Deceased CD45RA T Cells in B-Cell Chronic Lymphatic Leukemia Patients: Correlation With Disease Stage

By Shoshanna Peller and Suzanna Kaufman

T-cell subsets CD4, CD8 and suppressor-inducer (CD45RA) were determined in 20 patients with B-cell chronic lymphatic leukemia (B-CLL). The proportion of CD4 and CD45RA was decreased when compared with T cells from normal subjects. CD8 was markedly increased. The activity of concanavalin A-induced suppressor cells was not significantly different from that of normal controls and was negatively correlated to the percentage of CD4 of B-CLL patients. The selective loss of CD45RA cells was more prominent in patients in advanced Rai stages of the disease (III to IV) than in early stages (0 to II). Six patients of the advanced stages group suffered from autoimmune hemolytic anemia, whereas no patient in the early stages of disease showed an autoimmune phenomenon. Our results may indicate a mechanism of autoimmune in B-CLL similar to that of patients with autoimmune diseases.

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MATERIALS AND METHODS

Patients and controls. This study included 20 patients having the clinical and immunophenotype criteria for B-CLL. Table 1 summarizes the data on the patients, including their staging according to Rai et al^{22} and the absolute numbers of lymphocytes. These data present patients at the time this study was performed. Disease duration ranged from 1 to 18 years. Most of the patients were off therapy. Six patients received chlorambucil, steroids, or a combination of the two. In these cases, care was taken to withdraw blood for this study a few weeks before treatments. Patients no. 12, 13, 15, 17, and 19 had hemolytic anemia with a positive direct antiglobulin test (DAT). Patients 12 and 19 had a positive DAT for the first time 5 years before the study was performed; the others had positive DAT for the first time 6 months before the study. Patient no. 20 had DAT-negative hemolytic anemia responsive to steroid therapy six months before the study. Patients no. 1, 3, 5, 6, and 9 had normal serum protein electrophoresis. All other patients were hypogammaglobulinemic with patient no. 10 having an IgM spike and patient no. 15 an IgM spike.

There was no clinical indication in our patients to test for autoantibodies to DNA, nuclear factors, or rheumatoid factor. Results from patients were compared with those of 15 age- and sex-matched healthy control subjects studied in parallel. A group of six patients and four normal controls were studied again only for T-cell subsets by flow cytometry.

Separation of lymphocytes. Lymphocytes were separated from 20 mL of heparanized blood by density gradient centrifugation on lymphoprep and recovered by lysing the SRBCs with Tris-NH,Cl pH 7.2 solution. T cells were washed three times with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 (GIBCO, Grand Island, NY) containing 25 mmol/L HEPES, L-glutamine, and combined antibiotics.

T lymphocytes were separated by rosetting with sheep red blood cells (SRBCs) previously treated with 2-aminoethylisothiouronium bromide (AET; Sigma, St Luis, MO). One volume of lymphocytes at a concentration of 5 × 10^6 cells/mL was mixed with 1 vol of 1% AET-SRBC and 0.25 vol of inactivated fetal calf serum (FCS; GIBCO). Rosetted T cells were pelleted by centrifugation on lymphoprep and recovered by lysing the SRBCs with Tris-NH,Cl pH 7.2 solution. T cells were washed three times with PBS and resuspended in RPMI. The suspension contained 95.2% ± 4.2% T lymphocytes of patients or 97.8% ± 2.9% T cells of normal controls. These cell suspensions were used to determine T-cell subsets and Con A-induced suppressor cells at the same time.

Immunophenoqing. Indirect immunofluorescence was used. Briefly, 10^6 T cells were incubated with 10 to 20 μL of either CD4, CD8 (Dakopatts, A/S, Denmark) or CD45RA (2H4 Coulter Clone, Hialeah, FL) for 30 minutes at 4°C. The cells were washed twice with PBS, resuspended in RPMI, and stained with monoclonal antibodies. The cells were counted in a counting chamber and the mean fluorescence intensity (MFI) was determined by flow cytometry.

