Identification and Characterization of a New Surface Membrane Antigen Found Predominantly on Malignant B Lymphocytes

By Roger D. Gingrich, Christopher E. Dahle, Kent F. Hoskins, and Martha J. Senneff

A monoclonal antibody, 1D10, was derived that identifies a new antigenic epitope on the surface of malignant B lymphocytes. Normal resting and stimulated lymphocytes do not express the antigen. The majority of individuals with acute Epstein-Barr virus infection express the antigen on their lymphocytes, and in these patients, the T lymphocyte may also be antigen positive. The antigen was found on B-lymphoid neoplasia from the early pre-B cell stage through terminally differentiated plasma cells, a characteristic not reported for other B cell-associated antigens. Studies on homozgyous typing cells and cells from individuals with known HLA phenotypes indicate that the antigen does not segregate in a pattern characteristic for major histocompatibility antigens. The molecule is a heterodimeric polypeptide with the molecular weight and isoelectric points of the α and β chains being 32,000 d/4 and 28,000 d/6, respectively. Evidence is presented that the 1D10 molecule is not HLA-DR, -DP, or -DQ. By extrapolation, we suggest that this novel molecule may represent HLA D-region gene expression of a gene(s) not normally expressed. Potential candidates are D-region pseudogenes. We conclude that the antigenic epitope identified by the 1D10 monoclonal antibody is unique among previously described B-lymphocyte antigens. Further studies of the factors controlling the expression of this molecule, as well as studies designed to look at the possible cellular function, may provide insights for understanding crucial events in the malignant transformation of lymphocytes.

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THE CELL MEMBRANE of lymphocytes is a dynamic mosaic of molecules uniquely constructed so as to precisely define and determine such diverse cellular phenotypic characteristics as the suppressor, inducer, or cytolytic function of the cell, the state of activation or stage of differentiation of the cell, and whether the cell belongs to a population that is monoclonal or polyclonal. The vast majority of cellular membrane antigens thus far described on malignant lymphocytes are represented on nonmalignant lymphocytes at some stage of differentiation or activation.

In this article, we describe an antigen found predominantly on the surface of malignant B lymphocytes that is not expressed on resting lymphocytes or B and T cells activated in vitro by a variety of inductive stimuli. Analysis of fresh human tissue reveals that the antigen can be expressed when lymphocytes undergo malignant transformation or, in some cases, when they are perturbed by the Epstein-Barr virus (EBV). We also show that while the molecular weight and isoelectric point of the antigen are very similar to the HLA class II molecules, the antigenic epitope identified by the monoclonal antibody 1D10 is present on a molecule that is separate and distinct from HLA-DR, -DP, and -DQ.

MATERIALS AND METHODS

Cells and cell culture. HO-85 is a lymphoma cell line derived from our laboratory with a peripheralizing diffuse large cell lymphoma. It grows well in suspension culture in RPMI 1640 with 10% fetal calf serum (FCS), with a doubling time of approximately 24 hours. The cell line is CD20, mu, delta (weakly), kappa, HLA class I and II antigen positive. It does not react with monoclonal antibodies (MoAbs) detecting CALLA, T cell, myeloid, or monocytic cell antigens. The cells react with the SFR7 DR7 and B7/21 MoAbs, indicating that they express DR 7 and DP antigens, respectively. Monoclonal reagents (Leu-10, SFR16 PI.2, Genox 3.53, and IV D12) were used to determine cell expression of DQ 1 and 2.

Raji, Daudi, Jiyoye, Ramos (Burkitt lymphomas), IM-9 (multiple myeloma), ARH-77 (plasma cell leukemia), U-937 (histiocytic lymphoma), HS445 (Hodgkin's lymphoma), HUT 102 (mycosis fungoides), MC116 (undifferentiated lymphoma), RPMI 8866 (EBV-transformed B lymphoblast), K562, KG-1, HL-60 (myeloid leukemias), THP (monoblastic leukemia), MOLT-3, and CEM-CM3 (T lymphoblasts) were obtained from the ATCC (Rockville, MD) and grown in the medium recommended by the supplier. Arent (EBV-transformed B lymphoblast) and OC-1-Ly8 (large cell lymphoma) were supplied by Dr Louis Vaikus (University of Iowa College of Medicine, Iowa City, IA). Homozygous typing cells (3104 [DR 1, DQ w1], 3107 and 3161 [DR 2, DQ w1], 3098 [DR 3, DQ w2], 3164 [DR 4, DQ w3], WET and Burkhart [DR7, DQ w2], Fisher [DR 7, DQ w3], WT46 [DR213, DQ w1], and Swel [DR w1, DQ w3]) were provided by Dr Robert Karr (University of Iowa College of Medicine).

Peripheral blood cells were obtained from healthy volunteers by phlebotomy. Blood was collected in heparin and centrifuged over ficoll-sodium triazoate (Histopaque 1077; Sigma, St Louis, MO). B lymphocytes were enriched for by treating mononuclear cells depleted of monocytes by plastic adherence with a lytic MoAb cocktail (B-Kwik; Lympho-Kwik, Los Angeles, CA). Blood from donors whose HLA-DR and -DQ determinants were known was provided by Dr Nancy Goeken (Histocompatibility Laboratory, Veteran's Administration Medical Center, Iowa City, IA).

Bone marrow cells were collected at the time of marrow harvests for bone marrow transplantation. Mononuclear cells were obtained by density gradient centrifugation. All peripheral blood and bone marrow samples were obtained via human tissue acquisition protocols approved by the Institutional Review Board of the University of Iowa College of Medicine.

