We demonstrate that human neoplastic B cells (B cells) contain a cytoplasmic protein of molecular mass 60 Kd that exhibits B-cell growth factor (BCGF) activity on growth factor-dependent long-term human B cells as well as on autochthonous tumor cells. This 60-Kd protein is recognized by antibodies against a similar intracellular 60-Kd protein derived from normal human lymphocytes. These results demonstrate that the two proteins share epitope homology. Microculture bioassays indicate that neoplastic and normal 60-Kd proteins are capable of driving neoplastic B cells through S-phase. Western immunoblot analysis indicates that neoplastic B cells secrete 60- as well as 14-Kd protein. Immunoaffinity-purified proteins secreted by B cells exhibit BCGF activity in anti-IgM or dextran sulfate-preactivated human B cells. In addition, a double-antibody immunofluorescence staining technique was used to demonstrate that B cells express cell surface receptors for BCGF molecule(s). These studies provide support for the autocrine growth model for neoplastic human B cells and suggest that the autocrine growth factor derived from such tumor cells is similar if not identical to normal BCGF molecules.

Isolation and Characterization of Growth Factor(s)
From a Human B-Cell Lymphoma

By Chintaman G. Sahasrabuddhe, Sudhir Sekhsaria, Linda Yoshimura, and Richard J. Ford

THE RELATIONSHIP of neoplastic B cell growth to that of normal B cell counterparts is of considerable interest both biologically and clinically. Two major forms of human B cell growth factor (BCGF), a low-molecular-weight (mol wt) form (12 to 20 Kd), and a high-mol-wt form (50 to 60 Kd), have been purified and characterized to some extent. The low-mol-wt form has been purified from the medium conditioned by lectin-activated human peripheral blood lymphocytes (PBLs). The high-mol-wt form is secreted by T-T hybridomas, neoplastic T lymphocytes, neoplastic B lymphocytes, Epstein-Barr virus (EBV)-transformed B lymphocytes, and human T-lymphotrophic virus (HTLV)-transformed T lymphocytes. Recent studies have also shown that human PBLs secrete 50- to 60-Kd BCGF when cultured with phytohemagglutinin (PHA) and sodium azide. Thus, secretion of 50- to 60-Kd BCGF appears to be associated primarily with neoplastic and virally transformed lymphocytes but can be released from normal human lymphocytes when cell lysis or cell death occurs in culture.

Our studies showed that a high-mol-wt form of human BCGF is localized within the intracellular compartment of human lymphocytes. Several reports have demonstrated that a 50- to 60-Kd protein secreted by neoplastic or virally transformed lymphocytes induces one round of DNA synthesis in B cells in an anti-IgM costimulatory assay. However, we showed that homogeneously purified intracellular BCGF (IC-BCGF), a 60-Kd protein, has consistently lacked this ability. IC-BCGF on the other hand is effective in stimulating growth in factor-dependent cycling B cells as well as in neoplastic B cells derived from hairy cell (chronic B cell) leukemia patients. It is therefore necessary to determine whether high-mol-wt BCGF derived from neoplastic B cells is related to the IC-BCGF from normal PBLs.

We demonstrated that neoplastic B-cell-derived high-mol-wt BCGF is antigenically similar to normal human IC-BCGF. These two proteins appear to share common epitopes recognized by the polyclonal antibodies against normal lymphocyte-derived IC-BCGF. This protein secreted by neoplastic B cells exhibits apparent autocrine BCGF activity and is also present in the cytoplasmic compartment of the tumor cells. Neoplastic B cells also bound and proliferated in response to normal lymphocyte-derived IC-BCGF, suggesting that the neoplastic B cells display functional growth factor receptors.

MATERIALS AND METHODS

Cells and Cell Lines

Fresh human B lymphocytes were isolated by a negative selection procedure from peripheral blood of normal donors as described previously. BCGF-dependent B-cell lines derived from normal B cells were established after initial activation with either dextran sulfate or anti-IgM. These normal B-cell lines were maintained in log-phase growth in RPMI 1640 medium supplemented with 5% (vol/vol) heat-inactivated fetal calf serum (FCS) and highly purified BCGF preparations.

