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Multidrug resistance after retroviral transfer of the human *MDR1* gene correlates with P-glycoprotein density in the plasma membrane and is not affected by cytotoxic selection

(membrane transport/flow sorting/gene amplification/vinblastine/colchicine)

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ABSTRACT **Multidrug resistance (MDR) in mammalian cells is associated with the expression of the *MDR1* gene encoding P-glycoprotein (P-gp), an active efflux pump for various lipophilic compounds. MDR transfectants can be isolated after *MDR1* gene transfer and selection with cytotoxic drugs; low levels of drug resistance have also been observed in unselected NIH 3T3 mouse cells after retrovirus-mediated transfer of mouse *mdr1* cDNA. MDR cell lines possess multiple phenotypic changes, suggesting that P-gp function could be complemented by some additional mechanisms associated with cytotoxic selection. To determine whether cytotoxic selection contributes to the MDR phenotype of *MDR1*-expressing cells, NIH 3T3 cells infected with a recombinant retrovirus carrying the human *MDR1* gene were selected by two different procedures: (*i*) noncytotoxic selection for increased P-gp expression on the cell surface by multiple rounds of immunofluorescence labeling and flow sorting or (*ii*) one or more steps of selection with a cytotoxic drug. The levels of MDR in both types of infectants showed an excellent correlation with the P-gp density in the plasma membrane, expressed as immunoreactivity with a P-gp-specific antibody normalized by reactivity with an antibody against an unrelated antigen. Cytotoxic selection conferred no additional increase in resistance relative to P-gp density. These results indicate that P-gp density in the plasma membrane may be sufficient to determine the level of MDR.**

Selection of mammalian cells resistant to a lipophilic cytotoxic drug usually results in the isolation of multidrug-resistant (MDR) cell lines, resistant to various compounds that share little structural similarity with the primary selective agent and act at different intracellular targets (1–3). These compounds include many drugs used in cancer chemotherapy. Human MDR cell lines selected with different drugs overexpress the same gene, termed *MDR1* (4, 5) or *PGY1* (ref. 6, pp. 37 and 43), whereas rodent cells contain two *MDR1*-like genes, either or both of which may be overexpressed in MDR cell lines (7, 8). The product of the *MDR1* gene is P-glycoprotein (P-gp; ref. 9), a transmembrane protein believed to function as an ATP-dependent efflux pump that extrudes lipophilic compounds from MDR cells (10). An increase in the cellular levels of *MDR1* mRNA and P-gp correlates with increased drug resistance in cell lines isolated by multistep selection with different drugs (3).

The functional role of the *MDR1* gene in MDR has been demonstrated by gene-transfer experiments in which the entire coding sequence of the mouse or human *MDR1* cDNA was inserted into an expression vector and transfected into drug-sensitive recipient cells; transfection with *MDR1* cDNA made it possible to obtain MDR colonies after single-step selection with one of the P-gp-transported drugs (11–14). The functional role of P-gp in MDR was also indicated by the finding that an altered pattern of cross-resistance to different drugs, observed in a MDR human cell line, was the result of a mutational substitution of a single amino acid in P-gp (13). This mutation alters the specificity of drug binding and transport by the mutant P-gp (15).

In addition to increased *MDR1* expression, MDR cell lines are characterized by a number of other biochemical and enzymatic changes (16, 17). Such multiple changes could signify that MDR is a multifactorial phenotype in which P-gp expression is just one of the components. The *MDR1* cDNA transfection studies, while demonstrating a functional role for the *MDR1* gene, did not prove that *MDR1* expression alone was sufficient for MDR, since they could not rule out that some other mechanisms of resistance, unrelated to P-gp expression, could have also been selected in the transfectants by growth in medium with cytotoxic drugs. Evidence that cytotoxic selection may not be required for the acquisition of the MDR phenotype came from Guild *et al.* (18), who infected drug-sensitive NIH 3T3 mouse cells with a recombinant retrovirus carrying the mouse *mdr1* gene and then subcloned the infectants either with or without prior selection with cytotoxic drugs. Most of the unselected infectants had slightly elevated levels of drug resistance, but these levels were lower than in any of the infectants selected with cytotoxic drugs in a single step (19). Although this discrepancy could be explained by differences in the levels of *mdr1* mRNA expressed by the two groups of infectants, it was also consistent with a hypothesis that some P-gp-independent mechanisms of resistance could have been activated in the infectants by drug selection.

