21. Tsuda T, Tahara E, Kajiyama G, Sakamoto H, Terada M, Sugimura T. High incidence of coamplification of *hst*-1 and *int*-2 genes in human esophageal carcinomas. *Cancer Res* 1989, **49**, 5505–5508.
22. Sakamoto H, Mori M, Taira M, *et al.* Transforming gene from human stomach cancers and a noncancerous portion of stomach mucosa. *Proc Natl Acad Sci* 1986, **83**, 3997–4001.
23. Miyagawa K, Sakamoto H, Yoshida T, *et al.* *hst*-1 transforming protein: expression in silkworm cells and characterization as a novel heparin binding growth factor. *Oncogene* 1988, **3**, 383–389.
24. Liscia DS, Merlo GR, Garrett C, French D, Mariani-Costantini R, Callahan R. Expression of *int*-2 mRNA in human tumors amplified at the *int*-2 locus. *Oncogene* 1989, **4**, 1219–1224.
25. Fantl V, Richards MA, Smith R, *et al.* Gene amplification on chromosome band 11q13 and oestrogen receptor status in breast cancer. *Eur J Cancer* 1990, **26**, 423–429.
26. Schuuring E, Verhoeven E, Brookes S, *et al.* Identification and cloning of two new chromosome 11q13 proto-oncogens, involved in human breast cancer and squamous cell carcinomas. Proceedings of the IV Symposium on Tumor Research in Oto-Rhino-Laryngology, Düsseldorf, Germany, 12–14 September, 1991 (Abstract).
27. Berenson JR, Koga H, Yang J, Pearl J, Holmes EC, Figlin R & the Lung Cancer Study Group. Frequent amplification of the *bcl*-1 locus in poorly differentiated squamous cell carcinomas of the lung. *Oncogene* 1990, **5**, 1343–1348.
28. Tsuda H, Hirohashi S, Shimosato Y, *et al.* Correlation between long-term survival in breast cancer patients and amplification of two putative oncogene-coamplification units: *hst*-1/*int*-2 and c-*erb*B-2/*ear*-1. *Cancer Res* 1989, **49**, 3104–3108.

Eur J Cancer, Vol. 29A, No. 3, pp. 389–394, 1993.
Printed in Great Britain

0964–1947/93 $6.00 + 0.00

Derivation and Characterisation of a Mouse Tumour Cell Line with Acquired Resistance to Cyclosporin A

Karen A. Wright and Peter R. Twentyman

Cyclosporin A (CsA) is an effective modifier of multidrug resistance. We have studied (a) the possibility that cells grown in increasing concentrations of CsA acquire cellular resistance to the agent and, (b) whether such cells have a multidrug resistant phenotype. Sublines of the EMT6 mouse tumour cell line were developed which were able to grow in 75 and 200 μg/ml of CsA, respectively. The resistant sublines grew slowly in the presence of CsA but reverted to control growth rates, whilst maintaining resistance, when the drug was removed. P-glycoprotein (Pgp) was not detectable in the resistant sublines by immunocytochemistry. The CsA-resistant cells were not cross-resistant to doxorubicin or vincristine but showed a clear degree of cross-resistance to the calcium transport blocker, verapamil. Cellular accumulation of both [^{3}H]CsA and [^{3}H]daunorubicin was significantly increased in the EMT6/CsA200R subline compared with the parent line. In the EMT6 parent line, which expresses very low levels of Pgp, 10–30-fold sensitisation to doxorubicin may be achieved using 0.1–5 μg/ml of CsA. Similar sensitisation by CsA was also seen in the CsA-resistant sublines.
Eur J Cancer, Vol. 29A, No. 3, pp. 389–394, 1993.

INTRODUCTION

A MAJOR FACTOR limiting the effectiveness of cancer chemotherapy is the emergence of drug-resistant cells. Several mechanisms can lead to cross-resistance to broad groups of agents, one of which is known as classical multidrug resistance (MDR) [1, 2]. This mechanism involves overexpression of P-glycoprotein, a membrane transport glycoprotein thought to act as a drug efflux "pump" [1, 2]. A number of chemical agents, including verapamil (VRP) and cyclosporin A (CsA) have been shown to act as modifiers of this form of resistance in that they are able, at least partially, to restore drug sensitivity to MDR cells [3–5]. Both VRP and CsA have been shown to bind to P-glycoprotein and to compete for binding with drugs involved in the MDR phenotype [6–8]. In addition, both agents have shown to accumulate to a lesser extent in some MDR cells than in wild-type drug sensitive cells [9, 10]. As agents involved in the MDR phenotype are generally able themselves to induce such a phenotype, we are interested in the question of whether such resistance modifiers possess this property. We have previously described the derivation and characterisation of a mouse tumour cell line with acquired resistance to VRP [11]. In this paper we describe the independent derivation and characterisation of CsA-resistant subline.

