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Profound Differences in the Transport of Steroids by Two Mouse P-Glycoproteins

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ABSTRACT. There are two mouse P-glycoproteins that convey multidrug resistance, mdr1 (mdr1b) and mdr3 (mdr1a), by serving as drug efflux transporters. These proteins each exhibit tissue-specific expression. There is relatively high expression of the *mdr*1 gene in the adrenals, the site of glucocorticoid and mineralocorticoid hormone synthesis. We previously demonstrated that *mdr*1 gene expression in murine thymoma cells correlated well with a decrease in their ability to accumulate the glucocorticoid dexamethasone and their increased resistance to glucocorticoid-induced apoptosis. Additional evidence is presented that supports the proposition that the mdr1 P-glycoprotein can transport glucocorticoids. Specifically, introduction and expression of the mouse *mdr*1 gene in the human HEK 293T cell line conveys a multidrug resistance phenotype that includes a reduced capacity to accumulate dexamethasone. Moreover, isolation of additional *mdr*1-expressing mouse lymphoid cells, without using steroids in the selection, confirms the linkage between multidrug resistance conveyed by the mdr1 P-glycoprotein and resistance to dexamethasone. In contrast, two newly isolated lymphoid lines, selectively expressing the *mdr*3 gene, were not found to have increased dexamethasone resistance or the capacity to accumulate significantly lower levels of hormone. The results support the concept that the mdr1 and mdr3 P-glycoproteins may serve alternative roles in the transport of endogenous substances such as steroids. BIOCHEM PHARMACOL **58**;7:1191–1199, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. P-glycoprotein; glucocorticoid; multidrug; *mdr*1; *mdr*3; steroid

Glucocorticoid-induced transcriptional changes are initiated by hormone activation of cytoplasmic receptors that subsequently translocate into the nuclear compartment. The extent of glucocorticoid receptor activation depends upon intracellular hormone accumulation, a process believed to be driven by steroid diffusion across the plasma membrane. However, there has been evidence, since as early as 1968, that steroid accumulation may be regulated by transporters within the plasma membrane [1]. More recently, a variant of the murine T-thymoma cell line W7TB was found to exhibit reduced capacity to accumulate dexamethasone, increased resistance to dexamethasone-induced apoptosis, and increased resistance to a number of other hydrophobic drugs [2]. Acquisition of the mdr† phenotype in this variant, MS23, coincided with expression of the *mdr*1 gene [3, 4]. P-Glycoproteins are plasma membrane-spanning proteins that have been shown to cause an ATP-dependent efflux of hydrophobic drugs from cells [5–15].

Two closely related murine P-glycoproteins, mdr1 and mdr3, can cause multidrug resistance, and a great deal of overlap exists in the spectrum of drugs transported by the two proteins [16]. For instance, expression of either P-glycoprotein imparts resistance to colchicine, daunomycin, paclitaxel, puromycin, and vincristine. Actinomycin D resistance, on the other hand, is much more characteristic of mdr3 than mdr1 activity. The two mouse *mdr* genes also differ in their patterns of relative expression. The *mdr*1 gene is expressed at a high level in the adrenals and kidney, while the *mdr*3 gene is expressed preferentially in the intestine and testes [17]. During pregnancy, the uterus also expresses very high levels of the mdr1 P-glycoprotein [18]. The patterns of differential expression suggest that the two proteins may play alternative roles in selectively regulating the intracellular levels of endogenous substrates in the various tissues. A direct comparison of the relative capacity of the two mouse P-glycoproteins to reduce intracellular steroid levels has not been reported.

Only one human P-glycoprotein, MDR1, has been shown to convey multidrug resistance, and there is conflicting evidence about its ability to transport steroids. Ueda *et al.* [19] reported that expression of the *MDR*1 gene caused increased movement of dexamethasone across a layer of kidney epithelial cells. Fojo *et al.* [20], on the other hand, found that expression of the *MDR*1 gene in human KB cells does not result in decreased intracellular accumulation of dexamethasone. Moreover, the evidence supporting the transport of steroids by the mouse mdr1 P-glycoprotein, while considerable, is not conclusive. For instance, the

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† *Abbreviations*: mdr, multidrug resistance; RT–PCR, reverse transcription–polymerase chain reaction; and 5βPodo, 5β-pregnane-17α-ol-3,20-dione.

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murine MS23 cell line was isolated through a prolonged selection (4 weeks) in the presence of a moderately toxic concentration of dexamethasone [2]. The glucocorticoid receptors in MS23 cells were found to be fully functional, and the multidrug resistance phenotype was reversed by the P-glycoprotein inhibitor verapamil. Verapamil also reversed the increased resistance to dexamethasone. However, this behavior does not rule out the participation of a verapamil-sensitive steroid transporter that functions independently of P-glycoproteins. Kralli and Yamamoto [21] reported evidence for the existence of a transporter that decreased the level of dexamethasone accumulation in mouse L929 cells, but they could not detect *mdr*1 expression in these cells.

We report, here, two additional lines of evidence that the murine mdr1 P-glycoprotein is capable of causing a reduction in intracellular dexamethasone accumulation. In contrast, we have found that the murine mdr3 P-glycoprotein, if it can transport steroids, does so with a much lower efficiency than the mdr1 P-glycoprotein.

