A Single Monoclonal Antibody Identifies T-Cell Lineage of Childhood Lymphoid Malignancies

By Michael Link, Roger Warnke, Jonathan Finlay, Michael Amylon, Richard Miller, Jeanette Dilley, and Ronald Levy

Immunophenotyping studies with monoclonal antibodies have revealed the heterogeneity of childhood acute lymphoblastic leukemia (ALL) and non-Hodgkin’s lymphoma (NHL). The lymphoid malignancies of T-cell lineage are particularly heterogeneous and, until now, no single monoclonal antibody has been found to identify all cases of T-ALL and T-NHL. A monoclonal antibody, 4H9, recognizes an antigen of 40,000 molecular weight on normal and malignant T cells. Thirty-six cases of childhood T-ALL and T-NHL were tested, and in all cases, the malignant blast cells were reactive with 4H9, whereas malignant cells from 61 cases of non-T ALL and NHL were not reactive with 4H9. Monoclonal antibody 4H9 is a sensitive and specific reagent for the identification of childhood T-cell ALL and NHL and should be extremely useful in immunophenotyping studies of lymphoid malignancies.

STUDIES OF IMMUNOLOGIC membrane markers of malignant cells from children with acute lymphoblastic leukemia (ALL) and non-Hodgkin’s lymphoma (NHL) have provided considerable insight into the heterogeneity of these diseases. At least five subgroups of childhood ALL have been defined by immunologic markers: T-ALL, B-ALL, common ALL, pre-B-ALL, and unclassified or null ALL.1-6 The T-cell phenotype is found in 15%-25% of cases of childhood ALL, and almost 50% of childhood NHLs.1,2,4-8 Cases of T-cell ALL and NHL typically present with clinical characteristics associated with unfavorable outcome, including male preponderance, anterior mediastinal mass, high numbers of circulating blast cells, and early relapse. Although the independent prognostic value of immunophenotyping studies in childhood lymphoma and leukemia has not yet been demonstrated conclusively,5,9-11 recognition of the association of the T-cell phenotype and adverse prognosis has led some investigators to select the T-ALL subgroup for more intensive treatment strategies.4 Accurate identification of the patient with T-cell ALL and NHL has thus become an important priority for selection of appropriate therapy.

The earliest immunophenotyping studies identified T-cell lymphoid malignancies by the capacity of blast cells to form spontaneous rosettes with sheep erythrocytes.12 Refinements in technology with the advent of heteroantisera and, more recently, of monoclonal antibodies, have increased the sensitivity for detecting T-cell disease and have revealed a marked heterogeneity in surface phenotype among the T-cell malignancies.13-20 A significant percentage of patients with T-cell malignancies identified by anti-T-cell monoclonal antibodies would be incorrectly classified by E-rosette testing alone.

Because of the heterogeneity of T-cell surface phenotypes, no single monoclonal antibody has been found that accurately identifies all patients with T-cell disease. We,14 and others,17 have previously reported the utility of the monoclonal antibody, anti-Leu-1, for the identification of T-cell ALL. While the blast cells from almost all cases of T-ALL and T-NHL tested have been found to be reactive with anti-Leu-1 by immunofluorescence assay, the reactivity is often weak and difficult to detect without the aid of flow cytometry. We report a novel monoclonal antibody, 4H9, which is specific for identifying childhood T-ALL and T-NHL. Moreover, the 4H9 antigen, when present, is usually expressed in high density on the blast cells, and reactivity is thus easily detected.

MATERIALS AND METHODS

Patients and Cells

Malignant cells were obtained from patients at the time of initial presentation or relapse, after informed consent was obtained. Only specimens containing greater than 80% blast cells were analyzed. The diagnosis in each case was established by standard morphologic and histochemical parameters.

Leukemia and lymphoma cells were isolated from heparinized bone marrow, peripheral blood, and malignant effusions by Ficoll-Hypaque density sedimentation. Lymphoma masses and lymph nodes were gently teased and passed through a stainless steel mesh to establish single-cell suspensions. In the majority of cases, immunophenotyping studies were performed on cells when first obtained. In some cases, immunophenotype analysis was performed on cells cryopreserved using standard techniques and stored at -196°C in 10% dimethylsulfoxide (DMSO) and 10% fetal calf serum. There was no difference in cell surface markers when performed sequentially on fresh and frozen cells.

Normal human thymus glands were obtained from fresh surgical specimens. Mononuclear cells were separated from the blood of normal volunteers by Ficoll-Hypaque density sedimentation. Granulocytes were purified from mononuclear-cell-depleted blood by dextran sedimentation followed by ammonium chloride lysis of residual
red blood cells. Mononuclear cell populations enriched for T cells were prepared with a sheep erythrocyte rosetting technique previously described, and the sheep red blood cells were lysed with ammonium chloride.

