CONCISE REPORT

Characterization of the Phytohemagglutinin-Induced Proliferating Lymphocyte Subpopulations in Chronic Lymphocytic Leukemia Patients Using a Clonogenic Agar Technique and Monoclonal Antibodies

By Stephen Davis

Peripheral blood lymphocytes from normal donors and patients with chronic lymphocytic leukemia, B-cell type, were purified into T, helper T, and suppressor T lymphocytes by fluorescence-activated cell sorting using OKT3, OKT4, and OKT8 monoclonal antibodies. The maximum response of the purified subpopulations to stimulation by phytohemagglutinin (PHA) was determined by measuring the production of colonies when the stimulated cells were grown on agar. The helper T cells in normal and CLL patients were the most responsive to PHA stimulation, although the responsiveness of helper T cells to PHA was decreased in CLL. Purified CLL B cells responded minimally to PHA stimulation, but normal B lymphocytes did not. The abnormal response of CLL lymphocytes to PHA appears to be due to abnormal helper T cells, and, to a smaller extent, to the ability of CLL B lymphocytes to respond.

PHYTOHEMAGGLUTININ (PHA) induces the proliferation of human lymphocytes in vitro. When normal lymphocytes are stimulated in culture by PHA, maximal proliferation occurs at 72 hr, as determined by 3H-uridine incorporation into RNA. The lymphocytes of patients with chronic lymphocytic leukemia (CLL) respond less well than normal lymphocytes when observed at 72 hr; the maximal response is seen at 5-7 days. The delayed response to PHA is greater in patients with more advanced disease.

CLL is said to be a B-cell disease, since the majority of circulating lymphocytes have monoclonal immunoglobulin (Ig) on their membrane. However, patients in all stages of CLL have an increased number of T cells. Furthermore, the T-cell population in these patients is both functionally and biochemically different from that in normal patients.

Available data have suggested that the delayed and diminished response of CLL lymphocytes to PHA is the expected response of B cells, although the ability of lymphocytes to respond to PHA is regarded as a function of normal T cells. The delayed response of CLL lymphocytes to PHA has also been regarded as the result of dilution of normal T cells by a large population of mitogen-unresponsive CLL B cells. Our previously published data suggest that the responsiveness of CLL lymphocytes to PHA is independent of the surface markers classically used to identify normal lymphocytes, i.e., E rosettes (T cells), surface immunoglobulin (B cells). In fact, PHA responsiveness appears to be a function of the absolute level of Tg and Tγ cells rather than B cells. Tγ and Tα cells supposedly represent suppressor and helper cells, respectively.

Because stimulation of lymphocytes by PHA in culture medium involves cell–cell interaction and the possible recruitment of B cells into a state of proliferation, the characterization of the PHA-stimulated cell in CLL has not been rigorously defined. Similarly, the exact nature and role of Tγ and Tα as suppressor and helper T cells, respectively, is controversial. In this study, we stimulated normal and CLL lymphocytes purified by monoclonal antibodies specific for helper and suppressor T cells using a clonogenic agar technique. The data show that the lymphocyte response to PHA is a function of helper T cells and that the response is decreased in helper T cells from CLL patients; B cells from CLL patients respond minimally to PHA whereas normal B cells do not.

MATERIALS AND METHODS

Twelve male patients with classic CLL were studied. Their mean age was 71 yr (range 59-90). No patient had received chemotherapy prior to the study. Peripheral blood lymphocyte counts ranged between 46,000 and 250,000/cu mm. Ten sex and age-matched (mean 67 yr) healthy subjects were used as normal controls. A high lymphocyte count was the only selection criteria for CLL patients. One patient was clinical stage 2; the remaining patients were stage 3–4 (Rai staging).

Phytohemagglutinin stimulation was analyzed by the clonogenic agar technique of Mercola and Cline. The plating medium consisted of McCosys 5A with 25% heat-inactivated fetal calf serum with 125 μg PHA-P/ml. Dose–response curves determined this as the optimal PHA concentration. The agar mixture contained a 3:2 ratio of medium to agar concentration of 0.3% for the top layer and

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0.5% for the bottom layer using 35-mm Petri dishes. The top layer contained agar, autologous erythrocytes, and lymphocytes. Lymphocytes were added in a concentration of \(10^5\) cells/ml. Linearity in colony formation was observed with lymphocyte concentrations between \(5 \times 10^3\) and \(3 \times 10^5\). Cultures were prepared in triplicate and incubated for 7 days at 37°C in humidified air with a 5% CO\(_2\) concentration. The cells in agar were fixed in methanol and colonies were counted at a magnification of 100 x. Essentially, there is no colony formation without PHA (<2.0 colonies).