Immunization and hybridoma formation. Female BALB/c mice aged 6 to 10 weeks were given four to six intraperitoneal (IP) inoculations at 2 week intervals with 5×10⁷ cells from the human large cell lymphoma cell line HO-85. Animals were killed 5 days after the last inoculation, and the spleen cells were fused with the human myeloma cell line SP2/0-Ag14 (American Type Culture Collection, Rockville, MD) using polyethylene glycol (PEG) 6500 (Life Technologies, Grand Island, NY). Fusion products were cloned by limiting dilution. Hybridomas were selected by indirect immunofluorescence for binding to a panel of human cell lines and normal peripheral blood lymphocytes. Hybridomas secreting antibodies were cloned by limiting dilution. Monoclonal antibodies were purified by size-exclusion chromatography on a Sephadex G-200 column.

Expression cloning of the 1D10 antigen. Peripheral blood lymphocytes were stimulated with immobilized antibodies, and the cell surface was analyzed by flow cytometry. The 1D10 antibody reacts with a new antigen expressed in the majority of the lymphoblastoid cell lines listed above. The reactive putative antigen has a molecular weight of 30,000 and is a heterodimeric 2-chain polypeptide.

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Monoclonal antibodies. All commercial antibodies were purchased as fluorochrome-conjugated reagents unless otherwise noted and were used at the concentration specified by the manufacturer. Leu-10 (Becton-Dickinson), which reacts with a determinant on the DO molecule (DO w1/w3), was purchased as unconjugated antibody. MoAbs SFR7 DR7 (anti-DR7), SG157 (anti-DR), B7/21 (anti-DR), SFR16 PL2 (anti-DQ w2/w3), Genox 3.53 (anti-DQ w1), and 1VD12 (anti-DQ w3) were provided as culture supernatants by Dr Robert Karr. An anti-DR MoAb referred to in this article as anti-DR was purchased from Cappel Laboratories (catalog no. 0201-2007; Westchester, PA). The CD 3, 5, 19, and 20 antibodies were purchased as conjugated antibodies (Coulter Immunology, Hialeah, FL). Asics formed by the B-1 (CD20) hybridoma was a gift from Dr Lee Nadler (Dana Farber Cancer Institute, Boston, MA). W6/32 is an IgG1 MoAb that reacts with the framework of HLA A, B, and C and served as a positive control. MOPC 21A served as an isotype-specific negative control.

Screening of cell fusions. Anti-HO-85 antibody binding activity was determined by a whole cell, indirect radioimmunoassay using fresh HO-85 cells as targets as described below. The following day a second, identical assay was done using as targets HO-85, Raji, MOLT-3, HL-60, and fresh peripheral blood mononuclear cells (PBMC). Wells that showed binding activity greater than five times that of tissue culture medium alone to HO-85 and Raji, but were nonreactive with MOLT-3, HL-60, and PBMC, were harvested.

Cloning of hybridomas and purification of antibody. Five antibodies meeting the above criteria were identified in two fusions and have been cryopreserved. One antibody, hereafter referred to as 1D10, has been cloned by limited dilution as previously described.7 Radial immunodiffusion assay in 1% agarose with murine isotyping antisera (Miles Laboratories, Inc, Elkhart, IN) revealed 1D10 to be of the IgG subclass. The hybridoma grew well in ascites of primed BALB/c mice. Ascites from these mice commonly have antibody titers greater than 1:10^6. Most of the studies reported in this article were done with antibody purified from DEAE--Affi-gel Blue (Bio-Rad, Richmond, CA) columns. By Coomassie blue staining of polyclonal gels, 5 to 8 mg of antibody with greater than 95% purity could be obtained from each milliliter of ascites.

Biostimulation of 1D10. Purified 1D10 antibody was diluted to 2.5 mg/mL in phosphate-buffered saline (PBS). Forty microliters of biotinyl-N-hydroxysuccinimide (BNHS, Sigma, St Louis, MO) 1.7 mg/mL in N,N-dimethyl formamide (formamide) was added to 1 mL of 1D10, mixed at 20°C, and then dialyzed against PBS before a 1-hour incubation.

Indirect, whole cell radioimmunoassay. Target cells were taken from exponentially growing cultures, washed in PBS, counted, and diluted to 4 x 10^5 cells/mL in PBS containing 0.5% bovine serum albumin (BSA). Cell suspension (50 µL) was dispensed to U-bottom wells of a 96-well plate. Culture supernatant (25 to 50 µL) was added to each well and incubated on ice for 30 minutes. After two washes in PBS with 0.1% BSA, 50 µL of rabbit anti-mouse IgG iodinated by the chloramine-t method9 at 500 µCi/25 µg of antibody protein (diluted to approximately 3 x 10^5 cpm per well) was added to each well for a 30-minute incubation on ice. The cells were harvested by a cell harvester (Brandel, Rockville, MD) and counted in a gamma counter (Packard, Downers Grove, IL).

Flow cytometry. Flow cytometry analyses were done on a Becton-Dickinson FACS IV or 440 instrument modified to permit collection of five optical parameters. Data were fed to a VAX 11/750 computer with software that permits integration of areas under two or three dimensional plots of fluorescence intensity.

Studies on peripheral blood lymphocytes were done with forward and right angle light scatter profiles used to allow setting of gates to examine the reactivity of antibodies with the lymphocyte fraction only. Once the gates were set, the cell population was checked for positive reaction with a pan-leukocyte antibody (HL-Le-1, Becton-Dickinson, Mountain View, CA) and for negative reaction with an anti-monocyte antibody, LeuM3 (Becton-Dickinson).

