Response Patterns of Hairy Cell Leukemia to B-Cell Mitogens and Growth Factors

By Bruce A. Barut, Maria K. Cochran, Carl O’Hara, and Kenneth C. Anderson

The effect of mitogens and/or recombinant B-cell growth factors (M/GFs) on the in vitro growth of hairy cells was examined. Tumor cells were isolated from the spleens of four patients with hairy cell leukemia (HCL) by Ficoll-Hypaque sedimentation and E-rosetting. Enrichment for tumor cells was confirmed with intracytoplasmic immunoglobulin (Ig) staining, tartrate resistant acid phosphatase (TRAP) staining, and staining using monoclonal antibodies (MoAbs) directed at B, T, myeloid, and monocytoid antigens (Ag) in indirect immunofluorescence assays. Tumor cells were isolated from the spleens of 2R(CD25)+ PCA-1 TRAP+. HCLs neither synthesized DNA nor secreted Ig in response to culture with granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1α (IL-1α), IL-1β, IL-2, IL-3, IL-4, IL-5, or IL-6. However, a proliferative response (stimulation index ≥3.0) without Ig secretion was triggered in HCLs by mitogens or combinations of GFs. Specifically, DNA synthesis was induced at 3 days in three of four HCL samples cultured with Staphylococcus aureus Cowan A (SAC) or the combination of phorbol ester (TPA) and the calcium ionophore A23187 (Ca²⁺); DNA synthesis was triggered later (day 7) by tumor necrosis factor (TNF) or by IL-4 and IL-5. In contrast, the fourth patient, a nonresponder to SAC or TPA/Ca²⁺, demonstrated increased DNA synthesis at day 3 when cocultured with IL-4 and IL-5. Both autoradiography and staining with antibromodeoxyuridine (BrdU) MoAb conjugated to fluorescein confirmed DNA synthesis by only a minority (5% to 23%) of tumor cells within each patient. Dual staining confirmed that responsive cells were both BrdU+ and TRAP+. DNA synthesis induced by TPA/Ca²⁺ was blocked specifically by anti–IL-6 Ab; in contrast, the HCL proliferative response to SAC, TNF, or IL-4 and IL-5 was not inhibited by anti–IL-6 Ab. α-Interferon inhibited the response to TPA/Ca²⁺, TNF, or IL-4 and IL-5 without any effect on response to SAC. Finally, peroxidase–anti-peroxidase staining demonstrated that HCLs are induced by TPA/Ca²⁺, but not by SAC, to produce intracytoplasmic IL-6. These data demonstrate IL-4, IL-5, and IL-6 mediated DNA synthesis by HCLs in vitro and suggest a possible in vivo role for these growth factors in the pathophysiology of HCL.

© 1990 by The American Society of Hematology.

Historically, due to heterogeneity in cell surface antigen (Ag) expression and functional repertoire, the lineage of derivation of hairy cell leukemias (HCLs) has been a topic of controversy. However, on the basis of phenotypic and gene rearrangement profiles, it is now generally accepted that the majority of HCLs are of B-cell origin. To date, few studies have examined the effects of B-cell mitogens (Ms) on HCL. Anti-IgM antibody, Staphylococcus aureus Cowan A (SAC), or phorbol myristate acetate (PMA), all of which trigger proliferation of normal B cells, have been reported to be inducers of DNA synthesis in either freshly isolated tumor cells or an HCL-derived cell line. Previous work in our laboratory has demonstrated that HCL cells may correspond to late stage B (preplasma) cells on the basis of their pattern of response to B-cell Ms as well as their cell surface phenotype. Finally, numerous investigators have demonstrated that HCL cells can be stimulated with B-cell growth factors (BCGFs) to proliferate in vitro, and a single study suggests that a BCGF may be involved in an autocrine growth pattern in HCL. In aggregate, these studies support the view that HCLs are of B-cell lineage and further suggest that HCLs may be responsive to normal B-cell growth and differentiation.