Neoplastic B (lymphoma) cells were obtained from a cell line B, derived in our laboratory from a patient with large-cell (immunoblastic) B-cell lymphoma that was initially established with and dependent on low-mol-wt (12 Kd) BCGF for in vitro cell growth. This line subsequently became independent of exogenously provided growth factor after ~1 month in vitro. This cell line was originally established in RPMI 1640 and 20% FCS, but has been adapted to growth under serum-free conditions in 1% Nutridoma (Boehringer-Mannheim, Indianapolis). These cells, which have been described in detail, show common B-cell markers, including cell surface Ig(Slg) B1 and HLA-DR, and are negative for T cell (T3, T3) and myelomonocytic (MO2, MY4) cell surface antigens. In addition, the cultured tumor cells display a characteristic nonrandom cytogenetic abnormality associated with human lymphoid neoplasms involving a T(8;14) (q24;q32) translocation. Characteristics of the B, cell line are summarized in Table 1.

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**Preparation of Cytosolic Extracts**

Cytosolic extracts were prepared from lectin-activated or nonactivated freshly isolated human peripheral blood lymphocytes (PBLs) and from neoplastic B lymphoma cells as described previously.8

**Polyclonal Antibodies Specific to Normal IC-BCGF**

Polyclonal anti–IC-BCGF antibodies were developed by immunizing a goat with homogeneously purified normal IC-BCGF.14 Anti–IC-BCGF antibodies were affinity purified with immobilized IC-BCGF and crosslinked to Reacti-gel 6x (Pierce, Rockford, IL) to construct an immunoaffinity matrix as described earlier.14 This immunoaffinity matrix was used to purify intracellular and extracellular proteins exhibiting autocrine BCGF activity from neoplastic B cells.

**Cell staining**

Cells were exposed to various BCGF samples for ten minutes at 4°C. The cells were then washed twice with ice-cold RPMI 1640 medium and exposed to purified polyclonal goat anti-IC-BCGF antibodies for ten minutes. Excess unbound anti-IC-BCGF antibodies were removed by washing the cells twice with cold RPMI 1640 medium. These cells were then exposed to fluorescein-conjugated rabbit anti-goat immunoglobulins for ten minutes. The cells were washed thoroughly to remove excess unbound fluorescent rabbit anti-goat Ig and immediately fixed in 1.0% formaldehyde. The fixed cells were photomicrographed or analyzed for fluorescence with flow cytometry, essentially as described by Loken.26 Control cells were treated exactly the same as the experimental cells except that either the exposure to BCGF or to primary goat anti-IC-BCGF antibodies was eliminated.

**Biologic assays.** GF preparations either from neoplastic human B cells or from normal human PBLs were assayed for BCGF activity on cycling GF-dependent human B-cell lines or on the autochthonous tumor cells from which the GF was derived. The B cells maintained in log-phase growth were washed extensively in medium to remove any residual bound GF. The BCGF assays were performed in triplicate in flat-bottom 96-microwell plates (Corning, Corning NY) exactly as described previously.14 Autocrine BCGF activity was assessed by using neoplastic B cells in an exactly similar manner. Anti-IgM or dextran sulfate costimulatory assays were performed as described previously.14 Each data point is the mean of triplicate cpm with <10% SE. The samples were tested at multiple serial dilutions, and units of BCGF activity were determined by probit analysis.14 One unit of BCGF activity, by definition, induced a 50% of the maximal proliferative response under the given culture conditions.

**Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).** The protein samples were analyzed under denaturing conditions on a slab gel (0.075 × 18 × 18 cm) with 12% acrylamide for separating gel and the SDS-Tris-glycine buffer system.27 The gels were prepared in a BioRad gel-casting apparatus, (BioRad, Richmond, CA), and samples were electrophoresed at constant voltage until the marker dye migrated through 15 cm. Protein bands were visualized by a sensitive silver-staining proce-
purified protein derived from neoplastic B-cell cytosol exhibited BCGF activity. The estimated specific activities of BCGF in crude cytosolic extract and immunoaffinity-purified BCGF were of the order of $2.7 \times 10^{5}$ U/mg and $1.2 \times 10^{2}$ U/mg respectively. Electrophoretic analysis of this protein on SDS-polyacrylamide gel under denaturing conditions followed by silver staining showed one major band at 60 Kd (Fig 3, lane 2), which corresponds to the affinity-purified cytosolic protein from normal T lymphocytes (Fig 3, lane 3). We observed that the affinity-purified protein has slightly low activity. The elution of 60-Kd protein from the affinity gel is achieved by lowering the pH to 3.5, which may cause some reduction in biologic activity although the pH is brought back to 7.5 quickly. This has caused some concern in determining the specific activity of the molecule. Our efforts to obtain biologically active material from SDS-gel have not been successful.

**Purification of GF From B-Lymphoma Cells by Anti-IC-BCGF Antibodies**

The next series of experiments was designed to determine if the GF secreted from neoplastic B cells is also recognized by the antibodies against normal IC-BCGF. In these experiments, overnight culture supernatants were concentrated with a YM-5 filter in an Amicon concentrator, and the concentrated sample was loaded onto the immunoaffinity column. The bound and the unbound samples were assayed for BCGF activity in factor-dependent cycling B cells as well as for possible autocrine activity on the neoplastic B lymphocytes. Results shown in Fig 4 demonstrate that the protein having apparent autocrine BCGF activity secreted by neoplastic B lymphocytes also modulates S-phase entry of BCGF-dependent normal cycling B cells.

**Demonstration of GF Binding to B-,Lymphoma Cells**

In most instances, a proliferative signal from a GF is mediated through its binding to receptor on the target cell surface, which also appears to be the case for low-mol-wt M, BCGF on human B cells.23 Because our results demonstrated that neoplastic B cells responded to normal IC-BCGF together with autocrine BCGF, we wished to determine if the GF bound specifically to these cells. A double-antibody immunofluorescence staining technique was used to determine if the antibody could identify putative GF molecules that were actually bound on the cell surface of the B lymphoma cells. Results indicate that the antibodies against IC-BCGF specifically recognized a BCGF-like molecule bound on the cell surface of neoplastic B cells (Fig 5). Photomicrographs clearly demonstrated that washed cells
Factor % (v/v)

Fig 2. B, cells contain an intracellular protein with BCGF activity. Cytosolic extract from $2 \times 10^8$ B, cells was prepared and passed over immunoaffinity matrix containing anti–IC-BCGF antibodies crosslinked to Reacti-gel-6 $\times$. The bound protein was eluted with 0.1 mmol/L Na-citrate (pH 3.5), concentrated, dialyzed against PBS, and assayed for BCGF activity with long-term normal B cells maintained in log-phase; $10^5$ B cells/well were plated in triplicate microcultures in 200 µl RPMI 1640 medium supplemented with 5% FCS or 1% nutridoma. The factor preparations were assayed at serial dilutions, and the cultures were labeled for the last 16 hours with 1 µCi/well [3H]-Tdr. At the end of the 40-hour culture period, cells were harvested and incorporated. [3H]-Tdr was measured in a scintillation counter, and cpm were plotted against percentage (vol/vol) of factor concentration: Commercial BCGF preparation (○), affinity-purified intracellular protein from B, cells (x-x).

Detection of B, GF by Western Blot Analysis

Presence of high-mol-wt protein in the cytoplasmic extract of B, cells was also demonstrated by Western blot analysis. As shown in Fig 6A (lane 1), cytosolic extract from B, cells exhibited a single 60-Kd protein detectable by anti–IC-BCGF antibodies. The culture supernatant, in contrast, exhibited a 60-Kd and a 14-Kd protein, both detectable by anti–IC-BCGF antibodies (Fig 6, lane 2). Figure 6B demonstrates the immuno-Western blot analysis of cytosolic extracts and secreted proteins from the T-ALL (T cells, from acute lymphocytic leukemia patient) cell line that did not exhibit BCGF activity. Lane 1 is positive control using affinity-purified normal IC-BCGF, lane 2 is cytosolic extract from T-ALL cells, and lane 3 is concentrated conditioned medium from T-ALL cells. Our earlier study showed that the affinity-purified high-mol-wt protein from normal lymphocytes does not exhibit BCGF activity in anti-IgM or dextran sulfate costimulatory assay, yet the affinity-purified low-mol-wt protein scores positive in such assays. Therefore, we tested the affinity-purified material from culture supernatants of B, cells for efficacy in anti-IgM or dextran sulfate costimulatory assay. Results indicated that the affini-

