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Immunology

Evidence for an intracellular precursor for human B-cell growth factor

(lymphokine/cell proliferation/*in vitro* culture)

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ABSTRACT **Human B-cell growth factor has been described as a trypsin-sensitive protein of M_r 12,000–14,000. Evidence is provided herein that this relatively low molecular weight product may be released from a larger precursor molecule of M_r 60,000–80,000. The precursor protein is confined to the cytosol of freshly isolated T lymphocytes, and only the M_r 12,000–14,000 moiety is released upon lectin stimulation. The precursor protein was subjected to limited tryptic digestion, which demonstrated that the biologically active fraction of the moiety resided in a relatively low molecular weight fragment. The T lymphocyte routinely possessed an intracytoplasmic pool of the precursor protein, the amount of which cyclically varied depending upon its depletion by the secretion process of a lower molecular weight product. Analysis of the mRNA size coding for the majority of B-cell growth factor activity, determined by translation in *Xenopus leavis* oocytes, suggested that the B-cell growth factor-specific mRNA resided in the ≥15S range. This value is consistent with the size of the larger precursor. Therefore, it is proposed that a precursor–product relationship exists for the processing of human B-cell growth factor, analogous to that which has been described for several other cytokines.**

Human B-cell growth factor (BCGF) has been shown to be released by T lymphocytes after either lectin or antigen stimulation (1). This growth factor promotes S-phase entry in human B lymphocytes activated by antigen, lectin, or anti-immunoglobulin stimulation (2–5). The BCGF moiety predominantly released by T lymphocytes in the presence of lectin and an obligate number of monocytes is a relatively heat-labile, trypsin-sensitive protein of M_r 12,000–14,000 (3, 6). The size of the released growth factor is consistent with numerous other biologically active cytokines, such as interleukin 1 of M_r 12,000, interleukin 2 of M_r 15,500, platelet-derived growth factor I of M_r 35,000, platelet-derived growth factor II of M_r 32,000, epidermal growth factor of M_r 6,000, and β nerve growth factor monomeric chains of M_r 13,500 (7–11). It recently has been documented that the cellular processing of several of these growth-regulating, secretory proteins involves the proteolytic cleavage of larger precursor compounds (11–14). In the cases of nerve growth factor and epidermal growth factor, this cleavage reaction occurs at both the amino-terminal and carboxyl-terminal ends of their respective precursor molecules. It has been suggested that the proteolytic cleavage reaction is, at least in part, due to the action of arginine-specific peptidases.

Several lines of evidence are now available in the literature indicating the possibility of precursor proteins for lymphocyte growth regulatory proteins. Under conditions in which human peripheral blood T lymphocytes were stimulated with the phorbol ester phorbol 12-myristate 13-acetate, in addition to the lectin phytohemagglutinin, it has been reported that the cells will release, in addition to the low molecular weight form of BCGF, a larger form of the growth factor whose size is estimated at M_r 50,000 (4). Furthermore, murine T-T hybridomas have been isolated that apparently release several molecular weight forms of BCGF, with the two major forms being at M_rs 80,000–90,000 and 15,000–20,000 (15). Therefore, the present study was directed at determining whether precursor forms of human BCGF may be isolated and how these forms are related to the relatively low molecular weight BCGF protein that is the primary one released by lectin-activated T cells. These experiments have included an examination of the cytosolic compartment of T lymphocytes both for localization of the putative precursor and for the isolation of mRNA specific for the growth factor.

MATERIALS AND METHODS

Preparation of Lymphocyte-Conditioned Medium. Human peripheral blood lymphocytes (PBL) (60–65% T cells, 10–15% monocytes, 10–15% B lymphocytes) were isolated as described (3). The PBL from multiple donors were pooled and cultured at 1×10^6 per ml in RPMI 1640 medium supplemented with 2 mM glutamine, 0.25% bovine serum albumin, and 0.75% phytohemagglutinin M (GIBCO). At the times indicated in the text, the cells were recovered by centrifugation, and the clarified supernatants were used as a source of extracellular cytokines, which included BCGF. Each preparation of conditioned medium was processed through ion-exchange chromatography on DEAE-Sepharose (3) prior to testing in a biological assay for BCGF.

