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REDUCED TOPOISOMERASE II AND ELEVATED α CLASS GLUTATHIONE *S*-TRANSFERASE EXPRESSION IN A MULTIDRUG RESISTANT CHO CELL LINE HIGHLY CROSS-RESISTANT TO MITOMYCIN C

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Abstract—We have isolated a multidrug-resistant derivative of Chinese hamster ovary CHO-K1 cells by exposure to progressively increasing concentrations of Adriamycin®. This cell line, designated CHO-Adr^r, was 27-fold more resistant than the parental line to Adriamycin and showed similar degrees of cross-resistance to several other topoisomerase II (topo II) inhibitors, including mitoxantrone, daunomycin and etoposide. CHO-Adr^r cells showed a lower (4-fold) level of cross-resistance to vincristine and colchicine, drugs associated with the multidrug-resistant phenotype. While CHO-Adr^r cells showed no enhanced resistance to several mono- and bi-functional alkylating agents or to UV and ionizing radiation, they were greater than 80-fold resistant to mitomycin C (MMC). There was a 5-fold decreased level of daunomycin accumulation in CHO-Adr^r cells compared to CHO-K1 cells and this was associated with increased drug efflux. The resistant cells had amplified multidrug resistance gene (*mdr*) sequences and overexpressed (*mdr*) mRNA. Verapamil was able to completely reverse Adriamycin resistance but reversal of MMC resistance was only partial, with residual 23-fold resistance. CHO-Adr^r cells expressed a 4-fold reduced level of topo II protein but overexpressed an α class (basic) glutathione *S*-transferase (GST). Analysis of cell hybrids showed that while the level of resistance to Adriamycin dropped by a factor of 3 in CHO-K1/CHO-Adr^r hybrids compared to CHO-Adr^r/CHO-Adr^r hybrids, resistance to MMC dropped 10-fold. Thus, CHO-Adr^r cells appear to exhibit simultaneously several different drug resistance mechanisms including MDR and GST overexpression, and topo II reduction.

Many common solid tumours respond only partially to chemotherapy and intrinsic or acquired resistance is a major problem preventing effective treatment. Resistance commonly occurs simultaneously to multiple classes of drugs, particularly antibiotics and *Vinca* alkaloids. The major mechanism observed for pleiotropic drug registance is via increased expression of P-glycoprotein, a membrane protein that appears to act as an energy-dependent efflux pump [1, 2]. Most cell lines that develop this mechanism of drug resistance show variable degrees of cross-resistance to other natural products or antibiotics. One reason for this has been shown recently to be the development of point mutations within the multidrug resistance (*mdr*‖) gene, which codes for the P-glycoprotein. Such mutations, caused the preferential development of resistance to colchicine, the selecting agent, in human KB cells [3].

A number of alternative mechanisms of resistance to anticancer agents have been described. In particular, the GSTs have been implicated in acquired resistance to alkylating agents, Adriamycin® and cisplatin [4–6]. There are three major classes of GST in man (α, μ and π), each with a different patten of preferred substrates [7]. They appear to act via conjugation of cytotoxic compounds to the major non-protein thiol compound, GSH.

Another key target for several drugs is the nuclear enzyme, topo II. The majority of drugs which interact with topo II, e.g. Adriamycin, VP16, *m*-AMSA, mitoxantrone and ellipticine [8–10] are also transported by the P-glycoprotein. Mutants with reduced levels of topo II or with abnormal enzyme activity are generally cross-resistant to this range of intercalating agents and epipodophyllotoxins [11–15].

Because relatively few resistant cell lines have been examined for all these potential contributing mechanisms, we have undertaken a detailed characterization of a multidrug-resistant CHO cell (CHO-Adr^r) selected by exposure to Adriamycin. Our aim was to correlate the pattern of cross-resistance in CHO-Adr^r cells with expression of the different potential resistance mechanisms. In contrast to 'classical' multidrug-resistant cells, CHO-Adr^r cells showed an extremely high level of resistance to MMC.

