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Genetic transfer of non-P-glycoprotein-mediated multidrug resistance (MDR) in somatic cell fusion: Dissection of a compound MDR phenotype

ELISABETH W. H. M. EIJDEMS*, PIET BORST*†, ANS P. M. JONGSMA*, STEVEN DE JONG‡§, ELISABETH G. E. DE VRIES‡, MARJON VAN GROENIGEN¶, CAROLINA H. M. VERSANTVOORT‖, AGGIE W. M. NIEUWINT**, AND FRANK BAAS*¶

*Division of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands; ‡Division of Medical Oncology, University Hospital, 9713 EZ Groningen, The Netherlands; ¶Division of Neurology, Academical Medical Center, 1105 AZ Amsterdam, The Netherlands; ‖Department of Medical Oncology and **Cytogenetics Laboratory, Free University Hospital, 1080 HV Amsterdam, The Netherlands

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ABSTRACT **A non-P-glycoprotein-mediated mechanism of multidrug resistance (non-Pgp MDR) has been identified in doxorubicin-selected sublines of the human non-small cell lung carcinoma cell line SW-1573. These sublines are cross-resistant to daunorubicin, VP16-213, *Vinca* alkaloids, colchicine, gramicidin D, and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (m-AMSA). They accumulate less drug than the parental cells and their resistance is not due to the *MDR1*-encoded P-glycoprotein, as the resistant cell lines have lost the low amount of *MDR1* mRNA detectable in parental cells. Here we show that the resistant cell lines also contain less topoisomerase II mRNA and enzyme activity than the parental cells. This might contribute to the resistance of these lines to drugs interacting with topoisomerase II, such as doxorubicin, daunorubicin, and VP16-213, but cannot account for the resistance to the other drugs. We have tested whether all properties of the non-Pgp MDR cell lines cosegregate in somatic cell fusions between lethally γ-irradiated, resistant donor cells and drug-sensitive acceptor cells. Whereas a MDR phenotype with reduced drug accumulation and the loss of *MDR1* P-glycoprotein mRNA were cotransferred to the acceptor cells, the decrease in topoisomerase II gene expression was not. We conclude that the MDR phenotype, the reduced drug accumulation, and the loss of *MDR1* P-glycoprotein mRNA are genetically linked. They might be due to a single dominant mutation, which does not cause the alteration in topoisomerase II.**

Cells selected for resistance to a natural, cytotoxic product may become cross-resistant to a series of large, hydrophobic, cytotoxic drugs that do not share a common structure or target (1, 2). Such multidrug-resistant (MDR) cells usually contain increased levels of P-glycoprotein, a membrane protein that can act as a drug efflux pump, actively lowering the intracellular drug concentration (3–7). Not all MDR cell lines contain increased levels of P-glycoprotein, however. Some of these lines are only resistant to drugs that interact with topoisomerase II (8–11). These lines contain either an altered topoisomerase II (10, 11) or a decreased level of topoisomerase II (8, 9). Other cell lines have been described, however, that are resistant to many of the drugs extruded by P-glycoprotein even though they do not detectably overexpress P-glycoprotein (12–19). The molecular basis of resistance in these non-P-glycoprotein-mediated MDR (non-Pgp MDR) cell lines has not been identified.

Clinical studies to date have failed to indicate a consistent correlation between the level of *MDR1* P-glycoprotein with nonresponse to chemotherapy in some of the major human cancers, such as lung and breast cancer (20, 21). Therefore elucidation of the molecular basis of non-Pgp MDR should provide the tools to test whether this form of drug resistance can account for therapy failure in these tumors.

To clarify the mechanism of such a form of non-Pgp MDR we have studied three resistant cell lines, independently derived from the human non-small cell lung cancer cell line SW-1573 (12, 22, 23). These lines, obtained by doxorubicin selection, are resistant to a wide range of MDR drugs (Table 1 and ref. 12) and they have a decreased accumulation of daunorubicin, vincristine (24), and VP16-213 (36). Both cannot be reversed by verapamil. Another remarkable feature of these lines is the loss of the *MDR1* P-glycoprotein mRNA, whereas this is readily detectable in the parental SW-1573 cells (12). Transcripts of the other human P-glycoprotein gene, the *MDR3* gene (3), are not detectable either in the parental cell line or in the non-Pgp MDR cell lines (12). A contribution of the *MDR1* and *MDR3* P-glycoproteins to the resistance of the non-Pgp MDR cell lines is therefore excluded.

