Expression of an *mdr* Gene is Associated with a New Form of Resistance to Dexamethasone-Induced Apoptosis

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A variant, MS23, of murine thymoma W7 cells, previously selected for its resistance to low concentrations of dexamethasone, is cross-resistant to unrelated drugs such as puromycin and colchicine. We report here that transcription of the mouse *mdr*1 gene is activated and P-glycoprotein is expressed in MS23 cells. Moreover, additional variants with increased resistance to dexamethasone and other drugs can be isolated from MS23 by stepwise selections in dexamethasone and colchicine. In one such variant (S7CD-5), the *mdr*1 gene is amplified and the *mdr* protein overexpressed. These variants have classical *mdr* characteristics: they accumulate reduced concentrations of drugs (including dexamethasone), and both drug sensitivity and intracellular accumulation can be restored by verapamil. The variants are most resistant to glucocorticoids with both 11- and 17-hydroxyl groups. The results indicate that we have identified a new form of glucocorticoid resistance, one associated with expression of the mouse *mdr*1 P-glycoprotein. (Molecular Endocrinology 7: 840–851, 1993)

INTRODUCTION

Glucocorticoids induce programmed cell death or apoptosis of immature thymocytes (reviewed in Refs. 1, 2). This phenomenon accounts, at least in part, for the immunosuppressive action of these steroids and for their use in combination chemotherapies of some types of leukemias and lymphomas. There is evidence that induction of apoptosis requires RNA and protein synthesis and, therefore, involves steroid-regulated changes in the expression of, as yet, unknown genes. This process is mediated by the glucocorticoid receptor, a member of a superfamily of receptors acting as hormone-dependent transcription factors (reviewed by Evans; 3). Since this receptor is intracellular, the entry and accumulation of hormone within the cell is obviously required for receptor binding. Little is known about the regulation of intracellular steroid levels. It has been generally assumed that steroids enter cells by passive diffusion through the plasma membrane by virtue of their small size and lipophilic properties. However, any process that can influence the intracellular accumulation of steroids would directly affect receptor occupancy and the magnitude of the hormonal response.

A variety of T-lymphoid cell lines, derived from lymphomas or leukemias, have been used to study apoptosis and the role of the glucocorticoid receptor in this process (4–7). The sensitivity of these human or rodent cell lines provides a powerful selection for glucocorticoid-resistant variants *in vitro*. We have used the glucocorticoid-sensitive murine thymoma cell line WEHI-7 (W7) to demonstrate that resistance can result from mutations affecting either the quality or the quantity of receptor, and the role of cAMP in modulating glucocorticoid receptor function (6, 8–10). In the course of our investigations we isolated a collection of W7 variants resistant to low concentrations (<10 nM) of dexamethasone (8). We demonstrated that most of these variants expressed only half the amount of functional receptor present in the parental line. However, one of the variants resulting from that selection, MS23, had acquired a rare new form of dexamethasone resistance (11). The glucocorticoid receptor appeared normal when assayed in MS23 cell extracts, but binding to whole cells was greatly reduced. We showed that this phenomenon was due to a defect in dexamethasone accumulation inside the MS23 cell. Since drug accumulation had been implicated in multidrug resistance, we tested the sensitivity of this variant to other drugs and found that it had acquired some resistance to unrelated drugs, puromycin and colchicine in particular. The phenotype of the MS23 variant was also unusual in that these cells had remained fully sensitive to triamcinolone acetonide, a synthetic glucocorticoid closely related to dexamethasone.

The classical form of multidrug resistance (*mdr*) in-

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volves unrelated lipophilic drugs, including some widely used in chemotherapy. Typically, pleiotropic resistance develops by selection for resistance to a single drug, an indication that the same event can lead to resistance to different drugs. Cell line model systems have been successfully used to identify the basis for this phenomenon. Multidrug-resistant cells consistently express in the plasma membrane increased amounts of P-glycoproteins, which have been extensively characterized and shown to play a direct role in the *mdr* phenotype (reviewed in Refs. 12–16). P-Glycoproteins cause energy-dependent efflux of a variety of hydrophobic drugs, and their increased expression in *mdr* cells results in reduced intracellular accumulation of the drugs to sublethal levels. Genes encoding P-glycoproteins have been cloned and characterized and constitute a small gene family in mammals. The mouse has three multidrug resistance genes, *mdr*1, *mdr*2, and *mdr*3, but only the *mdr*1 and *mdr*3 genes confer multidrug resistance in transfection experiments (17–19). A number of investigators have examined the possible role of *mdr* proteins in the transport of steroids. Several reports demonstrate that steroids can bind to both the human and mouse P-glycoproteins (20–22). There have been conflicting reports as to whether steroids can be transported out of *mdr* cells overexpressing the efflux proteins (21, 23–25). In this report, we present evidence that the unique dexamethasone-resistant phenotype of the MS23 variant and its derivatives is associated with expression of the *mdr*1 P-glycoprotein. We also examine the structural basis for the differential resistance of these variants to specific steroids.

RESULTS

Isolation of Dexamethasone-Resistant Variants

The original characterization of the MS23 variant revealed that it was resistant not only to dexamethasone but also to a variety of unrelated drugs such as puromycin, colchicine, and daunomycin (11). The increased resistance of MS23 cells to dexamethasone and colchicine is illustrated in Fig. 1, A and B. Figure 1, A, C, and D, also demonstrates that the change in steroid resistance had a very unique characteristic. While the resistance to dexamethasone increased 3-fold and the resistance to triamcinolone increased 10-fold in MS23 cells, these cells show a 2-fold increased sensitivity, rather than resistance, to triamcinolone acetonide. This is particularly striking, since all three of the steroids are strong agonists and are closely related structurally (see Fig. 2A). Moreover, our earlier studies had shown that the amount of glucocorticoid receptor and its affinity for dexamethasone did not differ in the W7TB and MS23 cell lines (11).

The fact that the MS23 variant had been obtained by selection for resistance to dexamethasone alone suggested that its resistance to the other drugs was related to the mechanism producing its dexamethasone resistance. To further analyze the basis for this new form of steroid resistance, highly resistant derivatives of MS23 cells were obtained by stepwise selection *in vitro* with increasing concentrations of colchicine and dexamethasone. This combination of drugs was chosen to avoid the glucocorticoid receptor mutants that could arise from selection in dexamethasone alone and possible microtubule mutants that could result from selection for resistance to colchicine alone (26). The final stage, S7CD-5, showed increased resistance to the same drugs to which MS23 cells were resistant. Moreover, like MS23, S7CD-5 did not develop significant resistance to triamcinolone acetonide (11). The observation that the pattern of resistance in S7CD-5 cells was the same as in MS23 cells indicated that the difference between the two cell lines was quantitative, not qualitative, and again suggested that the same event was responsible for the spectrum of drug resistance.

The combined resistance to a variety of drugs suggested that a mechanism producing reduced intracellular drug concentrations (including specific steroids) could be involved. One form of *mdr* has recently been shown to be caused by drug efflux proteins (P-glycoproteins). Multidrug resistance caused by such an efflux mechanism can be reversed by chemosensitizing drugs such as verapamil and quinidine (23, 27–29). These agents are thought to act by competing with the other drugs for binding to P-glycoproteins. Consequently, the toxic drugs are not as efficiently transported, and their resulting intracellular levels are raised.

We compared the ability of verapamil to alter the sensitivities of W7TB, MS23, and S7CD-5 cells to triamcinolone. The results are shown in Fig. 3. In the parental line W7TB (3A), verapamil reduced the concentration of triamcinolone needed to cause a 50% loss of proliferation (LD_{50}) by 1.8-fold. In the MS23 and S7CD-5 variants, the effect of verapamil was progressively greater. The LD_{50} levels were reduced by 13- and 32-fold in MS23 and S7CD-5 cells, respectively. Verapamil had a similar capacity to reverse the resistance to colchicine and puromycin in the MS23 and S7CD-5 cell lines (data not shown).

