Impaired T-cell Transformation in Chronic Lymphocytic Leukemia (CLL): Demonstration of a Blastogenesis Inhibitory Factor

By Peter D. Utsinger

A noncytotoxic serum factor was found in CLL serum which interfered with the response of normal lymphocytes to phytohemagglutinin (PHA) and pokeweed mitogen (PWM). This factor was not produced by B-lymphocytes during short-term culture. This factor may contribute to the impaired lymphocyte responsiveness in CLL. In five of six patients, T-lymphocytes which were purified by passage over nylon wool columns exhibited an impaired response to both PHA and PWM, suggesting that a dilutional effect alone does not fully explain the impaired lymphocyte transformation in CLL.

Although chronic lymphocytic leukemia (CLL) is classified as a B-cell malignancy,1-5 CLL patients show evidence of T-cell dysfunction, particularly in the T-cell response to phytohemagglutinin (PHA).6-9 Such abnormal lymphocyte transformation has been described in a number of lymphoproliferative diseases,10-11 but, at present, the reasons for the impaired response are unclear. However, in some disorders, a plasma factor appears to interfere with normal lymphocyte transformation.12-14

In this paper, we present data on the mitogen responsiveness of peripheral blood lymphocyte- and T-cell-enriched populations from 11 patients with CLL. In addition, we investigated a serum factor produced by CLL lymphocytes which interfered with the transformation of lymphocytes in vitro.

MATERIALS AND METHODS

Patients

The study group consisted of 11 untreated patients with chronic lymphocytic leukemia, 20 normal subjects with an average age of 37 (range, 23-45), and 16 normal subjects with an average age of 69 (range, 62-75).

Mononuclear Cell Isolation

Peripheral blood was drawn into a heparinized syringe and sedimented at room temperature. The leukocyte-rich supernatant was centrifuged and the cells washed in 10% fetal calf serum (FCS)-RPMI 1640. Washed cells were recentrifuged and suspended in 5 cc of cold 10% FCS-RPMI 1640, layered over 3 cc of Ficoll-Hypaque solution,15 and centrifuged at 300 g at room temperature for 25 min. Only preparations with a mononuclear cell yield of greater than 70%, were used. Cells from the interface were then washed three times in cold 10% FCS-RPMI 1640 at 200 g at 4°C for 5 min and were finally resuspended in 10% FCS-RPMI at a concentration of 1-2 × 10⁶/ml.
T-cell Enumeration

Spontaneous cold sheep erythrocyte rosettes (E) were assayed by a variation of the method previously described by Ross. Sheep red blood cells (SRBC) stored less than 2 wk were washed twice with phosphate-buffered saline (pH 7.2) at 4°C and 200 g. Then equal volumes of 1 x 10^6 lymphocytes in 25% FCS-RPMI and 100-200 x 10^6 SRBC per ml were incubated in 10 x 75-mm glass tubes at 37°C for 5 min, centrifuged at room temperature at 200 g for 5 min, and incubated in an ice water bath at a temperature of 10°C-15°C for 60 min. An 8-10-mm column of cells was withdrawn from the sedimented red cells and lymphocytes with a long Pasteur pipette and gently agitated. A drop was expressed onto a glass slide, overlaid with a cover slip, and 100 cells were counted. A mononuclear cell with more than two adherent SRBC was considered to be a T-rosette.

Antiserum Preparations

Antisera polyvalent for human immunoglobulins were produced in rabbits, goats, monkeys, and baboons using purified monoclonal proteins as previously described. Fluorescein-labeled antisera were passed through a Sephadex G-25 column, dialyzed against 0.01 M phosphate-buffered saline, pH 7.4, and absorbed with mouse liver powder before use.

Enumeration of Surface Immunoglobulin-bearing Lymphocytes (Sig)

0.05 ml of the lymphocytes in 10% FCS-RPMI were incubated with 0.05-0.10 ml of fluorescein-conjugated polyvalent antihuman immunoglobulin in 10 x 75-mm glass tubes for 1 hr at 4°C. To eliminate the possibility that aggregated immunoglobulin was adhering to the Fc receptor of B-lymphocytes, antiserum was routinely centrifuged at 100,000 g for 2 hr and the supernatant used for cell staining. The cells were then washed three times with 10 cc of cold 10% FCS-RPMI, mounted on glass slides, and overlaid with a cover slip. One thousand cells were counted by epifluorescence using a Leitz ultraviolet microscope equipped with a mercury arc HBO-200 lamp using BG 38 and 490 nm (FITC) excitor filters and a x510 nm barrier filter.

