Production of B Cell Growth Factor(s) by Neoplastic B Cells From Hairy Cell Leukemia Patients

By Richard J. Ford, Deborah Kwok, Jorge Quesada, and C.G. Sahasrabuddhe

Recent studies have shown that normal human T cells contain a high-molecular-weight (mol wt) protein exhibiting B cell growth factor (BCGF) activity. Other studies have shown that virally transformed human B cells also secrete a high-mol-wt BCGF-like molecule in vitro. We have studied neoplastic B cells from patients with untreated hairy cell leukemia (HCL) to ascertain whether such cytoplasmic BCGF activity is present in the tumor cells. Studies on HCL cells from four patients indicated that BCGF-like activity was in fact present in the cytosolic extracts when tested on autochthonous HCL cells as well as on normal BCGF-dependent human B cell lines. Chromatographic analysis indicated that the BCGF activity from HCL cells was similar in mol wt as well as function to the normal T cell-derived cytosolic BCGF activity. These studies suggest that HCL cells contain and, in some cases, secrete a high-mol-wt growth factor that can be autostimulatory and appears to resemble a similar growth factor molecule found in normal human T cells.

NORMAL HUMAN B CELL GROWTH has been shown to be dependent on cellular activation by antigen or mitogen, followed by the stimulatory action of the T cell lymphokine, B cell growth factor (BCGF). Human BCGF has been reported to be heterogeneous with both low (14 to 16 kd) and high (50 kd) molecular weight (mol wt) forms described. Recently, a high-mol-wt form (>50 kd) of BCGF has been described in the cytosol of normal human T cells that may represent a precursor molecule for the low-mol-wt secreted form of BCGF. Neoplastic human B cell growth, however, has long been an enigma, since the usual requirements for growth stimulation appear to have been circumvented by apparently autonomous cell growth. We have recently shown, however, that a variety of human non-Hodgkin’s lymphomas (NHL) can proliferate in vitro to greatly purified BCGF, indicating that these neoplastic B cells have retained the functional capacity to respond to the homologous normal growth factor. We report here that hairy cell leukemia (HCL) cells, a novel type of human B cell neoplasm that expresses the Tac antigen and rearranges Ig genes, contain a high-mol-wt BCGF molecule in the cytoplasm and, in at least some cases, secrete a molecule(s) with BCGF activity. This HCL-associated growth factor can stimulate in vitro cell growth in both autochthonous HCL cells and growth factor (BCGF)-dependent normal human B cell lines.

MATERIALS AND METHODS

Patients. Newly diagnosed untreated patients with HCL were studied at the time of initial presentation. Diagnosis was usually made by bone marrow needle biopsy and aspiration using conventional histopathologic criteria, including cytochemical staining for tartrate-resistant acid phosphatase (TRAP). Four patients with the leukemic form of the disease (WBC > 20,000/μL) with high numbers of HCL cells in the peripheral blood were either leukapheresed or phlebotomized to obtain large numbers of neoplastic B cells.

Cell preparation. Peripheral blood collections from either leukapheresis or venipuncture were separated into peripheral blood mononuclear cells (PBMCs) by Ficoll-Hypaque (FH) density gradient centrifugation. The PBMCs were washed and resuspended using neuraminidase-treated sheep red blood cells (SRBC; E0 overnight at 4°C. The resuspended PBMC population was then separated into SRBC rosette positive (E0+) or negative (E0-) populations on FH gradients. E0+ cells were then placed onto plastic Petri dishes to remove adherent cells. The nonadherent E0+ cell population was then washed and prepared for immunophenotyping.

HCL phenotyping. The E0+ cell population was examined by immunofluorescence with a standard battery of monoclonal antibodies (MAb) for human T and B lymphocytes. The MAb included pan T cell (OKT11, T3; Ortho, Raritan, NJ); pan B cell, B1, B4 (Dr. L. Nadler, Dana Farber Cancer Center, Boston); myeloid-monocyte (OKM1; Ortho); and the HCL-associated MAb HCl and HC2 (Dr. D. Posnett, Rockefeller University, New York). HCL cases were also studied with the combination of the MAb Leu-14 (pan B) and Leu-M5 (monocyte subset) (kindly provided by Dr. N. Warner, Becton Dickinson, Mountain View, Calif.), which, when expressed together, can discriminate HCL cells from normal B cells present in a leukemic cell population. The anti-Tac MAb was a gift from Dr. T. Waldmann (National Institutes of Health, Bethesda, Md).