Reduction in Steroid Accumulation and its Reversion by Verapamil

If the classical form of P-glycoprotein-mediated multidrug resistance is involved, it should result in the reduced accumulation of toxic drugs within the cells. We had previously demonstrated a reduced accumulation of puromycin in both the MS23 and S7CD-5 lines (11). Figure 4 illustrates the relative accumulation of dexamethasone in the cell lines used in Fig. 3. The concentration of dexamethasone (60 nM) that was used was chosen because it could discriminate between the sensitivities of the different cell lines. In comparison to W7TB, both the total amount of dexamethasone, as well as that specifically bound to GR, were reduced in the MS23 and S7CD-5 cell lines. The reduction in dexamethasone bound to receptors reflects the re-

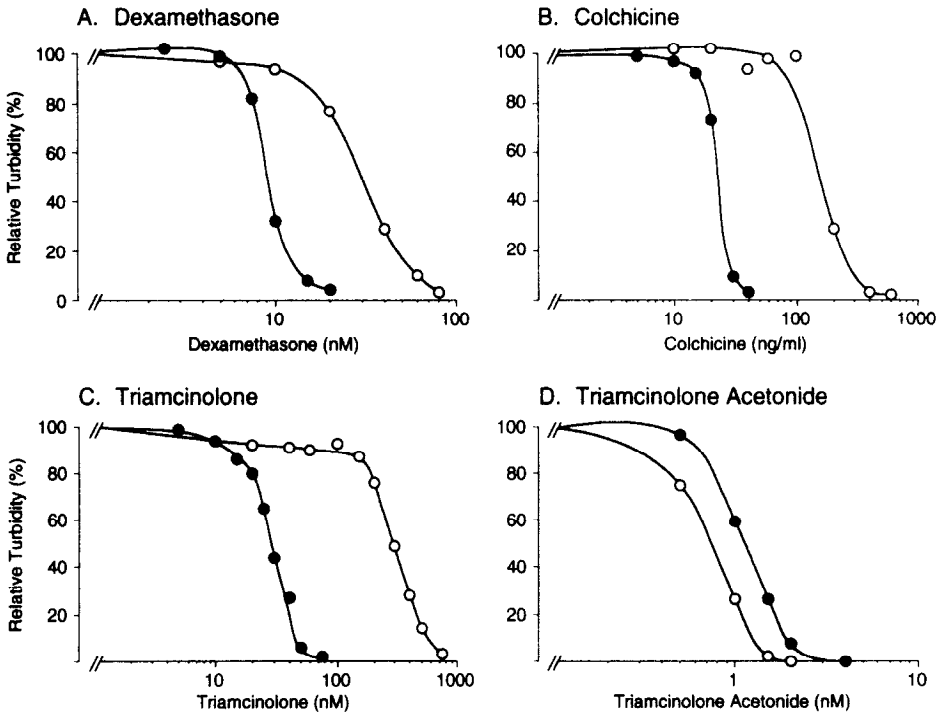


Fig. 1. Sensitivity of W7TB and MS23 Cell Lines to Colchicine and Three Glucocorticoids

Cell cultures, initially containing 5×10^4 cells/ml, were incubated in medium containing the indicated concentrations of drug for 5 days. At the end of the incubation period, the turbidities (660 nm) of the cultures were measured and expressed relative to cultures containing no drug. Each *point* is the average of two determinations. ●, W7TB cells; ○, MS23 cells;

duced accumulation of steroid rather than a receptor defect, since previous experiments using cytoplasmic extracts had shown that the number of glucocorticoid receptor binding sites were not different in the W7TB, MS23, and S7CD-5 cell lines (11). Moreover, the receptors of the variants are functional, since they mediate the induction of VL30 (data not shown), a gene that was found to be induced by glucocorticoids in the parental cell line W7 (30).

If verapamil has the potential to influence the efflux of steroids, then the results shown in Figs. 3 and 4 predict that the drug would have a pronounced effect on specifically bound dexamethasone in S7CD-5 cells but not in wild type cells. Furthermore, verapamil should cause little change in the accumulation of triamcinolone acetonide within either cell line, since there is little difference in their sensitivity to this agonist. Figure 5 shows that both predictions are correct. W7TB and S7CD-5 cells were incubated with either dexamethasone or triamcinolone acetonide in the presence and absence of verapamil. Without verapamil, very little dexamethasone was found within the S7CD-5 cells (less than 10% of that found within W7TB cells). In the case of triamcinolone acetonide, a considerable amount of hormone accumulates in the variant (approximately 70% of that found in W7TB cells). Addition of verapamil caused the level of dexamethasone to increase more than 10-fold within S7CD-5 while having only a slight effect on wild type cells. In comparison, verapamil had no significant effect on the levels of specifically bound triamcinolone acetonide within the wild type or S7CD-5 cells. Taken together, the results support the hypothesis that we have observed a new form of dexamethasone resistance in lymphoma cells—one that is associated with multidrug resistance.

Detection of *mdr* Proteins in MS23 and its Derivative S7CD-5

The multidrug resistance and reduced dexamethasone accumulation seen in the MS23 cell line and S7CD-5 suggest that a progressive overexpression of proteins causing drug efflux is responsible. To test the validity of this possibility, a monoclonal antibody (C219) recognizing the mouse *mdr*1 and *mdr*3 gene products was used to look for the presence of these proteins. Quantitative measurements were obtained by indirect immunofluorescence using a fluorescein isothiocyanate (FITC)-conjugated secondary antibody and fluorescence cytometry. The histograms in Fig. 6A show data from one such experiment and are typical of the results that we have obtained. Measurements were made with three cell lines: W7TB (the parental line), MS23, and S7CD-5. In each case, the experiment was carried out as a comparison between preparations incubated in the presence or absence of the primary antibody. No significant C219-dependent fluorescence was seen with the W7TB cells, indicating that if there is any P-glyco-

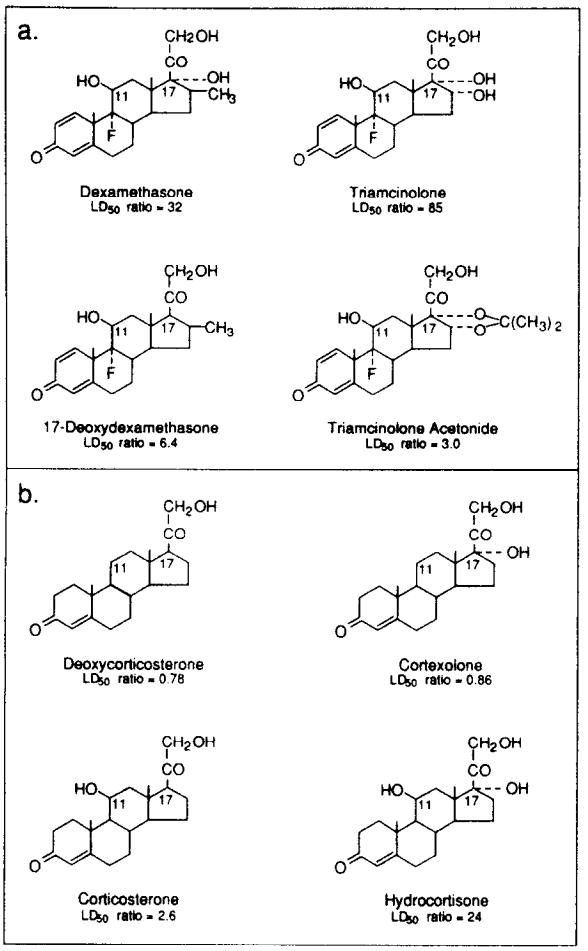


Fig. 2. Effect of the 11-OH and 17-OH Groups on the Relative Resistance of the S7CD-5 Variant

a, Triamcinolone acetonide differs from triamcinolone only by the acetonide group blocking the 17-OH; 17-deoxydexamethasone is identical to dexamethasone except for the absence of the 17-OH group. All four of these steroids have an 11-OH group. b, The structures of these four glucocorticoids are identical except for the presence of a 17-OH group (cortexolone), 11-OH group (corticosterone), or both OH groups (hydrocortisone). The LD_{50} ratios represent the relative resistance of the variant to each steroid calculated by dividing the drug concentration required to inhibit the growth of the variant cells by 50% by that required for the parental cells.