Monocyte Identification

Monocytes were identified by their ability to phagocytose 0.81-μ polystyrene beads (1 hr incubation at 37°C) and by their histology utilizing Wright’s stain preparations. An attempt was made to eliminate all monocytes from the subsequent enumeration of T- and B-lymphocytes.

Purification of T-cells

T-cells were purified by layering the Ficoll-Hypaque-separated lymphocyte population over sterile washed nylon wool columns (Fenwal leukopak) as described by Julius et al. A second cell passage was needed to provide a population of 96.0%, pure T-lymphocytes, determined as above by the ability to form E-rosettes.

Purification of B-cells

B-cells were purified by a modification of the method of Greaves. E-rosettes formed in 25% FCS-RPMI were layered over Ficoll-Hypaque and spun at 400 g for 40 min at room temperature. The cells at the interface were aspirated and washed twice for 5 min at 200 g at 4°C.

Serum Inhibitors

To study the effects of CLL serum on the PHA response of normal lymphocytes, 0.2 ml of pooled normal human AB serum or CLL serum was added to each culture tube.

Preparation of B-cell Culture Supernatants

B-lymphocytes were prepared for culture by centrifugation of SRBC-rosetted lymphocytes over Ficoll-Hypaque. The purified B-lymphocytes (> 97%, B-lymphocytes) were washed with 10% FCS-RPMI 1640 and cultured for 24 hr at 37°C in 5% CO₂ at a concentration of 1 x 10⁶ cells
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per ml. After centrifugation at 400 g, 0.5 ml of cell-free supernatant was used in place of 0.5 cc of media in the mitogen stimulation experiments described below.

Mitogen Stimulation

RPMI 1640 with 10\% FCS supplemented with 300 mM glutamine, 100 \( \mu \)g/ml penicillin, and 100 \( \mu \)g/ml streptomycin was the medium used in cell cultures. Three types of cell suspensions were used: Ficoll-Hypaque-separated CLL lymphocytes, isolated T-lymphocytes from CLL patients, and CLL lymphocytes which were washed at 37\(^\circ\)C and incubated for 24 hr in 10\% FCS-RPMI at 37\(^\circ\)C in a 5\% CO\(_2\) atmosphere. In all experiments, lymphocyte viability by trypan blue exclusion was greater than 90\%.

Triplicate cultures of 1 x 10\(^6\) lymphocytes in 1 ml medium were made in 10 x 75-mm sterile capped tissue culture tubes (Falcon) for each concentration of mitogen (PHA (Burroughs Wellcome), 1 \( \mu \)g and pokeweed mitogen (PWM; Grand Island Biological), 2 \( \mu \)g and 10 \( \mu \)g). Cultures were incubated at 37\(^\circ\)C in a 5\% CO\(_2\) atmosphere for 3 days, 5 days, and 7 days in every experiment. One microcurie of tritiated thymidine (New England Nuclear, Boston, Mass.) was added to each tube for the last 5 hr of culture. The trichloroacetic acid-insoluble radioactivity of cells collected on glass fiber filters (Gelman type E) or Millipore filters (HAWK) was measured in a liquid scintillation counter. All data are expressed as the maximal stimulation index (counts per minute in stimulation culture/counts per minute in control culture).

RESULTS

Lymphocyte Populations

The total lymphocyte counts and T- and B-lymphocyte populations are outlined in Table 1.

Mitogen Stimulation

The maximal stimulation index is outlined in Table 2. The mean PHA stimulation index in the 11 CLL patients was 9.3 ± 0.7 compared to 84.0 ± 5.1 in 20 normal and 63.0 ± 4.6 in 16 normal aged subjects. The mean PWM stimulation index in the 11 CLL patients was 5.7 ± 0.8 compared to 42.0 ± 2.1 in normal subjects and 31.0 ± 1.6 in normal aged subjects. The decreased response could not be related solely to the percentage of T-lymphocytes. For example,

