Dual B and T Markers in Acute and Chronic Lymphocytic Leukemia

By Kenneth A. Foon, Ronald J. Billing, and Paul I. Terasaki

Leukemic cells from 20 patients with chronic lymphocytic leukemia (CLL) and 60 patients with acute lymphocytic leukemia (ALL) were studied for T- and B-lymphocyte cell surface membrane markers. B-cell markers included surface membrane immunoglobulin, erythrocyte-antibody complement rosette formation, and B-cell (Ia-like or HLA-DR) antigens detected by a B-cell antiserum. T-cell markers included spontaneous sheep red blood cell rosette formation and a cytotoxic reaction to a specific T-cell antiserum. Seven patients with CLL and two with ALL had dual B and T markers. We propose that dual B- and T-cell markers are more common in CLL and ALL patients than previously reported. With newer and more sensitive tests for identification of B and T cells, this observation may be recognized more frequently.

It is widely accepted that the lymphoid system is composed of two major subtypes of lymphocytes. T cells can be identified by their ability to form spontaneous rosettes with sheep red blood cells (SRBC) and to react with T-cell antisera. B cells are distinguished by surface membrane immunoglobulin (SmIg), receptors for the Fc portion of IgG and the C3 component of complement (C'), and Ia-like antigens detected by specific antisera. Lymphocytes with both T- and B-cell markers have been reported to comprise 2%-4% of normal peripheral blood lymphocytes.1-12 In addition, cells with dual markers have been reported in patients with chronic lymphocytic leukemia (CLL),12-15 lymphosarcoma cell leukemia,16,17 lymphocytic lymphoma with or without leukemia,12,18-20 hairy cell leukemia,21 a Sézary-like syndrome,1 and cultured lymphoid cell lines.9,22,23 We studied cell surface markers on leukemic cells from patients with CLL and acute lymphocytic leukemia (ALL). These studies indicate a high incidence of CLL cells and a rare ALL cell with both B- and T-cell surface markers.

MATERIALS AND METHODS

Patients and Cell Preparation

The diagnosis of CLL and ALL was based on clinical features and Romanowsky-stained peripheral blood and bone marrow samples. In some instances, myeloperoxidase, periodic acid-Schiff, and neutral lipid stains24-26 were performed to confirm the diagnosis. Patients with CLL had a white cell count greater than 20 x 10^9/liter and most counts were greater than 50 x 10^9/liter. Peripheral blood was obtained in heparinized syringes and prepared on a Ficoll-Hypaque gradient.26 These preparations contained >95% mature lymphocytes. Patients with ALL had >70% blasts in their peripheral blood or bone marrow specimens. Ficoll-Hypaque preparation yielded test cells that were >80% blasts.

B-cell- and T-cell-enriched lymphocyte populations were prepared by rosette formation with neuraminidase-treated SRBC followed by repeat centrifugation on Ficoll-Hypaque.27 After centrifugation, the rosetted T cells sedimented to the bottom of the tube, and B cells remained at the interface. The T cells were then recovered and erythrocytes lysed by incubation with hypotonic Hanks' balanced salt solution. Purity of cell populations was >90% when prepared by this method.

Antisera

An antiserum with B-cell specificity was produced by immunization of rabbits with papain digests of cell membranes of involved spleens from patients with a variety of hematologic malignancies.28 An antiserum with T-cell specificity was prepared by intravenous immunization of a New Zealand white rabbit with 1.6 x 10^8 human thymus cells followed in 3 wk by a boost of 2.4 x 10^8 thymus cells. Two weeks later, the rabbit was sacrificed, and the serum was collected, heat-inactivated at 56°C, and extensively absorbed with B-cell lines.

Immunofluorescence

SmIg was detected with Fab', fragments of a goat anti-human immunoglobulin (Cappel Laboratories, Cochranville, Pa.) and/or goat anti-human whole immunoglobulin (Meloy Laboratories, Inc., Springfield, Va.) Fifty microliters of test cells at 10^6/ml were mixed with 5ul of fluorescein-labeled reagent and allowed to incubate for 30 min at 4°C. The cells were then washed and the number of labeled cells determined by ultraviolet microscopy. Immunofluorescence was recorded as percent positive cells.

