Studies in Chronic Lymphocytic Leukemia: Further Studies of the Proliferative Abnormality of the Blood Lymphocyte

ARNOLD D. RUBIN,* KLAUS HAVEMANN,† AND WILLIAM DAMESHEK

A NUMBER OF STUDIES have demonstrated that peripheral lymphocytes from patients with advanced chronic lymphocytic leukemia (CLL) show a greatly reduced transformation to blast cells ("immunoblasts") in response to phytohemagglutinin (PHA) in vitro.1,6 The metabolic events involved in lymphocyte transformation were investigated by an analysis of the kinetics of RNA and DNA synthesis employing this method.6,7 Rubin et al. demonstrated that about 26 per cent of CLL lymphocytes become transformed only after 5–7 days rather than in 2–3 days, at which time 60–80 per cent of normal lymphocytes respond. In the present study, the late-responding lymphocytes of CLL were identified as abnormal cells distinct from any population thus far detected in normal individuals.

CLL is a disease of many variations. Many cases are apparently "benign" and asymptomatic.8 Such cases may go along for many years without symptoms and with no or slight enlargement of lymph nodes or spleen. These cases may exhibit a slight to moderate leukocytosis of 15,000 to 50,000 per cu.mm., a marked lymphocytosis of 60–90 per cent, and a marrow containing a considerable proportion (50–90 per cent) of small lymphocytes. As these cases are observed over a 10 to 25 year period, there is almost invariably a gradual increase in leukocytes of the lymphocytic variety to levels of 100,000 per cu.mm. or greater, with a simultaneous marked increase in lymph node enlargement and splenomegaly. Symptoms such as fatigue and night sweats are usually present at this stage. Some cases, apparently more "aggressive" from the beginning, show marked lymphadenopathy and systemic symptoms as first manifestations.

The cases of CLL which served as the basis for this report could be divided into those with relatively low lymphocyte counts ("low count cases") and those
with high lymphocyte counts ("high count cases"). On the basis of previous experience, it was presumed that the "low count" cases represented early examples of the later or "high count" form of the disease. A third type of case, characterized by marked splenomegaly without lymphadenopathy but with modest lymphocytosis and minimal bone marrow involvement was also studied. Correlation in these cases between lymphocyte kinetics and quantitative immunoglobulin abnormalities demonstrated group differences which may be of importance in the further estimation of the metabolic defect in CLL.

**METHODS**

**Patient Material**

Only those patients with CLL were studied if no therapy with corticosteroids or alkylating agents had been administered during the previous three months. The degree of peripheral lymphadenopathy and hepatosplenomegaly was highly variable. Patients were divided into "high count" and "low count" cases. High count CLL patients exhibited blood lymphocyte counts consistently greater than 40,000 per cu.mm., and showed well-defined lymph node enlargement and/or hepatosplenomegaly. In the "low count" cases, the blood lymphocyte counts were consistently below 40,000 per cu.mm. and little, if any, lymphadenopathy or hepatosplenomegaly were present. One patient studied was assigned the diagnosis of probable lymphosarcoma of the spleen* on "splenic" lymphocytic leukemia, according to the following criteria: (a) splenomegaly was detected early and the organ descended 10 cm. below the left costal margin when the blood lymphocyte count was less than 40,000 per cu.mm.; (b) many circulating lymphocytes were morphologically atypical, exhibiting abundant cytoplasm, indented nuclei, and well-defined nucleoli; these cells have been described in cases of lymphosarcoma;* (c) there was no detectable lymphadenopathy.

**Lymphocyte Studies**

Peripheral lymphocytes were isolated in 92-98 per cent purity according to previously described methods.11 Short-term lymphocyte cultures contained $3 \times 10^6$ lymphocytes in 3.4 cc. of medium 199 containing antibiotics and 0.6 cc. human group AB serum in stoppered conical centrifuge tubes. Cells were not removed from the original culture vessels during the entire procedure. PHA (PHA-P Difco 75 $\mu$g.) was introduced at the beginning of the incubation period. Two hours before harvest, each culture received either 4 $\mu$c. of cytidine-H$^3$ (2C/mmol) to label newly synthesized RNA or 4 $\mu$c. thymidine-H$^3$ (3C/mmol) to label the newly synthesized DNA. RNA and DNA were quantitatively extracted from each culture and the specific activity expressed as C.P.M./$\mu$g nucleotide RNA or DNA. Verification of the extraction technic as described was established in a previous publication.7 All points represented the average of triplicate cultures. Under these conditions of incubation, RNA and DNA synthesis in unstimulated cultures, both normal and CLL, was virtually nil.