The lymphocyte membrane phenotype was determined using monoclonal antibodies for circulating T cells (OKT3), helper T cells (OKT4), and suppressor T cells (OKT8). The preparation and specificity of these antibodies is well established. Lymphocytes separated by a Ficoll-Hypaque density gradient were incubated with antibody and the percentage of positive cells determined by cytofluorography after labeling with fluorescein-conjugated goat anti-mouse serum.

Cytocentrifugation was performed on lymphocytes from normal and CLL patients. Briefly, T lymphocytes were treated with OKT3, OKT4, and OKT8 antisera (Ortho Diagnostic, N.J.); the cells were then reacted with fluorescein-conjugated goat anti-mouse IgG (Meloy Labs, Va.) at 4°C for 30 min and washed 4 times in media. These cells were analyzed on a Coulter TPS-1 fluorescence-cells sorter and the intensity of fluorescence per cell recorded on a pulse-height analyzer. The viability and purity after the sorting procedure were greater than 94% in all experiments.

B lymphocytes were reacted with fluorescein-conjugated F(ab')\(_2\) fragments of antisera specific for human IgM (Coppel Labs, Pa.). B cells were fractionated on the fluorescence cell sorter. The viability and purity after the sorting procedure were greater than 96% in all experiments.

Statistical significance was determined by the Fisher's t test.

**RESULTS**

**Cell Membrane Phenotyping**

The mean percentage of peripheral blood lymphocytes (PBL) reacting with OKT3 (T cells) in the 10 normal age-matched controls was 74.3 ± 7.4% (SD); 10.9% ± 4.1% of PBL had demonstrated immunofluorescent Ig staining using F(ab')\(_2\) fragments of anti-human IgM. The mean total lymphocyte count of the normal controls was 1850/cu mm ± 670. OKT4-positive cells (helper T cells) and OKT8-positive cells (suppressor T cells) comprised 72.1% ± 10.4% and 25.4% ± 5.2% of the T cells, respectively. The total T-cell pool in controls was 1390/cu mm. These data on normal donors is comparable to that indicated in a number of studies, including our own.

The mean total lymphocyte count in CLL cases was 78,000/cu mm. The mean percentage of PBL reacting with OKT3 antibody in the 12 CLL patients was 9.2% ± 6.2%; 86.9% ± 6.9% of PBL stained positively for Ig. Thus, the mean total T-cell pool, 7200/cu mm, was higher than seen in normals (p 0.01). Helper T cells (OKT4) comprised 50.7% ± 9.0% of total CLL T-cell pool (p 0.01 compared to normal), whereas suppressor T cells represented 46.8% ± 15.2% of the T cells from CLL patients (p 0.025 when compared to normal).

**Formation of Colonies**

The mean colony formation/100 OKT3+ cells was 49.7 ± 14 (SD) for normal donors, whereas the mean colony formation/100 Ig+ cells was 3.0 ± 2.2 (Table 1). Thus, it could be clearly seen that the ability to form colonies in agar was essentially a function of normal T cells. The OKT3+ cells obtained from the CLL patients formed 22.4 colonies ± 6.7/100 cells plated. The CLL B cells formed 9.6 colonies ± 3.2/100 cells plated. PHA-stimulated CLL T cells, therefore, formed colonies in agar but at a lower level than normal T cells (p 0.01). There was an unexpectedly significant difference in the mean colony count between normal and CLL B cells (p 0.05).

Using purified normal helper (OKT4) and suppressor (OKT8) T-lymphocyte preparations, it was clearly shown that the ability to proliferate in agar following exposure to PHA was a function of helper T cells (Table 1). Helper T cells (OKT4) formed 79.6 colonies ± 15.9/100 cells plated; whereas the mean for suppressor T cells (OKT8) was 16.1 ± 7.3/100 cells plated (p 0.001). Helper T cells purified from CLL patients formed colonies at a level approximately 40% of that observed with normal helper T cells (37.4 ± 16.2 versus 79.6 ± 15.9; p 0.025). Suppressor T cells from CLL patients had similar mean colony counts as normal suppressor T cells (12.2 ± 6.1 versus 16.1 ± 7.3%, p 0.4).

