; LMW, low molecular weight; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; SAC, *Staphylococcus aureus* Cowan I; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/85/10/1319/17 $1.00
Volume 162 October 1985 1319–1335

although a monoclonal antibody has recently been developed to a murine, low molecular weight (LMW) BCGF termed BSF-I (22).

We have recently described production of a high molecular weight (HMW) BCGF by the human B lymphoma line, Namalva, and a human T cell line, T-ALL (9). The HMW-BCGF-containing supernatants produced by these lines in serum-free media lack IL-1, IL-2, and IFN activity. Thus, these supernatants represent an ideal starting material for the purification of HMW-BCGF. In this paper, we describe purification of this HMW-BCGF to apparent homogeneity and the production of a monoclonal antibody to the purified protein.

Materials and Methods

Cell Lines and HMW-BCGF Production. Namalva has been grown in our laboratory for several years, and T-ALL was kindly provided by Dr. Jun Minowada (Loyola University, Chicago, IL). Both lines were grown in HB102 media (Hana Biologics, Inc., Berkeley, CA) without serum at a cell density of 10^6 cells/ml. Cells were stimulated with phytohemagglutinin (PHA) (Burroughs Wellcome Research Laboratories, Beckenham, England) at a concentration of 1 μg/ml for 72 h, and then cell-free supernatant was harvested, as described (9).

BCGF Assay. Human B cells were purified as previously described (9) from tonsils or peripheral blood. Supernatants or column fractions to be assayed for BCGF activity were added to B cells (5×10^4/ml) in 96-well, flat-bottomed microtiter plates (Costar 3596; Data Packaging Corp., Cambridge, MA) in the presence of 15 μg/ml of $F(ab')_2$ goat anti-human IgM heavy chain–specific anti-μ (Cappel Laboratories, Cochranville, PA). Over the last 16 h of the 72-h culture period, 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) was added to each well. Cells were then harvested using a Skatron cell harvester (Skatron, Inc., Sterling, VA), and the incorporation of [^{3}H]thymidine was measured by standard liquid scintillation counting techniques.

T Cell Proliferation Assay. T cells were purified from peripheral blood by rosetting once with aminoethylisothiouronium bromide–treated sheep erythrocytes as previously described (24). Supernatants or column fractions to be assayed for T cell proliferation–inducing activity were added to 96-well, flat-bottomed microtiter plates containing 10^5 T cells in RPMI 1640 with 10% fetal calf serum. Culture was maintained for 96 h, and cells were pulsed with [^{3}H]thymidine for the last 18 h of culture. Cells were then harvested and [^{3}H]thymidine incorporation was measured.

IL-2 Assay. IL-2 activity was assessed as previously described (25) using the IL-2-dependent T cell line HT-2 kindly provided by Dr. David Volkman (National Institutes of Health).

Production of Anti-HB102 Affinity Matrix. Although serum-free HB102 medium contains some proteins, the type and amount are not public information. Antibodies to these proteins were produced in rabbits by immunization with concentrated HB102 proteins in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO). Serum was collected after three immunizations, and IgG was purified by octanoic acid precipitation (23). The IgG was coupled to cyanogen bromide–activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) by a minor modification of the method of Tan-Wilson et al. (31).

HMW-BCGF Purification Scheme. The method for purification of HMW-BCGF is shown schematically in Fig. 1. 10 liters of PHA-stimulated T-ALL supernatant was concentrated to a volume of 10 ml using a Minitan concentrator with 10,000-mol-wt exclusion membranes (Millipore Corporation, Bedford, MA).

Concentrated T-ALL supernatant (1 ml) was introduced onto a 15 ml column containing rabbit anti-media IgG and incubated with the resin for 120 min at 4°C. Material containing BCGF was washed through the column with phosphate-buffered saline (PBS). Bound material was eluted with 0.2 M glycine (pH 3.0) and the column was washed with PBS before the next sample was applied.

The BCGF-containing pool from the anti-media column was concentrated to 5 ml by

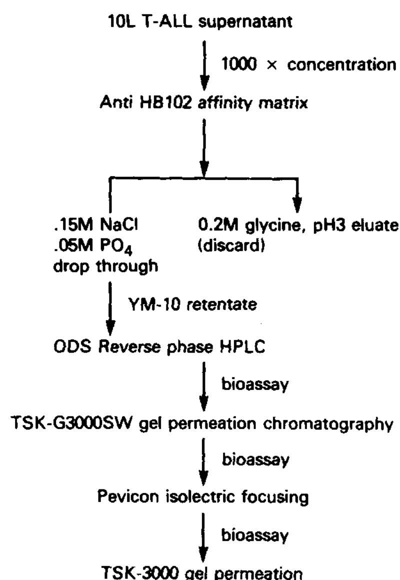

FIGURE 1. Protocol for purification of HMW-BCGF from T-ALL supernatant.

positive pressure using a YM10 membrane (Amicon Corp., Scientific System Division, Danvers, MA). Samples of 500 μl were then entered repetitively onto a 1.5 × 15 cm ultrasphere-ods reverse phase, high performance liquid chromatography column (HPLC) (Beckman Instruments, Irvine, CA) and eluted with a gradient of 10–100% acetonitrile in water at a buffer flow rate of 0.5 ml/min. Fractions were collected every 2 min. Each fraction was dialyzed individually against RPMI 1640 and then assayed for BCGF activity. Active fractions were pooled and again concentrated to 5 ml using a YM10 membrane. This material was applied in 1-ml aliquots to a TSK-G3000 SW HPLC sieving column (Toyo Soda Co., Ltd., Tokyo, Japan) and eluted with PBS at a flow rate of 1 ml/min. 60 fractions were collected and assayed for BCGF activity. Active fractions were pooled and applied to a preparative isoelectric focusing bed containing Pevicon (Accurate Chemical and Scientific Corp., Westbury, NY). Isoelectric focusing was performed as previously described (9). The bed was then separated into 24 slices, and the pH of each section measured. After removal of the Pevicon by centrifugation, individual fractions were dialyzed in RPMI 1640 and again assayed for BCGF activity. Active fractions between pH 6.7 and 7.8 were pooled. The active fraction at pH 4.9 was discarded because this was believed to be primarily albumin with some nonspecifically bound HMW-BCGF. This pool was concentrated to 0.5 ml, and the entire sample entered onto a TSK-3000 HPLC sieving column (Toyo Soda Co., Ltd.). The column was again eluted with PBS. One major peak of activity was obtained. Some of the material from this fraction was iodinated using the Bolton-Hunter reagent (26). The iodinated material was evaluated for purity by autoradiography after electrophoresis in a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel (27).