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cells were washed four times with PBS and resuspended in RPMI
lymphocyte samples. Incubation was performed at 37°C with
were added. After 72 hours of incubation, 0.5 pCi of 'H-thymidine
minutes with each monoclonal antibody (MoAb) and an additional
performed. After three washes, the cells were fixed with 3%
cytes were counted with a Zeiss epifluorescent microscope (Zeiss,
CD4-fluorescein isothiocyanate (FITC) and 2H4-phycoerythrin
Israel). A control test without Con A was also performed. T cells
Lymphocyte transformation was performed in microtiter plates.
compared with control lymphocytes incubated without Con A.

A-induced suppressor cells or control cells (lo5 cells in 100 pL)
with each monoclonal antibody (MoAb) and an additional
RPMI 1640. Fifty micrograms of mitomycin C was added and the

Viability of cells exceeded
minutes at 37°C. Subsequently, the
Con A (Biomakor, Rehovot, Israel) was added to each well for the last 4 hours. The plates were
was harvested and radioactivity (counts per minute) was counted in a beta counter. Percentage suppression was calculated as follows:

% Suppression

Results are mean ± SD.
Abbreviation: NS, not significant.

Twice with PBS, resuspended in 100 μL PBS, and incubated for 30 minutes at 4°C with 10 μL of fluorescein-conjugated F(ab), fragment of antimouse Ig (Dakopatts). After three washes with PBS, the cells were resuspended in PBS and fluorescent lymphocytes were counted with a Zeiss epifluorescent microscope (Zeiss, Germany).

For flow cytometry, we used direct immunofluorescence with CD4-fluorescein isothiocyanate (FITC) and 2H4-phycerythrin (Coulter Clone). Cells (10⁶) were incubated separately for 30 minutes at 4°C with 10 μL of fluorescein-conjugated F(ab), fragment of antimouse Ig (Dakopatts). After three washes with PBS, the cells were resuspended in PBS and fluorescent lymphocytes were counted with a Zeiss epifluorescent microscope (Zeiss, Germany).

Con A-induced suppressor cells. For induction of suppressor
cells, T lymphocytes were incubated in 1 mL of RPMI 1640 with
10% autologous plasma and 45 μg of Con A (Biomakor, Rehovot,
Israel). A control test without Con A was also performed. T cells
from healthy subjects were incubated in parallel with each patient's
lymphocyte samples. Incubation was performed at 37°C with 5% CO₂, for 24 hours. T lymphocytes to be used as responders were kept for 24 hours at 4°C. After induction of suppressor cells, the
lymphocytes were washed once with PBS and resuspended in 1 mL
RPMI 1640. Fifty micrograms of mitomycin C was added and the
test tubes were incubated for 45 minutes at 37°C. Subsequently, the
cells were washed four times with PBS and resuspended in RPMI 1640 at a concentration of 1 x 10⁶/mL. Viability of cells exceeded 80%.

Suppressor cell activity was measured by the reduction of
lymphocyte transformation of the responders with phytohemagglu-
tinin (PHA) in the presence of Con A-induced suppressors
compared with control lymphocytes incubated without Con A.
Lymphocyte transformation was performed in microtiter plates.
Each well contained 10⁶ responders, 10% autologous plasma, 10 μg/mL of PHA, and RPMI 1640 to a total volume of 100 μL. Con
A-induced suppressor cells or control cells (10⁵ cells in 100 μL)
were added. After 72 hours of incubation, 0.5 μCi of 'H-thymidine
(specific activity, 49 Ci/mmol; Nuclear Research Center, Negev,

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CD45RA CELLS IN B-CLL

Fig 2. T-cell subsets and suppressor cell activity in early and advanced stages of B-CLL patients and normal controls. (■) Patients at stages 0 to II; (■) patients at stages III to IV; (■) normal controls.

Fig 3. Immunophenotyping by flow cytometry of normal T lymphocytes. (a) CD4⁺ cells, (b) CD45RA⁺ cells, and (c) CD45RA⁺ CD4⁺ cells by double coloring.

Fig 4. Immunophenotyping by flow cytometry of T lymphocytes from a B-CLL patient. (a) CD4⁺ cells, (b) CD45RA⁺ cells, and (c) CD45RA⁺ CD4⁺ cells by double coloring.