A variety of recombinant lymphokines/growth factors (GFs) that regulate normal human B-cell differentiation have been described, but the role that these GFs may play in the regulation of malignant B-cell growth is largely undefined. Of great interest are in vitro studies which suggest a pathophysiologic role for GFs in multiple myeloma (MM) and HCL. We and others have reported responsiveness of myeloma cells to interleukin-6 (IL-6) and HCL. We and others have reported responsiveness of myeloma cells to interleukin-6 (IL-6). Kawano et al have proposed an autocrine growth mechanism in MM based on the observation that these cells express IL-6 messenger RNA (mRNA), secrete IL-6, express receptors for IL-6, and proliferate in a specific manner to exogenous recombinant IL-6. Moreover, reports of elevated serum IL-6 levels in patients with active MM suggests an in vivo role for IL-6 in the regulation of tumor cell growth. Tumor necrosis factor (TNF) may play an analogous autostimulatory role in HCL, because in vitro culture with TNF induces HCL cells to both proliferate and generate TNF mRNA and protein. Finally, recombinant IL-4 has been reported to induce DNA synthesis in a single case of HCL, whereas IL-2 induced a similar response in only two of seven patients. However, to date the response of HCL to GFs has not been extensively characterized, and their role in regulation of HCL growth in vivo remains undefined.

In the present report we have attempted to define the response of HCL tumor cells to M/GFs. E-rosette depletion of T cells and adherence to plastic was used to prepare homogeneous populations of tumor cells. Phenotypic and histochemical techniques confirmed that cells triggered in vitro to synthesize DNA were hairy cells. Our studies demonstrated that 12-O-tetradecanoylphorbol-13-acetate (TPA) and the calcium ionophore A23187 (Ca²⁺) can induce IL-6–mediated DNA synthesis, a response which can be inhibited by α-interferon (IFN). SAC, IL-4 and IL-5, and...
TNF induced DNA synthesis that does not appear to be related to IL-6. IFN inhibits these responses to IL-4 and IL-5, and to TNF, but does not block responsiveness to SAC. These studies characterize the in vitro response of HCLs to GFs and support the view that GFs may play a role in the pathophysiology of HCL.

**MATERIALS AND METHODS**

**Preparation of enriched HCL and normal B-cell populations.** Spleenic samples were obtained from four patients with HCL. The diagnosis of HCL was made on the basis of clinical presentation, as well as histopathologic (Wright-Giemsa) and histochemical (tartrate-resistant acid phosphatase, TRAP) staining of peripheral blood, bone marrow, and spleen. Patient samples were immediately placed in medium containing 5% fetal bovine serum (FBS), minced, extruded through stainless steel mesh, and cryopreserved until use. Ficoll-Hypaque cell preparations were enriched for tumor cells by E-rosette depletion of T cells and removal of monococytes by adherence to plastic Petri dishes (1 hour, 37°C). Normal spleens were obtained from two patients without any systemic or malignant disease. B cells were prepared as described above.

**Characterization of HCL populations.** HCL-enriched populations were characterized morphologically, histochemically, and phenotypically before and after culture with M/GFs. Morphology was examined by Wright-Giemsa staining, and HCL-associated histochemistry was defined by TRAP staining.14 The populations of enriched HCLs were also examined using monoclonal antibodies (MoAbs) directed against B, T, and myeloid Ags by indirect immunofluorescence assays as previously described.15 Monoclonal reagents used to characterize the HCLs included the following: anti-B4(CD19), B1(CD20), B2(CD21), interleukin-2 receptor (IL-2R, CD25), and PCA-1, which are reactive with B cells at various stages of differentiation; anti-T3 (CD3), T4 (CD4), and T8 (CD8), which identify T cells; and anti-Mo1(CD11) and Mo2(CD14), which stain monocyte/mast cells.15-22

**Culture of enriched hairy or normal B-cell populations with M/GFs.** The recombinant GFs, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1α, IL-1β, IL-2, IL-3, IL-5, and IL-6, were either obtained from Genzyme (Boston, MA) or generously provided by Dr Steven Clark at Genetics Institute (Cambridge, MA). TNF was a gift from Dr Donald Kufe (Dana-Farber Cancer Institute, Boston, MA). These factors were used at titers known to stimulate normal B cells. One hundred-microliter aliquots containing 5 x 10⁴ purified HCLs or normal B lymphocytes in RPMI/10% FBS medium were dispensed into 96-well round-bottomed tissue culture plates (Costar, Cambridge, MA). Cells were cultured in the presence of media or M/GFs at appropriate concentrations to a final culture volume of 200 μL/well.

