Glutathione Depletion in Chronic Lymphocytic Leukemia B Lymphocytes

By Robert Silber, Charles M. Farber, Esperanza Papadopoulos, Denise Nevrla, Leonard Liebes, Michael Bruck, Richard Brown, and Zoe Nakos Canellakis

Glutathione (GSH) content may be the major determinant of a cell's sensitivity to cytotoxic alkylating agents. In the present study, the GSH concentration was determined in lymphocytes isolated from the blood of normal subjects and patients with chronic lymphocytic leukemia (CLL). Comparable levels were found in both types of cells. Incubation for 20 hours led to a decrease in GSH to 51% of baseline values in CLL B cells. Under the same conditions, normal B or T-lymphocyte GSH content remained constant. GSH depletion was shown to be a characteristic of the B-CLL B lymphocyte. It was not found in the T cells of patients with B-CLL or in cells from patients with T-CLL. Chlorambucil (CLB) contributes to the decrease in GSH in B-CLL lymphocytes; after incubation with the drug, lower levels of GSH were found than in the normal B or T lymphocytes, B-CLL T cells, or T-CLL (CD4 or CD8) cells. GSH depletion of CLL B lymphocytes may be related to the greater therapeutic efficacy of CLB in B-CLL than in T-CLL.

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MATERIALS AND METHODS

Lymphocyte isolation. Heparinized blood was obtained after obtaining informed consent from 30 normal subjects, 36 patients with B-CLL, and 2 patients with T-CLL. All but 8 of the patients were untreated; none had received therapy in the month before study. Results with cells from treated and untreated patients were similar.

Blood was diluted with RPMI 1640 medium (GIBCO, Grand Island, NY) and mononuclear cells were isolated by ficoll-Hypaque centrifugation followed by three washes with medium.

Cells were counted and purity assessed with a Model ZBI Coulter Counter (Coulter, Hialeah, FL). The mononuclear cells were then resuspended in medium containing heat-treated 10% fetal calf serum (FCS; GIBCO) at a concentration of 10^7 cells/mL and were depleted of monocytes by adherence to Falcon plastic culture dishes (Becton Dickinson, Lincoln Park, NJ) at 37°C for 45 minutes. The nonadherent cells were gently decanted from the dishes and then washed twice with RPMI 1640. In general, fewer than 5% monocytes and only a very rare platelet were found in the final preparations. In some experiments, magnetic "immunobeads" (Dynal, Great Neck, NY) were used to further deplete the monocyte population.

B- and T-lymphocyte subpopulations were enriched as described previously. Cell subtype homogeneity was greater than 82% and greater than 89% for B and T lymphocytes, respectively. Cell viability, as determined by erythrosin B exclusion, was consistently greater than 90%.

Cell incubation. Lymphocytes were incubated in culture tubes at a concentration of 5 x 10^6 cells/mL for 20 hours in RPMI 1640 medium containing 10% FCS and 1% penicillin/streptomycin. At the end of incubation, cell viability was again determined by erythrosin B exclusion. Cells were then washed three times with RPMI 1640. GSH and glutathione disulfide (GSSG) were measured as described below. When used, CLB was added at the beginning of incubation. For the measurement of GSH export, the incubation medium contained 2 mmol/L L-serine/2 mmol/L sodium borate to inhibit γ-glutamyl transpeptidase.

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Supported by Grant No. CA41666 from the National Cancer Institutes of Health and VAMC Merit Review Funds.

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0006-4971/92/8008-0001$3.00/0

Blood, Vol 80, No 8 (October 15), 1992; pp 2038-2043
Table 1. GSH Concentration in Blood Lymphocytes

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>GSH</th>
<th>GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>24.8 ± 2.0 (n = 36)</td>
<td>3.7 ± 1.0 (n = 13)</td>
</tr>
<tr>
<td>CLL</td>
<td>25.2 ± 1.8 (n = 30)</td>
<td>2.0 ± 0.9 (n = 11)</td>
</tr>
</tbody>
</table>

Values are in nanomoles per milligram of protein (mean ± SEM).

