Antigenic Changes on the Surface of Lymphocytes from Patients with Chronic Lymphocytic Leukemia

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Summary

The antigenic characteristics of the surface of lymphocytes from patients suffering from chronic lymphocytic leukemia (CLL), from normal human adults, from patients with other neoplastic and nonneoplastic diseases, and from normal newborns were studied comparatively.

Rabbit antisera were prepared against the white blood cells of two CLL patients. Their reactivity against the different target cells was assayed by means of a cytotoxicity test and by the binding of labeled antibody preparations purified by absorption with normal human white blood cells and enriched by adsorption onto and elution from the leukemic target cells. The reactivity of normal rabbit serum and of a rabbit antiserum against normal adult white blood cells was investigated in parallel.

It was found that the surface of CLL white blood cells, which consist very largely of lymphocytes, is characterized by antigenic properties not expressed, at least in quantities of the same order of magnitude, on normal adult lymphoid cells or on lymphoid cells from patients with other pathologies. However, the lymphocytes of a proportion of normal newborns appeared to share these antigenic characteristics.

The tumor-associated antigenicity of CLL lymphoid cells could not be accounted for by adsorption onto their surface of serum proteins but appears to reside instead in the nature of the cell surface of the neoplastic variants.

The findings are discussed in the light of the possible nature of tumor-associated antigens in general.

Introduction

CLL of man appears to represent a good model for the study of the immunological parameters of host-tumor relationships in humans. The disease is marked by frequent instances of chronicity and by considerable and often prolonged fluctuations in its course, and the general pathogenetic picture is suggestive of an ongoing interaction between the cells involved in host defenses and the neoplastic lymphocytes, in the course of which the host is capable of offering appreciable, and quite frequently durable, resistance. The neoplastic cells are inherently susceptible to immunological attack (20), and their dispersed state would be expected to render them susceptible to the cytotoxic action of humoral antibodies as well as of sensitized lymphoid cells.

A systematic investigation has been initiated in our laboratories on the immunology of this neoplastic disease. This 1st communication reports the occurrence of antigenic changes on the surface of lymphocytes from CLL patients. Experiments were also performed to determine whether the surface of normal lymphocytes from normal human newborns is distinguishable by antigenic characteristics similar to those of CLL lymphocytes. These experiments were undertaken partly in view of the reports that tumor-associated antigens may be identical with antigens which also appear normally, but only during fetal or neonatal life (1, 10, 22, 29), and partly because of the marked similarities of the lymphoid tissues of CLL patients and of normal individuals during the 1st months of life.

It was found that rabbit antiserum against CLL lymphocytes can discriminate between such cells and normal lymphocytes from some newborn children on the one hand and lymphocytes from healthy adults and from patients with a variety of other diseases, both neoplastic and nonneoplastic, on the other. Antibodies apparently specific to individual, long-transplanted cell lines of human myelocytic and acute lymphocytic leukemia have been reported by other workers (28). Observations with unabsorbed heterologous sera have also suggested that CLL lymphocytes may express more frequently or in larger quantities than normal adult human lymphocytes antigen(s) also possessed by the lymphocytes of some other species (27). These findings, together with a number of other reports (2, 8, 9, 12, 16), support the possibility that human leukemic cells may indeed express unique antigenic properties. The present study demonstrates the high degree of specificity of the reaction of immune rabbit sera with CLL and some newborn normal lymphocytes, with only freshly obtained lymphocytes as target cells and with both a cytotoxicity assay and the specific adsorption of labeled, enriched antibody preparations as test systems.

Materials and Methods

Human Sera and Cells. Sera and WBC were obtained from normal adult volunteers, from patients with CLL, or other neoplastic and nonneoplastic disease, and from fetal cord
blood. The CLL patients were receiving either no treatment or only small daily steroid medication at the time their blood was obtained.