In the present work, we have followed up on the study of Guild *et al.* (18) by using a flow cytometric protocol for noncytotoxic selection for increased P-gp expression in NIH 3T3 cells infected with a recombinant retrovirus carrying human *MDR1* cDNA. Comparison of infectant clones isolated by noncytotoxic selection or by single-step or multistep selection with a cytotoxic drug showed that the levels of cellular resistance in both types of infectants correlated equally well with P-gp density in the cell membrane. These

Abbreviations: DHFR, dihydrofolate reductase; FACS, fluorescence-activated cell sorter; ID_{10}, drug concentration resulting in 90% inhibition of cell growth; MDR, multidrug resistance (resistant); VBL, vinblastine.

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results indicate that P-gp density in the cell membrane may be sufficient to determine the level of MDR.

MATERIALS AND METHODS

Generation of *MDR1*-Containing Recombinant Retrovirus. An *MDR1* cDNA insert containing the entire coding sequence of P-gp was excised from the plasmid pUCFVX-mdr1(gr) (13) with *Xba* I and *Cla* I. A *Cla* I–*Hin*dIII adaptor was then added onto the *Cla* I end of the cDNA insert, which was then subcloned into pGEM-3Zf(−) plasmid vector (Promega) that had been linearized with *Xba* I and *Hin*dIII. *MDR1* cDNA was subsequently excised from pGEM-3Zf(−) by partial *Eco*RI and complete *Cla* I digestion. This insert was then used to replace the neomycin phosphotransferase (*neo*) gene in the Moloney murine sarcoma virus-based retroviral vector pLNL6 (19). The resulting construct was termed pLMDR1L6.

The *MDR1*-containing recombinant amphotropic retrovirus was prepared essentially as described by Bender *et al.* (19). pLMDR1L6 plasmid DNA (10 μg) was introduced into the ψ-2 ecotropic packaging cell line (20) by calcium phosphate-mediated DNA transfection (21). Forty-eight hours after transfection, virus-containing supernatant was collected and clarified by centrifugation at 3000 × *g* for 5 min. The supernatant was used to infect the amphotropic packaging cell line PA317 (22) to produce an amphotropic *MDR1*-containing retrovirus. After infection, PA317 cells were selected for *MDR1* expression with vinblastine (VBL, 20 ng/ml). Colonies were picked 14 days later, expanded, and used to determine the virus titer in the culture supernatant. For this assay, virus-producing cells were seeded at 5×10^6 per 10-cm plate. On the next day, the medium was replaced with 5 ml of fresh medium, and after 16 hr of further growth the virus-containing supernatant was harvested, diluted, and used to infect NIH 3T3 cells (seeded 1 day before at 5×10^5 cells per plate) in the presence of Polybrene (4 μg/ml). After 24 hr of infection, cells were split 1:10, and 24 hr later selection with VBL (20 ng/ml) was initiated. Colonies were scored after 10–14 days of selection, and virus titer was calculated as the number of drug-resistant colonies per ml of virus supernatant. The cell line giving rise to the highest virus titer ($1–2 \times 10^5$ per ml) was used as the source of the virus.

Derivation of Infectant Cell Lines. P-gp-expressing infectants were isolated by two different procedures. In the first approach, the infected cell population was labeled by indirect immunofluorescence with the human P-gp-specific antibody MRK16 (IgG2a) (23), as described below, and the reactive cells were isolated using a fluorescence-activated cell sorter (FACS) (Epics V, Coulter). The selected population of primary infectants was expanded in culture and again labeled with MRK16. The most reactive 5–10% of the population was isolated and expanded. After five rounds of sorting, cells were subcloned by end-point dilution and individual subclones were isolated. In the second approach, the infected cells were selected for resistance to VBL at 20 ng/ml and individual resistant colonies were isolated. One of the resulting subclones, VBL-1, was subjected to further cytotoxic selection with VBL at 100 ng/ml and then at 200 ng/ml.