MMC is one of the few chemotherapeutic drugs with which responses are seen in colorectal and gastric carcinoma, and which has activity in squamous

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‖ Abbreviations: GST, glutathione *S*-transferase; topo II, topoisomerase II; MMC, mitomycin C; MDR, multidrug resistance; CHO, Chinese hamster ovary; VP16, etoposide; DCMMC, decarbamoyl mitomycin C; PBS, phosphate-buffered saline; *mdr*, multidrug resistance (gene); *m*-AMSA, amsacrine; GSH, glutathione; BSA, bovine serum albumin.

lung cancer [16] and in breast cancer patients pretreated with several other classes of anticancer drugs [17], including anthracyclines. However, responses are frequently limited in duration. This spectrum of activity, together with the general lack of cross-resistance to other drugs, suggests that mechanisms of resistance to MMC may differ from those for many other antitumour antibiotics, in particular those associated with the MDR phenotype.

Since clinical patterns of resistance are often more complex than those seen with increased expression of the *mdr* gene alone, investigation of multiple mechanisms is important for the rational development of strategies to circumvent resistance to multiple drugs.

MATERIALS AND METHODS

Cell culture and media. Cells were maintained as described previously [18, 19].

Isolation of CHO-Adr^r cells. The Adriamycin-resistant line was selected by growing CHO-K1 cells for 3 months in the presence of progressively higher concentrations of Adriamycin. Cells were exposed to Adriamycin for 3–4 days before subculturing and growing in drug-free medium for 24 hr. Following this, the drug treatment was repeated at a higher dose. The final concentration of Adriamycin used was 400 ng/mL. Resistant cells were cloned twice and expanded into bulk cultures. A subline, designated CHO-Adr^r, was taken for further study.

Survival curves. Survival determinations were carried out as described previously using a clonogenic assay [18]. Plating efficiencies were typically 85% for CHO-K1 cells and 70% for CHO-Adr^r cells. Treatment with cytotoxic agents was as described previously [18, 19]. Where indicated, verapamil was present at the stated concentration during the 24 hr exposure to a cytotoxic drug.

The D_{37} value of the dose of drug required to reduce cell survival to 37% of control.

Construction of cell hybrids. Cell hybrids were constructed as described previously [20]. Briefly, one or other of the dominant selectable markers *gpt* or *neo* was introduced into each cell line by transfection [21] using pSV5*gpt* and pSV5*neo*, respectively. The resultant mycophenolic acid- and G418-resistant transfectants were then mixed and fused using polyethylene glycol (6000), essentially as described by Davidson and Gerald [22]. Hybrids were selected in medium containing both mycophenolic acid and G418. Flow cytometry was used to confirm that hybrid populations were tetraploid.

Drug accumulation. Accumulation of [^{3}H]daunomycin was measured essentially by the method of Bates *et al.* [23].

Drug efflux. Efflux was measured by a modification of the method of Giavazzi *et al.* [24]. Exponentially growing cells were harvested into 10 mL PBS containing 0.02% EDTA. The cells were centrifuged and resuspended at a density of 10^7/mL in PBS containing 1% BSA and 10 mM sodium azide. The cells were incubated for 1 hr at 37° in the presence of 7.9 μM [^{3}H]daunomycin, after which 4 mL of ice-cold wash buffer (PBS/1% BSA) were added. The cells were centrifuged at 1500 rpm for 5 min, washed twice in ice-cold buffer, and the pellets resuspended in 1 mL wash buffer supplemented with 10 mM glucose. The cell suspensions were then incubated with shaking at 37°. At fixed times, 100-μL aliquots (10^6 cells) were removed and added to 4 mL ice-cold wash buffer. The cells were centrifuged at 1500 rpm for 5 min and the pellets solubilized in 1 mL 1% SDS before scintillation counting. The amount of drug retained in the cell in pmol was calculated and expressed as % drug retained (sp. act. 2.1 μCi/nmol).

Immunoblotting. Nuclear extracts, prepared by the method of Glisson *et al.* [14] and equalized for protein content were electrophoresed on an 8% polyacrylamide gel and transferred to a nitrocellulose membrane by electrophoretic blotting. The nitrocellulose was then washed in TTBS (50 mM Tris–HCl, pH 7.9, 150 mM NaCl, 0.05% Tween 20) containing 3% BSA for 16 hr. The membrane was incubated at room temperature for 24 hr with rabbit antiserum against calf thymus topo II (kindly supplied by Dr L. F. Liu, The Johns Hopkins University, Baltimore), which was diluted 1:3000 in TTBS with 1% gelatin before extensive washing with TTBS. Immunocomplexes were reacted with ^{125}I-labeled protein A (in TTBS plus 1% gelatin) before washing and autoradiography. To confirm that loadings were comparable, a duplicate set of identically loaded gel lanes was stained with Coomassie blue.