Evaluation of Specific Binding of HMW-BCGF. ^{125}I-HMW-BCGF produced as described above was added at ~50,000 cpm to tubes containing 10^5 unactivated B cells, SAC-activated B cells (48 h after SAC activation), unactivated T cells, or PHA-activated T cells (48 h after PHA stimulation) in a total volume of 500 μl RPMI 1640. Unlabeled HMW-BCGF or IL-2 were added to different tubes at different concentrations and each tube briefly shaken. Tubes were allowed to sit at 4°C for 8 h after which they were washed three times with PBS. After washing, cell-associated radioactivity was quantitated.

Production of Monoclonal Antibody to HMW-BCGF. BALB/c mice were immunized with purified HMW-BCGF in complete Freund's adjuvant at 2-wk intervals for a total of three

immunizations. 4 d after the third immunization, the spleens were harvested and the mononuclear cells dispersed by passage over a fine wire mesh. Mononuclear cells were fused to the mouse myeloma NS-1 at a ratio of 2:1 using a standard polyethylene glycol fusion technique (28). Hybridomas were selected in hypoxanthine/aminopterin/thymidine-containing media as described (28) and then clones were established by growing cells in 96-well, flat-bottomed plates seeded at 0.3 cells per well. Supernatants from clones were assayed for their ability to block anti-μ and HMW-BCGF-driven B cell proliferation. Positive wells were recloned, and then the clone that optimally blocked anti-μ and HMW-BCGF-driven B cell proliferation was evaluated for its ability to bind to HMW-BCGF by Western blotting. Western blotting was performed as described previously (29). The clone BCGF/1/C2 was then grown in the ascites of pristane-treated BALB/c female mice as previously described (30). Ascites was harvested, and IgG was purified by precipitation with octanoic acid (23) as previously described. The quantity of IgG present was estimated by measuring the absorbance at 280 nm ($E_{280}^{1\%}$ 1 cm IgG = 14). Some of this IgG anti-BCGF was coupled to Sepharose as described above.

Internal Labeling of HMW-BCGF. Internal labeling of HMW-BCGF was accomplished by growing T-ALL to 10^6/ml in HB102. Cells were then washed with PBS and placed in RPMI 1640 deficient in leucine, lysine, and glycine at a cell density of 10^6 cells/ml in a total volume of 4 liters. Then, 5 μCi each of [^{3}H]leucine (>140 Ci/mmol), [^{3}H]lysine (>5 Ci/mmol), and [^{3}H]glycine (30–60 Ci/mmol) (New England Nuclear) were added to the culture flask and the cells were stimulated with PHA at 1 μg/ml. Cell-free supernatant was harvested 72 h later, concentrated, and applied to monoclonal anti-BCGF–Sepharose columns. Internally labeled HMW-BCGF was detected in polyacrylamide gels by fluorography after treatment with Enhance (New England Nuclear).

Results

Purification of HMW-BCGF. The HMW-BCGF produced by Namalva has been described previously in detail (9). T-ALL stimulated with PHA produces a BCGF of similar molecular weight whose activity on anti-μ–stimulated, unfractionated peripheral blood B cells is demonstrated in Table I. The optimal BCGF activity was seen at a 25% vol/vol concentration of supernatant, a dose response similar to the HMW-BCGF produced by Namalva. For purposes of assessing functional recovery during purification, we have defined one unit as the amount of HMW-BCGF necessary to give half-maximal stimulation, with maximum defined as the proliferation in 25% crude Namalva supernatant. Half-maximal stimulation of B cell proliferation by unfractionated T-ALL supernatant occurred at ~15% vol/vol (equivalent to 30 μl per 200-μl well). Thus, since 1 U is contained in 30 μl of crude supernatant, our starting material of 10 liters for purification contains ~330,000 U of BCGF activity. Incubation of B cells with PHA alone in the functional assay caused no significant proliferation, which demonstrates that PHA does not play a significant role in the proliferation-inducing activity of the crude supernatant.

Because the T-ALL supernatant lacks IL-1, IL-2, and IFN activity, purification was concerned with removing media proteins (primarily albumin and transferrin), other T-ALL secretory products, and PHA. The PHA used shows some size and change heterogeneity, with the primary species having a molecular weight of 100,000 and a pI of 5.3 (data not shown).

After 1,000× concentration, the HMW-BCGF was absorbed with an affinity matrix containing antibodies to the proteins in HB102. This significantly reduced the quantity of contaminating protein. ~36.7 OD units of protein were applied

TABLE I

BCGF Activity of Supernatant from T-ALL and Namalva Lines after Stimulation wth PHA

Factor added		[^{3}H]Thymidine incorporation
None		1,063 ± 184
PHA		1,676 ± 4
T-ALL (F)	50% (vol/vol)	12,747 ± 119
	25%	23,893 ± 1,100
	10%	6,891 ± 1,061
	5%	1,297 ± 456
Namalva	25% (vol/vol)	21,082 ± 913

B cells purified as described in Materials and Methods were stimulated with 15 μg/ml anti-μ and placed in 96-well Costar plates at a concentration of 5 × 10^4 cells per well in 10% fetal calf serum with RPMI and factors added. Cells were kept in culture at 37°C in 5% CO_2 for 72 h and pulsed with 1 μCi/well of [^{3}H]thymidine for the last 18 h. Cells were harvested at 72 h, and incorporation of [^{3}H]thymidine was measured with a scintillation counter.

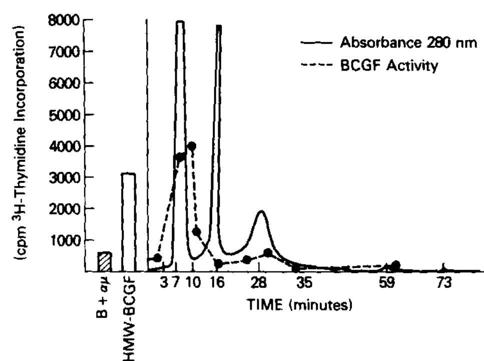

FIGURE 2. BCGF-containing T-ALL supernatant entered on a 1.5 × 15 cm ultrasphere-ods reverse phase HPLC column and eluted with a 10–100% gradient of acetonitrile. Fractions were individually dialyzed and then assayed for BCGF activity. (——) Absorbance, (---) BCGF activity. The bar graphs at the left represent the assay controls: β + αμ and β + αμ + HMW-BCGF.

to the immunoabsorbant; the drop-through containing the HMW-BCGF activity had only 11.8 OD units. The glycine eluate had 21.5 OD units.