**Human WBC and Purified Lymphocyte Suspensions.** A standard procedure (5) was used, with slight modifications, to obtain preparations of WBC and of lymphocytes freed of granulocytes. Ten ml of heparinized venous blood (25 units heparin per ml in 0.15% chlorocresol; this solution was not toxic) were mixed with 2.5 ml Plasmagel (Laboratoire Roger Bellon, Neuilly, Hauts-de-Seine, France), and the RBC were allowed to sediment for 30 min at room temperature. The supernatant containing leukocytes and platelets was centrifuged at 200 X g for 10 min at room temperature, and the plasma was removed. For use as WBC, the cells were resuspended in barbital buffer, pH 7.4 (19). To obtain purified lymphocytes, we resuspended the cells in 1.5 ml of the plasma, and the suspension was introduced into a Pasteur pipet packed with nylon previously cleaned with 4% Duponol (Du Pont Co., Wilmington, Del.). The filled pipets were incubated for 30 min at 37°, and the cells were then eluted from the nylon columns with 2.5 ml barbital buffer. The eluted cells, now containing no more than 1 to 3% granulocytes, were centrifuged for 10 min at 200 X g at room temperature, the supernatant was discarded, and the cells resuspended in 1 ml. Tris buffer:NH4Cl (0.83%), pH 7.2 to 7.4, was used to lyse the remaining RBC. The cell suspension was placed in a 37° water bath for 5 or 10 min and centrifuged for 6 min at 100 X g. The cell pellet was resuspended in barbital buffer to the desired concentration.

Suspensions of WBC from CLL patients consisted of 75 to 100% lymphocytes and were not passed through nylon columns for removal of any granulocytes. These preparations are referred to in this communication as CLL WBC.

**Other Animal Cells.** WBC preparations were obtained from a mongrel dog, in a manner similar to that described for human WBC (see above). Spleen cells were obtained from randomly bred Swiss albino mice and rats, both of local origin, by a standard procedure (7). All the cells were finally suspended in barbital buffer, pH 7.4.

**Antiserum.** Antisera to CLL WBC and normal lymphocytes were prepared in adult randomly bred rabbits obtained locally. Groups of 3 animals each were used to produce 2 pooled antisera against CLL WBC obtained from 2 CLL patients, N. C. and R. S., respectively (these antisera are referred to as RALL1 and RALL2), and 1 pooled antisera against pooled normal lymphocytes (freed of granulocytes) derived from 10 normal donors (this antisera is referred to as RANL).

Two procedures of immunization were used. In the one, the animals were given 4 or 5 i.v. injections at weekly intervals, each of 10⁸ cells in a total volume of 1 ml, and were bled 10 days after the last immunization. In the other, the rabbits were first given 2 X 10⁸ cells suspended in 1 ml of 0.9% NaCl solution and emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Inc. Detroit, Mich.); the emulsion was injected intradermally in multiple sites. Three booster injections, each of 10⁸ cells in 0.9% NaCl solution without adjuvant, were then administered intradermally in multiple sites at weekly intervals. The animals were bled 10 days after the last immunization.

The sera were inactivated at 56° for 30 min and stored in aliquots at —20° until use. Sera derived from the same rabbits prior to immunization were used as NRS controls.

**HL-A Typing of Lymphocytes.** HL-A antigenicity was determined as described by Cohen and Kozaki (5), by use of 44 isoantisera (including monospecific sera obtained from the NIH Serum Bank) with a spectrum of antibodies directed against the antigens HL-A1, HL-A2, HL-A3, HL-A9, HL-A10, and HL-A11 of the first locus; HL-A5, HL-A8, and HL-A12 of the second; and the so-called long-reacting antigens, 4a, 4b, 6a, and 6b.

**Cytotoxicity Test.** A modification of the technique described by Gorer and O'Gorman (11) was utilized. Fresh rabbit serum was used as the source of complement. The complement-serum was absorbed with 1.2 X 10⁷ pooled normal human adult WBC per ml at room temperature for 30 min and stored in aliquots at —20° until use.

One- to 3-μl quantities of antisera diluted with barbital buffer were mixed with equal volumes of cell suspensions to a concentration of 3 X 10⁶ cells/ml in Terasaki microtiter plates; complement was added to a final dilution of 1:6. The plates were incubated at 37° for 30 min; the supernatant was then removed and replaced with 0.25% trypan blue in buffer. Four hundred cells were counted, and the percentage of cells stained by the dye was calculated. Controls included cells incubated with complement in the absence of antiserum, antisera without complement, and undiluted NRS with complement. These controls never contained more than 15% stained cells and were considered negative. In titration experiments, a positive end point was taken as that dilution of serum which resulted in staining of more than 15% of the target cells.

**Absorption of Antisera.** Absorption of the antisera was carried out either with pooled WBC derived from normal donors of known HL-A antigenic type and including all the known HL-A antigens (in similar proportion as in the pool of cells against which RANL was prepared) of the cells against which the antisera were directed, or with nontyped normal WBC derived from several donors. RBC were not removed from the absorbing WBC preparations to permit removal of anti-RBC antibodies as well.

