Lymphocyte Lysosomes and Lysosomal Enzymes in Chronic Lymphocytic Leukemia

By Steven D. Douglas, Georg Cohnen, Erika König, and Günter Brittinger

Electron microscopic cytochemical and biochemical studies of lysosomal markers have been performed in unstimulated normal and chronic lymphocytic leukemia (CLL) lymphocytes. Decreased activities of the lysosomal enzymes acid phosphatase and β-glucuronidase but not of the nonlysosomal enzyme malate dehydrogenase were observed in CLL lymphocytes as compared to normal cells. At the electron microscopic level, the number of membrane-bounded acid phosphatase-positive organelles was diminished in CLL cells. (Average 1.07 per cell profile in normal cells and 0.17 in CLL lymphocytes). The findings indicate that the diminution of acid hydrolase activities in CLL lymphocytes is most likely due to a reduced number of lysosomes, rather than to a diminished enzyme content of these organelles.

In previous studies, the existence of structures fulfilling the criteria of lysosomes has been demonstrated in normal human peripheral blood lymphocytes. Thus, acid hydrolase activity has been found in the lysosome-rich granular fraction after differential centrifugation of homogenates derived from normal lymphocytes. The enzyme activity showed appropriate latency, as determined by its dose-dependent release from the granular fraction into the corresponding supernatant, after exposure to sublytic concentrations of membrane-disruptive agents. Furthermore, electron microscopic examination of the granular fraction, as well as of the intact lymphocytes, revealed the presence of membrane-bounded acid phosphatase-positive organelles. The electron microscopic features of such organelles have previously been reported.

Biochemical studies on unstimulated (“resting”) peripheral blood lymphocytes from patients with chronic lymphocytic leukemia (CLL) with high lymphocyte counts have shown decreased activities of the lysosomal enzymes acid phosphatase and β-glucuronidase as compared to normal lymphocytes.

From the Department of Medicine, Laboratory of Cellular and Subcellular Immunology, Mount Sinai School of Medicine of The City University of New York, New York, N.Y., and the Department of Medicine, Division of Hematology, Klinikum Essen, Ruhr-University, Essen, Germany.

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Reduced β-glucuronidase activity in CLL cells has also been found by light microscopic cytochemistry. Thus far, electron microscopic examinations have failed to distinguish circulating CLL lymphocytes from normal cells by any significant criteria. However, as yet no data have been reported concerning the content of lysosomes in CLL cells. The diminution of lysosomal hydrolases in unstimulated CLL lymphocytes could be the consequence either of a reduced number of lysosomes and/or of a diminished enzyme content of each organelle. Therefore, both electron microscopic cytochemical and biochemical studies of lysosomal markers were performed in the present investigation.

MATERIALS AND METHODS

Peripheral blood lymphocytes from four healthy individuals and five patients with CLL (lymphocyte counts ranging from 27,000/μl to 210,000/μl) were studied. Freshly drawn heparinized venous blood was allowed to sediment spontaneously for 1–2 hr at 37°C. The leukocyte-rich plasma (LRP) was then separated from the sedimented red cells. To remove the majority of the platelets, adenosine-5′-diphosphate (final concentration of 10–15 mg/ml) was added, and the resulting platelet clumps and agglomerates were removed by decanting and filtration. The LRP was divided into two parts. One part was used as “unseparated lymphocytes.” The remainder of the LRP was passed through a column of thoroughly washed nylon wool (Lenko-Pak; Fenwal/Travenol) at 37°C to remove adherent cells. The plasma was collected, and the column was washed with Eagle’s Minimum Essential Medium, Spinner-Modification (MEM-S; Grand Island Biological Co., Grand Island, N.Y.).

After centrifugation (740 g for 15 min), the cells were washed three times in MEM-S, resuspended in complete medium (MEM-S containing 20% fetal calf serum, 0.2 mmoles L-glutamine, 10,000 IU penicillin, and 10,000 μg streptomycin/100 ml), and allowed to stand overnight at room temperature. White blood counts were performed, and the purity of column-separated cell suspensions was measured by differential counts of stained smears (98%–99% mononuclear cells were present). Cell viability was determined by dye exclusion using trypan blue (the viability obtained was 95%–100%).

