Mitogen Stimulation of Chronic Lymphocytic Leukemic Lymphocytes: Defective Phytohemagglutinin Stimulation Independent of Immunologic Cell Surface Markers

By Stephen Davis and Pietro Rambotti

Peripheral blood lymphocytes (PBL) from 107 untreated patients with chronic lymphocytic leukemia (CLL) were analyzed for the presence of surface immunoglobulin (Ig) and the ability to form rosettes with sheep erythrocytes (SRBC). Four groups were identified based on the cell surface markers: (1) 81 patients’ PBL expressed primarily IgM kappa or IgM lambda, 4 further patients’ PBL expressed IgM with equal percentages of kappa and lambda surface markers; (2) 13 patients had equal percentages of PBL expressing Ig and SRBC receptors; (3) 6 patients’ PBL primarily formed rosettes with SRBCs, and (4) in 3 patients and the majority of cells had no detectable markers (null cells). Lymphocytes from all patients within each group were tested for their ability to respond to phytohemagglutinin (PHA) and pokeweed mitogen (PWM). The maximum response in PHA-stimulated normal cell cultures appeared at 2–3 days; for PWM-stimulated cultures, maximal response was at 3–5 days. CLL cultures from all patients in each of the four groups required 5–7 days to develop a maximal PHA response. The response of CLL lymphocytes in all groups to PWM stimulation was similar to normal lymphocytes. Thus, the abnormal PHA response of CLL lymphocytes was independent of the presence or pattern of cell surface markers.

PLANT MITOGENS such as phytohemagglutinin (PHA) are routinely used to induce in vitro proliferation of human lymphocytes. When stimulated by PHA, maximal enlargement and proliferation in normal lymphocytes appears at 2–3 days; whereas, chronic lymphocytic leukemia (CLL) lymphocytes respond maximally at 5–7 days. In addition, some CLL cells fail to respond at all.

Chronic lymphocytic leukemia has, by most investigators, been stated to represent an accumulation of monoclonal malignant B lymphocytes, as defined by the presence of normal B-cell markers, i.e., surface immunoglobulin, on circulating mononuclear cells. However, it is becoming increasingly apparent that the peripheral blood lymphocytes (PBL) of small numbers of patients with classic CLL can demonstrate predominantly T-cell markers. In addition, the number of SRBC cells may fluctuate greatly in CLL patients who have predominance of Ig-bearing cells at the time of diagnosis.

To encompass all of the available data relating to CLL lymphocytes, working hypotheses have suggested the delayed and diminished PHA response as the expected reaction of B cells, although PHA-stimulated proliferation is regarded as a function attributed exclusively to T cells. The delayed response to PHA has been alternatively regarded as the reaction of normal T cells diluted by a large population of mitogen-unresponsive B cells. Our previous reported data are not in accord with either interpretation. We suggested, from studying a small number of patients, that the delayed response of CLL lymphocytes is independent of surface markers classically used to classify normal lymphocytes.

The present study analyzes cell surface markers and mitogen responsiveness in a large series of CLL patients. Marker heterogeneity is demonstrated, and the impaired CLL mitogen response, unique to PHA and independent of cell markers, is confirmed.

MATERIALS AND METHODS

One-hundred-seven patients with classic CLL were studied. There were 62 males and 45 females. Mean age was 61 yr (range, 39–90). No patient had received chemotherapy prior to study. Peripheral blood lymphocyte counts ranged between 24,000 and 187,000/cu mm. Fifty-two healthy subjects aged 30–64 were used as normal controls.

Blood was obtained from each donor by phlebotomy. Normal peripheral blood lymphocytes were isolated 95%–98% free of granulocytes and monocytes by dilution of lymphocyte-rich plasma 1:1 with Eagle’s no. 2 medium followed by rapid elution from a loosely packed nylon fiber column (Leukopak, Fenwal Laboratories, Morton Grove, Ill.) according to previously described methods. Cell surface marker analysis of lymphocytes pre and post column elution showed no significant difference. CLL lymphocytes were obtained 84%–99% pure from lymphocyte-rich plasma.

Spontaneous rosette formation with sheep erythrocytes (E rosettes) was used as a T-cell marker as previously described. Immunofluorescent staining with fluorescein-conjugated F(ab’)_2 fragments of antiserum specific for human kappa (K) and lambda (\lambda) light chains and human IgM were performed as previously described. The absence of Ig was supported by an immunohistochemical peroxidase technique.