The remainder of the purification scheme is demonstrated by representative column and isoelectric focusing runs in Figs. 2–5. The first step (Fig. 2) consisted of fractionating the immunoabsorbant drop-through on a reverse phase HPLC column. The BCGF activity along with a large amount of additional protein eluted from the column at an acetonitrile concentration between 22 and 25%. The next step involved gel permeation on TSK-G3000 SW column. This separated the BCGF activity into two peaks, associated with two major absorbance peaks (Fig. 3). These peaks occur in the molecular weight ranges of 150–200,000 and 60–80,000. This may imply that an even higher (i.e., ~200,000 vs. 60,000) molecular weight BCGF exists, but we have not yet explored this possibility.

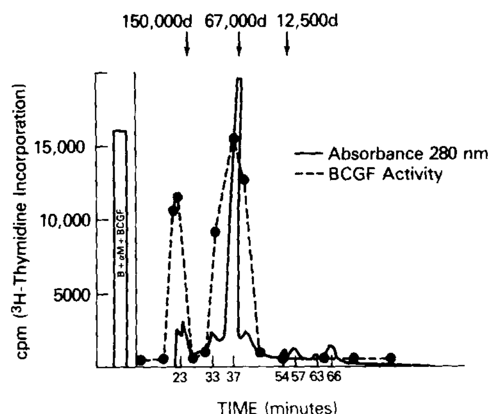

FIGURE 3. BCGF-containing fractions from reverse phase HPLC were pooled and entered on a TSK-G3000 SW HPLC sieving column. Fractions were eluted with PBS and assayed for BCGF activity. (——) Absorbance, (---) BCGF activity. The bar graph at the left represents the controls of the BCGF assay.

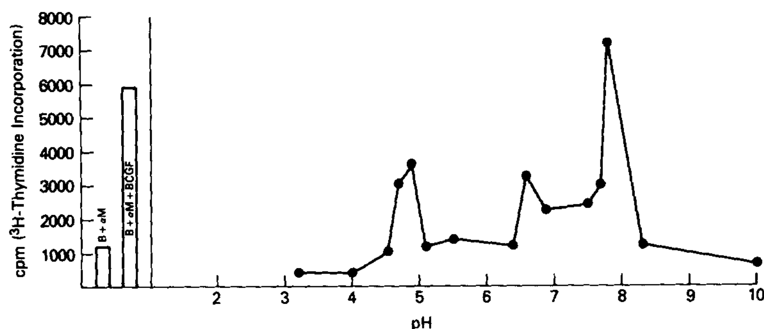

FIGURE 4. BCGF-containing fractions from TSK-G3000 SW column were pooled and subjected to isoelectric focusing in Pevicon as described in Materials and Methods. The focusing bed was then sliced into 24 sections, and the pH of each slice measured. After removal of Pevicon by centrifugation, individual fractions were dialyzed in RPMI 1640 and then assayed for BCGF activity. (——) BCGF activity. The bar graphs at the left represent the controls for the BCGF assay.

Active fractions from the G3000 SW column were pooled and subjected to isoelectric focusing in a bed of Pevicon (Fig. 4). Three activity peaks were obtained. Two peaks, with isoelectric points of 6.7 and 7.8, are identical to activity peaks described for Namalva-synthesized HMW-BCGF (9). The activity peak at pH 4.9 presumably represents a BCGF albumin complex, since albumin has an isoelectric point of 4.8–4.9. Therefore, this low pH activity peak was discarded. The peaks with isoelectric points of 6.7 and 7.8 were pooled for further purification.

The final purification step involved a second, smaller HPLC sieving column (Fig. 5), which resolved one major BCGF activity peak at 60,000 with very little absorbance at 280 nm. This activity peak lacked the ability to enhance the proliferation of resting T cells (data not shown), but contained ~1,000 U of BCGF activity. This represents an overall yield of the purification of ~0.3%. Table II outlines the approximate yield from the various purification steps.

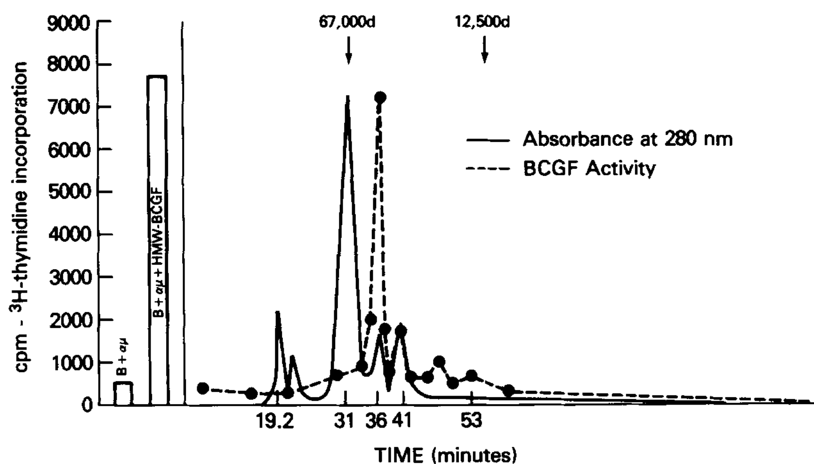

FIGURE 5. BCGF-containing fractions at pH 6.7 and 7.8 from isoelectric focusing were pooled and entered on a TSK-3000 HPLC sieving column. Fractions were eluted with PBS and assayed for BCGF activity. (——) Absorbance, (---) BCGF activity. The bar graphs at the left represent the controls for the BCGF assay.

TABLE II
Purification of HMW-BCGF

Step	Total activity*	Total protein‡	Specific activity	Times purified
	U	*mg*	*μ/mg*	
Crude supernatant§	330,000	145.0	2.5×10^3	—
Anti-HB102 affinity matrix	250,000	44.0	5.6×10^3	2.2
Reverse phase HPLC	46,000	0.55	83×10^3	32.2
TSK-G3000SW HPLC	40,000	0.30	133×10^3	56.0
Pevicon isoelectric focusing	1,200	ND‖	—	—
TSK-3000 HPLC	1,000	<0.002	$\sim 500 \times 10^3$	>200.0

* Activity is expressed in half-maximal stimulation units as described in Results.
‡ Protein was measured by absorbance at 280 nm either manually (steps 1 and 2) using a Beckman DU50 spectrophotometer or by the spectrophotometer monitoring the chromatographic separations; integration units from the HPLC spectrophotometer were converted to protein weight by comparing integration units from known amounts of bovine serum albumin chromatographed under the same conditions.
§ Starting material was 10 liters of crude supernatant.
‖ Not done.