Table 3. Immunophenotyping by Flow Cytometry of Lymphocytes From B-CLL Patients and Normal Controls

<table>
<thead>
<tr>
<th>Clinical Stage</th>
<th>% CD4</th>
<th>% CD45RA</th>
<th>% CD45RACD4⁺</th>
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<tr>
<td>Patient</td>
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<td></td>
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</tr>
<tr>
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<td>32</td>
</tr>
<tr>
<td>2</td>
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<td>IV</td>
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<tr>
<td>5</td>
<td>III</td>
<td>48</td>
<td>23</td>
</tr>
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<td>6</td>
<td>III</td>
<td>46</td>
<td>20</td>
</tr>
<tr>
<td>Normal control</td>
<td></td>
<td>58.3 ± 4.5</td>
<td>37.3 ± 6.4</td>
</tr>
</tbody>
</table>

*Counted as percentage of CD4⁺ cells.
†Results are mean ± SD.

patients in early and advanced stages of the disease because the numbers in each group were too small. The differences between the cell subsets in one B-CLL patient and one control subject by flow cytometry can be seen in Figs 3 and 4.

DISCUSSION

Our study showed a decreased percentage of CD4 lymphocytes in B-CLL patients and an increased percent-
age of CD8 cells. These results confirm previous reports on these T-cell subsets in B-CLL.

The percentage of CD45RA-positive T lymphocytes in the B-CLL patients we studied was markedly reduced. This decrease was more prominent in the advanced Rai stages. This result contradicts a previous report showing an increase in the percentage of CD45RA-positive CD3 lymphocytes in a group of B-CLL patients when compared with normal controls with no correlation to the stage of the disease. Our study determined CD45RA-positive T cells in 20 patients and counted by dual-color cytomtery the CD45RA-positive CD4 cells in six B-CLL patients. In both cases we observed a decrease in the CD45RA population, which was a result of a decrease in CD4+CD45RA+ cells. This finding agrees with the results of Totterman et al., who demonstrated a decrease in CD45RA+CD4+ cells with correlation to Rai staging.

CD45RA cells were found to generate in vitro Con A-induced suppressor cells. Therefore, we studied these suppressor cells in T cells from B-CLL patients. Our results show no significant difference between the suppressor cell activity of patients and normal controls. Most patients had suppressor activity within the normal range. Nine patients showed excessive suppression. Excessive suppression was found earlier when suppressor cell activity of B-CLL patients was studied on B-lymphocyte function.

Selective loss of CD45RA cells plays a role in the pathogenesis of autoimmune diseases. Unlike our results and the results of others on normal or excessive suppressor cell activity in B-CLL, patients with autoimmune diseases like SLE and idiopathic thrombocytopenic purpura (ITP) had impaired suppressor cell activity. When we looked for autoimmune phenomena in our patients we observed that five patients had a positive DAT hemolytic anemia, whereas one patient had autoimmune hemolytic anemia with a negative DAT. As can be seen in Table 1, all these patients were in an advanced Rai stage (III to IV), a group that also had a prominent decrease in CD45RA. A previous report by Hamblin et al showed that autoimmunity in CLL prevails in higher percentages in patients with advanced disease stages than in patients with early stages.

The excessive activity of suppressor cells observed by us may be explained by the presence of activated CD8 cells, as was recently reported. The presence of a certain percentage of activated suppressor cells in our B-CLL patients may also explain the negative correlation between the percentage of CD4 and the suppressor cell activity observed by us. Excessive suppression can also be explained if CD4 cells other than CD45RA act as suppressor-inducers in B-CLL. This explanation awaits further studies.

Except for the imbalance of the immunoregulatory T cells in B-CLL patients shown here, it has been recently reported that the expanded CD5-positive B cells of B-CLL patients can produce in vitro, after stimulation, autoantibodies. These autoantibodies could be demonstrated in sera from these patients, but only in very low levels.

Our results show a decrease in CD45RA cells in B-CLL patients progressing in the more advanced clinical stages of the disease. These results and the observation that 6 of 10 patients in the advanced stages had autoimmune phenomena may imply an autoimmune mechanism in B-CLL triggered by selective loss of CD45RA similar to that of autoimmune diseases.

The pathogenesis of autoimmunity in B-CLL may be more complex because of other impaired immune components like CD8 cells and dysfunction of B lymphocytes.

Sequential studies currently being performed on our patients may further enlighten the mechanism of onset of autoimmunity in B-CLL patients.

REFERENCES


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