The cell lines HPB-ALL, JM, 8402, Jurkat, Sager, and SS were used in these studies. Characteristics of these lines have been previously described.

Preparation of Monoclonal Antibody 4H9

The protocol utilized for production of monoclonal antibody 4H9 has been described. Leukemia cells used for immunization were obtained from the peripheral blood of a 17-yr-old male with T-cell acute lymphoblastic leukemia (TOR-ALL). The patient had a leukocyte count of 350,000/cu mm and prominent hepatosplenomegaly but no anterior mediastinal mass. His blast cells formed spontaneous rosettes with sheep erythrocytes. In addition, the blast cells were reactive with anti-Leu-1, Leu-2a, Leu-3a, Leu-5, Leu-6, and OKT10, but nonreactive with anti-Ia, Leu-4, OKT9, and cALLa. BALB/c mice were used, and the mouse myeloma line X63-Ag.8.653 was utilized as the fusion partner. Immunization with 5 x 106 TOR-ALL cells and 5 x 106 HPB-ALL cells were carried out 16 days, 9 days, and 3 days prior to fusion. Fusion was accomplished with 38% polyethylene glycol. Methods for growing, screening, and maintaining hybrids were as previously described. Culture supernatants from a single hybridoma (4H9), produced from this fusion, were utilized in these studies.

Other Reagents

Monoclonal antibodies Leu-1 (pan-T), Leu-2a (T cytotoxic suppressor), Leu-3a (T helper), Leu-4 (pan-T), Leu-5 (E-rosette receptor), Leu-6 (thymocyte), and anti-HLA-DR were generously supplied by the Becton-Dickinson Corporation (Mountain View, CA). Monoclonal antibodies OKT9 and OKT10 were purchased from Ortho Pharmaceuticals (Raritan, NJ), and monoclonal anti-cALLa (JS) was kindly furnished by Dr. Jerome Ritz (Sidney Farber Cancer Institute, Boston, MA). The method of development of these reagents and the distribution of the antigens detected by these reagents on normal tissues and in childhood ALL have been reported elsewhere.

Immunophenotyping Studies

Antigens on the cell surface were identified by the binding of monoclonal antibody detected by indirect immunofluorescence. One million cells were placed in plastic tubes and incubated with 1 μ of purified monoclonal antibody or with 100 μ of hybridoma culture supernatant at 4°C for 20 min. Cells were washed twice in the cold in phosphate-buffered saline plus 0.02% sodium azide to remove excess antibody, and incubated for 20 min at 4°C with 100 μ of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Tago), followed by avidin-horseradish peroxidase conjugate (Vector Laboratories, Inc., Burlingame, CA). Individual cells were identified on the basis of characteristic histologic features and antigen expression.

The proportion of E-rosette-forming cells after 1 hr of incubation at 4°C and 37°C was determined by previously described methods. Surface immunoglobulin was detected using the F(ab')2 fragment of FITC-conjugated goat anti-human immunoglobulin (Tago).

RESULTS

Reactivity With Leukemias and Lymphomas

Reactivity of various subclasses of ALL and NHL with antibody 4H9 is shown in Table 1. The T-cell lineage of the blast cells from all cases of T-ALL and T-NHL was confirmed by the demonstration of reactivity with at least one T-cell-specific monoclonal antibody and/or spontaneous rosette formation with sheep erythrocytes (Table 2). In case 3, T-cell lineage was confirmed by reactivity with a xenogeneic anti-T-cell antiserum. The majority of cases demonstrated reactivity with multiple anti-T-cell-specific monoclonal antibodies, and none demonstrated reactivity with anti-Ia. Some were reactive (usually weakly) with anti-cALLa. All cases of T-cell disease were reactive with 4H9, and the blast cells from most cases demonstrated strong reactivity. In 30/36 cases of T-cell disease, more than 50% of the blast cells were reactive with 4H9 by immunofluorescence assay, and in 20/36, greater than 75% of the blast cells stained. The T-cell lines 8402, HPB-ALL, JM, Molt 4, Jurkat, Sager, and SS were all reactive with 4H9.

By contrast, blast cells from patients with non-T

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<th>Table 1. Reactivity of 4H9 With Leukemia/Lymphoma Cells</th>
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*Positive is >20% of cells staining.
†Number reactive/number of cases tested.
‡Phenotype E+ or reactive with T-specific monoclonal antibody.
§Phenotype la+, CALLa+, T+, E-.
‖Phenotype E-, T-, la+, CALLa-.
¶Phenotype la+, Slg+.
Table 2. Surface Antigens on Blast Cells From Patients With T-Cell Malignancies

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*Positive is more than 25% blasts rosette.
†Percentage of positive cells using immunofluorescence method: (+) 20%–50% positive cells, (++) 50%–75% positive cells, (+++) >75% positive cells, (–) <20% positive cells.
‡Case 3 presented with large anterior mediastinal mass—blast cells reactive with xenogeneic anti-T-cell antiserum.