GSH content. Total GSH and GSSG concentrations were determined, as described by Griffith,19 using aliquots of 1 × 10⁷ cells. Lymphocytes were washed twice with saline and lysed with 1.0 mL 0.2% Triton X-100. An aliquot of lysate was used for protein determination.20 For the GSH measurement, 25% sulfosalicylic acid was added to make a final concentration in the lysate of 2.5%. Precipitated protein was removed by centrifugation at 5,000g for 5 minutes. The supernatant fluid was stored at −80°C for up to 5 days. Assay reagents were added in the following order: 140 μL of 100 mM NaH₂PO₄/5 mM NaEDTA buffer (pH 7.5), 25 μL of 6 mM 5,5'-dithio-bis-2-nitrobenzoic acid, 1 U of GSH reductase (type III; Sigma, St Louis, MO), and 12.5 or 25 μL lysate in a final volume of 250 μL. The reaction was started by adding 50 μL of 1.1 mM NADPH and absorbance at 340 nm was monitored for 5 minutes. The amount of total GSH was determined from a standard curve that was linear from 2.5 to 100 ng.

GSSG content was determined as follows. For every 100 μL of lysate, 2 μL of 2-vinylpyridine were added, followed by gentle agitation and the addition of 6 μL of triethanolamine. After mixing for 1 minute, the tube was sealed with parafilm and allowed to stand at room temperature for 1 hour, and the precipitate was removed by centrifugation at 5,000g for 5 minutes. Standards were prepared with GSSG, 2-vinylpyridine, and triethanolamine. The same methods were used to assay GSH and GSSG in the culture medium. FCS, L-serine, and sodium borate were included in the blanks.

Bound GSH estimation. Total GSH was measured after reduction of bound GSH with dithiothreitol (DTT) as follows.21 The experimental sample of (10⁷ cells) was suspended in 0.05 M tris buffer, pH 8.5, Tris buffer containing 0.02 M DTT, and maintained with shaking for 30 minutes at room temperature. Acidification of the sample to pH 2 (with sulfosalicylic acid) was followed by extraction of residual DTT with water-saturated ethylacetate. GSH in the aqueous layer was measured as described above.

GSH biosynthesis enzymes. Lymphocytes (2 × 10⁸ cells/mL) were lysed by standing on ice in 5 mM/L phosphate buffer for 15 minutes. The lysate was centrifuged at 10,000g for 10 minutes and the supernatant used for assays.

γ-Glutamylcysteine synthetase assay. The reaction mixture in a final volume of 0.1 mL contained 0.15 M Tris-HCl (pH 8.2), 50 mM/L KCl, 10 mM/L [¹⁴C]D-α-aminoxybutyrate (ICN Biomedicals, Irvine, CA), 10 mM/L glutamate, 10 mM/L ATP, 25 mM/L MgCl₂, 10 mM/L phosphoenol pyruvate, and 0.8 U pyruvate kinase.22

Table 2. GSH Concentration in Blood Lymphocyte Subsets

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>T-Enriched</th>
<th>B-Enriched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>20.6 ± 3.0 (n = 9)</td>
<td>15.3 ± 1.8 (n = 9)</td>
</tr>
<tr>
<td>CLL</td>
<td>13.8 ± 0.8 (n = 6)</td>
<td>16.4 ± 0.9 (n = 6)</td>
</tr>
</tbody>
</table>

Values are in nanomoles per milligram of protein (mean ± SEM).

Lysate was added last. The reaction mixtures were incubated for 30 to 90 minutes at 37°C and stopped by the addition of 900 μL of 20 mM/L acetic acid. The solution was applied to Dowex-1-acetate columns (Sigma, St Louis, MO), which were then washed with 8 mL 20 mM/L acetic acid. The substrates eluted within the first 5 mL. The labeled tripeptide product was eluted with 4 mL of 1.5 M/L ammonium acetate and radioactivity in the eluate measured by liquid scintillation counting. Substrate and product elution volumes were verified by the addition of GSH standards.
Table 3. Activity of GSH Biosynthesis Enzymes

<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>Normal (n = 4)</th>
<th>CLL (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B 1.2 ± 0.6</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>y-Glutamylcysteine synthetase</td>
<td>1.9 ± 0.3</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>T 4.7 ± 0.4</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>GSH synthetase</td>
<td>B 1.9 ± 0.5</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>T 2.0 ± 0.3</td>
<td>1.6 ± 0.4</td>
</tr>
</tbody>
</table>

Values are nanomoles per minute per milligram of protein (mean ± SE).