Unless stated otherwise, absorption of each serum was always by 2 consecutive exposures, each to either 5 X 10⁸ typed or 7.5 X 10⁸ nontyped absorbing cells/ml, for 20 min at room temperature.

One antisera (RALL1) was absorbed also with CLL WBC derived from a number of CLL patients. For each absorption, the antisera was exposed to cells obtained from 1 patient only. In other experiments, this antisera was also absorbed with xenogenic WBC and spleen cells. These absorptions were carried out in a manner similar to that described for the absorptions with normal human WBC, but different numbers of cells were used, as described in the text.

**Isolation of IgG.** The globulin fraction was obtained from unabsorbed sera by precipitation with 33% saturated (NH₄)₂SO₄. The IgG component was isolated by column chromatography on DEAE-cellulose (4). Protein concentra-
tions were determined by absorbance measurements at 280 nm assuming an extinction coefficient (E\_1\%\,\text{cm}^-1) of 14.0 for rabbit γG (23).

Iodination of IgG with \textsuperscript{125}I. The chloramine method of Greenwood et al. (13) was used. One mCi of carrier-free \textsuperscript{125}I was used to iodinate 10 to 100 μg IgG. After removal of the nonbound iodine, the protein retained approximately 85% of the radioactivity.

Binding of Radioiodinated IgG and Antibody Preparations to Cells. CLL WBC, normal WBC, or purified lymphocyte suspensions were washed 3 times in Saline G (14), in 15-ml sterile disposable plastic tubes (Falcon Plastics, Oxnard, Calif.). To 1 to 50 × 10⁶ packed cells were added 0.1 ml each of undiluted inactivated NRS and inactivated NHS. A 0.9% NaCl solution of radioiodinated protein [1 × 10⁶ to 5 × 10⁷ cpm IgG or 3 × 10⁸ cpm of specifically enriched antibody preparation (see below)] was added to a final volume of 1.0 ml. The mixtures were rotated on a roller drum (7 rpm) or gently shaken on a horizontal shaker for 60 min at room temperature. After the cells were washed 3 times with Saline G, their residual radioactivity was determined in an Elron Model E1-5U-C well-type scintillation counter.

Specific Enrichment of Immunoglobulin Binding to CLL WBC. The radioiodinated IgG obtained from the anti-CLL WBC antisera was enriched by adsorption onto, and elution from, living CLL WBC. In some instances, the radioiodinated IgG preparations were first adsorbed with normal adult WBC, as described above; in others, such previous absorption with normal cells was omitted.

Five to 50 μg of the labeled IgG (20 to 50 × 10⁶ cpm) were adsorbed onto 1 to 2 × 10⁸ CLL WBC. Approximately 5% of the radioactivity remained in the cell sediments after washing. For elution of the presumed antibodies now attached to the cells, either the technique of Harris et al. (15) or that of Woodruff (30) was used. Both techniques are based on elution of antibodies by lowering the pH by use of 0.1 M glycine buffer, pH 2.5, or citric acid:phosphate buffer, pH 3 to 3.5. The elution medium (2 to 3 ml) contained 1% NRS. After elution, the cell mixtures were centrifuged for 10 min at 12,000 × g. The supernatants were removed, neutralized with 0.5 M K₃PO₄, dialyzed overnight against borate-buffered 0.9% NaCl solution (pH 7.4) at 4°C, and centrifuged again at 12,000 × g for 20 min. Approximately 30% of the radioactivity which had been adsorbed onto the cells was eluted by the procedure of Harris et al., and approximately 40% was eluted by that of Woodruff. The adsorption-elution procedure was in some instances repeated to obtain still more enriched preparations termed "doubly enriched."

RESULTS

Cytotoxic Activity of Rabbit Sera against CLL WBC and against Lymphocytes from Normal Adults, Adults with Other Diseases, and Normal Newborns

Before Absorption. Before absorption with normal adult WBC, the cytotoxic titers of the 2 anti-CLL WBC sera RALL₁ and RALL₂, and of the antinormal lymphocyte serum RANL, against CLL WBC from 7 patients, lymphocytes from 5 normal adult donors, and lymphocytes from 3 patients with other pathologies were similar, ranging from 1:640 to 1:2560 in several consecutive assays. There was no indication of a greater susceptibility of CLL than of other target cells to a given dilution of the sera. The percentage of the different cells damaged was similar at the same serum dilution in any one experiment. The cytotoxic titers of these antisera against lymphocytes from normal newborns were somewhat lower, ranging from 64 to 512.