Correspondence to P.R. Twentyman.
The authors are at the MRC Clinical Oncology and Radiotherapeutics Unit, Hills Road, Cambridge CB2 2QH, U.K.
Revised 17 July 1992; accepted 6 Aug. 1992.

MATERIALS AND METHODS

Cell lines

The EMT6/Ca/VJAC mouse mammary carcinosarcoma cell line has previously been described [12, 13], as have the resistant sublines EMT6/AR 1.0 [14] and EMT6/VRP [11].

All cell lines were grown in Eagle's minimum essential medium (MEM) with Earle's salts supplemented with glutamine (0.5 mmol/l), penicillin (100 U/ml), streptomycin (100 μg/ml) and 20% new-born calf serum (NBCS) (Life Technologies).

Cells were maintained as stock cultures as attached monolayers in plastic tissue culture flasks (Falcon), incubated at 37°C in an atmosphere of 8% CO_2 and 92% air. They were disaggregated using a solution of trypsin (0.1%) (Life Technologies) in PBS.

The CsA resistant sublines EMT6/CsA75R and EMT6/CsA200R were derived by continuous growth of cells in increasing concentrations of CsA in the growth media. Flasks of EMT6/P cells were originally set up at concentrations of 50 and 100 μg/ml. A small effect on the growth rate was seen at 50 μg/ml and there was a considerable effect at 100 μg/ml. After 14 days the concentration of CsA in the flasks originally exposed to 50 μg/ml was increased to 75 and 100 μg/ml and the subline EMT6/CsA75R originated from the former of these. Cells in the original flask containing 100 μg/ml CsA failed to proliferate during a drug-free period of 14 days following 14 days of CsA exposure. Cells in the flask originally exposed to 50 μg/ml and subsequently to 100 μg/ml continued to proliferate, and by further stepwise increases in the concentration of CsA, an additional subline (EMT6/CsA200R) able to survive in 200 μg/ml was derived.

Drugs

CsA (MW = 1203) was kindly supplied by Sandoz (Basel). It was dissolved in absolute ethanol at 50 mg/ml and stored at 4°C. For maintenance of resistant cell lines and for clonogenic assays, CsA was added directly to growth medium in a small volume of ethanol. For use in tetrazolium (MTT) assays, dilution in medium was carried out immediately before use in experiments and the final ethanol concentration did not exceed 0.1%. Appropriate solvent controls were used in all experiments.

Doxorubicin (DOX) (Farmitalia) and vincristine (VCR) (David Bull Labs) were dissolved in sterile water at a concentration of 500 μg/ml and stored at −20°C. Dilutions of these drugs was made in sterile phosphate-buffered saline (PBS) immediately before use. VRP (Cordilox, Abbot Labs) was obtained as an aqueous solution at 2.5 mg/ml and stored at 4°C, dilutions were made in PBS immediately before use. Appropriate solvent controls were again used in all cases. Tritium-labelled daunorubicin (DNR) and CsA ([^{3}H]DNR, [^{3}H]CsA) were obtained from New England Nuclear and Amersham International plc, respectively, and stored at −70°C until required.

Sensitivity testing

To determine the response of these cell lines to cytotoxic agents and to confirm resistance, we used the MTT colorimetric assay [15] as adapted for use in our laboratory [16]. Briefly, single cell suspensions were prepared from exponentially growing cultures and inoculated into wells on 96 well tissue culture plates at concentrations between 6×10^2 and 1×10^3 cells/well in a volume of 200 μl. Cytotoxic agents were added 2 h later in a volume of 20 μl. After an incubation period of 3 days, 20 μl of a 5 mg/ml solution of MTT (Sigma) in PBS were added to each well. The plates were reincubated for 5 h. At the end of this period the medium was aspirated from each well, 200 μl of dimethylsulphoxide (DMSO) were added and the plates were shaken on a plate shaker for 10 min. The optical densities of the wells were read on a Titretek Multiskan MCC340 plate reader at a wavelength of 540 nm and at a reference wavelength of 690 nm. For sensitisation assays the above protocol was followed with the additional step of adding CsA in a volume of 10 μl approximately 2 h after the cells had been plated and 1 h before the addition of the drug.

Clonogenic survival assays were also carried out on cells exposed to CsA or VRP. Cells in drug-containing medium were plated onto 9 cm Petri dishes (Nunc). They were incubated at 37°C in a humidified atmosphere of 8% CO_2 for various periods of time. At the end of the incubation period the medium was removed, the plates rinsed in saline, and then fixed and stained simultaneously in a solution of 5% crystal violet (Gurr) in 95% alcohol. Colonies with greater than 50 cells were counted using an Olympus stereo zoom microscope.