GSH synthetase assay. The reaction mixture (0.1 mL) contained 0.1 mol/L Tris-HCL buffer (pH 8.2), 50 mmol/L KCl, 5 mmol/L L-y-glutamyl-L-α-aminobutyrate (Chemical Dynamics Corp, South Plainfield, NJ), 10 mmol/L ATP, 5 mmol/L [14C] glycine 8 × 10⁴ cpm/μmol (Amersham, Arlington Heights, IL), 20 mmol/L MgCl₂, 2 mmol/L EDTA, 5 mmol/L phosphocreatine, and 1 U creatine kinase. Lysate was added last. The reaction mixtures were incubated for 30 to 90 minutes at 37°C and stopped by the addition of 900 μL of 20 mmol/L acetic acid. The solution was applied to (0.7 x 7 cm) Dowex-1-acetate columns, which were then washed with 8 mL 20 mmol/L acetic acid. The substrate eluted within the first 5 mL. The labeled tripeptide product was eluted with 4 mL of 1.5 mol/L ammonium acetate and radioactivity in the eluate measured by liquid scintillation counting. Substrate and product elution volumes were verified by the addition of GSH standards.

Statistical methods. Results were analyzed for their statistical significance using the unpaired Student's two-tailed t-test, except for the results in Fig 1, in which the paired t-test was applied. All data were expressed as mean ± standard error of the mean.

RESULTS

GSH content. There was no significant difference in the total GSH content of lymphocytes from normal donors and patients with CLL (Table 1). These results are in agreement with those reported in the literature. The finding that GSSG was about 10% of the total GSH in normal and CLL lymphocytes is probably an overestimate, due to oxidation during extraction. Similar concentrations of GSH were also found in B or T cells from either source (Table 2). The lower GSH values shown in Table 2 were within the range found for the unseparated lymphocyte population shown in Table 1.

Effect of incubation on GSH content. The cellular GSH concentration reflects the interaction of numerous enzymatic and nonenzymatic reactions. Because earlier studies from our laboratory had shown that B lymphocytes were more susceptible to H₂O₂ toxicity than T cells and oxidant stress is known to deplete erythrocytes of GSH, we...
determined the effect of incubation on the GSH concentration in "unseparated" (T and B) lymphocytes. At the end of a 20-hour incubation period, the GSH content decreased to 42% of baseline in CLL lymphocytes compared with 77% of baseline for normal controls (Fig 1). This significant difference was not due to cell death, because viability was greater than 90% in both. The percent GSSG was similar in normal and CLL lymphocytes at the end of incubation (data not shown). The intracellular and extracellular occurrence of protein mixed disulfides with low molecular weight thiols is well documented.26 We found that less than 5% of cellular glutathione was present as a disulfide derivative in lymphocytes either before or after incubation (data not shown). Thus, the diminished cellular GSH level cannot be explained by its conversion to protein-bound disulfides.

To test the possibility that increased export may cause the loss of GSH in CLL lymphocytes, the GSH content of the incubation medium was measured. After a 20-hour incubation, 12% of the total initial cellular GSH was recovered in the medium with either normal or CLL lymphocytes. Greater export, therefore, did not account for the lower GSH in the CLL lymphocytes.

To establish whether the loss of GSH merely reflected the altered subpopulation ratio of B and T cells in CLL or was a characteristic of the CLL B lymphocyte, GSH was assayed in enriched lymphocyte subpopulations. After incubation for 20 hours, the GSH in CLL B cells decreased to 51% of baseline, while the GSH concentration in normal B or T lymphocytes or in the T cells of patients with B-CLL was significantly greater (Fig 2). The possibility was considered that contaminant monocytes could account for the persistence of GSH in normal B cells, because after incubation for 20 hours, monocytes double their GSH content (data not shown). While typical normal B-cell preparations had less than 3% monocytes, greater than 20% contamination would be required for the observed difference. Monocyte contamination of normal B-cell preparation, therefore, does not account for the maintenance of GSH content.

**GSH biosynthesis enzymes.** The specific activity of both γ-glutamylcysteine synthetase and GSH synthetase was significantly greater in CLL B lymphocytes than in normal B lymphocytes (Table 3). In contrast, no difference in these activities was observed between normal and CLL T cells.