Preparation of Cytoplasmic Extracts. The cell pellet obtained from cultured lymphocytes (either peripheral blood mononuclear cells, long-term-cultured T cells, long-term-cultured B cells, T cells from the CCRF-CEM cell line) was suspended in hypotonic buffer (16) at pH 7.5 containing 20.0 mM Hepes, 1.4 mM $Mg(OAc)_2$, 3.6 mM $CaCl_2$, and 2 mM 2-mercaptoethanol (10 ml of buffer per 10^9 cells). Cells were lysed with 40 strokes of a glass homogenizer, the lysate was centrifuged at 3000 rpm for 10 min to remove nuclei, and the resultant supernatant was centrifuged at 10,000 *g* for 10 min to remove mitochondria. The post-mitochondrial supernatant was centrifuged at 100,000 × *g* in a Ti 60 rotor for 90 min to remove cellular membranes and polysomal material and to obtain the soluble cytosolic proteins in the supernatant.

Isolation of RNA. RNA to be used for *in vitro* translation was isolated by two independent protocols. In the first, a polysomal pellet was prepared from activated lymphocytes and dissolved in 100 mM Tris·HCl, pH 9.0/100 mM NaCl/1

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Abbreviations: BCGF, human B-cell growth factor; PBL, peripheral blood lymphocytes.

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mM EDTA (TES buffer) and extracted twice with equal volumes of TES buffer saturated with phenol. The polysomal RNA from the extracted aqueous layer was precipitated with 2 vol of ethanol. The RNA pellet was washed twice with 70% chilled ethanol and redissolved in TES buffer containing 0.1% $NaDodSO_4$. The RNA was fractionated directly on a 10–28% sucrose gradient. The gradients were centrifuged at 35,000 rpm for 19 hr with an SW 40 rotor, and 40 fractions were subsequently collected. The RNA, recovered from each gradient fraction by precipitation with ethanol, was used for *in vitro* translation. In the second protocol, poly(A)$^+$ RNA was isolated by oligo(DT)-cellulose chromatography as described (17). The poly(A)$^+$ RNA was fractionated on 1.5% low-melting agarose gels in the presence of 10 mM methylmercuric hydroxide (18). The gel was sliced according to size distribution, and mRNA was eluted from the agarose and used for *in vitro* translation.

***In Vitro* Translation of RNA.** Poly(A)$^+$ RNA fractionated on agarose gels or RNA obtained from sucrose gradient fractions was translated *in vitro* in *Xenopus leavis* oocytes (19). Each RNA sample (30 ng) was injected into an oocyte in 30–60 nl. Forty of the injected oocytes, for each RNA sample, were incubated at 25°C for 48 hr in groups of 10 eggs per 100 μl of Barth's medium in flat-bottom microculture plates. At the end of the incubation period, supernatants from each group were collected along with a medium wash of each group of eggs. The pooled samples were dialyzed against RPMI medium and assayed for BCGF activity.

Column Chromatography. Ion-exchange chromatography was performed with DEAE-Sepharose essentially as described (3). Aliquots of either conditioned medium or cytosolic soluble proteins were dialyzed against 10 mM Tris·HCl (pH 8.0) containing 0.2 mM phenylmethylsulfonyl fluoride and 2 mM 2-mercaptoethanol; each aliquot was loaded onto a DEAE column equilibrated in the same buffer. The growth factor activity was removed from the column by elution at 0.12 M NaCl as described (3). For the purpose of further characterization of the growth factor activities, samples, either directly after preparation or after ion-exchange chromatography, were loaded onto molecular-sieving columns (Bio-Gel P-30 or Sephacryl S-200) as described (3). The gel filtration columns were 95 × 2.5 cm in size, and the elution buffer contained 10 mM sodium phosphate (pH 7.0), 0.2 mM PMSF, 2 mM 2-mercaptoethanol, and 0.15 M NaCl.

Biological Assays. Routine assays for interleukin 2 and BCGF activities were done in a 96-well flat-bottom microtiter plates. Cells from growth factor-dependent long-term cultures of B or T lymphocytes (20) were cultured in a final volume of 200 μl in RPMI 1640 medium supplemented with 2% heat-inactivated fetal calf serum. A total of 1.5–3.0 × 10^4 cells were used per well. The cultures were routinely incubated for a total of 40 hr in a humidified incubator purged with 5% CO_2/95% air. Sixteen hours prior to harvest, the cells were labeled with 1 μCi of [^{3}H]thymidine (6 Ci/mol; 1 Ci = 37 GBq). The growth factor-containing fractions were tested at multiple dilutions.