Detection of E-Rosette-Forming Cells

Twenty microliters of lymphocytes (or leukemia cells) (5 x 10^6/ml) and 20 µl of neuraminidase-treated SRBC (10^6/ml) were spun for 5 min at 500 g. The cells were incubated for 30 min at 5°C, then gently resuspended and 200 cells were counted for rosettes.

Detection of Erythrocyte-Antibody Complement (EAC) Rosettes

SRBC were incubated with the IgM fraction of rabbit anti-SRBC for 30 min at 37°C. The cells were washed and incubated for an additional 30 min at 37°C with 100 µl of mouse C' and then washed again. Twenty microliters of lymphocytes or leukemia cells (5 x 10^6/ml) and 20 µl of the EAC preparation (10^6/ml) were centrifuged at 500 g for 1 min and then incubated for an additional 60 min at 37°C. The cells were gently resuspended and 200 cells were counted in a hemacytometer. Cells surrounded by >3 EAC were scored as positive. The majority of cells were also incubated with
uncoated SRBC and SRBC coated with rabbit IgM anti-SRBC without C' for 30 min at 37°C as controls.

**Cytotoxicity**

Cells were tested with B-cell and T-cell antiserum in a standard microcytotoxicity assay. Rabbit serum was used as a source of C'. A reaction was considered positive when ≥80% of the cells were killed and negative when ≤30% of cells were killed. The C' used was unabsorbed but extensively screened against normal and malignant hematopoietic cells. In addition, all cells studied were tested with C' alone and C' and nonimmune serum as negative controls. None of the cells reported had ≥20% cytotoxicity in these control wells.

**Absorption Studies**

The T-cell antiserum (dilution 1:8) was absorbed with normal T lymphocytes and various CLL cells. Twenty-five microliters of antiserum were absorbed for 1 hr at 22°C with 2 × 10^6 or 4 × 10^6 cells. The absorbing cells were removed by centrifugation and the absorbed serum was tested in triplicate for cytotoxicity against T lymphocytes from 3 normal persons and 2 dual marker CLL cells. Specificity of the B-cell antiserum has been previously reported.

**RESULTS**

**Specificity of the B- and T-Cell Antiserum**

Data regarding the specificity of the B-cell and T-cell antiserum are indicated in Table 1. Both antisera showed specificity for their respective cell types as shown by testing against normal B and T cells, various cultured cell lines, thymocytes, T-ALL, and Sézary cells. The abbreviation, NK, denoted no kill, which means less than 30% of cells were killed with undiluted serum. Only a very rare B cell was killed up to 30% with undiluted T antiserum and usually ≤10%. Titers shown denote the highest titer where there is ≥80% cytotoxicity. Additional data on the B-cell antiserum have been previously reported.

**Cell Surface Marker Studies**

Twenty patients with CLL were studied. Cells from 7 patients (patients 1–7) demonstrated both T- and B-cell antigenic markers (Table 2). Six of 7 patients tested in this group had a high percentage of SmIg, and the seventh had a high percentage of EAC rosettes with undetectable SmIg. None of these cells formed E rosettes. All of them reacted with both T and B antisera. Patient 8 had cells with typical T-cell markers, including E rosettes and sensitivity to the T-cell antisera. Patient 8 had cells with typical T-cell markers, including E rosettes and sensitivity to the T-cell antisera, but not the B-cell antisera. Cells from patients 9–20 had B-cell markers. A number of these patients' cells were retested at different bleedings and the surface markers remained the same. Cells from 2 of 60 patients with ALL demonstrated both T- and B-cell surface antigenic markers, although one cell was both E, EAC, and SmIg negative. The remaining 58 cells had either T-cell markers, the B-cell antigen, or no markers. Details of the surface markers of these patients are not shown, but are described in the discussion section. A normal B cell and T cell are shown for comparison (23 and 24).