Because we were unable to quantitate protein in the final pure preparation, exact specific activity of the purified HMW-BCGF could not be determined. This final pool was radiolabeled using the Bolton-Hunter reagent because methods that iodinated the tyrosine (5) in the molecule destroyed functional activity (data not shown). The radiolabeled, functionally active fraction from the TSK3000 column was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. Fig. 6 demonstrates that radiolabeled protein showed only a single band at 60,000 mol wt. Thus, by this criterion, the HMW-BCGF was biochemically as well as functionally pure.

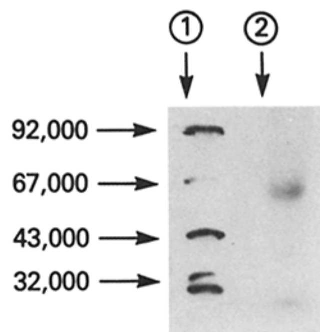

FIGURE 6. A small amount of purified HMW-BCGF was iodinated using the Bolton-Hunter reagent. The iodinated material was electrophoresed in a 10% SDS-polyacrylamide gel and then evaluated by autoradiography. (*1*) Protein standards; (*2*) iodinated HMW-BCGF.

Selective Binding of Purified HMW-BCGF to Activated B Cells. We investigated whether the purified protein bound to B cells, which is a requirement for a true BCGF (20, 32, 33). Iodinated HMW-BCGF was incubated with both resting and activated B and T cells, and cell-associated radioactivity was assessed. Nonspecific binding of iodinated HMW-BCGF was assessed by simultaneous incubation of cells with radiolabeled HMW-BCGF and a 20-fold excess of unlabeled purified protein (Fig. 7*A*). B cells were activated with SAC, and T cells were activated with PHA for 48 h before the binding assay. Neither resting B or T cells nor activated T cells showed any specific binding of the ^{125}I-HMW-BCGF, while activated B cells bound the labeled factor. Binding of ^{125}I-HMW-BCGF to activated B cells was inhibited in a dose-dependent manner by increasing amounts of unlabeled HMW-BCGF (Fig. 7*A*). Moreover, IL-2 purified from the Jurkat line (kindly provided by the Food and Drug Administration, Bethesda, MD) failed to inhibit the binding of ^{125}I-HMW-BCGF to activated B cells (Fig. 7*B*). This supports the notion that the association between ^{125}I-HMW-BCGF and activated B cells involved specific binding of factor to a cell surface receptor. Furthermore, these data demonstrate that the radiolabeled protein we had purified indeed bound to activated B cells quite specifically, as required of a BCGF.

Monoclonal Antibody to HMW-BCGF. The data suggested that the 60,000 mol wt protein we had purified contained the HMW-BCGF biologic activity. Our next goal was to develop a monoclonal antibody to the factor in order to allow rapid purification of additional HMW-BCGF, rapid screening of supernatants for the presence of HMW-BCGF, and studies of the chemistry and biochemistry of this and other B cell regulatory lymphokines. Female BALB/c mice were immunized using material obtained from purifications performed as described above. The first two immunizations (2 wk apart) were subcutaneous and used HMW-BCGF in complete Freund's adjuvant. The third immunization was performed 2 wk after the second and was intravenous and intraperitoneal. Hybridomas obtained from fusion of the spleens of these mice with NS-1 were screened for their ability to inhibit HMW-BCGF-induced proliferation of anti-μ–activated B cells. Of several screened hybridomas, BCGF/1/C2 was selected because of its

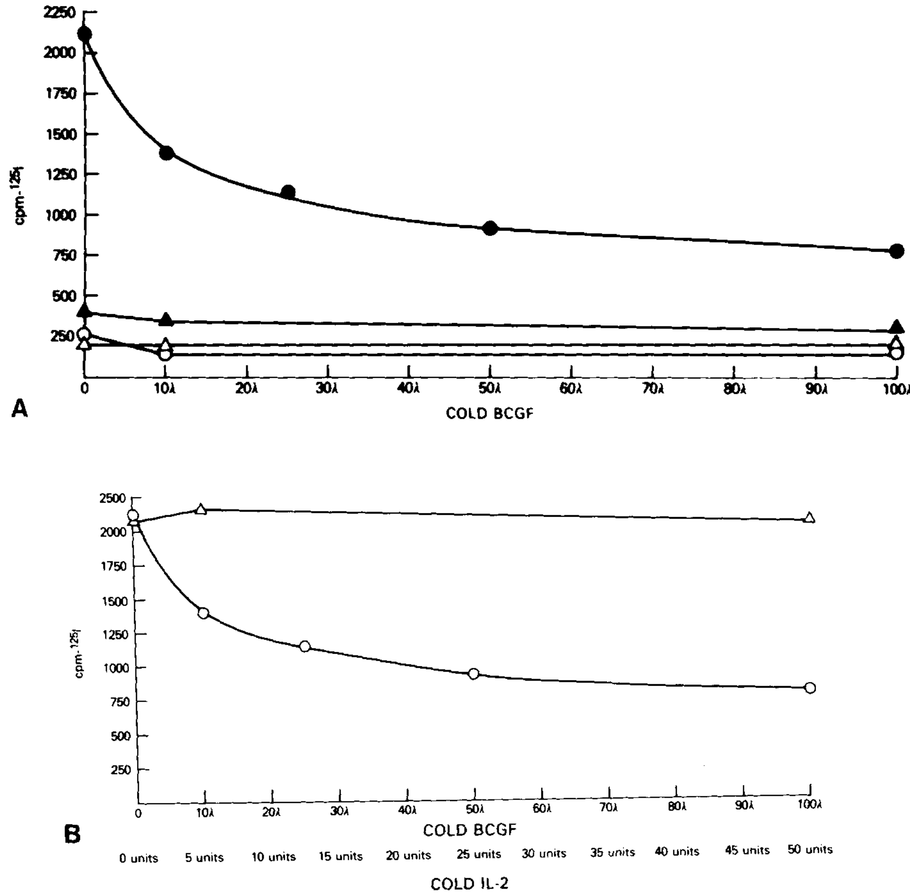

FIGURE 7. (*A*) ^{125}I-HMW-BCGF was added at 50,000 cpm to tubes containing 10^5 unactivated B cells, SAC-activated B cells, unactivated T cells, or PHA-activated T cells in RPMI 1640. Unlabeled HMW-BCGF was added to different tubes at different concentrations and each tube shaken briefly. Tubes were allowed to sit at 4°C for 8 h after which they were washed three times with PBS. After washing, cell-associated radioactivity was quantitated. (●) Activated B cells, (▲) unactivated B cells, (○) activated T cells, (△) unactivated T cells. (*B*) Same experiment as *A* except only cell-associated radioactivity on activated B cells is shown. Inhibition of binding of ^{125}I-HMW-BCGF is shown by cold HMW-BCGF (○ from *A*) and by cold IL-2 (△).