Fig 3. SDS-PAGE analysis under reducing conditions of affinity-purified intracellular proteins from B, cells and from normal T lymphocytes. Gel was silver-stained after electrophoresis and photographed. Lane 1, Molecular-weight markers (Pharmacia); lane 2, affinity-purified intracellular protein from B, cells; lane 3, affinity-purified intracellular protein from normal T lymphocytes.
CHARACTERIZATION OF BCGF

Fig 4. Secretion of autocrine BCGF activity from B cells. B cells were cultured, and the conditioned medium was obtained as described in the legend to Fig 1. The conditioned medium was concentrated 20-fold, with a YM-5 filter in an Amicon concentrator, and was then loaded onto immunoaffinity matrix containing anti-IC-BCGF antibodies crosslinked to Reacti-gel 6 x. Immunoadsorbed and unadsorbed protein samples were collected and thoroughly dialyzed against PBS. Dialyzed samples were assayed for BCGF activity with B cells (A) and BCGF-dependent cycling B cells (B) as described. [3H]Tdr incorporation was monitored, and cpm was plotted against factor concentration. Commercial BCGF preparation (-o-), immunoadsorbed protein samples (-a-), unadsorbed protein sample (-x-).

Fig 5. Double-antibody staining of B cells. B cells were successively exposed to IC-BCGF goat anti-IC-BCGF antibodies and fluorescent-conjugated rabbit anti-goat Ig. The control cells were treated exactly the same except that the exposure to BCGF was eliminated. Cellular fluorescence of the stained cells was detected either by cytofluorographic analyzer (Becton Dickinson) (A and B) or photomicrographs (C and D). (A) Relative fluorescence intensity of control cell sample and (B) of positively stained cell sample. (C) Photomicrographs of control cells, (D) positively stained cells.

DISCUSSION

Regulation of cell proliferation in neoplastic human B cells is an area of considerable interest and importance to the immunobiology of these neoplasms that may also influence their clinical behavior. Activated normal human B cells proliferate in response to a group of secreted soluble GFs called BCGF (B cell stimulatory factor [BSF]) molecules. Our previous studies showed that neoplastic human B cells from a variety of non-Hodgkin's lymphomas and chronic B-cell leukemias can be stimulated to proliferate in vitro with exogenously provided BCGF. These studies suggest that the normal B lymphotrophic lymphokine growth factor can at least modulate growth of neoplastic B cells in vitro. Proliferation of normal B lymphocytes appears to be tightly regulated through various types of accessory cell interactions and their soluble secreted products. Neoplastic B cells, however, appear to escape such immune regulation, possibly by synthesizing their own GF, which normal B cells cannot provide under normal circumstances.