After Absorption. After absorption with either typed or nontyped normal human WBC, all the antisera failed to exhibit cytotoxic activity against cells from any adult donor not suffering from CLL. However, marked activity towards all CLL WBC and against a proportion of normal newborn lymphocytes was retained by both RALL₁ and RALL₂. In contrast, the absorbed RANL retained no cytotoxic activity against any of the normal newborn lymphocytes or CLL WBC tested. The results of these experiments with absorbed, undiluted sera are depicted in Chart 1, A, B, and C.

It is seen from Chart 1, A and B, that following absorption with normal adult WBC the 2 undiluted anti-CLL WBC antisera remained highly cytotoxic for the CLL WBC from all of 18 CLL patients tested and for the lymphocytes from 11 of 37 samples of cord blood. The cytotoxic titers of both absorbed antisera were 1:161 and 1:2, against CLL WBC from 8 CLL patients and against cord blood lymphocytes from 4 newborns, respectively. Both sera were negative against lymphocytes from all 21 normal adults and from 28 patients with other neoplastic and nonneoplastic diseases (including 2 with acute myeloid leukemia, 2 with Hodgkin's disease, 2 with infectious mononucleosis, and 2 with infectious mononucleosis).

Following similar absorption of the antinormal lymphocyte serum with normal adult WBC, the antiserum became negative against lymphocytes from all individuals tested: 6 CLL patients, 16 normal newborns, 10 normal adults, and 18 patients with other neoplastic and nonneoplastic diseases (including 2 with infectious mononucleosis and 1 with Hodgkin's disease) (Chart 1C).

Experiments were now performed to test the cytotoxic activity of one of the antileukemic antisera, RALL₁, following absorption with different quantities of normal adult WBC and CLL WBC. Pools of normal WBC, HL-A typed to include within the absorbing population all the known HL-A antigens of the immunizing donor, or untyped pooled cells, were used. The CLL WBC for absorption came from individual donors.

The results of these experiments are shown in Chart 2.

With nearly similar numbers of absorbing cells, 1.5 × 10⁸ or more HL-A-typed as well as untyped normal WBC were equally effective in the removal of cytotoxic activity against normal lymphocytes from the undiluted antiserum. Absorption with up to 3 × 10⁹ normal adult WBC/ml of antiserum did not abolish the activity of the undiluted serum vis-à-vis CLL WBC from different donors. In contrast, absorption with even smaller numbers of CLL WBC removed completely the cytotoxic activity against all normal adult lymphocytes, as well as against some of the CLL WBC tested. Differences were observed in the cytotoxic activity of the undiluted antiserum towards CLL WBC following its absorption with WBC from different leukemic donors. Thus, absorption with WBC from
Chart 1. Cytotoxic activity of absorbed, undiluted rabbit antiserum against CLL WBC, lymphocytes from normal newborns, and lymphocytes from normal human adults and from patients with other pathologies (control). Each point represents a test with cells from a different donor. Note: Different donors for the newborn target cells were used in the experiments presented, but there was considerable overlap of the CLL target cell donors and some overlap of the non-CLL adult donors for these experiments.

ABSORPTION OF SERUM RALL₁ BY

NORMAL CELLS LEUKEMIC CELLS

Chart 2. Cytotoxic activity of undiluted rabbit antiserum RALL₁ against CLL WBC and normal adult lymphocytes following absorption with different numbers of CLL and normal WBC. The absorbing CLL WBC were derived from 3 patients, M. L., R. S., and S. S. The numbers of absorbing cells are the cumulative numbers used in the 2 consecutive absorptions. Each point represents a test with cells from a different donor; normal adult target lymphocytes (*) and CLL WBC targets (°). There was considerable overlap in the origin of the target cells for the different experimental groups.

Patient M. L. was effective in removing all cytotoxic activity from the serum against CLL WBC from 7 different leukemic donors. In contrast, after similar absorption of the antiserum with WBC from leukemic patients S. S. and R. S., marked cytotoxic activity still remained against CLL WBC from several of the leukemic patients.