very potent inhibitory activity and was expanded in mouse ascites. The IgG from this ascites was purified and assayed for inhibitory activity (Table III). The BCGF/1/C2 antibody inhibited the HMW-BCGF- but not the LMW-BCGF-induced B cell proliferation in a dose-dependent manner, while a control mouse ascites with a similar concentration of immunoglobulin had no effect. Thus, BCGF/1/C2 antibody was functionally specific for HMW-BCGF. Table III also demonstrates that the BCGF/1/C2 antibody used at a concentration maximally suppressive for B cell proliferation induced by HMW-BCGF, had no effect on either PHA-stimulated T cell proliferation or IL-2-induced proliferation of the IL-2-dependent T cell line, HT-2. To determine actual binding of BCGF/1/C2 antibody to HMW-BCGF, we performed Western blot analysis of HMW-BCGF,

TABLE III
Ability of IgG Antibody Produced by BCGF/1/C2 to Inhibit HMW-BCGF Activity

Cells	Factor added*		Antibody added*‡	[^{3}H]Thymidine incorporation
Anti-μ–stimulated B cells	None		—	1,905 ± 343
	HMW-BCGF§	25%	—	32,211 ± 1,588
		12.5	—	28,185 ± 1,146
		5	—	8,324 ± 1,366
		2.5	—	3,607 ± 1,350
		0.5	—	1,530 ± 90
		25	BCGF/1/C2 0.15 μg*	5,718 ± 3,593
		25	0.10	9,116 ± 3,872
		25	0.05	13,691 ± 2,365
		25	0.02	16,192 ± 2,510
		25	0.01	17,500 ± 1,202
		25	0.002	18,448 ± 7,895
		25	Control ascites 25% vol/vol	30,259 ± 585
	LMW-BCGF‖	25%	—	23,579 ± 1,732
		5	—	16,220 ± 802
		25	BCGF/1/C2 0.10 μg	17,765 ± 2,740
		25	0.02	18,880 ± 1,753
T lymphocyte¶	—		—	1,887 ± 166
	PHA (1 μg/ml)		—	35,262 ± 1,303
	PHA (1 μg/ml)		BCGF/1/C2 0.1 μg	31,188 ± 100
HT-2**	—		—	1,936 ± 230
	2 U IL-2		—	29,335 ± 1,909
	2 U IL-2		BCGF/1/C2 0.1 μg	23,939 ± 2,733

* Factor and antibody were added as indicated at time zero to human peripheral B cells stimulated with 15 μg/ml anti-μ as described in Materials and Methods. Cells were pulsed with 1 μCi/well of [^{3}H]thymidine for the last 18 h of a 72-h culture and then harvested.

‡ The antibody was at 2.5 mg/ml.

§ Namalva was used as the source of HMW-BCGF here although similar results were obtained with T-ALL (F).

‖ The T-T hybridoma 3B3 is the source of LMW-BCGF.

¶ T lymphocytes were purified from peripheral blood mononuclear cells by rosetting once with 2-aminoethylisothiuronium. PHA and/or antibody was added on day 0 as indicated to 96-well Costar plates (100,000 cells per well) with [^{3}H]thymidine added for the last 18 h of a 96-h culture period.

** HT-2 cells were washed three times and then put in culture with IL-2 (from the MLA-144 gibbon line) and/or antibody, as indicated, in 96-well Costar plates at 5,000 cells per well. Cells were pulsed with 1 μCi/well for the last 4 h of an 18-h culture period and then harvested for evaluation of [^{3}H]thymidine incorporation.

LMW-BCGF (3B3), IL-2, and PHA, using BCGF/1/C2 IgG. Fig. 8 demonstrates that BCGF/1/C2 IgG bound to the HMW-BCGF produced by Namalva and T-ALL but failed to bind to LMW-BCGF produced by 3B3 or to purified IL-2 produced by the Jurkat line. BCGF/1/C2 showed no binding to PHA by a similar analysis (data not shown). BCGF/1/C2, therefore, demonstrated antigenic as well as functional specificity for HMW-BCGF, and we concluded that BCGF/1/C2 was a monoclonal antibody which blocked the cell-binding site of HMW-BCGF.

Having demonstrated specificity of BCGF/1/C2 for HMW-BCGF, we next investigated whether BCGF/1/C2 could absorb HMW-BCGF from supernatants

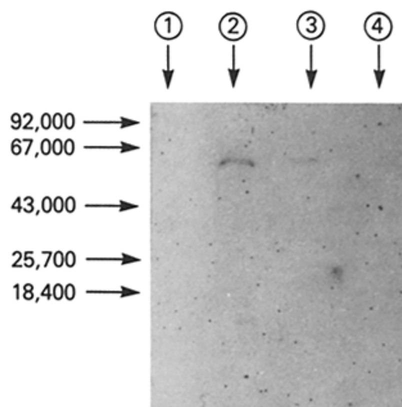

FIGURE 8. Western blotting using BCGF/1/C2 antibody. (*1*) LMW-BCGF (3B3), (*2*) HMW-BCGF (Namalva), (*3*) HMW-BCGF (T-ALL), (*4*) IL-2 (purified from Jurkat line).

