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 (A) 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

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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 (3H-T) or tritiated uridine (3H-U, 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 min, 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 trichloroacetic acid precipitates of whole culture lysates after dissolving the precipitates in hyamine. Probability values were determined by the Fisher's t test.

Using this in vitro system, greater than 30% of incubated lymphocytes must be normal T cells in order to have detectable mitogen-induced 3H-U incorporation by PHA into acid-precipitable RNA.1

RESULTS

Normal Donor Lymphocyte Mitogen Stimulation

Incorporation of 3H-U 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-U 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-U 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 normal lymphocytes showed a rise in incorporation by day 3 (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.1

Cell Surface Markers and Cytoplasmic Immunoglobulin Staining

The mean (±SD) percentage of PBL rosetting with SRBC in the 52 control (normal) patients was

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Table 1. Kinetics of DNA, RNA, and Protein Synthesis in Normal Lymphocytes Stimulated With Phytohemagglutinin (PHA) and Pokeweed Mitogen (PWM)

<table>
<thead>
<tr>
<th>Days in Culture</th>
<th>DNA* (cpm)</th>
<th>RNA† (cpm)</th>
<th>Protein‡ (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7.1 ± 1.1$</td>
<td>9.1 ± 1.9</td>
<td>12,750 ± 1,342</td>
</tr>
<tr>
<td>5</td>
<td>5.2 ± 0.9</td>
<td>5.7 ± 0.8</td>
<td>6,041 ± 1,096</td>
</tr>
<tr>
<td>7</td>
<td>2.7 ± 0.3</td>
<td>4.2 ± 0.8</td>
<td>2,785 ± 911</td>
</tr>
<tr>
<td>PWM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.6 ± 0.9</td>
<td>3.9 ± 0.6</td>
<td>4,712 ± 982</td>
</tr>
<tr>
<td>5</td>
<td>3.7 ± 0.8</td>
<td>4.8 ± 1.1</td>
<td>7,862 ± 1,250</td>
</tr>
<tr>
<td>7</td>
<td>1.4 ± 0.4</td>
<td>2.9 ± 0.9</td>
<td>3,211 ± 842</td>
</tr>
</tbody>
</table>

*Specific activity of thymidine = 3H/μg nucleotide (x 10^-3) following 2-hr incubation.
†Specific activity of uridine = 3H/μg nucleotide (x 10^-3) following 2-hr incubation.
‡Acid precipitable count (cpm)/culture.
§All values represent the mean ± standard deviation for 14 normal patients.
were stained with IgMK or IgMA but not both. (3) In 3
SRBC rosettes; whereas, 11% had demonstrable
markers (null cells). Additional evidence support-
Table I
expressed simultaneously on a single “set” of cells or
Thirteen percent of PBL from this group contained
by other investigators
been used
five patients PBL expressed equal percentages
of chains only, an additional 4 patients PBL expressed
membrane \( \text{Ig} \) with either 
(1) groups were identified: (1) Eighty-one patients PBL expressed membrane \( \text{Ig} \) with either \( \kappa \) or \( \lambda \) light chains only, an additional 4 patients PBL expressed equal percentages of \( \kappa \) and \( \lambda \) 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 \( \kappa \) and \( \lambda \) 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 \( \text{Ig} \). In all 6 patients, the Ig-bearing cells were stained with \( \text{IgM} \) or \( \text{IgM} \) but not both. (3) In 3 patients, the majority of cells (>70%) had no detectable markers (null cells). Additional evidence supporting these PBL as null cells was obtained from immunoperoxidase staining for cytoplasmic \( \text{Ig} \). Eleven percent \( \pm 4\% \) of PBL had detectable cytoplasmic \( \text{Ig} \). Thirteen percent of PBL from this group contained \( \text{IgM} \) or \( \text{IgM} \) and 9% SRBC-rosetting cells (Table 2). The absence of surface \( \text{Ig} \) and cytoplasmic \( \text{Ig} \) has been used by other investigators to classify lymphoproliferative diseases as null cell. (4) In 13 patients,
PHA stimulation of CLL lymphocytes

Fig. 2. Kinetics of RNA synthesis in PHA- and PWM-stimulated lymphocyte cultures in patients with chronic lymphocytic leukemia with (A) equal percentages of SRBC and Ig-bearing cells, (B) predominantly "null cells," (C) predominantly SRBC-bearing cells, and (D) predominantly Ig-bearing cells.

values ($p \leq 0.01$) for all time periods studied. When compared to PHA-stimulated normal lymphocyte cultures, the magnitude of the CLL incorporation at 3 days was significantly lower than normal ($p \leq 0.0005$) and no greater than normal at day 5 or day 7. Thus, the time required for the development of maximal incorporation was clearly delayed. The incorporation of PWM-stimulated lymphocyte suspensions from CLL patients was indistinguishable from normal lymphocytes (Fig. 1), with both magnitude and the time at which maximal incorporation developed. Incorporation by PWM-stimulated CLL cultures reached 482.4 ± 73.8 cpm/μg by day 3, increased further to 518.8 ± 97.6 cpm/μg on the fifth day, and then declined by day 7 to a level of 417.3 ± 61.4 cpm/μg. Thus, the abnormal response seen with PHA could not be reproduced with PWM as the stimulant at the times studied. When divided into the four groups based on immunologic markers, the data were similar: PHA-stimulated CLL lymphocytes with "T and B" cells (Fig. 2A), no detectable markers (null cell, Fig. 2B), "T" cells only (Fig. 2C), and "B" cells only (Fig. 2D) were abnormal when compared to the PHA response of normal cells in magnitude and time of maximal response. PWM stimulation of all four CLL groups (Fig. 2A–D) were similar to its normal counterparts in timing of maximal response and magnitude of response. Thus, each immunologic subgroup behaved similarly in response to PHA as the entire CLL taken as a whole (Fig. 1B).

**DISCUSSION**

In the 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. It has been shown that the absolute number of SRBC-cells does not correlate with clinical staging but might reflect a favorable prognostic indicator. 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. 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. Rowlands et al. 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. 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 \(^3\)HU. The normal response of CLL lymphocytes to PWM suggest specificity to the abnormal PHA response. These data confirm our earlier findings.

Contamination by normal T lymphocytes in PBL suspensions from CLL has been suggested by Wybran et al.\(^6\) 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.\(^1\) The delayed response of CLL cells to PHA appears unrelated to the above findings, as CLL cells clearly produce lymphokines in culture.\(^5\) In addition, the normal PWM response of CLL suspensions further testify to the potential reactivity of the majority of CLL cells. We do not know 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,\(^4\) 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.\(^4\) to find normal responses of 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;\(^3\) 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. Faguet\(^7\) showed that the response of CLL PBL to PHA was due to excessive suppressor activity. Kay et al.\(^8\) 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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