For the GSTs, rabbit antiserum to human acidic, basic or neutral GSTs was used at a 1:1000 dilution.

Topo II assay. Topo II activity was measured by a specific filter binding assay, essentially as described previously [25].

DNA extraction. High molecular weight was prepared essentially by the procedure of Maniatis *et al.* [26].

RNA extraction. RNA was prepared from cells by guanidium thiocyanate extraction. A human *mdr*1 cDNA clone was obtained from Dr P. Borst. The probe consisted of the 3 kb insert from the clone HepG2-26 and was radiolabeled by the random priming method.

DNA hybridization. Restricted DNA (10 μg) was electrophoresed in 0.8% agarose and transferred to nitrocellulose by the method of Southern [27]. Filters were prehybridized at 65° for 6 hr in a solution of 6 × SSC, 5 × Denhardts, 0.5% SDS and 100 μg/mL denatured salmon sperm DNA. Following prehybridization, the solution was replaced with fresh prehybridization solution containing 50 ng of radiolabeled probe (sp. act. up to 1×10^8 dpm/μg) and the filter hybridized for 18 hr at 65°. Filters were washed with 2 × SSC/0.1% SDS for 1 hr at 65° and then with 1 × SSC/0.1% SDS for 30 min at 65°, and exposed to X-ray film at −70°.

RNA hybridization. Total RNA (15 μg) was denatured by glyoxalation, electrophoresed through a 1.2% agarose gel and transferred to nitrocellulose as described previously [27]. Filters were prehybridized at 42° in a solution cointain 5 × SSPE, 50% formamide, 5 × Denhardt's, 0.5% SDS and 100 μg/mL denatured salmon sperm DNA for 6 hr. The solution was replaced with prehybridization

Table 1. Degree of drug resistance in CHO-Adr^r cells

Drug	Resistance factor
Adriamycin	27
Mitoxantrone	30
Daunomycin	27
Actinomycin D	15
VP16	12
m-AMSA	3
Vincristine	4
Colchicine	4
MMC	84
DCMMC	61*
BMY 25282	41
MMS	1
EMS	1
Cisplatin	1
BCNU	1
X-rays	1
UV light	1

Resistance factors are based on D_{37} values for CHO-Adr^r cells relative to those of CHO-K1 cells.
* Based on D_{60} values.

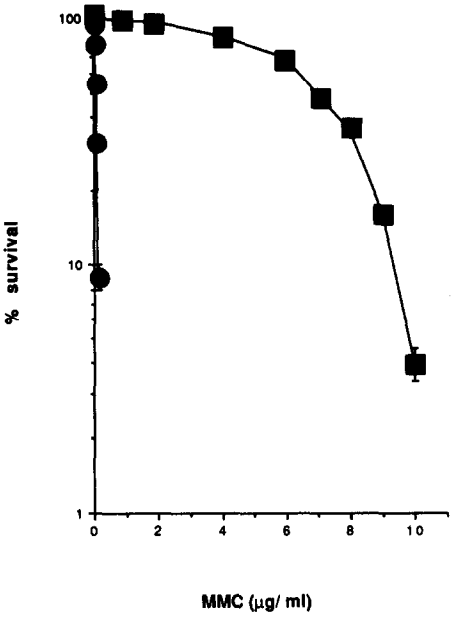

Fig. 1. Survival of parental CHO-K1 (●) and CHO-Adr^r (■) cells following a 24-hr exposure to MMC. Points represent the means of three independent experiments. Bars, SE.

buffer containing 25 ng radiolabeled probe (sp. act. up to 1×10^8 dpm/μg) and hybridization continued for 18 hr at 42°. Filters were then washed in 2 × SSPE 0.1% SDS for 40 min at 65° with one change. The solution was then replaced with 1 × SSPE, 0.1% SDS and washing and washing continued for 1 hr at 65°. Filters were then exposed to X-ray film at −70°.