MATERIALS AND METHODS

Cell Culture

WEHI-7 is a thymoma cell line obtained from a female BALB/c mouse after exposure to x-irradiation [22]. W7TG is a derivative of WEHI-7 that is resistant to thioguanine. W7TB is a derivative of WEHI-7 and is resistant to bromodeoxyuridine. Both thioguanine resistance and bromodeoxyuridine resistance are unrelated to multidrug resistance. MS23 is a variant selected from W7TB through prolonged growth in low levels of dexamethasone [2]. S7CD-5 is a derivative of MS23 that was selected through a series of steps for resistance to the combination of dexamethasone and colchicine. The Pur^r-1.2 and MDRCP-3 cell lines were isolated from W7TB cells through single step selections with puromycin (5 μM). The PurcE-12.2 cell line was isolated through a multistep selection of W7TG cells for resistance to increasing concentrations of a combination of colchicine and puromycin. HEK 293T is a human embryonal kidney epithelial line, transformed with the adenovirus E1a and SV40 large T antigen oncogenes. All of the cell lines were grown in suspension in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The incubator was maintained at 37° and had a humidified atmosphere of 13% CO_2 and 87% air.

Isolation of Human Cells Expressing the Mouse mdr1 P-Glycoprotein

The murine *mdr*1 gene was introduced into the pMT2 [23, 24] expression vector (obtained from Dr. R. Kaufman) as follows: The pMT2 vector was digested with the *Eco*RI and *Bsa*BI enzymes to excise a fragment containing the *DHFR* gene. An *Eco*RI linker was ligated to the blunt end *Bsa*BI site. The *mdr*1 gene was excised from the pGEM7Zf plasmid (provided by Dr. Phillipe Gros) using *Eco*RI and ligated into the modified pMT2 vector. The *mdr*-containing plasmid was introduced into the bacterial strain SOLR, and the resulting colonies were evaluated for a plasmid containing the *mdr*1 gene in the proper orientation (pMT-MDR). Purified pMT-MDR plasmid was linearized by digestion with the enzyme *Ahd*I and used to transfect the human HEK 293T cell line by the calcium phosphate precipitation method. HEK 293T cells exhibiting increased drug resistance were isolated by exposing the cells to 6 μM puromycin. A colony of resistant cells was isolated and designated as 293T-MDR. These cells have increased resistance to puromycin and vincristine, but not to actinomycin D.

Quantification of Drug Effects on Cellular Proliferation

The effect of drugs on cell proliferation was measured as previously described [25]. Briefly, cell cultures were set up (5×10^4 cells/mL) in varied concentrations of drugs and incubated for 5 days. The amount of accumulated cellular material was assayed by measuring the turbidity of the cultures (660 nm) and by expressing the values as normalized to those from cultures grown in the absence of drug. These Relative Turbidity values reflect the amount of cellular material synthesized during the period of incubation and provide a sensitive measure of the capacity of the cells to proliferate, even if a large portion of them are killed. Typically, Relative Turbidity values $< 5\%$ represent situations where all of the cells have lost viability. The LC_{50} value is defined as the concentration of drug that produces a Relative Turbidity value of 50%.

The relative ability of a non-toxic pregnane (5βPodo) to reverse P-glycoprotein-dependent drug resistance in cells was evaluated as follows: MS23 and MDRCP-3 cells (5×10^4 cells/mL) were grown with a fixed concentration of puromycin (5 μM) to which they are normally resistant based upon their P-glycoprotein expression. Increasing concentrations of the pregnane were included in the culture medium, and the Relative Turbidity values of the cultures were evaluated after 5 days. The inhibitory efficiency of the pregnane is expressed by an EC_{50} value defined as the concentration of chemosensitizer that reduces the Relative Turbidity value to 50%.

RNA Preparation and Analysis

Total cellular RNA was prepared as described by Chomczynski and Sacchi [26]. For northern blot analysis, the RNA was fractionated in 1% agarose, 2.2 M formaldehyde gels. After electrophoresis, the RNA was transferred to nylon filters (Hybond-N from Amersham) and incubated with the desired probe. Probes were generated from cDNA clones encoding portions of the *mdr*1 or *mdr*3 genes. The pcDR1.3 plasmid, obtained from Dr. James Croop, was used to generate a probe that hybridizes to both *mdr*1 and *mdr*3 sequences. The BBpG4 and H3pG3 plasmids, obtained from Dr. Phillipe Gros, were used to generate probes

specific for *mdr*1 and *mdr*3 sequences, respectively. The probes were synthesized using a multiprime labeling kit and [α-^{32}P]dATP (Amersham). Probes for a constitutively expressed *CHO-B* gene [27] were synthesized in a similar fashion. The hybridization reactions were carried out for 18 hr at 42° in a solution of 50% formamide, 1% bovine serum albumin, 1 mM EDTA, 5% SDS, and 0.5 M $NaPO_4$ (pH 7.4). Hybridization was evaluated by autoradiography.

Intracellular Steroid Accumulation

Measurements of steroid accumulation in intact cells were carried out by a method outlined elsewhere [4]. Briefly, lymphoid cells were collected by centrifugation and resuspended (1×10^7 cells/mL) in fresh medium. For the measurements involving the HEK 293T cells, the cells were first treated with trypsin and resuspended with fresh medium in untreated Petri dishes to prevent their reattachment. ^{3}H-Labeled dexamethasone was added (2×10^{-8} M final concentration), and the samples were incubated for 60 min at 37° in a CO_2 incubator. After the incubation period, the cells were washed free of unbound hormone using cold (0°) PBS. Retained hormone was measured with a scintillation counter.