**DISCUSSION**

Previously, it has been shown that the delayed reaction of lymphocytes from CLL patients to PHA stimulation resulted from a time lag of 48–96 hr before these cells entered the S-phase of the mitotic cycle. The mechanism(s) involved in the CLL lymphocyte response to PHA was poorly understood. Kay observed that CLL Tγ cells (supposedly suppressor T cells) were capable of suppressing spontaneous DNA synthesis in normal B cells and that CLL non-Tγ cells (supposedly helper T cells) were not efficient in controlling spontaneous B-cell DNA synthesis in vitro. He suggested that T cells in CLL have deficient helper and excessive suppressor cell activity. Faguet suggested that the delayed response of CLL PBL to PHA stimulation was due to excessive suppressor activity in the CLL lymphocyte population. We had

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**Table 1. Colony Formation By Normal And CLL Lymphocytes**

<table>
<thead>
<tr>
<th></th>
<th>T3-Positive</th>
<th>T4-Positive</th>
<th>T8-Positive</th>
<th>Ig-Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>49.7 ± 14</td>
<td>79.6 ± 15.9</td>
<td>16.1 ± 7.3</td>
<td>3.0 ± 2.2</td>
</tr>
<tr>
<td>CLL</td>
<td>22.4 ± 6.7</td>
<td>37.4 ± 16.2</td>
<td>12.2 ± 6.1</td>
<td>9.6 ± 3.2</td>
</tr>
</tbody>
</table>

*Expressed as mean colonies formed/100 plated cells ± standard deviation.*
PHA stimulation of CLL lymphocytes

Previously shown that in CLL lymphocytes, the delayed response to PHA stimulation was correlated with the absolute Tγ cell level and with increasing clinical stage, but since the Tγ cell population was decreased, we could not delineate the cause(s) of the phenomenon.2 Chiorazzi et al.13 found that the allo-geneic helper activity of T cells from CLL patients was defective in an in vitro plaque-forming assay. Fauci et al.10 demonstrated that B cells from CLL patients could be made to differentiate into immunoglobulin-secreting cells only in the presence of normal T cells, data also suggesting a defect in the helper T cells in lymphocytes from CLL patients. Excessive suppressor cell activity could not be demonstrated by either group of investigators.1,3,15

In this study we used a clonogenic agar technique to determine the PHA-responsive cells in normal and CLL lymphocytes. This assay has been shown to be clonogenic; it avoids mitogen-induced agglutination and direct cell-to-cell interaction. To increase specificity, we purified each cell population by fluorescence cell sorting. We showed that in normal patients PHA responsiveness in agar was predominantly found in the helper T cells. Suppressor T cells (T8 positive) were less responsive. In lymphocytes obtained from CLL patients, helper T cells were the predominant cell responding to PHA. However, helper T-cell responsiveness was less in CLL patients than it was in helper T cells from normal donors. Suppressor T-cell responses to PHA was similar in normal and CLL patients.

Since B lymphocytes from normal donors did not respond to PHA in agar, it was an unexpected finding to detect minimal but significant increase in colony formation with B cells from CLL patients. Boumsell et al.16 have demonstrated that B cells from CLL patients share membrane proteins unique to T lymphocytes from normal controls. We had previously hypothesized that CLL represents a disease of all bone marrow lymphocytes, with the defect in maturation occurring at an early stage of lymphocyte development.17 It is conceivable that the PHA response detected in B lymphocytes from CLL patients is due to retained T-cell membrane proteins, which allow for some responsiveness to PHA. It is important to realize that the agar assay system used does not permit the determination of the role of lymphokines or cell–cell interaction in the lymphocyte response to PHA as seen in liquid culture medium.

We and others have demonstrated that in patients with CLL there is an increase in the absolute number of T, Tγ cells, and Tμ lymphocytes when compared to normal, although the proportion of T and Tμ cells is decreased and Tγ cells are increased.2,12 In our present study we clearly confirm by the use of monoclonal antibodies that the absolute T, helper T, and suppressor T-cell pools are increased in patients with CLL, although helper T cells are decreased in percentage when compared to normal. Whether these changes are a primary component of CLL, B-cell type, is at present unknown.

REFERENCES

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