Immunofluorescence Analysis and Microscopic Measurements. For indirect immunofluorescence labeling, $1–2 \times 10^6$ cells were dissociated with 20 mM EDTA in phosphate-buffered saline (pH 7.4) and incubated in 100 μl with the primary antibody MRK16 (final concentration, 100 μg/ml) for 1 hr on ice. Cells were then washed and incubated in the dark for 1 hr in 100 μl with fluorescein-conjugated goat anti-mouse IgG (20 μg/ml). After washing and addition of 500 μl of medium containing propidium iodide (133 μg/ml), cell fluorescence was analyzed by flow cytometry (Epics V). Propidium iodide accumulation was used to exclude the dead cells from the analysis. Mean fluorescence values were calculated on a linear scale. As a negative control, cells were incubated with total mouse IgG or IgG2a as primary antibodies instead of MRK16. A fluorescein-labeled antibody against Thy-1.2, an antigen expressed on the surface of NIH 3T3 cells (24), was obtained from Sigma and used for direct immunofluorescence labeling as a control for changes in cell surface area.

Microscopic measurements of cell size were carried out by placing dissociated cells in suspension, depositing them on microscopic slides, and measuring the apparent diameter of rounded viable cells (based on trypan blue exclusion) prior to their attachment. Measurements were carried out at ×400 magnification with a phase-contrast objective lens and an eyepiece grid delineated into 2.5-μm^2 areas. The mean cell projection area was calculated from measurements of 100 cells in each cell line.

Drug Sensitivity Assay. Two types of assays were used to measure drug resistance in the infectant cell lines. In a rapid growth-inhibition assay (25, 26), 5000 cells were plated in each well of a 24-well microtiter plate containing cytotoxic drugs (VBL or colchicine) with concentrations increasing in 2-fold increments. After 7–10 days, cells were fixed with May–Grünwald reagent and stained with Giemsa reagent. Drug concentrations resulting in ≈90% inhibition of cell growth (ID_{10}) were estimated for each cell line, and the relative resistance was calculated as the ratio of ID_{10} for the drug-resistant line to that of the parental NIH 3T3 cells. For the most resistant cell lines, drug resistance was also determined by the more precise colony formation assay (13); there was a good correlation between the results of the two assays.

Nucleic Acid Hybridization. Cellular DNA and RNA were extracted using an Applied Biosystems nucleic acid extractor (model 370A). Southern hybridization of DNA and dot blot RNA hybridization were performed as described (4, 5). A human *MDR1* genomic clone, pMDR1 (4), was used as a probe for Southern hybridization. cDNA clone pHDRV15′ (13) was used as a probe for human *MDR1* mRNA, and the entire coding sequence of the mouse *mdr1* (*mdr1b*) cDNA (27) was used as a probe for the expression of mouse *mdr1* genes. A mouse dihydrofolate reductase (DHFR) cDNA clone (28) was used as a control for equivalent loading of DNA and RNA. The hybridization signal was quantitated using an LKB Ultroscan XL laser densitometer.

RESULTS

A defective recombinant amphotropic retrovirus for high-efficiency transfer of the human *MDR1* gene was prepared using the retroviral vector LNL6 (19), into which a full-length protein-coding sequence of the wild-type human *MDR1* cDNA (13) was inserted. The resulting virus, LMDR1L6, was used to infect NIH 3T3 cells. Cells expressing the human P-gp on their surface were detected and isolated on the basis of their reactivity with the monoclonal antibody MRK16, which recognizes an extracytoplasmic domain of human P-gp (23) and does not react with mouse P-gp (29). Indirect immunofluorescence labeling of virus-infected NIH 3T3 cells with MRK16, followed by flow cytometry, revealed that MRK16-reactive cells comprised ≈25% of the infected cell population (Fig. 1*A*); no such cells were detected in the uninfected control population or among the infected cells incubated with mouse IgG instead of MRK16. The MRK16-reactive cells were isolated by flow sorting and maintained in culture as a mass population (primary infectants) (Fig. 1*B*).

To obtain cells expressing increased amounts of human P-gp, primary infectants were labeled by indirect immunofluorescence with MRK16, and ≈5% of the cells showing the strongest fluorescence were isolated by flow sorting and expanded in cell culture. This procedure was repeated for a total of five rounds, with an increase in the mean MRK16 reactivity observed after each round (Fig. 1 *C* and *D* and data not shown). After the fifth round of sorting, single-cell