In these and in other, repeated experiments, absorption with smaller numbers of normal adult WBC (5 × 10⁶ to 1 × 10⁷) also revealed differences in the degree of cytotoxic activity of the undiluted absorbed antiserum towards normal adult lymphocytes from several different donors. These differences could well have accrued from variations in the expression of antigenic determinants on the diverse normal cells. Absorption with the smaller numbers of normal adult WBC did not, on the other hand, reveal differences in the cytotoxic activity of the antiserum towards CLL WBC, suggesting that the distribution of normal antigens on the surface of the leukemic cells was more uniform, or that any variability was overshadowed by the presence of leukemia-associated antigens.

To determine whether the differences in reactivity of the antileukemic antisera against normal lymphocytes and CLL WBC could have arisen from the presence in the sera of antibodies to serum constituents attached in different quantities to the surface of the normal and neoplastic target cells, we subjected the antisera to immunoelectrophoresis against NHS and against CLL serum. Lines of precipitation were not detected. Neither was there any evidence of antihuman globulin activity in the antisera as determined by direct and indirect Coombs’ tests. Moreover, rabbit and goat antihuman globulin (Hyland Laboratories, Los Angeles, Calif.) revealed either no or only very low cytotoxic activity and titer (titers of 1:2 or 1:4) against normal adult lymphocytes and CLL WBC. It would appear, therefore, that the differences in cytotoxic activity of absorbed anti-CLL WBC sera for normal lymphocytes and CLL WBC reflected differences inherent in the surface of the normal and the leukemic cells.
Experiments were also undertaken to determine whether similar antigens are present on cells of other species. Serum RALL was subjected to further absorption by rat and mouse spleen cells and by dog WBC. For these absorptions, a total of $3 \times 10^9$ cells/ml were used of each cell type. No significant reduction in cytotoxic activity or titer of the absorbed antiserum against either normal or leukemic cells was found.

In most of the cytotoxicity experiments, sera taken from the rabbits prior to their immunization with CLL WBC were used as controls. These NRS were uniformly negative against all of the CLL WBC tested.

### Binding of Radioiodinated IgG and Enriched Antibody Preparations to CLL WBC

Table 1 shows the results of several experiments undertaken to determine the increased binding of labeled IgG and enriched antibodies from both anti-CLL WBC sera to leukemic target cells.

It is seen from Table 1 that a 4- to 20-fold increase in cell-bound radioactivity resulted from a 1st elution enrichment; a further 2-fold increase was obtained by repeating the enrichment procedure.

<table>
<thead>
<tr>
<th>Antiserum preparation</th>
<th>% binding (to $1 \times 10^6$ CLL WBC)</th>
<th>No. of experiments performed</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG of RALL b, 1 mCi/16 µg</td>
<td>1 - 1.5</td>
<td>4</td>
</tr>
<tr>
<td>IgG of RALL b, 1 mCi/100 µg</td>
<td>3.7 - 5</td>
<td>10</td>
</tr>
<tr>
<td>IgG of RALL b, 1 mCi/100 µg</td>
<td>4.5 - 5</td>
<td>2</td>
</tr>
<tr>
<td>Enriched antibodies from RALL b, 1 mCi/16 µg</td>
<td>9.5 - 21</td>
<td>8</td>
</tr>
<tr>
<td>Enriched antibodies from RALL b, 1 mCi/100 µg</td>
<td>10 - 22</td>
<td>20</td>
</tr>
<tr>
<td>Enriched antibodies from RALL b, 1 mCi/100 µg</td>
<td>14 - 22</td>
<td>2</td>
</tr>
<tr>
<td>Doubly enriched antibodies from RALL b, 1 mCi/100 µg</td>
<td>25 - 30</td>
<td>2</td>
</tr>
</tbody>
</table>

### Table 2

Table 2 shows the results of several experiments undertaken to determine the increased binding of labeled IgG and enriched antibodies from both anti-CLL WBC sera to leukemic target cells.

<table>
<thead>
<tr>
<th>% binding when no. of target cells was:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiserum preparation b &amp; c</td>
</tr>
<tr>
<td>IgG</td>
</tr>
</tbody>
</table>

* a Antiserum preparations in some of the experiments were previously absorbed with normal human WBC.
* b RALL b and RALL c are 2 distinct rabbit antisera against WBC of patients with CLL (see text for details).

Increase in the number of CLL WBC used in the attachment tests with crude and enriched IgG heightened the proportion of cell-bound radioactivity (Table 2). Most of the binding experiments were subsequently performed with 1 to 3 $\times$ 10^6 target cells.