In vitro growth factor production. Fresh HCL cells obtained from FH gradients were enriched for B cells by double E0+ rosetting, followed by adherence to plastic Petri dishes. The resulting cell populations were >90% HCL B cells by immunophenotyping and were 90% viable by trypan blue staining. The HCL B cells were then set up in vitro in RPMI 1640 (Irvine, Irvine, Calif) at 2 x 10^6 cells per milliliter in 1% fetal calf serum (FCS; Hyclone, Logan, Utah) or 1% bovine serum albumin (BSA; Sigma Chemical Co, St Louis) and incubated at 37°C for 24 hours in 5% CO2 atmosphere. The culture supernatants were collected by pelleting the cells in a refrigerated centrifuge at 800 g for ten minutes and were tested for BCGF activity on growth factor-dependent human B cell lines or on autochthonous HCL B cells.

Cytoplasmic BCGF activity from HCL cells. Cytoplasmic extracts from neoplastic HCL B cells were prepared and assayed for by the methods described by Sahasrabuddhe et al for normal human T lymphocytes. Briefly, the tumor cells were pelleted and resuspended in hypotonic buffer containing 20 mmol/L Hepes (pH 7.5), 1.4 mmol/L Mg (AC02), 3.6 mmol/L CaCl2, and 2 mmol/L 2-mercaptoethanol. The tumor cells were lysed with 40 strokes in a glass homogenizer and the lysate was centrifuged at 1,500 g for ten minutes to remove nuclei. The supernatant was centrifuged at 10,000 g for ten minutes to remove mitochondria and then ultracentrifuged at 100,000 g for 90 minutes to remove cell membranes and...
to obtain soluble cytosolic proteins in the supernatant. The cytosolic extract was concentrated by lyophilization and either assayed for BCGF activity or fractionated on a Sephacryl 200 (Pharmacia Fine Chemicals, Piscataway, NJ) gel filtration column.

**Microassays for in vitro growth factor activity.** Growth factor preparations from neoplastic human HCL B cells were assayed either on continuous BCGF-dependent human B cell lines or on the autochthonous tumor cells from which the growth factor was derived. The normal B cell lines were maintained in log phase growth in the presence of RPMI 1640 medium, 3% (vol/vol) heat-inactivated FCS, and greatly purified BCGF preparations. For the assay, B cells (>95% slg, B1') cultured for 72 hours were washed extensively in medium (four to five times) to remove any residual bound growth factor. The assays were performed in triplicate in flat-bottom 96-microwell plates (Corning Glass, Corning, NY), using a total of 1.0 to 1.5 x 10^6 cells per well in a final volume of 200 

The assays in which the target cells were exposed to serial dilutions of growth factor preparations were performed in RPMI 1640 supplemented with 2% (vol/vol) FCS for 48 hours. Sixteen hours before harvest the cells were labeled with 0.5 

**Gel filtration chromatography.** Tumor cell supernatants and cytosolic extracts from HCL cells were chromatographed on a Sephacryl 200 column (2 x 100 cm) that had been calibrated with a 4 x 89 90/2 83 75 80 3

<table>
<thead>
<tr>
<th>HCL Patient</th>
<th>slg*</th>
<th>s/A</th>
<th>B1</th>
<th>Leu-14/M5†</th>
<th>HC2</th>
<th>Tac</th>
<th>OKM1</th>
<th>T,</th>
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<tr>
<td>1</td>
<td>94</td>
<td>93/2</td>
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<td>81</td>
<td>4</td>
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<tr>
<td>4</td>
<td>89</td>
<td>90/2</td>
<td>83</td>
<td>78</td>
<td>88</td>
<td>75</td>
<td>80</td>
<td>3</td>
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</table>

PBMCs from untreated HCL patients were separated on FH gradients and then rosetted with neuraminidase-treated SRBC (E,) overnight at 4 °C. The E, negative cells were then depleted of adherent cells and immunophenotyped for cell surface antigens.

*E,- HCL cells were assayed using mouse monoclonal antibodies by indirect immunofluorescence with appropriate controls. The number shown represents the percentage of positive cells staining with each of the designated reagents.

†Leu-14, Leu-M5 double-staining percentage with FITC and phycoerythrin conjugated reagents, respectively.

**Production of growth factor activity by HCL cells.** HCL cell populations were set up for in vitro culture in either low-serum (1%) or serum-free (1% BSA) conditions and incubated for 24 hours at 37 °C in a 5% CO, atmosphere. At the termination of the culture period, the cells were centrifuged and the culture supernatants were removed. Cell viability at this time was ≥80%. The supernatants were then either frozen for future studies or used immediately in vitro proliferative assays using either long-term growth factor-dependent human B cell lines or autochthonous HCL cells that had been maintained in vitro for 24 to 36 hours. Table 2 shows a representative example of these studies, where it can be seen that the populations of HCL B cells

**RESULTS**

**Characterization of HCL cells.** Four patients with the clinical and pathologic diagnosis of HCL were studied. All had the leukemic form of the disease (>20,000 WBC/μL; ≥80% leukemic cells by morphology), and the cells studied were collected either by leukapheresis or by venipuncture. The tumor cells were enriched for by double E rosetting, followed by plastic adherence. The E, negative cell populations obtained appeared to contain only HCL cells by morphological criteria and were ≥90% TRAP positive.

