Isolation and Characterization of a Chinese Hamster Ovary Cell Line Resistant to Bifunctional Nitrogen Mustards

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ABSTRACT

A drug-resistant derivative of a Chinese hamster ovary cell line has been generated by chronic exposure to progressively higher concentrations of chlorambucil. The cells exhibit greater than 20-fold resistance to the cytotoxic effects of chlorambucil and comparable levels of cross-resistance to mechlorethamine and melphalan. These drugs all belong to a class of bifunctional alkylating agents which generate DNA cross-links by reaction at the N-7 position of guanine. However, no resistance is observed to several other drugs which possess a similar mechanism of action, to cis-platinum diamine dichloride or to bischloroethylnitrosourea and mitomycin C, which cross-link DNA via the O6 position of guanine. Lack of resistance to vincristine, colchicine, or Adriamycin coupled with the failure of the calcium channel blocker verapamil to reverse the phenotype, indicates that the mechanism of resistance is distinct from that characterized by the multidrug-resistant phenotype. Support for this view comes from the finding that no significant alteration in melphalan uptake could be demonstrated. The phenotype is very stable and has been maintained during 12 months of continuous culture without further selection. A slightly elevated basal level of glutathione is present in the resistant cells, but resistance is not overcome by depletion of intracellular glutathione with buthionine sulfoximine. A cytosolic protein with a molecular weight of approximately 25,000 is constitutively overexpressed in the resistant cells. Although these cells have an abnormal karyotype, no conclusive evidence for any cytogenetic indicator of gene amplification could be detected.

INTRODUCTION

One of the major drawbacks to effective cancer chemotherapy is the emergence of tumor cell subpopulations with intrinsic or acquired resistance to anticancer drugs. A common clinical observation is that tumor cells which acquire resistance to a particular anticancer agent also exhibit resistance to a range of other drugs which may be structurally or functionally unrelated.

In vitro studies implicate alterations in intracellular drug accumulation with the "so-called" multidrug resistance phenotype. An increasing level of cellular resistance to agents such as the Vinca alkaloids, colchicine, or the anthracyclines is accompanied by increased expression of a family of high molecular weight membrane glycoproteins (1-6). This appears to be a common mechanism for acquired drug resistance in several different human and rodent cell lines.

Although this mechanism has been reported to be responsible for induced cellular resistance to some alkylating agents, such as melphalan and mitomycin C (4), other potential mechanisms exist for mediating alkylating agent resistance, including increased efficiency of DNA repair, or enhanced intracellular drug detoxification. For example, cell lines selected on the basis of resistance to certain DNA-damaging agents, such as the chloronitrosoureas, appear not to be cross-resistant to other drugs unless they generate adducts recognized by a common DNA repair pathway (7-9). Conversely, the Walker carcinoma cell line, which exhibits resistance to bifunctional nitrogen mustards, is collaterally sensitive to nitrosoureas (10), despite the fact that both these classes of agent generate DNA interstrand cross-links.

Although chlorambucil is widely used in the curative therapy of Hodgkin's disease (11), in modified cyclophosphamide-methotrexate-5-fluorouracil protocols for breast cancer (12), in ovarian cancer (13), and in small cell lung cancer (14), cell lines isolated on the basis of chlorambucil resistance have rarely been reported (15).

Here, we describe the isolation of a CHO cell line which exhibits elevated levels of resistance to the cytotoxic effects of chlorambucil and appears to exhibit a novel profile of drug resistance. The cells are cross-resistant only to a strictly limited number of bifunctional alkylating agents. Acquired resistance has been accompanied by marked overexpression of a cytosolic protein with a molecular weight of approximately 25,000. Wild-type levels of sensitivity to Adriamycin, vincristine, and colchicine have been maintained, suggesting that an alternative mechanism to the membrane glycoprotein-mediated multidrug-resistant phenotype is responsible for the observed drug resistance of these cells.

MATERIALS AND METHODS

Cell Culture and Media. Cells were maintained as described previously (16).