The kinetics of RNA and DNA synthesis as outlined above have been shown to be reliable indicators of cell growth in cultures of human lymphocytes stimulated by PHA. The morphologic assessment of the PHA response, as judged by the percentage of blast cells in smears prepared from these cultures, was not included in this study, since, in our previous work, this estimation had failed to present an accurate reflection of the dynamic aspects of lymphocyte growth. Thus, although cultures incubated with PHA for 7 days contained many blast cells, nucleic acid synthesis had fallen to near prestimulation levels.7 Furthermore, in cultures which eventually showed a good growth in response to PHA, RNA synthesis was very active long before there was any evidence of morphologic evidence of blast cell formation.11 Finally, it could never be ascertained in stained smears whether the cells present were representative of the original culture.

** Autoradiography**

Lymphocyte cultures to be analyzed autoradiographically received 4 $\mu$c. thymidine-H$^3$...
two hours before harvest. Air dried smears were prepared from each culture and were fixed in methanol. Fixed smears were dipped in Kodak NTB-2 emulsion and then exposed for 21 days. Autoradiographs were developed in Kodak D-19 and counterstained with Giemsa.

For pulse-chase studies, cultures received 4 µc. thymidine-H³ at 70 hours. Two hours later, a fifty-fold excess of unlabeled thymidine was introduced and the cells recovered by centrifugation at room temperature. The incubation medium was then replaced with unlabeled medium in the original concentrations and the incubation continued at 37 C. At appropriate intervals, autoradiographs were prepared.

Few labeled blasts contained less than 100 grains after a two hour pulse of thymidine-H³. In the slides prepared from pulse-chase experiments, a blast was considered labeled if it contained greater than 3 grains. Between 500 and 1,000 blast cells were counted and categorized as labeled or unlabeled.

**RESULTS**

**Chronic Lymphocytic Leukemia (CLL)**

Normal lymphocytes in culture, when exposed to PHA, showed a prompt rise in the rate of RNA synthesis, which reached a peak on day 2 and then fell to baseline levels by day 9 (Fig. 1). DNA synthesis occurred as a wave beginning by day 2, with a maximum on day 3, followed by a steady decline (Fig. 2). In cases of high count CLL, the results were quite in contrast (Figs. 1 and 2). RNA synthesis showed little increase during the first three days of incubation with PHA. Thereafter, the rate of RNA synthesis rose to maximum levels between 5-7 days and then decreased towards baseline. The magnitude of the peak CLL response was consistently about one-third or less of the maximal normal response. DNA synthesis was near baseline over the first three days of incubation, following which a well-defined increase took place with a peak at 7 days.

In mild, low count CLL, particularly in those patients showing the lowest counts (Fig. 1), PHA led to a well-defined increase in the rate of RNA synthesis, occasionally approaching normal values. On the other hand, the time required for the development of maximal rates of RNA synthesis was consistently delayed, although the delay was variable and somewhat less than in the high count cases. Thus, the pattern of increased RNA synthesis following PHA in these low count cases may be regarded as intermediate between the patterns observed in normal individuals and in high count cases, both of which yielded more homogeneous results. DNA synthesis following PHA stimulation in the low count cases (Fig. 2) seemed to show two distinct patterns: two patients (J.S. and J.L.) with minimal lymphocytosis (Table 1) exhibited near normal patterns; the other low count patients, excepting L.L., showed a pattern more akin to high count CLL, the main difference being a slightly greater magnitude of response in the low count cases. In patient L.L., DNA synthesis reached a maximum at 5 days. This intermediate response was more in keeping with the pattern of RNA synthesis in low count cases.