TABLE IV
Absorption of BCGF Activity from Culture Supernatant by Sepharose-coupled BCGF/1/C2 Antibody

Supernatant	Concentration (vol/vol)	[^{3}H]Thymidine incorporation
	%	
None		1,274 ± 214
T-ALL crude supernatant	25	10,833 ± 1,269
PBS washed BCGF/1/C2 Sepharose*	25	2,496 ± 54
Glycine washed BCGF/1/C2 Sepharose‡	25	9,620 ± 815
Control Sepharose PBS wash§	25	8,138 ± 224
Control Sepharose glycine wash	25	1,530 ± 199

* Effluent from BCGF/1/C2 Sepharose column washed with PBS and then concentrated to original sample volume.
‡ Effluent from BCGF/1/C2 Sepharose column washed with 0.2 M glycine (pH 3.0), dialyzed against PBS, and then concentrated to original sample volume.
§ Control Sepharose contained rabbit antibody to media proteins; protocol was as for BCGF/1/C2 Sepharose column.

containing large amounts of other proteins. Again, this was examined both functionally and chemically. Table IV demonstrates that a column containing BCGF/1/C2 coupled to Sepharose bound the HMW-BCGF activity specifically. After loading a supernatant containing HMW-BCGF on the column, washing with PBS eluted minimal if any BCGF activity. However, washing with 0.2 M glycine, pH 3.0, eluted most of the original BCGF activity from the supernatant. In contrast, a column containing antibody directed towards media proteins of HB102 coupled to Sepharose did not specifically bind the HMW-BCGF in the supernatant. The majority of BCGF activity was eluted with PBS in this case, rather than with glycine. To test the specificity of the absorption by BCGF/1/C2 Sepharose, we internally labeled proteins produced by T-ALL using [^{3}H]-amino acids in amino acid–deficient media at the time that the cell line was stimulated to produce HMW-BCGF with PHA. The supernatant was then concentrated and entered onto the column containing BCGF/1/C2 IgG coupled

to Sepharose. The material eluted by the 2 M glycine, pH 3.0 wash migrated to the region of 60,000 mol wt by SDS-PAGE, with a retardation factor (R_f) identical to the purified HMW-BCGF (data not shown). The PBS wash from this column contained many other internally labeled proteins while the glycine wash from the column with anti-media immunoglobulin coupled to Sepharose did not contain this internally labeled, 60,000 mol wt protein. Thus, the 60,000 mol wt, internally labeled protein that eluted with glycine from the BCGF/1/C2 antibody column was HMW-BCGF. Furthermore, the BCGF/1/C2 antibody did not bind any other internally labeled proteins produced by T-ALL.

Discussion

In a previous study (9), we demonstrated the production of HMW-BCGF activity by certain human B and T cell lines. In the present study, we have purified this active material to biochemical homogeneity, using immunoabsorption, HPLC, and preparative isoelectric focusing. Despite the low overall yield of this procedure, sufficient HMW-BCGF was generated to immunize mice and produce a monoclonal antibody. The iodinated product of the purification scheme produced a single band by SDS-PAGE and demonstrated specific binding to activated B cells that could be inhibited by unlabeled HMW-BCGF but not by purified IL-2. One of the monoclonal antibodies developed by immunizing mice with the purified HMW-BCGF was demonstrated to inhibit the proliferation of activated B cells stimulated with HMW-BCGF, but had no affect on PHA-induced proliferation of normal T cells nor on IL-2-induced proliferation of the IL-2-dependent T cell line, HT-2. The monoclonal antibody was demonstrated by Western blotting to bind to BCGF from two different sources but not to LMW-BCGF, IL-2, or PHA. Finally, when coupled to Sepharose, the monoclonal antibody specifically absorbed all BCGF activity and recognized only a single protein in T-ALL supernatants that comigrated with purified HMW-BCGF by SDS-PAGE.

The purification of various lymphokines by different investigators has revealed that certain biochemical procedures were associated with poor functional lymphokine recovery. Smith et al. (13) and Yoshizaka et al. (34) noted major losses of functional lymphokine with isoelectric focusing in the purification of IL-2 and B cell differentiation factor (BCDF), respectively. Henderson et al. (35) noted moderate loss of IL-2 activity in a one-step purification of IL-2 using reverse phase HPLC. We found that both isoelectric focusing and reverse phase chromatography were essential to separate HMW-BCGF from other proteins in the culture supernatant. This explains why we obtained an overall functional yield of only 0.3%. Nevertheless, we generated sufficient HMW-BCGF to use for immunizing mice to make monoclonal antibodies and to label for investigation of the specific binding of HMW-BCGF to lymphocytes. This represents a considerable improvement over other isolation procedures, since previously published techniques were able to obtain only enough protein to perform selected functional studies with the final product (36–38).

The end result of the purification was a 60,000 mol wt protein that, when iodinated, produced a single band by SDS-PAGE. This protein bound specifically to activated but not resting B cells, suggesting that a receptor for HMW-BCGF

is present only on activated B cells. This supports the model of B cell function in which resting B cells must be activated by antigen or a surface crosslinking signal before they can respond to BCGF (20, 32, 33). Furthermore, the lack of binding of our labeled HMW-BCGF to resting or activated T cells parallels previous studies (39, 40) demonstrating that anti-μ–activated B cells but not T cells absorb BCGF activity from culture supernatants. The existence of purified HMW-BCGF in sufficient quantities to perform binding studies has allowed us to put these previous phenomenologic observations on a quantitative, biochemical basis. Further studies evaluating the nature of the receptor(s) for HMW-BCGF and its expression on subpopulations of B cells are currently in progress.

The use of monoclonal antibodies to study B cell function in the past (41) has generally involved evaluation of surface antigens expressed by different subpopulations of B cells. Recently, Jung and Fu (42) described a monoclonal antibody that blocks growth factor–dependent B cell proliferation. This antibody may be directed towards a growth factor receptor, since it was produced by immunizing mice with pokeweek mitogen–stimulated lymphocytes. One other antibody that recognizes a BCGF has been reported (22); however, the one described here is the first reported monoclonal antibody to a human BCGF. Antibodies to HMW-BCGF can be used to help clarify the physiologic roles of HMW-BCGF as well as to aid in identification and quantitation of HMW-BCGF in vivo and in vitro.

