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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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 non-cleaved 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 mol/L) for 20 minutes followed by several changes of fixative (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
al, 1986). Karyotype descriptions are according to ISCN nomenclature (1985).12

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.8 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 10% fetal calf serum (FCS; Hyclone, Logan, UT). BCGF (12 Kd) was used either in the partially purified state as purchased from Genentech, South San Francisco, CA). Cell lines were obtained from Genentech, South San Francisco, CA.)

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.19

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 by Ig gene rearrangement, and for cytokenetic 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 7 days.
5-day intervals by assaying for tritiated thymidine ($^{3}H$-TdR) incorporation, and in representative wells, for increase in viable cell growth by hemacytometer counts using trypan blue dye exclusion. Other lymphoid growth factors such as IL-2, IL-4 (BSF-1), IL-6, or lymphotoxin (LTN) did not stimulate $^{3}H$-TdR incorporation in fresh lymphoma cells, or could not support the growth of freshly explanted or cultured lymphoma cells within the first 10 days in vitro. Once established in vitro, the lymphoma cell cultures showed similar reactivities to the initial responses to growth factors obtained. Established B-cell lymphoma cell lines were then assayed for proliferative responses to BCGF, IL-2, IL-4, IL-6, and LTN in $^{3}H$-TdR incorporation assays. The results of a representative experiment for the B-cell lymphoma cell lines RR, CJ, and RT are shown in Table 2, where it can be observed that BCGF (12 Kd) stimulates a strong proliferative response, while the other cytokines show only weak reactivity.

Large cell lymphomas, B-cell type. Large cell lymphomas are a heterogenous 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 IA and R) 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 2. Giemsa-banded karyotype of RR cell line from early in vitro passage, showing 44, XY, -17, -19, del(2)(q14q32), t(8;14)(q22q32), del(17) t(11;17)(q13;p13), del(19)t(19;7)(q10;q10).
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-
Production of Growth Factor Activity by B-Cell Lymphoma Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Supernatant</th>
<th>Autologous Lymphoma Cells</th>
<th>Normal B Cell Line</th>
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<tr>
<td></td>
<td>SN %</td>
<td>cpm *</td>
<td>SN %</td>
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<tr>
<td>RR</td>
<td>0</td>
<td>2.418</td>
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<td>42.968</td>
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<td>76.200</td>
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<td>20</td>
<td>6.190</td>
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Cell lines were cultured at 10⁵/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. Autologous lymphoma cells or normal BCGF-dependent human B cells (10⁴ per well) were washed, exposed to serial dilutions of the above supernatants, and cultured for 72 hours at 37 °C. The microplates 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 microtiter 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-
hensive immunophenotypic analysis of any group of human tumors. When such extensive immunophenotyping is combined with genotypic analysis by current cytogenetic and molecular techniques, the resulting biologic data base is probably the most comprehensive available for any type of human tumor. Such a comprehensive data base for tumor cell characterization can in turn allow for correlation with a wide variety of experimental parameters.

Given the experimental potential of human NHL systems, the availability of long-term cell lines from representative subtypes of NHL-B should be particularly useful in delineating the biology of these lymphomas. Since studies by many groups, including our own, have shown that NHL cells do not usually grow spontaneously in vitro, under standard in vitro culture conditions for human lymphoid cells, the use of exogenous lineage-specific growth factor (BCGF) for initial growth stimulation is of considerable importance from a technical perspective. Our studies over the past 2 years have indicated that we could grow the majority (70%) of fresh NHL-B for at least 4 weeks in vitro in a growth factor-dependent manner. It has been possible to establish a significant number of the intermediate and high grade (aggressive) NHL-B into long-term cell lines that subsequently become independent of exogenously provided BCGF, due at least in part to the secretion of a BCGF-like autocrine growth factor molecule by the lymphoma cells. We have used one of these cell lines to purify and characterize this growth factor molecule, which cross-reacts with the high 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 in our experience have shown moderate amounts of H-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 H-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-
mement 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 resis-
tant 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.

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