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 autochthonous 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.

THE NON-HODGKIN’S lymphomas (NHL) are a heterogeneous group of human lymphoid neoplasms, principally derived (≈80%) from the B-lymphoid lineage (NHL-B). The NHL are among the most common human neoplasms, being of particular interest to both hematologists as well as immunologists due to their close phenotypic similarity and association with the lymphoid cells of the immune system. Although these tumors are often readily accessible to biopsy in peripheral lymphoid tissues, or by bone marrow aspiration, splenectomy, etc, and amenable to suspension cell culture by routine tissue culture techniques, the tumor cells have been difficult if not impossible to reproducibly grow in vitro. The single possible exception to this general rule is Burkitt’s lymphoma (BL; small noncleaved cell lymphoma), which usually contains the Epstein-Barr virus (EBV) genome that is thought to impart in vitro immortalization as one of the manifestations of the expression of the EBNA gene(s). The establishment of other NHL cell lines has been sporadically reported since the original studies of Epstein and Kaplan, but these cell lines have generally arisen "spontaneously" and have, for the most part, provided little information regarding the necessary and sufficient conditions for in vitro establishment. We have reported that fresh NHL-B, obtained from lymph node biopsies, etc, can be stimulated to proliferate in vitro in response to partially purified 12 Kd B-cell growth factor (BCGF). Furthermore, we have also shown that in the chronic B-cell leukemia, hairy cell leukemia (HCL), BCGF will not only stimulate tritiated thymidine incorporation in vitro, but will also support the growth of the leukemic cells in culture for extended periods. In the studies reported here, we show that a spectrum of high grade NHL-B, extending from relatively immature to mature B cell phenotypes, can be established in vitro to yield long-term cell lines, using the lineage-specific growth factor, 12 Kd BCGF. These cell lines appear to be representative of the original lymphomatous disease process from which they were derived, and as such provide valid experimental model systems for studying immunologic, molecular, and cell biologic aspects of human B cell lymphoma.

MATERIALS AND METHODS

Patients. Both untreated and relapsing and/or refractory treated patients with NHL-B were studied. Patient samples included bone marrow aspirations, fine needle aspirations (FNA) from involved lymph nodes, and body cavity fluids containing lymphoma cells. The specimens were usually obtained at the time of initial biopsy or when the patient presented in relapse. A brief description of the patients from which the cell lines were derived is presented in Table 1.

Characterization of lymphoma cells. Patient samples were studied for histopathologic type of lymphoma, using the International Working Formulation (IWF) and modified Rappaport classifications by comparing initial cell suspension cytospin (Shandon Southern, Pittsburgh, PA) preparations with the permanent biopsy paraffin sections, or from the cytologic prepared specimens in the case of fluids or aspirations. Immunophenotypic analysis of single cell suspensions were performed as previously described, using standard T and B lymphocyte-associated panels of murine monoclonal antibodies (MoAbs). Neoplastic B lymphoid cell populations stained for immunofluorescence were analyzed either by fluorescence microscopy (Leitz Orthoplan with Ploem illumination) or using flow cytometry (Becton-Dickinson Fluorescence Analyzer, B-D FACS systems, Mountainview, CA). Immunophenotypic profiles of the established cell lines are also shown in Table 1.

Cytogenetics. Cell cultures were enriched for mitotic cells by a 4°C cold shock inhibition of growth and subsequent release by incubation at 37°C. Aliquots were harvested after 16 hours at 2- to 4-hour intervals, using 0.004 µg/mL colcemid for 15 minutes to disrupt spindles. Cells were exposed to hypotonic KCl (0.075 methanokacetic acid, 3:1). Air dried slides were made from the cell suspension. Harvests showing the optimal mitotic indices were banded using conventional Q, G, and C banding techniques (Trent et
typed, and prepared for cell culture. Duplicate cell cultures were nonadherent cell population was cell surface-phenotyped, karyo-

RNA polymerase II, 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, methotrexate, 6-mercaptopurine, 6-thioguanine.

grown in low (fetal calf serum [FCS]) conditions and exposed on X-ray film for 24 hours at 70°C.

immunoglobulin gene rearrangement studies. 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 BamH1 and HindIII digestions for IgH, and CK and/or CX for IgL.