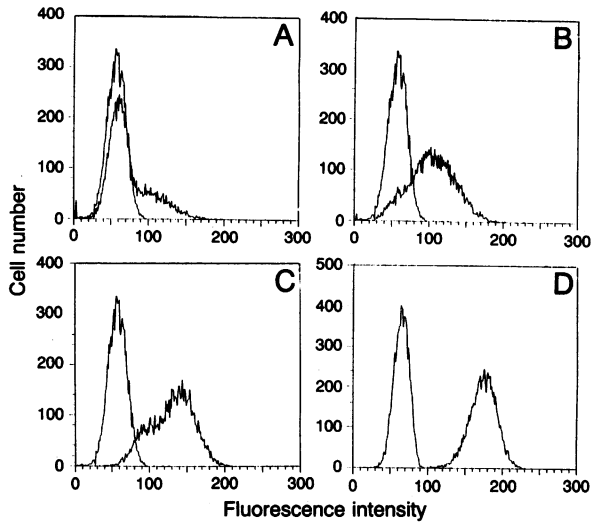

FIG. 1. Selection of cells with increased P-gp expression by flow sorting. Panels compare flow profiles after MRK16 immunofluorescence labeling of the parental NIH 3T3 cells and the following cell populations: NIH 3T3 cells immediately after infection with LMDR1L6 (*A*), MRK16-reactive primary infectants after flow sorting and expansion in culture (*B*), and infectants after one round (*C*) or five rounds (*D*) of flow sorting for increased MRK16 reactivity.

subclones were isolated and characterized with regard to their resistance to VBL and colchicine and P-gp expression on the cell surface, assumed to be proportional to mean fluorescence intensity after indirect immunofluorescence labeling with MRK16. There was significant variability in the levels of P-gp expression and drug resistance among the FACS-selected subclones (Fig. 2*A*). P-gp expression in the sorted mass populations was unstable, judging from the gradual increase in heterogeneity and decrease in the mean fluorescence during the propagation of these populations; these changes became apparent after just 2 or 3 weeks in culture (data not shown). The subcloned cell lines were more stable, but clones with the highest levels of P-gp also showed a decrease in MRK16 reactivity and drug resistance after propagation in tissue culture for 3–6 months.

To determine whether the increased expression of P-gp in flow-sorted cells was due to gene amplification, we analyzed DNA from the parental NIH 3T3 cells, the primary infectants, and the FACS-selected subclones by Southern hybridization with an *MDR1* probe (Fig. 3). The intensity of the 4.9-kb *Xba* I band, corresponding to the LMDR1L6 sequences, relative to endogenous mouse *mdr*-specific bands suggests that the majority of primary infectants contained a single copy of the integrated provirus. Similar hybridization signals were observed for FACS-1 (Fig. 3) and two other subclones with lower levels of P-gp expression (data not shown). In contrast, clones with the highest levels of P-gp expression (FACS-2, FACS-14, and FACS-17) showed low-level amplification of the exogenous *MDR1* gene relative to the primary infectants. No amplification of the endogenous mouse *mdr* sequences was detected in any of the clones. Gene amplification was associated with a 6- to 7-fold increase in the amounts of *MDR1* mRNA in FACS-2 and FACS-14 cell lines relative to the primary infectants, as determined by slot blot RNA hybridization (Fig. 4).

In addition to noncytotoxic MRK16/FACS selection, we have also selected LMDR1L6-infected NIH 3T3 cells with VBL at 20 ng/ml. Although all the infectants were selected with the same concentration of VBL, they expressed differ-