**Assays of DNA synthesis.** In vitro DNA synthesis by HCLs cultured in response to M/GFs was measured at days 3, 5, 7, and 10 of culture using 3H-thymidine ([3H]TdR) uptake. Cells were pulsed with [3H]TdR during the last 6 hours of incubation (0.2 μCi/well), harvested onto glass filters with the aid of an automatic cell harvester (Cambridge Technology, Cambridge, MA), and counted on a liquid scintillation counter (Packard Tri-Carb 4500, Downers Grove, IL). Significant proliferation was defined as a stimulation index (SI, [3H]TdR uptake of sample/[3H]TdR uptake of control) >3.0. At various intervals, [3H]TdR-labeled cells were also harvested by cytocentrifuge onto glass slides and prepared for autoradiography. The slides were coated with Kodak (Rochester, NY) NTB-2 nuclear emulsion, exposed at 4°C for 2 to 4 weeks, and then developed and stained with Giemsa. The percentage of [3H]TdR-labeled cells (greater than five grains per nucleus) was determined. Slides were prepared in duplicate, and 200 cells were counted on each slide.

Cells were also examined at various intervals for bromodeoxyuridine (Brdu) incorporation as another measure of DNA synthesis. Before and after culture with M/GFs, cells were incubated with 10 μmol/L Brdu (Sigma, St Louis, MO) for 30 minutes at 37°C. Cytocentrifuged preparations of cells were then fixed in ethanol, incubated with anti-Brdu Ab (7.5 μg/sample, courtesy of Dr Nick Gonchoroff, Mayo Clinic, Rochester, MN) for 30 minutes, washed, and stained with goat anti-mouse immunoglobulin G (IgG) fluorescein isothiocyanate (FITC) for 30 minutes. The percentage of intranuclear Brdu-labeled cells was enumerated using a fluorescent microscope.

**Radioimmunoassay for IgG.** Supernatants were harvested at various intervals after culture with M/GFs and assayed for IgG using a solid-phase radioimmunoassay as previously described.23 This assay can detect ≥5 ng/mL IgG.

**Characterization of M/GF-responsive hairy cells.** To confirm that those cells responding to M/GFs were HCLs, dual staining for Brdu incorporation and TRAP activity was performed on cells after culture. One hour before sampling, 10 μmol/L Brdu was added to culture media. The cells were harvested by cytocentrifugation onto glass slides and stained for TRAP positivity.14 The cells were further incubated with an anti-Brdu murine MoAb, which does not require denaturing,24 and then developed by a standard alkaline phosphatase anti-alkaline phosphatase (APAAP) technique.25 Cell surface phenotype of responsive HCLs was delineated using the panel of MoAbs described above.

**M/GF-responsive cells containing cytoplasmic immunoglobulin (clg)** were identified by immunofluorescence with either anti-κ or anti-λ directly conjugated to fluorescein or rhodamine (DAKO Immunoglobulins, Westminster, PA), as previously described.26 These directly labeled reagents were tested on myeloma cells of known κ and λ isotype and were found to be specific before use. Cells containing clg were examined using a Zeiss fluorescent microscope (Carl Zeiss, Inc, New York, NY) to assure that responsive cells were monoclonal tumor cells.

**Elucidation of the mechanisms of response by hairy cells to M/GFs.** Recombinant α-IFN (Schering Plough Corp, Kenilworth, NJ) and Abs directed against the recombinant GFs GM-CSF, IL-3, and IL-6 (Genetics Institute) were used to determine the mechanisms of response. Purified HCLs were cultured at 5 x 10⁴ cells per 200 μL of media and M/GFs. Either α-IFN at a concentration (1,000 U/mL) known to be cytotoxic to HCLs, or Abs to GM-CSF, IL-3, or IL-6 were added at the initiation of culture. Cells were harvested at intervals of maximal DNA synthesis. The percent inhibition by the IFN or Abs, defined as (SI to M/GF - SI to [M/GF + blocking agent])/SI to M/GF, was determined.