Trypsin Digestion of Cytosolic Proteins. The cytosolic BCGF prepared by gel-filtration chromatography was used for testing trypsin sensitivity of the protein. The protein samples were digested with 50 μg of trypsin per ml for various times indicated in the text, with trypsin action terminated by the addition of fetal calf serum to the reaction mixture. The digestion samples were tested for BCGF activity at various dilutions in microtiter assays. A standard preparation of BCGF prepared from that released by PBL (3) was used as a positive control.

RESULTS

Identification of Precursor BCGF Molecules. Given the prior observations that high molecular weight precursors are frequently confined to the intracellular environment and are not released, the initial experiments examined whether BCGF activity could be localized in the cytosolic fraction of lectin-stimulated PBL. Postmitochondrial supernatants were isolated from these lymphoid cells, and this material was then assayed for its efficacy in promoting the long-term growth of cultured B lymphocytes. Fig. 1 shows that the cytosolic fraction from PBL contained a soluble intracellular compound with BCGF activity. Given previous experimentation documenting the T-cell-dependent release of BCGF, several cell lines also were examined for the presence of BCGF activity in the cytosolic compartment. Along with the data on total PBL, long-term-cultured, lectin-stimulated normal T cells contained cytoplasmic BCGF activity, whereas long-term-cultured, lectin-stimulated normal B cells did not (Fig. 1). The malignant T-cell line CCRF-CEM-NK served as a second negative control. This particular CEM variant does not normally release BCGF activity with lectin stimulation and did not show the presence of a cytoplasmic factor.

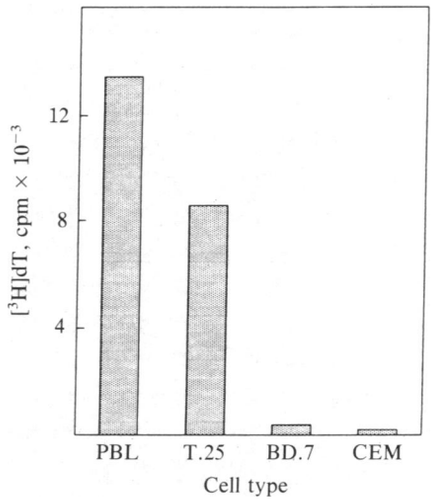

FIG. 1. Relative BCGF activity of soluble cytosolic proteins isolated from a variety of lectin-stimulated human lymphoid cells. The cytoplasmic extracts were prepared from human PBL, a long-term-cultured factor-dependent T-cell line (T.25), a long-term-cultured factor-dependent B-cell line (BD.7), and a human T-cell lymphoma line (CCRF-CEM-NK). The volumes of the cytoplasmic extracts were adjusted to represent 10^6 cells per ml and were assayed for BCGF activity at a final concentration of 10% (vol/vol). Bars represent tritiated thymidine incorporation of a representative microtiter culture.

Biosynthesis of Intracellular BCGF. We examined the kinetics of appearance of the intracellular versus secreted extracellular BCGF activity. Freshly isolated PBL were placed in culture in the presence of lectin (0.75% phytohemagglutinin). At 3-hr intervals for the first 24 hr after the initiation of culture, both an aliquot of conditioned medium and an aliquot of lymphoid cells were isolated. The conditioned medium was processed by DEAE-Sepharose ion-exchange chromatography (3) and tested for BCGF activity. The cytosolic fraction was isolated from the withdrawn cells, and the sample volume was equalized to that of the conditioned medium source and tested for BCGF activity. At time zero, there was no detectable BCGF activity in the conditioned medium; yet, there was an apparent pool of intracellular BCGF activity (Fig. 2). During the first several hours of stimulation, the intracellular compartment decreased precipitously in association with a rapid rise in secreted material. Interestingly, the intracellular pool began to reaccumulate after the initial fall, in association with a continued, but slower than initial, rise in extracellular material.