Drug accumulation

The ability of the cell lines to accumulate drug was measured using [^{3}H] DNR and [^{3}H]CsA. Labelled DNR was used in these studies in common with many others because of its greater availability compared to labelled DOX.

Cells were inoculated into 6-well plates (Falcon) 48 h before experiments, initial numbers being adjusted so as to achieve equal numbers at the time of experimentation. All drug resistant sublines were grown in the absence of drug during this time. To commence experiments, medium was aspirated from the wells and replaced with 2 ml of medium containing [^{3}H]DNR or [^{3}H]CsA at 3.7 KBq/ml together with unlabelled drug to give a final concentration of 1 μmol/l. The plates were then incubated for the appropriate time period. At the end of this time, the plates were rinsed three times with ice-cold PBS, 1 ml of distilled water was added to each well and the plates were left at room temperature for 2 h to allow cell lysis to occur. At the end of this time the contents of each well were pipetted several times and 500 μl was removed and placed in a plastic scintillation vial together with 5 ml scintillant (Quicksafe, Zinsser Analytic). Radioactivity was measured the following day on a Beckman LS 5000CE liquid scintillation counter. For each cell line used in each experiment, cells in a replicate set of three wells were disaggregated and cell number determined. This allowed values of drug uptake to be corrected to uptake per cell.

Growth curves

To assess the population doubling time of the CsA-resistant sublines compared to the parent, growth curves were constructed. Cells from exponentially growing cultures were disaggregated and inoculated in T25 tissue culture flasks at a seeding density of 5×10^4 cells/flask. Each day for the following three days a pair of flasks was trypsinised and the cell number determined by counting viable cells under phase contrast microscopy.

Cell size and DNA distribution

The distributions of DNA content of EMT6/P, EMT6/CsA75R and EMT6/CsA200R cells were determined using a flow cytometer. Cells from exponentially growing cultures were resuspended in medium containing ethidium bromide and triton X [17] and analysed on the Cambridge flow cytometer [18] using an argon laser operating at 488 nm. DNA content per nucleus was measured on the basis of fluorescence output from each nucleus.

Size distributions were determined on cell suspensions diluted in Isoton (Coulter) and analysed using a Coulter ZB1 particle counter.

Detection of P-glycoprotein

Single cell suspensions of the EMT6/P and the CsA-resistant sublines were washed in PBS and resuspended in PBS at a concentration of 3×10^5 cells/ml. Double-frosted microscope slides previously coated with poly-L-lysine (to enhance

attachment) were placed in a Cytospin 2 (Shandon) along with 0.5 ml of the cell suspension. They were then centrifuged at a speed of 500 rpm for 1 min. The slides were allowed to air-dry at room temperature for at least 2 h and then fixed in acetone (10 min at room temperature). Slides were then stained using the alkaline phosphatase anti-alkaline phosphatase (APAAP) procedure. The primary antibody used was C219 (Centocor) at a concentration of 10 μg/ml. The secondary linking antibody used was rabbit immunoglobulins to mouse immunoglobulins (Dakopatts) at 1:25 dilution and the final APAAP complex, soluble complexes of alkaline phosphatase and monoclonal mouse anti-alkaline phosphatase (Dakopatts) was used at 1:50 dilution. All antibodies were diluted in 1% rabbit serum (Dakopatts) in Tris-buffered saline.

RESULTS

Morphology, size, growth rate and DNA distribution

EMT6/CsA75R and EMT6/CsA200R cells growing in CsA showed some morphological change from the parent line (Fig. 1). Following the removal of CsA from the medium, however, the cells reverted to the parental appearance within a period of 2 days. The growth rates of the CsA-resistant lines maintained in CsA were considerably slower than that of the parent (without CsA) but, following the removal of the drug the growth rate became similar to that of the parent cells within three days (Table 1).

EMT6/CsA75R cells growing in CsA did not differ significantly in size from the parent cells, but EMT6/CsA200R cells were considerably larger as shown in Fig. 2. The DNA distributions obtained by flow cytometry of the EMT6/CsA75R and EMT6/CsA200R cells were unchanged from the distribution given by EMT6/P (data not shown).

Sensitivity to cytotoxic drugs—MTT assay

The response of the resistant sublines to DOX, VCR, VRP and CsA was determined using a 3-day MTT assay. The VRP-resistant subline EMT6/VRP, which exhibits drug resistance via a different mechanism, was also included in this study. Table 2 and Fig. 3 summarise the results obtained. The EMT6/CsA75R and EMT6/CsA200R sublines gave resistance factors to CsA of 3.7 and > 6, respectively (the insolubility of CsA in medium prevented the use in the MTT assay of concentrations above 100 μg/ml). They did not, however, show any clear cross-resistance to either DOX or VCR. A degree of cross-resistance to VRP was seen for each of the CsA-resistant sublines (resistance factors = 1.7 and 2.8, respectively for EMT6/CsA75R and EMT6/CsA200R). Conversely, the EMT6/VRP subline was 3.5-fold resistant to CsA.