**Staining of hairy cells for intracytoplasmic GFs.** The purified HCLs were cultured with M/GFs and harvested at various intervals onto glass slides. Hairy cells were probed for intracytoplasmic IL-3 or IL-6 using polyclonal Abs to these growth factors in a horseradish peroxidase antiperoxidase (PAP) technique.27 Cultured monocytes and the U266 cell line served as positive controls for intracytoplasmic IL-3 and IL-6 staining, respectively.28

**RESULTS**

**Morphologic, phenotypic, and histochemical characterization of hairy cell enriched populations.** Ficoll Hypaque mononuclear cells were obtained from the spleens of four patients with HCL. T cells and monocytes were depleted by

From www.bloodjournal.org by guest on October 23, 2017. For personal use only.
Table 1. Phenotypic and Histochemical Characterization of Hairy Cell Enriched Populations

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>78.9</td>
<td>30.8</td>
<td>79.5</td>
<td>48.1</td>
<td>31.9</td>
<td>80.9</td>
<td>80.4</td>
</tr>
<tr>
<td>2</td>
<td>76.1</td>
<td>23.8</td>
<td>78.4</td>
<td>24.2</td>
<td>70.2</td>
<td>62.9</td>
<td>82.0</td>
</tr>
<tr>
<td>3</td>
<td>78.0</td>
<td>11.5</td>
<td>72.2</td>
<td>32.3</td>
<td>80.2</td>
<td>77.9</td>
<td>74.7</td>
</tr>
<tr>
<td>4</td>
<td>86.9</td>
<td>16.0</td>
<td>87.1</td>
<td>32.3</td>
<td>87.2</td>
<td>86.5</td>
<td>63.2</td>
</tr>
</tbody>
</table>

*Mononuclear cells were isolated from the spleens of patients with HCL by Ficoll-Hypaque density sedimentation. E-rosetting and adherence were used to remove T cells and macrophages, respectively. The resulting hairy cell enriched populations were examined using indirect immunofluorescence and flow cytometric analysis.

†Hairy cells were tested for TRAP staining and the percentage of cells expressing positive staining was determined.

E-rosetting followed by adherence to plastic. The E-rosette negative nonadherent cell population demonstrated morphologic features of hairy cells by Wright-Giemsa staining. Stained cells contained an oval nucleus with a reticular nuclear chromatin pattern and had abundant, pale cytoplasm. Cytoplasmic projections were not distinguishable on the cytocentrifuged cells. Cell surface phenotypic analysis in all cases was compatible with the diagnosis of HCL (Table 1, Fig 1). The B-cell restricted (pan B cell) Ags B1 and B4 were strongly expressed on a majority (80%) of cells; in contrast, only a minority of cells (10% to 30%) bore cell surface B2 Ag. The IL-2R, Mol, and the plasma cell associated PCA-1 Ag were present on 60% to 85%, 30% to 90%, and 25% to 50% cells, respectively. T-cell restricted (T3, T4, T8) and monocyte restricted (Mo2) Ags were minimally (4%) expressed on HCL enriched populations. TRAP positive staining was present in all cases (63% to 82%).

Response of hairy cells to M/GFs. The enriched HCL populations were cultured with M/GFs either singularly or in combination and assayed for DNA synthesis after 3, 5, 7, and 10 days of culture. Spontaneous [3H]TdR uptake by the HCLs cultured in media alone was minimal (<500 cpmp) (Table 2). At day 3, cells from three of four patients (patients 1 through 3, Table 2) demonstrated increased DNA synthesis when cultured with SAC (SI 3.4 to 10.3) or the combination of TPA and Ca2+ (SI 3.3 to 5.6). Maximal [3H]TdR uptake occurred at day 3, decreased by day 5, and was absent on days 7 and 10 (Fig 2A). Neither TPA nor Ca2+ alone resulted in significant DNA synthesis by HCLs.