A number of experiments were now conducted to determine the specificity of binding of the anti-CLL sera to the leukemic target cells. In a 1st experiment, WBC and purified lymphocyte suspensions, both obtained from the same normal donor, were incubated with enriched antibody preparation derived from 1 antileukemic antiserum (RALL b) (previously absorbed with normal WBC). It can be seen from the results presented in Table 3 that there was only a low and identical degree of binding over a 3-fold range of either normal adult WBC or lymphocytes.

In a 2nd series of experiments, both crude labeled IgG and single-elution enriched labeled antibodies (not previously absorbed) derived from 1 of the anti-CLL antisera (RALL b) were tested in parallel for ability to bind to normal adult WBC, normal adult RBC, CLL WBC, and mouse spleen cells. The results are depicted in Table 4. RALL b had the expected species specificity as indicated by a preferential binding of the labeled antiserum preparations to the human normal and CLL WBC.

Chart 3 presents the results of 6 further experiments undertaken to investigate the consistency of the increased binding of enriched radioiodinated anti-CLL WBC antibody preparations to the neoplastic cells as compared to the normal adult WBC. Preparations derived by elution from the leukemic WBC were not absorbed with normal human WBC prior to enrichment. In the experiments with sheep red blood cells and mouse spleen cells the labeling was with 1 mCi/100 µg; in the other experiments, labeling was either 1 mCi/16 µg or 1 mCi/100 µg.
cells without prior absorption with normal WBC as well as those which were subjected to this step were examined. At the same time, the binding of radioiodinated absorbed and enriched anti-normal lymphocyte antibodies (derived from RANL) to leukemic and normal WBC was determined. This antibody preparation was subjected to the same enrichment procedure as that used with the anti-CLL sera, i.e., adsorption and elution from leukemic cells after prior absorption with normal WBC.

It can be seen from Chart 3 that there was a consistently elevated binding of both the previously absorbed and nonabsorbed enriched anti-CLL antibody preparations to the neoplastic cells. In contrast, no such effect was apparent with antibodies derived from the anti-normal WBC serum. Preceding absorption with normal WBC reduced the degree of attachment of the labeled enriched anti-CLL antibodies to the normal WBC. It also appeared that freezing and storage of the antibody preparations at —20° for several days reduced the effectiveness of the binding.

Also, preceding absorption with CLL WBC in this and in other experiments virtually eliminated the attachment of labeled anti-CLL antibodies to the neoplastic target cells.

Additional, similar experiments were performed with labeled normal rabbit serum. For simulation of the enrichment procedure the IgG fraction was similarly adsorbed and eluted from human leukemic cells. No significant binding of the labeled preparations was found to either normal or leukemic target cells.

Two other types of experiments were performed to show clearly that there is an element of specificity to the binding of the radioiodinated absorbed and enriched anti-CLL WBC antibodies to these cells. The first was a competition experiment, in which binding was carried out in the presence of an excess of unlabeled, unabsorbed IgG from RALL1 (ca. 0.003 μg purified antibody and 35 to 50 μg crude IgG). Table

### Chart 3

** Binding of 125I-labeled, enriched antibodies from rabbit sera directed against CLL WBC (RALL1 and RALL2) and against normal human adult WBC (RANL) to normal and CLL WBC target cells. As indicated, in some instances the labeled IgG preparations were absorbed with normal human adult WBC prior to enrichment. Each point represents a test with cells from a different donor; normal adult WBC (o) and CLL WBC targets (•). The preparation in Experiment 3 (*) was doubly enriched by 2 consecutive adsorptions and elutions.

### Table 5

**Binding of 125I-labeled enriched antibodies from serum RALL1 to CLL and normal adult WBC target cells in the presence and absence of unlabeled IgG from the same serum**

<table>
<thead>
<tr>
<th>Test system</th>
<th>% binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enriched antibodies, 10⁶ CLL WBC target cells</td>
<td>16.8</td>
</tr>
<tr>
<td>Enriched antibodies + unlabeled IgG, 10⁶ CLL</td>
<td>6.7</td>
</tr>
<tr>
<td>WBC target cells</td>
<td></td>
</tr>
<tr>
<td>Enriched antibodies, 10⁶ normal human adult</td>
<td>2.0</td>
</tr>
<tr>
<td>WBC target cells</td>
<td></td>
</tr>
<tr>
<td>Enriched antibodies + unlabeled IgG, 10⁶ normal</td>
<td>1.0</td>
</tr>
<tr>
<td>human adult WBC target cells</td>
<td></td>
</tr>
</tbody>
</table>

a The labeled enriched antibodies were prepared from IgG previously absorbed with normal human adult WBC; labeling with 1 mCi/100 μg.
b The unlabeled IgG (from serum RALL1) had not been previously absorbed.
5 shows the results of 1 of 8 such experiments, each one of which yielded similar findings.