Isolation of a Chlorambucil-resistant Cell Line. The chlorambucil-resistant cell line was selected by growing cells for 3 months in the presence of progressively higher concentrations of chlorambucil. Cells were exposed to chlorambucil for 3-4 days before subculturing and growing in drug-free medium for 24 h. Following this, the drug treatment was repeated but at a higher dose. The final concentration of chlorambucil used was 50 µg/ml. This culture was plated for single colonies which were overlaid with medium containing 0.4% Noble agar. After 2 days, when the cells were growing up into the agar, they were transferred onto the surface of an agar plate (0.5% agar in medium). Single colonies of cells that grew were dispersed into liquid medium and expanded into large scale cultures.

Survival Curves. Survival determinations were performed as described previously (16). Treatments with cytotoxic agents were as outlined below.

Radiation. Exposure to UV or X-rays was as described previously (16).

Drug Treatments. The DNA-damaging drugs were generally purchased from Sigma. BCNU and busulfan were from Dr. Narayanam, National Cancer Institute, Bethesda, MD, and Professor Fox, Christie Hospital, Manchester, United Kingdom, respectively. The activated cyclophosphamide and ifosfamide were from Dr. Chapman, Boehringer, Ingelheim, Federal Republic of Germany.

Drug storage conditions were as described previously (16), with the following additional. CdCl2 was dissolved in H2O prior to use and stored at 4°C. Mechlorethamine, thiotepa, melphalan, BSO, verapamil, and colchicine were prepared freshly in H2O. 4-Hydroxyperoxycyclophosphamide and 4-hydroxyperoxycyclophosphamide were freshly prepared in serum-free medium. Etoposide was dissolved in dimethyl sulfoxide and stored at 4°C. Busulfan was freshly prepared in dimethyl sulfoxide.

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1 Supported by the North of England Cancer Research Campaign.
2 To whom requests for reprints should be addressed.

3 The abbreviations used are: CHO, Chinese hamster ovary; BCNU, bischloroethyl nitrosourea; cis-Pt, cis-platinum diamine dichloride; BSO, buthionine sulfoximine; D20, dose required to reduce cell survival to 37% of control values.
NITROGEN MUSTARD-RESISTANT CHO CELLS

Normally, cells were exposed to a drug for 24 h before being washed with phosphate-buffered saline and returned to fresh growth medium. The exceptions were mechlorethamine and BCNU, with 0.5-h exposures, and cis-Pt, for which a 2-h exposure was used. The cells were then incubated at 37°C until colonies visible by eye developed (up to 12 days). These were fixed in methanol-acetic acid (3:1), stained with crystal violet (400 µg/ml), and counted. Colonies containing more than 50 cells were considered survivors.

Verapamil was included in selected experiments at a concentration of 100 µM. This represented the maximum tolerated dose before verapamil toxicity was observed. Cells were simultaneously exposed to verapamil and either chlorambucil or melphalan for 24 h.

For BSO treatments, cells were incubated for 8 h in medium containing 25 µM BSO. Following this, mechlorethamine was added and incubation was continued for a further 0.5 h, after which the cells were returned to drug-free medium.

Each point on a survival curve represents the average of at least 3 independent experiments. In every case, wild-type CHO-K1 cells were treated in parallel. The D37 represents the average dose required to kill a cell. The D37 values were estimated directly from the survival curves.

Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis was carried out essentially as described by O’Farrell (17), with the exception that isoelectric focusing gels were loaded at the anode. Polyacrylamide slab gels (9%) were used for the second dimension.

Following electrophoresis, the gels were soaked in 10% acetic acid for 15 min, dried onto Watman No. 3MM paper, and exposed to Kodak DEF-59 film.

Gel samples were prepared by labeling approximately 5 x 10^5 cells with 10 µCi [35S]methionine for 2 h. The cells were then harvested, washed with phosphate-buffered saline, and resuspended in sample buffer (0.8 g Nonidet P-40, 0.5 ml 2-mercaptoethanol, 2.0 ml pH 3.5–10 ampholines, 5.5 g urea, and H2O to 10 ml). Approximately 20 µg of soluble protein were loaded onto each gel.

Melphalan Uptake. Accumulation of [3H]melphalan was measured essentially as described by Bates et al. (18).

Glutathione Levels. Determination of intracellular glutathione levels was kindly performed by Dr. A. Hall using the method of Griffith (19).