IL-1α, IL-1β, IL-2, IL-3, IL-5, IL-6, and GM-CSF alone failed to stimulate significant [3H]TdR incorporation by HCLs at intervals up to 10 days of culture. When combinations of two GFs were examined, a significant response to IL-4 and IL-5 was noted in three patients studied: one patient (patient 4, Table 2) demonstrated markedly increased DNA synthesis (SI 4.7) to IL-4 and IL-5 on day 3, which decreased thereafter (Fig 2B); in contrast, two patients (patients 2 and 3, Table 2) did not respond at day 3 to IL-4 and IL-5, but did so on day 7 (Fig 2A). A single patient (patient 1, Table 2) did not respond to IL-4 and IL-5 at day 3 of culture, and was not studied at longer intervals. The response pattern observed to TNF was similar to that triggered by IL-4 and IL-5: [3H]TdR uptake was significantly increased at day 7 for patients 2 and 3 (Fig 2A) and at day 3 for patient 4 (Fig 2B).

No single GF or combinations of GFs other than IL-4 and IL-5 enhanced [3H]TdR uptake by HCLs. Similarly, no enhancement of the SAC or TPA/Ca2+-induced proliferation of HCLs was triggered by the addition of growth factors. Secretion of Ig by HCLs was not induced by M/GFs, even when cultures were extended to 10 days.

Characterization of hairy or normal B cells after culture with B cell M/GFs. DNA synthesis by HCLs in response to SAC, TPA/Ca2+, or IL-4 and IL-5 was confirmed using...
A time course of responses of purified hairy cells to B-cell Ms and recombinant Gfs. Purified hairy cells were suspended at 5 × 10^6/mL in RPMI/10% FBS and 100 μL aliquots were dispensed in 96-well round bottom tissue culture plates with 100 μL of either media or GF. Cells were pulsed with 0.2 μCi/well [3H]Tdr after 48 hours in culture and were harvested onto glass filters and counted on a scintillation counter (mean ± SEM) 18 hours later. Stimulation index = [%]Tdr uptake of sample/[%]Tdr uptake of control. Values represent a minimum of three experiments.

Fig 2.

Table 2. Responses of Hairy Cells to B-Cell Ms and Recombinant Gfs

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>[3H]Tdr Uptake</th>
<th>Stimulation Indices</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient</td>
<td>Media</td>
</tr>
<tr>
<td>1</td>
<td>256 ± 172</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>184 ± 102</td>
<td>10.3 ± 7.3</td>
</tr>
<tr>
<td>3</td>
<td>187 ± 94</td>
<td>5.4 ± 1.3</td>
</tr>
<tr>
<td>4</td>
<td>119 ± 35</td>
<td>1.4 ± 0.7</td>
</tr>
</tbody>
</table>

Purified hairy cells were suspended at 5 × 10^6/mL in RPMI/10% FBS and 100 μL aliquots were dispensed in 96-well round bottom tissue culture plates with 100 μL of either media or GF. Cells were pulsed with 0.2 μCi/well [3H]Tdr after 48 hours in culture and were harvested onto glass filters and counted on a scintillation counter (mean ± SEM) 18 hours later. SI = [%]Tdr uptake of sample/[%]Tdr uptake of control. Values represent a minimum of three experiments.

[3H]Tdr incorporation by scintillation counting, autoradiography, and bromodeoxyuridine incorporation. As can be seen in Table 3, HCLs that responded to M/GFs comprise a minor subpopulation (2.3% to 23.3%) of cells, with the greatest response occurring in patient 2 to SAC (7.7% of cells BrdU positive at 30 minutes; 23.3% of cells with [3H]Tdr uptake at 18 hours). Up to 14.3% cells (patient 2) responded to TPA/Ca^{2+}. The combination IL-4 and IL-5 also triggered only a minor subpopulation of cells: 4.1% BrdU at day 3 (patient 4, Table 3), and a mean of 6.5% in patients 2 and 3, who were responsive to IL-4 and IL-5 at day 7.