RESULTS

P-Glycoprotein Dependency of Dexamethasone Resistance

The MS23 cell line was isolated using dexamethasone as the selective agent. While cells with increased dexamethasone resistance were being sought, the multidrug resistance component of the phenotype was unanticipated. Additional studies indicated that levels of multidrug resistance, dexamethasone resistance, and *mdr*1 expression were directly related [3]. Co-expression of the P-glycoprotein and another non-P-glycoprotein steroid transporter, however, could not be ruled out. Moreover, dexamethasone does not induce transcription of the *mdr*1 gene (unpublished observations) in the parental W7TB cell line. Thus, the causal link between the phenotype of multidrug resistance through P-glycoprotein expression and dexamethasone resistance has not been demonstrated unambiguously. In an effort to address this question, we have isolated additional drug-resistant variants of W7TB. Instead of dexamethasone, puromycin was used to select variants without requiring that they exhibit increased steroid resistance. Figure 1 depicts the results of a northern blot analysis of the P-glycoprotein mRNA levels in two of the new cell lines, Pur^r-1.2 and MDRCP-3, in comparison to expression in the W7TB and MS23 cell lines. In each of the three sections of the figure, the lower set of bands represents hybridization to a probe for a constitutively expressed gene, *CHO-B*. In panel A, the filter was also incubated with a probe that hybridizes to both *mdr*1 and *mdr*3 sequences. Evidence of P-glycoprotein expression was observed in all samples but W7TB. The band of hybridization associated with the

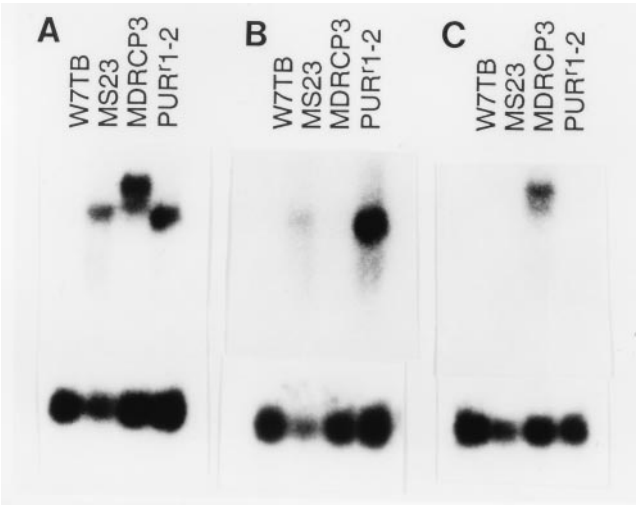

FIG. 1. Northern blot analysis of P-glycoprotein expression in the W7TB, MS23, MDRCP-3, and Pur^r-1.2 cell lines. RNA samples from each of the cell lines were analyzed by northern blotting. Three filters were generated. The results shown in panel A were obtained from the filter that was incubated with a probe that hybridizes to both the *mdr*1 and *mdr*3 sequences. The results shown in panel B were obtained with a probe that is specific for only the *mdr*1 gene. The results in panel C were obtained with a probe that is specific for the *mdr*3 gene. The hybridization shown at the bottom of each panel represents hybridization by a constitutively expressed gene (*CHO-B*) and reflects the amount of RNA present in each lane that was transferred to the filter.

MDRCP-3 sample indicated that this RNA migrated more slowly than those detected in the MS23 and Pur^r-1.2 samples. In panel B, the probe used was specific for the *mdr*1 gene. Hybridization was observed with the MS23 and Pur^r-1.2 samples, but not the MDRCP-3 sample. Panel C represents the hybridization observed when a probe specific for the *mdr*3 gene was employed. Only the MDRCP-3 sample demonstrated expression of the *mdr*3 gene. The slower migration rate of the mdr3 RNA is in agreement with observations made in previous studies [17]. Thus, the two new cell lines each express one, but not the other, of the P-glycoproteins.

Figure 2A represents the results of an evaluation of the puromycin sensitivities of the four cell lines used in Fig. 1. All three of the P-glycoprotein-expressing cell lines exhibited increased drug resistance relative to the W7TB cells. In each case, the resistance could be reversed by verapamil (data not shown). The degree of puromycin resistance associated with the MS23 and MDRCP-3 cell lines was the same, approximately 6-fold. The resistance seen with Pur^r-1.2 cells was approximately 25-fold. Figure 2B illustrates the sensitivities of the cell lines to actinomycin D. Neither MS23 nor Pur^r-1.2 cells exhibited a significant difference in sensitivity compared with W7TB cells. MDRCP-3 cells, on the other hand, had a 4-fold increase in actinomycin D resistance (LC_{50} = 2.5 ng/mL vs LC_{50} = 0.6 ng/mL for W7TB). This observation is consistent with the *mdr*3 expression in these cells demonstrated in Fig. 1. Figure 3