All reactions of antibodies with cells were done on ice for 30 to 60 minutes. Analyses of reactivity with an antibody required acquisition of a minimum of 50,000 fluorescent events. In most fluorescent antibody assays, diluted ascites from the MOPC 21A plasmacytoma and PBS were used as negative controls and produced comparable results. However, with particular reference to fresh tissue or cells, a high level of nonspecific reaction with the control antibody was evident. In these instances, the background was always taken as that produced by the MOPC rather than by PBS.

Staining of tissue sections. Freshly biopsed tissues were frozen and sectioned and obtained through cooperation with the Immunopathology Laboratory at the University of Iowa Hospitals and Clinics, in accordance with regulations established by the Institutional Review Board. Sections were air-dried and fixed by a 20-second immersion in cold acetone. Reactions with antibody reagents were carried out for 48 to 72 hours with parallel control and test wells monitored for [H]-thymidine uptake by a 16-hour pulse at 0.2 µCi/mL. Cultures carried longer than 72 hours showed a marked increase in cell death secondary to toxicity from the phorbol myristate acetate (PMA) (Sigma), which was used at a concentration of 1.6 x 10^-3 mol/L.

T-cell conditioned medium was used as a source of lymphocyte growth factors. It was produced by incubating fresh PBMC at 1 x 10^6 cells/mL in RPMI 1640 (containing 2 mmol/L glucose, 10% fetal calf serum [FCS], and 5 µg/mL gentamycin) plus phytohemagglutinin (PHA) at 10 µg/mL for 72 hours at 37°C. The culture supernatant was filtered and then concentrated fivefold. It was used at a final concentration of 4% by volume.

Labeling of cell membranes. HO-85 cells were taken from culture and washed in 10 mL of methionine-free RPMI 1640 containing 1% FCS. Cells (20 to 30 x 10^6) were resuspended in 5 mL of the same medium, and, after 30 minutes of incubation at 37°C, 0.5 mCi of [35S]-methionine (Amershams, Arlington Heights, IL) was added. The cells were washed and lysed 4 hours later.

HO-85 cell membranes were iodinated by suspending 2 x 10^9 cells in 250 µL of PBS containing 20 mmol/L d-glucose. NaI (500 µCi) was added to the cell suspension together with 20 µg glucose oxidase (1 mg/mL, Sigma) and 200 µg lactoperoxidase (5 mg/mL, Calbiochem, LaJolla, CA), and then mixed and incubated for 15 minutes at room temperature. The reaction was stopped by diluting the cell suspension with 10 cc of iced PBS containing 1 mmol/L phenylmeth-

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lysufonyl fluoride (PMSF). The cells were washed twice more with PBS/PMSF before being lysed.

Whole cells were washed in PBS and centrifuged to a pellet. They were lysed by adding 1 mL of 1% NP40 in 10 mmol/L Tris, pH 8.2, 150 mmol/L NaCl, 1 mmol/L PMSF for 30 minutes on ice. Centrifugation at 12,000g for 10 minutes produced a supernatant containing labeled membrane proteins but free of nuclei.

**Immunoprecipitation.** Labeled cell lysate was precleared by a 30-minute incubation over ice with 50 μL of 10% *Staphylococcus aureus* Cowan strain I (SAC; Pansorbin, Calbiochem, LaJolla, CA) and 20 μL of goat anti-human Ig (Sigma). The SAC-antihuman Ig was then centrifuged out, and the lysate was divided. SAC (50 μL) was mixed with 20 μL of rabbit anti-mouse (RAM) IgG (heavy and light chain) (Cappel, Malvern, PA) and incubated on ice for 60 minutes, then washed twice with PBS/azide. SAC-RAM (10 μL) was mixed with 1 to 2 μg of antibody, incubated for 60 minutes on ice, and washed twice. The SAC-RAM MoAb was added to the precleared lysate, incubated on ice for 2 hours, and then washed twice in 1% NP40/10 mmol/L Tris, pH 8.2, 500 mmol/L NaCl, and once in 1% NP40 10 mmol/L Tris, pH 8.2, 150 mmol/L NaCl.

**Single and two dimensional gel electrophoresis and autoradiography.** Immunoprecipitates were analyzed by single or two-dimensional gel electrophoresis. Single dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) used 12.5% polyacrylamide, 1 mm slab gels with the Laemelli buffer system. Two-dimensional gels were run with the isoelectric focusing gel (pH 4 to 7) subjected to nonequilibrium conditions, the latter as modified by Rudd et al. Gels were air-dried between two sheets of cellophane membrane (Bio-Rad). Dried gels were then used to expose X-ray film (X-OMAT AR5; Eastman Kodak Co, Rochester, NY) using intensification screens (Cronex Lighting Plus; Dupont, Wilmington, DE).

**RESULTS**

**1D10 reaction with peripheral blood or bone marrow cells from normal donors.** Peripheral blood lymphocytes were examined from 15 normal individuals by flow cytometry and were found to be nonreactive with 1D10. Myeloid and monocyte cell populations were also examined by altering the gating parameters and were also nonreactive. Figure 1 shows a representative flow cytometric contour plot of 1D10 reactivity with peripheral blood lymphocytes enriched for B lymphocytes by pre-incubation with a lytic MoAb cocktail containing anti-T-lymphocyte, -monocyte, and -granulocyte activity.