Hairy cells that responded to the M/GFs were characterized phenotypically and histochemically. In all four patients studied, incubation with TPA/Ca^{2+}, SAC, or IL-4 and IL-5 resulted in no consistent changes in B1, B2, B4, Mol, IL-2R, and PCA-I cell surface Ag expression. A representative cell surface phenotype of patient 3 before and after culture with Ms is shown in Table 4. To confirm that those cells responsive to M/GFs were HCLs, dual staining was performed for TRAP reactivity and BrdU incorporation. In all HCL samples responding to SAC, TPA/Ca^{2+} or IL-4 plus IL-5, there were minor populations (<7.7%) of cells which were positive for BrdU staining; a majority (61%) of BrdU positive cells were also TRAP positive (Fig 3). Within responsive HCL populations, >93% cells expressed cIg of identical light chain isotype, thus assuring that responsive cells were of B-cell origin and were monoclonal; the remainder of cells did not express any cIg. Culture of normal B cells with TPA/Ca^{2+} or SAC for 3 days or with TNF or IL-4 plus IL-5 for 7 days did not induce TRAP positivity.

Mechanisms of response of B-cell Ms to B-Cell Gfs.

Hairy cells were cultured with M/GFs and either α-IFN or antibodies to IL-6, IL-3, or GM-CSF to define potential mechanisms of response. α-IFN was able to inhibit DNA synthesis induced by TPA/Ca^{2+} (42.3% ± 24.8% inhibition) (Fig 4) as well as that induced by IL-4 and IL-5 (69.1% ± 13.4% inhibition). In contrast, α-IFN did not block SAC-induced DNA synthesis by HCLs (11.8% ± 6.8% inhibition). Anti–IL-6 (1:1,000) blocked the response of HCLs to TPA/Ca^{2+}: DNA synthesis by patients 1, 2, and 3 was inhibited by 22.0% ± 4.5%, 45.0% ± 38.2%, and 47.0% ± 8.5%, respectively, with no abrogation of response by either anti-IL-3 or anti-GM-CSF. In contrast, DNA synthesis noted after culture of HCLs with SAC was not reduced by coculture with Abs directed against IL-6 (Fig 4), IL-3, or GM-CSF.

Because the response of HCLs to TPA/Ca^{2+} could be blocked by Ab to IL-6, this population of stimulated HCLs was probed for intracytoplasmic IL-6 using anti–IL-6 Ab and a PAP staining technique. Cytospin slides confirmed the presence of intracellular IL-6 protein in more than 90% HCLs responsive to TPA/Ca^{2+}. In contrast, HCLs triggered by SAC or by IL-4 and IL-5 did not contain intracytoplasmic IL-6.
TABLE 3. Characterization of M or GF Responsive Hairy Cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>Stimulus*</th>
<th>Stimulation Indices</th>
<th>Percentage of BrdU Positive Cells</th>
<th>Percentage of ³H-Thymidine Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Media</td>
<td>1.0</td>
<td>0.9</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>TPA/Ca²⁺</td>
<td>3.3</td>
<td>4.6</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>SAC</td>
<td>3.4</td>
<td>2.3</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>Media</td>
<td>1.0</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TPA/Ca²⁺</td>
<td>4.6</td>
<td>6.5</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>SAC</td>
<td>10.3</td>
<td>7.7</td>
<td>23.3</td>
</tr>
<tr>
<td>3</td>
<td>Media</td>
<td>1.0</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>TPA/Ca²⁺</td>
<td>5.6</td>
<td>5.1</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>SAC</td>
<td>5.4</td>
<td>4.1</td>
<td>13.7</td>
</tr>
<tr>
<td>4</td>
<td>Media</td>
<td>1.0</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>IL-4 + IL-5</td>
<td>4.7</td>
<td>4.1</td>
<td>--</td>
</tr>
</tbody>
</table>

*Cells were cultured at 2.5 x 10⁷/mL in the presence of SAC, TPA/Ca²⁺, IL-4 + IL-5, or media alone. [³H]-thymidine was added to the cultures after 48 hours of culture and the cells were harvested 18 hours later onto glass filters or slides. Thymidine incorporation was determined by a scintillation count of the glass filters. SI = [³H]Tdr uptake of sample/[³H]Tdr uptake of control. The slides were processed for autoradiography and the percentage of labeled cells was determined. At least 200 cells per slide were counted. Cells were incubated with BrdU for 30 minutes harvested onto slides by cytopsin, and stained with directly counting at least 200 cells per slide.