Table 1 shows the phenotypic characterization of the HCL cells, which demonstrate that the tumor cells had standard B cell membrane markers, including cell surface immunoglobulin (slg) and the characteristic pan B cell antigen recognized by the MAb B1. In addition, however, the myeloid-monocytic MAb, OKM1, and Leu-M5 were also present on most, if not all, of the neoplastic cells. The HCL cell population also contained the Tac antigen, which is associated with the receptor to human T cell growth factor but has also been found on some normal human B cells. The combination of the pan B cell MAb, Leu-14 labeled with fluorescein (FITC), and Leu-M5 labeled with phycoerythrin (PE) stained HCL cells simultaneously, while single-stained cells identified occasional residual normal B cells or monocytes, respectively. Further analysis by slg light chain typing for assessing monoclonality of B cells and nonspecific esterase for monocytes indicated that ≤5% of the total HCL cell population represented these contaminating cell populations.

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>Bkgd</th>
<th>5%</th>
<th>10%</th>
<th>20%</th>
<th>30%</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCL Patient 1</td>
<td>132</td>
<td>990</td>
<td>1263</td>
<td>1725</td>
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<tr>
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<td>156</td>
<td>680</td>
<td>1560</td>
<td>2580</td>
<td>ND</td>
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<tr>
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<td>981</td>
<td>1867</td>
<td>4332</td>
<td>10175</td>
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<tr>
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<td>320</td>
<td>684</td>
<td>1424</td>
<td>4427</td>
<td>9564</td>
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</table>

HCL supernatants (SN) were generated at a final density of 2 x 10^6 cells per milliliter from E rosette and adherent cell-depleted tumor cell populations that were cultured for 24 hours in RPMI 1640 + 1% BSA. Two SN from each patient were obtained and tested. HCL cells are the B cells isolated from the patient’s peripheral blood lymphocytes by negative selection procedure described in Materials and Methods. BD23 and BDk9 are BCGF-dependent normal B cell lines. HCL cells (1 x 10^6, >95% viable) or normal B cells (1.5 x 10^6, BD23 or BDk9 cell line) were cultured in microwells for 48 hours with varying dilutions of HCL-derived supernatants. Microwell cultures were labeled with 1 μCi of 3H-Tdr for the final 24 hours of the 72-hour culture period. Results are expressed as mean cpm of triplicate assays. Standard errors were less than 10%.
release a soluble factor into culture supernatants that stimulate proliferation in both long-term human B cell lines as well as autologous, freshly obtained HCL cells in vitro. The release of the growth factor appears to be spontaneous in all patients studied, although the amount of activity varies somewhat from patient to patient. Growth factor activity does not seem to require lectin stimulation. In fact, lectin (PHA, protein A) stimulation does not augment the release of the growth factor. Also, it was found that the BCGF cytoplasmic activity from normal T cells would also stimulate \(^3\)H-Tdr incorporation in the HCL cells (data not shown).

**High-mol-wt growth factor activity in the cytosol of HCL cells.** Normal human T lymphocytes have been shown to contain a high-mol-wt (>60 kd) BCGF activity in their cytosol, which may be a precursor molecule for the low-mol-wt (14 to 16 kd) secreted form of human BCGF. We were interested to ascertain whether neoplastic human B cells, such as HCL, contained such a molecule. For these studies, we purified populations of HCL cells as before and prepared cytoplasmic extract to obtain a cytosolic protein fraction. The cytosolic fraction was assayed on long-term human B cell lines as before to determine whether BCGF activity was present. Table 3 shows examples of the cytosolic fraction from two patients' HCL B cells that were positive when tested for BCGF activity on long-term BCGF-dependent cell lines. This BCGF activity was also found to titrate in a dose-response manner similar to the cytosolic BCGF obtained from normal human PBMCs. The cytoplasmic BCGF activity from normal PBMCs has been previously shown to be exclusively present in T lymphocytes.

**Chromatographic characterization of cytosolic BCGF activity from HCL cells.** Because we had identified BCGF activity in the cytosolic compartment of HCL B cells, it was of interest to compare this factor with the cytosolic fractions obtained from lectin-activated normal human PBMCs. Therefore, the concentrated cytosolic extract from HCL B cells was chromatographed on a Sephacryl 200 column that had been calibrated with a variety of standard protein mol wt markers. Figure 1 shows the elution profile of the cytosolic and secreted BCGF-like activity from HCL B cells as compared with the similar cytosolic activity obtained from normal human T lymphocytes. It can be observed that the BCGF activities from both sources eluted from the column in a similar manner, suggesting that the growth factor activities obtained may share biochemical as well as functional similarities.