It is seen from Table 5 that the unlabeled globulin preparation from the same antiserum strongly inhibited binding of the labeled one.

In the 2nd approach, the affinity for 2 unrelated target cells by 2 specific antisera was tested. The target cells were CLL and normal human adult WBC, and hamster kidney cells transformed in tissue culture by the SV40 virus; the antiserum preparations were enriched antibodies from anti-CLL WBC serum RALL, and a similarly prepared antibody preparation from an antiserum against cell membrane of the SV40-transformed cells (18).

It is seen from Table 6 that the enriched anti-CLL antibodies attached to a very significant extent to the CLL WBC but not to the neoplastic hamster cells. Conversely, the anti-hamster cell membrane antibodies bound strongly to the normal WBC but not to the neoplastic hamster cells. Binding of the anti-CLL antibodies to transformed hamster cells was not enhanced by use of intact transformed hamster cells, rather than CLL WBC, in the adsorption and elution procedure.

**DISCUSSION**

The results of the present experiments with 2 rabbit antisera directed against the WBC of 2 patients with CLL point to the occurrence of antigenic properties on the surface of these cells, mostly lymphocytes, which are characteristic in adulthood to this neoplastic disease but which also seem to distinguish the surface of the lymphocytes of at least a proportion of normal newborns. This latter observation is consistent with demonstrated similarities in behavior of CLL and normal infant lymphocytes (17).

The specificity of the reaction of the 2 distinct heterogenic antisera with the leukemic target cells was shown in 2 test systems, a cytotoxicity assay and the binding onto CLL WBC of labeled antibody preparations concentrated by prior adsorption and elution.

In the former system, it was found that after complete absorption with normal adult WBC, the antiserum retained cytotoxic efficacy against all CLL WBC tested and against normal newborn lymphocytes from some donors. NRS and a rabbit anti-normal human adult lymphocyte serum had no such activity after absorption with normal cells.

The cytotoxic activity of the anti-CLL sera against CLL target cells here observed cannot be ascribed, therefore, to natural heteroantibodies against such cells which some investigators have occasionally found in NRS (25). With nearly similar numbers of absorbing cells, HL-A typed and untyped normal WBC were equally effective in removing cytotoxic activity against normal lymphocytes from 1 of the antileukemic sera tested. Absorption of this serum with varying numbers of normal adult WBC revealed differences in the degree of its cytotoxic activity towards normal adult lymphocytes from several different donors but not towards CLL WBC of diverse origin. Absorption of the antiserum with CLL WBC from a number of patients showed that the absorbing cells of different origin were differentially effective in removing anti-CLL target cell reactivity. In contrast, absorptions with similar numbers of leukemic cells from different patients were equally effective in abrogating all anti-normal lymphocyte reactivity.

These observations could be interpreted to indicate that the antigenic difference between normal and CLL lymphocytes is merely a quantitative one, with variations in the presence of “normal” lymphocyte-expressed antigens among both normal and leukemic individuals, but with the spectrum of differential expression in the latter lying at a considerably higher order of magnitude. It is also conceivable that different normal isoantigens on the leukemic cells are recognized by the rabbit and that HL-A typing does not reveal all the determinants to which the heterogenic antibody producer responds. On the other hand, our findings are consistent, and perhaps even more compatible, with the possibility that distinct leukemia-associated antigens are responsible, at least in part, for the observed differences in the antigenic profile of normal and leukemic lymphocytes. These leukemia-associated antigens may be expressed to different extents, quantitatively and perhaps even qualitatively, on the neoplastic cells of different subjects, and there may be at the same time a more uniform appearance of normal antigens on the leukemic than on normal lymphocytes. A reduction in the complexity of normally represented antigenic components on the surface of cancer cells, side by side with the appearance of a spectrum of tumor-related antigens, has been demonstrated for other instances of neoplasia (6, 24).
The WBC of adults suffering from a variety of other neoplastic and nonneoplastic diseases behaved in the cytotoxicity test in the same manner as target cells from normal adults, thus pointing further to the specificity of the antibody reaction against the CLL targets.