DISCUSSION

In this report, we have examined the response of hairy cell enriched populations to a variety of cytokines. Enrichment for tumor cells was confirmed by characteristic phenotypic and histochemical staining. No significant proliferation was noted to GM-CSF, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, or IL-6. However, HCLs did synthesize DNA in response to SAC, TPA/Ca²⁺, TNF, as well as the combination of IL-4 and IL-5. In the majority of cases, maximal response was noted early (day 3) to SAC and TPA/Ca²⁺; in contrast, peak proliferation was triggered by TNF or IL-4 and IL-5 later (day 7). BrdU staining and autoradiography confirmed that a minority (<23%) of HCLs within each patient responded. Responsive cells demonstrated TRAP positivity and bore the cell surface phenotype of HCL. Moreover, dual staining in an immunoenzymatic technique (APAAP) confirmed that BrdU positive cells were also TRAP positive. To elucidate the mechanism of response, either α- or Abs directed to GFs were added to HCL cultures. Both anti-IL-6 Ab and α-IFN inhibited the response to TPA/Ca²⁺ without any effect on the response to SAC; Abs to GM-CSF or IL-3 did not affect the response to either TPA/Ca²⁺ or SAC. Finally, PAP staining demonstrated that HCL populations could be induced by TPA/Ca²⁺, but not by SAC, to produce intracytoplasmic IL-6. These observations give insight to both the biology of hairy cell growth in vitro and the pathophysiology of HCL in vivo.

It has been suggested that the effects of M/GFs on HCLs reported in previous studies are due to "contaminating" normal cells present within the tumor cell populations examined. Most commonly, the increased ³H-Thymidine incorporation by hairy cells in vitro has been attributed to contaminating T cells. In our studies, the enrichment technique used was E-rosetting to deplete T cells and adherence to plastic to deplete monocytes. Such purification techniques are critical because T cells, macrophages, and B cells may respond to exogenous GFs and confound the analysis. Resultant populations were enriched for tumor cells, confirmed by both the lack of cell surface staining for either T or monocyte Ags and by the expression of cell surface phenotype and

Table 4. Cell Surface Phenotype of M Responsive Hairy Cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>Percentage of Cells Expressing Antigens*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Day 0</td>
</tr>
<tr>
<td></td>
<td>TPA/Ca²⁺</td>
</tr>
<tr>
<td></td>
<td>SAC</td>
</tr>
</tbody>
</table>

*Mononuclear cells were isolated from the spleens of HCL patients and enriched for hairy cells by E-rosetting and adherence. Populations of cells were used immediately or cultured at 2.5 x 10⁷/mL in the presence of TPA/Ca²⁺ or SAC for 3 days. Viable cells were isolated by Ficol-Hypaque density sedimentation. The populations were examined using indirect immunofluorescence and flow cytometric analysis. None of the patients studied displayed significant changes in cell surface phenotype after culture with Ms or GFs.
histrochemical profile characteristic of HCL. The lack of proliferation to GM-CSF suggests that myeloid cells and macrophage stem cells were not present in the hairy cell enriched fractions. Similarly, lack of response to TPA or IL-2 supports the view that T cells, B cells, or monocytes were not present. Although the depletion techniques used would not remove normal B cells, the lack of Ig secretion to any lymphokine(s) (ie, SAC, IL-4, IL-5) suggests that normal B cells were only a minor subpopulation within tumor cell populations studied. Moreover, hairy cell enriched populations before and after culture with M/GFs were predominantly CD20+ CD21− CD19+ PCA-1+ CD11+ IL-2R+, the phenotype characteristic of HCL. In our previous studies, the majority of normal human splenic B cells coexpress CD21; ≤30% of B cells express either PCA-1 or IL-2R, and few, if any, coexpress CD11. Finally, dual staining after culture demonstrated that approximately two-thirds of the responsive (BrdU positive) cells were also TRAP positive. Because not all hairy cells are TRAP positive, 3 it is impossible to histochemically confirm that BrdU+ , TRAP− cells are tumor cells. Nonetheless, our data provide direct evidence of DNA synthesis in hairy cells triggered by SAC, TPA/Ca2+, and IL-4/IL-5.