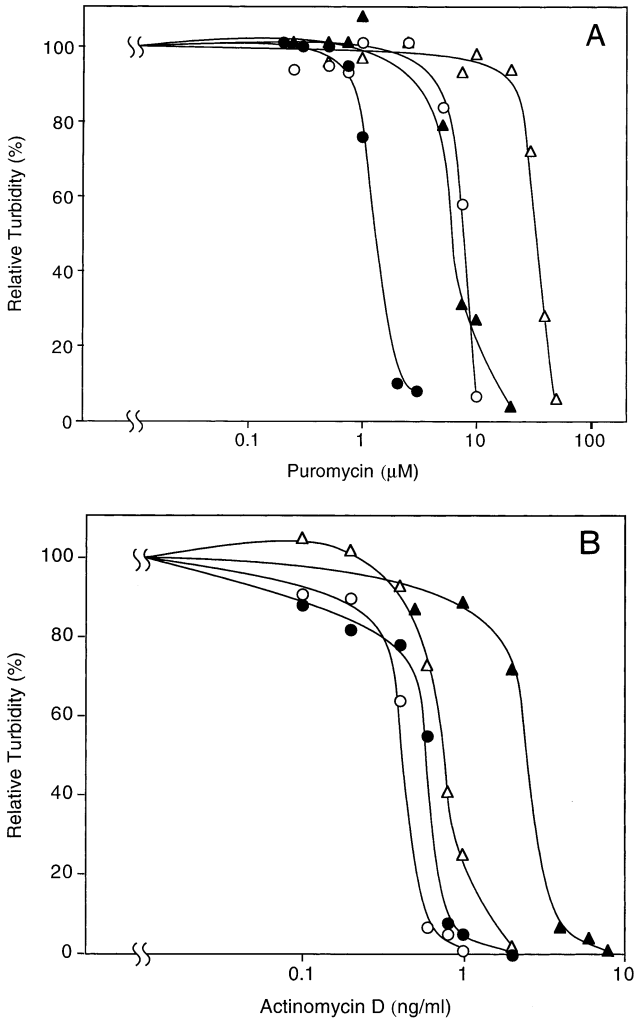

FIG. 2. Evaluation of puromycin (A) and actinomycin D (B) sensitivity in the W7TB, MS23, MDRCP-3, and Pur^r-1.2 cell lines. A series of cell cultures (5 × 10^4 cells/mL) from each cell line were grown for 5 days in increasing concentrations of the indicated drug. At the end of the incubation period, the turbidities of the cultures (660 nm) were measured and expressed relative to the values obtained from the cultures grown without drug. Each point represents the average of duplicate determinations. Similar results were obtained in two independent experiments. Key: W7TB (●); MS23 (○); MDRCP-3 (▲); and Pur^r-1.2 (Δ).

illustrates the sensitivities of the cell lines to dexamethasone. MDRCP-3 cells, expressing *mdr*3, did not show any change in sensitivity to dexamethasone. The MS23 and Pur^r-1.2 cells exhibited an increased resistance of 4- and 14-fold, respectively. Thus, by comparison, expression of the *mdr*3 gene in these lymphoid cells did not impart measurable glucocorticoid resistance.

The ability of the four cell lines to accumulate dexamethasone in the presence or absence of the P-glycoprotein inhibitor verapamil is shown in Fig. 4A. In this instance, the MS23 and Pur^r-1.2 cell lines displayed a similar (approximately 75%) reduction in accumulated steroid compared with W7TB cells. MDRCP-3 cells exhibited only a 20% reduction, potentially indicative of a relatively weak capacity by the mdr3 P-glycoprotein to transport dexamethasone. Verapamil increased the dexamethasone accumulation in the cell lines (Fig. 4B), having the greatest effect on Pur^r-1.2 cells and a much smaller effect on W7TB and MDRCP-3 cells.

Steroids also have been found to inhibit the P-glycoprotein transport of other drugs. Progesterone was shown to be more effective at reversing drug resistance in cells expressing the mdr1 than the mdr3 P-glycoprotein [28]. We have found that the compound 5βPodo is a more efficient inhibitor of the murine mdr1 P-glycoprotein than is progesterone. The pregnane also has the advantage that it does not act as a glucocorticoid agonist or antagonist. Progesterone is a partial agonist and can induce apoptosis in these lymphoid cell lines. Therefore, to test the relative capacity of steroids to inhibit the two P-glycoproteins, we have evaluated the ability of 5βPodo to reverse the puromycin resistance seen in the MS23 and MDRCP-3 cell lines. Both cell lines were derived from W7TB and exhibited a similar degree of resistance to this drug (Fig. 2A). The test was carried out by growing a series of cultures in the presence of a fixed concentration of puromycin that was not toxic to either cell line (5 μM). Progressively increasing concentrations of 5βPodo were added to the cultures to inhibit the P-glycoproteins expressed in each cell line. The results are shown in Fig. 5. The EC_{50} for 5βPodo with MS23 cells was 0.6 μM, whereas the EC_{50} with MDRCP-3 cells was 14 μM, a 23-fold difference. The control, MDRCP-3 cells grown in the absence of puromycin, demonstrated that the cells were not affected significantly by concentrations of 5βPodo as high as 30 μM. A comparable control with MS23 cells yielded the same result (data not shown). The data indicate that the interaction of 5βPodo with the mdr3 P-glycoprotein is much less effective than its interaction with the mdr1 P-glycoprotein.

