Malignant B cells from hairy cell leukemia (HCL) patients are unable to proliferate when stimulated with standard B cell mitogens. Using chromatographically purified B cell growth factor (BCGF), HCL can be stimulated to proliferate as assessed by incorporation of tritiated thymidine (3H-TdR) into DNA. Proliferation was found to be time dependent, with no detectable 3H-TdR incorporation in up to three days of culture, and significant stimulation evident at days 6 and 10. The presence of 10% BCGF in culture was an absolute requirement for HCL proliferation; however, this BCGF-induced DNA synthesis could be further augmented by the addition of anti-immunoglobulin heavy chain antibodies. BCGF-induced proliferation was abrogated in six of six patients by addition of 1,000 U/mL of recombinant \( \alpha \)-interferon (IFN) at day 0, although 1,000 U/mL of recombinant \( \gamma \)-IFN had no inhibitory effect in five of six patients studied. Specific cellular receptors for type I IFN were demonstrated in HCL by inhibition of binding of \( ^{125I} \alpha \)-IFN by a 40-fold excess of unlabeled \( \alpha \) or \( \beta \) IFN with no inhibition by unlabeled \( \gamma \)-IFN. These data demonstrate that malignant HCL lymphoblasts express specific type I IFN receptors and that type I, but not type II IFN, can inhibit growth factor-induced DNA synthesis by hairy cells in vitro. They further suggest a direct antiproliferative mechanism of action for IFN in HCL and predict equivalent clinical activity by either \( \alpha \) or \( \beta \), but not \( \gamma \) IFN in this malignancy.

**MATERIALS AND METHODS**

**Reagents.** BCGF was obtained from Cellular Products, Inc., Buffalo. This factor has been chromatographically purified from crude mitogen-stimulated peripheral lymphocyte supernatants and is devoid of interleukin 2 (IL-2) activity but capable of sustaining the growth of normal B lymphocytes. Polyconal rabbit anti-human \( \mu \) and \( \gamma \) chain antibodies coupled to polyacrylamide beads were purchased from Bio-Rad Laboratories, Rockville Centre, NY. Recombinant \( \alpha \)-IFN was obtained from Schering Plough Corporation, Kenilworth, NJ; \( \beta \)-IFN was obtained from Cetus Corporation, Emeryville, Calif; and \( \gamma \)-IFN was obtained from Biogen Inc., Cambridge, Mass. All interferons (\( \alpha \), \( \beta \), and \( \gamma \)) were recombinant bacterial products purified to \( \geq 99\% \) homogeneity.

**Preparation of affinity-purified rabbit anti-human IgM (soluble anti-\( \mu \)).** Pure IgM was obtained by passage of the ammonium sulfate-precipitated immunoglobulin fraction of an IgM macro-globulinemia patient's serum over a Sephareryl-200 (Pharmacia Fine Chemicals, Piscataway, NJ) column. Recovered IgM was determined to be free of IgG and IgA by ELISA and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This IgM was used to immobilize a rabbit to human IgM and to couple to an affinity column for recovery of anti-IgM. The affinity column was prepared by coupling IgM to cyanogen-bromide-activated Sepharose-4B (Pharmacia) according to manufacturer's directions. The IgM-coupled gel was packed into a 3-mL syringe, and serum from the IgM-immunized rabbit was recirculated through the gel overnight at room temperature by means of a peristaltic pump. The syringe was washed with at least 10 bed vol of phosphate-buffered saline (PBS), and the coupled anti-human IgM was eluted with 0.1 mol/L of glycine HCl containing 1 mol/L of NaCl (pH 2.5). The eluted fractions were pooled and dialyzed against PBS. Any reactivity to IgG was removed by absorbing the anti-IgM eluate with IgG coupled to Sepharose-4B.

**Cell separation.** Eight patients with HCL in leukemic phase were used as peripheral blood donors for these studies. All were men...

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ranging in age from 37 to 75 years. Mononuclear cells were separated from heparinized blood samples by Ficoll-Hypaque density gradients. Recovered cells were washed and resuspended in RPMI 1640 medium supplemented with antibiotics, 25 mmol/L of HEPES buffer and 20% fetal calf serum (FCS). This cell population was T cell depleted by rosetting overnight at 4 °C with AET-modified sheep red blood cells (SRBCs). Nonrosetting cells (hereinafter referred to as B cells) were removed from the interface of a second Ficoll gradient. The percentage of hairy cells in this population was used as the source of normal B cells.

