Transport, Metabolism, and DNA Interaction of Melphalan in Lymphocytes from Patients with Chronic Lymphocytic Leukemia

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ABSTRACT

We investigated the transport of [chloroethyl-14C]melphalan with lymphocytes from three groups of patients with chronic lymphocytic leukemia (untreated, treated sensitive, and treated resistant). There was no significant difference in the $K_m$ or $V_{max}$ of melphalan transport in lymphocytes from the three groups. In addition, there were no significant differences in intracellular melphalan levels after a 35-min incubation with 5.4 $\mu$m melphalan among the three groups. There was no evidence of intracellular metabolism of melphalan to dihydroxymelphalan except in lymphocytes from one treated sensitive patient. DL-2-Aminobicyclo[2,2,1]heptane-2-carboxylic acid, a specific analogue of the sodium-independent leucine-prefering amino acid transport system, inhibited the uptake of melphalan to a greater extent in lymphocytes from resistant patients than in those of untreated patients. Glutathione levels were not significantly different in lymphocytes from resistant patients as compared to those of untreated patients. The percentage of DNA cross-links as determined by an ethidium bromide fluorescence assay was 2-5-fold greater in lymphocytes from untreated patients than in those of resistant patients. These results suggest that resistance to the nitrogen mustards in patients with chronic lymphocytic leukemia is secondary to neither a transport defect nor alteration in intracellular melphalan levels but rather due to some other mechanism responsible for decreased DNA cross-links.

INTRODUCTION

The nitrogen mustards are an important group of alkylating agents with activity against several human tumors (1-4). Many of the nitrogen mustard analogues enter cells by carrier-mediated transport systems and alkylate DNA, RNA, and proteins (5-7). It has been suggested that the alkylation of DNA and, more specifically, the cross-linking of DNA correlates with cytotoxicity (8,9). However, resistance to the nitrogen mustards does not necessarily imply resistance to the chloroethynitrosoareas, another group of bifunctional alkylating agents that cross-link DNA (10).

Resistance to the nitrogen mustards in human and murine tumor cell lines has been correlated with (a) an alteration in the transport of these agents (11), (b) cytoplasmic metabolism of the chloroethyl alkylating moiety to the inactive hydroxyethyl derivative associated with an increase in the intracellular GSH concentration (12), or (c) alterations in the kinetics of DNA cross-links formed by these agents (9). Recently, resistance to the nitrogen mustards with a human ovarian cell line has also been reported to be associated with an increase in GSH concentration. Reduction of the GSH levels decreased the resistance of the ovarian cancer cell line to alkylating agents (13). Buthionine sulfoximine which can decrease GSH levels is now being utilized in vivo to sensitize tumor cells to MLN (14). Increased GSH levels may also be involved in quenching DNA:cis-platinum monoadducts in resistant L1210 cells (15). Buthionine sulfoxide treatment can reduce elevated GSH levels and restore sensitivity to cis-platinum (16). We have reported the partial metabolism of MLN to the inactive dihydroxymelphalan in breast cancer cells isolated from a patient resistant to multiple combinations of chemotherapy (17). The precise mechanism(s) responsible for the development of resistance to the nitrogen mustards in humans is (are) not known.

We have previously described the cytotoxicity and transport of MLN in human bone marrow cells obtained from normal subjects and adenocarcinoma cells isolated from pleural or peritoneal effusions (17). BCH, a specific substrate of the L (leucine-prefering) neutral amino acid system significantly reduced MLN uptake into the human bone marrow cells but did not alter the uptake of MLN into human tumor cells obtained from previously untreated patients. In contrast, BCH significantly inhibited the uptake of MLN into the tumor cells obtained from the one patient that had been extensively pretreated with chemotherapy. The results of our study were for the most part in agreement with previous investigations utilizing tumor cell lines. MLN is transported by the neutral amino acid transport systems L and/or ACS in these cell lines. However, BCH partially suppressed MLN uptake in all the tumor cell lines (18-21). Therefore, there may be minor but significant differences of MLN transport into freshly obtained human cells as compared to cell lines. Our preliminary results with freshly obtained human cells were difficult to interpret because the cell population was not homogeneous (the effusions contained 50-90% malignant cells).