Overall, the present studies have several important implications. They provide definitive evidence that HMW-BCGF is a lymphokine distinct from LMW-BCGF or IL-2, both antigenically and functionally. We have previously shown (9) that, by biochemical and functional criteria, HMW-BCGF can be distinguished from IL-1, IL-2, IFN, and LMW-BCGF. Nevertheless, variability in the size of many lymphokines (43–45) makes distinction based on size alone difficult, especially when their activities can be similar or overlapping. Glycosylation, which affects Stokes radius and migration in SDS gels, can affect the activity of a lymphokine as well. IgE-binding factors that have identical protein backbones can have suppressive or enhancing activity for the production of IgE antibody depending upon their degree of glycosylation (46). Thus, purification of the various growth factors and direct comparison of their binding to cells is required for definitive distinction among them. Using purified HMW-BCGF and IL-2, we have demonstrated that IL-2 does not inhibit the binding of HMW-BCGF to activated B cells. Furthermore, BCGF/1/C2 blocks the activity of HMW-BCGF but does not block either the activity of LMW-BCGF or the ability of IL-2 to enhance the proliferation of an IL-2-dependent T cell line. Finally, BCGF/1/C2 binds to HMW-BCGF in Western blots, but fails to bind to LMW-BCGF or IL-2. Thus, these studies have clearly demonstrated that HMW-BCGF is a unique growth factor, distinct from IL-2 and LMW-BCGF. HMW-BCGF is in fact a significant product of both T-ALL and Namalva, as it can be internally labeled by the addition of [^{3}H]amino acids to both cell lines grown in amino acid–deficient media. Moreover, HMW-BCGF produced by the two cell lines is apparently identical since the product of both lines is recognized by a single monoclonal antibody, and the two BCGFs have identical molecular weights. The monoclonal antibody data suggest also that the very high molecular weight BCGF (150,000–200,000) found on the TSK G3000 SW column is antigenically related to the

HMW-BCGF that we have purified, since BCGF/1/C2 absorbs all BCGF activity from crude T-ALL supernatants. Since only the 60,000 mol wt species was seen on SDS-PAGE of the BCGF/1/C2-adsorbed material from T-ALL, it is likely that the HMW species seen on gel permeation chromatography represents a complex of the species that we have purified, formed either by self-aggregation or by binding to other proteins in the supernatant.

Finally, purification of HMW-BCGF will allow amino acid sequencing and subsequent cloning of the gene responsible for the production of HMW-BCGF. The monoclonal antibody BCGF/1/C2 will help in rapid purification of HMW-BCGF, rapid screening of HMW-BCGF-producing cells, and further exploration of the precise role of HMW-BCGF in the regulation of human B cell function.

Summary

High molecular weight B cell growth factor (HMW-BCGF) produced by a T cell line was purified to homogeneity and demonstrated to bind specifically to activated human B cells. A monoclonal antibody to HMW-BCGF was developed that (*a*) specifically inhibited the activity of HMW-BCGF in enhancing B cell proliferation, (*b*) specifically bound to HMW-BCGF in Western blots, (*c*) specifically absorbed HMW-BCGF activity from culture supernatants, and (*d*) specifically absorbed an internally labeled protein from T-ALL supernatant which comigrates with HMW-BCGF on sodium dodecyl sulfate–polyacrylamide gels. This antibody should help in cloning the gene for HMW-BCGF and further exploring the physiologic roles of HMW-BCGF.

We thank Ms. Norma Witzel, Mr. Howard S. Mostowski, and Dr. Shoken Tomita for their expert assistance, and Ms. Ann C. London for editorial assistance.

Received for publication 6 May 1985 and in revised form 25 June 1985.

References

1. Falkoff, R. J. M., A. Muraguchi, J.-X. Hong, J. L. Butler, C. A. Dinarello, and A. S. Fauci. 1983. The effects of interleukin 1 on human B cell activation and proliferation. *J. Immunol.* 131:801.
2. Marrack, P., S. D. Graham, E. Kushmir, J. H. Leibson, N. Roehm, and J. W. Kappler. 1982. Nonspecific factors in B cell responses. *Immunol. Rev.* 63:33.
3. Muraguchi, A., J. H. Kehrl, D. L. Longo, D. J. Volkman, K. A. Smith, and A. S. Fauci. 1985. Interleukin 2 receptors on human B cells. Implications for the role of interleukin 2 in human B cell function. *J. Exp. Med.* 161:181.
4. Harada, H., K. Shiori-Nakano, M. Mayumi, and T. Kawai. 1983. Distinction of two subtypes of human leukocyte interferon (IFN-alpha) on B cell activation. B cell proliferation by two subtypes of IFN-alpha. *J. Immunol.* 131:238.
5. Howard, M., J. Farrar, M. Hilfiker, B. Johnson, K. Takatsu, T. Homeoka, and W. E. Paul. 1982. Identification of a T cell-derived B cell growth factor distinct from interleukin 2. *J. Exp. Med.* 155:914.
6. Swain, S. L., M. Howard, J. Kappler, P. Marrock, J. Watson, R. Booth, G. D. Wetzel, and R. W. Dutton. 1983. Evidence for two distinct classes of murine B cell growth factors with activities in different functional assays. *J. Exp. Med.* 158:822.
7. Okada, M., N. Sakaguchi, N. Yoshimura, H. Hara, K. Shimizu, N. Yoshida, K. Yoshizaka, S. Kishimoto, Y. Yamamura, and T. Kishimoto. 1983. B cell growth

factors and B cell differentiation factor from human T hybridomas. Two distinct kinds of B cell growth factor and their synergism in B cell proliferation. *J. Exp. Med.* 157:583.