Characterization of Intracellular BCGF. Previous data had

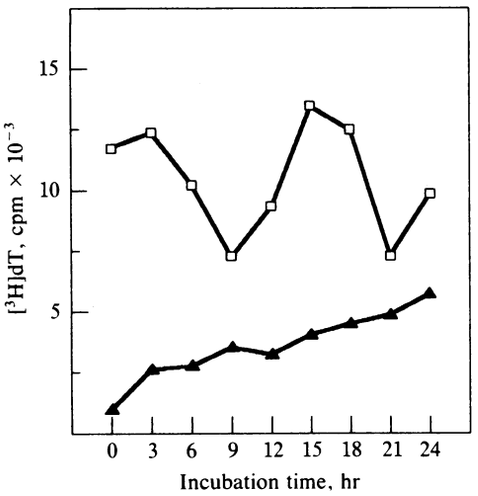

FIG. 2. Kinetics of intracellular and extracellular BCGF activities. Lectin-activated human PBL were cultured as described in the text. Cultures were harvested at the time intervals indicated, and both conditioned medium and cytoplasmic extracts were subsequently partially purified by ion-exchange chromatography. Shown is a representative microtiter culture of partially purified conditioned medium (▲) and partially purified cytoplasmic extract (□) assayed at a final 10% concentration. The results are those from a single experiment with multiple replicates indicating the same finding.

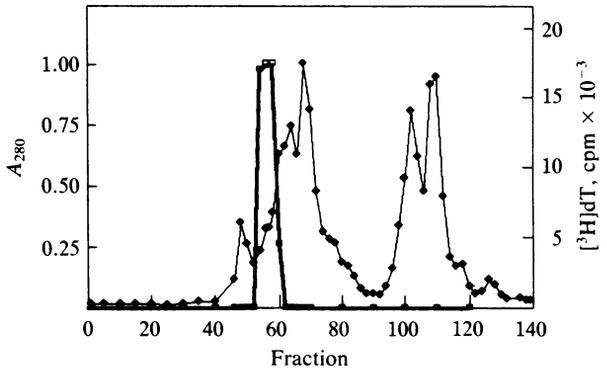

FIG. 4. Sephacryl S-200 chromatogram of cytosolic proteins from human PBL. Fractions (5 ml) were collected from a 95 × 2.5 cm column and bioassayed for BCGF activity. ◆, Absorbance at 280 nm; □, tritiated thymidine incorporation of a representative microtiter culture.

indicated that the BCGF activity released by lectin-activated T cells resided in a M_r 12,000–14,000 fraction when examined in the presence of 0.1% $NaDodSO_4$ on a 15% polyacrylamide gel (3). Therefore, the next series of experiments were directed at determining the size characteristics of the intracellular BCGF activity. Cytoplasmic extracts from several preparations of lectin-activated PBL were pooled, concentrated, and applied to a Bio-Gel P-30 gel filtration column. Previous experiments had revealed that the extracellular form of BCGF was eluted as a relatively broad peak of activity within the included volume on such a column (3). In contrast, the Bio-Gel P-30 chromatogram of the cytoplasmic fractions revealed that the major peak of BCGF activity resided in the leading edge of the void volume, and only a minor peak of activity appeared within the included volume (Fig. 3). The minor peak of activity was eluted in a manner corresponding to that normally seen with the excreted form of the growth factor activity (3). To further examine the high molecular weight cytoplasmic activity, active fractions from the Bio-Gel P-30 chromatographic void volume were further fractionated by application on a Sephacryl S-200 gel filtration column. The Sephacryl S-200 chromatogram (Fig. 4) revealed a relatively sharp peak of activity in the M_r 60,000–80,000 range. It should be emphasized that the intracellular high molecular weight peak of BCGF activity was essentially devoid of either T-cell growth factor activity or interleukin 1 activity. This was determined by the fact that the column fractions containing the high molecular weight BCGF could not support either the growth of cultured T cells or thymocyte mitogenesis.

In the next series of experiments, we examined whether the biological activity of the high molecular weight form of BCGF required the entire intact molecule or whether the activity resided in a smaller fragment. The cytosolic fractions possessing BCGF activity after Sephacryl S-200 chromatography (i.e., M_r 60,000–80,000 fractions) were subjected to trypsin digestion for periods of time ranging from 0 to 120 min. Aliquots of the digest were removed at 30-min time intervals and simultaneously tested for BCGF activity and analyzed for protein content on 15% polyacrylamide/$NaDodSO_4$ gels. The data indicated that a 30- to 60-min trypsin digestion, which removed the high molecular weight protein species (i.e., all stainable bands were reduced to $\leq M_r$ 40,000), did not result in the complete loss of biological activity (Fig. 5). The complete absence of biological activity