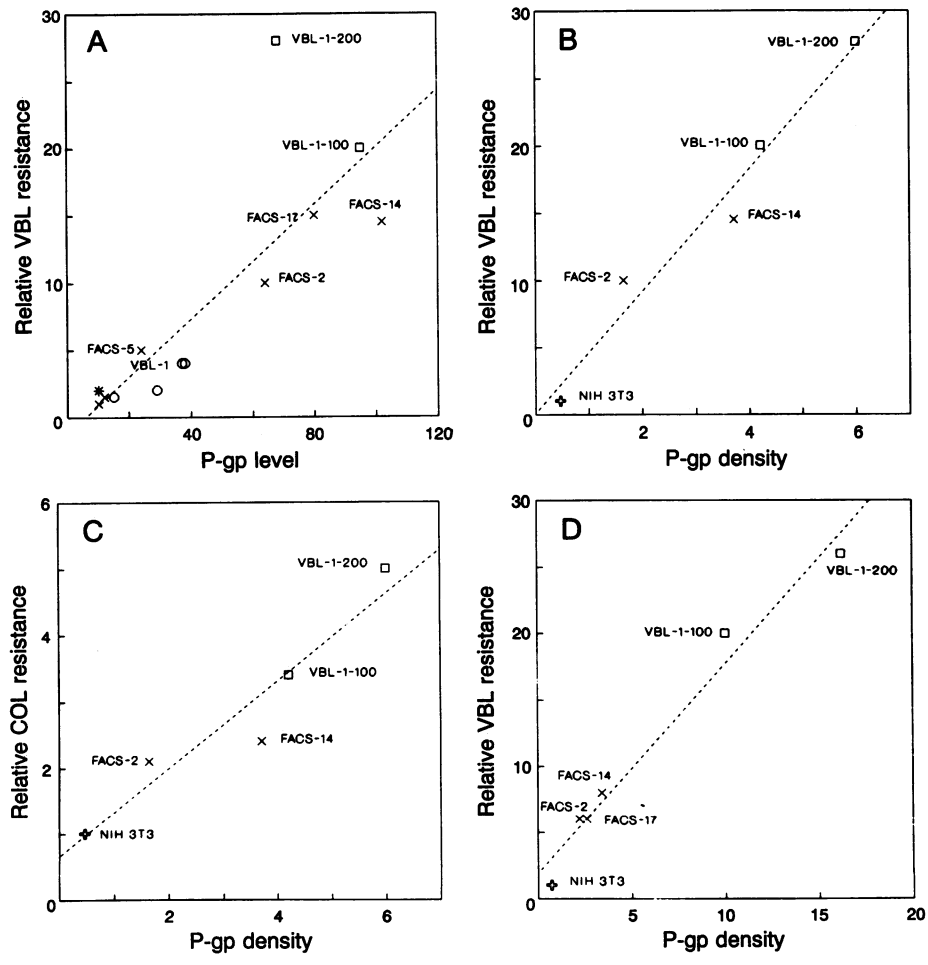

FIG. 2. Correlation of drug resistance and P-gp expression in FACS- and VBL-selected infectant clones. Drug resistance is expressed as the ratio of ID_{10} for a given cell line relative to the recipient NIH 3T3 cells. Each point corresponds to an average of three measurements of drug resistance. , NIH 3T3 cells; *, primary infectants; ×, FACS-selected subclones; ○, single-step VBL-selected subclones; □, multistep VBL-selected populations. Correlation coefficients (r^2) for linear fit, determined by least-squares analysis, are given below. (*A*) Correlation of relative VBL resistance with the amount of P-gp per cell (difference between mean MRK16 and total IgG immunofluorescence). r^2 = 0.675. (*B*) Correlation of relative VBL resistance with P-gp density in the cell membrane (ratio of MRK16 to Thy-1.2 immunofluorescence). r^2 = 0.966. (*C*) Correlation of relative colchicine (COL) resistance with P-gp density in the cell membrane. r^2 = 0.915. (*D*) Same as in *B*, but after 4 weeks of continued growth of the cell lines (see Table 1). r^2 = 0.973.

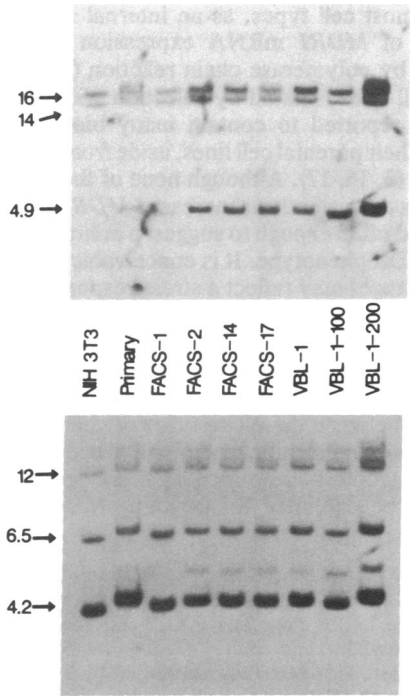

FIG. 3. Southern hybridization analysis of the copy number of *MDR1* in the infectants. (*Upper*) Hybridization of *Xba* I-digested DNA from the indicated cell lines with the pMDR1 probe. The 4.9-kilobase (kb) band corresponds to the proviral sequences of LMDR1L6. The 14-kb and 16-kb bands correspond to the endogenous mouse *mdr1a* and *mdr1b* genes. (*Lower*) Control hybridization of the same blot (without removal of the pMDR1 probe) with the mouse DHFR cDNA. Sizes of the DHFR-specific bands are indicated.

