Phenotypic Analysis of Established Diffuse Histiocytic Lymphoma Cell Lines Utilizing Monoclonal Antibodies and Cytochemical Techniques

By Jane N. Winter, Daina Variakojis, and Alan L. Epstein

Ten diffuse histiocytic lymphoma (DHL) cell lines were extensively characterized with monoclonal antibodies and histochemical techniques. The original biopsy specimens, representing nine of ten cases from which the cell lines were derived, were reviewed utilizing the International Working Formulation. Eight of ten cell lines reacted with antimmunoglobulin reagents and/or a subset of B-lymphocyte surface markers, supporting a B-cell derivation. Only U-937, a monotypoid DHL cell line reactive with OKT4 and 6, displayed any T-cell markers. Cytochemical analysis alone proved to be of little value in the subclassification of the DHls. The pathologic review revealed that, despite disparate immunologic phenotypes, five of the diffuse large cell lymphomas were subclassified as large, noncleaved lymphomas. Our analysis confirms the phenotypic diversity of this subgroup of malignant lymphomas and underscores the value of monoclonal reagents for the immunologic evaluation of the hematologic malignancies. These well-characterized cell lines constitute a valuable resource for the laboratory investigation of the lymphomas.

The diffuse histiocytic lymphomas (DHL), as described by Rappaport, represent a morphologically diverse group of neoplasms. The heterogeneous nature of these lymphomas has been confirmed by immunologic, cytochemical, and ultrastructural studies. The majority of these tumors are lymphoid in origin; only rarely do they display characteristics of histiocytes, such as lysozyme production and nonspecific esterase content. They commonly bear surface immunoglobulins characteristic of B lymphocytes, but a percentage appear to be null or T cell in derivation when studied with conventional techniques.

The advent of somatic cell hybridization methodology for the production of monoclonal antibodies has furnished immunologic reagents with improved sensitivity and specificity with which to study lymphoma cell lines and biopsy specimens. Monoclonal antibodies directed against newly discovered markers of T- and B-cell lineage permit a far more extensive phenotypic analysis than previously possible with conventional procedures. Cytochemistry provides additional information which may, in concert with the immunologic phenotype, help elucidate the histogenesis and basic biologic nature of these tumors.

The study of the hematologic malignancies has been greatly facilitated by the development of cell lines derived from these neoplasms. In this report, ten DHL cell lines have been extensively characterized utilizing monoclonal antibody reagents. Histochemical analysis has also been performed to provide a detailed biologic profile. The original biopsy specimens have been reviewed utilizing the International Working Formulation.

MATERIALS AND METHODS

Pathologic Review of Biopsy Specimens

The original materials, representing nine of the ten cases from which the cell lines were established, were reviewed and the original diagnoses confirmed. The International Working Formulation was applied.

Cell Lines

The establishment of cell lines SU-DHL-1, 2, and 4-9, has been previously reported. The SU-DHL-6 cell line was recently re-established from an ampule of the original peritoneal fluid. NU-DHL-1, a newly established DHL cell line derived from a malignant lymph node, will be reported separately. The U-937 line was the generous gift of Dr. K. Nilsson, Uppsala, Sweden.

All cell lines were grown in RPMI 1640 medium supplemented with 15% fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. All the DHL cell lines, except for the newly established NU-DHL-1 cell line, are currently grown without human serum. NU-DHL-1 requires supplementation with 10% heat-inactivated pooled human serum collected from healthy adult donors. All cultures were maintained at 37°C in a humidified 5% CO2 incubator and were passaged twice weekly. Microbiologic tests, using the mycotrim culture technique (Hana Media, Inc., Berkeley, CA), revealed that, except for U-937, all the DHL cell lines were free of mycoplasma contamination.

Cytochemistry

The following stains and reactions were performed on cytopsins of the cell lines using standard cytochemical techniques: periodic


**Monoclonal Antibodies**

The following commercially available monoclonal antibody reagents were used: mouse anti-human kappa, lambda, IgA, D, E, G, and M (Bethesda Research Laboratories, Rockville, MD); OKM1, OKT3, OKT4, OKT6, OKT8, and OKT11 (Ortho Pharmaceutical Corp., Immunobiology Branch, Raritan, NJ); J5, Bl, and B2 (Coulter Immunology, Hialeah, FL); and BA-1 and BA-2 (Hybritech, La Jolla, CA). Antibody LN-1, a mouse anti-human monoclonal antibody directed against follicular center cells, was generated in our laboratory and has been reported in abstract form.

Monoclonal antibodies SC2 and SC3 identify the human Ia antigen (p28,34) and were the kind gifts of Dr. Robert Fox, Scripps Clinic and Research Foundation, La Jolla, CA.