Table 1 indicates the clinical findings in these CLL patients at the time of study. It should be noted that every case demonstrated a well defined depression in at least one of the components of serum gamma globulin. In most cases, all three components were depressed. Hypogammaglobulinemia was most marked in the high count cases. With the exception of J.R. and A.O. the
Days of Incubation with PHA

From the kinetic analyses of RNA and DNA synthesis, it appeared that even

Fig. 1.—Kinetics of RNA synthesis in PHA stimulated lymphocyte cultures. After an appropriate length of incubation with PHA, each culture received 4 µc. of Cytidine-H³ two hours before harvest. RNA was extracted and specific activity was expressed as counts per minute per µg. nucleotide RNA. (Top) Shaded area depicts the range of 9 normals studied in triplicate. Each point represents the average ± I.S.D. Single curves depict the responses of patients J.S. ( - O - ), J.L. ( - Δ - ), and P.M. ( - □ - ); examples of low count cases. (Bottom) Shaded area depicts the range of 9 cases of high count CLL studied in triplicate. Each point represents the average ± I.S.D. Single curves depict the responses of patients M.E. ( - □ - ), L.L. ( - O - ) and R.B. ( - Δ - ); examples of low count cases.

degree of hypogammaglobulinemia distinguished high count from low count cases.

"Lymphosarcoma" of the Spleen: Leukemia with Marked Splenomegaly

From the kinetic analyses of RNA and DNA synthesis, it appeared that even
Fig. 2.—Kinetics of DNA synthesis in PHA-stimulated lymphocyte cultures. After an appropriate length of incubation with PHA each culture received 4 µc thymidine-H³ two hours before harvest. DNA was extracted and specific activity was expressed as count per minute per µg. nucleotide DNA. (Top) Shaded area depicts the range of 9 normals studied in triplicate. Each point represents the average ± I.S.D. Single curves depict the responses of J.S. ( -Δ- ), J.L. ( -O- ), and P.M. ( -□- ); examples of low count cases. (Bottom) Shaded area depicts the range of 9 patients with high count CLL. Each point represents the average ± I.S.D. Single curves depict the responses of M.E. ( -□- ), L.L. ( -O- ), and R.B. ( -Δ- ); examples of low count cases.

In low count CLL, lymphocytes responded to PHA as a single, delayed wave. A wave of normal reactivity could not be detected by this method. On the other hand, the patient with probable splenic lymphosarcoma and leukemic blood picture showed two distinct waves of reactivity. When he was first seen in February, 1967, splenomegaly was marked while peripheral lymphocytosis was lacking (Table 2). Splenectomy was decided upon as the therapeutic
Table 1.

