Establishment and Characterization of Human B-Cell Lymphoma Cell Lines Using B-Cell Growth Factor

By Richard J. Ford, Angela Goodacre, Irma Ramirez, Shashikant R. Mehta, and Fernando Cabanillas

B-cell non-Hodgkin's lymphomas (NHL-B) have been difficult to establish in long-term cell culture using standard techniques. We report the establishment of five representative cell lines from high grade NHL-B using B-cell growth factor (BCGF). The five NHL-B cell lines display the morphologic, immunophenotypic, genotypic, and biologic characteristics of the lymphoma cells present in the original diagnostic specimen. The cell lines showed at least a sevenfold dose-dependent increase in proliferation in vitro over background in the presence of BCGF. Other putative B-cell growth-stimulating cytokines showed no significant proliferative activity or were inhibitory in some cases. NHL-B cell lines secreted growth factor(s) into culture supernatants that mediated at least a fivefold dose-dependent increase in cell proliferation in autologous lymphoma cells and a 10-fold or greater stimulation in growth factor-dependent normal B cell lines in vitro. The cell lines show monoclonal rearrangements of IgH genes and nonrandom chromosomal abnormalities characteristic of NHL-B, while the expression of Epstein-Barr virus associated antigen (EBNA-I) is present in two of the five cell lines. The studies show that lineage-specific growth factors may be used to establish neoplastic B cell lines in vitro, which are important experimental systems for cellular and molecular studies in the NHL-B.

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typed, and prepared for cell culture. Duplicate cell cultures were nonadherent cell population was cell surface-phenotyped, karyotyped as previously described. The SRBC negative (E-), arabinoside, methotrexate, 6-mercaptopurine. 6-thioguanine. prednisone; Cis-plat: cis-platinum; MIME: methyl-GAG, ifosfamide, methotrexate, etoposide: ARA-C: cytosine arabinoside: CVP: cytoxan, vincristine, prednisone; M-BACOD: methotrexate, bleomycin, Adriamycin, prednisone, dexamethasone; ALINC-13: acute leukemia in children 13 (POG), cytoxan, vinristine, L-asparaginase, prednisone, cytosine arabinoside, methotrexate, 6-mercaptopurine, 6-thioguanine.

Table 1. Description of Lymphoma Patients From Whom NHL-B Cell Lines Were Derived

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Age [yr]</th>
<th>Sex</th>
<th>Dx</th>
<th>Immunophenotype</th>
<th>Cytogen</th>
<th>Therapy</th>
<th>Clinic Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>CJ</td>
<td>64/F</td>
<td></td>
<td>NHL-DLCL (NSCL)</td>
<td>CD19, 20, 10, Slg DR+, CD2, 3; Tac (IL-2R)-</td>
<td>t(8, 14)</td>
<td>CHOP/COP</td>
<td>PR, died of refractory lymphoma</td>
</tr>
<tr>
<td>RR</td>
<td>38/M</td>
<td></td>
<td>DLCL</td>
<td>CD19, 20, 22, DR+, Ig, CD2, 3; Tac (IL-2R)</td>
<td>t(10, 14)</td>
<td>MIMET/VP</td>
<td>CR, relapse/died of refractory lymphoma</td>
</tr>
<tr>
<td>JT</td>
<td>70/H</td>
<td></td>
<td>DLCL</td>
<td>CD19, 20, Slg DR+, CD2, 3, 4, 8, IL-R</td>
<td>t(14, 18)</td>
<td>CHEP/Bl</td>
<td>PR, relapse of refractory lymphoma</td>
</tr>
<tr>
<td>FM</td>
<td>43/W</td>
<td></td>
<td>DMCL/FCC</td>
<td>CD19, 20, Ig DR+</td>
<td>t(14, 18)</td>
<td>CVP, M-BACOD</td>
<td>PR, progress transformation to high grade refractory lymphoma</td>
</tr>
<tr>
<td>RT</td>
<td>16/W</td>
<td></td>
<td>ALL (pre-B) with lymph node involvement</td>
<td>CD19, 20, 10, Ig+, CD2, 3; Slg-</td>
<td>t(11, 17)</td>
<td>Vincristine</td>
<td>PR, CNS relapse/died of ALL</td>
</tr>
</tbody>
</table>