**Live Cell Indirect Immunofluorescence**

Cells were washed twice with phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin (BSA) and 0.02% sodium azide. Single-cell suspensions, containing 1.0 x 10⁶ cells in a volume of 200 μl, were incubated for 30 min with specific antibody at 4°C. Cells were then washed to remove excess antibody and incubated with a 1:20 dilution of fluorescein-conjugated goat anti-mouse IgG, F(ab')² fragment specific (Cappel, Cochranville, PA), for 30 min at 4°C. Following 2 additional washes, 2 drops of mounting solution composed of 1:1 glycerol and PBS, pH 8.0, and 2% formaldehyde (4018, Polysciences, Warrington, PA) were added to each tube. The cells were mounted onto a glass slide and examined within 24 hr by epi-fluorescence microscopy using a Leitz Orthoplan microscope with a Ploemopak 2.1 fluorescence illuminator, HBO 100 mercury lamp, and 50x water immersion objective. A minimum of 200 cells were examined for immunofluorescence by two independent observers. Supernatants from NS1 myeloma cultures were used to determine background staining.

**Fixed Cell Immunofluorescence**

To examine cells for the presence of cytoplasmic immunoglobulin and nuclear terminal transferase (TdT), fixed cell preparations were used. TdT was assessed using commercially available heterologous antisera (P-L Biochemicals, Inc., Milwaukee, WI). Cells were washed in PBS containing 1 mg/ml BSA and 0.02% sodium azide and pipetted dropwise at a concentration of 5.0 x 10⁶ cells/ml onto Teflon-coated printed microscope slides containing 10 5-mm wells/ slide (Cel-Line Associates, Inc. Newfield, NJ). After the cells settled to the surface of the glass, the overlying fluid was slowly removed by aspiration and the cells dried to the slide by a gentle stream of warm air. The slides were then immediately fixed in 2% formaldehyde (4018, Polysciences) in PBS for 15 min at room temperature. After fixation, the slides were rinsed in PBS and placed in aceton at 20°C for 3 min to make the cells permeable.

After a final PBS rinse to remove the aceton, the slides were stored at 4°C in PBS containing 0.02% sodium azide.

For the immunofluorescence assay, 35 μl of each antibody preparation was pipetted onto a well of the printed microscope slide preparations. After 60 min of incubation at 37°C in a humidified chamber, the slides were rinsed 3 times in PBS and again incubated for 30 min at 37°C with 20 μl of a 1:20 dilution of fluorescein-conjugated goat anti-mouse IgG, F(ab')² fragment specific (Cappel), or fluorescein-conjugated goat anti-rabbit IgG for the TdT assays. The slides were then rinsed 3 times in PBS, counterstained with Evans Blue for 5 min at room temperature, using a freshly prepared solution containing 50 μl of a 1% stock solution of Evans Blue in 80 ml of PBS, rinsed a final time in PBS, and mounted with coverslips using a 1:1 solution of glycerol and PBS, pH 8.0.

The cell lines were also examined for the presence of the Epstein-Barr virus-associated nuclear antigen (EBNA) using the procedure of Reedman and Klein.

**RESULTS**

**Morphological Classification**

Original biopsy specimens representing nine of the ten cases from which the cell lines were established were reviewed, and these results are summarized in Table 1.

Only a scalp biopsy could be found on the patient from whom SU-DHL-6 was derived. Whereas the scalp biopsy showed diffuse lymphoma, mixed small and large cell type, a lymph node biopsy on that patient is reported as having shown "histiocytic lymphoma." The presence of multiple histologies occurring in the same patient with lymphoma is well documented.

**Table 1. Pathologic Review of Histiocytic Lymphoma Cell Lines**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Source of Cell Line</th>
<th>Tissue Reviewed</th>
<th>Diagnosis (Working Formulation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SU-DHL-1</td>
<td>Pleural effusion</td>
<td>Lymph node</td>
<td>DLNC</td>
</tr>
<tr>
<td>SU-DHL-2</td>
<td>Pleural effusion</td>
<td>NA*</td>
<td></td>
</tr>
<tr>
<td>SU-DHL-4</td>
<td>Pleural effusion</td>
<td>Lymph node</td>
<td>DLC</td>
</tr>
<tr>
<td>SU-DHL-5</td>
<td>Lymph node</td>
<td>Lymph node</td>
<td>DLNC</td>
</tr>
<tr>
<td>SU-DHL-6</td>
<td>Peritoneal effusion</td>
<td>Scalp†</td>
<td></td>
</tr>
<tr>
<td>SU-DHL-7</td>
<td>Pleural effusion</td>
<td>Lymph node</td>
<td>FL</td>
</tr>
<tr>
<td>SU-DHL-8</td>
<td>Pleural effusion</td>
<td>Lymph node</td>
<td>DLNC</td>
</tr>
<tr>
<td>SU-DHL-9</td>
<td>Peritoneal effusion</td>
<td>Pleural biopsy</td>
<td>DLNC</td>
</tr>
<tr>
<td>U-937</td>
<td>Pleural effusion</td>
<td>Spleen</td>
<td>DLC</td>
</tr>
<tr>
<td>NU-DHL-1</td>
<td>Lymph node</td>
<td>Lymph node</td>
<td>DLNC</td>
</tr>
</tbody>
</table>