B lymphocytes were identified by an anti-CD20 antibody conjugated to phycoerythrin, while 1D10 reactivity was detected by a fluorescein conjugated polyclonal goat antihuman antiserum. MOPC 21A, rather than PBS, was used as a control in all such studies, since these cells showed a great deal of nonspecific adsorption of immunoprotein to the cell membrane. No reaction with 1D10 was detected when compared with MOPC 21A.

Bone marrow cells isolated by density gradient centrifugation were examined from six healthy donors. Figure 2 shows a representative flow cytometric contour plot of 1D10 reactivity compared with that of MOPC 21A. B and T lymphocytes were identified in the cell population with anti-CD19 and -CD5 fluorochrome-conjugated MoAbs. Again, no significant expression of 1D10 was detected.

**1D10 reactivity with malignant lymphoid and nonlymphoid cells.** Exponentially growing cells were taken from culture or freshly drawn venous blood and examined for expression of the 1D10 antigen by flow cytometry. Figure 3 shows representative fluorescent histograms for a B cell line (Raji), blasts from a patient with pre-B acute lymphocytic leukemia (ALL), a T cell line (MOLT 3), and a terminally differentiated multiple myeloma cell line (IM-9). Table 1 shows that 1D10 was expressed on the majority of cell lines derived from malignant B-cell disorders, while cells from T-cell disorders were negative. Reactivity of 1D10 with nonlymphoid, malignant cell lines is also shown in Table 1. KG-1, a very primitive myeloblast, did show a weak expression of the 1D10 antigen.

The relative antigen density compared with other prominent cell surface molecules as determined by fluorescence intensity in the flow cytometer indicates that on HO-85, DR > 1D10 > HLA class I > DP > DQ (Fig 4, left panel). Ramos, a Burkitt cell lymphoma line, was also tested for the relative quantitative expression of these antigens. While the relative intensity of antibody fluorescence is different for Ramos (Fig 4, right panel), it is of interest to note that the DQ antigen is not expressed on this cell line.

**1D10 reactivity with lymphoid tissue.** Fresh peripheral blood cells, lymph node biopsies, or cadaver donor specimens were examined for the presence of the 1D10 antigen by
immunoperoxidase staining or flow cytometry. Leukemia cells were obtained from the peripheral blood of patients before therapy and were examined by flow cytometry. Cells from patients with monospot-positive mononucleosis were obtained at the time of diagnosis of the acute illness.

Table 2 shows that the majority of B-cell neoplastic processes express the ID10 antigen, while nonmalignant cells, T-cell lymphomas, and nonlymphocytic leukemias did not.

Fresh cells from peripheral blood or bone marrow were studied by flow cytometry. A number of patients with typical B cell chronic lymphocytic leukemia (CLL) were studied because of the ease of obtaining cells. Figure 5 shows two antibody flow cytometric studies indicating that the majority of the ID10 positive cell population was also CD20 positive.

Bone marrow from a patient with nodular lymphoma showed 2% of cells to be "suspicious for lymphoma." Figure 6 shows a series of two-antibody flow cytometric studies on this marrow identifying a small fraction of CD19 positive cells as 1D10-positive. However, these cells were DQ-negative.

The finding of ID10 expression on peripheral blood lymphocytes from patients with acute infectious mononucleosis was both surprising and of great interest. Further analysis with dual fluorescence labeling revealed that of the 17 total patient samples, 13 were positive for 1D10. Twelve of these expressed the 1D10 antigen on CD3-positive cells.

Fig 2. Fresh bone marrow cells from a healthy donor were reacted with 1D10 or MOPC 21A as indicated in Fig 1. Orthogonal and forward scatter gates were set to identify the lymphoid cell population. Lymphocyte subsets were identified with phycoerythrin-labeled anti-CD5 or CD19. Each population was then examined for reaction with 1D10 and compared with MOPC. Computer-aided integration of the areas under the contoured histograms revealed less than 1% of cells to be reactive with 1D10.

Fig 3. Cells were taken from culture (MOLT 3, Raji, IM-9) or peripheral blood (patient with pre-B ALL) and examined for reactivity with 1D10 (——) compared with MOPC (----) by indirect immunofluorescence. The flow cytometric histograms show that the T lymphoblast cell line MOLT 3 is negative, while the three B-cell malignancies are positive for 1D10.
Table 1. 1D10 Reactivity With Cultured Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Tissue of Origin</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-85</td>
<td>DLCL</td>
<td>+</td>
</tr>
<tr>
<td>OC-1-LyB</td>
<td>DLCL</td>
<td>+</td>
</tr>
<tr>
<td>MC-116</td>
<td>DLCL (t8:14)</td>
<td>+</td>
</tr>
<tr>
<td>Raji</td>
<td>BL</td>
<td>+</td>
</tr>
<tr>
<td>Ramos</td>
<td>BL</td>
<td>+</td>
</tr>
<tr>
<td>Daudi</td>
<td>BL</td>
<td>-</td>
</tr>
<tr>
<td>Jiyoye</td>
<td>BL</td>
<td>-</td>
</tr>
<tr>
<td>IM-9</td>
<td>MM</td>
<td>+</td>
</tr>
<tr>
<td>ARH-77</td>
<td>PCL</td>
<td>+</td>
</tr>
<tr>
<td>HS 445</td>
<td>HL</td>
<td>+</td>
</tr>
<tr>
<td>HUT 102</td>
<td>MF</td>
<td>-</td>
</tr>
<tr>
<td>MOLT-3</td>
<td>T-ALL</td>
<td>-</td>
</tr>
<tr>
<td>CEM-CM3</td>
<td>T-ALL</td>
<td>-</td>
</tr>
<tr>
<td>ARENT</td>
<td>Lymphocyte-EBV transformed</td>
<td>-</td>
</tr>
<tr>
<td>8866</td>
<td>Lymphocyte-EBV transformed</td>
<td>-</td>
</tr>
<tr>
<td>KG-1</td>
<td>Early myeloblast</td>
<td>Weakly +</td>
</tr>
<tr>
<td>HL-60</td>
<td>Promyelocyte</td>
<td>-</td>
</tr>
<tr>
<td>K562</td>
<td>Erythroblast</td>
<td>-</td>
</tr>
<tr>
<td>THP</td>
<td>Monoblast</td>
<td>-</td>
</tr>
<tr>
<td>U937</td>
<td>Monoblast</td>
<td>-</td>
</tr>
</tbody>
</table>