To investigate whether the entire phenotype of these cell lines is due to a single, dominant mutation, we have tested here whether it could be transferred by somatic cell fusion from lethally γ-irradiated, resistant donor cells to drug-sensitive parental acceptor cells.

MATERIALS AND METHODS

Cell Culture. Doxorubicin-resistant cell lines were derived from the human non-small cell lung cancer cell line SW-1573 and were isolated as described (12, 22, 23). The non-Pgp MDR cell lines 1R50b and 2R50 were isolated from the drug-sensitive cell line S1 by a multistep doxorubicin selection up to 50 nM, whereas cell line 3R80 was isolated by a single step at 80 nM doxorubicin and was subcloned, resulting in cell line 3R8. In the selection procedure the 1R50b and 2R50 cell lines were cloned once, and karyotypic analysis of these lines shows that they are homogeneous (A.W.M.N., unpublished data). For the cell fusion experiments the cell lines were recloned. As acceptor, a subclone of the drug-sensitive parental cell line S1 transfected with pSV2α3.6 (25), which encodes the α-1 subunit of the mouse sodium/potassium exchanger, conferring >1000-fold resistance to ouabain, was used, S1ou. As donor, we used a subclone of the non-Pgp MDR cell line 1R50b transfected with pCDneo (26), conferring resistance to G-418 sulfate, 1R50bneo. For

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Abbreviations: MDR, multidrug resistance; non-Pgp MDR, non-P-glycoprotein-mediated mechanism of MDR.

†To whom reprint requests should be addressed.
§Present address: Department of Genetics, University of Groningen, 9751 NN Haren, The Netherlands.

selection of these clones, 1 μM ouabain or 0.8 mg of G-418 per ml was used. The transfected, subcloned cell lines showed the same sensitivity to other cytotoxic drugs as their nontransfected parents. All cells were free of *Mycoplasma* as tested by the use of the Gen-Probe rapid *Mycoplasma* detection system (Gen-Probe, San Diego).

Assay of Drug Resistance. The clonogenic survival assay was carried out as described by Baas *et al.* (12). The relative resistance was calculated as the ratio of IC_{10} (inhibitory concentration where 10% of the cells survive) of the resistant cell line to the IC_{10} of the parental cell line.

Topoisomerase II Catalytic Activity. Nuclear extracts were isolated as described by Pommier *et al.* (11). The catalytic activity of topoisomerase II was measured in a decatenation assay (27, 28). The decatenation of kinetoplast DNA (29) was visualized on agarose gels stained with ethidium bromide and analyzed using IMAGE, a public domain program by Wayne Rasband (National Institutes of Health, Bethesda, MD). Ratios between the densities of each decatenated end product of a non-Pgp MDR cell line and of the same decatenated end product of cell line S1 at each nuclear extract amount were plotted against the extract amounts and used to calculate the catalytic activity.

RNase Protection. Cytoplasmic RNA was isolated by a Nonidet P-40 lysis procedure (30). The RNase protection was carried out according to Zinn *et al.* (31), modified by Baas *et al.* (12). The following probes were used: *MDR1*, a 301-nucleotide *MDR1* cDNA fragment [nucleotide positions 3500–3801 according to Chen *et al.* (32)]; topoisomerase II, a 174-nucleotide topoisomerase II cDNA fragment [nucleotide positions 1343–1517 according to Wang and coworkers (33)]. In all experiments a probe for γ-actin (34) was included to measure RNA recovery. The intensity of the topoisomerase II and γ-actin mRNA bands in each sample was determined by scanning the autoradiographs with a Beckmann DU-8 scanning system; topoisomerase II mRNA levels were corrected for the amount of γ-actin mRNA.