In order to further elucidate the transport of MLN into freshly obtained human tumor cells and the mechanism of resistance to the nitrogen mustards in man, we studied the uptake, metabolism, and DNA cross-link formation of MLN in lymphocytes obtained from patients with CLL. Patients with CLL were chosen because a homogeneous population of abnormal cells is readily obtained and the majority of these patients respond well to treatment with one of the nitrogen mustards.

MATERIALS AND METHODS

Materials. Unlabeled and [chloroethyl-14C]MLNs (10.9 mCi/mmol) were kindly supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Silver Spring, MD (NSC 8806). Unlabeled MLN solutions were prepared daily in 75% ethyl alcohol containing an equimolar concentration of hydrochloric acid. Further dilutions were made in aqueous medium immediately prior to use to minimize hydrolysis. Radiolabeled MLN was dissolved in absolute ethanol and stored at -20°C. Thin-layer chromatography revealed that the drug was stable for weeks at this temperature.

Bovine serum albumin was obtained as serum fraction V from Miles Laboratories (Elkhart, IN). Phosphate-buffered saline (Dulbecco’s modification) and preservative-free heparin were purchased from...
of ethidium bromide. The DNA in the resultant lysates was denatured by heating at 100°C for 5 min and rapidly cooled and 0.4 mM EDTA (pH 12.0). The ethidium bromide solution was a solution containing ethidium bromide (10 pg/ml), 20 mM K2HPO4, ethidium bromide fluorescence assay as described previously (23, 24). Corrected for the 2-component interaction by the equations derived by the Lineweaver-Burk plot (1/v versus 1/s). A similar Km result was determined in quadruplicate. The Km and Vm were calculated from the sample and this percentage did not differ significantly among the three groups of patients' lymphocytes. Nonspecific adsorption of labeled melphalan was estimated by layering 200 μl of cells (1.2 × 10⁶/ml) onto 200 μl of media containing [chloroethyl-14C]melphalan followed by immediate centrifugation as described above. Kinetic studies were performed utilizing 2, 4, 6, 9, 12, 20, 40, 60, and 100 μM melphalan. The uptake at 1 min after drug addition was determined in quadruplicate. The Km and Vmax were calculated from the Lineweaver-Burk plot (1/v versus 1/s). A similar Km result was obtained with platelet melphalan and melphalan. With some of the drugs, the kinetic parameters were best described by biphasic curves and were corrected for the 2-component interaction by the equations derived by Neal (22). Detection of DNA Cross-Links. The lymphocytes at 2 × 10⁶ cells/ml were incubated at 37°C with unlabeled MLN or vehicle for 35 min in PAG. The cell suspensions were then washed once with PAG and resuspended in an equivalent volume of PAG for a 4-h incubation at 37°C to allow for development of DNA cross-links as described previously (8). The original samples were frozen in 10% dimethyl sulfoxide in liquid nitrogen until analysis. However, in the five patients that had detailed concentration-dependent DNA cross-link analysis, the samples were processed without delay. Cross-linking of DNA by melphalan was detected by utilizing an ethidium bromide fluorescence assay as described previously (23, 24). Forty μl of cells (7 × 10⁶ cells) were added to 200 μl of a lysing solution [4 mM NaCl, 50 mM KHPO4, 10 mM EDTA, and 0.1% (w/v) Sarkosyl (pH 7.2)]. Twenty μl of heat-inactivated bovine pancreas RNase (2 mg/ml) were added to the lysates which were then incubated at 37°C for 16 h. Following the incubation, 25 μl of heparin (500 IU/ml) were added for 20 min at 37°C. Each of the resulting lysates was added to 3 ml of a solution containing ethidium bromide (10 μg/ml), 20 mM K3HPO4, and 0.4 mM EDTA (pH 12.0). The ethidium bromide solution was placed in test tubes wrapped in aluminum foil to prevent light-induced cleavage of DNA by ethidium bromide. The DNA in the resulting lysates was denatured by heating at 100°C for 5 min and rapidly cooled to 22°C. Fluorescence was measured in 1-cm² cuvets at 22°C in a SLM-Aminco spectrofluorometer. The excitation wavelength was 525 nm and the emission wavelength was 580 nm. Fluorescence was measured prior to and after denaturation. The percentage of cross-linked DNA was determined by measuring the difference in fluorescence of denatured control cell lysates and the denatured MLN-treated samples by the formula