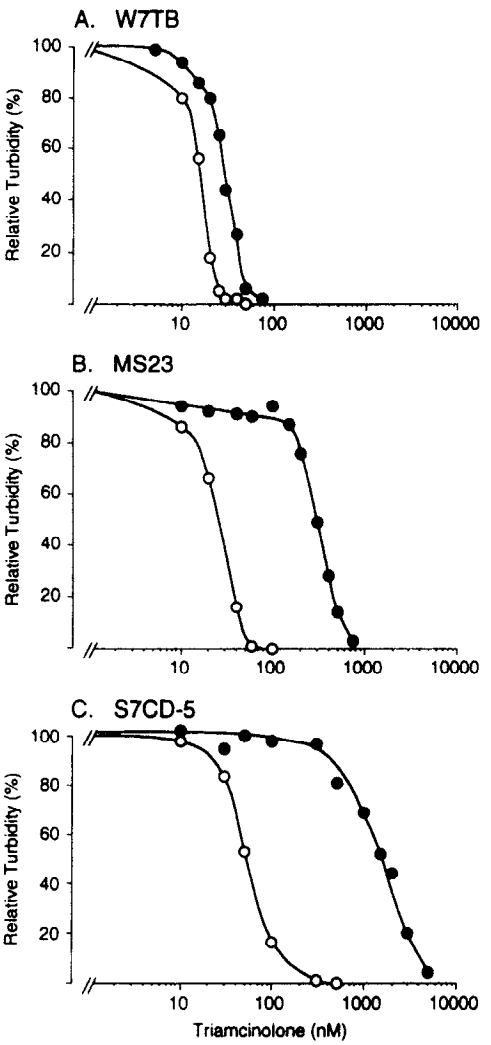


Fig. 3. Reversion of the Triamcinolone Resistance in the W7TB, MS23, and S7CD-5 Cell Lines by Verapamil

Two sets each of W7TB MS23 and S7CD-5 cells were plated out at 5×10^4 cells/ml in increasing concentrations of triamcinolone. One set from each cell line (O) contained 5 μM verapamil. The cultures were incubated for 5 days and their turbidities measured (A_{660}). Each *point* represents the average of duplicate samples, and the data are normalized to the values obtained without steroid for each set.

protein present in W7TB, it is below the threshold of detection of our assay. On the other hand, both MS23 and S7CD-5 demonstrated a C219-dependent increase in fluorescence, with S7CD-5 displaying the greatest shift. The ratio of mean fluorescence (±C219 antibody) can be used to compare the differences between the cell lines. The averages of values obtained from three separate experiments are shown Fig. 6B and verify that both MS23 and S7CD-5 express an *mdr* epitope which is not detected in the parental cell line.

Expression and Amplification of *mdr* Genes in the Variants

The possible involvement of drug efflux proteins in the phenotype of the cell lines shown above was further evaluated by testing for their expression of the mouse *mdr* genes. Figure 7A shows a Northern blot analysis of total RNA isolated from the parental W7TB cells, the MS23, and the S7CD-5 variants. The individual blots were hybridized with three different *mdr* probes. The first probe (*left panel*) was generated from the entire pcDR1.3 cDNA clone and does not discriminate between the three different mouse *mdr* genes (generic probe). The *middle* and *right* panels reflect hybridization to probes generated from oligonucleotides specific for the *mdr*1 and *mdr*3 genes, respectively. No hybridization was observed with RNA from the W7TB cells by any of the probes, even at longer times of autoradiographic exposure. A 5-kb transcript (migrating imme-

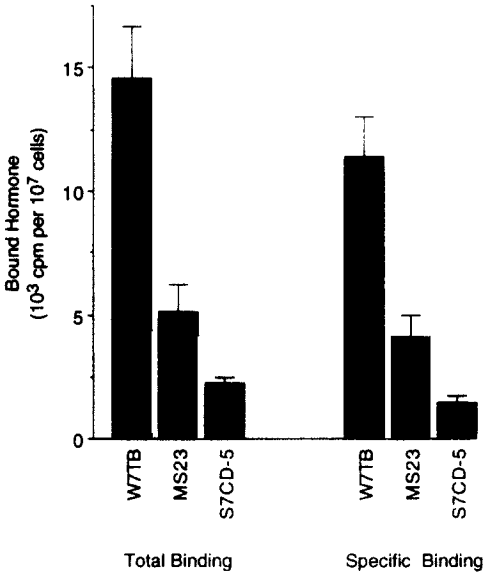


Fig. 4. Relative Accumulation of Dexamethasone in the W7TB, MS23, and S7CD-5 Cell Lines

The cell lines characterized in Fig. 3 were incubated with [^{3}H]dexamethasone (60 nM) for 1 h at 37 C. After the incubation, the cells were washed free of unbound steroid in cold (0 C) PBS. Bound hormone (total) was measured in a scintillation counter. Parallel cultures containing 150-fold excess of unlabeled triamcinolone acetonide were used to evaluate nonspecific binding. The difference between total and nonspecific binding is defined as specific binding. The data represent the means + SE from three independent experiments.

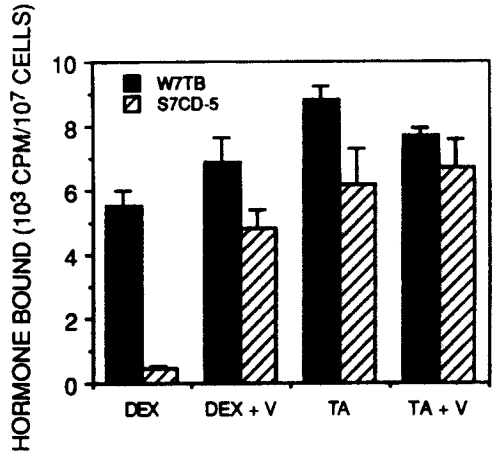


Fig. 5. Effect of Verapamil on the Accumulation of Glucocorticoids in W7TB and S7CD-5 Cells

Two sets each of wild type W7TB cells and drug-resistant S7CD-5 cells were incubated with either ^{3}H-labeled dexamethasone or triamcinolone acetonide (10 nM) for 1 h in the presence or absence of 10 μM verapamil. At the end of the incubation the cells were washed free of unbound hormone Nonspecific binding was measured using parallel samples containing 5 μM unlabeled hormone. The hormone specifically bound to each sample is shown. The *bars* represent the means + SE from two independent experiments.