RESULTS

Cross-resistance to DNA damaging drugs

Following the above selection procedure, a clonal population of Adriamycin-resistant cells was isolated (designated CHO-Adr^r cells) and its resistance to other cytotoxic drugs compared with that of the parental CHO-K1 cell line (Table 1). Resistance to the topo II-specific drug, *m*-AMSA, was 3-fold. CHO-Adr^r were 27-fold resistant to Adriamycin, based on D_{37} values. Cross-resistance was observed to vincristine and colchicine in CHO-Adr^r cells, a characteristic of typical multidrug-resistant cell lines. However, the degree of resistance exhibited (3–4-fold), was somewhat lower relative to Adriamycin than is normally observed in MDR cell lines. There was a more marked degree of resistance to a group of drugs which interact with topo II and which are transported by the P-glycoprotein including mitoxantrone, daunomycin, actinomycin D and VP16 (Table 1). An unusual feature of CHO-Adr^r cells was the degree of cross-resistance to MMC (Fig. 1). This was greater than 80-fold, based on D_{37} values (Table 1). A similar degree of resistance was found to be the monofunctional analogue, DCMMC (Table 1). Survival analyses following single low drug exposures gave similar levels of drug resistance in CHO-Adr^r cells compared to CHO-K1 cells as those seen following 24 hr exposures (Table 1).

Mechanism of resistance: MDR

To evaluate functional expression of the *mdr* genes, the transport of daunomycin was measured. There was a 5-fold decreased drug accumulation in CHO-Adr^r cells compared to CHO-K1 cells (Fig. 2). This appeared to be due to increased drug efflux. The time taken for 50% of the drug to efflux was 2 min in the resistant cells and 10 min in the wild type cells, i.e. a 5-fold difference (Fig. 3).

Southern blots probed with the human *mdr*1 gene showed an approximately 4-fold amplification of homologous hamster sequences in the CHO-Adr^r cell line compared to the parental cell (Fig. 4). There was also over-expression of *mdr* mRNA (Fig. 5). Immunocytochemistry showed much higher expression of P-glycoprotein in CHO-Adr using C219 antibody (gift from V. Ling, Toronto). Thus, gene amplification and overexpression of the Chinese hamster homologue of *mdr*1 presumably accounts for part of the observed cross-resistance pattern.

Reversal of resistance

The reversal of resistance by the calcium channel blocker verapamil is a common feature of multidrug-resistant cells [28]. A maximal concentration of verapamil that was non-toxic in the clonogenic assays was chosen to try to reverse completely the resistant phenotype of CHO-Adr^r cells. There was no difference in the sensitivity of wild type and resistant CHO cells to verapamil. Verapamil (100 μM) for 24 hr reversed Adriamycin and vincristine resistance to the level in parental cells (Table 2). There was also a substantial sensitization to both Adriamycin and vincristine in wild type CHO-K1 cells by

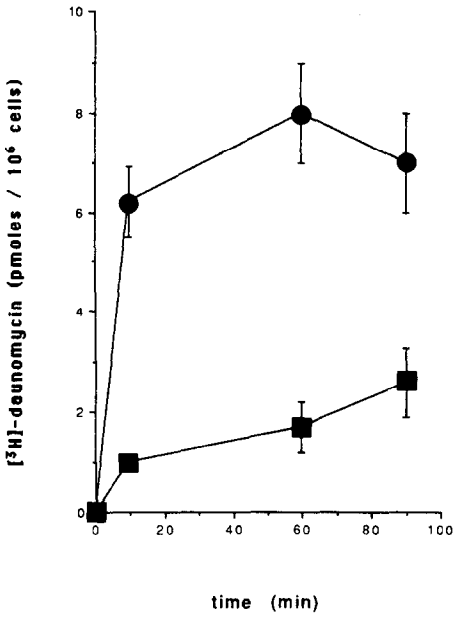

Fig. 2. Accumulation of [^{3}H]daunomycin by CHO-K1 (●) and CHO-Adr^r (■) cells. Cells in suspension (10^7/mL) were exposed to [^{3}H]daunomycin (7.9 μM) for the times indicated and then processed as described in Materials and Methods. Points represent means of three independent experiments. Bars, SE.