<table>
<thead>
<tr>
<th>Age</th>
<th>Patient</th>
<th>Lymphocytes/mm³</th>
<th>Hemoglobin (Gm. %)</th>
<th>Platelets/mm³</th>
<th>Lymphadenopathy*</th>
<th>Hepatosplenomegaly†</th>
<th>Immune complications</th>
<th>Immunoglobulins‡ (mg. %)</th>
<th>Abnormal Antibodies</th>
<th>Marrow lymphocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IgG IgA IgM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>61 M</td>
<td>J.R.</td>
<td>57,040</td>
<td>11.6</td>
<td>96,000</td>
<td>++</td>
<td>Liver 4 cm. Spleen -0-</td>
<td>-0-</td>
<td>950 63 41</td>
<td>-0-</td>
<td>present</td>
</tr>
<tr>
<td>43 M</td>
<td>A.R.</td>
<td>139,650</td>
<td>14.5</td>
<td>208,000</td>
<td>++++</td>
<td>Liver 6 cm. Spleen -0-</td>
<td>Mikulicz syndrome</td>
<td>gamma globulin 600</td>
<td>-0-</td>
<td>present</td>
</tr>
<tr>
<td>52 F</td>
<td>B.F.</td>
<td>51,000</td>
<td>12.8</td>
<td>210,000</td>
<td>++</td>
<td>-0-</td>
<td>-0-</td>
<td>485 62 45</td>
<td>Anti-thyroid antibody positive</td>
<td>present</td>
</tr>
<tr>
<td>49 F</td>
<td>I.W.</td>
<td>60,808</td>
<td>13.2</td>
<td>174,000</td>
<td>++++</td>
<td>-0-</td>
<td>-0-</td>
<td>-0-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>67 M</td>
<td>A.O.</td>
<td>52,250</td>
<td>10.0</td>
<td>298,000</td>
<td>++++</td>
<td>Liver -0- Spleen 8 cm.</td>
<td>-0-</td>
<td>gamma globulin 950</td>
<td>-0-</td>
<td>present</td>
</tr>
<tr>
<td>76 M</td>
<td>C.B.</td>
<td>190,000</td>
<td>13.2</td>
<td>172,000</td>
<td>-0-</td>
<td>Liver ? Spleen 16 cm.</td>
<td>-0-</td>
<td>270 34 17</td>
<td>-0-</td>
<td></td>
</tr>
<tr>
<td>66 M</td>
<td>I.K.</td>
<td>168,000</td>
<td>14.0</td>
<td>128,000</td>
<td>++++</td>
<td>Liver 8 cm. Spleen 8 cm.</td>
<td>Myas. gravis hemol. anemia</td>
<td>330 44 130</td>
<td>Positive Coombs, Latex 1/11520</td>
<td>present</td>
</tr>
<tr>
<td>26 F</td>
<td>L.B.</td>
<td>72,000</td>
<td>9.5</td>
<td>75,000</td>
<td>-0-</td>
<td>Liver 12 cm. Splenectomy</td>
<td>Infections</td>
<td>70 85 0</td>
<td>Positive cold agglutinins in high titer</td>
<td>present</td>
</tr>
<tr>
<td>62 F</td>
<td>S.J.</td>
<td>114,000</td>
<td>10.0</td>
<td>90,000</td>
<td>++++</td>
<td>Liver 6 cm. Spleen 8 cm.</td>
<td>-0-</td>
<td>-0-</td>
<td></td>
<td>present</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56 M</td>
<td>R.B.</td>
<td>29,440</td>
<td>13.9</td>
<td>104,000</td>
<td>+</td>
<td>Liver 4 cm. Spleen 6 cm.</td>
<td>525</td>
<td>240</td>
<td>22</td>
<td>-0-</td>
</tr>
<tr>
<td>66 M</td>
<td>M.E.</td>
<td>36,000</td>
<td>13.5</td>
<td>68,000</td>
<td>+</td>
<td>Liver 4 cm. Spleen 4 cm.</td>
<td>ITP</td>
<td>520</td>
<td>105</td>
<td>58</td>
</tr>
<tr>
<td>68 M</td>
<td>L.L.</td>
<td>26,520</td>
<td>12.8</td>
<td>260,000</td>
<td>+</td>
<td>Liver 4 cm. Spleen 2 cm.</td>
<td>-0-</td>
<td>640</td>
<td>128</td>
<td>24</td>
</tr>
<tr>
<td>65 F</td>
<td>J.S.</td>
<td>6,328</td>
<td>12.3</td>
<td>230,000</td>
<td>-0-</td>
<td>Liver 4 cm. Spleen -0-</td>
<td>-0-</td>
<td>-0-</td>
<td>610</td>
<td>53</td>
</tr>
<tr>
<td>67 M</td>
<td>P.M.</td>
<td>16,279</td>
<td>14.3</td>
<td>200,000</td>
<td>±</td>
<td>Liver 4 cm. Spleen -0-</td>
<td>gamma globulin</td>
<td>950</td>
<td>-0-</td>
<td>equivocal</td>
</tr>
<tr>
<td>41 M</td>
<td>J.L.</td>
<td>5,120</td>
<td>15.7</td>
<td>270,000</td>
<td>-0-</td>
<td>Liver 3 cm. Spleen -0-</td>
<td>-0-</td>
<td>760</td>
<td>84</td>
<td>29</td>
</tr>
</tbody>
</table>

*arbitrary scale 0-4+.*

† cms below costal margin.

Summary of Clinical Data on CLL Patients

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG 1200 ± 300 (normal values)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgA 280 ± 100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM 88 ± 22</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
procedure most likely to succeed. At laparotomy, the spleen weighed 2,000 Gm. and the organ was totally replaced by small lymphocytes. No enlarged intra-abdominal nodes were found. Lymphocyte kinetic studies were carried out before splenectomy and again two weeks and seven months after splenectomy. Following the introduction of PHA, cultures of peripheral lymphocytes prepared before splenectomy yielded two distinct waves of increased RNA
synthesis (Fig. 3); a peak on day 2 and a peak between days 8–9. Peaks of increased DNA synthesis were observed at day 3 and between days 8–9, i.e., both at the normal and at a distinctly delayed time. This type of biphasic response had not been observed in the cases of typical CLL.