NA, not available. DLNC, diffuse large cell lymphoma, noncleaved cell type; DLC, diffuse large cell lymphoma, cleaved cell type; FL, follicular large cell lymphoma.

*The original diagnosis, based on a gastrectomy specimen, was "histiocytic lymphoma."

†Only a scalp biopsy was available for review and showed diffuse lymphoma, mixed small and large cell type. A lymph node biopsy from this patient is reported as having shown "histiocytic lymphoma."

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The morphology of the cytospin from SU-DHL-6 is similar to that of the other cell lines supporting a "large cell" derivation.

Wright's-Giemsa stained cytospin preparations of all the cultured cell lines were examined and were morphologically similar to the cell lines as they were first described in 1978.5

Cytochemistry

The cytochemical analysis of the cell lines is summarized in Table 2. The overall intensity and quantity of the staining and reaction products varied among the cell lines. Furthermore, there was great cell-to-cell variability within the individual cell lines.

The acid phosphatase (ACP) reaction product was uniformly positive in all cell lines. It appeared predominantly in a granular form diffusely scattered throughout the cytoplasm, although in SU-DHL-1 and -2 and NU-DHL-1, it demonstrated a somewhat more polarized distribution. All the cell lines were tartrate-sensitive, except NU-DHL-1, in which case a small number of cells had a positive reaction product after tartrate inhibition. Both the ANBE and ACP reactions were intensely and diffusely positive in nearly all U-937 cells.

Immunologic Characterization

The DHL cell lines were phenotyped utilizing monoclonal antibody reagents. Table 3 summarizes these results.

Although IgG with kappa light chains was detected in the cytoplasm of SU-DHL-7, only light chains were identified on the cell surface. SU-DHL-8 and 9, previously reported to have neither surface nor cytoplasmic light nor heavy chains,5 were both shown to have trace amounts of cytoplasmic light chain only, supporting a B-cell derivation for these two cell lines.

Whereas SU-DHL-6 was previously reported to produce lambda light chains,6 we found 100% of the cells to be kappa positive. Subsequent to the original publication, cytogenetic analysis revealed that the SU-DHL-6 cultures had been contaminated with SU-DHL-5, and they were discarded. SU-DHL-6 has now been reestablished from an ampule of the original peritoneal fluid and is reported here. A second discrepancy involves SU-DHL-7, which was previously reported to have both cytoplasmic IgG and IgA as well as lambda light chains. Using highly specific monoclonal reagents, we found this cell line to be uniformly IgG, kappa positive. The reason for this discrepancy is presently unknown.

BA-1, B1, B2, and LN-1, newly developed monoclonal antibodies reactive with differentiation antigens characteristically associated with lymphocytes of B-cell lineage, were also used to study the cell lines. All DHL lines, including SU-DHL-1 and -2 and U-937, reacted with BA-1, suggesting a common lymphoid derivation. However, neither SU-DHL-1 nor U-937 reacted with B1 or with either of two anti-Ia monoclonal antibodies, and none of the cells lines reacted with B2. The majority of the DHL cell lines were positive when screened against LN-1, a monoclonal antibody produced in our laboratory that identifies a subset of B cells.

The ten cell lines were tested with a panel of monoclonal antibodies identifying T-cell-related antigens. Only U-937 displayed any reactivity with these reagents. Despite its lysozyme production and phagocytic activity, which have caused some investigators to label it as "monocytoid," U937 did not react with OKM1.

TdT, a nuclear enzyme characteristic of immature lymphocytes found in normal thymus and bone marrow and certain leukemias, was evaluated by indirect immunofluorescence using commercially available heterologous antisera (P-L Biochemicals). All ten DHL cell lines were negative. NS1 supernatant was used to assess background staining. The null-ALL cell line REH was used as a positive control.