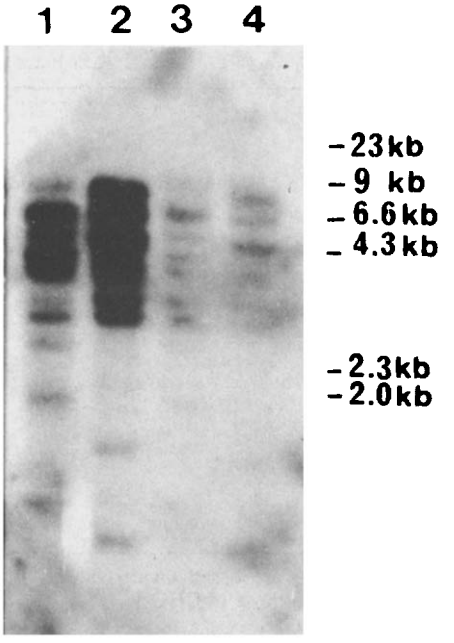

Fig. 4. Analysis of MDR sequences in CHO-Adr^r and CHO-K1 cells. Each lane contained 10 μg of digested genomic DNA: CHO-Adr^r DNA digested with *Eco*RI (Lane 1), CHO-Adr^r DNA digested with *Hind* III (Lane 2), CHO-K1 DNA digested with *Eco*RI (Lane 3), CHO-K1 DNA digested with *Hind* III (Lane 4). Equal loading of the DNA samples was confirmed by spectrophotometric readings and by ethidium bromide staining. DNA size markers of phage λ digested with *Hind* III are shown. Radiolabeled human MDR cDNA containing 3 kb of the 3′ end of the structural gene was used as probe.

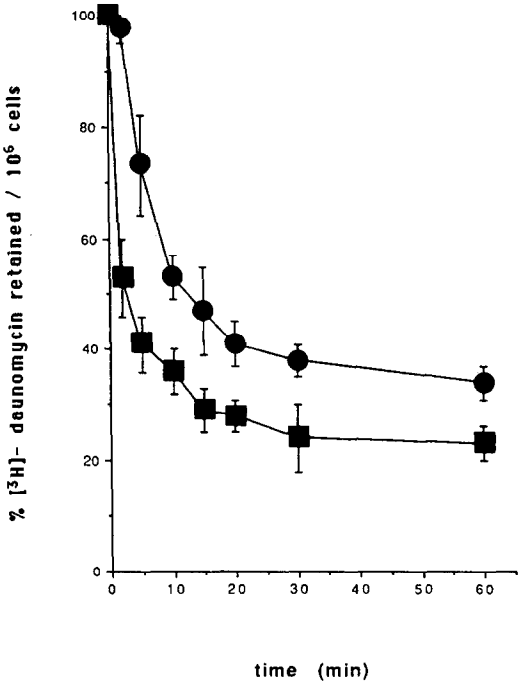

Fig. 3. Efflux of [^{3}H]daunomycin by CHO-K1 (●) and CHO-Adr^r (■) cells. Results are expressed as % of [^{3}H]-daunomycin retained in the cells as a function of time. Points represent means of three independent experiments. Bars, SE.

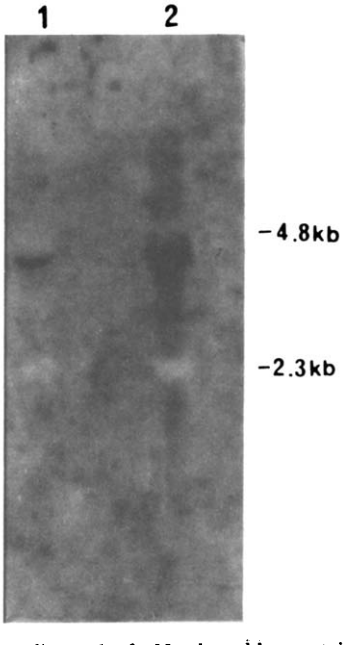

Fig. 5. Autoradiograph of a Northern blot containing 15 μg total RNA from CHO-K1 (Lane 1) and CHO-Adr^r (Lane 2) cells. The probe was that used in Fig. 4. The positions of the 28S (4.8 kb) and the 18S (2.3 kb) ribosomal RNA subunits visualized by endothelium bromide staining are shown.

Table 2. Effect of verapamil on drug resistance in CHO-Adr^r cells

Drug	Cell line	
	CHO-K1 (D_{60} ng/mL)	CHO-Adr^r (D_{37} ng/mL)
Adriamycin (ng/mL)	90	3600
+ 10 μM verapamil	28	126
+ 100 μM verapamil	9	12
MMC (ng/mL)	92	7850
+ 10 μM verapamil	89	745
+ 100 μM verapamil	21	525

Cells were co-incubated with a cytotoxic drug plus verapamil (at the concentrations stated) for 24 hr.

verapamil, consistent with a recent report of high basal expression of *mdr* in hamster cells compared with human cells [29]. Lower concentrations of verapamil (<10 μM) gave only a partial reversal of Adriamycin resistance in CHO-Adr^r cells (Table 2).