Two weeks after splenectomy (Fig. 4), the first wave of RNA synthesis persisted; but the second wave was distinctly reduced. The pre- and post-splenectomy patterns of DNA synthesis displayed the same phenomenon. Thus, splenectomy appeared to be responsible for diminishing the second wave of reactivity, although the number of circulating lymphocytes was only slightly reduced and the percentage of atypical lymphocytes remained unchanged (Table 2).

Seven months later, with increasing hepatomegaly, there was little evidence of a normal response while the secondary or delayed response accounted for the entire reaction (Fig. 4). Two components could now be distinguished in the delayed response, one at day 6 and the other at day 9. It was apparent that the 9 day component had been most affected by splenectomy and it was this component that became evident at the time of relapse. With CLL lymphocytes, the delayed response usually took place between 5–7 days. The abnormal component, which in this case, reached a maximum on day 6, closely resembled the pattern seen in typical CLL.

Immunoelectrophoretic studies of this patient's circulating immunoglobulins shed further light on the biphasic phenomenon. In February, 1967, two components were observed migrating in the region of IgA. By March, 1968, the faster of these components increased in amount to raise the total IgA to 640 mg. per cent (Table 2). The IgA comprising this dense arc on immunoelectrophoresis contained only type 2 light chains, suggesting a monoclonal or "neoplastic" pattern. These findings would correlate well with the lymphocyte kinetic data which also suggested at least two populations of reactive lymphocytes. Thus, with respect to immunoglobulin production and lymphocyte reactivity, the initial coexistence of normal and abnormal function later gave way to predominantly abnormal function.

It should be stressed that this one case in no way establishes a pattern common to most cases of lymphosarcoma, in which the kinetics of the PHA response of circulating lymphocytes are highly variable. It is clear from the few studies available that both normal and late reacting lymphocytes can simultaneously occur in the circulation, suggesting clonal abnormalities of presumably neoplastic cells.

**Autoradiographic Studies**

The kinetic data from lymphocyte cultures in CLL established that a delayed reaction to PHA was a characteristic feature and suggested that few, if any, normally reactive lymphocytes were present. However, the reactivity of individual cells could not be demonstrated by this technic. Despite indirect evidence to the contrary, the possibility remained that a small population of normally reactive lymphocytes, undetected by this technic, might have expanded by repeated mitosis until their number was large enough to manifest a peak in specific activity at 5–7 days. To eliminate this possibility, the follow-
### Table 2.—Primary Lymphosarcoma of Spleen

<table>
<thead>
<tr>
<th>Date</th>
<th>WBC/mm³</th>
<th>% lymph.</th>
<th>Hgb. G%</th>
<th>Platelets/mm³</th>
<th>Lymphadenopathy</th>
<th>Marrow</th>
<th>Spleen*</th>
<th>Liver*</th>
<th>Abnormal Immuno. Function</th>
<th>Abnormal Antibodies</th>
<th>IgG</th>
<th>Serum. Glob. IgA (mg%)</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/67</td>
<td>25,900</td>
<td>79</td>
<td>15.1</td>
<td>99,000</td>
<td>0</td>
<td>normal</td>
<td>12 cm.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>800</td>
<td>400†</td>
<td>67</td>
</tr>
<tr>
<td>3/67</td>
<td>25,600</td>
<td>63</td>
<td>12.8</td>
<td>460,000</td>
<td>0</td>
<td>—</td>
<td>†</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8/67</td>
<td>25,900</td>
<td>53</td>
<td>16.2</td>
<td>360,000</td>
<td>0</td>
<td>—</td>
<td>8 cm.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3/68</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>9 cm.</td>
<td>0</td>
<td>0</td>
<td>1350</td>
<td>640†</td>
<td>—</td>
<td>57</td>
</tr>
</tbody>
</table>

* cms. below costal margin  
† splenectomy  
† double arc on immunoelectrophoresis—see text.
Fig. 4.—Kinetics of nucleic acid synthesis in PHA stimulated lymphocyte cultures prepared from patient A.G.—Lymphosarcoma. Each point represents the average of triplicate determinations. Curves represent response of A.G. before splenectomy (-O-), and seven months post-splenectomy (-□-). Note absence of determination on day 2.

ing autoradiographic analyses were performed. The results provided evidence of a small population of normally reactive lymphocytes circulating in CLL and a distinct but considerably larger population of late reacting cells.