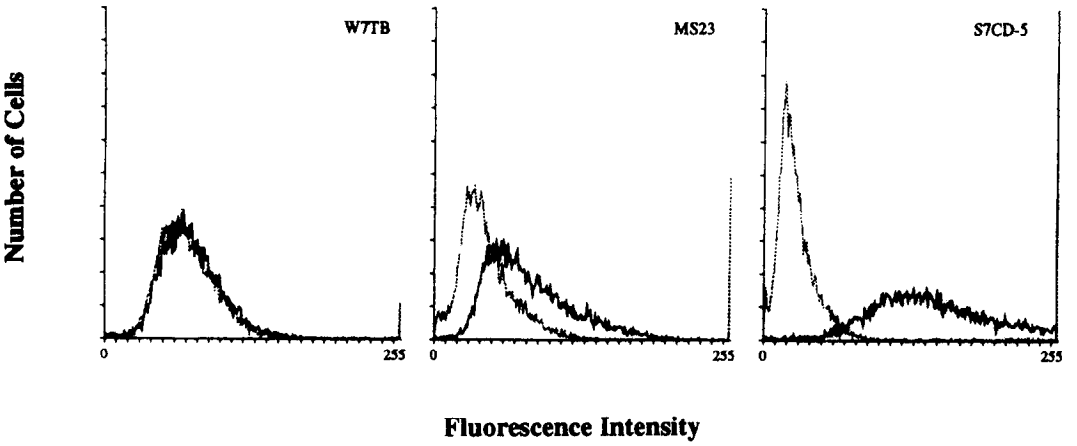
diately above the 28S rRNA species) was detected with the generic probe in the RNA from MS23 cells. The concentration of the 5-kb species was approximately 10-fold higher in the S7CD-5 RNA. An identical pattern of hybridization was observed with the *mdr*1-specific probe (*middle panel*), while no hybridization was found with the probe specific for the *mdr*3 gene (*right panel*). The size (5 kb) of the hybridizing RNA is in agreement with that described by others using an *mdr*1 specific probe (19). In addition, we have not detected hybridization using cDNA probes specific for the *mdr*2 and *mdr*3 genes and poly (A)$^+$ RNA preparations from S7CD-5 cells (data not shown).

Figure 7B shows a Southern blot analysis of DNA isolated from the W7TB, MS23 and S7CD-5 cell lines, digested with *Eco*RI and hybridized with the generic cDNA probe. There were no differences apparent in the hybridization patterns obtained from the W7TB and MS23 samples. However, all seven of the hybridizing bands were amplified in the S7CD-5 DNA. Densitometric scans of the autoradiograms indicated that each band was amplified approximately 8-fold. Taken together, our results demonstrate that the original selection for the MS23 variant resulted in the isolation of cells expressing the *mdr*1 gene. Furthermore, this expression was increased, through a gene dosage effect, during the subsequent selection of the S7CD-5 cells.

Steroid Specificity of the Phenotype of S7CD-5 Cells

The differences observed between dexamethasone and triamcinolone acetonide in S7CD-5 cells are significant since the two steroids are closely related structurally, and changes in resistance to one had always been found to parallel changes in resistance to the other. The results outlined above suggested that the basis for the selective dexamethasone resistance observed in S7CD-5 involved a structural feature in this steroid molecule that is not present in triamcinolone acetonide. With this in mind, the relative resistance of S7CD-5 cells to a variety of steroids with agonist activity was examined. The results of some of the studies are shown in Fig. 8. Two glucocorticoids very closely related to dexamethasone and triamcinolone acetonide were tested, namely 17-deoxydexamethasone and triamcinolone. Figure 2A illustrates the structural relationship between these four steroids. Triamcinolone acetonide differs from triamcinolone by the presence of an acetonide group replacing the 16- and 17-hydroxyl groups. Similarly, 17-deoxydexamethasone is identical to dexamethasone except for the absence of a 17-hydroxyl group. The S7CD-5 variant has developed considerably more resistance to the steroids having a 17-hydroxyl group (dexamethasone and triamcinolone; Fig. 8, A and B) than to the steroids in which the 17-hydroxyl group is absent (17-deoxydexamethasone, Fig. 8C) or modified (triamcinolone acetonide, Fig. 8D). The degree of resistance of S7CD-5 cells to those steroids can be

A



B

Cell Line	Ratio of Mean Fluorescence +/- Primary Antibody
W7TB	0.99 +/- 0.04
MS23	1.46 +/- 0.31
S7CD-5	3.10 +/- 1.48

Fig. 6. MDR Protein Expression in the W7TB, MS23, and S7CD-5 Cell Lines

A, Indirect immunofluorescence measurements were made by flow cytometry using a mouse monoclonal, *mdr*-specific, primary antibody and a secondary antibody conjugated with FITC. Comparisons were made for each cell line between samples incubated in the absence (*dotted line*) or presence (*solid line*) of the primary antibody. The data are presented as histograms with the fluorescence intensity plotted on a linear scale. B, Ratios of the mean fluorescence values (±the primary antibody) for each cell line. The values represent the means + SE from three independent experiments.

expressed quantitatively as the ratios of the LD_{50} for S7CD-5 cells to the LD_{50} for parental cells. The values of these ratios are only 3.0 for triamcinolone acetonide and 6.4 for 17-deoxydexamethasone but are 85 and 32 for triamcinolone and dexamethasone, respectively (Fig. 2A). These results point to the presence of a 17-hydroxyl group as one of the structural features important in the specificity of the resistance phenotype.

Additional agonists were tested, and the results are shown in Table 1. The steroids can be divided into three classes based on the relative level of resistance observed in the S7CD-5 cells: class I steroids to which the variant had no increased resistance (LD_{50} ratio < 1.0); class II steroids to which the variant had modest resistance ($2.4 \leq LD_{50}$ ratio ≤ 6.4); class III steroids to which the variant acquired considerable resistance (LD_{50} ratio > 14) up to as much as 85-fold. With no exception, all of the class III steroids have hydroxyl groups in both the 11- and 17- positions. The class II steroids have a hydroxyl group in only the 11- position. The class I steroids either lack both 11- and 17-hydroxyl groups or, in the case of cortexolone, has only a hydroxyl group at the 17- position. Figure 2B illustrates the structure of four natural ligands that differ only by the presence of 11- and/or 17-hydroxyl groups and which fall into the three different classes of steroids. Deoxycorticosterone lacks both 11- and 17-hydroxyl groups and belongs to class I steroids (LD_{50} ratio = 0.78). The addition of a 17-hydroxyl group to deoxycorticosterone yields cortexolone, a glucocorticoid that still belongs to class I steroids, since the S7CD-5 variant has developed no resistance to cortexolone either (LD_{50} ratio = 0.86). This indicates that the presence of a 17-hydroxyl group may be necessary but is not sufficient to generate a steroid to which the S7CD-5 variant is resistant. However, the addition of an 11-hydroxyl group to cortexolone yields hydrocortisone, a steroid of class III to which the variant has developed considerable resistance (LD_{50} ratio = 24). Corticosterone, on the other hand, differs from deoxycorticosterone only by the presence of an 11-hydroxyl group and belongs to class II steroids (LD_{50} ratio = 2.6). These results indicate that the presence of both the 11- and 17-hydroxyl groups contribute synergistically to the specificity of the resistance phenotype.