Abbreviations: PR, partial response; CR, complete remission; NHL-DLCL, nodular poorly differentiated lymphocytic; DLCL, diffuse large cell lymphoma; NSCL, nodular small cleaved cell lymphoma; NHL-LCL, nodular large cell lymphoma; ALL, acute lymphoblastic leukemia; CHOP: cytotoxan, Adriamycin, oncovin, prednisone; COP: cytoxan, oncovin, prednisone; Cis-plat: cis-platinum; MIME: methyl-GAG, ifosfamide, methotrexate, etoposide; ARA-C: cytosine arabinoside; CVP: cytoxan, vinristine, prednisone; M-BACOD: methotrexate, bleomycin, Adriamycin, prednisone, dexamethasone; ALINC-13: acute leukemia in children #13 (POG), cytoxan, vinristine, L-asparaginase, prednisone, cytosine arabinoside, methotrexate, 6-mercaptopurine, 6-thioguanine.

al, 1986). Karyotypes are described according to ISCN nomenclature (1985).

Cell culture. Single cell suspensions from biopsy or fluid specimens were depleted of T cells by sheep red blood cell (SRBC) E rosetting, and of mononuclear cells (mostly monocytes and/or macrophages) as previously described. The SRBC negative (E-), nonadherent cell population was cell surface-phenotyped, karyotyped, and prepared for cell culture. Duplicate cell cultures were established in RPMI 1640 media (Irvine Scientific, Irvine, CA) and established in RPMI 1640 media (Irvine Scientific, Irvine, CA) and monitored for cell growth, cell viability, and growth factor activity were the lymphoma cell line cells and BCGF-dependent

Monoclonality of neoplastic B cells was assessed by standard Ig gene rearrangement analysis essentially as described by Cleary et al. using Southern blot analysis for DNA restriction fragments from BamHI and HindIII digestions for IGH and JH. The tumor cells obtained from the patients were analyzed for functional activities such as proliferation and/or differentiation (Ig secretion) or plated for in vitro cell growth in the presence or absence of the lineage-specific growth factor, BCGF (12 Kd). Cell growth was assessed at 3- to 5 days post-collection.

Southern blot analysis for the presence of EBV genome in lymphoma cell lines. High molecular weight DNA from each of the lymphoma cell lines (10 μg) was digested with the restriction endonuclease BamHI, size-fractionated in 0.8% agarose gel, and transferred to a nitrocellulose filter. The filter was hybridized to an EBNA 1 probe (pEBNA) oligolabeled with P3* to a specific activity of 1 x 10⁶ cpm/μg. The filter was washed under high stringency conditions and exposed on X-ray film for 24 hours at 70°C.

In vitro immunoglobulin secretion by lymphoma cells. Constitutive secretion of immunoglobulins was assayed for by measuring IgM and IgG levels in cell culture supernatants at 48 or 72 hours in vitro. These assays were performed using a standard micro-enzyme linked immunosorbent assay (ELISA) method.

RESULTS

Establishment of human NHL-B cells in vitro. Freshly obtained specimens of grade high human NHL-B from various types of biopsy or other diagnostic procedures were received under sterile conditions and processed within 1 hour of removal from the patient. These specimens were representative of the spectrum of high grade type of human B-cell lymphomas. The tumor cells obtained from the patients were usually greater than 90% viable and could be maintained in standard RPMI media and 20% FCS for up to 24 hours without significant loss of tumor cell viability. Cells were aliquoted at this time for immunophenotyping, genotyping by Ig gene rearrangement, and for cytogentic karyotyping. Tumor cell populations were depleted of T cells by E-rosetting and of monocytes and/or macrophages by plastic adherence. Characteristics of patients' initial lymphoma cell populations are shown in Table 1. Tumor cells were then assayed for functional activities such as proliferation and/or differentiation (Ig secretion) or plated for in vitro cell growth in the presence or absence of the lineage-specific growth factor, BCGF (12 Kd). Cell growth was assessed at 3- to 5 days post-collection.
Large cell lymphomas, B-cell type. Large cell lymphomas are a heterogeneous group of tumors, arising either as spontaneous lymphomas or as transformed variants of primary small cell B-cell lymphomas or B-cell chronic lymphocytic leukemias, as in Richter's syndrome. In the present study, three of the cell lines that were derived represented different histopathologic subtypes (eg, large noncleaved cell, immunoblastic, etc) of large cell lymphoma. While the tumors showed some morphologic (Fig 1A and B) and immunophenotypic heterogeneity, typical of these lymphomas, they also usually displayed quite stable nonrandom chromosomal abnormalities (NRCA) associated with NHL-R, as shown in Figs 3 through 5. In these cell lines, the cell surface immunophenotype and karyotype remained stable in vitro for at least 1 year.