Confirmation of resistance

To confirm the resistance to CsA indicated by the MTT assay data, clonogenic assay was used. Initially the CsA-resistant sublines were plated into their maintenance concentration of CsA. They were incubated for 9 days (our standard assay duration for EMT6/P) and colony formation examined. There was evidence of colony formation in the CsA sublines but the colonies were very small compared with those formed by EMT6/P. The experiment was repeated increasing the incubation period to 17 days and the EMT6/CsA200R cells gave a surviving fraction of 40%. To confirm that the increased growth seen with the longer incubation period was not due to degradation of CsA, an additional experiment was carried out in which the CsA was replenished after 9 days in a duplicate set of

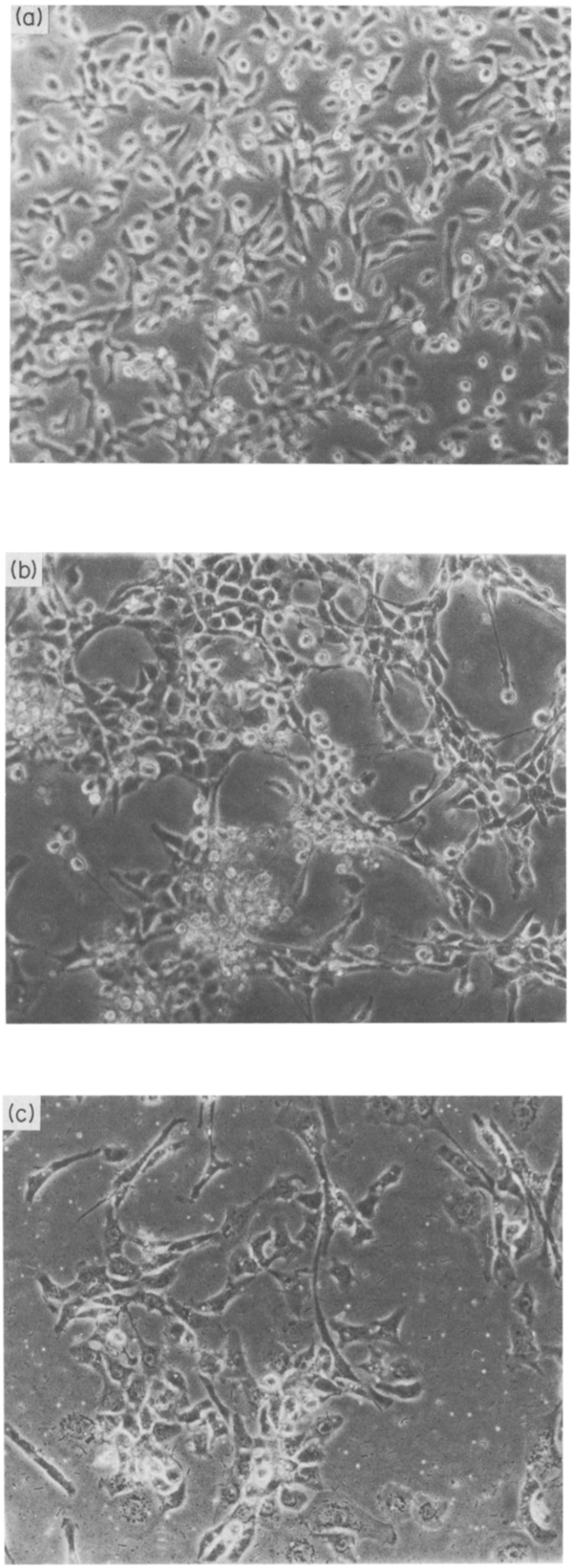

Fig. 1. Photomicrographs of growing cultures of (a) EMT6/P, (b) EMT6/CsA75R and (c) EMT6/CsA200R cells. Resistant cells were growing in their maintenance concentrations of CsA.

Table 1. Growth of cell lines

Line	CsA	Number of cells per flask (× 10^5) Day 0	Day 1	Day 3
EMT6/P	−	0.5	1.0	12.5
EMT6/CsA75R	+	0.5	0.3	3.6
EMT6/CsA75R	−	0.5	0.4	11.1
EMT6/CsA200R	+	0.5	0.2	0.4
EMT6/CsA200R	−	0.5	0.3	8.6

Values are mean counts from two replicate flasks.
Two independent repeat experiments produced similar data.