In the 2nd test system, large differences (up to 10-fold) were found in the binding to CLL and normal adult WBC of enriched, labeled antibody preparations obtained from the rabbit antisera by preceding adsorption onto the leukemic cells and subsequent elution. In some experiments, the enriched preparations were also purified by a prior absorption with normal adult WBC. This affinity for the surface of the leukemic cells was not seen in similar preparations derived from rabbit anti-normal human lymphocyte serum. Moreover, the enhanced attachment of the labeled, enriched anti-CLL antibody preparations to the CLL cells could be inhibited by introducing unlabeled antibody to the system. Other animal cells, including transformed hamster kidney cells, had only very low avidity for the anti-CLL preparations and, conversely, anti-transformed hamster kidney cell serum preparations attached well to the corresponding target cells but not to CLL WBC.

The difference in reactivity of the anti-leukemic antisera against normal lymphocytes and CLL WBC could conceivably have arisen from the presence in the sera of antibodies to serum constituents attached in different quantities to the surface of the normal and leukemic target cells (3, 21). However, the failure of rabbit and goat anti-human globulin sera to react with CLL WBC, the negative direct and indirect Coombs’ tests with the 2 rabbit anti-CLL sera, and the negative findings in the immunoelectrophoretic analyses of the rabbit antisera against normal human serum and against several sera from CLL patients indicate that the antigenic differences between the normal and leukemic lymphocytes here detected lie with the cell surface itself and are not caused by any differential absorption of serum proteins. This was also borne out by the binding experiments with the labeled antibody preparations, which were performed in excess of NRS and NHS.

It has been postulated that human leukemic lymphocytes may be derived from 1 type of precursor, the “T” WBC, which is apparently present in greater numbers in normal rat than in normal human blood, and a related antigenicity between human CLL and normal rat lymphocytes may thus not be unlikely (26). It was also suggested by Rapaport (24) that there exists a common or related heterophilic antigen in human leukocytes and rat erythrocytes. Schrek et al. (27) recently described the occurrence of a heterophilic antigen in human leukemic and in normal dog lymphocytes, which was considered not to be of the classic Forssman type. Our present experiments showed that absorptions of one of the anti-CLL WBC sera with rat, mouse, and dog lymphoid cells did not remove or decrease its anti-human CLL cytotoxicity. Moreover, labeled anti-CLL antibody preparations were not found to attach significantly to cells from various other species (Tables 4 and 6). These results would seem to indicate that the different antigenicity of CLL WBC demonstrated in our study is not likely to be of Forssman or of other common heterogenic origin.

Even doubly enriched anti-CLL antibody preparations exhibited only a limited degree of attachment to the leukemic cells. This limitation may reflect an inherently low avidity of these antibodies or may be an artifact of the technique. The chemical conditions under which elution takes place may damage antibody molecules in such a manner as to reduce their avidity, and in addition it may be the low-avidity antibodies which are preferentially removed. It is also possible that considerable quantities of cell proteins are removed during the procedure, leading to the formation of antigen-antibody complexes, and perhaps even to the destruction of the labeled antibody by cell enzymes. We have initiated experiments in which the adsorbing target cells are first fixed with glutaraldehyde, a procedure which reduces markedly the loss of cellular proteins; preliminary findings indicate that this modification provides antibody eluates with considerably higher binding activity.

The present findings may constitute another example of the relationship between antigens associated with the neoplastic state on the one hand and with normal tissues in early life on the other. Such observations, together with the characterization of other tumor antigens as determinants which also appear normally, but in smaller quantities, on parenchymal cells of the corresponding or of other organs, point to the need of a revised view of the nature of “tumor-specific” antigens. Many, and perhaps all, such antigens may not in fact represent bona fide new molecular entities but may be normally occurring antigens expressed in very different quantities, or displaced ontogenically, architecturally, and perhaps even phylogenetically.

It would appear that the technique of purifying antibodies to tumor antigens by means of absorption with corresponding normal cells and then enriching the labeled preparations by repeated adsorption onto and elution from the neoplastic target cells provides a ready means of quantifying the immune response to such antigens and may also facilitate their eventual isolation and characterization. Experiments are already under way in this direction with the use of highly enriched labeled antibody preparations from RALL₁ and RALL₂ and CLL WBC membrane fractions.

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Antigenicity of CLL Lymphocytes

Antigenic Changes on the Surface of Lymphocytes from Patients with Chronic Lymphocytic Leukemia

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