Cytochemistry

The cells were fixed for 5 min at 4°C in 1.5% glutaraldehyde in 0.05 M sodium cacodylate containing 7.5% sucrose. The pellets were washed six times (10 min each) in 0.08 M sodium cacodylate, were rapidly frozen for 3 min in a mixture of dry ice and ethanol, and were thawed at room temperature. Following this procedure, the pellets were incubated for 30 min at room temperature in a modified Gomori substrate mixture. The substrate was prepared as follows: 0.42 g lead nitrate was dissolved in 300 ml 0.05 M acetate buffer (pH 5.0). To 50 ml of this solution, 5 ml of 0.1 M D, L-beta-glycerophosphate disodium salt pentahydrate grade 1 (Sigma Chemical Co., St. Louis, Mo.) were added. This mixture was filtered prior to use. Following incubation, the pellets were washed six times (15 min each) in 0.05 M acetate buffer (pH 5.0) containing 7.5% sucrose and 4% formaldehyde. The pellets were rinsed overnight in 0.05 M acetate buffer (pH 5.0). Controls consisted of incubations in which glycerophosphate substrate was omitted or 0.01 M sodium fluoride was added to the media.

The cells were dehydrated in graded concentrations of ethyl alcohol and in propylene oxide and were then embedded in Epon 812 epoxy resin. Thin sections were cut with an LKB Ultrotome III, were placed on carbonized copper grids, and were stained with 1% aqueous uranyl acetate, lead citrate, or a combination of these stains. The material was examined in a Siemens-101 electron microscope at primary magnifications from 3000 to 40,000. The number of acid phosphatase-positive organelles was estimated by counting at least 100 cell profiles in uniformly thin sections (approximately 800 Å) at a primary magnification of 8000 X.
**Biochemical Analyses**

Cell suspensions were cooled in ice water, and 15 ml of MEM-S (4°C) were added. The cells were then centrifuged (740 g for 15 min) at 4°C. Erythrocytes were lysed by repeated hypotonic shock (two to three times). The cells were resuspended in 3.7 ml of 0.34 M cold sucrose solution containing 0.01 M EDTA (pH 7.0) and were homogenized in a grinder of the Potter-Elvehjem type. For release of organelle-bound enzyme activity at 4°C for 15 min, the homogenates were treated with Triton X-100 (final concentration 0.1%). After centrifugation (20,000 g for 20 min), assays were performed on the supernatants.

Acid phosphatase activity was determined by a modification of the method of Valentine and Beck.\(^{13}\) β-Glucuronidase activity was assayed by a modification of the method of Talalay et al.\(^{14}\) Malate dehydrogenase (MDH) activity was determined by the method of Mehler et al.\(^{15}\) Protein was determined according to the method described by Lowry et al.\(^{16}\)

**RESULTS**

Acid phosphatase cytochemistry on normal lymphocytes showed the presence of some lead reaction product in many cell profiles (Fig. 1, Table 1). Some normal cells showed the reaction product in Golgi vesicles and sacculi (Fig. 1). In contrast, preparations of separated and unseparated CLL cells showed infrequent reaction products (Figs. 2–6). In Table 1, the results of cell profile analysis of the acid phosphatase reaction product are shown. The activities of the lysosomal enzymes, acid phosphatase and β-glucuronidase, but not of nonlysosomal enzyme (malate dehydrogenase) were diminished in CLL lymphocytes as compared to normal cells (Table 2). No significant differences in the enzyme activities of unseparated and column-purified (separated) CLL lymphocyte preparations were observed.
Table 1. Acid Phosphatase-positive Organelles Per Cell Profile Determined Cytocemically*

<table>
<thead>
<tr>
<th>Patients</th>
<th>Unseparated Lymphocytes</th>
<th>Separated Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>0.26</td>
<td>0.28</td>
</tr>
<tr>
<td>K</td>
<td>0.19</td>
<td>0.08</td>
</tr>
<tr>
<td>B</td>
<td>0.17</td>
<td>0.09</td>
</tr>
<tr>
<td>Y</td>
<td>0.13</td>
<td>0.17</td>
</tr>
<tr>
<td>G</td>
<td>0.11</td>
<td>0.21</td>
</tr>
<tr>
<td>Normals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>—</td>
<td>1.46</td>
</tr>
<tr>
<td>Z</td>
<td>—</td>
<td>0.72</td>
</tr>
<tr>
<td>J</td>
<td>—</td>
<td>1.16</td>
</tr>
<tr>
<td>S</td>
<td>—</td>
<td>0.92</td>
</tr>
</tbody>
</table>

*In each instance at least 100 cell profiles were examined at a magnification of 8000 X.