<table>
<thead>
<tr>
<th>Patients Number</th>
<th>Age</th>
<th>Total</th>
<th>Ig-bearing</th>
<th>T</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>64</td>
<td>135,000</td>
<td>114,750 (85)</td>
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<td>81,060 (84)</td>
<td>12,545 (13)</td>
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<td>59</td>
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<td>51,800 (70)</td>
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<td>62</td>
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<td>7,440 (12)</td>
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<td>6</td>
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<td>67</td>
<td>41,500</td>
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<td>19,720 (58)</td>
<td>9,860 (29)</td>
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<td>4,840 (22)</td>
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<td>10</td>
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<td>11</td>
<td>64</td>
<td>18,500</td>
<td>10,730 (58)</td>
<td>7,400 (40)</td>
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<tr>
<td>Normal†</td>
<td>16</td>
<td>2,360 ± 176</td>
<td>572 ± 82 (24)</td>
<td>1,699 ± 81 (72)</td>
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*Lympocytes per cubic millimeter and per cent lymphocytes.
†Expressed as absolute number of cells per cubic millimeter ± SEM.
Table 2. Maximal Mitogen Stimulation of Total Peripheral Blood Lymphocytes

<table>
<thead>
<tr>
<th>Patients</th>
<th>Number</th>
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<td>8</td>
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<tr>
<td>11</td>
<td>8</td>
<td>5</td>
</tr>
</tbody>
</table>

Patient mean ± SEM: 11
Normal mean ± SEM: 20
Normal aged mean ± SEM: 16

Patient mean vs. normal mean or normal aged mean: p < 0.01

For some patients with a relatively high percentage of T-lymphocytes (such as patient 11) had a relatively poor mitogen response, and some with a relatively low percentage of T-lymphocytes (such as patient 5) had a relatively good mitogen response. If the CLL cells were washed five times at 37°C and incubated for 24 hr at 37°C in an attempt to elute any serum factors absorbed to the cell surface, there was no significant increase in the stimulation index (p > 0.05). Cell viability before mitogen stimulation was greater than 90%.

Table 3. PHA Stimulation of Purified CLL T-Cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>Per Cents T-cells</th>
<th>Per Cents B-cells</th>
<th>Number</th>
<th>Mean Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>3</td>
<td>4</td>
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<tr>
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<td>8</td>
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<tr>
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<td>3</td>
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<tr>
<td>11</td>
<td>97</td>
<td>1</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

Patient mean ± SEM: 6
Mean normal purified T-lymphocytes: 24
Mean normal aged population T-lymphocytes: 12

For some patients with a relatively high percentage of T-lymphocytes (such as patient 11) had a relatively poor mitogen response, and some with a relatively low percentage of T-lymphocytes (such as patient 5) had a relatively good mitogen response. If the CLL cells were washed five times at 37°C and incubated for 24 hr at 37°C in an attempt to elute any serum factors absorbed to the cell surface, there was no significant increase in the stimulation index (p > 0.05). Cell viability before mitogen stimulation was greater than 90%.

To investigate the possibility that a dilution of T-lymphocytes by the proliferating B-cell clone was responsible for the impaired PHA response, T-lymphocytes from six patients and two groups of control subjects were purified by passage of cells over Fenwal leukopak columns. Greater than 3% monocytes were present in the effluent, and 96% of the lymphocyte population were T-lymphocytes. As shown in Table 3, the mean (± SEM) stimulation of the T-lymphocytes of the six leukemia patients was significantly less than the mean (± SEM) stimulation of the T-lymphocytes of both normal (p < 0.01) and normal aged subjects (p < 0.01) prepared in the identical fashion.
It should be noted that in the relative absence of monocytes, such as one obtains by leukopak filtration, the PHA response of normal lymphocytes is diminished.\textsuperscript{21,23}

To investigate the possibility that a plasma factor was responsible for the poor response, experiments comparing the PHA response of lymphocytes cultured in the presence of CLL serum or pooled normal AB serum were performed. The serums of five patients (patients 1, 5, 6, 8, and 11) were studied. These serums consistently depressed the blastogenic responses of normal subjects ($p < 0.01$). The stimulation indices to PHA were 51\%, 46\%, 44\%, 35\%, and 28\% and to PWM, 58\%, 74\%, 85\%, 77\%, and 77\% of those obtained using pooled normal plasma (Table 4). This effect was not due to cytotoxicity as assessed by trypan blue exclusion.

It is unlikely that the difference in stimulation indices is due to enhancement of lymphocyte transformation by the AB serum, since the stimulation index of the control subjects' lymphocytes cultured in FCS (Table 2) was also significantly higher than when cultured in CLL serum ($p < 0.01$).

To determine if the interference was due to a B-cell product, the B-lymphocytes of these five patients were isolated by sequential passage over Ficoll-Hypaque and cultured for 24 hr. The cell-free supernatant of these cultures, prepared as described in Materials and Methods, did not inhibit the PHA response of lymphocytes of normal patients ($p > 0.05$).