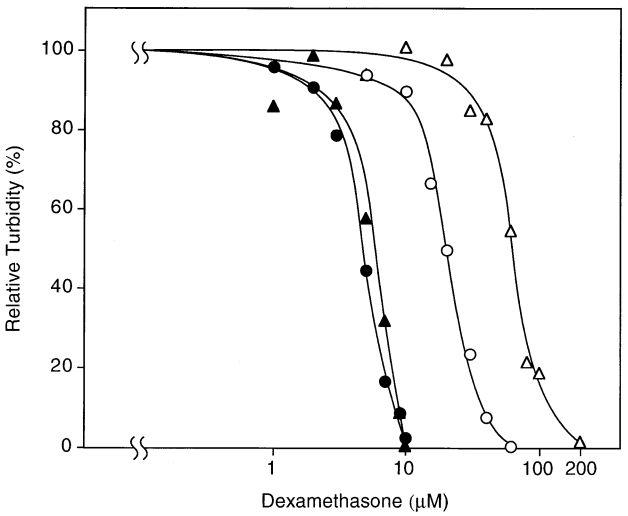

FIG. 3. Evaluation of dexamethasone sensitivity in the W7TB, MS23, MDRCP-3, and Pur^r-1.2 cell lines. A series of cell cultures were set up and incubated with increasing concentrations of dexamethasone. The results were evaluated as in Fig. 2. Key: W7TB (●); MS23 (○); MDRCP-3 (▲); and Pur^r-1.2 (Δ).

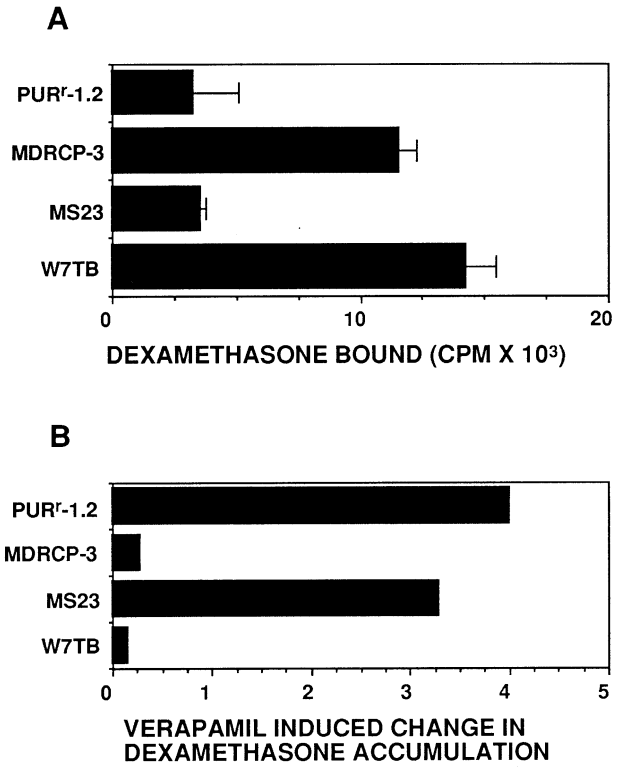

FIG. 4. Dexamethasone accumulation in W7TB, MS23, MDRCP-3, and Pur^r-1.2 cells. The ability of the four cell lines to accumulate dexamethasone in the presence or absence of verapamil was measured as described in Materials and Methods. (A) This panel depicts the amount of hormone bound to the cells of each line (per 10^7 cells) in the absence of verapamil. Thus, the amount bound reflects the relative ability of the P-glycoprotein expressed in each line to exclude 2×10^{-8} M dexamethasone. Values are the means ± SEM for triplicate determinations. (B) This panel depicts the amount of bound dexamethasone in the presence of verapamil divided by that bound in its absence (Fig. 4A). Thus, the values represent the relative increase in bound hormone when the P-glycoprotein function is inhibited. Similar results were obtained in two independent experiments.

The relatively modest levels of puromycin resistance observed with the MS23 and MDRCP-3 cells were indicative of low expression of the P-glycoprotein genes. This situation could limit detection of steroid transport by the mdr3 P-glycoprotein if it was less efficient than that caused by the mdr1 P-glycoprotein. In an effort to explore this issue more thoroughly, additional variants were isolated from W7TG cells using a selection involving increasing concentrations of the combination of puromycin and colchicine. A previous selection with the combination of dexamethasone and colchicine had led to the isolation of a variant, S7CD-5, that overexpressed the *mdr*1 gene. One of the new variants, PurcE-12.2, was found to overexpress only the *mdr*3 gene (evaluated by northern blotting, data not shown). Moreover, flow cytometry analysis of permeabilized cells exposed to the C219 monoclonal antibody (specific for P-glycoproteins) indicated that the PurcE-12.2 line expresses slightly higher levels of P-glycoprotein than does the S7CD-5 line. Figure 6A shows a comparison of the puromycin sensitivities observed with the PurcE-12.2 and S7CD-5 cell lines relative to W7TB cells. The LC_{50} ratios (relative to W7TB) were virtually the same (S7CD-5, 36x; PurcE-12.2, 39x). Figure 6B illustrates the sensitivities to dexamethasone. The LC_{50} ratio observed with S7CD-5 was 40x. In comparison, the LC_{50} ratio of PurcE-12.2 was only 1.8x, indicating little if any significant shift in dexamethasone resistance. Table 1 lists a summary of the LC_{50} values obtained with W7TB, S7CD-5, and PurcE-12.2 cells for a variety of drugs. The results confirm that both S7CD-5 and PurcE-12.2 are significantly multidrug resistant, with PurcE-12.2 exhibiting a definitive 14-fold increase in actinomycin D resistance, which is typical of *mdr*3-expressing cells. Consistent with the results shown in Fig. 6B, there was very little indication of increased resistance to dexamethasone in PurcE-12.2 cells.