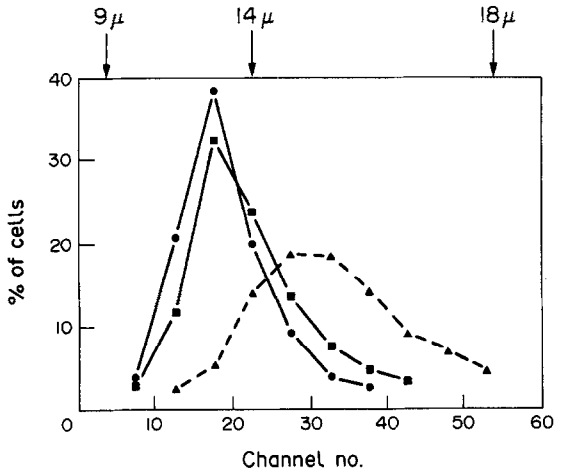

Fig. 2. Size distributions of cells from exponential phase cultures of EMT6/P (●); EMT6/CsA75R (■); and EMT6/CsA200R (▲). Resistant cells disaggregated from cultures containing their maintenance concentrations of CsA.

Table 2. Response of cell lines to cytotoxic drugs

Cell line	IC_{50} (μg/ml)* DOX	VCR	CsA	VRP
EMT6/P	0.055†	0.048	16.7	35.3
	(0.009)	(0.020)	(6.4)	(16.8)
EMT6/CsA75R	0.073	0.048	56.7	51.0
	(0.020)	(0.032)	(4.2)	(7.9)
	RF = 1.4‡	RF = 1.0	RF = 3.7	RF = 1.7
EMT6/CsA200R	0.063	0.033	> 100§	80.0
	(0.025)	(0.019)	RF > 6	(0.0)
	RF = 1.2	RF = 0.7		RF = 2.8
EMT6/VRP			60.0, 55.0‖	90¶
	—	—	RF = 3.5	

*IC_{50} = dose of drug to reduce final optical density to 50% of control.
† Unless otherwise stated, data are mean values (S.D.) from 3 independent experiments, each based on 4 replicate wells at each dose point.
‡ RF (Resistance Factor) = $\frac{IC_{50}\text{ (subline)}}{IC_{50}\text{ (EMT6/P)}}$.
§ The three individual values were > 100, 95.0, > 100.
‖ Two individual values are given.
¶ Single experiment, confirmatory of previously published data [11]

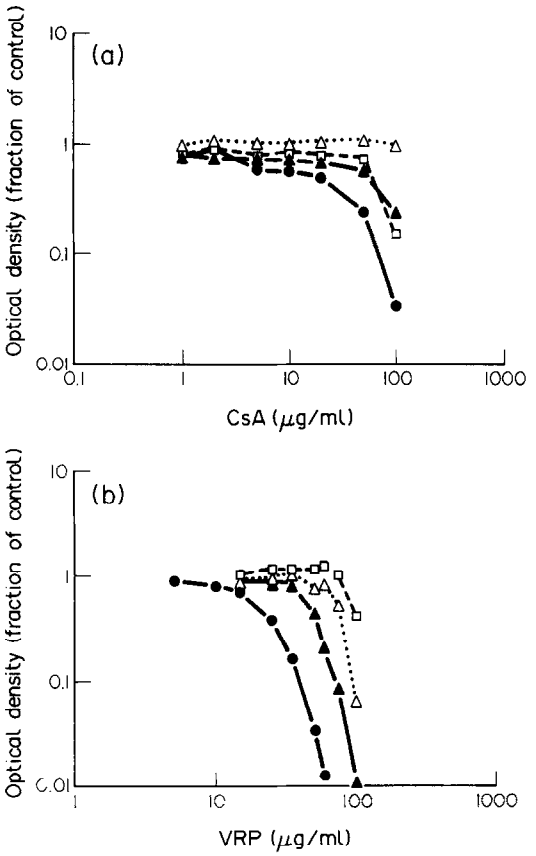

Fig. 3. Response curves obtained in the MTT assay for cells exposed to (a) CsA or (b) VRP. EMT6/P (●); EMT6/CsA75R (▲); EMT6/CsA200R (△); EMT6/VRP (□). Data are from a typical single experiment results of which are included in Table 2.

dishes. The surviving fraction was 35 compared with 33% with no medium change. The EMT6/P line was also plated into CsA at a concentration of 200 μg/ml, for incubation periods of between 9 and 21 days. No colonies were detected on any of the dishes.

Resistance was further examined by allowing the EMT6/CsA200R cell line to proliferate for 1 week in the absence of CsA. Flasks were then set up to compare the growth of these cells with the growth of EMT6/CsA200R which had remained in CsA and also with the growth of the parental line. Table 3 shows the results obtained. The parental line was unable to proliferate in the presence of CsA whereas the CsA-resistant lines had both increased in number by a factor of 6, thereby confirming a resistant phenotype.