**DISCUSSION**

Our 11 patients had a range in T-lymphocyte percentage of 10\%-40\% and in B-lymphocyte percentage of 58\%-85\%. This difference may reflect a higher percentage of T-lymphocytes than in other studies on CLL, but it is difficult to compare directly our data with prior studies for a number of reasons. First of all, there are very few studies which have measured both SIg-bearing lymphocytes and E-receptor lymphocytes in CLL patients. In the study of Mellstedt and Pettersson, however, both were determined in eight patients, and the range of E-lymphocytes was 11\%-61\% and of SIg-bearing lymphocytes, 33\%-86\%.\textsuperscript{24} These values closely approximate the range in our study. Second, in most studies where SIg-bearing lymphocytes alone were determined, the percentage of T-lymphocytes would range from 4\% to 80\%, if determined simply by subtraction.\textsuperscript{13-5} In these studies, it is not clear what attempt was made to eliminate monocytes from enumeration. Since monocytes will also stain with fluorescent
antiserums, the percentage of B-lymphocytes may have been somewhat overestimated and the percentage of T-lymphocytes underestimated. Third, studies where aggregate receptors were determined cannot be used as a direct indication of the number of B-lymphocytes, since T-lymphocytes may also have aggregate receptors. Fourth, by using a higher percentage of FCS in the incubation of lymphocytes with sheep red blood cells, the maximal number of T-lymphocytes are detected. Most previous studies have used less than 25% FCS. It is also extremely unlikely than non-T-lymphocytes are being detected by our method, since other investigators using similar methods did not find a significant number of non-T-lymphocytes binding and since none of our immunoglobulin-producing cell lines (UM 56, UM 61, T 5-1, IM-9, IM-1, DG, BU-1) form any E-rosettes.

This study confirms prior reports that most CLL patients have a reduced ability to respond to mitogens in vitro. Because of the advanced age of our patients with CLL and the known age-dependent decline in mitogen stimulation, age-matched normal controls were studied. The in vitro response of CLL cells to mitogen was significantly lower than that of both control groups. However, especially in the case of PHA, the reasons for the reduced response are not clear. The impairment has been attributed to the dilution of normally responding T-cells by the large percentage of nonresponding B-cells present as a result of the disease.

In our experiments, any possible B-cell dilutional effect was largely, but not completely, eliminated by using lymphocyte populations that were 96% T-cells. When such enriched populations from six patients were tested, the PHA responsiveness of only one (patient 11) was within normal limits. Therefore, while the purified T-lymphocytes of many patients with CLL have a normal PHA response as documented by Wybran et al., there are other patients whose purified T-lymphocytes do not have a normal response.

Plasma factors that interfere with normal lymphocyte transformation have been described in a variety of tumors and lymphoproliferative diseases. All five CLL patients tested displayed an inhibitory serum factor in multiple experiments, producing a highly significant reduction in the ability of normal lymphocytes to incorporate tritiated thymidine when cultured with CLL serum and stimulated with PHA and PWM. This impairment is unrelated to cell death. This inhibitory factor apparently is not produced by leukemic B-cells, since it cannot be detected in supernatants from 24-hr B-cell cultures. The fact that the CLL serum did not reduce the PHA and PWM stimulation of normal lymphocytes down to the level of CLL lymphocytes suggests that other factors may be involved or that the CLL serum should be preincubated with the homologous normal lymphocytes to approximate more closely the in vivo situation.

There seem to be, then, at least two factors which contribute to the decreased mitogen response of CLL T-cells. The first is an inhibitory factor in the serum. An attempt to eliminate this factor in experimental situations may be made through the use of normal plasma and careful washing of the test cells. Washing at 37°C and incubation at that temperature for 24 hr does not increase mitogen response. Therefore it is less likely that the plasma factor plays a role in the decreased response that is also seen in cultures prepared in nonautologous
plasma. The decreased response in this second experimental situation appears to be caused by a dilutional effect or possibly by an inhibitory substance strongly bound to the responding lymphocyte. The increased PHA response of one of the six patients tested using enriched T-cell preparations would point to a dilutional effect. However, the lack of an increased response in the other patients suggests that a serum factor may be incorporated into or onto the lymphocyte, interfering with lymphocyte transformation.

In summary, the etiology of the impaired lymphocyte transformation in CLL is complex and seems to involve at least two factors. These include a serum inhibitory factor and a dilutional effect resulting from a decreased percentage of T-lymphocytes.

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Impaired T-cell transformation in chronic lymphocytic leukemia (CLL): Demonstration of a blastogenesis inhibitory factor

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