Effects of Murine mdr1 Gene Expression in Human Cells

We have evaluated the effect of expressing a cloned *mdr*1 gene on steroid accumulation in a human embryonal kidney cell line, HEK 293T. The pMT2-MDR expression vector (see Materials and Methods) was transfected into the cells, and those cells exhibiting resistance to 6 μM puromycin were isolated and tested for expression of the mouse *mdr*1 gene. Expression was evaluated using RT–PCR to generate a specific fragment of the mouse *mdr*1 gene. A

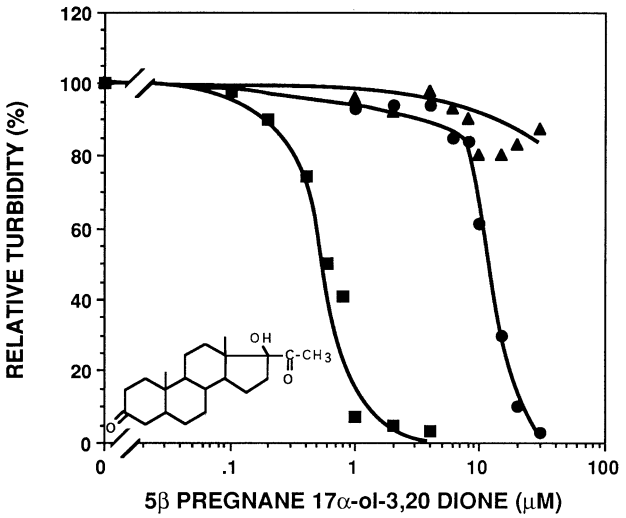

FIG. 5. Reversal of puromycin resistance by 5βPodo in cells expressing *mdr*1 or *mdr*3. A series of cultures were set up (5×10^4 cells/mL) with MS23 (■) and MDRCP-3 (●) cells. Each culture contained 5 μM puromycin. The indicated concentrations of 5βPodo were added to all of the cell cultures. A separate set of cultures containing MDRCP-3 cells without puromycin (▲) were used as a control. After incubating the cells for 5 days under these conditions, the turbidities of the cultures were measured and evaluated as for Fig. 2. Each value is the average of duplicate determinations. Similar results were obtained in two independent experiments.

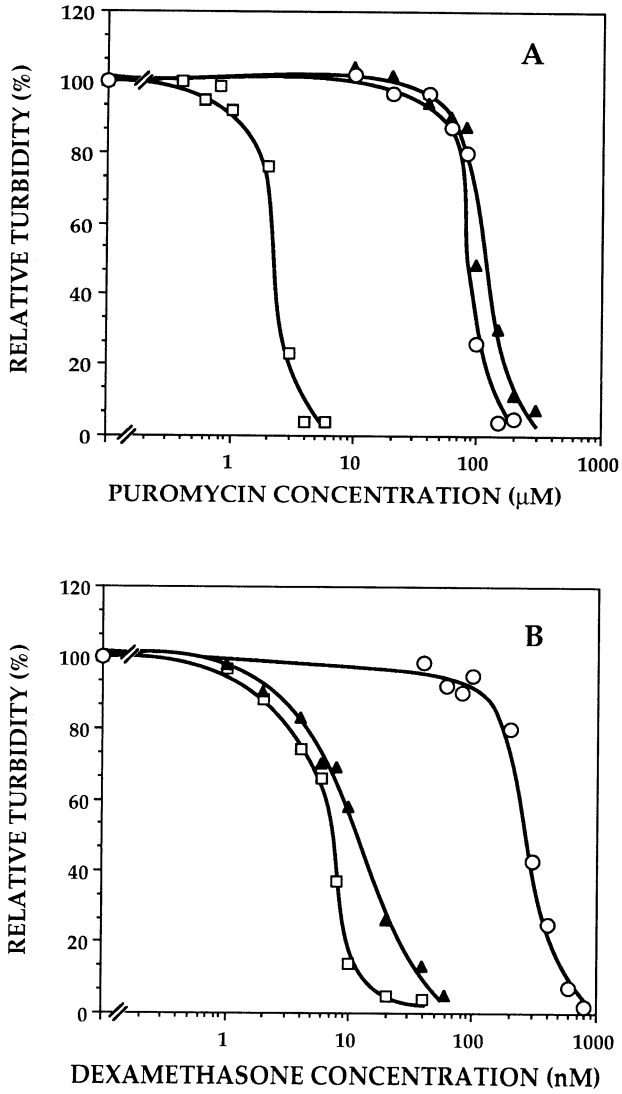

FIG. 6. Evaluation of puromycin (A) and dexamethasone (B) sensitivities in W7TB, S7CD-5, and PurcE-12.2 cell lines. A series of cell cultures (5×10^4 cells/mL) from each cell line were grown for 5 days in increasing concentrations of the indicated drug. The cultures were processed to evaluate LC_{50} values as described for Fig. 2. Each value represents the average of duplicate determinations. Key: W7TB (□); S7CD-5 (○); and PurcE-12.2 (▲).

second round of PCR, employing a nested set of primers, was used to generate a smaller subfragment (301 bp) from the samples. The sample from the drug-resistant 293T-MDR cells produced the expected fragments, whereas the sample from the non-transfected HEK 293T cells did not. The smaller subfragment was sequenced, which confirmed that it contained the appropriate portion of the mouse *mdr*1 gene. Steroid accumulation was measured in the two cell lines by incubating the cells with labeled dexamethasone in the presence or absence of verapamil. Figure 7 illustrates the results. Compared with the non-transfected HEK 293T cells, the 293T-MDR cells accumulated only 38% as much dexamethasone. The addition of verapamil had little effect on the hormone accumulation in the HEK 293T cells, but completely restored the ability of the 293T-MDR cells to accumulate dexamethasone.