To confirm the resistance to VRP seen in the MTT assay for

Table 3. Response of EMT6/P and CsA resistant sublines to CsA

Cell line	CsA (μg/ml)	Total number of cells/flask (× 10^5)†
EMT6/P	0	100
EMT6/P	200	0.74
EMT6/CsA200R	200	3.8
EMT6/CsA200R*	200	3.2

* Grown in absence of CsA for previous 7 days.
† Values shown are means of two replicate flasks, set up at 5 × 10^4 cells/flask at day 0 and counted at day 3.

the CsA-resistant sublines, clonogenic assays were also carried out. The various lines were plated into a range of concentrations of VRP and dishes incubated for 9 days before counting of colonies. The IC_{50} for VRP in the parent cell line was 21 μg/ml (mean from 2 experiments, 3 dishes per dose point), compared with 29 μg/ml for EMT6/CsA75R, 30 μg/ml for EMT6/CsA200R and > 50 μg/ml for EMT6/VRP. These data therefore support the finding of a degree of cross-resistance to VRP in the CsA-resistant lines.

Accumulation of [^{3}H]*DNR and* [^{3}H]*CsA*

The results of experiments to determine the accumulation of [^{3}H]DNR and [^{3}H]CsA are summarised in Table 4. Accumulation of [^{3}H] CsA at 1 h was increased by 17% in the EMT6/CsA75R line and by 64% in the EMT6/CsA200R line compared to the parent. Accumulation of CsA was also increased by 34% in the EMT6/AR1.0 subline. The data for [^{3}H]DNR however show a large decrease in accumulation in the EMT6/AR1.0 to only 14% of control whereas the EMT6/CsA75R and the EMT6/CsA200R sublines showed increases of 53 and 72%, respectively.

Detection of P-glycoprotein

The cytospin preparations were examined using a light microscope. No positive staining was detected on EMT6/P cells or on cells of either of the CsA-resistant lines. In contrast, intense positive staining was observed on the preparations from the EMT6/AR1.0 cell line (data not shown).

Sensitisation to ADM

The ability of CsA to sensitise EMT6/P, EMT6/AR1.0, EMT6/CsA75R and EMT6/CsA200R cells to DOX was determined in a number of experiments. The data are summarised in Table 5. The sensitisation ratios for the parent cell line and EMT6/AR1.0 were similar to those which we have previously reported [14]. There was greater sensitisation in the parent line at very low doses of CsA, with EMT6/AR1.0 showing greater sensitisation at the higher concentration. In the two CsA-resistant lines clear sensitisation was seen and the sensitisation ratios were not significantly different from those seen in the parent cell lines at any of the doses of CsA studied.

DISCUSSION

Cells exhibiting a classical (i.e. P-glycoprotein overexpressing) MDR phenotype show a very characteristic pattern of cross-resistance to a group of agents which includes DOX, DNR, VCR, vinblastine, colchicine, actinoymycin D and etoposide. Conversely, it is generally believed that selection with any of these cytotoxics may result in the emergence of a subline with the classical MDR phenotype. Resistance modifiers such as VRP and CsA are thought to exert their effects primarily by interaction with P-glycoprotein. Both compounds have been shown, by photo affinity labelling, to bind to P-glycoprotein and to compete for binding with drugs involved in the MDR phenotype [6–8]. The binding affinity of CsA is considerably greater than that of VRP or quinidine [19]. The possibility therefore arises that these agents may themselves be capable of inducing overexpression of P-glycoprotein and an MDR phenotype.

Relative sensitivity to, and accumulation of, VRP and CsA by parent and MDR cells are the subject of conflicting data. Three different studies showed that MDR cells expressing P-glycoprotein were more sensitive to the cytotoxic effects of VRP alone than the corresponding parent cells [10, 20, 21]. In one of these [10], data were also presented showing a large reduction in accumulation of VRP by the resistant cells. Two subsequent studies [22, 23], however, reported that MDR cells were cross-resistant to VRP. One of these [22] was from our own laboratory and involved a number of MDR sublines of EMT6, including the EMT6/AR1.0 subline which is also involved in some of the work described in this paper. We also found that accumulation of VRP was similar in EMT6/P and EMT6/AR1.0. The relative sensitivity of parent and MDR cells to CsA alone has also been studied in our laboratory [24]. We found that the human lung cancer cell line H69/LX4 which overexpresses P-glycoprotein and is approximately 100-fold resistant to DOX and 1000-fold resistant to VCR compared to the parent line showed a low, but significant (1.5–2-fold) resistance to CsA. In contrast, two MDR cell lines which did not overexpress P-glycoprotein were unchanged in sensitivity from their respective parent lines [24].

