Lack of Drug-induced DNA Cross-Links in Chlorambucil-resistant Chinese Hamster Ovary Cells

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ABSTRACT

Chlorambucil (CLB) is an alkylating agent commonly used in the treatment of several neoplastic disorders. The mechanisms underlying resistance to this drug are not fully defined. We used the DNA alkali elution technique to study cross-link formation in the wild type (Kl) and a CLB-resistant (ChlR) Chinese hamster ovary cell line. [14C]CLB was used to measure drug uptake. The CLB-resistant cells were found to have negligible DNA cross-link formation compared to Kl cells at all time points tested. There was a correlation between the resistance to CLB and the decreased ability of resistant cells to form DNA cross-links. Results of drug uptake experiments excluded altered CLB accumulation as the basis for these findings. Assays of O6-alkylguanine transferase and topoisomerase II provide evidence against a role of these enzymes in CLB resistance. These studies suggest that the mechanism of CLB cytotoxicity involves the formation of DNA cross-links. Reduced cross-link formation may confer resistance to CLB.

INTRODUCTION

CLB is a bifunctional alkylating agent commonly used in the treatment of several neoplastic disorders, including ovarian carcinoma and chronic lymphocytic leukemia (1, 2). Based on results with other alkylating drugs such as nitrogen mustard, busulfan, and cis-diaminedichloroplatinum, it has been suggested that chlorambucil exerts its antineoplastic effect by binding to DNA in target cells (3–5). The mechanisms by which tumors become insensitive to this agent are not fully understood, and the development of drug resistance in patients treated with chlorambucil remains a serious clinical problem.

Recently, Robson et al. (6) developed a CHO cell line with a 20-fold relative resistance to chlorambucil. In the present report, we show the correlation between the formation of CLB-induced DNA lesions and drug sensitivity in CHO cells. The results indicate that drug transport, O6-alkylguanine DNA transferase, and topoisomerase II do not appear to play a role in conferring resistance to CLB.

MATERIALS AND METHODS

Cell Culture. Two CHO cell lines, Kl (parental) and ChlR (chlorambucil-resistant) were kindly provided by Dr. Ian Hickson. The cells were maintained in continuous culture in Ham's F-10 medium as described (6).

Measurement of Drug Sensitivity. The clonogenic assay of Robson et al. (6) was used. Exponentially growing cells were harvested by trypsinization and seeded into 8.5-cm culture dishes, at a cell density of 2000 in 10 ml of medium per dish. After a 4-h incubation to allow cell attachment, CLB was added and the cells were incubated for an additional 24 h. Each dish was washed twice with 10 ml of cold PBS, fresh medium was added, and the incubation continued for 10 days. Colonies consisting of 50 or more cells were counted after fixation and staining with crystal violet as described (6).

Measurement of DNA Damage. The DNA alkaline elution technique (7) was used. Cells were grown with [14C]thymidine for 24 h, and in medium without label for another 6 h. After a 1-h exposure to CLB (16.5 or 33 µM), cells were incubated for 6 h in drug-free medium.

Cells were then harvested, resuspended in ice-cold medium, and irradiated with 300 rads at 4°C. Three × 10^6 cells were layered on polyvinyl chloride filters with a pore size of 2 µm (Millipore or Omega). Similar results were obtained with filters from either source.

Each filter was washed with 5 ml ice-cold PBS, after which cells were lysed with 5 ml of Sarksosyl-EDTA (pH 10.0). After the lysing solution was eluted, 5 ml of 2% SDS containing 0.5 mg/ml proteinase K were added to selected chambers, and allowed to drip through by gravity. Fifty-five ml of tetratrapylammonium hydroxide elution buffer (pH 12.1) were then added to each chamber. Elution was carried out at 2.3 ml/h. Five-mI fractions were collected and each chamber was purged with 10 ml of 0.4 N NaOH after removal of the filter. The DNA remaining on each filter was solubilized by incubating in 0.4 ml of 1.0 N HCl for 1 h at 60°C. Following the addition of 2.5 ml of 0.4 N NaOH, the radioactivity in each sample was determined. Results were expressed as fraction of total radioactivity remaining on the filter at each time point and DNA cross-links quantitated (7).