All cases were Ia–.

ND, not done.

Leukemia and lymphoma did not react with 4H9 (Table 1). The non-T lineage in these cases was determined by lack of reactivity with T-cell antibodies and failure to rosette with sheep erythrocytes. The non-T lineage was confirmed by reactivity with anti-Ia. We, and others,2,16,17,37 have noted that reactivity with anti-Ia is unusual in childhood T-cell disease.

None of the antibodies utilized, other than 4H9, successfully identified all cases of T-ALL and T-NHL. The “pan-T” monoclonals, anti-Leu-4 and anti-Leu-5, failed to identify more than half of the cases in this study. All but three of the T-lymphoid malignancies tested demonstrated reactivity with anti-Leu-1, although in many cases, fluorescent staining was dull (see Figs. 1 and 2). To assess whether 4H9 would be superior to anti-Leu-1 for detecting T-cell lineage, the degrees of reactivity (as determined by the mean fluorescence intensity) of the blast cells from 31 of the patients with anti-Leu-1 and 4H9, were compared (Fig. 1B). The fluorescence intensity for the two antibodies was determined at the same photomultiplier tube voltage and gain settings for an individual patient, so direct comparison was possible. In 27/31 cases of T-cell disease, the mean fluorescence intensity was higher for 4H9 than for Leu-1, and was significantly higher in the majority of cases (Fig. 1B). However, no
correlation was evident between degree of reactivity with Leu-1 and reactivity with 4H9.

Reactivity With Normal Tissues

The reactivity of antibody 4H9 with normal tissues was tested by immunofluorescence staining of cell suspensions or by immunoperoxidase staining of frozen tissue sections where appropriate. More than 80%, but not all, of E-rosette-positive cells from normal peripheral blood stained with 4H9; a small percentage of the E-rosette-negative population appeared to be reactive with 4H9 as well. Granulocytes demonstrated no reactivity with 4H9. Further studies of the 4H9+ and 4H9− cell populations in normal blood are currently underway in our laboratory.

Thymocytes analyzed in cell suspension demonstrated heterogeneous reactivity with 4H9. Approximately 50% of thymocytes were reactive with 4H9. Thymus analyzed in frozen section revealed that approximately 50% of cortical and 50% of medullary

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**Fig. 1.** Comparison of reactivity of malignant blast cells from individual patients with T-ALL and T-NHL with Leu-1 and 4H9. (A) Comparison of percent of cells reactive with Leu-1 and 4H9. (B) Comparison of mean fluorescence intensity of specimens obtained by indirect immunofluorescence staining with Leu-1 and 4H9.

**Fig. 2.** FACS histograms obtained from indirect immunofluorescence staining of malignant cells from patient no. 20 with Leu-1 and 4H9. Reactivity with 4H9 is significantly stronger than with Leu-1. Reactivity with a nonreactive myeloma protein (control) is shown for comparison.
thymocytes stained with 4H9. Only occasional cells in sections of normal bone marrow demonstrated reactivity with 4H9.

**Characteristics of 4H9 Antigen and Antibody**

The target antigen of monoclonal antibody 4H9 was examined by SDS-polyacrylamide gel electrophoresis of 125I-labeled cell surface proteins after immunoprecipitation with 4H9 antibody. A polypeptide of molecular weight 40,000 was precipitated from the surfaces of normal peripheral blood T cells as well as from malignant T cells. Antibody 4H9 did not inhibit E-rosette formation by normal peripheral blood T cells.

The chain composition of antibody 4H9 was determined by the Ouchterlony technique and demonstrated kappa light chain and gamma 2a heavy chain.

**DISCUSSION**

The murine monoclonal antibody 4H9 recognizes an antigen of 40,000 molecular weight that is found on thymocytes and the majority (but not all) of circulating T cells. Apparently, this antigen is present on the malignant blast cells from all cases of childhood T-ALL and T-NHL. Moreover, the antigen is not found on the blast cells from cases of common ALL, B-ALL, and Burkitt's lymphoma—all lymphoid malignancies shown to be of non-T lineage. 4H9 is thus a discriminating and extremely useful diagnostic reagent for immunophenotyping studies of childhood leukemia and lymphoma. In our studies, 4H9 correctly identified all cases of T-cell lymphoid malignancy; conversely, cases of non-T lineage could be confirmed by lack of reactivity with 4H9.