**DISCUSSION**

The control of cell growth in malignant human B cells is an area of considerable interest and importance to the immunobiology of these neoplasms as well as to their clinical behavior. The description and characterization of human B cell growth factor for normal B cells \(^7\) has greatly increased our understanding of how normal B cell proliferation is

### Table 3. Cytoplasmic BCGF Activity From HCL Cells

<table>
<thead>
<tr>
<th>Source of</th>
<th>Cytoplasmic Growth Factor</th>
<th>1(^\text{H})-Tdr Incorporation (cpm)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Added to Cultures (vol/vol)</td>
<td>Normal PBMCs</td>
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<tr>
<td>Target Cells</td>
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</tr>
<tr>
<td>BD23 B cells</td>
<td>0*</td>
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<tr>
<td></td>
<td>20.0</td>
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</table>

*Target cells for evaluating cytoplasmic BCGF activity consisted of long-term BCGF-dependent B cell lines used at 13,000 cells per well.

*Cytoplasmic growth factor activity obtained from fractionated E\(_2\)-negative HCL that were >90% TRAP, sig. \(x\), B1, Leu-14/M5, Tac positive.

†Mean cpm of triplicate assays of 72-hour cultures, labeled for final 24 hours with 1 \(\mu\)Ci \(^3\)H-Tdr. Standard errors were less than 10%.

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regulated through the secretion of soluble factors. Our recent studies have shown that neoplastic human B cells from a variety of non-Hodgkin's lymphomas and chronic leukemias can be stimulated to proliferate in vitro with BCGF. These studies suggest that normal homologous (ie, B cell lineage) growth factor(s) can at least stimulate neoplastic B cell growth in vitro experimentally. The secretion of BCGF under the usual immunologic circumstances appears to be a tightly regulated control mechanism, similar to interleukin 2 regulation that is mediated through various types of accessory cell interactions and their soluble cytokine products. It would therefore seem unlikely that the reactive (normal) accessory cells usually present either in neoplastic lymphoid lesions or in the peripheral blood in the chronic B cell leukemic state could account for the necessary BCGF activity, which is presumably needed to drive the neoplastic proliferative process. A likely source for such growth factor activity might then be the neoplastic B cells themselves. Since ample experimental precedence for autocrine growth factor mechanism(s) in tumor cells exists and recent studies have indicated that Epstein-Barr virus-transformed human B cells can secrete a BCGF-like factor, the prediction would be that at least some type of growth factor activity should be present in the culture supernatants if the tumor cells can in fact secrete the factor. Similarly, such a factor(s) could be found in the cytoplasm of the tumor cell itself, as is the case in the normal T lymphocyte.

The important recent observation that a high-mol-wt BCGF molecule is present in the cytoplasm of normal T cells, possibly representing a precursor molecule for the low-mol-wt secreted form of BCGF, suggested the possibility that a similar type of system might occur in neoplastic B cells. We have subsequently observed that both secreted and cytoplasmic factors with BCGF activity could be demonstrated in fresh HCL cells. The cytoplasmic BCGF from the HCL cells appears to be similar, if not identical, to that found in normal human T cells, but the HCL BCGF activity does not show as sharp a peak after elution from Sephadryl 200 gel filtration chromatography. Also, the elution profile appears to indicate that the mol wt of both the normal PBMCs as well as the HCL cytoplasmic growth factor activity is greater than 100,000 when compared with the elution of the mol wt markers used to calibrate the column. However, recent studies indicate that when the normal cytoplasmic BCGF is purified to homogeneity, it has a mol wt of approximately 60,000, as determined more accurately by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The elution profiles shown in Fig 1 compare the normal and HCL cytoplasmic growth factors, but this technique gives only the general range of mol wt. When taking these considerations into account, we believe that the actual mol wt of the HCL cytoplasmic growth factor will correspond to that found in normal PBMCs.

These findings suggest an autocrine model of neoplastic B cell proliferation exemplified by HCL, which is shown in Fig 2, and contrasted to a current conceptual model for the control of normal B cell proliferation. In this model, neoplastic B cells (HCL) contain a high-mol-wt BCGF molecule in their cytoplasm that is secreted, in most cases, as either a high- or a low-mol-wt BCGF molecule. This model implies that the neoplastic B cells possess functional receptors for the BCGF molecule that do not require in vitro activation in freshly prepared tumor cells. This type of autocrine stimulation also implies that the gene for BCGF, which does not appear to be expressed in normal, nonvirally transformed B cells, is expressed in neoplastic B cells such as HCL. Neoplastic B cell proliferation driven by an autostimulatory growth factor should circumvent the control mechanisms regulating normal B cell proliferation. This model appears to be consistent with the growth kinetics of most B cell neoplasms and suggests a number of interesting possible approaches to the control of neoplastic B cell growth in the future.

ACKNOWLEDGMENT

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REFERENCES


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