RESULTS AND DISCUSSION

Following the selection procedure described in “Materials and Methods,” a clonal population of cells, designated CHO-Ch1, was recovered and tested for resistance to a range of DNA-damaging agents and anticancer drugs.

Fig. 1 shows the survival response to chlorambucil of CHO-Ch1 cells and wild-type CHO-K1 cells. A comparison of D37 values indicates that the CHO-Ch1 cell line is greater than 20-fold resistant to the cytotoxic effects of chlorambucil. CHO-Ch1 cells were also tested for resistance to mechlorethamine (Fig. 2) and to melphalan (Fig. 3) and were found to be approximately 34-fold and 14-fold resistant, respectively. In addition, mild cross-resistance (2-fold) to 4-OH hydroxyurea was evident, but no significant resistance to radiation, mitomycin C, hydroxyperoxycyclophosphamide, BCNU, vincristine, colchicine, ethylmethane sulfonate, CdCl2, thiotepa, cis-Pt, busulfan, Adriamycin, or etoposide was observed (data not shown).

This pattern of resistance differs from that of another chlorambucil-resistant cell line, the Walker carcinoma, which is cross-resistant to a much wider range of bifunctional alkylating agents, including cis-Pt and mitomycin C (10). Although a number of other cell lines previously isolated do show limited phenotypic similarities to CHO-Ch1 cells (7, 15, 20–24), none exhibits an identical profile of cross-resistance (Table 1).

CHO-Ch1 cells were grown for 12 months in continuous culture without reexposure to chlorambucil and then retested for drug resistance. No changes in drug resistance levels were observed (data not shown).

Cross-resistance to melphalan has been described in the multidrug-resistant phenotype resistant phenotype (25). However, CHO-Ch1 cells show no resistance to a number of agents generally associated with this phenotype, such as Adriamycin, colchicine, and vincristine. Also, the calcium channel blocker verapamil is usually able to reverse the phenotype of multidrug-resistant cells (26), but in CHO-Ch1 cells verapamil produced only the same low level of potentiation of melphalan and...
NITROGEN MUSTARD-RESISTANT CHO CELLS

A 4-fold dose enhancement of mechlorethamine toxicity was achieved in our wild-type cells following depletion of intracellular glutathione levels by treatment with BSO (Table 2). The concentration of BSO used was the highest which could be tolerated by the CHO cells before the toxicity of the BSO contributed significantly to cell survival. This level of BSO depleted glutathione levels in both lines by approximately 80% (data not shown). Although CHO-Chl1 cells were sensitized around 1.5-fold more than wild-type cells, they were nonetheless approximately 25-fold more resistant than CHO-K1 cells to the combined cytotoxic effects of mechlorethamine and BSO.

The differential effect of BSO-mediated glutathione depletion is probably a consequence of a slightly (approximately 1.8-fold) elevated basal glutathione level in CHO-Chl1 cells (Table 4).

Because this CHO cell line shows wild-type resistance to X-rays, Adriamycin, and BCNU, BSO does not reverse the resistant phenotype, and basal glutathione levels are similar to those of wild-type, the mechanism of resistance is unlikely to be solely a consequence of an alteration in intracellular glutathione levels.

Metallothioneins are ubiquitous low molecular weight proteins with an unusually high cysteine content (30 mol%) (29). They can bind cadmium, zinc, and copper; and are induced by these metals, and resistance to cadmium can be mediated by gene amplification of metallothioneins (30). Resistance to chlorambucil and melphalan has also been associated with zinc pretreatment (31, 32). Since these CHO cells are not resistant to cadmium, metallothionein induction is unlikely to cause such a high degree of drug resistance.

All of the drugs to which this cell line is resistant produce DNA intrastrand cross-links, and in some cases interstrand cross-links, via N-7-guanine (33). If this cell line was more proficient at repairing N-7-guanine monoadducts or cross-links than the wild-type CHO cell line, it may be expected that the cells would also be resistant to busulfan, cis-Pt, and thiotaope and almost as resistant to oxazaphosphorines as to aniline mustards. However, these cells are not cross-resistant to busulfan, cis-Pt, or thiotaope. The range of lesions produced by these compounds does, however, differ markedly from the mustards (34–39). All of these N-7 binding compounds may have different repair pathways or different lethal lesions. The critical lesions are unlikely to be monoadducts, since the CHO cell line was not cross-resistant to monofunctional alkylating agents.