Normal human B cells were isolated fromuffy coats as follows: peripheral blood buffy coat concentrate was withdrawn from a heparinized syringe and centrifuged for 30 minutes at 1,500 rpm over a Ficoll gradient. Mononuclear cells from the interface were depleted of adherent cells by incubating them for one hour at 37 °C in a 30-mL vol of RPMI 1640/10% FCS in a 75-cm² plastic flask (Corning 25110). The nonadherent cells were poured off, washed, and subsequently depleted of T cells by AET-SRBC rosetting for 90 minutes on ice. After Ficoll separation, the T-depleted lymphocyte population was put through a second one-hour adherence step at 37 °C and a second rosetting step at 4 °C.

Cells were diluted in RPMI 1640 containing 5% CO₂ in ambient air, cells were washed and cyto centrifuge preparations were made. Slides were stained with Wright’s-Giemsa, and cells ingesting two or more latex particles were assessed as positive for phagocytic activity.

Normal human B cells were isolated from buffy coats as follows: peripheral blood buffy coat concentrate was withdrawn from a sterile transfer pack into a heparinized syringe and centrifuged for 30 minutes at 1,500 rpm over a Ficoll gradient. Mononuclear cells from the interface were depleted of adherent cells by incubating them for one hour at 37 °C in a 30-mL vol of RPMI 1640/10% FCS in a 75-cm² plastic flask (Corning 25110). The nonadherent cells were poured off, washed, and subsequently depleted of T cells by AET-SRBC rosetting for 90 minutes on ice. After Ficoll separation, the T-depleted lymphocyte population was put through a second one-hour adherence step at 37 °C and a second rosetting step at 4 °C for 18 hours. After removal of rosetted T cells by Ficoll, the resulting population was used as the source of normal B cells.

**Proliferation assay for HCL B cells and normal B cells.** Cultures were performed in 96-well flat-bottomed microtiter plates (Corning 25860) with 1 x 10⁴ cells per well in a 0.2-mL vol. Factors were added to cultures as described in legends to Figs 1 through 5. Incubations for indicated time periods were carried out at 37 °C in a humidified 5% CO₂-air atmosphere; 2 μCi per well of methyl-³H-thymidine (6.7 Ci/mmol) (New England Nuclear, Boston) were added, and harvesting of cells onto glass microfiber filters was performed 24 hours later. Filters were placed in scintillation vials containing 7 mL of a mixture of 170 mL of Spectrafluor PPO-POP (Amersham Corporation, Arlington Heights, Ill) to 4 L of toluene (J.T. Baker Chemical Co., Phillipsburg, N.J) and counted in a beta scintillation counter (Tri-Carb 460 C, Packard Instrument Co, Inc, Downers Grove, Ill).

**TCGF assay.** BCGF was confirmed to be free of IL-2 activity by both a thymidine incorporation assay and a five-day growth assay, comparing BCGF with a TCGF standard for activity on activated T cells.

Target T cells were prepared by a seven-day activation of human peripheral lymphocytes prepared from Ficoll-separated buffy coats. T cells were stimulated with 0.5% vol/vol phytohemagglutinin-p (PHA-P) (Difco Laboratories, Detroit) in RPMI 1640 containing 15% FCS and 50 μg/mL of gentamycin at a cell density of 1 x 10⁶/mL. Cultures were held at 37 °C for seven days, at which time activated T cells were recovered and washed three times in RPMI 1640.

Thymidine incorporation was assessed by placing 1 x 10⁴ activated cells per well in a 96-well plate along with twofold dilutions of either BCGF or a TCGF standard, beginning at 25% concentration of factor. Cells were diluted in RPMI 1640 containing 15% FCS, 50 μg/mL of gentamycin, and 2 mmol/L of glutamine. After 48 hours of incubation at 37 °C, cultures were pulsed with 0.25 μCi of tritiated thymidine per well and were harvested after 24 hours.

Five-day growth assays were carried out, using the same population of activated T cells: 2 x 10⁴/mL T blasts were placed in 1-mL aliquots in 24-well trays with final concentrations of 0% to 20% BCGF or TCGF standard. Cultures were counted after five days at 37 °C and compared with the TCGF standard.