8. Gordon, J., S. C. Ley, M. D. Melamed, L. C. English, and N. C. Hughes-Jones. 1984. Immortalized B lymphocytes produce B cell growth factor. *Nature (Lond.).* 310:145.
9. Ambrus, J. L., Jr., and A. S. Fauci. 1985. Human B lymphoma cell line producing B cell growth factor. *J. Clin. Invest.* 75:732.
10. Butler, J. L., A. Muraguchi, H. C. Lane, and A. S. Fauci. 1983. Development of a human T-T cell hybridoma secreting B cell growth factor. *J. Exp. Med.* 157:60.
11. Schmidt, J. A. 1984. Purification and partial biochemical characterization of normal human interleukin 1. *J. Exp. Med.* 160:772.
12. Auron, P. E., A. C. Webb, L. J. Rosenwasser, S. M. Mucci, A. Rich, S. M. Wolff, and C. A. Dinarello. 1984. Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. *Proc. Natl. Acad. Sci. USA.* 81:7907.
13. Smith, K. A., M. F. Favata, and S. Orozzlon. 1983. Production and characterization of monoclonal antibodies to human interleukin 2: strategy and tactics. *J. Immunol.* 131:1808.
14. Toniguchi, T., H. Matsui, T. Fujita, C. Takaoka, N. Kashima, R. Yoshimoto, and J. Hamura. 1983. Structure and expression of a cloned cDNA for human interleukin 2. *Nature (Lond.).* 302:305.
15. Devos, R., G. Plaetinck, H. Cheroutre, G. Simons, W. Degrave, J. Tavermier, E. Remaut, and W. Fiers. 1983. Molecular cloning of human interleukin 2 cDNA and its expression in *E. coli. Nucleic Acids Res.* 11:4307.
16. Goeddel, D. V., D. W. Leung, T. J. Dull, M. Gross, R. W. Lawn, R. McCandliss, P. H. Seeburn, A. Ullrich, E. Yelverton, and P. W. Gray. 1981. The structure of eight distinct cloned human leukocyte interferon cDNA's. *Nature (Lond.).* 290:20.
17. Derynck, R., J. Cantent, E. DeClency, G. Volckoert, J. Tavermier, R. Devos, and W. Fiers. 1980. Isolation and structure of a human fibroblast interferon gene. *Nature (Lond.).* 285:542.
18. Grey, P. W., and D. V. Goeddel. 1982. Structure of the human immune interferon (IFN-gamma) gene. *Nature (Lond.).* 298:859.
19. Muraguchi, A., T. Kasahara, J. J. Oppenheim, and A. S. Fauci. 1982. B cell growth factor and T cell growth factor produced by mitogen-stimulated normal human peripheral blood T lymphocytes are distinct molecules. *J. Immunol.* 129:2486.
20. Muraguchi, A., J. L. Butler, J. H. Kehrl, and A. S. Fauci. 1983. Differential sensitivity of human B cell subsets to activation signals delivered by a monoclonal B cell growth factor. *J. Exp. Med.* 157:530.
21. Muraguchi, A., and A. S. Fauci. 1982. Proliferative responses of normal human B lymphocytes. Development of an assay system for human B cell growth factor. *J. Immunol.* 129:1104.
22. O'Hara, J., and W. E. Paul. 1985. Production of a monoclonal antibody to and molecular characterization of B cell stimulatory factor I. *Nature (Lond.).* 315:333.
23. Steinbuck, M., and R. Audron. 1969. The isolation of IgG from mammalian sera with the aid of caprylic acid. *Arch. Biochem. Biophys.* 134:279.
24. Falkoff, R. M., M. Peters, and A. S. Fauci. 1982. T cell enrichment and depletion of human peripheral blood mononuclear cell preparations. Unexpected findings in the functional activities of the separated populations. *J. Immunol. Methods.* 50:39.
25. Kappler, J., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J. Exp. Med.* 153:1198.

26. Langone, J. J. 1980. Radioiodination by use of the Bolton-Hunter and related reagents. *Methods Enzymol.* 70:221.
27. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.).* 227:680.
28. Oi, V. T., and L. A. Herzenberg. 1980. Immunoglobulin-producing hybrid cell lines. *In* Selected Methods in Cellular Immunology. B. B. Mishell and S. M. Shiigi, editors. W. H. Freeman and Co., San Francisco. 351–367.
29. Taubin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350.
30. Oi, V. T., and L. A. Herzenberg. 1980. Immunoglobulin-producing hybrid cell lines. *In* Selected Methods in Cellular Immunology. B. B. Mishell and S. M. Shiigi, editors. W. H. Freeman and Co., San Francisco. 367–368.
31. Tan-Wilson, A. L., M. Reichlin, and R. W. Noble. 1976. Isolation and characterization of low and high affinity goat antibodies directed to single antigenic sites on human hemoglobin. *Immunochemistry.* 13:921.
32. Yoshizaki, Y., T. Nakagawa, T. Kaieda, A. Muraguchi, Y. Yamamura, and T. Kishimoto. 1982. Induction of proliferation and Ig production in human B leukemic cells by anti-immunoglobulins and T cell factors. *J. Immunol.* 183:1296.
33. Dutton, R. W. 1975. Separate signals for the initiation and differentiation in the B cell response to antigen. *Transplant Rev.* 23:66.
34. Yoshizaki, K., T. Nakagawa, K. Fukunaga, L. T. Tseng, Y. Yamamura, and T. Kishimoto. 1984. Isolation and characterization of B cell differentiation factor (BCDF) secreted from a human B lymphoblastoid cell line. *J. Immunol.* 132:2948.
35. Henderson, L. E., J. F. Hewetson, R. F. Hopkins III, R. C. Sowder, R. H. Neubauer, and H. Rabin. 1983. A rapid, large scale purification procedure for gibbon interleukin-2. *J. Immunol.* 131:810.
36. Thoman, M. L., and W. O.Weigle. 1983. Identification and partial purification of a B cell growth-promoting material utilizing the BCL_1 cell line. *J. Immunol.* 130:233.
37. Farrar, J. J., M. Howard, J. Fuller-Farar, and W. E. Paul. 1983. Biochemical and physiochemical characterization of mouse B cell growth factor: a lymphokine distinct from IL-2. *J. Immunol.* 131:1838.
38. Dutton, R. W., G. D. Wetzel, and S. L. Swain. 1984. Partial purification and characterization of a BCGF II from EL-4 culture supernatants. *J. Immunol.* 132:2451.
39. Butler, J. L., J. L. Ambrus, Jr., and A. S. Fauci. 1984. Characterization of monoclonal B cell growth factor (BCGF) produced by a human T-T hybridoma. *J. Immunol.* 133:251.
40. Butler, J. L., R. J. M. Falkoff, and A. S. Fauci. 1983. Development of a human T cell hybridoma secreting separate B cell growth and differentiation factors. *Proc. Natl. Acad. Sci. USA.* 81:2475.
41. G. Möller, editor. 1982. B Cell Differentiation Antigens. *Immunol. Rev.* Vol. 69.
42. Jung, L. K. L., and S. M. Fu. 1984. Selective inhibition of growth factor-dependent human B cell proliferation by monoclonal antibody AB1 to an antigen expressed by activated B cells. *J. Exp. Med.* 160:1919.
43. Farrar, J. J., W. R. Benjamin, M. L. Hilfiker, M. Howard, W. L. Farrar, and J. Fuller-Farrar. 1982. The biochemistry, biology, and role of interleukin 2 in the induction of cytotoxic T cell and antibody-forming B cell responses. *Immunol. Rev.* 63:129.
44. Lackman, L. L., M. B. Hacker, G. T. Blyden, and R. E. Handschumacker. 1977. Preparation of lymphocyte-activating factor from continuous murine macrophage cell lines. *Cell Immunol.* 34:416.

45. Stewart, W. E., II. 1979. The Interferon System. Springer-Verlag Inc., New York, New York. 134–183.
46. Ishizaka, K., and T. Ishizaka. 1983. Immunology of IgE-mediated hypersensitivity. *In* Allergy. Principles and Practice. E. Middleton, Jr., C. E. Reed, and E. F. Ellis, editors, C. V. Mosby Co., St. Louis. 43–73.