DISCUSSION

The ultrastructural features of unstimulated normal human blood lymphocytes have previously been described.\textsuperscript{17-20} In a detailed assessment of organelle development, no significant differences have been found between resting normal and CLL lymphocytes.\textsuperscript{10,11} However, in the present investigation a reduced number of lysosomelike organelles has been demonstrated in CLL lymphocytes, as compared to normal cells. Furthermore, parallel biochemical analyses of homogenates derived from lymphocytes of the same CLL patients

Fig. 2. Lymphocyte from CLL patient showing a single lysosome containing acid phosphatase reaction product. × 20,000.
Figs. 3–6. Acid phosphatase cytochemistry showing electron-opaque reaction product in lysosomes of lymphocytes from CLL patients. When present, these organelles show reactivity comparable to lymphocytes from normal individuals. × 38,000.
Table 2. Cytochemical and Biochemical Studies of CLL and Normal Lymphocytes

<table>
<thead>
<tr>
<th>Patients</th>
<th>Cytochemistry—No. Acid Phosphatase-positive Organelles</th>
<th>Acid Phosphatase Activity(t)</th>
<th>(\beta)-Glucuronidase Activity(t)</th>
<th>Malate Dehydrogenase Activity(t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unseparated</td>
<td>0.17 ± 0.03(g)</td>
<td>67.69 ± 25.80</td>
<td>7.15 ± 1.49</td>
<td>225.74 ± 117.84</td>
</tr>
<tr>
<td>Separated</td>
<td>0.17 ± 0.04</td>
<td>50.45 ± 13.83</td>
<td>9.43 ± 1.79</td>
<td>201.31 ± 37.27</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unseparated</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Separated</td>
<td>1.07 ± 0.16</td>
<td>146.15 ± 5.17</td>
<td>26.05 ± 1.96</td>
<td>222.53 ± 15.45</td>
</tr>
</tbody>
</table>

\(\text{*Mean} \pm \text{SEM.}\)

\(\text{tMillimicromoles phosphorus released/hr/10^7 cells.}\)

\(\text{tMillimicromoles phenolphthalein released/hr/10^7 cells.}\)

\(\text{\textbar Millimicromoles NADH_2 oxidized/min/10^7 cells.}\)

revealed decreased activities of the lysosomal enzymes, acid phosphatase and \(\beta\)-glucuronidase, but not of the nonlysosomal enzyme MDH. The most likely interpretation of these observations is that the diminution of lysosomal hydrolase activities in CLL lymphocytes, which has also been observed in previous studies\(^6\)–\(^8\) is due to a reduced number of lysosomes, rather than to a diminished enzyme content of the single organelle. The possibility that individual lysosomes in CLL lymphocytes may, in addition, have a decreased enzyme content cannot be excluded, and its investigation requires quantitative cytochemistry. The enzyme activities and the number of lysosomes in CLL lymphocytes were not significantly different in lymphocytes obtained from column-purified preparations or leukocyte-rich plasma. This comparison has not been possible in preparations from normal individuals, because the high percentage of granulocytes in the unseparated leukocyte-rich plasma did not permit precise determinations of lysosomal enzymes present in the lymphocytes.

Alteration of the lysosomal apparatus is an early event in the activation of normal lymphocytes by phytomitogens.\(^21\)–\(^24\) Thus, in homogenates derived from normal lymphocytes incubated with PHA and pokeweed mitogen (PWM) for 30–120 min, redistribution of lysosomal hydrolases from the lysosome-rich, 20,000 g for 20 min, granular fraction into the corresponding supernatant was observed.\(^21\)–\(^24\) The labilization of lysosomal membranes in PHA-treated normal cells has been further demonstrated by treatment of lysosomes with membrane-disruptive agents such as lysolecithin, streptolysin S, and Filipin, which results in further release of enzyme activity from the lysosomes that is greater than that released by resting lymphocyte lysosomes.\(^21\)–\(^28\)

Lymphocytes from most CLL patients show a diminished and/or delayed, or even absent, in vitro response to PHA\(^7\)–\(^8\),\(^25\) and PWM\(^26\)–\(^27\) as compared to normal lymphocytes. Redistribution of acid phosphatase and \(\beta\)-glucuronidase failed to occur in homogenates from PHA- and PWM-treated CLL cells that had an impaired response to these mitogens after 3 days of incubation.\(^6\) The release of enzyme activity following lysolecithin exposure, from lysosomes derived from PHA-treated CLL lymphocytes, was not greater than that
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released by lysosomes of resting CLL cells. Moreover, the susceptibility of lysosomes from PHA-treated CLL cells to lysolecithin correlated with the mitogenic response of these cells to PHA.

Thus CLL lymphocytes have a reduction in the number of lysosomes and in the total content of the lysosomal enzymes, acid phosphatase and β-glucuronidase. These lysosomes have impaired labilization, an early event in lymphocyte transformation. The precise relationship between these abnormalities and the impaired response of CLL lymphocytes to mitogens awaits an understanding of the role of lysosomes in normal lymphocyte activation.

REFERENCES


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