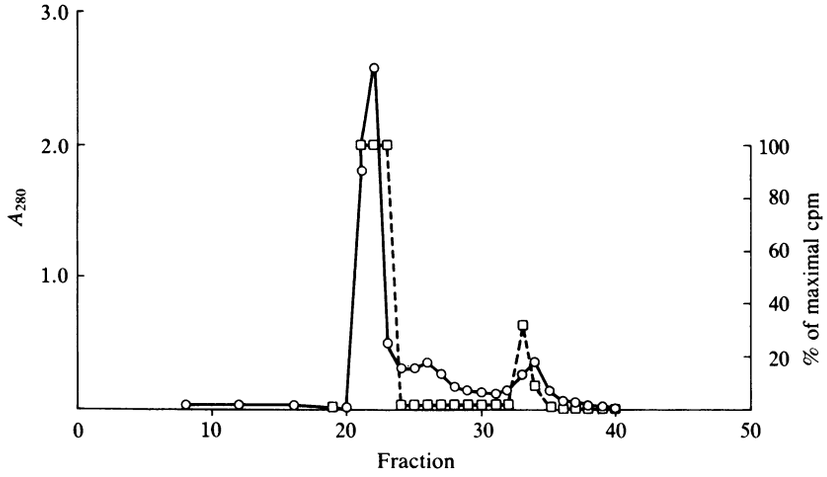

FIG. 3. Bio-Gel P-30 chromatogram of cytosolic proteins from human PBL. Fractions (5 ml) were collected from a 45 × 2.5 cm column and bioassayed for BCGF activity. ○, Absorbance at 280 nm; □, tritiated thymidine incorporation of a representative microtiter culture.

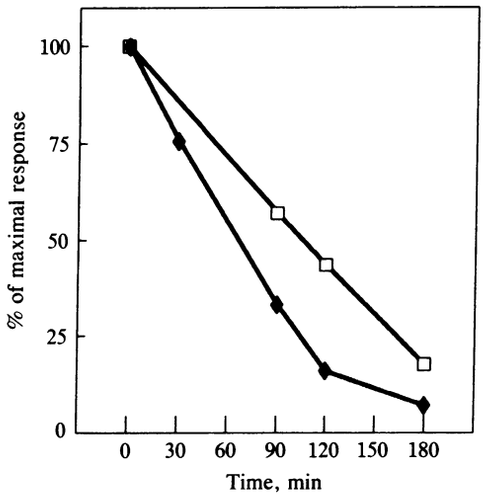

FIG. 5. Trypsin digestion of cytosolic and extracellular BCGF activity. Sephacryl S-200-purified cytosolic BCGF and DEAE ion-exchange-purified extracellular BCGF were exposed to trypsin at 50 μg/ml. Trypsin was inactivated with a final concentration of 50% fetal calf serum at the indicated time intervals, and samples were subsequently bioassayed for BCGF activity. Shown is a representative microtiter culture of trypsin-digested cytosolic BCGF (□) and trypsin-digested extracellular BCGF (◆) assayed at a final 10% concentration.

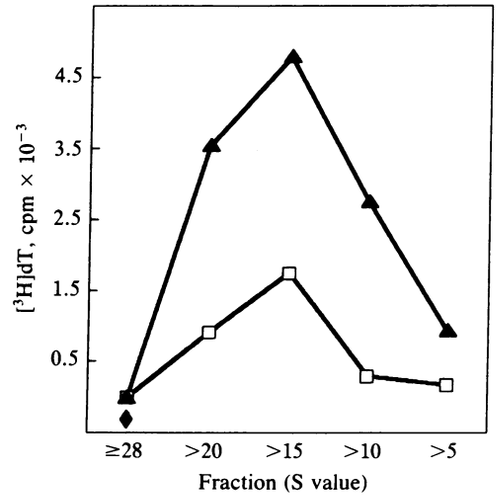

FIG. 6. Relative size determination of mRNA for BCGF. Purified poly(A)$^+$ RNA was applied to a 1.5% low-melting agarose gel; the gel was subsequently sliced, and RNA was eluted from each fraction and injected into *Xenopus leavis* oocytes. Shown is a representative microtiter culture of those supernatants conditioned by the injected oocytes and subsequently assayed for BCGF activity at a final 2.5% (□) and 5% (▲) concentration.

occurred after complete protein digestion, which occurred within 180 min of exposure to trypsin. In another series of experiments examining this question, the cytosolic fractions possessing BCGF activity were again subjected to limited tryptic digestion. The material exposed to the proteolytic enzyme for 60 min was subsequently passed through a Bio-Gel P-30 column, and those fractions corresponding to M_rs ≤ 30,000 were tested for BCGF activity. Under these conditions, the fractions corresponding to relatively low molecular weights possessed BCGF activity, in contradistinction to undigested cytosolic proteins, which did not possess activity in the low molecular weight range chosen.