Although there was a marked effect of verapamil on MMC resistance, the CHO-Adr^r cells still showed 23-fold higher resistance to MMC in the presence of verapamil compared to the wild type cells under the same conditions (Table 2). This suggests that more than one mechanism may be responsible for MMC resistance in CHO-Adr^r cells. BMY 25282, a more easily activated analogue of MMC [30, 31], was tested for its ability to reverse the MMC resistance in CHO-Adr^r cells. BMY 25282 was 3-fold more toxic to the resistant cells than was MMC on a molar basis, but was also more toxic to CHO-K1 cells, with the result that CHO-Adr^r cells were still 41-fold more resistant than CHO-K1 cells to BMY 25282 (Table 1).

Mechanism of resistance: topo II

Apart from resistance to MMC, the most striking feature of CHO-Adr^r cells was resistance to drugs which interact with topo II. Cellular topo II levels were therefore quantified by Western blotting (Fig. 6). There was a 4-fold reduction in the expression of topo II in the resistant line, based on densitometric scanning of autoradiograms (three separate assays showed 3.4-, 3.9- and 4.3-fold differences). The cell line was only 3–4-fold resistant to *m*-AMSA, a lower level of resistance than to other topo II inhibitors. This difference in sensitivity is apparently due to the difference in level of topo II. The amount available for complexing with DNA and *m*-AMSA was assessed using a filter binding assay (Fig. 7). To produce similar covalent complex formation in wild type and resistant extracts, four times more *m*-AMSA was required in the latter case. Shown for comparison is increased trapping of topo II complexes in another CHO cell line that overexpresses topo II 3-fold. The ratio of covalent complex is proportioned to the ratio of topo II in the cell lines.

Mechanism of resistance: GSTs

Another potential mechanism of resistance to

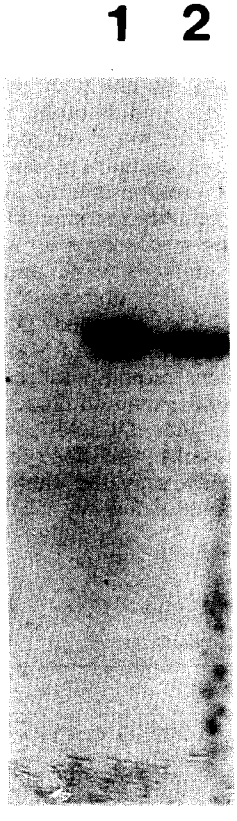

Fig. 6. Autoradiograph of a Western blot of nuclear extracts from CHO-K1 (Lane 1) and CHO-Adr^r (Lane 2) cells, using specific topo II antibodies.

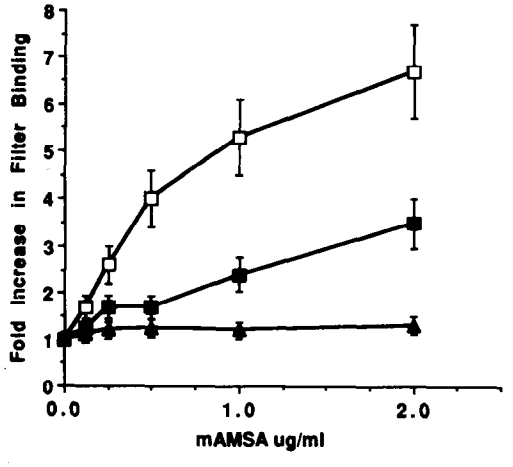

Fig. 7. Filter binding assay of topo II activity in CHO-Adr^r (▲), CHO-K1 (■) and a mutant that overexpresses topo II (Adr^1) (□).

Fig. 8. Autoradiograph of a Western blot of total cell extracts of CHO-K1 (Lane 1) and CHO-Adr^r (Lane 2) cells using specific antibodies to α-class GSTs.

Adriamycin is an increase in the GSH metabolizing system and/or the GSTs [5]. High GSH has also been associated with resistance to MMC [32]. Total GSH levels were very similar in the parental and resistant cells (data not shown). GST activity using the general substrate CDNB showed a small elevation (1.5-fold) in the resistant cells. Western blots showed no change in π- or μ-class GSTs, but the presence of a new α-class GST was observed in extracts from CHO-Adr^r cells (Fig. 8). Previous work has identified the two proteins cross-reacting with the antisera used in the experiment as the Ya-type (lower band) and the Yc-type (upper band) subunits. [33]. The expression of the Ya-type is apparently unchanged in CHO-Adr^r cells, but that of the Yc-type is elevated to a level equivalent to that of the Ya subunit.