The reaction of each cell line was determined by flow cytometry, where the mean channel of greatest fluorescence was to the right of the channel containing 99% of all fluorescent events from cells reacted with the negative control antibody.

Abbreviations: DLCL, diffuse large cell lymphoma; DUL, diffuse undifferentiated lymphoma; BL, Burkitt lymphoma; MM, multiple myeloma; PCL, plasma cell leukemia; HL, Hodgkin lymphoma; MF, mycosis fungoides; T-ALL, T-lymphoblast acute leukemia.

Table 2. 1D10 Reactivity With Fresh Tissue

<table>
<thead>
<tr>
<th>Histologic Diagnosis</th>
<th>Tissue of Origin</th>
<th>Positive Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal†</td>
<td>Lymph node</td>
<td>0/1</td>
</tr>
<tr>
<td>Normal</td>
<td>PB lymphocytes</td>
<td>0/15</td>
</tr>
<tr>
<td>Benign lymphoid</td>
<td>Hyperplasia</td>
<td>Lymph node</td>
</tr>
<tr>
<td>Mononucleosis</td>
<td>Lymph node</td>
<td>1/1</td>
</tr>
<tr>
<td>Reactive hyperplasia</td>
<td>Tonsil</td>
<td>1/1†</td>
</tr>
<tr>
<td>Sjogren's disease</td>
<td>PB lymphocytes</td>
<td>0/2</td>
</tr>
<tr>
<td>Normal, neonatal</td>
<td>Thymus</td>
<td>0/1</td>
</tr>
<tr>
<td>CLL/WDLL (B-cell)</td>
<td>PB lymphocytes/lymph node</td>
<td>14/15</td>
</tr>
<tr>
<td>CLL/WDLL (T-cell)</td>
<td>PB lymphocytes/lymph node</td>
<td>0/2</td>
</tr>
<tr>
<td>PDL</td>
<td>Lymph node</td>
<td>3/8</td>
</tr>
<tr>
<td>IDL</td>
<td>Lymph node</td>
<td>1/1</td>
</tr>
<tr>
<td>LCL</td>
<td>Lymph node</td>
<td>11/14</td>
</tr>
<tr>
<td>HL</td>
<td>Lymph node</td>
<td>0/2</td>
</tr>
<tr>
<td>HCL</td>
<td>PB lymphocytes</td>
<td>0/2</td>
</tr>
<tr>
<td>TCL</td>
<td>Lymph node</td>
<td>0/3</td>
</tr>
<tr>
<td>CML</td>
<td>PB cells</td>
<td>0/2</td>
</tr>
<tr>
<td>ANLL (M2)</td>
<td>PB blasts</td>
<td>0/1</td>
</tr>
<tr>
<td>ALL (pre-B)</td>
<td>PB blasts</td>
<td>3/3</td>
</tr>
<tr>
<td>Mononucleosis</td>
<td>PB lymphocytes</td>
<td>13/17</td>
</tr>
</tbody>
</table>

*Reaction determined on frozen sections stained by the immunoperoxidase technique and examined by light microscopy; flow cytometry used on cell suspensions as described in Table 1.
†Tissue from a cadaver organ donor taken at the time of organ harvest.
‡Positive reaction was weak and focal.

Fig. 4. Two B-cell lymphoma cell lines were examined for the relative antigen density as determined by the immunofluorescent intensity with flow cytometry. The relative antigen density on HO-85 is DR (---) > 1D10 (----) > HLA class I (-- --) > DP (-----) > DG (-----). MOPC is shown for comparison (-----). On the Burkitt lymphoma cell line, RAMOS DR > DP > HLA class I > 1D10. DG is not expressed on this cell line.

Cytes could be provoked to express the 1D10 antigen. Lymphocytes from four different donors were exposed to various stimuli and then examined by flow cytometry for the 1D10 antigen.

Initial experiments in which lymphocytes were co-cultured with PHA or lipopolysaccharide (LPS) at 100 μg/mL for 96 hours produced no detectable expression of the 1D10 antigen (data not shown). Data from Freedman et al[5] have shown...
that interleukin-2 (IL-2) in combination with PMA produces much greater cell stimulation than the more commonly used mitogens. Accordingly, we tested the effect of T cell-conditioned media (TCM) combined with PMA. In some experiments, control wells were checked for proliferative activity by radioactive thymidine incorporation as indicated in Table 3, and by expression of IL-2 receptors. Figure 8 shows a representative experiment in which lymphocytes were stimulated for 72 hours with a combination of TCM and PMA, and the CD3-positive and -negative populations of lymphocytes were examined for 1D10 expression. Tritiated thymidine uptake and the enhanced expression of the IL-2 receptor clearly indicate that both CD3-positive and -negative cells are in a proliferative mode, yet no 1D10 expression can be detected. Seventy-two hours was as long as the experiment could be carried without excessive cell death. Thus, T cell, B cell, and mitogen receptor independent stimuli fail to provoke the expression of this antigen.