To further test the hypothesis outlined above, several other steroids were evaluated that have a 17-hydroxyl group but lack an 11-hydroxyl group (testosterone, prednisone, cortisone, 17-hydroxyprogesterone, and 17-hydroxypregnenolone) or lack both the 11- and 17-hydroxyls (pregnenolone). However, those steroids have only very low affinity for the glucocorticoid receptor, and little, if any, toxicity was observed at concentrations as high as 10 μM. It is, however, interesting to

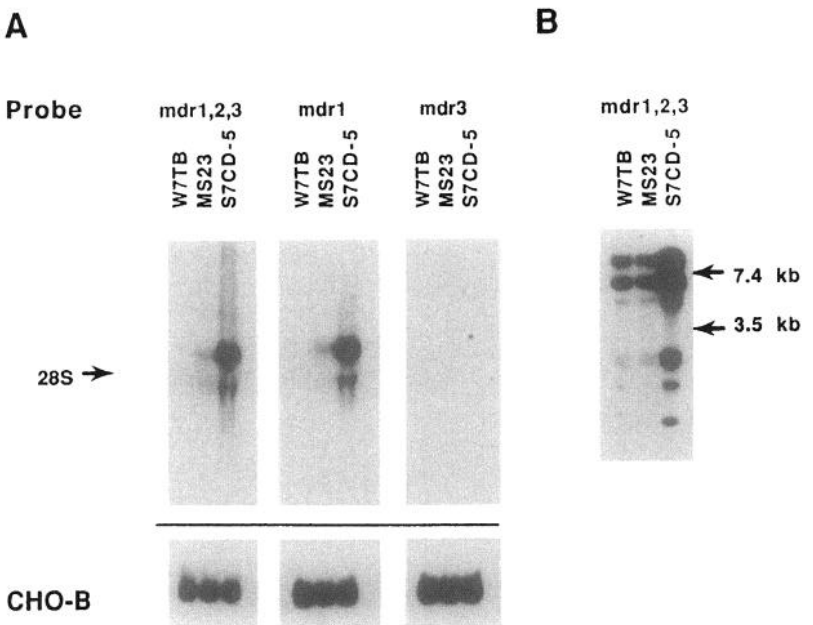


Fig. 7. Analysis of RNA and DNA from W7TB, MS23, and S7CD-5 Cells

A, Northern blot analysis of *mdr* RNA expression in the W7TB, MS23, and S7CD-5 cells. Total RNA was isolated from W7TB, MS23, and S7CD-5 cells and fractionated (20 μg/lane) by electrophoresis in a 1% agarose gel containing formaldehyde. After the RNA was transferred to nylon membrane filters, replicate blots were hybridized with: *left panel*, a probe generated from the pcDR1.3 clone (this probe does not distinguish between the individual *mdr* genes); *middle panel*, a probe generated from an oligonucleotide specific for the *mdr*1 gene; *right panel*, a probe generated from an oligonucleotide specific for the *mdr*3 gene. All of the filters were rehybridized with a probe for the constitutively expressed CHO-B gene. B, Southern blot analysis of genes encoding *mdr* genes in W7TB, MS23, and S7CD-5 cells. DNA was isolated from W7TB, MS23, and S7CD-5 cells, digested with *Eco*RI, and fractionated (5 μg/lane) by electrophoresis in a 1% agarose gel. The DNA was transferred to a nylon filter and hybridized with a probe generated from the generic pcDR1.3 cDNA clone.

note that the steroid to which the S7CD-5 variant has developed most resistance is triamcinolone (LD_{50} = 85), the only steroid tested that has a third hydroxyl group, in position 16 (Fig. 2A).

DISCUSSION

The results presented in this report constitute, to our knowledge, the first evidence of a possible role of P-glycoprotein in glucocorticoid resistance of T-lymphoma cells. Interestingly, Gross *et al.* (31, 32) presented evidence more than two decades ago that certain steroids were actively transported out of mouse fibroblasts, lymphomas, and adrenal cells growing *in vitro*. This transport process was shown to be energy dependent, temperature sensitive, and steroid specific. In a result similar to ours, cortisol, dexamethasone, and prednisolone were found to be the best substrates for the putative transport process and to be extruded from L cells. At that time, the P-glycoproteins and their role in drug efflux and multidrug resistance were unknown, and the steroid transport process involved remained unidentified.

Since the discovery of *mdr* P-glycoproteins, a number of investigators have examined their possible role in the transport of steroids. Several reports have convincingly demonstrated that steroids can bind to both the human and mouse P-glycoproteins. Progesterone effectively competes the photoaffinity labeling of the mouse *mdr*1 P-glycoprotein by azidopine and inhibits the binding of [^{3}H]vinblastine to membrane vesicles prepared from *mdr* mouse cells (20). Progesterone was also found to inhibit binding and efflux of vinblastine in *mdr* mouse cells and increased the drug sensitivity of these cells (21). Screening of steroids other than progesterone indicated that glucocorticoids were less efficient than progesterone (20). Finally, [^{3}H]progesterone itself has been used to directly photoaffinity label the human MDR1 P-glycoprotein (22). In spite of the substantial evidence that progesterone binds the mouse P-glycoprotein, steroids were not transported out of mouse *mdr* cells overexpressing the efflux proteins (21). Similarly, the human MDR1 protein was not found to export dexamethasone (23). More recently, Nelson and Hinkle (24) reported that a variant of rodent GH_4C_1 cells, selected for colchicine resistance, accumulated reduced amounts of cortisol. The variant was also shown to express *mdr* protein using the same C-219 monoclonal antibody that was employed in our flow cytometry experiments. Ueda *et al.* (25) introduced the human *mdr*1 gene into porcine LLC-PK1 cells and detected increased transepithelial transport of cortisol, aldoste-

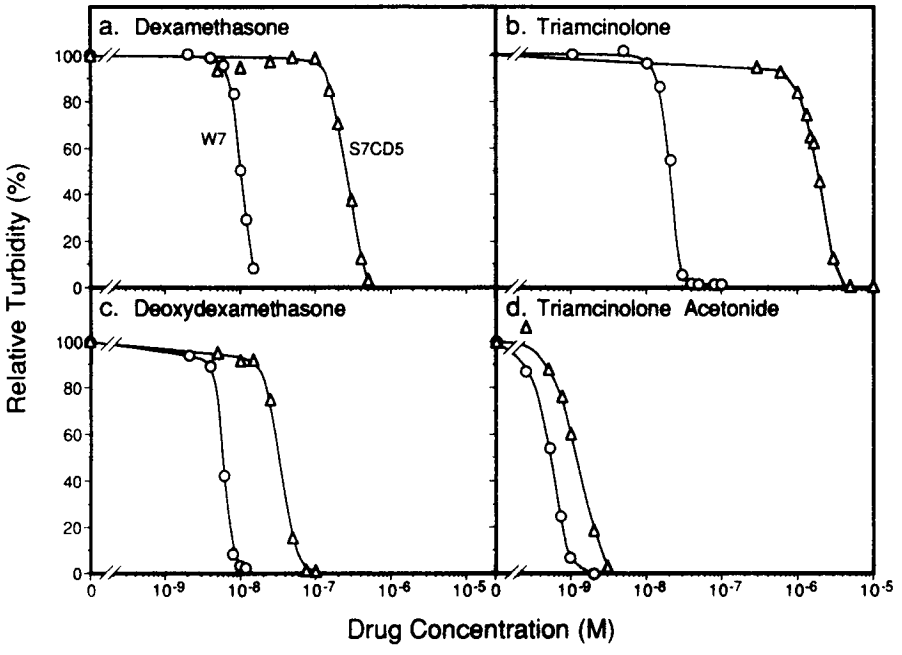


Fig. 8. Increased Resistance of the S7CD-5 Variant to Various Glucocorticoids

Dose-response curves were generated as described for Fig. 1. The cellular material present in the culture was monitored by turbidity at 660 nm. The results are expressed as a percentage of the turbidity reached in the control without drug. ○, Parental W7TB cell line; △, S7CD-5 variant.

rone, and dexamethasone, but not progesterone. These authors did not demonstrate reduced accumulation of steroid within the cells. It is interesting, however, that dexamethasone and cortisol are two of the steroids that we have found to be effectively excluded from S7CD-5 cells (class III in Table 1). Ueda *et al.* (25) did not test triamcinolone or triamcinolone acetonide in their assay.