Somatic Cell Fusion. The procedure for the somatic cell fusion experiments is shown in Fig. 1. 1R50bneo cells were lethally γ-irradiated with a dose of 15 Gy [with two ^{137}Cs sources of 415 Ci each (1 Ci = 37 GBq), Von Gahlen Nederland B.V., Didam, The Netherlands]. S1ou cells (0.7×10^6) and γ-irradiated 1R50bneo cells (3.3×10^6) were mixed, spun down, resuspended in Ham's F-10 culture medium, and plated on a 100-mm culture dish (Falcon 3003, Becton Dickinson). When the cells were attached to the tissue culture dish, they were fused using 2 ml of polyethylene glycol 1500 (PEG 1500; 783641 Boehringer Mannheim, according to the manufacturer's suggested conditions). The following day the cell population was trypsinized and 1/15th was reseeded per 150-mm tissue culture dish (Falcon 3025, Becton Dickinson). The next day one part of the cell population was selected with 1 μM ouabain and 800 μg of G-418 per ml for the first 5 days, followed by selection with 400 μg of G-418 per ml. The other part of the cell population was selected for the first 5 days with 1 μM ouabain and with one of the following drugs: vincristine (12 nM), doxorubicin (50 nM), or VP16-213 (100 nM). After 5 days the ouabain selection was stopped, and vincristine or doxorubicin selection continued at the same level whereas VP16-213 selection was reduced to 50 nM. Drug-resistant colonies were isolated after 2 weeks of selection.

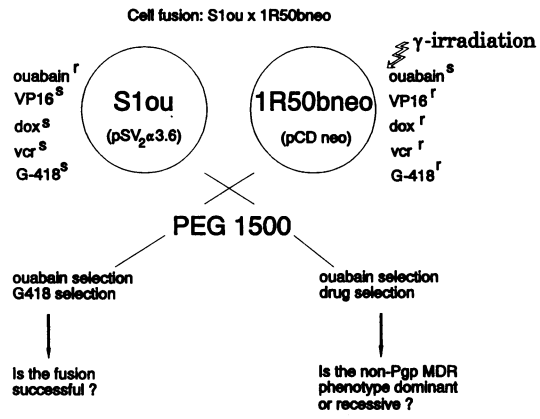

FIG. 1. Schematic representation of the somatic cell fusion experiment between γ-irradiated non-Pgp MDR cell line 1R50bneo and the drug-sensitive parental cell line S1ou. The combinations of drugs used for selection, after fusion with PEG 1500, are indicated. [s], Sensitive; [r], resistant; dox, doxorubicin; vcr, vincristine.

Cellular Drug Accumulation. Steady-state accumulation of drugs was measured according to Broxterman *et al.* (35). Cells in the logarithmic phase of growth ($0.2–1 \times 10^6$ cells per ml) were incubated at 37°C with [G-^{3}H]vincristine (diluted with unlabeled vincristine to a final vincristine concentration of 1 μM) or [14-^{14}C]daunorubicin (diluted with unlabeled daunorubicin to a final daunorubicin concentration of 0.5 μM). After 60 min ice-cold phosphate-buffered saline was added to the cells and after two cold washes, the cells were transferred to liquid scintillation fluid Opti-Phase III (LKB) and radioactivity was measured. Values were corrected for the amount of cell-associated radioactivity at time zero at 0°C.

RESULTS

Level of Topoisomerase II in the Non-Pgp MDR Cell Lines. In view of the high resistance of the non-Pgp MDR cell lines to drugs known to interact with topoisomerase II (see Table 1), we investigated whether topoisomerase II activity was decreased or altered in the non-Pgp MDR cell lines by decatenation of kinetoplast DNA in nuclear extracts. At equal protein concentrations, nuclear extracts from the non-Pgp MDR cell lines 1R50b and 2R50 contained less decatenating activity than the nuclear extracts derived from the parental cell line (Table 2). This difference was present in logarithmic phase and in confluent cells. The topoisomerase II activities of the cell lines 1R50b, 2R50, and S1 were equally sensitive to VP16-213 (data not shown), indicating that the lowered topoisomerase II of the non-Pgp MDR cell lines was functionally equivalent to topoisomerase II of the drug-sensitive parental cell line.

The reduction of topoisomerase II activity was accompanied by a reduction in topoisomerase II protein (immunoblot not shown). RNase protection experiments using an antisense topoisomerase II cDNA probe showed that the non-Pgp MDR cell lines (1R50b, 2R50, and 3R8) contained reduced topoisomerase II mRNA levels as well (Fig. 2). This decrease was found in logarithmic phase and in confluent cells (Table 2). The Pgp-overproducing cell line 1R500-0, derived from 1R50b by prolonged doxorubicin selection (23), also showed the decrease in topoisomerase II mRNA, whereas this was not present in cell line S1 1.1, which overproduces Pgp upon transfection of *MDR1* cDNA (Fig. 2).