Isolation of BCGF-Specific mRNA. Given the biological and biochemical data presented above, it appeared essential that a determination be made of the mRNA size responsible for the translation of BCGF activity. If, as the data appeared to suggest, the high molecular weight form of BCGF activity actually represented a precursor to the smaller secreted form, one would expect the mRNA species to be relatively large. Determination of mRNA size was accomplished by two independent protocols. In the first, polysomal RNA was isolated from lectin-stimulated PBL and sedimented through a neutral sucrose gradient, and fractions were isolated by sedimentation value. The fractions were injected into *Xenopus leavis* oocytes; 48 hr later the supernatants were collected and tested for BCGF activity. Assays across the sucrose gradient revealed a peak of BCGF activity occurring in the range ≥ 16S. Confirmation for this result was achieved by using a second method for actual mRNA isolation. Sixteen-hour lectin-stimulated T lymphocytes, cultured in the presence of autologous monocytes (10:1 ratio of T cells to monocytes), were prepared, and total RNA was isolated. Poly(A)$^+$ RNA was fractionated on an oligo(dT)-cellulose column and subsequently subjected to electrophoresis on a 1.5% low-melting agarose gel in the presence of 10 mM methylmercuric hydroxide. The gel was sliced, and mRNA was eluted according to migration distance and injected into *Xenopus* oocytes; the 48-hr supernatant was assayed for BCGF activity. Essentially the same profile of activity appeared from the agarose gels as had been seen with sucrose sedimentation—i.e., the RNA translating for BCGF with the highest efficacy resided in fractions of >15S yet <20S (Fig. 6). This size of the BCGF-specific mRNA appears to be appropriate for encoding a M_r 60,000–80,000 protein.

DISCUSSION

These experiments provide evidence for the existence of a high molecular weight protein that possesses BCGF activity and that appears to represent a precursor for a lower molecular weight molecule that is ultimately secreted from the cell. Biological precedence for this type of processing is readily available both in the literature on growth factors (11–14) and in the literature on hormonal agents (13). Evidence presented here for a precursor–product relationship for human BCGF includes: (*i*) the high molecular weight protein possessing BCGF activity is confined to the cytosolic compartment under the usual conditions of lectin stimulation; (*ii*) biologically active fragments of relatively lower molecular weight may be generated from the higher molecular weight cytosolic material; (*iii*) the intracellular BCGF activity and the secreted form show an inverse relationship during the initial phases of factor secretion; and (*iv*) the size of mRNA translatable as BCGF in a *Xenopus* oocyte translation system is compatible with the size of the high molecular weight cytosolic fraction.

It should be emphasized that, in the present report, BCGF activity was determined solely by its ability to support the long-term growth of cultured B lymphocytes. This is of interest in that preliminary experiments utilizing a BCGF assay based on freshly prepared, peripheral venous blood B lymphocytes has revealed that the cytosolic, high molecular weight factor, in its native form, possesses only minimal activity. This is in contradistinction to the low molecular weight secreted molecule, which possesses activity under both assay conditions. This differential sensitivity to growth-promoting agents in different assay systems is similar to reports that, in the murine system, BCGF activities derived from different cellular sources may be discriminated by their effects in two different assay systems (21). A question that readily becomes apparent is whether the multiple forms of BCGF reported in both murine and human experimental systems may simply reflect the recovery of the putative precursor molecule and/or intermediates from proteolytic cleavage reactions under specialized conditions of cell activation

(e.g., phorbol ester stimulation in addition to lectin activation).

The determination of mRNA size encoding for BCGF activity should be of significance for subsequent experiments on the cloning of BCGF-related genes. Enrichment of the >15S mRNA fractions from isolated T lymphocytes, stimulated with lectin and an obligate number of monocytes, should allow for the establishment of cDNA libraries and the subsequent procurement of the appropriate genomic segments specific for BCGF. The availability of this gene, in addition to purified BCGF, should greatly enhance our understanding of B-cell proliferation and B-cell immunocompetence.

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