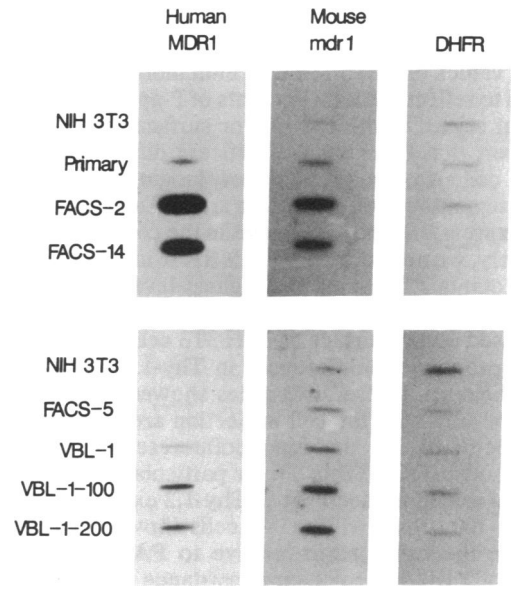

FIG. 4. Analysis of *MDR1* mRNA expression in FACS-selected and VBL-selected infectants by slot blot hybridization. Each slot contained 10 μg of total cellular RNA from the indicated cell line. The upper and lower blots were hybridized separately to the indicated probes. The signal for the mouse *mdr1* probe represents hybridization with the mouse *mdr1b* mRNA and cross-hybridization with the mouse *mdr1a* and human *MDR1* transcripts.

ent amounts of P-gp (Fig. 2*A*). One of the subclones (VBL-1) was further selected for increased drug resistance by multistep selection with increasing concentrations of VBL. A mass population of VBL-1 derivatives isolated after selection with VBL at 100 ng/ml was designated VBL-1-100. These cells showed increased expression of human P-gp (Fig. 2*A*) and *MDR1* mRNA (Fig. 4) relative to VBL-1 cells but showed no further increase in the copy number of the exogenous or endogenous *MDR1* genes (Fig. 3). In contrast, VBL-1-200 cells, obtained at the next step of selection (VBL at 200 ng/ml), showed no further increase in P-gp expression (Fig. 2*A*), *MDR1* mRNA level (Fig. 4), or *MDR1* gene copy number (Fig. 3), although they were more resistant than VBL-1-100. There was no apparent increase in the expression of the endogenous mouse *mdr1* mRNA in VBL-1-200 cells relative to VBL-1-100 (Fig. 4). The VBL-1-100 and VBL-1-200 populations were unstable and showed a gradual increase in P-gp expression while maintained at VBL concentrations used for their initial selection.

Fig. 2*A* and Table 1 show comparisons of drug resistance and P-gp expression among the FACS- and VBL-selected infectants. There was a rough correlation between the amount of P-gp per cell and the levels of drug resistance in both groups of infectants, but this correlation was not maintained in all cases (Fig. 2*A*). Single-step VBL-selected infectants were no more resistant to VBL than were FACS-selected infectants with similar levels of P-gp. In contrast, VBL-1-100 and VBL-1-200 cells were more resistant to VBL than were FACS-selected lines expressing similar levels of P-gp (FACS-2, FACS-14, and FACS-17). The increase in drug resistance in VBL-1-200 relative to VBL-1-100 was in fact associated with a slight decrease in P-gp. Furthermore, the FACS-14 subclone was more resistant than FACS-2,

Table 1. Properties of FACS- and VBL-selected infectants

Cell line	Mean immunofluorescence			Mean cell projection	Relative resistance	
	IgG	MRK16	Thy-1.2		VBL	COL
NIH 3T3	9.05	8.90	19.48	1.00	1	1
	3.88	3.82	5.22	—	1	—
FACS-2	19.76	106.44	64.37	1.82	10	2.1
	8.62	31.38	14.46	—	6.0	—
FACS-14	11.02	112.93	30.41	1.04	14.5	2.4
	4.94	31.33	9.15	—	8.0	—
FACS-17	4.64	32.08	12.39	—	6.0	—
VBL-1-100	7.77	102.72	24.38	1.00	20	3.4
	3.76	64.13	6.39	—	20	—
VBL-1-200	4.00	72.04	11.99	0.78	27.7	5.0
	4.68	125.36	7.72	—	26	—

The upper and the lower row for each cell line (except FACS-17) correspond to two experiments carried out 4 weeks apart. Mean cell projection area and resistance to VBL and colchicine (COL) are shown relative to NIH 3T3 cells. Because of different conditions for flow cytometric assays, the values for mean immunofluorescence do not correspond between the two experiments.

although these cell lines expressed equal amounts of P-gp on the cell surface (Table 1).