Pre-B-cell leukemia/lymphoma. The cell line reported here (RT) was derived from the bone marrow of a 14-year-old patient with the pre-B cell type of acute lymphocytic
leukemia (ALL) with peripheral lymph node involvement. The tumor cells from the initial bone marrow aspirate were typical ALL blasts morphologically and immunophenotypically (Table 1). The tumor cells responded actively to exogenous BCGF on initial exposure, and have continued to respond vigorously to the growth factor for more than a year in vitro (Table 2). The ALL cells had a variety of characteristic cytogenetic abnormalities in the original diagnostic sample that are also present in the cell line (Fig 6).

Cytogenetic abnormalities. NRCA associated with the various histopathologic subtype diagnoses have become important parameters in the characterization of NHL-B. The cell lines described here all demonstrated characteristic NRCA, primarily of the t(14;18) and t(8;14) type in the fresh biopsy specimens, as well as in the subsequent cell line (Figs 2 through 6). The NRCA were concordant in all of the cell lines reported here. In addition to the NRCA, a variety of apparent random chromosomal abnormalities were also identified in the various cell lines (Figs 2 through 6).

Growth factor studies. In addition to providing the initial stimulatory conditions necessary for the establishment of in vitro cell growth in these cell lines, BCGF (12 Kd) sensitivity has been maintained for more than 1 year. Besides retaining growth factor sensitivity, the cell lines also produced a BCGF growth factor activity in the culture supernatants. This growth factor activity displayed autostimulatory

Fig 3. Q-banded karyotype of the large cell lymphoma, NHL-B cell line CJ showing 51, XX, + X, −4 + 12, + del (2) (p21), + del (2) (q21), + del (3) (q21), + del (3) (q21), der(4) t(14:18) (q21; q21), t(8:22) (q21; q21), del(10) (q21; q21), t(14;18) (q21; q21), and three unidentified markers.
activity, as well as growth promoting activity on BCGF-dependent normal B cell lines in vitro (Table 3). The characteristics of the purified autocrine growth factor produced by the RR cell line have been recently described in detail. No proliferative activity was observed on normal IL-2-depen-
Table 3. Production of Growth Factor Activity by B-Cell Lymphoma Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Supernatant</th>
<th>Autochthonous Lymphoma Cells</th>
<th>Normal B Cell Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR</td>
<td>2.418</td>
<td>0</td>
<td>940</td>
</tr>
<tr>
<td>RR</td>
<td>42.968</td>
<td>5</td>
<td>3.282</td>
</tr>
<tr>
<td>RR</td>
<td>60.682</td>
<td>10</td>
<td>10.644</td>
</tr>
<tr>
<td>RR</td>
<td>76.200</td>
<td>20</td>
<td>14.254</td>
</tr>
<tr>
<td>CJ</td>
<td>453</td>
<td>0</td>
<td>642</td>
</tr>
<tr>
<td>CJ</td>
<td>2.336</td>
<td>5</td>
<td>2.316</td>
</tr>
<tr>
<td>CJ</td>
<td>4.265</td>
<td>10</td>
<td>3.050</td>
</tr>
<tr>
<td>CJ</td>
<td>5.476</td>
<td>20</td>
<td>7.722</td>
</tr>
<tr>
<td>FM</td>
<td>8.020</td>
<td>0</td>
<td>860</td>
</tr>
<tr>
<td>FM</td>
<td>32.245</td>
<td>5</td>
<td>15.401</td>
</tr>
<tr>
<td>FM</td>
<td>44.487</td>
<td>10</td>
<td>17.230</td>
</tr>
<tr>
<td>FM</td>
<td>47.536</td>
<td>20</td>
<td>26.179</td>
</tr>
<tr>
<td>JT</td>
<td>1.512</td>
<td>0</td>
<td>330</td>
</tr>
<tr>
<td>JT</td>
<td>4.105</td>
<td>5</td>
<td>5.456</td>
</tr>
<tr>
<td>JT</td>
<td>5.137</td>
<td>10</td>
<td>7.228</td>
</tr>
<tr>
<td>JT</td>
<td>6.190</td>
<td>20</td>
<td>9.864</td>
</tr>
</tbody>
</table>

Cell lines were cultured at 10^6/ml for 24 hours in RPMI 1640, 5% FCS. The supernatants were collected, dialyzed overnight at 4°C against RPMI, and concentrated 10-fold with an Amicon concentrator, using YM 5 membranes. Autochthonous lymphoma cells or normal BCGF-dependent human B cells (10^6 per well) were washed, exposed to serial dilutions of the above supernatants, and cultured for 72 hours at 37°C. The microwells were pulsed with [3H]-Tdr for the final 18 hours in vitro. No activity above background was observed on IL-2-dependent normal human T cells or T-ALL cells exposed to the supernatants for 72 hours.