Genetic analysis of Adriamycin and MMC resistance

CHO-Adr^r and wild type cells were fused and the resulting hybrids were compared with the appropriate self-cross hybrids for MMC and Adriamycin resistance (Table 3).

Adriamycin resistance was clearly dominant in the hybrids, as expected from the evidence of *mdr*1 gene amplification. Similarly, mitoxantrone and VP16 resistance were only partially reversed in the hybrids (data not shown). However, MMC resistance was decreased markedly in the CHO-K1/CHO-Adr^r hybrid (Table 3).

DISCUSSION

We have described the isolation of a multidrug-resistant CHO cell line by exposure to Adriamycin. The CHO-Adr^r cell line showed an atypical pattern of cross-resistance to other drugs, being unusually resistant to MMC but lacking high level resistance to vincristine and colchine. CHO-Adr^r cells exhibited an MDR phenotype with amplification of *mdr* gene sequences, overexpression of *mdr* mRNA and increased drug efflux. The resistance mechanism(s) for Adriamycin and actinomycin D was codominant as shown by cell fusion experiments.

The CHO-Adr^r cell line is unusual in its degree of cross-resistance to MMC since in most MDR lines cross-resistance to MMC is usually 10-fold less than to the selecting drug [1, 28]. MDR as the predominant mechanism of MMC resistance has been reported in a drug-resistant L1210 cell line selected with MMC [34]. Part of the MMC resistance in our line was probably due to the elevation of *mdr* gene expression (this seems to be an important mechanism in the wild type cells also), as shown by the ability of verapamil to markedly potentiate toxicity. However, there was substantial residual MMC resistance which was presumably independent of *mdr*. The majority of the MMC resistance was reversed on fusion with wild type cells suggesting that a recessive mechanism (unlike MDR) is responsible for much of this resistance. These genetic experiments are compatible with two resistance mechanisms for MMC, one not reversed by verapamil (recessive) and a component that is reversible (the dominant *mdr*-mediated mechanism). Neither enhanced DNA cross-link repair nor reduced activation of MMC seem likely mechanisms for this recessive aspect of resistance because of the similar marked degree of resistance to the MMC analogues, DCMMC, which does not form DNA cross-links, and BMY 25282 which is much more readily activated than MMC. It is possible that complete deficiency in a drug-activating

Table 3. Degree of resistance of cell hybrids to cytotoxic drugs

Cytotoxic drug	Cell hybrid		
	CHO-K1/CHO-K1	CHO-Adr^r/CHO-Adr^r	CHO-K1/CHO-Adr^r
Adriamycin	1	25	8
Actinomycin D	1	19	7
Mitomycin C	1	166	15

Degree of drug resistance is defined as the D_{37} value for a given hybrid divided by the D_{37} value for the parental CHO-K1/CHO-K1 hybrid.

system would give similar degrees of resistance to MMC and BMY 25282. However, in preliminary analyses we have found no difference in the ability of CHO-K1 and CHO-Adr^r cells to activate MMC. Moreover, the activity of two enzymes that may be involved in activating MMC (DT diaphorase and NADPH cytochrome P450 reductase) [35, 36] does not appear to be altered in CHO-Adr^r cells (unpublished results).

The high MMC resistance and partial reversal by verapamil could be due to a mutated *mdr* gene with high selectivity for MMC. Since there are two homologous domains in the P-glycoprotein [37, 38], one domain may favour one class of drugs, the second another. If there was a difference in the affinity of the two domains for verapamil, then one set of drugs could have its resistance reversed more readily. Another possibility is co-expression of different *mdr* genes. The selective reversal for one class of drugs has been shown both for *Vinca* alkaloids and for Adriamycin. In a human carcinoma cell line, Adriamycin and intercalator resistance was reversed, but not *Vinca* alkaloid or MMC resistance [39]. Conversely, in MDR human leukemia cell lines resistance to *Vinca* alkaloids but not Adriamycin was reversed [40].

The mechanism of action of MMC is complex and may involve redox cycling, free radical generation, DNA monoadducts, cross-links and strand breaks [41–44]. Changes in protective mechanisms such as those of the selenium-dependent GSH peroxidase [45] and metallothionein [46] may be involved. GSH itself can protect against MMC toxicity [33], but the level was not significantly elevated in CHO-Adr^r cells. Clearly, further work is required to identify the underlying basis for the unusually high level of MMC resistance in CHO-Adr^r cells.