Lack of cross-resistance to mitomycin C and BCNU could

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Table 1: Cross-resistance patterns of 12 cell lines to alkylating agents

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Initial selective agent</th>
<th>Chlorambucil</th>
<th>Melphalan</th>
<th>Mechlorethamine</th>
<th>4-OOH CP</th>
<th>4-OOH IP</th>
<th>cis-Pt</th>
<th>BCNU</th>
<th>Thiotaope</th>
<th>Other agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO (this paper)</td>
<td>Chlorambucil</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>MMS&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>L1210 (20)</td>
<td>4-OOH CP</td>
<td>CS</td>
<td>CS</td>
<td>CS</td>
<td>R</td>
<td>R</td>
<td>CS</td>
<td>CS</td>
<td>CS</td>
<td>X-ray&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>L1210 (21)</td>
<td>4-OOH CP</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td></td>
<td></td>
<td>Adriamycin&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>L1210 (7)</td>
<td>Melphalan</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td></td>
<td>R</td>
<td>X-ray&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2080 Me (22)</td>
<td>Melphalan</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td></td>
<td>R</td>
<td>Adriamycin&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Raji 1 (23)</td>
<td>Mechlorethamine</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>Raji 1 (23)</td>
<td>Mechlorethamine</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raji 1 (23)</td>
<td>BCU</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walker (15)</td>
<td>Chlorambucil</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>CS</td>
<td>R</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>P388 (20)</td>
<td>4-OOH CP</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td></td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>Human SSC25 (23)</td>
<td>cis-Pt</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td></td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>Human melanoma  (24)</td>
<td>Melphalan</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td></td>
<td>S</td>
<td>MMS&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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* 4-OOH CP, 4-hydroxyperoxyisocyanamide; 4-OOH IP, 4-hydroxyperoxyisothiocyanamide; MMS, methylmethanesulfonate.

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Fig. 3. Survival of wild-type CHO-K1 (O) and CHO-Chl1 (•) cells after melphalan. Points, mean of 3 independent experiments.
be expected because of the cross-linking via O'-guanine in the case of these two drugs (40, 41) and because resistance to BCNU may be mediated by the specific repair enzyme O'-methylguanine methyltransferase (42).

To investigate whether increasing cellular resistance was accompanied by major alterations in the level of synthesis of specific proteins, 2-dimensional gel electrophoresis of [35S]-methionine-labeled polypeptides was undertaken. The results (Fig. 4) show that CHO-Chl cell synthesis greatly elevated levels of a polypeptide with a molecular weight of approximately 25,000 with an isoelectric point around 7.5. This level of enhanced protein synthesis was not dependent upon continued exposure of CHO-Chl cells to chlorambucil. This single major difference in the protein gel patterns for the 2 lines is also seen on Coomassie blue-stained gels (data not shown).

The 25,000 polypeptide does not appear to be a nuclear protein as it was absent from preparations of nuclei from CHO-Chl cells isolated by a number of different methods (data not shown). Based on its cellular location and isoelectric point, the possibility that this protein is a glutathione transferase is currently under investigation.

There are many different glutathione transferase isoenzymes which participate in a wide variety of intracellular reactions involving detoxification of drugs and carcinogens. If one specific transferase had increased in activity in CHO-Chl cells then the pattern of cross-resistance to alkylating agents and other drugs such as Adriamycin would depend on its substrate specificity. This has not yet been defined with regard to cytotoxic drugs for any glutathione transferase. There has been a report of a chlorambucil-resistant line in which glutathione transferase activity was elevated (43). Also, an MCF-7 cell line resistant to Adriamycin was shown to have a manifold increase in glutathione transferase but was not nearly as cross-resistant to alkylating agents (44).