DISCUSSION

The acute glucocorticoid sensitivity of W7TB cells has provided a unique tool to measure the consequences of P-glycoprotein expression upon steroid regulation. Earlier studies demonstrated that low levels (5 to 7.5 nM) of dexamethasone could be used to isolate variants of W7TB displaying an increase (2- to 5-fold) in steroid resistance. Nearly all of these variants had a partial loss of glucocorticoid receptor function. However, one variant, MS23, had a very different phenotype. It had no measurable loss in receptor function, exhibited a multidrug resistance phenotype, and expressed the *mdr*1 gene [2, 3]. Additional variants, such as S7CD-5, were isolated from MS23 cells using a combination of dexamethasone and colchicine. S7CD-5 was found to overexpress the *mdr*1 gene and have a further increase in resistance to dexamethasone as well as other drugs. In both cell lines, the level of steroid resistance was accompanied by a coordinate reduction in the ability of the cells to accumulate dexamethasone. Steroid resistance in the variant cell lines was reversed completely by verapamil, suggesting that P-glycoproteins were responsible for transporting dexamethasone out of the cells. No expression of the *mdr*3 gene was detected in either the MS23 or S7CD-5 cell lines. Thus, the ability of the mdr3 P-glycoprotein to convey dexamethasone resistance was not evaluated by these studies. The capacity of the mdr1 P-glycoprotein to cause reduced dexamethasone accumulation was demonstrated more definitively in this study by introducing the gene into the human embryonal kidney cell line HEK 293T. These cells contain glucocorticoid receptors, but do not undergo glucocorticoid-induced apoptosis. We found that the *mdr*1-expressing transfected cells, 293T MDR, accumulated 60% less dexamethasone than did HEK 293T cells and that the difference was abrogated completely by verapamil. Northern blot analysis did not detect any expression of the human *MDR*1 gene in either the HEK 293T or 293T-MDR cells (data not shown).

There are a number of other murine lymphoid lines that respond to glucocorticoids by undergoing apoptosis. Galski *et al.* [29] used S49 cells as a recipient for the human *MDR*1 gene in transfection experiments. The effect of expressing the P-glycoprotein upon S49 steroid sensitivity was not reported. A *mdr*1-expressing derivative of lymphoid L1210 cells, L1210 DN, has been studied [17], but not evaluated relative to altered ability to accumulate steroids. We have tested L1210 cells and found them to be much less sensitive to dexamethasone-induced apoptosis than W7TB cells. However, we did find evidence that the drug-resistant variant L1210 DN was more resistant to dexamethasone suppression of proliferation than L1210 cells. Moreover, L1210 DN cells exhibited a verapamil-sensitive reduction in dexamethasone accumulation (data not shown). In another study, Wolf and Horwitz [30] found that the

TABLE 1. Comparison of LC_{50} values of drugs transported by P-glycoproteins in cells expressing the *mdr*1 and *mdr*3 genes*

Cell line	LC_{50} values				
	Paclitaxel†	Colchicine‡	Puromycin§	Dexamethasone†	Actinomycin D‡
W7TB	10 ± 2.1	15.8 ± 6.7	2.5 ± 0.25	6.5 ± 0.55	0.90 ± 0.04
S7CD-5	175 ± 50 (18x)‖	286 ± 36 (18x)	88.7 ± 2.2 (35x)	237 ± 41 (36x)	1.12 ± 0.1 (1.2x)
PurcE-12.2	278 ± 38 (28x)	309 ± 63 (20x)	91.0 ± 11 (36x)	11.3 ± 4.3 (1.7x)	13.0 ± 1.9 (14x)

*Each value represents the mean ±SD of LC_{50} measurements made in three separate experiments.
†LC_{50} Values are expressed in nM.
‡LC_{50} values are expressed in ng/mL.
§LC_{50} Values are expressed in μM.
‖Values shown within the parentheses are relative LC_{50} values normalized to the LC_{50} values obtained with the W7TB cell line.

multidrug-resistant murine cell line J7.V-1 accumulated approximately one-third of the level of corticosterone seen in its progenitor line, J774.2. Verapamil (50 μM) caused a 50% reversal of the effect on steroid accumulation in the J7.V-1 cells. J7.V-1 cells overexpress the *mdr*1 gene and are highly resistant to vinblastine compared with the J774.2 cells. Thus, the effect on steroid accumulation was small compared to what might be expected based upon the overall drug resistance. Corticosterone, however, appears to be a relatively weak substrate for transport by the mdr1 P-glycoprotein compared with dexamethasone. Our murine thymoma variant S7CD-5 is 30- to 40-fold more resistant to dexamethasone, but only 2- to 3-fold more resistant to corticosterone [3].