In studies of CsA accumulation, the MDR Chinese hamster cell line CH^RC5 was found to accumulate only 50% of that by the parent line [9], whereas accumulation was similar in an MDR variant of the P388 leukaemia cell line and the parent line from which it was derived [25]. There must however be some doubt about the interpretation of drug accumulation studies where it is not directly shown that the drug is intracellular. The possibility exists that VRP and CsA molecules may become

Table 4. Accumulation of [^{3}H]*DNR and* [^{3}H]*CsA*

Cell line	Accumulation per cell as % of control*	
	[^{3}H]DNR	[^{3}H]CsA
EMT6/P	100	100
EMT6/AR1.0	14.0	134.0
	(6.2)	(21.9)
EMT6/CsA75R	153.0	117.0
	(57.3)	(24.4)
EMT6/CsA200R	172.0	164.0
	(39.0)	(5.7)

* As determined following 1 h exposure. Values are means (S.D.) from at least three separate experiments, each with three dishes per point.

Table 5. Sensitisation to DOX by CsA

Cell line	Sensitisation ratio*		
	CsA 0.1 μg/ml	CsA 1.0 μg/ml	CsA 5.0 μg/ml
EMT6/P	4.8 (0.7)	12.4 (3.6)	27.6 (11.9)
	$n = 6$	$n = 7$	$n = 6$
EMT6/AR1.0	1.3 (0.3)	10.4 (2.0)	137 (43)
	$n = 7$	$n = 8$	$n = 7$
EMT6/CsA75R	4.1 (0.9)	6.6 (2.2)	74 (31)
	$n = 9$	$n = 4$	$n = 4$
EMT6/CsA200/R	5.8 (2.9)	16.4 (5.0)	12.5 (4.8)
	$n = 7$	$n = 7$	$n = 5$

Values are means (S.E.) from n independent experiments, each carried out using the MTT assay with four replicate wells per point.

* Sensitisation ratio = $\frac{\text{DOX } IC_{50} \text{ in absence of CsA}}{\text{DOX } IC_{50} \text{ in presence of CsA}}$.

irreversibly bound to the cell membrane such that they are not removed by the washing procedure used. On the face of it, however, the data with respect to relative cellular accumulation of these compounds in parent and MDR cells must be regarded as equivocal and the concept that VRP and CsA are substrates for P-glycoprotein regarded as open to question.

In this paper we have described cell lines with acquired resistance to CsA. The cells grew very slowly in the presence of the drug but resumed essentially normal growth when it was removed. Data from MTT assay and clonogenic assay experiments clearly demonstrated the existence of a resistant phenotype which was maintained for at least a week in the absence of selecting drug. The CsA-resistant cells did not, however, show any cross-resistance to DOX or VCR, did not overexpress P-glycoprotein and did not show reduced accumulation of [^{3}H]DNR compared with the parent cell line. Indeed, accumulation of [^{3}H]DNR was rather higher in the CsA-resistant lines than in the parent lines. It would, therefore, be interesting to examine DNR cytotoxicity in the various lines. However, the two types of experiments are not directly comparable because of the very different drug exposure times involved. It is quite clear, however, that the CsA-resistant cells do not possess a classical MDR phenotype. Furthermore the ability of CsA to act as a modifier of DOX and VCR sensitivity appears similar in the EMT6/CsA200R and EMT6/P lines. It may be therefore that resistance modification and cytotoxicity by CsA involve different cellular targets. Whereas resistance modification may chiefly involve interaction with P-glyoprotein, cytostatis and cytotoxicity are likely to depend upon less specific membrane effects [26, 27].

It is interesting that the properties of the CsA-resistant cell lines and those of the EMT6/VRP line which we have previously described [11] are rather similar in most respects as detailed above. Also, we now find that the CsA-resistant cells show a degree of cross-resistance to VRP and VRP-resistant cells are cross-resistant to CsA. The possibility therefore arises that a similar mechanism of resistance, not involving P-glycoprotein, is induced by the two resistance modifiers. In conclusion, therefore, it does not appear that prolonged exposure of cells to CsA leads to hyperexpression of P-glycoprotein or to the emergence of resistance to drugs such as DOX or VCR. Furthermore, the emergence of a CsA-resistant phenotype does not appear to lead to a loss of efficacy of CsA as a resistance modifier. Successful clinical use of CsA in this context, therefore, should not be jeopardised by acquired CsA-resistance.