The specificity of the EAC receptor for C' was tested by incubating test cells at 37°C for 60 min with uncoated SRBC and SRBC coated with the IgM fraction of rabbit anti-SRBC without C'. The 12 CLL cells studied in this fashion included 3 from the dual

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### Table 1. Specificity of B-Cell and T-Cell Antiserum

<table>
<thead>
<tr>
<th>Target Cell</th>
<th>Cytotoxicity Titer*</th>
<th>T-Cell Antiserum (mean titer of 10 donor cells)</th>
<th>B-Cell Antiserum (mean titer of 10 donor cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal T cells</td>
<td>64</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Normal B cells</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>T-cell lines (Molt 4, 8402, HS2)</td>
<td>64</td>
<td>64</td>
<td>64</td>
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<tr>
<td>B-cell lines (LA 85, Raj, Daudi)</td>
<td>64</td>
<td>64</td>
<td>64</td>
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<tr>
<td>T-ALL (mean titer of 3 donor cells)</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Thymocytes (mean titer of 3 donor cells)</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Sézary cell leukemia</td>
<td>64</td>
<td>64</td>
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</table>

*Highest titer with ≥80% dead cells.

†No kill, ≤30% dead cells with undiluted antiserum.

### Table 2. Cell Surface Markers

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>EAC (E) (%)</th>
<th>SmIg (EAC) (%)</th>
<th>Cytotoxicity Titer†</th>
<th>T-Cell Antiserum (mean titer of 10 donor cells)</th>
<th>B-Cell Antiserum (mean titer of 10 donor cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytotoxicity Titer†</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Patient</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
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<td>90</td>
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<td>64</td>
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<tr>
<td>2</td>
<td>CLL</td>
<td>98</td>
<td>70</td>
<td>1</td>
<td>64</td>
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<tr>
<td>3</td>
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<tr>
<td>4</td>
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<td>67</td>
<td>74 (64)</td>
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<td>6</td>
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<td>50</td>
<td>2</td>
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<td>7</td>
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<td>23</td>
<td>94 (60)</td>
<td>0</td>
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</tr>
<tr>
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<td>CLL</td>
<td>91</td>
<td>54 (7)</td>
<td>0</td>
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<td>20</td>
<td>CLL</td>
<td>47</td>
<td>61 (17)</td>
<td>5</td>
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<td>ALL</td>
<td>50</td>
<td>0</td>
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<tr>
<td>23</td>
<td>Normal B</td>
<td>84</td>
<td>87 (72)</td>
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<td>NK</td>
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<td>64</td>
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*Number in parentheses denotes percent positive SmIg using Fab2 fragments of goat anti-human immunoglobulin.

†Highest titer with ≥80% dead cells.

‡No kill, ≤30% dead cells with undiluted antiserum.
marker group and 9 from the B-cell group. In addition, a normal B-cell control was also tested. Patient 16 rosetted 13% and the normal B cell 14% with uncoated SRBC. The remaining cells rosetted ≤2% with uncoated SRBC. Patients 5 and 9 rosetted 23% and 25%, respectively, with SRBC coated with rabbit anti-SRBC (EA), while the normal B cell and the remaining CLL cells rosetted ≤10% with EA. These data suggest that the EAC rosette is binding via the C' receptor and not nonspecifically via Fc or other receptors.

**Clinical Data**

The clinical course of the dual marker CLL patients was not unlike the typical B-lymphocyte CLL patients. All of them presented with high mature lymphocyte counts, rarely with lymphadenopathy or organomegaly, and followed a relatively benign clinical course. There is no evidence that they represent a clinical subset. Patient 8 was unique in that she had ataxia telangiectasia with marked neurologic disability. Details of this case have recently been reported.3

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The ALL cases also did not represent clinical subsets. Both patients 21 and 22 were adults without mediastinal masses whose disease followed an aggressive course.

**Absorption Experiments**

Table 3 shows the results of absorption studies with the T-cell antiserum. The cytotoxicity of this antiserum to two dual marker CLL cells and three normal T lymphocytes was completely removed by absorption with normal T lymphocytes and with dual marker CLL cells. Cells from two patients with CLL that had only B-cell markers and two B-cell lines failed to absorb out the T-cell antiserum activity. Results were similar when either 2 x 10⁶ cells or 4 x 10⁶ cells were used for absorption.