**1D10 expression on EBV-transformed lymphoblastoid cell lines and lymphocytes of known HLA class II type.** As will be shown below, the molecular nature of the 1D10 antigen is strikingly similar to HLA class II molecules. Therefore, it was of interest to ascertain whether the presence of the 1D10 antigen segregated with known class II determinants. Table 4 shows that 1D10 reacts with some but not all transformed cell lines; however, no pattern of reaction conforming with known supertypic specificities can be detected. The fluorescence intensity on positive cells was quite variable and could not be accounted for on the basis of cell size alone. All homozygous typing cells (HTC) expressing DQw2 were reactive with 1D10. However, fresh peripheral blood lymphocytes from four healthy donors known to be homozygous for DQw2 were tested and found to be nonreactive with 1D10.

**Anti-DR, -DP, and -DQ competition with 1D10.** HO-85 cells were incubated with saturating concentrations of B7/21 (anti-DP), SFR16 Pl.2 (anti-DQ), SG157, and Cappel anti-DR (anti-DR) followed by a fluoresceinated anti-mouse (or anti-rat in the case of SFR16 Pl.2) anti-serum. To determine if the 1D10 epitope had been blocked, biotinylated-1D10 followed by avidin-Texas Red, was added in sequence. The cells were then examined by two-color flow cytometry. No change in 1D10 binding could be detected when compared with controls (data not shown).

**Immunoprecipitation analyses.** In order to define the molecular characteristics of the 1D10 antigen and its relationship to the HLA class II antigen system, we radiolabeled HO-85 cells, solubilized the cell membranes with detergent and precipitated antigen using MoAb, anti-murine IgG anti-serum, and staphylococcal protein A. Initial studies using iodinated cells and single dimension SDS-PAGE.
Fig 7. PBMC were prepared from a patient with mononucleosis. Flow cytometric gating was used to examine the reactivity of lymphocytes to 1D10 and anti-CD3. The left panel shows CD3 positive lymphocytes, some of which react with 1D10 (compared with MOPC 21A in the right panel), while the remainder are nonreactive.

analysis of the precipitate revealed that the 1D10 antigen was composed of two bands with approximate molecular weights of 28 and 32 Kd. Figure 9, lane A shows a precipitate formed using MOPC 21A. Lane B is the precipitate formed with 1D10 consisting of the 28 and 32 Kd bands. The higher molecular weight band probably represents actin (at 44 Kd), nonspecifically present in most precipitates. In lane C, the precipitate was formed with B-1 (CD20) and shows the prominent expected band at 35 Kd.12 Lane D shows the DR antigen precipitate formed by SG157 with the characteristic heterodimeric bands at 32 and 28 Kd.

These data suggested a close relationship between 1D10 and the class II antigens13,14. Therefore, we wanted to compare the molecular characteristics of the class II antigens with those of 1D10. HO-85 cells were metabolically labeled, and the solubilized membrane was divided into four aliquots in a parallel precipitation experiment using 1D10, SG157 (anti-DR), Leu-10 (anti-DQ), B7/21 (anti-DP), and MOPC 21A (negative control). Analysis of each of the antigens on two-dimensional non-equilibrium pH gel electrophoresis (NEPHGE)/SDS-PAGE gels was done under identical conditions. Figure 10 shows the characteristics of the 1D10 molecule and each of the known class II molecules. The differences between 1D10 and the DR and DP antigens are evident, particularly in regard to the number, position, and labeling of the light chain spots. These differences strongly suggest that 1D10 is not HLA-DR or -DP.

Comparison of 1D10 and the DQ molecule reveals a number of similarities. Each appears to have three spots associated with the heavy and light chains. In addition, the DQ precipitate reveals several additional heavy chain spots slightly heavier than the more prominent cluster of three. Similarly, there are three additional light chain region spots, also slightly heavier than those spots most resembling 1D10. Whether these are part of the light and heavy chains or are part of a more complex pattern of invariant polypeptides unique to HO-85 is unknown. Even if the latter were true, there are also obvious labeling differences between the 1D10 and DQ antigens, suggesting they are not the same molecule.

To clarify further the relationship between 1D10 and DQ, a labeled membrane lysate from HO-85 was depleted of either the 1D10 or DQ antigen by serial immunoprecipitation with 1D10 or anti-DQ (in this experiment, Genox 3.53). The depleted lysates were then divided into two aliquots, one for immunoprecipitation with 1D10 and the other with anti-DQ. Figure 11 shows the 1D10 antigen (arrows in top left panel) from the initial precipitate exposed to autoradiography for 1 week. Also shown is the fourth of the serial anti-DQ precipitates (top right panel); the DQ antigen cannot be seen even with a 2-week exposure. 1D10 was then used to form a precipitate from the DQ-depleted lysate, and the antigen can clearly be identified after 1 week of autoradiography (bottom left panel). The final panel is the MOPC control.

Conversely, Fig 12 shows the DQ (Genox 3.53) antigen (top left panel). Similarly, the fourth precipitate of the serial 1D10 precipitations is shown (top right panel). This depleted lysate is then precipitated with anti-DQ, and the DQ antigen pattern is clearly present (bottom left panel). Again, the MOPC control is shown for comparison.