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 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 high grade 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 and 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

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Pt Age (yr)</th>
<th>Race/Sex</th>
<th>Dx</th>
<th>Immunophenotype</th>
<th>Cytogen</th>
<th>Therapy</th>
<th>Clinic Response</th>
</tr>
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<tbody>
<tr>
<td>CJ</td>
<td>64/W/F</td>
<td></td>
<td>NPDL-DLC (NSCL)</td>
<td>CD19, 20, 10, S IgM, DR+, CD2, 2- 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/W/M</td>
<td></td>
<td>DLCL (IB)</td>
<td>CD19, 20, 22, DR+, Ig, CD2, 2- Tac (IL-2R)</td>
<td>t(8, 14)</td>
<td>M-MIPE</td>
<td>CR, relapse/died of refractory lymphoma</td>
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<td>70/H/M</td>
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<td>DLCL</td>
<td>CD19, 20, S IgM, DR+, CD2, 3, 4, 8, IL-R</td>
<td>t(14, 18)</td>
<td>CHOP/Bleo</td>
<td>PR, relapse/died of refractory lymphoma</td>
</tr>
<tr>
<td>FM</td>
<td>43/W/M</td>
<td></td>
<td>DLCL (FCC)</td>
<td>CD19, 20, Ig (k) DR+</td>
<td>t(14, 18)</td>
<td>CVP, M-BACOD</td>
<td>PR, progress transformation</td>
</tr>
<tr>
<td>RT</td>
<td>16/W/M</td>
<td></td>
<td>ALL with lymph node involvement</td>
<td>CD19, 20, 10, S IgM, CD2, 3, S Ig-</td>
<td>t(11, 17) and multiple clonal abnormalities</td>
<td>ARA-C</td>
<td>PR, CNS/relapse/died of ALL</td>
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</table>

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

Abbreviations: PR, partial response; CR, complete remission; NPDL, nodular poorly differentiated lymphocyte; DLCL, diffuse large cell lymphoma; NSCL, nodular small cleaved cell lymphoma; NLCL, nodular large cell lymphoma; ALL, acute lymphoblastic leukemia; CHOP: cytotoxan, Adriamycin, oncovin, prednisone; COP: cytoxan, vincristine, 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, methotrexate, 6-mercaptopurine, 6-thioguanine.

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 P32 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.
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 (large noncleaved cell, immunoblastic, etc) of large cell lymphoma. While the tumors showed some morphologic 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-β-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
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. 18 No proliferative activity was observed on normal IL-2-depend
Table 3. Production of Growth Factor Activity by B-Cell Lymphoma Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Supernatant</th>
<th>Target Cells</th>
<th>SN %</th>
<th>cpm*</th>
<th>SN %</th>
<th>cpm*</th>
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<tr>
<td></td>
<td>Autogenous</td>
<td>Normal B Cells</td>
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<tr>
<td>Lymphoma Cells</td>
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<td>RR</td>
<td>0</td>
<td>2.418</td>
<td>0</td>
<td>940</td>
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<td></td>
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<td>6,190</td>
<td>20</td>
<td>9,864</td>
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Cell lines were cultured at 10^5/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. Autogenous lymphoma cells or normal BCGF-dependent human B cells (10^5 per well) were washed, exposed to serial dilutions of the above supernatants, and cultured for 72 hours at 37 °C. The microtiter wells 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%.

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-
Fig 8. Southern blot analysis showing IgH gene rearrangements in NHL-B cell lines. Isolated cell line DNA was cut with the restriction endonuclease BamHI, and hybridized with a Jy probe that recognizes a germline 17 kb BamHI fragment. Lane 1, placental DNA; lane 2, RR NHL-B cell line; lane 3, CJ NHL-B cell line; lane 4, FM NHL-B cell line; lane 5, JT NHL-B cell line.

Fig 9. Spontaneous secretion of ng/mL IgM (■) or IgG (□) secretion in vitro from 72-hour supernatants of NHL-B cell lines, measured by micro-ELISA assay.
ment of NHL-B cell lines. Finally, it should be noted that our greatest success in establishing NHL-B has been in those patients with relapsing, relatively chemotherapeutically resistant disease. The establishment of such lines has augured a particularly poor prognosis in these patients, in that all have died within 6 months of our establishing the cell lines. Whether the various treatment modalities used in these patients have contributed to the apparent increased in vitro 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.

ACKNOWLEDGMENT

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