In this study, a two hour exposure of normal lymphocytes to thymidine-\(^3\)H labeled approximately 50 per cent of the blast cells in cultures incubated with PHA for 3 days (Fig. 5). These labeled blast cells were normal, as the proliferative response to PHA took place at the anticipated time. The incubation was then continued in the absence of radioactive precursors. In autoradiographs prepared at subsequent times, normal lymphocytes could always be identified because their nuclei were labeled by radioactive DNA. Blast cells containing less than 3 grains were assumed to be unlabeled. Accepting a minimal cell cycle time of 12–16 hours,\(^{13}\) it is highly unlikely that a normal blast cell originally labeled with more than 100 grains could dilute its label to less than 3 grains by repeated division in a nonradioactive medium. Background (0–1 grains per area occupied by an average blast cell) together with reutilization from a labeled precursor pool could account for 1–3 grains occasionally observed in cultures incubated for 5 and 7 days in unlabeled medium.

During a 7 day incubation of normal lymphocytes, a transient increase in the proportion of labeled blast cells took place at day 5 (Fig. 5). By day 7 the percentage of labeled blasts was still not decreased below the level observed at day 3. Assuming that the label identified a blast as capable of incorporating thymidine (i.e., thymidine-\(^3\)H) on day 3, these normal cultures contained few, if any,
Fig. 5.—Pulse-chase studies of PHA stimulated lymphocyte cultures. Each culture was incubated with PHA for three days and then thymidine-H³ was introduced. After a two hour exposure to thymidine-H³ an excess of unlabeled thymidine was introduced and the cells were recovered by centrifugation. Each cell pellet was resuspended in fresh, unlabeled medium and the incubation was continued for an appropriate interval after which autoradiographs were prepared.

lymphocytes which transformed into blasts after day 3. Clearly a population of blasts arising after day 3 would have reduced the percentage of labeled blasts observed at days 5 and 7. This conclusion could have been anticipated from the kinetic studies which demonstrated a single wave of metabolic activity in normal cultures.

Autoradiographic analysis of CLL cultures (2 high count cases, Fig. 5) also demonstrated labeling in approximately 50 per cent of those blast cells present at day 3. This confirmed the presence of a small population of normal reactive lymphocytes even in high count CLL. However, in contrast to the normal cultures, continuing the incubation of CLL cultures to 5 and 7 days in an unlabeled medium resulted in a marked decrease in the percentage of labeled blast cells as the small pool of normally reactive cells became diluted by a much larger population undergoing transformation and DNA replication at the later times. It should, however, be emphasized that the absolute number
of blast cells in normal cultures was far greater than the number observed in corresponding CLL cultures, suggesting that relatively few lymphocytes of CLL become transformed with PHA. This does not alter the conclusion that blast cells arising at 3 days predominated in normal cultures and blast cells arising at 5 and 7 days predominated in CLL cultures. Therefore, the unlabeled blast cells in 5 and 7 day cultures could be distinguished as a population of late reacting or abnormal lymphocytes.

**DISCUSSION**

Utilizing the action of PHA in short-term tissue cultures, an in vitro test of lymphocyte function has been emphasized. This model appears to have some of the features of the normal in vivo immunoproliferative reaction, and thus provides an opportunity to evaluate those situations in which lymphocyte function may be abnormal. Previous data have established the normal pattern of RNA and DNA synthesis in PHA stimulated lymphocytes, a pattern which could not be altered by frequent replenishment of the incubating medium. Since plasma factors were eliminated by washing the isolated lymphocytes, deviations from the normal pattern could be said to reflect abnormalities intrinsic to the cell.