Responsiveness of hairy cells to M/GFs did not result in an absolute increase in the number of hairy cells in culture. In those three patients who responded to TPA/Ca2+ at day 3 (SI 3.3 to 5.6), only a minority of cells were either BrdU positive (4.6% to 6.5%) or incorporated 3[H]Tdr (13.8% to 14.3%). Similarly, only a minority of cells which responded to SAC at day 3 (SI 3.4 to 10.3) were either BrdU positive (2.3% to 2.7%) or incorporated 3[H]Tdr (13.7% to 23.3%). In the patients who responded to IL-4 and IL-5, DNA synthesis was also present only in a small subset of hairy cells (4.1% to 8.1% BrdU positive cells). The observation that a minor subset of tumor cells are responsive is analogous to results of our recent studies of purified myeloma cells, which demonstrated that ≤6% of cells within the malignant clone synthesize DNA after triggering with IL-3, IL-5, or IL-6. Although those hairy cells which synthesize DNA are not necessarily "clonogenic" cells that can be maintained in long term culture, it is interesting to note that "clonogenic" cells in acute myelocytic leukemia, for example, also comprise a small fraction of tumor cells. Future studies will determine whether M/GFs which trigger DNA synthesis by HCLs may also promote long-term growth and enhance the development of clonogenic assays.

We have previously reported that homogeneous populations of hairy cells proliferate in response to phytohemagglutinin-stimulated leukocyte-conditioned media.3 Korsmeyer et al have also documented a 1.5- to 3.0-fold increased in DNA synthesis in hairy cells triggered by T-cell GFs. Two other factors, B-cell growth factor (BCGF) and TNF, have been shown to induce hairy cell proliferation. Finally, Mongini et al have demonstrated responses of an HCL cell line to four known activators of normal human B cells. The availability of recombinant GFs, coupled with techniques for the isolation of homogeneous populations of hairy cells, has permitted us to examine more specifically the role of lymphokines in regulation of hairy cell growth. Our data suggest that IL-6, IL-4 and IL-5, and TNF may be GFs for hairy cells. In particular, hairy cell populations can be induced by TPA/Ca2+ to synthesize IL-6, and either anti-IL-6 or IFN can inhibit DNA synthesis induced in this setting. The response to IL-4 and IL-5 or to TNF, which can also be blocked by IFN, is not affected by anti-IL-6 Ab and is therefore unrelated to IL-6. Thus, it would appear that there are at least two HCL growth regulatory pathways involving GFs, one of which is IL-6 mediated and a second of which is IL-4 and IL-5 or TNF mediated. HCL proliferation induced by SAC, which appears to be unaffected by IFN, may represent a third mechanism of response. Thus, data to date suggest that some mitogens (ie, TPA/Ca2+) may affect hairy cell growth via GFs, but that other mitogens (ie, SAC) trigger hairy cells by as yet undefined mechanisms.

More detailed studies of the effects of GFs on hairy cells are needed to define mechanisms of response on both a cellular and molecular level. Availability of cDNA probes for GFs and GF receptors will permit assays for GF or GF receptor mRNA. Abs directed against GFs and their receptors will allow measurement of intracytoplasmic or cell surface GF and receptors. Finally, the availability of GF dependent cell lines, ie, the B9 IL-6 dependent cell,36 will permit assessment of GF secretion by HCLs. Such studies may not only augment our understanding of the biology of HCL but may also suggest new therapeutic options.

ACKNOWLEDGMENT

We thank Bernadette Miner for secretarial assistance.
REFERENCES


29. KLINUS-Nelemans HC, Jansen JH: Residual T lymphocytes, and not malignant B cells, proliferate upon mitogenic stimulation. Leukemia 3:715, 1989


Response patterns of hairy cell leukemia to B-cell mitogens and growth factors

BA Barut, MK Cochran, C O'Hara and KC Anderson