Genetic Transfer of the Non-Pgp MDR Phenotype. To examine whether the characteristics of the non-Pgp MDR cell lines—i.e., MDR phenotype, reduced drug accumulation, loss of *MDR1* P-glycoprotein mRNA, and lowered topoisomerase II expression—are all due to a single dominant mutation, we determined whether they are cotransferred to a sensitive recipient cell in somatic cell fusion experiments. For these experiments we used the non-Pgp MDR cell line 1R50b as donor and the parental cell line S1 as recipient. To allow selection for hybrids after the fusions, selectable markers were introduced into donor and acceptor cells. S1 was transfected with pSV2α3.6, resulting in the ouabain-resistant

Table 1. Drug resistance in SW-1573 derivatives and in the somatic cell hybrids

Selection	S1 IC_{10}, nM	Resistance factor*				
		1R50b	2R50	3R8	Non-Pgp fused cells†	Pgp fused cells‡
Doxorubicin	15	5	5	5.5	3.2 (2.0–4.3)	2.3–4.2
Daunorubicin	5	5	5	5.6	ND	ND
Vincristine	2.5	3	1.5	3	6.3 (4.0–8.0)	14.3–17.5
VP16-213	21	10	10	10	2.5 (1.8–4.0)	3.5–5.1
m-AMSA	50	2	2	2	0.2 (0.2–0.3)	ND
Colchicine	11	2	2	2	3.7 (3.4–4.1)	ND
Gramicidin D	100	3	3	ND	4.4 (3.7–4.9)	ND

ND, not determined. m-AMSA, 4'-(9-acridinylamino)methanesulfon-*m*-anisidide.
*Resistance factor = IC_{10} cell line/IC_{10} parental cell line S1 (IC_{10} = inhibitory concentration where 10% of the cells survive). Data are from at least two experiments, each performed in duplicate.
†The mean resistance and the range (in parentheses) of five somatic cell hybrids F6.1–F6.4 and F10.12.3, which have no detectable *MDR1* P-glycoprotein mRNA.
‡The range of resistance of the two independent somatic cell hybrids F10.10.1 and F10.10.2, which have a moderate level of *MDR1* P-glycoprotein mRNA.

cell line S1ou, and 1R50b was transfected with pCDneo, resulting in the G-418-resistant cell line 1R50bneo (see *Materials and Methods*). To control the direction of the gene transfer, the non-Pgp MDR cell line 1R50bneo was lethally γ-irradiated with 15 Gy prior to fusion.

Cells were fused with PEG 1500 and fused cells were selected with a combination of ouabain and G-418. Ouabain was included in the selection medium to rapidly kill the γ-irradiated 1R50bneo, allowing the fused cells to grow. The control experiments presented in Table 3 show that the γ-irradiated 1R50bneo cells did not survive at all and that the parental S1ou acceptor cells did not survive in the presence of G-418. After fusion of S1ou cells to γ-irradiated 1R50bneo cells, however, cells resistant to G-418 and ouabain were obtained (see Table 3), indicating that the fusions were successful.

To test for transfer of the non-Pgp MDR phenotype, similar experiments were performed in which doxorubicin, VP16-213, or vincristine each in combination with ouabain was used for selection after the cell fusion. The only cells that can be expected to grow under these selection conditions are S1ou cells that have received the mutation conferring the non-Pgp MDR from the 1R50bneo cells or in which MDR has been induced by the fusion procedure. The experiments summarized in Table 3 for vincristine/ouabain selection show that the frequency of drug-resistant cells obtained by PEG 1500 fusion is at least 15 times higher when a non-Pgp MDR donor cell is used instead of a drug-sensitive S1 donor cell. In these experiments no colonies appeared on tissue culture dishes containing the γ-irradiated 1R50bneo alone, and only zero to two colonies appeared on dishes containing the control S1ou cells (Table 3). These results indicate the transfer of a drug-resistant phenotype from cell line 1R50bneo to the drug-sensitive parental cell line S1ou by somatic cell fusion.