In high count CLL the maximal increase in RNA synthesis was consistently delayed, apparently characteristic of this stage of the leukemic condition. The addition of CLL cells to cultures of normal lymphocytes, as previously reported, did not retard the development of the normal response to PHA. Lymphocytes from patients with low count CLL also showed a delayed response to PHA, but the abnormality was less marked. Increased RNA and DNA synthesis developed in what was apparently a single wave, suggesting that mainly one cohort or population of cells was responding, and that few of the circulating lymphocytes were normal. Conversely, in the lymphosarcoma case, a biphasic response to PHA was observed initially, disappearing when the spleen was removed. This supported our contentions that when considerable numbers of normal lymphocytes were present their reaction could be detected by kinetic studies and that the delayed reaction could be attributed to a discrete population of abnormal, i.e., "leukemic" lymphocytes, cells with a metabolic defect resulting in reduced and delayed in vitro proliferative activity. The autoradiographic experiments separated normal from abnormal lymphocytes and therefore provided further confirmation that the late reacting lymphocytes in CLL formed a discrete, abnormal population. This eliminated the possibility that the small population of normal lymphocytes detectable by the autoradiographic technic, might have expanded by repeated mitoses until their numbers became large enough to detect as a wave of increased RNA and DNA synthesis in the whole culture kinetic analysis. A distinct population of abnormal lymphocytes in CLL was demonstrated by Goh who observed aneuploid karyotypes (pseudodiploid) in cultures of PHA stimulated CLL lymphocytes after 3–6 days of incubation. Normal lymphocytes incubated with PHA for 3–6 days, even in the presence of CLL cells, exhibited normal karyotypes.

Our results suggest that in CLL and in certain cases of LSA, some circulating lymphocytes respond to PHA, but in a delayed fashion, and many do not
respond at all. Demonstration of normal or near normal numbers of blast cells in PHA stimulated cultures from patients with low count CLL had previously led to the conclusion that many normal lymphocytes circulate in this mild or early form of the disease.\(^5\) The present study detected abnormal reactivity even in low count CLL. In advanced or high count CLL the delay in reactivity was more pronounced and the decreased magnitude of the metabolic response correlated well with the decreased percentage of blast cells previously reported.\(^7\) Perhaps lymphocyte reactivity in CLL becomes increasingly impaired as the disease progresses until most lymphocytes are totally unreactive. This intrinsic metabolic defect demonstrable even in low count—presumably early—cases of the disease, suggests a generalized process from the start. Perhaps the disorder could be congenital, a hypothesis supported by the finding of many familial cases of CLL.\(^8\)

The presence of a well-defined immunoglobulin deficiency state even in early cases of the disease may be correlated with the metabolic defect as demonstrated in PHA cultures. The more pronounced deficiency with advanced disease as seen in the high count cases may reflect progressive inability of CLL lymphocytes to respond to antigen. Accumulation of immunologically incompetent lymphocytes could well be the end result of the failure of many small lymphocytes to respond to antigens.\(^15\) Since it is sometimes difficult in a case presenting no lymphadenopathy or splenomegaly, but a well-defined small cell lymphocytosis of the blood and marrow, to make the diagnosis of CLL, the results of the reaction of the blood lymphocytes to PHA and of the immunoglobulin concentration could well be crucial. Circulating lymphocytes in Hodgkin's disease, a condition not association with lymphocyte accumulation, also show an impaired reactivity to PHA.\(^16\) However, normally reactive lymphocytes are present in large numbers until the terminal phases of the disease\(^17\) and similarly, it is only in these later phases that Hodgkin's patients display immunologic deficiency states.\(^18\) Therefore, the lymphocyte defect in CLL may represent a unique situation in which immunologic impairment results in a slow accumulation of incompetent cells.

Demonstration of an altered growth pattern in leukemic lymphocytes raises certain questions concerning the nature of the basic proliferative abnormality in this form of leukemia. Unstimulated small lymphocytes from leukemic individuals, like normal lymphocytes, do not proliferate in vitro.\(^7\) Only after stimulation with PHA are proliferative abnormalities brought out. Since the PHA reaction in short-term tissue culture may be considered as an in vitro model of one phase of the immunologic response, the slow response of CLL lymphocytes may reflect a primary defect in the capacity of the small lymphocyte to mount a proliferative response to appropriate immunologic stimulation.

The delay in increased RNA synthesis following PHA stimulation probably indicates that the metabolic defect in CLL lymphocytes influences that phase of the cell cycle prior to DNA replication. In the low count cases, the time required for the development of the peak in RNA synthesis was intermediate between the normal response (on day 2) and the response of high count cases (on day 7). The pattern of increased DNA synthesis was close to normal in two cases with the lowest count (J.L. and J.S.) while in two other low count
cases, the peak of the DNA response tended to be prolonged to seven days, much the same as in the high count cases.