DISCUSSION

The goal at the outset of this study was to create MoAb reagents that reacted predominantly or solely with malignant B lymphocytes. Our purpose was to use these as tools to examine cell membrane molecules that may conceivably determine important functional differences between malignant and nonmalignant cells. The strategy involved immunizing mice with a new cell line, termed HO-85, derived from a patient with a progressive and refractory large cell B lymphoma. The fusion wells were screened against a cell panel designed to identify antibodies with a high probability...
of satisfying the reaction criteria desired. One of the antibodies identified by this approach, 1D10, detects an epitope on a cell surface molecule that appears to be both novel and potentially informative.

A review of the literature concerning membrane molecules found predominantly on B lymphocytes reveals that, in most instances, the antigens are found on normal or mitogen-activated lymphocytes in addition to malignant lymphocytes. An exception to this generalization was reported by Howard et al: the antibodies LM-26 and 155 do not react with normal B lymphoid blasts. However, in contrast to the reactivity pattern of 1D10, they did not react with lymphocytes from 10 patients with CLL. At the time of the initial description, the authors had been unsuccessful in their attempts to characterize the molecular nature of the antigen(s). The expression of the 1D10 molecule on malignant B lymphocytes together with its distinct lack of expression on resting or mitogen-stimulated cells sets this antigen apart from those previously described.

Epstein et al have described two antibodies called Lym-1 and -2 that react with normal B lymphocytes, as well as with many malignant B-cell neoplasms. Of interest, the antigen detected by Lym-1 precipitates an antigen with molecular characteristics identical to HLA-DR as determined by one-dimensional SDS-PAGE. The epitope detected by Lym-1 (the antigen for Lym-2 was not characterized) is clearly not 1D10, but the similarities are intriguing.

In our efforts to characterize the 1D10 antigen, we noted striking similarities when compared with the characteristics of the HLA class II antigens. We have considered that the 1D10 epitope may be a polymorphic determinant on a class II molecule. The lack of competition or blocking between 1D10 and the anti-class II antibodies used in this study is not definitive. Rather, it indicates that 1D10 does not react with the specific epitope detected by those antibodies.

Resting T lymphocytes express essentially no class II antigens. However, with mitogenic stimulation, DR and DQ appear on the cell surface. In our studies, mitogen-stimulated lymphocytes or lymphocytes activated by surface immunoglobulin crosslinking (anti-Ig beads) (data not shown) or via the protein kinase C pathway by PMA do not express 1D10. This suggests that even if the 1D10 antigen is class

| Table 4. 1D10 Reactivity of Homozygous Typing Cells |
|----------------|----------------|
| DR Type | DR Type |
| 1 | 2 | 3 | 4 | 7 | w11 | w13 |
| w1 | 3,104 | 3,107 | 3,161 | | | |
| w2 | 3,104 | 3,098 | | | | WT46 |
| w3 | 3,164 | 3,161 | | | | |

Lymphoblastoid HTC were used fresh from culture for flow cytometric determination of reactivity with 1D10 compared with MOPC 21A.
NOVEL HLA D-REGION ANTIGEN

Fig 9. HO-86 cells were enzymatically labeled with $^{32}$P, and a membrane lysate was made by nonionic detergent solubilization. Shown is an autoradiograph of an SDS-PAGE analyzing the immunoprecipitates formed by MOPC 21A, 1D10, B-1 (anti-CD20), and SG157 (anti-DR) (lanes A, B, C, and D, respectively). Molecular weight markers are lysozyme 14.4 Kd, soybean trypsin inhibitor 31 Kd, ovalbumin 44 Kd, and BSA 68 Kd, respectively.

Fig 10. HO-86 cells were metabolically labeled with $^{35}$S-methionine and detergent lysed. Immunoprecipitation was done using 1D10, anti-DR (SG157), DP (B7/21), and DQ (Leu-10) antibodies. The precipitates were analyzed under identical conditions using the NEPHGE gel system and autoradiography. SG157 produced a relatively clean precipitate, whereas the other antibodies consistently produced precipitates with a number of faint and variable spots. Arrows indicate individual spots seen consistently in all such analyses. Asterisks identify prominent spots seen in all precipitates, including those formed by MOPC. L, light chains; H, heavy chains.

HLA-related, the regulation of its expression is under control mechanisms different from those that affect the class II antigens.

The presence of the 1D10 antigen on malignant lymphoid tissues from the majority of patients with malignancies of B lymphocyte origin and the lack of 1D10 of expression on nonmalignant B lymphocytes makes it improbable that the 1D10 epitope is a normal and/or known polymorphic D-region determinant. While the distinctive molecular nature of the antigen described in this paper provides a more definitive answer, the precise relationship of the 1D10 antigen to the antigens of the class II system awaits purification of the antigen and amino acid sequencing.

One of the most interesting findings of this study was the nearly uniform finding of 1D10-positive lymphocytes in the peripheral blood of patients during the initial acute phases of mononucleosis. In the majority of these individuals, the lymphocyte population that was reactive with 1D10 was CD3-positive. In some, 1D10 was found on both CD3 positive and negative cells, and in only one patient was the CD3 negative cell population (containing the majority of B lymphocytes) exclusively positive. This expression (presumably transient) of the 1D10 antigen evoked by lymphocytes reacting to the presence of the Epstein-Barr virus is in contrast to our inability to do the same in the laboratory with the most powerful proliferative stimuli known. Some, but not all, B lymphoblasts transformed by the Epstein-Barr virus express high levels of the antigen in the immortalized state.