**Iodination of α²-IFN.** Iodination was carried out by the iodogen method, essentially as described by Markwell and Fox. In brief, 10 μg of α²-IFN (5 x 10⁵ U) were combined with 0.5 mCi of Na¹²¹I in a 30-μl vol and allowed to react for 30 minutes at room temperature. Free Na¹²¹I was separated from bound α²-IFN by fractionation on a Sephadex G-25 column. The radioactive peaks were pooled and tested for antiviral activity by challenge of GM2504 cells with vesicular stomatitis virus, with assessment of protection from viral cytopathic effect according to Finter’s method. α²-IFN was iodinated to a final specific activity of 32 mCi/mg of protein and retained > 90% of its antiviral activity.

**Cellular binding of¹²⁵I-α²-IFN.** Specific binding of¹²⁵I-α²-IFN to hairy cells was performed by modification of the method of Branca and co-workers. In brief, 5 x 10⁴ or 1 x 10⁵ cells were incubated in plastic 12 x 75-mm tubes (Falcon, Oxnard, Calif) with 0.5 to 50 ng of¹²⁵I-α²-IFN in a final 1-mL vol of RPMI 1640/20% FCS for one hour in a 37 °C water bath. Duplicate tubes containing a 40-fold excess of unlabeled α²-IFN in addition to the labeled IFN were included to quantitate nonspecific binding. Unbound¹²⁵I-α²-IFN was removed by three washes with 0.1% BSA/PBS, and the dry cell pellets were counted in a Packard model 5260 gamma counter. Specificity of binding was determined by the formula: percentage of specific binding = un inhibited CPM - inhibited CPM/ inhibited CPM x 100.

**RESULTS**

**Cell populations.** HCL B cells (E rosette negative) in all cases were > 90% typical HCL cells by morphological criteria. TRAP positive cells in these populations ranged from 4% to 82%, and latex phagocytizing cells ranged from 33% to 92%.

**Thymidine incorporation of peripheral blood HCL B cells.** As seen in Fig 1, BCGF stimulated thymidine uptake of HCL cells in a time-dependent manner. No thymidine was...
incorporated in up to three days of culture. At day 6, proliferation was readily detected in BCGF-stimulated cultures, with $^3$H-TdR incorporation increasing from day 6 to day 10, the last measurement. A combination of BCGF and anti-$\mu$ or anti-$\gamma$ stimulation was also effective in initiating DNA synthesis, with increased activity of the combined stimuli on day 10. No proliferation was observed unless BCGF was present. The effect of anti-heavy-chain antibodies on different patients varied. Although no patient’s cells showed spontaneous proliferation in the absence of BCGF and seven of eight responded to addition of BCGF with proliferation of >1,000 cpm/10$^5$ cells, most patient samples demonstrated a significantly augmented response when anti-heavy-chain antibodies were added to cultures along with BCGF, and some showed similar levels of BCGF-stimulated DNA synthesis with or without anti-heavy-chain antibodies (Fig 2). In one patient who responded to BCGF with <1,000 cpm (patient 4, Table 1), significant proliferation was achieved when insoluble anti-$\mu$ was added. In addition, in two patients for whom soluble anti-$\mu$ was compared to insoluble anti-$\mu$, the insoluble form gave greater stimulation of DNA synthesis than did the soluble form. Overall, of seven patients tested with insoluble anti-heavy-chain antibodies, proliferation in five of the seven was significantly greater in the presence of the anti-heavy-chain antibodies. The remaining two patients showed only marginal augmentation in the presence of anti-heavy-chain antibodies.

Figure 3 shows the differential effect of $\alpha_2$-IFN and $\gamma$-IFN on thymidine uptake. Neither recombinant $\alpha_2$-IFN nor $\gamma$-IFN directly induced proliferative activity by HCL cells. However, the results indicate that 1,000 U/mL of $\alpha_2$-IFN was capable of inhibiting BCGF-induced proliferation by cultured malignant HCL B cells. In contrast, 1,000 U/mL of recombinant $\gamma$-IFN did not have an antiproliferative effect when added to HCL cells in the presence of BCGF. As seen in Table 1, six of six patients tested for inhibition of proliferation by different IFN species were severely reduced in their ability to synthesize DNA in response to BCGF when cultured in the presence of 1,000 U/mL of $\alpha_2$-IFN. Thymidine incorporation in the presence of both BCGF and $\alpha_2$-IFN ranged between 6% and 35% of values obtained with BCGF alone, with a mean value of 17.5%. By the Wilcoxon test, the reduction in thymidine incorporation was significant with $P = 0.028$ when comparing BCGF alone with BCGF plus $\alpha_2$-IFN. In contrast, $\gamma$-IFN was substantially inhibitory in only one case, patient 4. In the remaining five patients, $\gamma$-IFN enhanced proliferation in four and minimally reduced proliferation in one. The percentage of BCGF control ranged from a low of 21% in patient 4 to 70% to 190% in the remaining patients, with a