BCGF is functionally defined as a soluble protein that induces proliferation in normal activated B lymphocytes. BCGF induces proliferation in anti-IgM-activated B lymphocytes, which is usually scored by measuring [3H]thymidine incorporation in microculture assays. Anti-IgM-activated human B lymphocytes respond to various recombinant lymphokines such as IL-1, IL-2, IL-4, and interferon-γ (IFN-γ) in addition to BCGFs. These various lymphokines may be inducing proliferation in different B cell subsets (eg, it has been shown that only a fraction of anti-IgM-activated B cells express anti-Tac antigen and respond to IL-2). Recently, a cloned lymphokine IL-4 was shown to induce proliferation of an appropriately activated subpopulation of B cells. These studies also indicated that IFN-γ synergistically enhances the IL-4-induced proliferation of anti-IgM-preactivated B cells. On the other hand, IFN-γ alone does not induce proliferation in anti-IgM-preactivated B cells. The mode of action of these proliferation-inducing lymphokines is completely unknown at this time, but anti-IgM costimulatory assay clearly is not specific for a BCGF in vitro.
Several investigators have demonstrated production of putative autocrine BCGF activity by EBV-transformed or malignant B cells, yet none have previously demonstrated any direct relation between this protein and the BCGF produced by normal lymphocytes. Recently, EBV-transformed lymphoblastoid cells were shown to be stimulated to proliferate by either IL-6 or by ligation of the CD23 cell surface antigen. We demonstrated that a tumor cell-derived BCGF from human B lymphoma cells is antigenically related to a 60-Kd protein, believed to be a precursor to normal BCGF, from Namalva cells. Blazer et al and Gordon et al first postulated the central role of autonomous growth-promoting activity in transformed B cells. Production of growth-promoting activity in such transformed cells was proposed to be the direct result of the transformation process. Buck et al recently reported purification of putative autocrine GFs from EBV-transformed B cells with approximate molecular masses of 16, 34, and 68 Kd; the last two species were interpreted as multimers of 16 K monomer. Staphylococcus aureus Cowan strain (SAC)-activated normal B lymphocytes also secrete a 30-Kd protein that exhibited BCGF activity. This 30-Kd protein appears to be antigenically related to 60-Kd BCGF from Namalva cells. Our own results suggest that 60-Kd protein in normal lymphocytes may be a precursor to mature 14-Kd BCGF and that the two 50- and 30-Kd proteins may be the intermediate products produced during the activation step.

Neoplastic B cells respond to normal BCGF and to an apparent autocrine GF, which may be a form of BCGF. We showed that normal BCGF and the autocrine GF are antigenically similar and stimulate proliferation in vitro in a similar spectrum of normal and neoplastic human B cells. These observations imply that the GF receptors may play a significant role in transduction of a proliferative signal in

![Fig 6. Immuno-Western blot analysis of neoplastic B cells and T-ALL cell-derived protein components. (A) Cytosolic and secreted protein components from B cells were electrophoresed on a 12% SDS-polyacrylamide gel under reducing conditions. Proteins from the gel were then electrophoretically transferred onto nitrocellulose paper. Transferred proteins were then tested for the reactivity with affinity-purified anti-IC-BCGF antibodies as described in the Materials and Methods section. Lane 1, Cytosolic proteins; lane 2, secreted proteins. (B) Protein components from T-ALL cells were analyzed similarly. Lane 1, Affinity-purified IC-BCGF as a positive control; lane 2, cytosolic extract from T-ALL cells; lane 3, concentrated conditioned medium from T-ALL cells.

![Fig 7. Secretion of BCGF activity from B cells. Immunoaffinity-purified material obtained from products secreted by B cells was assayed in anti-lgM or dextran sulfate-activated normal human B cells. Assays were performed as described in the legend to Fig 1, except that freshly isolated B cells from the peripheral blood of normal human donors were used. Fifty thousand B cells, preactivated with anti-lgM (•-••) or dextran sulfate (•-•) were used per microculture well. [3H]-Tdr incorporation as cpm was plotted against the percentage (vol/vol) of sample: affinity-purified BCGF (•-••) and unbound protein component (•-•).}
neoplastic B cells, analogous to the presumed mechanism in normal B cells. Very little is now known about the biochemistry and biology of such receptors. Our initial approach in this study, although an indirect one, was to demonstrate the existence of BCGF receptors on neoplastic B cells by identifying cell surface-bound GF by immunofluorescence. Nonetheless, our results clearly suggest the presence of BCGF receptors on neoplastic B cells that appear to be functional and imply that GF receptors and their regulation may be an important step in the control of neoplastic B cell proliferation. Together, the results of these studies provide support for the autocrine growth model of neoplastic human B cells as previously suggested. The cross-reactivity of the anti-IC-BCGF antibody to BCFG molecules derived from neoplastic B lymphocytes, coupled with the similarity in mol wt and functional activities, suggest that the tumor cell-derived GF is similar if not identical to normal BCFG molecules.

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Isolation and characterization of growth factor(s) from a human B-cell lymphoma

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