A variety of diagnostic tests have been utilized to identify T-cell disease. The E-rosette test provided the first insight into the immunologic heterogeneity of ALL and has been the standard to which newer reagents have been compared. Methodological variability, the requirement of a single-cell suspension, and false positive results because of cross-reacting antigens, have limited the reliability and applicability of this test. Moreover, we and others have noted cases of T-ALL, as identified by monoclonal antibody testing, that were E-rosette negative.

The availability of monoclonal antibodies has overcome many of the limitations of the E-rosette test by providing unlimited quantities of homogeneous reagents for immunophenotyping studies. Unfortunately, no single reagent has been found to be satisfactory for the identification of all cases of T-lymphoid malignancy. In particular, the group of T-ALLs and T-NHLs demonstrating the early thymocyte phenotype have been a difficult group to classify. Monoclonal antibodies to the E-rosette receptor (Leu-5 and OKT11) have overcome some of the technical difficulties of E-rosette testing. While these antibodies have proven to be very sensitive detectors of T-cell lineage in some reports, this has not been our experience (Table 2), since more than half of the cases reported here failed to react with anti-Leu-5, and correlation with E-rosette testing was not accurate. This may reflect low expression of the Leu-5 antigen by T leukemic blast cells in many cases forming E-rosettes.

The T lineage of some cases of ALL that are E-rosette negative has been identified by reactivity with OKT10 and/or OKT9 (neither of which is T lineage nor lymphoid specific) and lack of reactivity with anti-Ia antibody. The T-subset-specific monoclonal antibodies, Leu-6 (OKT6), Leu-2a (OKT5 and OKT8), and Leu-3a (OKT4), are useful, but the majority of T-ALLs are not reactive with these reagents.

Another pan-T monoclonal antibody, anti-Leu-4 (OKT3), recognizes an antigen present on mature T lymphocytes, but is frequently absent on T-ALL and T-NHL cells. The antigen identified by anti-Leu-1 (L17F12) has been found on the blast cells from most cases of T-ALL and T-NHL, but expression of this antigen is often weak, and in many cases, is not detectable without the aid of flow cytometry. 4H9 should prove to be a useful addition to the panel of monoclonal antibodies available for phenotyping studies. Although a small percentage of peripheral blood T cells are not reactive with 4H9, all T-ALLs and T-NHLs tested are easily identified by this antibody. Malignant cells from these patients usually demonstrate strong reactivity with 4H9 and can be easily classified with conventional fluorescence microscopy or by immunoperoxidase techniques in tissue section. When 4H9 is used in conjunction with anti-Ia, anti-cALLa, and anti-immunoglobulin reagents, the major subclasses of childhood ALL and NHL can be readily identified.

The anti-T-cell murine monoclonal antibody 3A1, previously reported by Haynes, et al., also identifies a 40,000 molecular weight surface antigen on peripheral blood T cells. The reactivity pattern of 3A1 on normal tissues is similar to that of 4H9, and these two antibodies apparently define identical surface antigens. 3A1 has been reported to bind to 85% of normal peripheral blood T cells, including all suppressor/cytotoxic T cells and the majority of helper cells. Preliminary results of immunophenotyping studies of ALL and NHL utilizing 3A1 were reported by Haynes. All seven cases of childhood and young adult T-ALL tested were reactive with 3A1, as were eight T-ALL cell lines. It is noteworthy that 3A1 was the only antibody in the panel utilized that reacted with all
of the T-ALL cases, including one case that was E-rosette negative.

Some investigators have related the heterogeneous phenotypes of T-ALL and T-NHL to a model of T-cell ontogeny defined by monoclonal antibodies.\(^5\)\(^6\) In this model, malignant cells represent the progeny of a transformed cell frozen at one point in normal cellular differentiation. A corollary of this hypothesis is that each malignant phenotype corresponds to a phenotype expressed during normal lymphoid differentiation.

If this hypothesis is correct, the 4H9 antigen must be acquired early in T-cell ontogeny, since it is present on the blast cells of patients with even the most immature thymocyte phenotype. Furthermore, the antigen is conserved throughout T-cell maturation, since it is expressed by malignant cells corresponding to all stages of intrathymic differentiation and by most normal circulating T cells as well.

Detailed immunophenotypic analyses of the 36 cases of T-cell lymphoid malignancy reported here are not all easily reconciled with the model of T-cell ontogeny reported by Reinherz et al. As in some other reports, a number of the immunophenotypes expressed by the blast cells from our patients do not correspond to any of the proposed phenotypes in normal T-cell ontogeny. It is likely that the model will be modified as further studies utilizing newer reagents increase our understanding of thymocyte development.

Continued study of the T-lymphoid malignancies will undoubtedly contribute substantially to define better the stages of normal T-cell maturation.

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A single monoclonal antibody identifies T-cell lineage of childhood lymphoid malignancies

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