The values of mean MRK16 immunofluorescence are expected to reflect the total amounts of P-gp on the cell surface, without regard to the cell size or surface area. We noticed, however, that there were significant differences in the apparent cell size among subclones, judging from microscopic measurements of cell projection areas (Table 1). To correlate P-gp expression with differences in the cell surface area more precisely, we normalized mean MRK16 immunofluorescence of infectants expressing the highest levels of P-gp by their reactivity with an antibody against an unrelated antigen expressed on the surface of NIH 3T3 cells, Thy-1.2 (24). We found pronounced differences in Thy-1.2 reactivity among the subclones. These differences showed a general correlation not only with the cell projection area but also with the nonspecific background immunofluorescence observed with total mouse IgG (Table 1). In particular, VBL-1-200 cells showed a >2-fold decrease in Thy-1.2 expression relative to VBL-1-100 cells, and FACS-2 cells showed a similar difference in the cell surface relative to FACS-14 cells. When levels of VBL and colchicine resistance in the parental NIH 3T3 cells and the infectants were correlated with the density of P-gp on the cell surface, defined in relative terms as the ratio of MRK16 to anti-Thy-1.2 reactivity, an excellent correlation was observed for all the clones (Fig. 2 *B* and *C*). This correlation was maintained despite subsequent changes in the VBL-1-100 and VBL-1-200 populations, at which time VBL-1-200 cells manifested a significant increase both in cell surface area and in P-gp expression (Table 1 and Fig. 2*D*).

DISCUSSION

We have analyzed the role of cytotoxic selection in P-gp-mediated MDR by comparing the levels of drug resistance and P-gp expression in two types of MDR derivatives of mouse NIH 3T3 cells obtained after retrovirus-mediated transfer of the human *MDR1* gene. Infectants of the first type were selected for MDR by one or more steps of selection with a cytotoxic drug, and infectants of the second type were selected for increased P-gp expression by a noncytotoxic procedure using a FACS. The levels of drug resistance in both types of transfectants showed the same correlation with P-gp density in the plasma membrane. This result indicates that cytotoxic selection has not elicited any major changes complementing P-gp function in *MDR1*-expressing cells and that P-gp expression is sufficient to determine the level of cellular drug resistance. However, our results do not disprove the contribution of auxiliary cellular factors to the drug-resistance phenotype, as long as such factors are not limiting within the examined range of resistance.

P-gp-mediated MDR in different tumors and cell lines has usually been analyzed by measuring absolute amounts of *MDR1* mRNA or P-gp per cell or by normalizing the levels of *MDR1* mRNA to mRNA levels for an intracellular protein such as β-actin. The reported correlations of P-gp expression with the levels of drug resistance were not always precise (3). In the present study, we have measured P-gp expression on the cell surface by indirect immunofluorescence of intact cells reacted with an antibody recognizing the extracellular domain of P-gp. The fluorescence intensity in this assay depends not only on the amount of the antigen but also on the shape of the plasma membrane and the level of cellular autofluorescence. To account for these variables, we have normalized the mean fluorescence intensity with a P-gp-specific antibody to the value obtained with an antibody against an unrelated antigen (Thy-1.2). The normalized value, reflecting P-gp density in the cell membrane, has provided the best correlate for the level of drug resistance. In view of these results, we have chosen mRNA for β_2-microglobulin, a cell surface protein expressed at similar densities in the membranes of most cell types, as an internal standard for measurements of *MDR1* mRNA expression in human tumor specimens by polymerase chain reaction (30).

MDR cell lines isolated by selection with cytotoxic drugs have been reported to contain many biochemical changes relative to their parental cell lines, aside from increased *MDR1* expression (3, 16, 17). Although none of these alterations are observed as consistently as increased *MDR1* expression, they are reported often enough to suggest a nonrandom association with the MDR phenotype. It is conceivable that at least some of these changes may reflect a stress response upon exposure to cytotoxic drugs, whereas others may be a consequence of the presence of P-gp in cellular membranes. Comparison of P-gp-expressing cell lines isolated either by cytotoxic selection or by the flow-sorting procedure described in this study should distinguish between these two types of changes. Understanding the origin and significance of cellular changes associated with MDR would help to identify those properties that may potentially be exploited for targeting MDR cells in cancer therapy.

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