Abbreviation: SN, supernatant.

*Mean cpm of triplicate microwell cultures. SE were less than 10%.

dent human T cells or T-ALL cells in vitro when exposed to supernatant from the B-cell lymphoma cell lines.

EBV expression in NHL-B cell lines. Due to the ubiquitous nature of EBV expression in most well-known human B-cell lymphoma cell lines (particularly Burkitt's lymphoma cell lines), our cell lines were evaluated for EBNA-1 by Southern blot analysis. In the five lines studied, two were positive and three were negative (Fig 7).

Immunoglobulin secretion by lymphoma cell lines. The cell lines reported represent different apparent stages in B-cell differentiation showing characteristic Ig gene rearrangement by Southern blot analysis (Fig 8), including fairly late (mature) stages of differentiation, where Ig secretion occurs in normal B cells and many EBNA positive lymphoblastoid cell lines. One of the cell lines (Fig 8, lane 5) shows only a single allele present, probably indicating that one of the alleles has been deleted. It was also of interest to determine if the NHL-B cell lines secreted Ig in vitro. Micro-ELISA analysis for IgM and IgG secretion was performed on cell culture supernatants after various lengths of time in vitro. As shown in Fig 9, most of the cell lines did not spontaneously secrete IgM or IgG when cultured under standard conditions in vitro.

DISCUSSION

The non-Hodgkin's lymphomas are an important, heterogeneous group of common human lymphoid neoplasms that have provided a great deal of interest in a variety of areas of cancer research. The NHL are, in fact, among the most studied human tumors, due in part to the relative ease in obtaining representative specimens from lymph node biopsies and other commonly performed diagnostic procedures. Such specimens, if properly handled, will usually provide a source of fairly homogeneous, viable tumor cells that can be not only readily characterized in considerable detail and further purified if necessary, but can also provide excellent experimental systems for a wide range of studies. The recent development of extensive panels of lineage and in some cases stage-specific or associated MoAbs for antigens expressed on normal human B cells, which are also expressed on most if not all neoplastic B cells, has provided for the most compre-
molecular weight (70 Kd) intracellular BCGF molecule found in normal human T lymphocytes. These findings imply that the gene for BCGF, which is not usually expressed in normal B cells but which is apparently turned on in EBV-transformed lymphoblastoid cell lines, is activated in the high grade NHL-B. Preliminary studies on these NHL-B cell lines, using the cDNA for the 12 Kd BCGF gene, indicate that this may in fact be the case.

A further consideration involves the issue of BCGFs in general, which has sparked considerable controversy recently. A rather wide array of cytokines, including IL-2, IL-4, IL-6, and LTN in particular, have been reported to stimulate proliferation in human B cells of one source or another. We have tested all of these (as well as a wide spectrum of other natural products and recombinant cytokines on NHL-B), both from freshly plated biopsy specimens and on established NHL-B cell lines (data not shown), and find significant and reproducible growth stimulation only by low (12 Kd) and/or high (60 Kd) BCGF. Another consideration has been the role of the CD23 antigen and other agonistic MoAbs in human B cell growth stimulation.

The NHL-B cells and cell lines that have expressed EBNA-1 in our experience have shown moderate amounts of TdR incorporation in vitro after stimulation with anti-CD23 MoAb, but have not been able to support tumor cell growth in vitro. Those NHL-B that have been EBNA negative (greater than 50%) have been CD23 negative on immunophenotypic analysis and have shown neither TdR incorporation nor cell growth in vitro in the presence of anti-CD23 MoAb.

The presence of EBNA negative NHL-B cell lines suggests again, as in the Burkitt lymphoma cell lines previously described that have shown the lack of expression of this antigen, that transformation is not necessary for establish-
sensitivity to growth factor(s) or, alternatively, have led to aberrant expression of autocrine growth factor genes by the lymphoma cells is of particular interest.

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