\[ C_+ = \frac{F_+ - F_-}{1 - F_-} \times 100\% \]

where C+ is the percentage interstrand cross-linked DNA in treated cells; F+ is the fluorescence after denaturation divided by fluorescence before denaturation of treated cells; and F− is the fluorescence after denaturation divided by fluorescence before denaturation of control cells. Utilization of protease K (0.5 mg/ml) instead of heparin did not significantly alter the percentage of DNA cross-links formed. Intracellular GSH Concentrations. Intracellular GSH concentrations were determined in the following manner. Lymphocytes (10⁶ cells) were lysed in 900 μl of H2O by vortexing for 5 min, and 100 μl of 30% sulfosalicylic acid were then added. The resulting suspension was left at 0°C for 15 min and then centrifuged at 12,000 × g for 2 min. The supernatant was stored at −20°C until analysis. Total GSH content was assayed by the glutathione reductase assay (25). Patients. All patients had a diagnosis of CLL or the leukemic phase of small cleaved cell lymphoma (patients 15 and 19). We classified the patients into 3 groups: (a) untreated patients, the vast majority of whom will respond to one of the nitrogen mustards if and when they are treated; (b) treated sensitive patients, those who were or are being treated with chlorambucil (4–6 mg daily) and where peripheral lymphocyte count returned to ≤15,000/μl; and (c) treated resistant patients, those who were treated with chlorambucil (4–6 mg daily) or intermittent high dose cyclophosphamide for at least 3 months and failed to have a 25–30% reduction in their peripheral lymphocyte count (all these patients had a peripheral lymphocyte count ≥30,000/μl after treatment). We did not test the lymphocytes for in vitro resistance. In the treated sensitive group of patients, patients 8, 10, 11, and 13 were receiving chlorambucil when their lymphocytes were obtained (patient 8 was studied again after chlorambucil treatment was stopped). Patients 9 and 12 had stopped chlorambucil 18 and 3 months prior to being studied, respectively. In the treated resistant group, patients 14, 17, 20, and 21 were still receiving chlorambucil at the time their lymphocytes were studied (patients 20 and 21 were restudied in detail for DNA cross-links within 3 months of stopping chlorambucil). Patient 15 had stopped chlorambucil and was receiving no therapy for 1 month. Patients 18 and 19 were not receiving alkylating therapy for 5 and 2 months, respectively, prior to the study. Patient 16 was receiving cyclophosphamide at the time of the study. Statistics. Potential differences among the 3 groups were determined by analysis of variance with intergroup comparisons by least significant differences (26). The unequal t test was utilized for comparison of GSH levels.

RESULTS

Transport and Metabolism of [chloroethyl-14C]MLN at 37°C in Lymphocytes. The CLL patients were divided into three groups as described in “Materials and Methods” (untreated, treated sensitive, and treated resistant). The kinetic parameters of [chloroethyl-14C]MLN transport at 37°C into lymphocytes from these patients are shown in Table 1. Analysis of the Km and Vmax of MLN transport failed to indicate a significant difference among the groups. There were no significant differences in the intracellular MLN concentration following a 35-min incubation with 5.4 μM MLN in PAG (an amino acid-free medium) among the groups. Chromatographic analysis of the cellular samples obtained after a 35-min incubation with 5.4 μM radiolabeled MLN in...
PAG revealed that lymphocytes from only 1 of 21 patients were partially metabolizing MLN to dihydroxymelphalan (patient 8). This patient was receiving chlorambucil at the time of the original analysis. After the drug had been discontinued for 1 month, the patient's lymphocytes were again studied and they were no longer metabolizing MLN. Furthermore, approximately 70% of the total radioactivity associated with each group of CLL patients was greater than 70% of control (1 of 7 patients was less than 70% of control) from treated resistant patients as compared to untreated patients. In addition, concentration-dependent analysis of CV development with lymphocytes from 5 patients revealed that a logarithmic curve best described this relationship (Figs. 2 and 3). Furthermore, the slope of the curve clearly revealed that a logarithmic curve best described this relationship.