Normal and CLL lymphocyte suspensions were adjusted to a concentration of 3 × 10^6 cells/ml of Eagle’s no. 2 medium.
containing glutamine (2 μmole/liter), normal human type AB serum (15%), along with penicillin (100 U/ml) and streptomycin (100 U/ml). PHA-P (125 μg/ml, Difco Labs, Detroit, Mich.) or PWM (0.25 ml of 1/10 dilution, Gibco, Grand Island, N.Y.) was introduced, and the cultures were incubated for the specified times at 37°C. The concentration of mitogen selected yielded maximal stimulation as determined in each case by a dose-response curve. Control cultures contained no mitogens. At appropriate times, tritiated thymidine (3HT) or tritiated uridine (3HU, Schwarz-Mann, 1 μCi/ml) was introduced for 2 hr before harvesting. Tritium-labeled cells were isolated by centrifugation at 1000 rpm for 10 min at 4°C, and washed cells were fixed in a 3:1 alcohol-acetic acid mixture for 10 mm, centrifuged, and stored under 70% ethanol. Incorporation into acid-precipitable RNA or DNA was quantified as previously described and served as a reliable parameter of proliferative activity.

Kinetic studies of protein synthesis were carried out in an identical fashion except that isoleucine, lysine, and serine were omitted from the culture medium. At the specified times, 14C-labeled isoleucine, lysine, and serine (0.3 μCi) was added to each culture. Radioactivity incorporated into protein was determined in triplicate by specific activity of RNA nucleotides, measured by specific activity of RNA nucleotides, determined in trichloroacetic acid precipitates of whole culture lysates of proliferative activity. Kinetic studies of protein synthesis were carried out in an identical fashion except that isoleucine, lysine, and serine (0.3 μCi) was added to each culture. Radioactivity incorporated into protein was determined in triplicate by specific activity of RNA nucleotides, measured by specific activity of RNA nucleotides, determined in trichloroacetic acid precipitates of whole culture lysates of proliferative activity.

**RESULTS**

**Normal Donor Lymphocyte Mitogen Stimulation**

Incorporation of 3H into an insoluble RNA, as measured by specific activity of RNA nucleotides, proved to be the most reproducible and reliable parameter of in vitro growth response to mitogens in our laboratory. There was excellent and reproducible correlation with 3H-thymidine incorporation into DNA and 14C-labeled amino acids into protein (Table 1). Figure 1A shows a constant rate of 3H incorporation in unstimulated cultures from day 3 (25.5 ± 2.7 cpm/μg) to day 5 (36.7 ± 4.7 cpm/μg) and day 7 (40.2 ± 4.2 cpm/μg). In PHA-stimulated cultures of normal cells, incorporation of 3H rose to 1019 ± 162.4 cpm/μg at day 3 and subsequently declined at 5 days (665.2 ± 4.3 cpm/μg), reaching its lowest level (512.7 ± 89.9 cpm/μg). Similarly, PWM-stimulated lymphocytes showed a rise in incorporation by day 5 (441.6 ± 50.9 cpm/μg). This level increased to 662.6 ± 100.2 cpm/μg on the fifth day and subsequently declined to 517.4 ± 82.1 cpm/μg by day 7. These values were also significantly above control culture values determined at comparable times (p < 0.001). Parallel studies performed on non-column-separated leukocytes from normal individuals gave identical stimulation results (data not included). Previous studies in our laboratory show that PHA reactivity resides solely with T lymphocytes.

**Cell Surface Markers and Cytoplasmic Immunoglobulin Staining**

The mean (±SD) percentage of PBL rosetting with SRBC in the 52 control (normal) patients was...
66.2% ± 8.1%; 9.7% ± 3.5% of PBL had demonstrable immunofluorescent Ig staining using anti-human IgM. The techniques have been described previously.3

The mean number of circulating SRBC cells and Ig-containing cells in normal and CLL patients are shown in Table 2. The variability in the percentage allowed CLL patients to be divided into four distinct immunologic groupings (Table 2).