**Effect of CLB.** In a representative experiment (Fig 3), the addition of CLB resulted in greater cytotoxicity to B-CLL than to normal lymphocytes. It also led to a steeper dose-related decrease in the GSH content in CLL lymphocytes. A more detailed analysis shows that incubation of B-CLL lymphocytes in the presence of increasing concentrations of CLB resulted in a dose-dependent lowering of GSH, which became significant at 10 μg/mL (Fig 4). In contrast, CLB had a significantly lesser effect on lymphocytes from normal subjects in which even 50 μg/mL only reduced the GSH concentration by less than half. Increasing concentrations of CLB decreased viabilities in both CLL and normal lymphocytes (Table 4). CLL lymphocytes, however, were more susceptible to CLB, with viabilities of 76% and 47% compared with 92% and 75% for normal lymphocytes at the indicated doses of CLB.

The CLL B lymphocyte appeared to be more sensitive to GSH depletion by CLB than the normal B cell (Fig 5), although the difference was primarily due to the loss of GSH during the 20 hours of incubation. Intermediate sensitivity was noted in T cells from patients with B-CLL; B-cell contamination did not account for this finding. The

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**Table 4. Effect of CLB on Lymphocyte Viability**

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>CLB Concentration</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
<td>93 ± 1</td>
</tr>
<tr>
<td></td>
<td>10 μg/mL</td>
<td>92 ± 1*</td>
</tr>
<tr>
<td></td>
<td>50 μg/mL</td>
<td>75 ± 4t</td>
</tr>
<tr>
<td>(n = 14)</td>
<td>(n = 5)</td>
<td>(n = 12)</td>
</tr>
<tr>
<td>CLL</td>
<td>90 ± 1</td>
<td>76 ± 2*</td>
</tr>
<tr>
<td></td>
<td>50 μg/mL</td>
<td>47 ± 4t</td>
</tr>
<tr>
<td>(n = 24)</td>
<td>(n = 24)</td>
<td>(n = 24)</td>
</tr>
</tbody>
</table>

Cells were incubated for 20 hours as described in Materials and Methods (% viability ± SEM).

*P < .01.

†P < .001.

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Fig 5. Effect of CLB concentrations (μg/mL) on GSH content of T- and B-lymphocyte subsets from normal (NL) donors and patients with CLL. Results expressed as mean ± SE. The incubation time was 20 hours. (□) CLB 0 μg/mL; (■) CLB 10 μg/mL; (■) CLB 50 μg/mL.
GSH content of cells from two patients with T-CLL (CD4 or CD8) showed only a moderate decrease after incubation (Fig 6). The GSH in cells from a patient with CD8 CLL was particularly stable. The viability of these lymphocytes after exposure to the high CLB concentrations of 25 and 50 µg/mL was comparable to that of lymphocytes from normal subjects.

**DISCUSSION**

Our data showed a marked decrease in the cellular GSH content of B lymphocytes from patients with B-CLL after 20 hours of incubation in vitro. This was not observed with the T cells from patients with this disorder or with either B or T cells from normal subjects. The decrease was not caused by the interaction between the nuleophilic sulfhydryl group with proteins or with other macromolecules to form mixed disulfides, because a similar low level of mixed disulfide formation was noted in CLL and normal lymphocytes. The activities of the GSH biosynthesis enzymes were higher in CLL B cells than in normal B cells. GSH export differences provided no explanation for the GSH loss in B-CLL lymphocytes. A decreased γ-glutamyl transpeptidase activity in CLL B lymphocytes has been reported.27 Because this enzyme may be important for the conservation of GSH by reuse of the cysteine moiety, it is conceivable that the lack of GGT in CLL B lymphocytes may play a significant role in the observed GSH instability.

CLB, which is rapidly taken up by CLL lymphocytes,28 is useful in the therapy of B-CLL and other B-cell disorders. In contrast, it is generally ineffective in T-cell neoplasms. We have presented evidence that CLB contributed to the depletion of GSH in B-CLL lymphocytes. The present studies do not elucidate the mechanisms for the GSH depletion of B-CLL lymphocytes upon in vitro incubation. The significance of the in vitro results is that they mirror clinical responses. Normal T cells were very resistant to the effect of CLB on viability and GSH stability. Cells from two patients with T-CLL were studied, with one patient's cells yielding identical results on four different occasions. Even a high concentration of CLB had little effect on cell viability. These studies show that the leukemic T lymphocytes share neither the sensitivity to CLB nor the GSH depletion of the CLL B cell. Clinically, T-CLL is generally unresponsive to CLB therapy.

In an earlier report, GSH levels did not differ in lymphocytes from patients resistant to CLB than from those in untreated patients with CLL. The effect of CLB on GSH stability was not investigated.24

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