T-cell subsets CD4, CD8 and suppressor-inducers (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 immunity in B-CLL similar to that of patients with autoimmune diseases.

**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. 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 lambda 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 heparinized blood by density gradient centrifugation on lymphoprep (Nycomed, Oslo, Norway). The 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 Louis, MO). One volume of lymphocytes at a concentration of 5 x 10^6 cells/mL was mixed with 1 vol of 1% AET-SRBC and 0.25 vol of inactivated feline calf serum (FCS; GIBCO). Rosetted T cells were pelleted by centrifugation on lymphoprep and recovered by lysing the SRBCs with Tris-NH4Cl 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.

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

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1569
cells were washed four times with PBS and resuspended in RPMI kept for 24 hours at 4°C. After induction of suppressor cells, the lymphocyte samples. Incubation was performed at 37°C with from healthy subjects were incubated in parallel with each patient's lymphocytes were counted with a Zeiss epifluorescent microscope (Zeiss, Israel). A control test without Con A was also performed. T cells to be used as responders were incubated in 1 mL of RPMI 1640 with 10% autologous plasma and 45 μg of Con A (Biomakor, Rehovot, Israel) was added to each well for the last 4 hours. The plates were harvested and radioactivity (counts per minute) was counted in a beta counter. Percentage suppression was calculated as follows:

\[
\% \text{ Suppression} = 1 - \frac{\text{cpm With Con A-Induced Suppressors}}{\text{cpm With Control Lymphocytes}} \times 100
\]

Statistical analysis was performed by unpaired Student's t-test, the Pearson's correlation coefficient, and the Mann-Whitney test.

RESULTS

Table 2 summarizes T-cell markers and the percentage of suppression in both patients and control subjects. We have observed a significant decrease in the percentage of CD45RA cells in T cells from B-CLL patients compared with normal controls. The CD4 population in B-CLL was also significantly decreased, whereas the CD8 cells were increased. There was no significant difference between the percent suppression of patients' T cells and normal T cells, although there was a wider variability of results within the patients. Most patients had suppression within normal range, two had negative suppression meaning enhancement, and nine patients had suppressor cell activity greater than the upper range of normal cells.

Our analysis showed a negative significant correlation (r = -0.57, P < .015) between the percentage of CD4 and suppressor cell activity in the patients, as can be seen in Fig 1.

For further analysis, the patients were divided into two groups: a group of 10 patients at early stages of the disease

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Clinical Stage</th>
<th>Lymphocytes (x 10^6/L)</th>
<th>Disease Duration (y)</th>
<th>Therapy*</th>
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<tr>
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<tr>
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<td>M</td>
<td>IV</td>
<td>30.2</td>
<td>4</td>
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</tr>
</tbody>
</table>

*+, chlorambucil; ++, steroids; ++++, chlorambucil and steroids.

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-phycocerythrin (Coulter Clone). Cells (10^6) were incubated separately for 30 minutes at 37°C. Subsequently, the cells were fixed with 3% formaldehyde in PBS and counted using EPICS Profile Analyzer (Coulter Corp).

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 10^6/mL. Viability of cells exceeded 80%.

Suppressor cell activity was measured by the reduction of lymphocyte transformation of the responders with phytohemagglutinin (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 10^5 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 (10^5 cells in 100 μL) were added. After 72 hours of incubation, 0.5 μCi of 3H-thymidine (specific activity, 49 Ci/mmol; Nuclear Research Center, Negev.
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.

(0 to II) and a second group with 10 patients in advanced stages (III to IV).

The results of each group were compared with those of normal controls and showed the same significant differences observed with the group of 20 B-CLL patients, a decrease in CD4 and CD45RA and an increase in CD8. There was no significant difference between the suppressor cell activity in each group and the activity of normal lymphocytes.

A negative correlation between the percent of CD4 and percent of suppression was also observed both in patients with early disease stages and patients with advanced stages ($r = -0.6$, $P < 0.05$).

When T-cell subsets and Con A-induced suppression were compared between the two groups of patients, no difference was observed in the percent of CD4, CD8, and suppressor cell activity. The percentage of CD45RA, on the other hand, was markedly reduced in patients at stages III to IV compared with those at stages 0 to II ($31.4 \pm 7.8$ vs. $38.5 \pm 6$, $P < 0.03$) (Fig 2).

The results of determination of T-cell subsets CD4 and CD45RA by flow cytometry are summarized in Table 3. The patients had a marked decrease in CD4 cells, CD45RA cells, and cells bearing both markers (CD45RA"CD4") when compared with controls ($P < 0.05$, $P = 0.05$, and $P < 0.005$, respectively). We did not compare in this case 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-

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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical Stage</th>
<th>% CD4</th>
<th>% CD45RA</th>
<th>% CD45RA&quot;CD4&quot;</th>
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</thead>
<tbody>
<tr>
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<td>33</td>
<td>32</td>
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</tr>
<tr>
<td>6</td>
<td>III</td>
<td>46</td>
<td>20</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Normal controls* (n = 4) 58.3 ± 4.5 37.3 ± 6.4 24 ± 3

*Counted as percentage of CD4" cells.
†Results are mean ± SD.
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 cytometry 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 CD45RA+CD4+ 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.

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Decreased CD45RA T cells in B-cell chronic lymphatic leukemia patients: correlation with disease stage

S Peller and S Kaufman