A reduction in topo II has been demonstrated in other cell lines made resistant to topo II inhibitors, either in a single step or with repeated exposure [11–15, 47, 48]. Moreover, intrinsic sensitivity or resistance of different cell types has been related to the level of expression of topo II [42, 49]. In several cell lines showing marked degrees of resistance to topo II inhibitors there was only a 2-fold decrease in topo II [15]. In our cell line, the 5-fold decrease in drug accumulation and 4-fold decrease in topo II probably account for most, if not all, of the resistance to topo II inhibitors. In the case of *m*-AMSA, which does not appear to be transported via P-glycoprotein, the degree of resistance could be accounted for by the reduction in topo II alone. The complementation studies for Adriamycin resistance may be expected to show codominance of the topo II phenotype, based on reconstruction experiments *in vitro* with extracts from cells expressing high or low levels of topo [11]. The variation in degree of resistance between different topo II inhibitors could depend both on their interaction with topo II and on their affinity for the P-glycoprotein.

If a decrease in topo II level is important for resistance, it is interesting that high doses of verapamil reversed Adriamycin resistance completely. A possible explanation is related to the high basal level of MDR expression in CHO cells [29] and their tolerance of high verapamil doses. Although some MDR cell lines are hypersensitive to verapamil, these lines are much more resistant than is CHO-Adr^r [50, 51]. Suppression of basal as well as induced MDR in the resistant line may allow sufficient drug accumulation to overcome the problem of low topo II expression.

Previous studies have shown elevation of anionic (π-class) GST in MDR cells [15, 52], but there have been no reports of an elevation in an α-class GST. We detected an increase in a Yc-containing sub-unit of an α-class GST, which we have also shown recently to be grossly elevated in a melphalan- and chlorambucil-resistant CHO cell line, CHO-Adr^r [33]. A small increase in GSH activity was reported in another MMC-resistant cell line [53]. It is unlikely that the elevated Yc GST could account for the MMC resistance of CHO-Adr^r cells, as CHO-Chl^r cells are not cross-resistant to MMC in spite of their very high Yc expression. Thus, CHO-Adr^r cells differ from the resistant hepatocyte model in the subclass of GST that is expressed [54].

Our analysis of this Adriamycin-resistant line has shown that several mechanisms of drug resistance can be expressed simultaneously. It is possible that similar multiple changes may account for the differences in cross-resistance profiles reported for several other drug-resistant cell lines [54–59]. Recently, multiple resistance mechanisms have been reported in an Adriamycin-resistant MCF 7 cell line which was 250-fold resistant and showed amplification of the *mdr* gene, an increase in anionic GST and a decrease in topo II [15, 60]. We did not see any alteration in anionic GST in the CHO-Adr^r cell line. Deffie *et al.* [52] showed a 2-fold increase in both anionic GST and *mdr* gene expression in a P388 cell line. However, they found no change in drug transport or *mdr* amplification. Zijlstra *et al.* [61] showed decreased drug transport and decreased DNA double strand breaks in a small cell lung cancer cell line after adjusting for intracellular drug differences although the mechanism was not elucidated. Dank *et al.* [62] have recently described atypical multidrug resistance in which there is a marked cross-resistance between topo II inhibitors. Thus several studies indicate that there may be multiple mechanisms of resistance expressed simultaneously but there have been few studies detailing all three mechanisms—topo II, GSTs and *mdr* [63].

It is not clear whether all these mechanisms occurred in one step of development of resistance or during successive phases. It would be of interest to assay cell lines at each subculture to see if they developed sequentially and in which order. The finding that several drug resistance mechanisms can be expressed simultaneously also suggests the possibility of a coordinated, inducible stress response system. As part of a study of stress responses in mammalian cells, we have isolated drug hypersensitive mutants. In one case, we have found the opposite pattern of expression to that seen in CHO-Adr^r cells, that is, high topo II and low *mdr* expression [64]. However, the isolation of this drug-sensitive mutant did not involve chronic drug exposure. This may indicate that simultaneous changes in *mdr* and topo II expression are in some

way coordinated. Multiple mechanisms of resistance possibly account for the complex patterns of drug resistance found by several groups.

Effective modulation of resistance *in vivo* will need to take into account that multiple mechanisms may exist and that modifying only one, or even two, may be inadequate for successful reversal.

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