Studies of glucocorticoid receptor activation of transcription in yeast have produced additional evidence for the existence of steroid transport proteins. Expression of the yeast LEM1 protein, a putative drug transport protein, can cause reduced dexamethasone accumulation in yeast cells [31]. Moreover, the effect was steroid-specific; it did not affect deoxycorticosterone accumulation. Steroid specificity is a property that we had observed for the mdr1 P-glycoprotein. The results suggested that steroid efflux by drug transporters may be a more general phenomenon than is appreciated currently and may be part of the broad range of drug resistance seen with these proteins. One way to test this hypothesis was to select for additional cell lines expressing P-glycoproteins, but without using dexamethasone. Selections with puromycin resulted in the isolation of the Pur^r-1.2 and MDRCP-3 cell lines. Isolation of the Pur^r-1.2 cell line confirmed that steroid resistance was linked to mdr1 P-glycoprotein expression. Isolation of MDRCP-3, on the other hand, demonstrated that the link between steroid resistance and P-glycoprotein expression was gene-specific. No steroid resistance was observed in these cells, which suggests that the mdr3 P-glycoprotein does not transport dexamethasone effectively. This conclusion is supported by the observation that there was a profound difference in the ability of steroids to reverse puromycin resistance in cells expressing the mdr1 and mdr3 P-glycoproteins. 5βPodo was an efficient inhibitor of P-glycoprotein function in cells expressing the mdr1 P-glycoprotein. This pregnane was much less effective in cells expressing the mdr3 P-glycoprotein. While MS23 and MDRCP-3 exhibited very similar degrees of puromycin resistance, MDRCP-3 cells required 23-fold more 5βPodo to produce a comparable reversal of resistance. A similar distinction has also been reported with progesterone [28]. If this behavior reflects the relative ability of glucocorticoids to interact productively with P-glycoproteins, it explains why the mdr3 transporter does not cause as great a reduction in steroid accumulation and why *mdr*3 expression does not convey dexamethasone resistance to the MDRCP-3 cells.

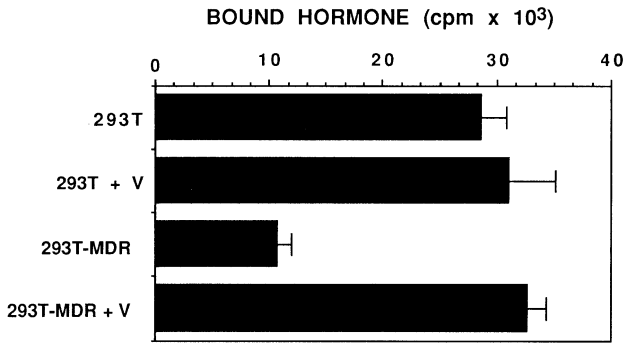

FIG. 7. Dexamethasone accumulation in HEK 293T and 293T-MDR cells. Samples of HEK 293T and 293T-MDR cells were brought into suspension by treatment with trypsin, placed into fresh medium, and dispensed into plastic Petri dishes (3.5 mL each, 10^7 cells total). [^{3}H]Dexamethasone was added to the cultures at a final concentration of 2×10^{-8} M, and the samples were incubated in a CO_2 incubator for 1 hr at 37°. In a parallel set of cultures, verapamil was added to a final concentration of 10 μM. After the incubation period, the cells were collected by centrifugation and washed two times in cold (0°) PBS. The amount of hormone remaining bound to the cells was measured in a scintillation counter. The values represent the means ± SEM for triplicate determinations. Similar results were obtained in two independent experiments.

In comparison to our results, Schinkel *et al*. [32] reported two lines of evidence that the mouse mdr3 P-glycoprotein can transport dexamethasone. Tissue distribution of a variety of drugs were measured comparing normal and *mdr*3 (−/−) "knockout" mice. Of the ten tissues studied, only the brain exhibited a significant increase (2.5-fold) in dexamethasone accumulation. This was interpreted as indicative of the participation of the mdr3 P-glycoprotein in the exclusion of steroids by the blood–brain barrier. In a second approach, the *mdr*3 gene was transfected into a

porcine kidney epithelial cell line, LLC-PK1. Cells exhibiting increased drug resistance (L-mdr1a) were selected in a relatively high concentration (640 nM) of vincristine. Since P-glycoproteins are expressed on the apical surface in these cells, monolayers of cells grown on porous membranes can be used to measure the rate of drug transport from the basal medium compartment to the apical compartment. These studies produced convincing evidence of dexamethasone transport by the *mdr*3-expressing L-mdr1a cells. No measure of the intracellular levels of dexamethasone was demonstrated. Several reasons could account for the apparent difference between our results and those obtained with the L-mdr1a cells. First, the transport assay used by Schinkel *et al.* may be more sensitive than measuring intracellular steroid accumulation. This sensitivity, combined with the high level of P-glycoprotein expression observed in the L-mdr1a samples, could account for a positive indication of transport even if the transport was relatively inefficient. These studies were not carried out as a direct comparison with cells expressing the mdr1 P-glycoprotein. A second possibility is that the selection of cells with increased vincristine resistance resulted in the isolation of cells with increased expression of a porcine P-glycoprotein along with that of the transfected mdr3 P-glycoprotein. No evidence was presented to verify that the porcine P-glycoprotein gene expressed in the LLC-PK1 cells was maintained at the same level in the L-mdr1a cells. If the porcine protein is similar to the mouse mdr1 protein, an efficient dexamethasone transporter, the contribution of a relatively small increase in its expression might be detected by a sensitive transport assay.

In summary, our studies have produced additional evidence that the mouse *mdr*1 expression can alter the ability of cells to respond to dexamethasone by limiting hormone available to their glucocorticoid receptors. We have not found this to be true for expression of the mouse mdr3 P-glycoprotein. Since the basis for the phenomenon of transporter-dependent multidrug resistance is the failure of drugs to accumulate at their intracellular targets, we conclude that only the mouse mdr1 and yeast LEM1 proteins have demonstrated this ability for steroids.

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