Table 3 describes the patterns of immunologic markers seen in 107 patients with untreated CLL. Using the ability of PBL to form SRBC rosettes and to stain for membrane Ig by immunofluorescence, four groups were identified: (1) Eighty-one patients PBL expressed membrane IgM with either κ or λ light chains only, an additional 4 patients PBL expressed equal percentages of κ and λ markers. Greater than 80% of staining cells showed low intensity staining typical of CLL. We did not perform studies that would enable us to determine whether κ and λ chains were expressed simultaneously on a single “set” of cells or independently on 2 “sets” of lymphocytes. (2) Six patients PBL formed SRBC rosettes. As can be seen in Table 1, 78% of lymphocytes in this group formed SRBC rosettes; whereas, 11% had demonstrable membrane Ig. In all 6 patients, the Ig-bearing cells were stained with IgMx or IgML but not both. (3) In 3 SRBC rosettes, whereas, 11% had demonstrable markers (null cells). Additional evidence supports us to determine whether κ and λ chains were expressed simultaneously on a single “set” of cells or independently on 2 “sets” of lymphocytes. (2) Six patients PBL formed SRBC rosettes. As can be seen in Table 1, 78% of lymphocytes in this group formed SRBC rosettes; whereas, 11% had demonstrable membrane Ig. In all 6 patients, the Ig-bearing cells were stained with IgMx or IgML but not both. (3) In 3 patients, the majority of cells (0–70%) had no detectable markers (null cells). Additional evidence supporting these PBL as null cells was obtained from immunoperoxidase staining for cytoplasmic Ig. Eleven percent ± 4% of PBL had detectable cytoplasmic Ig. Thirteen percent of PBL from this group contained IgMx or IgML and 9% SRBC-rosetting cells (Table 2). The absence of surface Ig and cytoplasmic Ig has been used by other investigators to classify lymphoproliferative diseases as null cell. (4) In 13 patients, equal percentages of PBL expressed Ig and SRBC receptors. The mean percentage of SRBC rosette-forming cells in this subset was 41% (Table 2). No assessment of clonality of these SRBC rosetting PBL could be made. The mean percentage of Ig membrane staining PBL was 56%. Membrane Ig staining revealed only IgMx or IgML, but not both.

**Chronic Lymphocytic Leukemia Lymphocyte Mitogen Stimulation**

CLL lymphocytes, harvested from leukocyte-rich plasma, were stimulated with PHA and PWM. Stimulation data were tabulated for all 107 CLL cases (Fig. 1B) and for each group of CLL patients based on immunologic markers (Fig. 2A–D). Following PHA stimulation, CLL lymphocytes, as a group, showed a moderate increase in incorporation above control on day 3 (469.4 ± 107.1 cpm/μg), but rose to a maximum level on the fifth day (578.3 ± 104.6 cpm/μg) and then declined slightly to 512.8 ± 72.6 cpm/μg on day 7. These levels were significantly above control

**Table 2. Mean Total and Percentage of SRBC-Rosetting and Immunoglobulin (lg) Bearing Cells in CLL**

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Mean Total Cell Count (cells/cu mm ± SD)</th>
<th>Mean (%)</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ig only (85; 43,000/cu mm)†</td>
<td>36,500 ± 14,500</td>
<td>85</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ig cells</td>
<td>5,200 ± 1,200</td>
<td>12</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>SRBC only (6; 32,000/cu mm)</td>
<td>3,500 ± 750</td>
<td>11</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>SRBC cells</td>
<td>25,000 ± 8,700</td>
<td>78</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>No markers (3; 68,000/cu mm)</td>
<td>8,800 ± 2,800</td>
<td>13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ig cells</td>
<td>6,100 ± 1,600</td>
<td>9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>SRBC cells</td>
<td>29,100 ± 7,400</td>
<td>56</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Controls (S2; 2,400/cu mm)</td>
<td>21,300 ± 4,800</td>
<td>41</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ig cells</td>
<td>240 ± 40</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>SRBC cells</td>
<td>1,580 ± 210</td>
<td>66</td>
<td></td>
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</tbody>
</table>

*Student's t test between mean of each CLL group of cells/cu mm compared to mean of controls.
†Numbers in parentheses represent number of patients; total cell count.
PHA STIMULATION OF CLL LYMPHOCYTES