As far as steroid resistance is concerned, this has been rarely tested, because, unlike many other drugs, glucocorticoids are not toxic to most cells. In contrast to most previous studies, the T-lymphoma cell line W7 used here is highly sensitive to glucocorticoids (8). Moreover, the selection conditions have been designed to yield variants with characteristics of *mdr*-associated glucocorticoid resistance rather than the usual variants defective in glucocorticoid receptor. It should be noted that the MS23 variant would have been eliminated at the high dexamethasone concentration ($>10^{-7}$ M) commonly used in selections for glucocorticoid resistance (see Fig. 1A). The initial result of our selection was a variant (MS23) with increased resistance to dexamethasone as well as unrelated drugs, and a concomitant expression of the *mdr*1 gene. The event responsible for activation of the *mdr*1 gene in MS23 is unknown but could be either a genetic (*e.g.* promotor mutation) or an epigenetic (*e.g.* DNA demethylation) change. Activation of an *mdr* gene as a first step in acquiring multidrug resistance has been well documented (14, 15). In the case of CHO cells, it was shown that selection for colchicine resistance yielded mutants that were cross-resistant to a number of drugs and displayed increased (collateral) sensitivity, rather than resistance, to steroids such as deoxycorticosterone (33). It has often been observed that multidrug-resistant cell lines are significantly more resistant to the drug used in the selection than to the other drugs, in spite of the fact that the same *mdr* gene is involved in the resistance to the various drugs. In two instances it has recently been shown that preferential resistance to the selecting agent was the result of *mdr* gene mutations increasing the ability of P-glycoprotein to extrude the drug used in the selection. A single amino acid substitution, Gly-185 to Val-185, in the human MDR1 P-glycoprotein was shown to occur during selection in colchicine and to coincide with the emergence of preferential resistance to colchicine (34). A similar example was found in the case of the mouse *mdr*1 P-glycoprotein, where a Ser-941 to Phe-941 substitution produced a mutant protein that had retained the capacity to confer vinblastine resistance but lost the ability to confer adriamycin and colchicine resistance (35). Whether an *mdr*1 mutation has occurred during selection of MS23 cells remains to be examined.

The observation that S7CD-5 (and MS23) cells have become dexamethasone resistant while remaining sensitive to triamcinolone acetonide is a phenomenon that is unique in the field of glucocorticoid resistance. It indicates that the mechanism responsible for this phenotype recognizes a specific feature of dexamethasone, the steroid used in the selection. This preferential resistance to the selective drug appears especially striking because dexamethasone and triamcinolone acetonide are so closely related structurally. Even though

Table 1. Relative Resistance of S7CD-5 Cells to Various Steroids

	n	$LD_{50} \times 10^8$ M[a]		LD_{50}[b] S7CD-5 / LD_{50} W7TB	11-OH[c]	17-OH[d]
		W7TB	S7CD-5			
Class I						
Progesterone	4	240	145	0.60	–	–
Deoxycorticosterone	3	18	14	0.78	–	–
Cortexolone	3	145	125	0.86	–	+
Class II						
Corticosterone 21-acetate	2	5.4	13	2.4	+	–
Corticosterone	4	5.8	15	2.6	+	–
Triamcinolone acetonide	4	0.05	0.15	3.0	+	Blocked
Fluocinolone acetonide	3	0.02	0.07	3.5	+	Blocked
D-Aldosterone	4	15	55	3.7	+	–
17-Deoxydexamethasone	3	0.56	3.6	6.4	+	–
Class III						
Hydrocortisone 21-phosphate	3	5.0	68	14	+	+
Fluorometholone	3	0.41	7.3	18	+	+
Betamethasone	5	0.24	5.5	23	+	+
Hydrocortisone (cortisol)	2	4.7	111	24	+	+
Dexamethasone	9	0.60	19	32	+	+
6 α-Methyl prednisolone	2	2.5	82	33	+	+
Prednisolone	3	2.7	107	40	+	+
Prednisolone 21-acetate	3	2.8	163	58	+	+
Hydrocortisone 21-acetate	4	4.6	325	71	+	+
Triamcinolone	3	2.5	213	85	+	+

Dose-response curves to the various steroids were generated as described in *Materials and Methods* and illustrated in Fig. 1. The results represent the average of the number of determinations (n).

[a] Drug concentration required to inhibit the growth by 50%.

[b] Relative resistance calculated by dividing the drug concentration required to inhibit the growth of the S7CD-5 variant cells by 50% by that required for the parental W7TB cells.

[c] The absence (–) or presence (+) of a hydroxyl group in position 11 is indicated for each steroid.

[d] The absence (–) or presence (+) of a hydroxyl group in position 17 is indicated for each steroid. In the cases of triamcinolone acetonide and fluocinolone acetonide, the 17-OH is blocked by an acetonide group.

drug efflux pumps may seem relatively indiscriminate, minor differences in drug structure, such as between colchicine and colcemid, have been shown before to cause wide variations in relative resistance (33). The structural basis for such differences in resistance has not been examined in detail. It is fortunate that, in the case of steroids, a wide variety of analogs are available that can all bind the glucocorticoid receptor and trigger killing of W7 cells. Therefore, this phenomenon could be analyzed here in some detail. Our results (see Table 1) indicate, in particular, that the three hydroxyl groups in positions 11, 16, and 17 contribute synergistically to the specificity of the resistance phenotype. Yang *et al.* (20) concluded from their study of steroid specificity that the most hydrophobic steroids, such as progesterone, had the highest affinity for binding the mouse *mdr*1 P-glycoprotein. Our results are not necessarily in contradiction with that observation, since there is evidence on the human MDR1 P-glycoprotein for two distinct sites: one site for the initial binding of the drug, and another site associated with drug release outside of the cell (36). The second site is thought to play a significant role in determining the capacity to efflux a specific drug.

Finally, glucocorticoids, including prednisone (converted to prednisolone *in vivo*) and dexamethasone, are important components of chemotherapies especially for various types of leukemias and lymphomas (37–40). Recent results indicate that the expression of P-glycoprotein is related to clinical drug resistance in acute leukemia (41, 42). The variants described in this study provide not only the first evidence for a role of P-glycoprotein expression in glucocorticoid resistance but also represent the first instance of a form of steroid resistance that has a specificity entirely different from the specificity of the glucocorticoid receptor. A most outstanding example of this is triamcinolone acetonide, a steroid that has a higher affinity than dexamethasone for the receptor but that appears not to be transported in our variants (see Fig. 5). The observation that a cell can become resistant to prednisolone or dexamethasone while retaining its sensitivity to other steroids, such as triamcinolone acetonide, indicates that the choice of the steroid may influence the outcome of combination therapies.

MATERIALS AND METHODS

Steroids

All steroids were obtained from Sigma Chemical Company (St. Louis, MO), except for 17-deoxydexamethasone, which was a gift from Roussel Uclaf (Romainville, France).

Cell Lines

W7TB is a 5-bromodeoxyuridine-resistant derivative of the glucocorticoid-sensitive BALB/c murine thymoma line WEHI-7 (7). The isolation of the glucocorticoid-resistant variants MS23 and S7CD-5 has been described elsewhere (11). Briefly, the MS23 variant was selected for partial resistance to dexamethasone after 6 days in the presence of 5×10^{-9} M dexamethasone and after 23 days upon transfer into medium containing 7.5×10^{-9} M dexamethasone. The MS23 cell line was then cloned in the absence of steroid. The S7CD-5 variant was obtained by submitting the MS23 variant to seven rounds of selection in increasing doses of a combination of dexamethasone and colchicine. After selection the population was subcloned in the absence of drug.

Cell Culture and Drug Sensitivity Tests

Cells were grown in suspension in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, and drug sensitivity measurements were carried out as previously described (8). Briefly, cells were plated out (5×10^4cells/ml) in medium containing the appropriate concentration of drug to be tested. After 5 days of incubation the turbidity (660 nm) of the cultures was measured. This gives an estimate of the total amount of cell proliferation during that period of time. The results are expressed relative to cultures without drug.