Inhibition of [chloroethyl-14C]MLN Transport by Amino Acids

The kinetic parameters of [chloroethyl-14C]MLN transport and the GSH levels in lymphocytes from each group of CLL patients are shown. The K_m and V_max were determined following exposure to various concentrations of MLN for 1 min in PAG (an amino acid-free medium) at 37°C. The pmol of MLN/10^6 cells was determined after a 35-min incubation in PAG at 37°C with 5.4 μM MLN. GSH levels were determined as described in "Materials and Methods."

Table 1 MLN transport and GSH levels in lymphocytes from CLL patients

| Patient | K_m (μM) | V_max (pmol/10^6 cells/min) | pmol of MLN/10^6 cells at 35 min | GSH levels (nmol/10^6 cells)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>10</td>
<td>2.3</td>
<td>5.1</td>
<td>0.73</td>
</tr>
<tr>
<td>Patient 2</td>
<td>0.8/24&quot; (24)</td>
<td>0.03/10 (9.9)</td>
<td>7.9</td>
<td>0.90</td>
</tr>
<tr>
<td>Patient 3</td>
<td>80</td>
<td>12</td>
<td>6.5</td>
<td>0.94</td>
</tr>
<tr>
<td>Patient 4</td>
<td>95</td>
<td>18</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Patient 5</td>
<td>4/177 (105)</td>
<td>0.23/25 (22)</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>Patient 6</td>
<td>4</td>
<td>2.0</td>
<td>5.5</td>
<td>0.63</td>
</tr>
<tr>
<td>Patient 7</td>
<td>22</td>
<td>5.5</td>
<td>7.2</td>
<td>1.12</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>49 ± 16</td>
<td>10 ± 2.9</td>
<td>6.4 ± 0.5</td>
<td>0.86 ± 0.09</td>
</tr>
</tbody>
</table>

Table 2 Inhibition of MLN uptake by amino acids

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>BCH suppressibility</th>
<th>L-Threonine suppressibility</th>
<th>L-Leucine suppressibility</th>
<th>L-Glutamine suppressibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes from</td>
<td>1 min</td>
<td>3 min</td>
<td>1 min</td>
<td>3 min</td>
</tr>
<tr>
<td>Untreated CLL patients</td>
<td>77 ± 4.8</td>
<td>92 ± 6.7</td>
<td>67 ± 11</td>
<td>80 ± 7.7</td>
</tr>
<tr>
<td>Treated sensitive</td>
<td>69 ± 19</td>
<td>64 ± 14</td>
<td>60 ± 12</td>
<td>58 ± 7.1</td>
</tr>
<tr>
<td>Treated resistant</td>
<td>54 ± 16</td>
<td>59 ± 7.1</td>
<td>49 ± 12</td>
<td>46 ± 7.8</td>
</tr>
</tbody>
</table>

* Values represent percentage of control ± SE.

Table 1 demonstrates that there was no significant difference in GSH levels in the lymphocytes from resistant patients [1.13 ± 0.16 (SE) nmol/10^6 cells] as compared to lymphocytes from untreated patients [0.86 ± 0.09 nmol/10^6 cells].

DNA Cross-Links. An ethidium bromide fluorescence assay was utilized to determine the percentage of interstrand DNA cross-links (C_v) as described previously (23, 24). Lymphocytes were incubated at 37°C and then for 4 h in drug-free medium to allow for formation of DNA cross-links as noted previously (8). Fig. 1 demonstrates that in the presence of 5.0 μM MLN, lymphocytes from untreated patients developed a significantly (P < 0.05) greater percentage of cross-links than those from resistant patients. In addition, concentration-dependent analysis of C_v development with lymphocytes from 5 patients revealed that a logarithmic curve best described this relationship (Figs. 2 and 3). Furthermore, the slope of the curve clearly separated the lymphocytes of the 3 untreated patients (≥2.7) from those of the 2 resistant patients (≤1.6). The concentration of MLN necessary to produce a C_v of 5% was <25 μM in the lymphocytes of untreated patients in contrast to >60 μM for those of the 2 resistant patients.
RESISTANCE TO NITROGEN MUSTARDS IN CHRONIC LYMPHOCYTIC LEUKEMIA

Fig. 1. Percentage of DNA cross-links ($C_1$) formed following a 35-min incubation of 5.0 $\mu$M melphalan and a 4-h drug-free incubation in PAG at 37°C with lymphocytes isolated from each patient of the 3 groups. Shaded area, area occupied by the mean ± SE.