Consistent with previous results, all of the cell lines

Table 2. Cytochemical Analysis

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>PAS</th>
<th>SBB</th>
<th>PEROX</th>
<th>ACP</th>
<th>ACPT</th>
<th>ANAE</th>
<th>ANBE</th>
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<tbody>
<tr>
<td>SU-DHL-1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
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<td>+</td>
</tr>
<tr>
<td>SU-DHL-2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>SU-DHL-4</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>SU-DHL-5</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>SU-DHL-6</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>SU-DHL-7</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>±</td>
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<tr>
<td>SU-DHL-8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SU-DHL-9</td>
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<td>-</td>
<td>+</td>
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<tr>
<td>U-937</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+++</td>
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<td>++</td>
</tr>
<tr>
<td>NU-DHL-1</td>
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<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

PAS, periodic acid-Schiff; SBB, Sudan black B; PEROX, peroxidase; ACP, acid phosphatase; ACPT, acid phosphatase with tartrate; ANAE, alpha-naphthyl acetate esterase; ANBE, alpha-naphthyl butyrate esterase.
were negative for the presence of the Epstein-Barr-virus-associated nuclear antigen.

DISCUSSION

Utilizing a panel of monoclonal antibody reagents and cytochemical techniques to characterize ten DHL cell lines, we have confirmed the phenotypic diversity of this subgroup of malignant lymphomas. Although a sample of ten is unlikely to represent the full spectrum of phenotypes found in DHL, our analysis provides important clues to the immunobiology of these tumors and underscores the value of monoclonal antibody reagents in the immunologic characterization of the hematologic malignancies.

Our studies with monoclonal antibody reagents expand upon earlier investigations that utilized conventional immunologic techniques. Studies of frozen biopsy sections and single-cell suspensions have previously demonstrated that most DHLs are lymphoid and of B-cell lineage.6,8 In the past, the B-cell derivation was established by the presence of cell surface immunoglobulin (SIg), usually of the IgM heavy chain subtype. Using monoclonal reagents, we have demonstrated that seven of the ten DHL cell lines are B-cell derived by virtue of the presence of partial or complete immunoglobulin. Both surface heavy and light chains were detected on four of the ten DHL cell lines, and of these, three of four heavy chain classes were IgM. Studies with fixed cell preparations suggested that these immunoglobulins were also present in the cytoplasm. One cell line lacked heavy chains on its surface but had both cytoplasmic heavy and light chains. Two cell lines, previously reported as null when studied with heterologous antisera,5 were found to have small but discrete amounts of intracytoplasmic light chains. The monoclonality of the cell lines was confirmed by their restricted expression of only one immunoglobulin light chain class.24 Neither cytoplasmic nor surface immunoglobulin was detected on cell lines SU-DHL-1 and -2 and U-937.

In the absence of SIg or sheep erythrocyte receptors (E), a subset of DHLs has, in the past, been classified as “null.”6-8 Some of these may be of true histiocytic derivation as evidenced by morphological and functional characteristics. Others may represent B cells that have lost characteristic surface markers in the process of malignant transformation. Monoclonal antibodies reactive with lymphoid antigens specific for distinct stages of B-cell differentiation are particularly useful in the phenotypic analysis of tumors that lack SIg or T-cell markers. For example, our panel of B-lymphocyte markers has helped to identify SU-DHL-2 as a B-cell-derived line, despite its lack of cytoplasmic or surface immunoglobulin. In addition, many lymphomas identified in the past as “null” using heterologous antisera may be found to have surface and/or cytoplasmic immunoglobulin when studied with more highly sensitive monoclonal reagents. The routine use of a battery of monoclonal antibodies, such as that used in this investigation, may generate important new information concerning the immunobiology of the DHLs.

Neither the common acute lymphoblastic leukemia antigen (CALLA)19 nor the BA-223 antigen, found on ALL cells, was identified on the DHL cell lines. CALLA has been detected on some B-cell lymphomas believed to be more mature than the primitive B lymphocytes of common ALL, and therefore may not represent a discrete stage of lymphoid differentiation.29 Its value for the subclassification of the lymphomas is uncertain.

Human Ia (immune-associated) antigens, polymorphic glycoproteins linked to the major histocompatibility complex, were detected on all the DHL cell lines.
with the exception of SU-DHL-1 and U-937. Whereas Ia antigens were initially thought to be restricted to B lymphocytes, they have been identified on other hematopoietic cells, such as immature myeloid and erythroid cells, activated T cells, and monocytes. Common and pre-B-ALL have been reported to possess Ia antigens. The Ia positivity of the DHL cell lines is consistent with their B-cell derivation. The presence of Ia-like antigens on U-937 cells has been reported by Nilsson et al., who were able to immunoprecipitate a dimeric protein with the expected molecular weights of approximately 29,000 and 34,000 daltons. A heterologous rabbit anti-human antisera was used in both their immunoprecipitation and immunofluorescence assays. The discrepancy between their data and ours may be attributable to the emergence of an Ia- clone or to differences between the antisera used.