1. Moscow JA, Cowan KH. Review: Multidrug resistance. *J Natl Cancer Inst* 1988, **80**, 14–20.
2. Endicott JA, Ling V. The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu Rev Biochem* 1989, **58**, 137–171.
3. Tsuruo T, Iida H, Tsukagoshi S, Sakurai. Overcoming of vincristine resistance in P388 leukaemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res* 1981, **41**, 1967–1972.
4. Slater LM, Sweet P, Stupecky M, Gupta S. Cyclosporin A reverses vincristine and daunorubicin resistance in acute lymphatic leukemia *in vitro*. *J Clin Invest* 1986, **77**, 1405–1408.
5. Twentyman PR. Cyclosporins as drug resistance modifiers. *Biochem Pharmacol* 1992, **43**, 109–117.
6. Cornwell MM, Pastan I, Gottesman MM. Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. *J Biol Chem* 1987, **262**, 2166–2170.
7. Tamai I, Safa AR. Competitive interaction of cyclosporins with the vinca alkaloid-binding site of P-glycoprotein in multidrug-resistant cells. *J Biol Chem* 1990, **265**, 16509–16513.
8. Foxwell BMJ, MacKie A, Ling V, Riffel B. Identification of the multidrug resistance-related P-glycoprotein as a cyclosporine binding protein. *Mol Pharmacol* 1989, **36**, 543–546.
9. Goldberg H, Ling V, Wong PY, Skorecki D. Reduced cyclosporin accumulation in multidrug-resistant cells. *Biochem Biophys Res Commun* 1988, **152**, 552–558.
10. Cano-Gauci DF, Riordan JR. Action of calcium antagonists on multidrug resistant cells: specific cytotoxicity independent of increased cancer drug accumulation. *Biochem Pharmacol* 1987, **36**, 2115–2123.
11. Twentyman PR, Wright KA, Fox NE. Characterisation of a mouse tumour cell line with *in vitro* derived resistance to verapamil. *Br J Cancer* 1990, **61**, 279–284.
12. Rockwell SC, Kallman RF, Fajardo LF. Characteristics of a serially transplanted mouse mammary tumor and its tissue-culture-adapted derivative. *J Natl Cancer Inst* 1972, **49**, 735–749.
13. Twentyman PR. Response to chemotherapy of EMT6 spheroids as measured by growth delay and cell survival. *Br J Cancer* 1980, **42**, 297–304.
14. Twentyman PR, Reeve JG, Koch G, Wright KA. Chemosensitisation by verapamil and cyclosporin A in mouse tumour cell lines expressing different levels of P-glycoprotein and CP22 (sorcin). *Br J Cancer* 1990, **62**, 89–95.
15. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immun Methods* 1983, **65**, 55–63.
16. Twentyman PR, Luscombe M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. *Br J Cancer* 1987, **56**, 279–285.
17. Taylor IW. A rapid single step staining technique for DNA analysis by flow microflorimetry. *J Histochem Cytochem* 1980, **28**, 1021–1025.
18. Watson JV. Enzyme kinetic studies in cell populations using fluorogenic substrates and flow cytometric techniques. *Cytometry* 1980, **1**, 143–151.
19. Naito M, Tsuruo T. Competitive inhibition by verapamil for ATP-dependent high affinity vincristine binding to the plasma membrane of multidrug resistant K562 cells without calcium ion involvement. *Cancer Res* 1989, **49**, 1452–1455.
20. Twentyman PR, Fox NE, Bleehen NM. Drug resistance in human lung cancer lines: cross-resistance studies and effects of the calcium transport blocker, verapamil. *Int J Radiat Oncol Biol Phys* 1986, **12**, 1355–1358.
21. Warr JR, Brewer F, Anderson M, Fergusson J. Verapamil hypersensitivity of vincristine resistant Chinese hamster ovary cell lines. *Cell Biol Int Rep* 1986, **10**, 389–399.
22. Reeve JG, Wright KA, Rabbitts PH, Twentyman PR, Koch GLE. Collateral resistance to verapamil in multidrug resistant mouse tumor cells. *J Natl Cancer Inst* 1989, **81**, 1588–1590.
23. Sehested M, Skovsgaard T, Buhl Jensen P, Demant EJF, Friche E, Bindslev N. Transport of the multidrug resistance modulators verapamil and azidopine in wild type and daunorubicin resistant Ehrlich ascites tumour cells. *Br J Cancer* 1990, **62**, 37–41.
24. Twentyman PR, Wright KA, Wallace HM. Effects of cyclosporin A and a non-immunosuppressive analogue, O-acetyl cyclosporin A, upon the growth of parent and multidrug resistant human lung caner cells *in vitro*. *Br J Cancer* 1992, **65**, 335–340.
25. Hait WN, Stein JM, Koletsky AJ, Harding MW, Hardschumacher RE. Activity of cyclosporin A and a non-immunosuppressive cyclosporin on multidrug resistant leukemic cell lines. *Cancer Commun* 1989, **1**, 35–43.
26. Haynes M, Fuller L, Haynes DH, Miller J. Cyclosporin partitions into phospholipid vesicles and disrupts membrane architecture. *Immunol Lett* 1985, **11**, 343–349.
27. Matyus L, Balazs M, Aszalos A, Mulhern S, Damjanovich S. Cyclosporin A depolarizes cytoplasmic membrane potential and interacts with Ca^{2+} ionophores. *Biochim Biophys Acta* 1986, **886**, 353–360.