Plasmids and Probes

The plasmid pcDR1.3 was kindly provided by Drs. James M. Croop (Harvard Medical School, Cambridge, MA) and Daniel Haber (Massachusetts Institute of Technology, Cambridge, MA). It contains a 1.3-kb *Bgl*II fragment from the middle third of the mouse *mdr*1 cDNA subcloned in pUC19 by Gros *et al.* (43). This probe cross-hybridizes extensively to homologous regions of the *mdr*2 and *mdr*3 genes or transcripts and gives an estimate of the total level of *mdr* sequences (19). The cDNA probes generated from pcDR1.3 were labeled using the multiprime labeling kit RPN.1600Z purchased from Amersham (Arlington Heights, IL) and following the procedure recommended by the manufacturer. [α-^{32}P]Deoxy-ATP was obtained from Amersham. Probes specific for *mdr*1 and *mdr*3 were generated from synthetic oligonucleotides 71 and 74 bases long, respectively, which correspond to cDNA sequences designated as a linker region (44). These oligonucleotides correspond to nucleotide positions 1915–1985 in the *mdr*1 gene (45) and positions 1906–1979 in the *mdr*3 gene (46). The oligonucleotide probes were labeled by phosphorylation with T4 polynucleotide kinase (47). The [γ-^{32}P]ATP was purchased from ICN (Irvine, CA).

Analysis of MDR Protein Expression by Flow Cytometry

Preparation of the Samples Samples of cells (1×10^7 total) were washed two times in PBS and fixed in a 4% solution of paraformaldehyde in PBS for 20 min at room temperature. The cells were then washed two times in PBS containing 10% sucrose followed by an extraction in PBS containing 0.3% Triton X-100. The fixed cells were further extracted in 100% acetone. After the acetone treatment, the samples were washed twice in PBS containing 0.05% Triton X-100 and resuspended in PBS containing 3% BSA, 0.01% Triton X-100, and horse serum as a blocking agent. The samples were divided into two portions, one of which received a monoclonal antibody (C219, Signet Laboratories Inc., Dedham, MA) specific for the *mdr*1 and *mdr*3 proteins of the mouse. The final concentration of the antibody was 20 μg/ml. The samples were incubated overnight at 4 C with continuous rotation of the tubes. After the incubation, the samples were washed four times in cold (0 C) PBS containing 0.01% Triton X-100. The samples were suspended in PBS containing 3% BSA, 0.01% Triton X-100, and a 1:100 dilution of an FITC-conjugated secondary antibody (FITC-conjugated rabbit antimouse immunoglobulin G, Sigma Chemical Co., St Louis, MO; F7506). The samples were incubated for 1 h at room temperature with continuous rotation. After the incubation with secondary antibody, the samples were washed three times at 0 C with PBS containing 0.01% Triton X-100 and finally resuspended in PBS without detergent.

Analysis by Flow Cytometry Fluorescein fluorescence was analyzed by flow cytometry using a Becton Dickinson (Mountain View, CA) FACScan. Fluorescence was measured in the FL1 channel with the standard 520-nm bandpass filter over a 4-decade log range. A minimum of 10^4 events was analyzed for each sample. The results were expressed as a histogram where both forward angle light scatter and side scatter were used to remove artifacts due to debris and cell clumping (48). The mean fluorescence values for each distribution were used to characterize the samples.

RNA Preparation and Analysis

Total cellular RNA was prepared as described by Chomczynski and Sacchi (49) and fractionated in 1.0% agarose, 2.2 M formaldehyde gels. After electrophoresis, the RNA was transferred onto nylon filters (Hybond-N from Amersham) and hybridized with the desired *mdr* probe. The filters were hybridized for 18 h at 42 C in 50% formamide, 1% BSA, 1 mM EDTA, 5% SDS, and 0.5 M $NaPO_4$, pH 7.2 (50). In order to normalize the data for quantitative variations in the RNA samples, the blots were also hybridized with a cDNA probe for the constitutively expressed CHO-B gene (51). After hybridization, the filters were washed and the amount of ^{32}P probe bound measured using autoradiography and scanning densitometry (Ultroscan XL Laser Densitometer, LKB, Bromma, Sweden).

DNA Preparation and Southern Blot Analysis

Nuclei were purified from W7TB, MS23, and S7CD5 cells as described by Gruol *et al.* (52). DNA was isolated by the method of Blin and Stafford (53). DNA samples (10 μg) were digested overnight with enzyme at 37 C, and additional enzyme was added for the final 4 h of incubation. The samples were fractionated in 1.0% agarose gels and blotted onto nylon filters (Hybond-N, Amersham) by capillary action with 20× SSPE (47). The DNA was fixed to the filters by 3 min UV irradiation. Hybridizations of the blots were carried out under the same conditions used for the Northern blot analysis.

Steroid Accumulation in Intact Cells

Measurements of steroid accumulation in intact cells were carried out by the method outlined in Pfahl *et al.* (54) with minor modifications. Briefly, cells were collected by centrifugation and resuspended ($1–2 \times 10^7$ cells/ml) in a mixture of medium and PBS (1:1). After dispensing the cells into individual tubes, ^{3}H-labeled hormone (dexamethasone, 38 Ci/mmol; triamcinolone acetonide, 29 Ci/mmol) was added and the samples placed for 1 h at 37 C in a CO_2 incubator. Parallel samples containing an excess of unlabeled triamcinolone acetonide (typically 5 μM) were used to assess nonspecific (*i.e.* not bound to glucocorticoid receptors) binding. After the incubation, the cells were washed free of unbound hormone using cold (0 C) PBS. Bound hormone was measured by scintillation counting. Specific binding was calculated by subtracting nonspecific binding (samples containing unlabeled hormone) from the total radioactivity bound without competitor.