It has been shown that the majority of "reactive" lymphocytes in patients with mononucleosis are CD3/CD8 positive, suppressor cells that also express HLA class II antigens. A satisfactory explanation for the variable expression of
1D10 on the different lymphocyte populations is not available. However, it may be related to the time during the course of the illness when the respective patients were tested. Data from Tosato et al. and Purtillo et al. suggest that early in the disease, B cell activation of a polyclonal nature predominates, while later, T cell suppressor activity would take over. Hypothetically, the patients with 1D10 found on CD3-negative cells would be early, while those in which it was found on the CD3-positive cells would be later in the course of the disease. These observations also suggest that the mechanisms leading to the expression of the 1D10 antigen (malignant transformation, Epstein-Barr virus transformation, or induced activation) use pathways that are different from those used by mitogens and phorbol esters. Further studies to examine convalescing patients whose cells express 1D10 in the acute phase of mononucleosis are needed. In addition, the effect of EBV transformation on resting B lymphocytes that do not express 1D10 must be...
examined. These experiments may provide valuable clues in deciphering the mechanism of malignant transformation of B lymphocytes.

The expression of this antigen on malignant B lymphocytes throughout the pathway of differentiation, from the precursor-B cell through the plasma cell stage, is unique among antigens described to date. HLA-DR has perhaps the most global expression in the B cell lineage but, in contrast to 1D10, even it is not found on the terminally differentiated plasma cell.14

The biochemical characteristics of a molecule will occasionally suggest function or a relationship to a family of molecules.43 Therefore, we were interested in defining the molecular nature of the 1D10 antigen. The initial one-dimensional SDS-PAGE characteristics revealed the molecule to be a noncovalently bound heterodimer, with the molecular weight of each band being 28 and 32 Kd. Two-dimensional gel analysis showed that the heavy chain had an approximate isoelectric point of 4, while that of the light chain was approximately 6. Each chain was composed of three distinct molecular species with slight variations in the pI. These findings are strikingly similar to those described for HLA D-region molecules, yet the expression of 1D10 seemed quite different from the class II antigens. Flow cytometric studies of antibody reactivity make the possibility that the 1D10 antibody binding site is a polymorphic determinant on a normal class II molecule highly unlikely. Nevertheless, we sought confirmation for this position from comparative molecular studies using NEPHGE/SDS-PAGE.

Monoclonal antibodies were obtained that react with the HLA-DR, -DP, and -DQ antigens. The HO-85 cell line was an appropriate tool for the study since each antibody reacted strongly with it. From the same 35S-methionine labeled cell lysate, antigen detected by the respective antibodies was precipitated and analyzed by NEPHGE/SDS-PAGE. The DR and DP antigens obtained in this way were strikingly different in most features relative to both the heavy and the light chains when compared with the 1D10 antigen. Comparison of the DQ and 1D10 antigens revealed both similarities as well as differences, such that an absolute differentiation between the two molecules based on the electrophoretic analyses alone was difficult.

In order to distinguish whether DQ and 1D10 were present on the same or different molecules, a two-way immunodepletion experiment was done. It was possible to show that when either no identifiable 1D10 or DQ antigen could be precipitated from a labeled cell lysate after multiple serial immunoprecipitations, the alternate molecule was still present. These findings support the contention that 1D10 detects an antigen that, while very much like the known HLA D-region molecules, is definitely distinct from them.

Our data are compatible with the conclusion that the 1D10 determinant is expressed as the result of transcription of genetic material not transcribed except in the context of transformation by EBV or by the poorly defined mechanisms of malignancy. Why this should be is not at all clear. It may relate to a cellular function of the 1D10 antigen analogous to one or more functions of the D-region antigens to which it appears to be molecularly closely related, including cell-cell recognition,40 hematopoietic regulation,41,42 cell proliferation consequent upon immune stimulation,43,44 and cell-cell adhesion.45 Recent studies have also indicated that D-region molecules can transmit anti-proliferation signals, supporting further the notion that these molecules are potentially important in the regulation of cell growth activities.46

Whatever the reason for its expression, the presence of high levels of the 1D10 antigen on the cell surface appears to be associated with B-cell malignancy. Clearly, it is not a prerequisite for malignancy, since some lymphoid tumor cell lines as well as tumor tissue from patients do not appear to react with 1D10.

In conclusion, the murine monoclonal antibody 1D10 described in this article identifies a novel membrane antigen found predominantly on the surface of malignant B lymphocytes whose clonal origins (as histologically distinct tumors) have occurred at all stages of differentiation. The antigen is not found on resting B lymphocytes, nor can it be induced with the most potent B cell stimuli. Within the limits of standard flow cytometric methods, neither is it found on other blood cells in bone marrow or peripheral circulation. The molecular characteristics of the antigen are strikingly similar, yet distinct from, the known HLA D-region antigens. It is possible that the 1D10 antigen in the gene product of D-region sequences not previously known to be transcribed. DX is a pseudogene without an identifiable genetic reason for its apparent nonexpression and would certainly be among the potential candidates.50,51

Whatever the identity of the 1D10 or of its function, its further study will likely yield information important to the understanding of process of malignant transformation of B lymphocytes. The precise definition of the role 1D10 may play as a diagnostic and therapeutic reagent will be important to establish.

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Identification and characterization of a new surface membrane antigen found predominantly on malignant B lymphocytes

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