Perhaps this observation reflects certain properties of the $G_1$ (the interval between the end of mitosis and the beginning of DNA replication) and the S phases (DNA replication time) of the cell cycle. If the intracellular events preparing for the S phase are delayed beyond a certain point in some cells, there may be a mandatory delay before DNA synthesis can begin. The data might also reflect an attenuation of the S phase, thus yielding the low, flat DNA curves manifested by patients M.E., P.M., and R.B. These aspects of the problem are now under investigation.

In view of the high incidence of family cases, as well as the appearance of mild, asymptomatic cases found ordinarily at routine examination, it is possible that the growth disturbance of small lymphocytes in CLL is, at least in some individuals, an inherited or congenital defect involving one of the tissues either producing or regulating lymphocytic proliferation.

**SUMMARY**

Peripheral lymphocytes from patients with chronic lymphocytic leukemia were exposed to phytohemagglutinin in culture. Quantitative assessment of the proliferative response provided an in vitro test of lymphocyte function. The results were correlated with several clinical parameters, both hematologic and immune. Lymphocytes from patients with modestly elevated lymphocyte counts and mild disease manifested slightly delayed and depressed reactions to PHA. All immunoglobulin levels in these patients were also moderately reduced. Further delay and depression of the PHA response showed a rough correlation with progressive lymphocytosis and reduction in circulating immunoglobulins.

Kinetic and autoradiographic analyses have suggested that late-reacting lymphocytes formed distinct populations of abnormal cells which did not seem to be appreciably contaminated with normal lymphocytes, even in instances of mild disease. Only in a patient with splenic lymphosarcoma were multiple circulating populations of normal and late reacting lymphocytes detected simultaneously. In that case, at least some of the late-reacting lymphocytes appeared to originate in parenchymal lymphoid masses.

A definition of CLL could well include, not only the findings in the blood and bone marrow, but a statement of the reaction of the lymphocytes to phytohemagglutinin and of the immunoglobulin characteristics.

**SUMMARIO IN INTERLINGUA**

Lymphocytos peripheric ab patientes con chronic leucemia lymphocytic esseva exponite in cultures al activitate de phytohemagglutinin. Le quantitativa evaluation del responsa proliferative provideva un test in vitro del function lymphocytic. Le resultatos esseva correlatae con plure parametros clinic, tanto hematologic como etiam immunologic. Lymphocytos ab patientes con moderatemente elevate numerationes lymphocytic e un forma leve del morbo manifestava paucaretardate e deprimite reactiones a phytohemagglutinin. Le nivellos de omne immunoglobulinas in iste patientes esseva etiam moderamente reducite. Retardos e depressiones additional del responsa a phytohemagglutinin monstrava un grossier correlazione con progresso del lymphocytose e reduction del nivellos de immunoglobulinas circulante.

Analyses cinetic e autoradiographic ha suggestionate que lymphocytos a reaction tardive
forma distincte populationes de cellulas anormal que non pare esser notabilemente contaminate con lymphocytos normal, mesmo in casos de leve grados de morbidade. Multiplica populationes circulante de lymphocytos normal e lymphocytos a reaction tardive esseva detegite simultaneemente solo in un patiente con lymphosarcoma splenic. In su caso, al minus certes del lymphocytos a reaction tardive pareva haber lor origine in parenchymatose massas lymphoide.

Un definition de chronic leucemia lymphocytic deberea includer non solmente le constations in le sanguine e le medulla ossee sed etiam un description del reaction del lymphocytos a phytohemagglutinin e del estimation immunoglobulinic.

REFERENCES

Studies in Chronic Lymphocytic Leukemia: Further Studies of the Proliferative Abnormality of the Blood Lymphocyte

ARNOLD D. RUBIN, KLAUS HAVEMANN and WILLIAM DAMESHEK

Updated information and services can be found at: http://www.bloodjournal.org/content/33/2/313.full.html
Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at: http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests
Information about ordering reprints may be found online at: http://www.bloodjournal.org/site/misc/rights.xhtml#reprints
Information about subscriptions and ASH membership may be found online at: http://www.bloodjournal.org/site/subscriptions/index.xhtml