**DISCUSSION**

In this study we found a high incidence of CLL cells with both B- and T-cell surface markers. There have been reports of dual markers in patients with CLL, lymphoproliferative disorders, lymphoblastic lymphoma, and convoluted lymphocytic lymphoma in adults. Two studies reported results similar to ours.14,15 In addition, a single case of ALL has been described in which two leukemic subpopulations were identified, one with T-cell and the other with B-cell surface markers.32

Previous studies have utilized the more routine B-cell markers, including immunofluorescent staining for SmIg, EAC rosette formation, and the use of aggregated immunoglobulin to detect Fc receptors. The majority of cells we tested formed EAC rosettes and had surface membrane immunoglobulin detected by goat whole antibody and Fab₂ fragments to human immunoglobulin. Fab₂ fragments exclude the possibility of nonspecific binding to the cell membrane via Fc receptors. Generally, the percent of positive cells is lower with Fab₂ fragments than with whole antibody. This may be due to some nonspecific binding to Fc receptors and/or because the Fab₂ fragments are prepared with a less intense fluorescein tag than the whole immunoglobulin.

A few of the CLL cells reported in this study, which by most criteria appeared to be B cells, had faint fluorescence patterns or no detectable SmIg with either whole immunoglobulin or Fab₂ fragments. This is not an unusual observation and is consistent with the hypothesis that CLL cells have a maturational arrest in their B-cell differentiation.33 In spite of these pitfalls, surface membrane immunoglobulin detected by direct immunofluorescence techniques is probably the most reliable B-cell marker.

The B-cell origin of the cells reported in this study were further investigated using a B-cell antiserum. The la-like or HLA-DR antigen(s) detected by this antiserum is also found on monocytes, ALL cells, acute myelogenous leukemia (AML) cells,28 and myeloid-monocytic34,35 and erythroid36 progenitor cells. It has recently been described on a small population of normal T lymphocytes, rare leukemic T cells, and T cells transformed in a mixed lymphocyte culture.31,32 Therefore, this antigen(s) is not restricted to B lymphocytes. Furthermore, C' receptors are also not unique to B lymphocytes and are found on a wide variety of nonlymphoid cells as well as a rare T
lymphocyte.  It is therefore necessary to study numerous surface membrane markers to determine the origin of a lymphocyte.

T cells are identified by rosette formation with SRBC and specific T-cell antisera. In order to determine if the T-cell antiserum was reacting with the same antigen on T lymphocytes and dual marker CLL cells, absorption studies were performed. Cytotoxicity to both dual marker CLL cells and T lymphocytes was completely removed by absorption with either of these cells. Absorption with CLL expressing only B surface markers or cultured B-cell lines did not remove this activity. These data indicate a common antigen(s) shared between the dual marker CLL cells and T lymphocytes.

It has been generally accepted that typical CLL cells demonstrate only B-cell surface markers. A rare T-cell variant is distinguished clinically by cutaneous involvement. The CLL patients with whom we report dual markers are not clinically distinguishable from those with only B-cell markers. Patient 9 had a T-CLL and was unique in that she had ataxia telangiectasia and a 14q- chromosomal translocation. She was the only CLL patient whose cells formed E rosettes.

Typical cases of ALL involve “null” cells. These cells are negative for SmIg, EAC, and E rosetting and 50% EAC-rosetting cells that also had markers in this disease. Patient 21 had 91% E- rosetting and 50% EAC-rosetting cells that also had Ia-like and T-cell antigens. Patient 22 had typical null cells, in that they did not have SmIg, C′, or SRBC receptors, however, they had both T and Ia-like antigens. The remaining 58 ALL cells studied will be reported separately. In brief, 48 were similar to patient 22 in that they did not have SmIg, C′, or SRBC receptors but had Ia-like antigens. About 80% of these null cells also had an ALL-associated antigen. None of them had the T antigen(s). The remaining ALL cells were mostly T lymphoblasts detected by SRBC rosetting and/or T antiserum and did not have any B-cell markers, including Ia-like antigens.

A number of theories have been proposed to explain the presence of dual markers on leukemic cells. These include the expression of otherwise inactive genes (gene depression), transformation in a stem cell prior to the divergence of B and T lymphocytes, or derivation from the subpopulation of lymphocytes that normally express dual markers.

We propose that cells with both B- and T-cell markers are more common than has previously been noted. With newer and more sensitive tests for identification of B and T cells, this observation may be recognized more frequently.

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