Table 2. Topoisomerase II activity and mRNA level of the non-Pgp MDR cell lines

Cell line	Decatenation activity, %		mRNA level, %	
	Confluent*	Logarithmic phase*	Confluent†	Logarithmic phase*
S1	100	100	100	100
1R50b	24 (20–27)	28 (23–34)	27	20 (15–23)
2R50	69 (63–74)	26 (24–28)	63	49 (41–58)
3R8	ND	ND	ND	28 (25–32)

The decatenation activity of the confluent cells was about 0.6× the decatenation activity of the logarithmic phase cells in these experiments. The mRNA level of the confluent cells was about 0.3× the mRNA level of the logarithmic phase cells in these experiments. ND, not determined.
*Number of experiments = three; the mean decatenation activity or mRNA level and the range (in parentheses) are given.
†Number of experiments = one.

Analysis of the Fused Cells. Cytogenetic analysis some weeks after fusion showed that the fused cells contained all karyotypic markers of the drug-sensitive parental cell line S1 and none of the alterations that were considered to be specific for non-Pgp MDR cell line 1R50b (data not shown). All cell lines obtained by cell fusion were cross-resistant to several drugs with different structures and cellular targets and thus have a MDR phenotype. The mean resistance levels of the fused cell lines (F6.1–F6.4, F10.12.3, F10.10.1, and F10.10.2) are shown in Table 1. Two fused cell lines (F10.10.1 and F10.10.2) were more resistant to vincristine compared to the other fused cell lines.

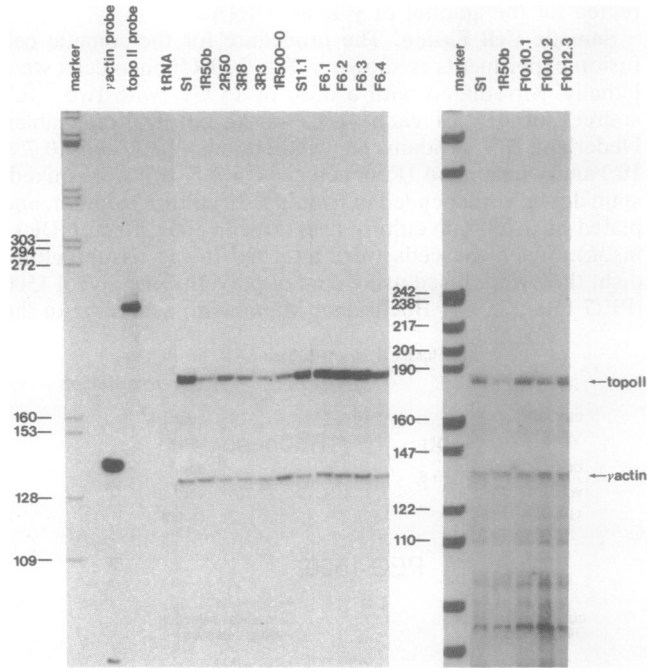

FIG. 2. RNase protection assay to quantify topoisomerase II mRNA levels in non-Pgp MDR cell lines and in MDR cells obtained after somatic cell fusion. Ten micrograms of total cytoplasmic RNA from each SW-1573 derivative was used. Positions of the protected fragments of topoisomerase II and γ-actin mRNA as well as the size (nucleotides) of the molecular weight markers are indicated. Probes for topoisomerase II, γ-actin, and a tRNA control are shown (lanes topo II, γ-actin, tRNA). Positions of the molecular weight markers are indicated.

Table 3. Colonies obtained in somatic cell fusion

Cells	Selection			
	G-418/ ouabain	G-418	Vincristine/ ouabain	Vincristine
Slou 1R50bneo* (γ-irradiated)	25 (20–30)	25 (20–30)	15 (12–18)	ND
Slou S1† (γ-irradiated)	ND	ND	1 (0–2)	1 (0–2)
Slou*	0	0	1 (0–2)	1 (0–2)
1R50bneo* (γ-irradiated)	0	0	0	0
S1† (γ-irradiated)	0	0	0	ND

ND, not determined. The mean number of colonies per dish per fusion and the range (in parentheses) are given.
*The number of independent fusion experiments was 10; per fusion experiment three dishes were analyzed at the selection conditions indicated.
†The number of independent fusion experiments was 2; per fusion experiment three dishes were analyzed at the selection conditions indicated.

The fused cells accumulated less daunorubicin and vincristine than the drug-sensitive parental cell line (see Table 4 for cell line F6.1–F6.4). The accumulation defect of the fused cells for vincristine, however, was smaller than that of the non-Pgp MDR donor cell line 1R50bneo, although they had a higher resistance level (Table 1). We attribute this discrepancy (in part) to the larger volume and thus the increased amount of binding sites for vincristine of the fused cells compared to the 1R50bneo donor cells. In a Coulter Counter calibrated with microbeads, the average volume of the fused cells was about 50% larger than that of the donor cells.