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REFERENCES

1. Munck A, Crabtree GR 1981 Glucocorticoid-induced lymphocyte death. In: Bowen ID, Lockshin RA (eds) Cell Death in Biology and Pathology. Chapman and Hall, New York, pp 329–359
2. Cohen JJ 1989 Lymphocyte death induced by glucocorticoids. In: Schleimer RP, Claman HN, Oronsky AL (eds) Anti-Inflammatory Steroid Action. Academic Press, Inc., San Diego, pp 110–131
3. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889–895
4. Yamamoto KR, Gehring U, Stampfer MR, Sibley CH 1976 Genetic approaches to steroid hormone action. Recent Prog Horm Res 32:3–32
5. Stevens J, Stevens Y-W, Haubenstock H 1983 Molecular basis of glucocorticoid resistance in experimental and human leukemia. Biochem Actions Horm X:383–446
6. Bourgeois S, Gasson JC 1985 Genetic and epigenetic bases of glucocorticoid resistance in lymphoid cell lines. Biochem Actions Horm XII:311–351
7. Thompson EB, Smith JR, Bourgeois S, Harmon JM 1985 Glucocorticoid receptors in human leukemias and related diseases. Klin Wochenschr 63:689–698
8. Bourgeois S, Newby RF 1977 Diploid and haploid states of the glucocorticoid receptor gene of mouse lymphoid cell lines. Cell 11:423–430
9. Gruol DJ, Campbell NF, Bourgeois S 1986 Cyclic AMP-dependent protein kinase promotes glucocorticoid receptor function. J Biol Chem 261:4909–4914
10. Gruol DJ, Harrigan MT, Bourgeois S 1989 Modulation of glucocorticoid-induced responses by cyclic AMP in lymphoid cell lines. In: Roy AK, Clark J (eds) Gene Regulation by Steroid Hormones. Springer Verlag, New York, pp 41–59
11. Johnson DM, Newby RF, Bourgeois S 1984 Membrane permeability as a determinant of dexamethasone resistance in murine thymoma cells. Cancer Res 44:2435–2440
12. Juranka PF, Zastawny RL, Ling V 1989 P-Glycoprotein: multidrug-resistance and a superfamily of membrane-associated transport proteins. FASEB J 3:2583–2592
13. Gottesman MM, Pastan I 1988 The multidrug transporter, a double-edged sword. J Biol Chem 263:12163–12166
14. Endicott JA, Ling V 1989 The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu Rev Biochem 58:137–171
15. van der Bliek AM, Borst P 1989 Multidrug resistance. Adv Cancer Res 52:165–203
16. Beck WT 1987 The cell biology of multiple drug resistance. Biochem Pharmacol 36:2879–2887
17. Gros P, Raymond M, Bell J, Housman D 1988 Cloning and characterization of a second member of the mouse mdr gene family. Mol Cell Biol 8:2770–2778
18. Hsu SI-H, Lothstein L, Horwitz SB 1989 Differential overexpression of three *mdr* gene family members in multidrug-resistant J774.2 mouse cells. J Biol Chem 264:12053–12062
19. Croop JM, Raymond M, Haber D, Devault A, Arceci RJ, Gros P, Housman DE 1989 The three mouse multidrug resistance (*mdr*) genes are expressed in a tissue-specific manner in normal mouse tissues. Mol Cell Biol 9:1346–1350
20. Yang C-PH, DePinho SG, Greenberger LM, Arceci RJ, Horwitz SB 1989 Progesterone interacts with P-glycoprotein in multidrug-resistant cells and in the endometrium of gravid uterus. J Biol Chem 264:782–788
21. Yang C-PH, Cohen D, Greenberger LM, Hsu SI-H, Horwitz SB 1990 Differential transport properties of two *mdr* gene products are distinguished by progesterone. J Biol Chem 265:10282–10288
22. Qian X-d, Beck WT 1990 Progesterone photoaffinity labels P-glycoprotein in multidrug-resistant human leukemic lymphoblasts. J Biol Chem 265:18753–18756
23. Fojo A, Akiyama S-I, Gottesman MM, Pastan I 1985 Reduced drug accumulation in multiply drug-resistant human KB carcinoma cell lines. Cancer Res 45:3002–3007
24. Nelson EJ, Hinkle PA 1992 Characterization of multidrug-resistant pituitary tumor cells. Endocrinology 130:3246–3256
25. Ueda K, Okamura N, Hira M, Tanigawara Y, Saeki T, Kioka N, Komano T, Hori R 1992 Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. J Biol Chem 267:24248–24252
26. Connolly JA, Kalnins VI, Ling V 1981 Microtubules in colcemid-resistant mutants of CHO cells. Exp Cell Res 132:147–155
27. Horio M, Gottesman MM, Pastan I 1988 ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. Proc Natl Acad Sci USA 85:3580–3584
28. Safa AR, Glover CJ, Sewell JL, Meyers MB, Biedler JL, Felsted RL 1987 Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for calcium channel blockers. J Biol Chem 262:7884–7888
29. Qian X-d, Beck WT 1990 Binding of an optically pure photoaffinity analogue of verapamil, LU-49888 to P-glycoprotein from multidrug-resistant human leukemic cell lines. Cancer Res 50:1132–1137
30. Harrigan MT, Baugman G, Campbell NF, Bourgeois S 1989 Isolation and characterization of glucocorticoid and cyclic-AMP-induced genes in T lymphocytes. Mol Cell Biol 9:3438–3446
31. Gross SR, Aronow L, Pratt WB 1968 The active transport of cortisol by mouse fibroblasts growing *in vitro*. Biochem Biophys Res Commun 32:66–72
32. Gross SR, Aronow L, Pratt WB 1970 The outward transport of cortisol by mammalian cells *in vitro*. J Cell Biol 44:103–114
33. Bech-Hansen NT, Till JE, Ling V 1976 Pleiotropic phenotype of colchicine-resistant CHO cells: cross-resistance and collateral sensitivity. J Cell Physiol 88:23–32
34. Choi K, Chen C-j, Kriegler M, Roninson IB 1988 An altered pattern of cross-resistance in multidrug-resistant human cells results from spontaneous mutations in the *mdr*1 (P-glycoprotein) gene. Cell 53:519–529
35. Gros P, Dhir R, Croop J, Talbot F 1991 A single amino acid substitution strongly modulates the activity and substrate specificity of the mouse *mdr*1 and *mdr*3 drug efflux pumps. Proc Natl Acad Sci USA 88:7289–7293
36. Safa AR, Stern RK, Choi K, Agresti M, Tamai I, Mehta ND, Roninson IB 1990 Molecular basis of preferential resistance to colchicine in multidrug-resistant human cells conferred by Gly-185 Val-185 substitution in P-glycoprotein. Proc Natl Acad Sci USA 87:7225–7229
37. British National Lymphoma Investigation 1975 Value of

prednisone in combination chemotherapy of stage IV Hodgkin's disease. Br Med J 3:413–414
38. Gee TS, Haghbin M, Dowling Jr MD, Cunningham I, Middleman MP, Clarkson BD 1976 Acute lymphoblastic leukemia in adults and children. Cancer 37:1256–1264
39. Jacobs AD, Gale RP 1984 Recent advances in the biology and treatment of acute lymphoblastic leukemia in adults. New Engl J Med 311:1219–1231
40. De Vita Jr VT, Hubbard SM, Longo DL 1987 The chemotherapy of lymphomas: looking back, moving forward—The Richard and Hinda Rosenthal Foundation Award Lecture. Cancer Res 47:5810–5824
41. Kuwazuru Y, Yoshimura A, Hanada S, Utsunomiya A, Makino T, Ishibashi K, Kodama M, Iwahashi M, Arima T, Akiyama S-I 1990 Expression of the multidrug transporter, P-glycoprotein, in acute leukemia cells and correlation to clinical drug resistance. Cancer 66:868–873
42. Marie J-P, Zittoun R, Sikic BI 1991 Multidrug resistantce (*mdr*1) gene expression in adult acute leukemias: correlations with treatment outcome and *in vitro* drug sensitivity. Blood 78:586–592
43. Gros P, Neriah YB, Croop JM, Housman DE 1986 Isolation and expression of a complementary DNA that confers multidrug resistance. Nature 323:728–731
44. van der Bliek AM, Baas F, Ten Houte de Lange T, Kooiman PM, Van der Velde-Koerts T, Borst P 1987 The human *mdr*3 gene encodes a novel P-glycoprotein homologue and gives rise to alternatively spliced mRNAs in liver. EMBO J 6:3325–3331
45. Gros P, Croop J, Housman D 1986 Mammalian multidrug resistant gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. Cell 27:371–380
46. Hsu SI-H, Cohen D, Kirschner LS, Lothstein L, Hartstein M, Band Horwitz S 1990 Structural analysis of the mouse *mdr*1a (P-glycoprotein) promoter reveals the basis for differential transcript heterogeneity in multidrug-resistant J774.2 cells. Mol Cell Biol 10:3596–3606
47. Maniatis T, Fritsch EF, Sambrook J 1982 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p 122
48. Lesley J, Hyman R, Schulte R, Trotter J 1984 Expression of transferrin receptor on murine hematopoietic progenitors. Cell Immunol 83:14–25
49. Chromczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenolchloroform extraction. Anal Biochem 162:156–159
50. Church GM, Gilbert W 1984 Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995
51. Harpold MM, Evans RM, Salditt-Georgieff M, Darnell JE 1979 Production of mRNA in Chinese hamster cells: relationship of the rate of synthesis to the cytoplasmic concentration of nine specific mRNA sequences. Cell 17:1025–1035
52. Gruol DJ, Kempner ES, Bourgeois S 1984 Characterization of the glucocorticoid receptor: comparison of wild type and variant receptors. J Biol Chem 259:4833–4839
53. Blin N, Stafford DW 1976 A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res 3:2303–2308
54. Pfahl M, Kelleher Jr RJ, Bourgeois S 1978 General features of steroid resistance in lymphoid cell lines. Mol Cell Endocrinol 10:193–207