In our present study, PBL from a large number (107) of untreated patients with CLL were characterized as to the presence of SRBC-rosetting cells and Ig-bearing cells. Eighty-five patients’ cells were predominantly (>70%) Ig-bearing, as determined by immunofluorescent analysis. However, the pattern of rosetting and Ig-bearing cells in the remaining 22 cases was so variable as to allow four groups of CLL to be established based on initial immunologic markers (Table 2): (1) Ig predominant, (2) SRBC predominant, (3) null cell predominant, and (4) mixed SRBC and Ig pattern. Of interest, in all groups the total number of SRBC cells and Ig-bearing cells were markedly increased over a healthy control population. This finding has been demonstrated by other investigators in early and late CLL.\(^{18,19}\) It has been shown that the absolute number of SRBC-cells does not correlate with clinical staging but might reflect a favorable prognostic indicator.\(^{18,19}\) At present, the significance of the expanded “T-cell” pool in CLL remains to be defined. The pathophysiologic significance of null cell CLL lymphocytes is unknown but might be related to the proposed ontogenic development of B lymphocytes.\(^9\) The CLL groups with predominance of T cells, mixed (SRBC and Ig) patterns, and null cells had only IgMx or IgMa staining on their detectable B cells. In the “T-cell” predominant and “null cell” predominant groups, too few Ig-staining cells were seen to make any comments regarding the nature of these B cells; however, in the mixed pattern group, 56% of PBL had detectable Ig, which had a single light chain specificity (κ or λ). This suggests that the B cells in the mixed pattern group were clonal in origin.

Previously, it has been demonstrated that the delayed reaction to PHA resulted from a time lag of 2–4 days before CLL cells entered the S-phase of the mitotic cycle.\(^3\) Rowlands et al.\(^10\) failed to confirm these data, suggesting CLL lymphocytes fail to mount any response to PHA. However, their technique employed colchicine which, at the concentration employed (1 μM), inhibits mitogenic activity independent of any effect on DNA synthesis.\(^11\) In our present study we
have shown that CLL lymphocytes respond to PHA with a delay in development of a maximal proliferative response, as reflected by the incorporation of \(^{3}H\)U. The normal response of CLL lymphocytes to PWM suggest specificity to the abnormal PHA response. These data confirm our earlier findings.1

Contamination by normal T lymphocytes in PBL suspensions from CLL has been suggested by Wybran et al.14 to be the sole source of PHA reactivity. Our previously published data showing that dilution of normal lymphocytes by mitomycin-C-treated cells or mitogen-unresponsive myeloblasts caused no delay in the development of a maximal PHA response refute their data.1 Recent reports suggest that a soluble product of lectin-stimulated lymphocytes may actually mediate the mitogenic event.12 The delayed response of CLL cells to PHA appears unrelated to the above findings, as CLL cells clearly produce lymphokines in culture.13 In addition, the normal PWM response of CLL suspensions further testify to the potential reactivity of the majority of CLL cells. We do not understand why PWM stimulation in CLL, in our hands, is normal or what subpopulation(s) are involved in the PWM in vitro phenomenon.

Schweitzer and coworkers,14 employing normal lymphocyte suspensions diluted with lymphoblasts from patients with acute lymphoblastic leukemia, demonstrated a delayed response to PHA. However, we have found that blasts themselves from ALL patients respond in a delayed fashion (data not included). These inconsistencies, together with the failure of Schweitzer et al.14 to find normal responses to PWM in CLL cells, is best reconciled by differences in culture techniques and conditions. Despite the cell surface marker analysis, normal T lymphocytes cannot be distinguished from abnormal T lymphocytes. Thus, it remains possible that an admixture of responsive and unresponsive cells might contain a small fraction of normal T cells that would respond to PHA in a delayed fashion,15 within the framework of our methodology, which fails to detect a PHA response at all with less than 30% T lymphocytes.1 the delayed response seen in our CLL cases does not reflect a few contaminating normal T cells.

Because a delayed PHA response is seen in null cell, T-cell, B-cell, and mixed (SRBC and Ig-bearing) CLL, we must conclude that the abnormal response of CLL PBL is independent of the immunologic markers described. The mechanisms involved in the CLL response to PHA remains to be elucidated. Faguet17 showed that the response of CLL PBL to PHA was due to excessive suppressor activity. Kay et al.18 recently reported imbalances in B-cell CLL circulating T-cell subpopulations. In their study Fc\(^{y}\)-receptor-bearing cells (supposedly suppressor T cells) were increased. The role of T-cell subsets requires further analysis.

Identification of the origin of neoplastic lymphocytes has received considerable attention. Implicit in this formulation is the concept that neoplasia arises from clonal proliferation of T or B cells. Although the majority of cases of CLL appear to be B-cell predominant, our data demonstrate the heterogeneity of CLL PBL as judged by immunologic markers.

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