To examine whether the alterations in *MDR1* and topoisomerase II gene expression are cotransferred with drug resistance from the 1R50bneo donor cells to the sensitive parental cells, the *MDR1* and topoisomerase II mRNA levels were determined in the MDR fused cells. As shown in Fig. 3, five of the seven fused cell clones analyzed (F6.1–F6.4 and F10.12.3) have no detectable *MDR1* P-glycoprotein mRNA, whereas *MDR1* P-glycoprotein mRNA is readily detectable in the drug-sensitive parental cell line S1. Thus, in these clones, the decrease of *MDR1* P-glycoprotein mRNA level is cotransferred with resistance to the parental cell line in these somatic cell hybrids. In contrast, all fused cells have topoisomerase II expression levels identical to the parental cell line S1 (Fig. 2), showing that down-regulation of topoisomerase II gene expression is not transferred in the somatic cell fusion. Two of the seven fused cell lines (F10.10.1 and F10.10.2) had an increased level of *MDR1* P-glycoprotein expression relative to the parental cell line (Fig. 3). These were the cell lines with the high vincristine resistance (Table 1).

Table 4. Accumulation of vincristine (1 μM, 60 min) and daunorubicin (0.5 μM, 60 min) in SW-1573 cell lines and in fused cells

Cell line	Vincristine,* pmol per 10^6 cells	%	Daunorubicin,† pmol per 10^6 cells	%
Slou	43.6 (43.4–44.0)	100	225 (224–226)	100
1R50bneo	16.6‡ (12.0–21.7)	38	118‡ (101–136)	52
F6.1	31.3‡ (29.0–21.7)	71	156‡ (129–184)	69
F6.2	27.9‡ (25.3–32.3)	64	ND	
F6.3	30.3‡ (18.2–41.9)	68	129‡ (114–145)	57
F6.4	32.2‡ (28.4–39.6)	73	ND	

ND, not determined.
*Data are from three experiments, each performed in quadruplicate; the mean accumulation and the range (in parentheses) are given.
†Data are from two experiments, each performed in quadruplicate; the mean accumulation and the range (in parentheses) are given.
‡Statistically different ($P < 0.05$) compared to Slou (unpaired Student's *t* test).

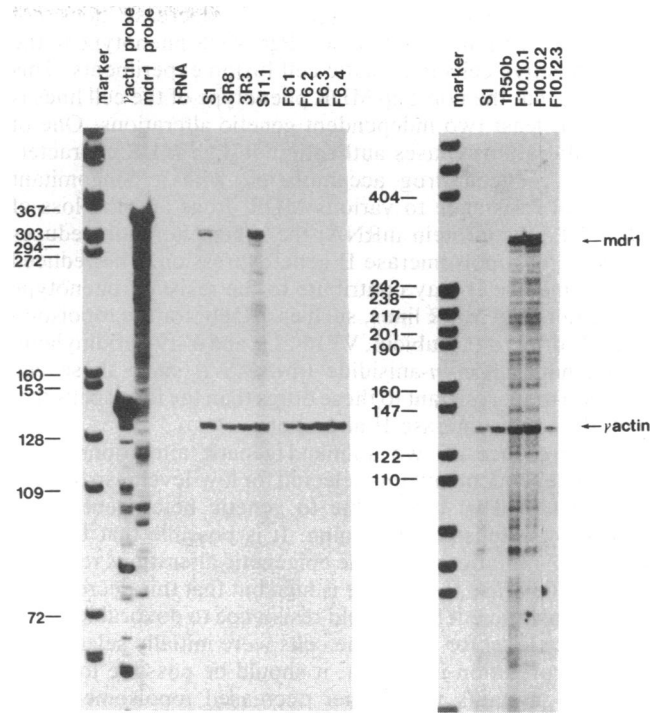

FIG. 3. RNase protection assay of *MDR1* P-glycoprotein mRNA levels in MDR cells obtained after somatic cell fusion of non-Pgp MDR cell line 1R50bneo with drug-sensitive parental cell line Slou. Ten micrograms of total cytoplasmic RNA from each SW-1573 derivative was used. Positions of the protected fragments of *MDR1* and γ-actin mRNA as well as the size (nucleotides) of the molecular weight markers are indicated. Probes for *MDR1*, γ-actin, and a tRNA control are shown (lanes *MDR1*, γ-actin, tRNA). Positions of the molecular weight markers are indicated.