Drug Uptake. [14C]CLB labeled in the chloroethyl groups was obtained from the National Cancer Institute and purified by high-pressure liquid chromatography as follows. The drug was dissolved in acetone-trif- tarate and injected into a Spectra-Physics 8000 high-pressure liquid chromatography apparatus equipped with a CI4 analytical column. Isocratic elution was with acetonitrile:water:acetic acid at 375:125:1 (v/v/v). To measure drug uptake, cells were incubated with [14C]CLB at a 33 µM drug concentration under conditions identical to those used for alkaline elution. At each time point, medium was removed, and each dish was rinsed 3 times with ice-cold PBS (10 ml). Three ml of 0.3 N KOH were then added and, after incubation overnight at room temperature, 2 ml were removed for counting and the remainder of the lysate was used for protein determination (8). The amount of cell-associated CLB was determined from a calculation of drug-specific activity in the starting medium corrected for trapped medium as measured by [H]inulin.

O6-Alkylguanine Transference and Topoisomerase II Assays. O6-Alkylguanine transferase activity was measured according to the method of Myrnes et al. (9). For the topoisomerase II assay the immunoblot analysis by using rabbit antiserum against human topoisomerase II was done with minor modifications as described previously (10). Briefly, logarithmically growing Kl and ChlR cells were lysed with SDS sample buffer (10% glycerol, 5% β-mercaptoethanol, 3% SDS, 62.5 mm Tris, pH 6.8). Following boiling at 100°C for 3 min, protein in cell lysates (approximately 2 × 10^6 cell equivalents/lane) was analyzed by a SDS-polyacrylamide electrophoresis in 7.5% gels and electroblotted onto nitrocellulose filters. The filters were immunoblotted with antiserum against human DNA topoisomerase II and stained with [3H]-labeled protein A.

Cell Cycle Analysis. Cell cycle analysis was performed as previously described (10).
RESULTS

Drug-induced Cytotoxicity. The results of a typical colony formation assay are shown in Fig. 1. The CH1R cell subline was 20-fold more resistant to CLB relative to the parental K1 cells, as determined by the dose required to reduce cell survival to 37% of control values (6). These data agree closely with the results of Robson et al. (6) described for nitrogen mustard in these cells.

Drug-induced DNA Cross-Links. The elution pattern of DNA from both types of cells is shown in Fig. 2. The elution rate for DNA from irradiated drug-treated sensitive cells was strikingly slower than that from resistant cells showing that DNA cross-links were formed in the sensitive but not in the resistant line. At no point was a significant number of lesions observed in the ChlR cells (Fig. 3). These results strongly suggest that the production of DNA cross-links correlates closely with drug sensitivity. Furthermore, the absence of cross-links at any time point in the resistant cells points to lack of formation, rather than accelerated excision of lesions as the underlying cause.

CLB Transport. Drug accumulation by K1 and ChlR cells incubated with [14C]CLB under conditions identical to those used for alkaline elution is shown in Fig. 4. Since no significant difference was observed between the two cell types, altered drug uptake is not a factor in the reduced DNA cross-linking in the resistant line. Drug accumulation was determined at early time points during incubation with [14C]CLB because the amount of cell-associated radioactivity remaining after transfer to drug-free medium decreased rapidly during subsequent incubation.

CLB toxicity was compared in multidrug-sensitive and -resistant KB cells (11) (KB-3-1 and KB-VI). Similar killing curves were observed, indicating no difference in sensitivity toward CLB.

O6-Alkylguanine Transferase Assay. O6-Alkylguanine transferase has been implicated in the repair of alkylating drug-induced DNA lesions in several systems (12). Both cell lines had barely detectable levels of this enzyme activity, suggesting that the transferase is not involved in the mechanism of CLB resistance (data not shown).

Topoisomerase II Content. A recent report showed a correlation between resistance to nitrogen mustard and elevated topoisomerase II levels in a human cell line (13). In contrast, immunoblots of cell lysates performed with an antibody raised against HeLa topoisomerase II showed no difference in the amount of enzyme protein between K1 and ChlR cells (Fig. 5). To determine if the cell cycle could have any effect on the topoisomerase II levels, cell cycle profiles of both cell types were obtained. Their similarity is shown in Table 1. Topoisomerase II content in synchronized K1 and ChlR cells was measured at multiple time points and was found not to vary through the late G1, S, G2, and M phases of the cell cycle (data not shown).