![Fig 2](image2.png)

**Fig 2.** Effect of anti-$\mu$ or anti-$\gamma$ on B cell growth factor (BCGF)-induced hairy cell proliferation. Cells from seven different hairy cell leukemia (HCL) patients were incubated in medium alone, with 10% BCGF, or 10% BCGF + 15 µg/mL soluble or insoluble anti-immunoglobulin heavy-chain antibodies for seven days. Thymidine was added for an additional 24 hours prior to harvesting. ▼—▼, insoluble; □—□, soluble.

![Fig 3](image3.png)

**Fig 3.** Effect of $\alpha_2$-interferon (IFN) and $\gamma$-IFN on $^3$H-thymidine incorporation. $1 \times 10^6$ hairy cell leukemia (HCL)-B cells per well (in triplicate) were incubated for seven days with medium alone, 10% B cell growth factor (BCGF), 1,000 U/mL of recombinant $\alpha_2$-IFN, 1,000 U/mL of recombinant $\gamma$-IFN, or BCGF in combination with $\alpha$-IFN or $\gamma$-IFN (cpm values represent the mean ± SE).
mean value of 114% (P < .60). It is clear that α2-IFN and γ-IFN have differential effects on BCGF-induced proliferation of malignant hairy cells.

Effect of IFN on normal peripheral blood B cell proliferation. The normal B cell response to BCGF and anti-μ was qualitatively similar to the proliferation seen with hairy cells (Table 2). As with hairy cells, addition of 1,000 U/mL of recombinant α2-IFN had an antiproliferative effect on BCGF/anti-μ-stimulated B cells. In addition, normal B cell proliferation was inhibited to some extent by γ-IFN, although α2-IFN reduced proliferation to a greater degree than did γ-IFN (65% inhibition with α2-IFN as compared with 20% to 39% inhibition by γ-IFN).

Lack of IL-2 activity in BCGF preparations. BCGF, supplied by Cellular Products, was confirmed to be free of IL-2 activity by both a 3H-thymidine incorporation assay (Fig 4) and by a five-day growth assay (Table 3). A TCGF standard stimulated proliferation of T cell blasts in both thymidine incorporation and growth assays, whereas BCGF had no stimulatory activity in either assay.

Specific binding of [125]α2-IFN by HCL cells. The Scatchard curve shown in Fig 3 was generated by incubating HCL cells with [125]α2-IFN in concentrations ranging from 0.5 to 50.0 ng per 10^7 cells, with or without a 40-fold excess of unlabeled α2-IFN. Binding of [125]α2-IFN increased up to the highest concentration tested and was inhibited by unlabeled recombinant α2-IFN with a specificity of 69% to 86%. Saturation of binding sites was approached with increasing doses of [125]α2-IFN (Fig 5). Scatchard analysis (Fig 5, inset) revealed the apparent dissociation constant (Kd) to be 7.1 x 10^-10 mol/L, indicating a high-affinity binding site. The approximate number of IFN receptors per cell (also based on Scatchard analysis) was calculated to be ~350.

A competitive inhibition experiment demonstrated that unlabeled recombinant α2-IFN or β-IFN but not γ-IFN can effectively compete with [125]α2-IFN for binding sites on HCL cells. α2-IFN inhibited binding by 75.4%, β-IFN inhibited it by 67.9%, and γ-IFN showed no inhibition. This strongly supports the concept that α2-IFN and β-IFN share a common receptor on malignant HCL cells and that the γ-IFN receptor, if present, is distinct, in agreement with the findings of Branca and Baglioni of unique receptors for types I and II IFNs on lymphoblastoid cell lines.14

DISCUSSION

In our experience, BCGF has proven to be useful in stimulating HCL cells to divide in vitro. This is an important finding because standard B cell mitogens are relatively ineffective in inducing HCL proliferation. To date, no consistent or diagnostic chromosomal abnormality has been found in HCL, possibly due to the difficulty encountered in obtaining analyzable metaphases. BCGF may prove useful in extending cytogenetic or molecular genetic studies of HCL by stimulating cell division to a more easily quantitated level.