Fewer than 15% of DHLs have T-cell markers. In our study, only U-937 reacted with any of the T-cell antibodies tested. Using conventional E-rosetting techniques to characterize this cell line, other investigators have concluded that it is not T-cell derived. We have found that OKT4 and OKT6, both markers of common thymocytes and activated monocytes, were reactive with the majority of U-937 cells. This cell line has been extensively studied and is generally thought to be derived from an immature monocyte. That OKM1, a marker of peripheral blood monocytes, did not react with U-937 is most likely a reflection of the cell line's poorly differentiated state. Characteristics cited as support for its monocytic lineage have included the secretion of lysozyme, the presence of cytoplasmic nonspecific esterases inhabitable by sodium fluoride, and its surface glycoprotein profile. Furthermore, it has been demonstrated that both morphological and functional differentiation of U-937 cells into monocyte-like cells can be induced in vitro. The presence of strong, diffuse acid phosphatase and alpha-naphthyl butyrate esterase staining also supports a monocytic lineage. Recently, it has been reported that alpha-antitrypsin, synthesized by cells of the monocyte/macrophage lineage, is a highly reliable marker of malignant histiocytes, and, in one large series of large cell lymphomas, it identified 10% as truly "histiocytic." The identification of alpha-antitrypsin in the DHL cell lines is presently being investigated.

Terminal deoxynucleotidyl-transferase (TdT) is recognized as a marker of immature lymphocytes. Elevated levels of TdT are found not only in lymphocytes but also in disorders of early B cells such as common ALL. Non-Hodgkin's lymphomas, with characteristics of more mature B cells, however, have negligible levels of this marker. The majority of DHL biopsy specimens studied, like the ten cell lines reported here, are negative when studied with TdT. Although TdT has been shown to be a useful marker of lymphoid derivation for the study of the leukemias, its precise role in the classification of the non-Hodgkin's lymphomas remains to be defined.

Consistent with previous cytochemical analyses of DHL, the cell lines were PAS, acid phosphatase, and alpha-naphthyl esterase positive. The intensity and pattern of staining was variable among the cell lines and did not correlate with morphological or immunologic features. Cytochemical analysis by itself appeared to be of little value in the subclassification of the DHLs. The significance of the various staining patterns may become evident after further cytochemical studies are performed.

Using the International Working Formulation, five of the diffuse large cell lymphomas were subclassified as large, noncleaved lymphomas. Despite similar morphologies, these cell lines had disparate immunologic phenotypes. This suggests that phenotypic analysis of the non-Hodgkin's lymphomas using monoclonal antibodies can provide information not conveyed by morphological studies alone.

Cell lines constitute an invaluable resource for the laboratory investigation of neoplastic disease. In the past, most of the DHL cell lines were established from malignant effusions. With new tissue culture techniques and greater experience, it has become possible to establish cell lines from biopsy specimens, a more available source of tissue in this disease. NU-DHL-1, and a new DHL cell line recently reported by Dillman et al., LNPL, were both derived from lymph node biopsies. The expanding library of lymphoma cell lines will be especially helpful to the molecular geneticist studying the relationship of karyotypic abnormalities and oncogenes. Cell line LNPL has been found to have the classic translocation between chromosomes 8 and 14, first described in Burkitt's lymphoma. The SU-DHL cell lines have also been karyotyped, and contain a number of 14 q abnormalities including the 14; 18 translocation. The existence of lymphoid cell lines also facilitates the study of immunoglobulin gene expression and lymphoid differentiation. They are ideal immunogens for use in generating monoclonal antibodies for the study and classification of the lymphoid malignancies. Our laboratory is currently engaged in producing monoclonal reagents to the DHLs, utilizing these cell lines for immunization and screening purposes. Well characterized cell lines will also aid in the evaluation of the specificity and usefulness of new monoclonal antibody reagents.

In summary, ten DHL cell lines have been phenotyped using monoclonal antibody reagents and cytochemical techniques. We have provided evidence that
the majority of these cell lines are derived from B cells and that, despite some morphological similarities, they differ cytochemically and in their degree of differentiation. Subclassification of this heterogeneous group of lymphoid neoplasms according to their immunologic phenotypes may prove to have important clinical ramifications.

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