**Table 2** Effects of BSO and verapamil on drug resistance

<table>
<thead>
<tr>
<th>Drug</th>
<th>CHO-K1 (µg/ml)</th>
<th>CHO-Chl (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechlorethamine</td>
<td>0.059</td>
<td>2.05</td>
</tr>
<tr>
<td>Mechlorethamine + BSO</td>
<td>0.014</td>
<td>0.33</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>3.3</td>
<td>80</td>
</tr>
<tr>
<td>Chlorambucil + verapamil</td>
<td>1.8</td>
<td>30</td>
</tr>
<tr>
<td>Melphalan</td>
<td>0.65</td>
<td>8.65</td>
</tr>
<tr>
<td>Melphalan + verapamil</td>
<td>0.33</td>
<td>3.10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug</th>
<th>CHO-K1 (µg/ml)</th>
<th>CHO-Chl (µg/ml)</th>
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<tr>
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<td>30</td>
</tr>
<tr>
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<td>0.65</td>
<td>8.65</td>
</tr>
<tr>
<td>Melphalan + verapamil</td>
<td>0.33</td>
<td>3.10</td>
</tr>
</tbody>
</table>

*Mean ± SE of 3 separate experiments. Cells (10⁷/ml) in suspension were exposed to [35S]-melphalan for the times indicated. The reaction was terminated by the addition of ice-cold phosphate-buffered saline-1% bovine serum albumin, the cells were washed 3 times with the same buffer, and the pellet was solubilized in 1% sodium dodecyl sulfate before scintillation counting.

**Table 3** Comparison of intracellular melphalan accumulation in wild-type and CHO-Chl cells

<table>
<thead>
<tr>
<th>Extracellular melphalan</th>
<th>Intracellular melphalan level (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (µM)</td>
<td>CHO-K1</td>
</tr>
<tr>
<td>15.4</td>
<td>0.19 ± 0.06*</td>
</tr>
<tr>
<td>15.4</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>15.4</td>
<td>0.34 ± 0.06</td>
</tr>
<tr>
<td>15.4</td>
<td>0.41 ± 0.08</td>
</tr>
<tr>
<td>15.4</td>
<td>0.35 ± 0.07</td>
</tr>
<tr>
<td>15.4</td>
<td>0.33 ± 0.08</td>
</tr>
<tr>
<td>15.4</td>
<td>0.79 ± 0.11</td>
</tr>
<tr>
<td>15.4</td>
<td>0.74 ± 0.12</td>
</tr>
</tbody>
</table>

*Mean ± SE of at least 3 independent assays.

Karyotype analysis on CHO-Chl cells (kindly performed by C. Ockey) failed to reveal conclusive evidence for cytogenic indicators of gene amplification, such as double minutes or homogeneous staining regions. However, it did reveal that CHO-Chl cells have an abnormal karyotype with a modal chromosome number of 29 (compared to 22 for parental cells). While this additional genetic information appears to have resulted largely from chromosome rearrangements, there does not appear to be evidence of significant heterogeneity among the population (data not shown).

Since glutathione transferases are inducible and up to a 26-fold increase in mRNA for a transferase has been reported (45), it may be unnecessary for gene amplification to occur in order to produce the level of resistance seen here. It is possible that a genetic rearrangement has constitutively activated an inducible protein, in a way similar to that of c-myc activation in lymphomas. Alternatively, the extra genetic information may simply code for extra copies of a glutathione transferase gene.

The pattern described in this CHO cell line is compatible with many clinical observations of alkylating agent resistance. Chlorambucil is commonly used as a single agent in ovarian cancer treatment, and cis-Pt is an effective second line agent (46). In multiple myeloma, patients relapsing or resistant to melphalan have responded to cyclophosphamide (47, 48). In addition, the cell line is stable, as is clinical resistance.

Considering the heterogeneity already described in different cell lines (Table 1) and the variety of mechanisms discussed above, it is clear that there are many "multidrug-resistant phenotypes" to alkylating agents. As these mechanisms are elucidated, it should be possible to biochemically profile a
relapsed tumor to select appropriate alkylating agent therapy (e.g., by measuring metallothionein, specific glutathione transferase isoenzymes, glutathione levels). This should also allow specific pharmacological antagonists to be developed.

ACKNOWLEDGMENTS

We thank Dr. Narayan for the gift of BCNU, Professor Fox for busulfan, Dr. Chapman for activated cyclophosphamide and ifosfamide, and Dr. Ockey for performing the karyotype analysis. We also thank Dr. Hall for carrying out the glutathione assay and Dr. Haugwitz, National Cancer Institute, for the radiolabeled melphalan.

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