The effect of anti-heavy chain antibodies on BCGF-induced hairy cell proliferation was variable but, in most patients, it augmented BCGF-associated proliferation. However, anti-heavy-chain antibodies were not an absolute requirement for proliferation, as BCGF alone gave significant amounts of thymidine incorporation in the absence of anti-μ or anti-γ in most patients. Two subsets of B cells have been described by Muraguchi et al: (a) a small, resting population of B cells that requires an activation step prior to becoming BCGF responsive; and (b) a larger, activated population that can respond directly to BCGF in the absence of prestimulation. This second subset may have been activated in vivo. It seems likely that some proportion of hairy cells fall into the second category, as they are large B cells that are capable of direct response to BCGF in the absence of activation by anti-heavy-chain antibodies.

BCGF may also prove useful in vitro drug sensitivity testing on hairy cells and possibly other B-derived lympho-

Table 2. Seven-Day Thymidine Incorporation of Normal B Cells

<table>
<thead>
<tr>
<th>Factor Added</th>
<th>Exp. 1 (cpm)</th>
<th>Exp. 2 (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,223</td>
<td>6,605</td>
</tr>
<tr>
<td>BCGF</td>
<td>14,161</td>
<td>16,955</td>
</tr>
<tr>
<td>Anti-μ</td>
<td>283</td>
<td>1,275</td>
</tr>
<tr>
<td>BCGF + anti-μ</td>
<td>11,543</td>
<td>13,079</td>
</tr>
<tr>
<td>BCGF, anti-μ, α-IFN</td>
<td>4,059</td>
<td>4,648</td>
</tr>
<tr>
<td>BCGF, anti-μ, γ-IFN</td>
<td>7,016</td>
<td>10,496</td>
</tr>
<tr>
<td>α-IFN</td>
<td>256</td>
<td>2,781</td>
</tr>
<tr>
<td>γ-IFN</td>
<td>259</td>
<td>2,997</td>
</tr>
</tbody>
</table>

Normal peripheral blood B cells were cultured for seven days at 1 x 10^6 cells per well (in triplicate) with RPMI 1640 and 20% FCS. Additions were as indicated: BCGF 10%, anti-μ 15 μg/mL (Bio-Rad 170-5120), α2-IFN 1,000 U/mL, and γ-IFN 1,000 U/mL.
proliferative malignancies. To test antiproliferative capacity of a cell cycle-specific drug on tumor cells, measurable proliferation must be obtained prior to drug addition. In our studies with BCGF in combination with a2-IFN and γ-IFN, a2-IFN appears to inhibit DNA synthesis by the dividing hairy cells directly, whereas γ-IFN had no inhibitory effect in most patients tested and may even increase proliferation in some patients. The differential effects of types I (α and β) and II (γ) IFNs may be related to the fact that type I and II receptors are distinct, each IFN binding specifically to its own receptor.14 It is likely that the biological effects and possible that the therapeutic effects of type I and II IFNs are also distinct. These observations may prove useful in determining the efficacy of different types of IFNs for therapeutic use, and the in vitro data predict that γ-IFN may have limited efficacy in this malignancy.

It is interesting to speculate on the mechanism of action of a2-IFN in its successful treatment of hairy cell leukemia as compared with the less dramatic responses seen with other B cell tumors.22 We have found a2-IFN receptors on other types of malignant B cells from patients whose disease is relatively unresponsive to IFN therapy (data not shown), indicating that simple presence of receptors does not confer sensitivity to the therapeutic effects of IFN. As suggested by Hannigan et al,13 resistance may be due to the lack of growth inhibitory effects of IFN on IFN-resistant cells, rather than to the absolute presence or absence of receptors. The fact that a2-IFN directly inhibits hairy cell proliferation and γ-IFN does not suggest the possibility that a2-IFN is exerting its therapeutic effects at least partially by direct inhibition of malignant cell growth rather than indirectly as a biological response modifier of non malignant cells.

Unlabeled a2-IFN and β-IFN were each able to inhibit binding of 125I-a2-IFN to hairy cells, whereas γ-IFN was not capable of competing for the a2-IFN binding site, indicating the presence of unique receptors for type I interferon on malignant B cells, as found on B cell lines.14 These data indicate that IFN’s therapeutic effects may be mediated by receptor-dependent phenomena in HCL. It may be possible to gain a clearer understanding of this interaction and its significance by attempting to modulate receptor expression in vitro by treatment with IFN and other lymphokines or by monitoring receptor expression in patients before and during IFN therapy.

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B cell growth factor-induced proliferation of hairy cell lymphocytes and inhibition by type I interferon in vitro

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