DISCUSSION

The correlation found between formation of DNA cross-links and sensitivity to CLB indicates that the cytotoxicity of this agent stems from the formation of these lesions. This concept is consistent with results obtained in these and in other cell types, with several other alkylating compounds, including melphalan, nitrogen mustard, cis-diaminedichloroplatinum, the...
antisera against human topoisomerase II was performed as described in “Materials and Methods.”

Fig. 5. DNA topoisomerase II in Kl and ChiR cells. Immunoblot analysis of the protein in whole cell lysates (2 × 10^5 cell equivalents/lane) using rabbit antisera against human topoisomerase II was performed as described in “Materials and Methods.” Lane A, Kl cells; Lane B, ChiR cells.

Table 1 Cell cycle analysis

Exponentially growing cells were stained with propidium iodide as described in “Materials and Methods” and were analyzed by flow cytometry. The percentage of cells in each phase of the cell cycle is shown.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>G1</th>
<th>S</th>
<th>G2 + M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kl</td>
<td>31</td>
<td>41</td>
<td>28</td>
</tr>
<tr>
<td>ChiR</td>
<td>36.8</td>
<td>29.5</td>
<td>33.7</td>
</tr>
</tbody>
</table>

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nitrosoureas, and cyclophosphamide (3, 14–17). The rationale for testing each alkylating drug individually and limiting conclusions to this agent is based on many earlier studies. For example, cellular uptake of nitrogen mustard occurs by active transport, melphalan by carrier-mediated diffusion, and CLB by passive diffusion (18–20). In a study of lipophilicity and alkylating activity differences were found among CLB, phenylacetic mustard, melphalan, and mitoclomine (21).

Various mechanisms have been found to be the basis of in vitro insensitivity to alkylating agents. Altered cellular drug accumulation has been implicated in several systems (18, 22). As seen in Fig. 4, drug uptake was not substantially different between the two cell lines and therefore it is not the cause of resistance to CLB. This is in keeping with the observations in other cell types that CLB influx and efflux occur by passive diffusion (19, 20, 23). The mdr gene, which confers the multidrug resistance phenotype by coding for a glycoprotein which reduces intracellular drug concentration (11), was found not to be associated with CLB efflux. In a cytotoxicity assay, KB cells genetically engineered to express the mdr gene had the same sensitivity to CLB as their wild-type counterparts.

Another mechanism of alkylating agent resistance is based on the ability to repair DNA damage caused by these drugs. The enzyme O6-alkylguanine transferase has been shown to be involved in the excision of toxic DNA-drug adducts in cells exposed to nitrosoureas, thereby conferring decreased sensitivity to this group of agents (12, 24). Both cell types contained similar low levels of this enzyme activity. It is therefore unlikely that CLB resistance is related to this enzyme. We have recently reported the occurrence of purine-CLB adducts in chronic lymphocytic leukemia lymphocytes (23), but the precise chemical nature of these adducts has not been established.

It has recently been proposed that topoisomerase II may play a role in resistance to nitrogen mustard, presumably by facilitating repair of damaged DNA (13). Increased levels of P4 DNA unknotting activity, as well as of topoisomerase II mRNA transcripts were observed in Raji cells selected for resistance to nitrogen mustard (25, 26). In contrast, we observed no difference in the amount of immunoreactive enzyme protein between the Kl and ChiR lines (Fig. 5), assayed throughout the cell cycle. The reason for the difference between our results and those reported by Tan et al. (25, 26) is not clear. It may be due to the different assays interspecies variation between the cell lines (i.e., human versus rodent) used, or the drugs (nitrogen mustard versus CLB) in the two studies.

There is abundant evidence for a relationship between intracellular molecules containing sulfhydryl groups and resistance to alkylating agents. Increased levels of metallothionein, a low-molecular-weight protein with a large number of cysteine residues, has been linked to chlorambucil resistance (27, 28). Several reports have established the connection between increased levels of glutathione, as well as elevated glutathione-S-transferase activity and alkylating agent insensitivity (14, 15, 29–31). Chlorambucil cytotoxicity may be modulated by these compounds through the direct interaction with sulfhydryl groups which could prevent the drug from reaching the DNA target or by quenching CLB-DNA monoadducts, thereby avoiding cross-linking (30). Further studies are needed to delineate these interrelationships.

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