DISCUSSION

In this paper we show that part of the non-Pgp MDR phenotype in our SW-1573 cells can be transferred to a sensitive recipient cell line in somatic cell fusion experiments. This indicates that the genetic alteration responsible for resistance acts in a dominant fashion. Therefore, it may be possible to identify the mutated gene by transfection of sensitive cells with DNA from resistant cells.

We have previously shown that the non-Pgp MDR cell lines accumulate less drug (24) and have lost the low level of *MDR1* P-glycoprotein mRNA present in the sensitive parental cells (12). In somatic cell fusion experiments we transferred these characteristics together with a modified MDR phenotype to the drug-sensitive parental cell line, showing these characteristics to be linked to the non-Pgp MDR phenotype. This could mean that the non-Pgp MDR cell lines are altered in a regulatory protein that affects drug accumulation and the level of *MDR1* P-glycoprotein mRNA. As the SW-1573 cell lines are aneuploid and contain three to five copies of chromosome 7q (A.W.M.N., unpublished results), where the *MDR1* gene is located, the down-regulation of *MDR1* mRNA in the non-Pgp MDR cell lines is probably mediated in trans. This down-regulation is reversible, since prolonged drug selection of the cell lines 1R50b and 2R50 resulted in the appearance of the stable *MDR1* overproducing cell lines 1R500 and 2R160 (12, 22, 23).

We show here that the expression of the topoisomerase II gene in the non-Pgp MDR cell lines is also decreased com-

pared to the parental cell line. This decrease does not cotransfer with the modified non-Pgp MDR phenotype to the drug-sensitive cells in somatic cell fusion experiments. This suggests that the non-Pgp MDR phenotype of the cell lines is due to at least two independent genetic alterations. One of these alterations causes authentic non-Pgp MDR characterized by reduced drug accumulation with a concomitant low-level resistance to various MDR drugs and the loss of *MDR1* P-glycoprotein mRNA; the other alteration reduces the level of topoisomerase II gene expression. The reduced topoisomerase II may contribute to the resistant phenotype of the non-Pgp MDR lines, such as 1R50b, for the topoisomerase II drugs doxorubicin, VP16-213, and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (m-AMSA) since these cell lines are more resistant to these drugs than the fused cells that lack the topoisomerase II alteration.

The presence of two unlinked genetic alterations in all three independent mutants selected for low-level resistance is unexpected. This is not due to genetic heterogeneity, as shown by extensive subcloning. It is possible that the frequency of mutations or stable epigenetic alterations resulting in topoisomerase II decrease is high but that this decrease by itself is insufficient for a 5-fold resistance to doxorubicin, the resistance level for which the cells were initially selected. If this interpretation is correct, it should be possible to select SW-1573 mutants with either decreased topoisomerase II alone or non-Pgp MDR mutants alone by using low concentrations of the appropriate drugs (see Table 1).

The increased resistance to non-topoisomerase II drugs in the fused cells may be due to selection. To completely kill sensitive cells we were forced to select for resistance with 12 nM vincristine, a concentration that already affects the 1R50b cell line used as fusion donor (see Table 1). We may therefore have selected for increased expression (e.g., by amplification) of the genetic change responsible for non-Pgp MDR. The high level of selection required in the somatic cell fusion experiments might also be the reason for the generation of hybrids with a moderate level of P-glycoprotein expression (F10.10.1 and F10.10.2) after somatic cell fusion. Since prolonged drug selection of the non-Pgp MDR cell lines resulted in *MDR1* overproducing cell lines (12, 22, 23), we might also have selected for P-glycoprotein expression in a subset of the non-Pgp MDR hybrids by the stringent selection. Whether these cell lines also contain the non-Pgp MDR remains to be investigated.

Several other cell lines with a non-Pgp type of MDR have been described in recent years (12–19). Most of these lines were selected for high degrees of resistance by many rounds of selection. Given our results, it seems likely that resistance in these cell lines is due to several additive mutations as well. The somatic cell fusion method used here to dissect a compound resistant genotype may also be of use for the further characterization of these other cell lines.

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