Fig. 2. Percentage of DNA cross-links ($C_1$) formed after a 35-min incubation with various concentrations of melphalan and a 4-h drug-free incubation at 37°C with lymphocytes in PAG from untreated patients. The curves generated by these points fit best with a log curve. Points, mean of quadruplicate assays; bars, SE. Upper left, patient 2 ($y = -2.9 + 2.7 \times 10^1$; $r = 0.86$); upper right, patient 7 ($y = -3.8 + 2.7 \times 10^1$; $r = 0.99$); lower, patient 3 ($y = -1.9 + 3.1 \times 10^1$; $r = 0.97$).

Fig. 3. Percentage of DNA cross-links ($C_1$) formed after a 35-min incubation with various concentrations of melphalan and a 4-h drug-free incubation at 37°C with lymphocytes in PAG from resistant patients. The curve generated by these points fits best with a log curve. The assay was done in quadruplicate for each value. Points, mean of duplicate experiments; bars, SE. Right, patient 20 ($y = -1.5 + 1.6 \times 10^1$; $r = 0.99$); left, patient 21 ($y = -0.3 + 1.3 \times 10^1$; $r = 0.96$).

DISCUSSION

It appears that the development of resistance in CLL patients to chlorambucil, a nitrogen mustard which enters cells by passive diffusion (27), does not induce a transport defect for MLN uptake into lymphocytes isolated from these patients. Also, there was no evidence of metabolism of MLN to inactive dihydrooxyzemphanal in the lymphocytes of resistant patients after a 35-min incubation. It is possible that metabolism of MLN would have been detected during a 3-h incubation as described previously (12). However, we utilized a 35-min incubation in order to evaluate drug accumulation, metabolism, and DNA cross-links under the same conditions. There were no significant differences in the GSH levels in lymphocytes of untreated patients as compared to those of treated resistant patients.

The percentage of DNA cross-links following a 35-min incubation of 5.0 $\mu$M MLN in amino acid-free medium at 37°C was significantly greater in the lymphocytes of untreated patients as compared to those of resistant patients. The detailed concentration-dependent analysis of DNA cross-links in 5 patients confirmed that DNA cross-links were decreased in lymphocytes from resistant CLL patients as compared to those of untreated patients. Since during the 35-min incubation neither a transport defect for MLN nor increased metabolism of MLN was detected, the decrease in DNA cross-links in resistant lymphocytes may be secondary to decreased formation or increased removal of these DNA cross-links as described previously (9). However, other possible mechanisms include decreased nuclear transport of MLN or decreased initial alkylation of DNA.

Our observations on the BCH-suppressible component of MLN transport support our previous investigation (17). BCH inhibited the transport of MLN in lymphocytes from resistant patients to a greater extent than those from untreated patients. Vistica (19) has reported that MLN is transported by 2 separate L-neutral amino acid systems in L1210 leukemia cells (a BCH-sensitive, sodium-independent L system and a BCH-insensitive sodium-dependent L system). Our results suggest that the BCH-sensitive L system is more active in the transport of MLN in lymphocytes from resistant patients as compared to those from untreated patients. These results could have interesting implications for a new nitrogen mustard analogue that has a high affinity for this transport system (28).

In summary, our observations suggest that the development of resistance to the nitrogen mustards in CLL patients is neither the result of a transport defect nor secondary to differences in the intracellular MLN levels. The resistant state in these patients appears to be characterized by an increased component of MLN transport by the BCH-sensitive L system and, most importantly, a decrease in the percentage of DNA cross-links in their lymphocytes. Further investigations will be necessary to determine the precise mechanism responsible for the decrease in DNA cross-links in the lymphocytes from resistant patients.

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