ABSTRACT

Two independent multidrug-resistant (MDR) sublines, AdR1.2 and SRA1.2, were developed from the established human colon carcinoma cell line LoVo. AdR1.2 was developed by a long-term continuous exposure of LoVo cells to Adriamycin in stepwise increments of concentration; SRA1.2 was selected/induced by pulse treatments by using a single concentration of Adriamycin. The two resistant sublines were cross-resistant and cross-sensitive to a similar spectrum of cytotoxic agents. However, AdR1.2 was most resistant to Adriamycin among the nine agents tested, and SRA1.2 was most resistant to Vinca alkaloids. Although SRA1.2 had biological characteristics similar to those of LoVo, AdR1.2 had remarkably altered biological properties, including non-detected carcinoembryonic antigen secretion, a smaller proportion of proliferating cells and a lower growth rate, lower fraction of cells in S phase, a lower colony-forming ability, and smaller colonies. In addition, the resistant phenotype of AdR1.2 was reversed when the cells were grown in a drug-free medium, whereas SRA1.2 maintained its resistance for at least 10 months under similar conditions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the plasma membrane proteins demonstrated overproduction of an M, 130,000 protein in both the resistant sublines. The M, 130,000 protein was not immunoreactive with C219 monoclonal antibody against p170, but the absence of M, 130,000 protein in an AdR1.2 revertant and the parental LoVo suggests that it is an MDR-related plasma membrane protein. The absence of a 46-kDa cytosolic protein and the presence of a M, 150,000 plasma membrane protein were found in AdR1.2 but not in SRA1.2. This M, 150,000 protein immunoreacted with C219. This protein was also present, although in a reduced amount, in an AdR1.2 revertant that retained three times the MDR of LoVo cells and was thus comparable to SRA1.2. The two MDR sublines thus may represent two independent subclones which may serve as two different models for the study of multidrug resistance in human colon cancer.

INTRODUCTION

Drug resistance is a common problem in cancer chemotherapy. Some tumors never respond to cytotoxic drug treatment; others initially respond well but eventually regrow and become resistant, possibly because of tumor subclones of a low drug sensitivity emerge. That phenomenon may result from epigenetic or genetic mutations induced by the administered antitumor agents, or it may represent the selection of preexisting resistant cell subpopulations in the cancer.

One of the better-studied types of drug resistance is that which develops after exposure to anthracycline derivatives (i.e., doxorubicin) or to certain antimitotic agents (i.e., Vinca alkaloids). Resistance to either of these two classes of agents is often associated with pleiotropic, or multidrug, resistance, a phenomenon manifested by collateral cross-resistance to other classes of agents. The basis for the cross-resistance seems to be a decreased net intracellular drug accumulation attributed to certain alterations in the cell membrane involving an energy-dependent efflux pump resulting from the overproduction of a M, 170,000 plasma membrane glycoprotein, namely, p170 (1-5). The reduced drug accumulation in the MDR² cells can be partially restored by treating the resistant cells with diverse agents such as verapamil or trifluoperazine (6-9). However, certain discrepancies have been reported in results obtained from the conventionally acquired MDR cell lines and those using fresh tissues from patients who developed drug resistance after receiving cancer chemotherapy, e.g., expression of p170 was not always associated with drug resistance in fresh human tumor specimens (10, 11) and the verapamil sometimes failed to enhance drug sensitivity against tumors from patients in relapse (12-14).

Our present knowledge about the hypothetical mechanisms responsible for MDR phenotypes was derived from the studies on highly resistant mammalian tumor cell lines in which the resistance was induced by continuous exposure to a drug that was selected for that subpopulation (4, 5, 15), or by a combination of drug treatment following an initial exposure to either a chemical mutagen (16) or irradiation (17). In the clinic, however, patients may develop drug resistance at relapse after only several courses of chemotherapy. Therefore, in terms of the method of induction, the drug resistance induced by a short-term exposure to antitumor agents may have more clinical relevance than that produced by a long-term exposure. It is unclear what are the factors causing the discrepancies in results between in vitro models of acquired MDR and from clinically encountered drug resistance. An ideal approach to study this problem is perhaps to study with tumor tissues from patients who receive prior chemotherapy and become refractory to chemotherapy subsequently and to compare their MDR properties with those of the conventional MDR models. However, such studies would be limited by the availability of tumor biopsy specimens in large quantity. In order to simulate the resistance confronted in the clinic, we have therefore pulse selected a MDR subline from a human colon tumor line. We chose a human colon tumor line in view of its intrinsic drug resistance properties and high rate of appearance of resistance population (18) which may provide greater possibility of obtaining MDR subclone by pulse selection. In this report, we described the characteristics of two independently selected MDR sublines derived from a human colon cancer cell line, LoVo, by two different methods with AdR.

MATERIALS AND METHODS

Cell Lines. LoVo cells were established from a metastatic nodule in a 56-year-old patient with adenocarcinoma of the colon. The patient had not received chemotherapy before surgery. The established cell line was normally propagated as a monolayer culture in Ham's F-10 medium (Hazelton, Denver, PA) supplemented with 10% fetal calf serum, vitamins, and glutamine. Under these conditions, the cells were able to form acinar structures and signet ring cells, and they secreted a moderate amount of CEA (19). AdR1.2 was produced by exposing LoVo...
Cells were exposed to another pulse treatment by the method described above. We with a 0.5% trypsin-EDTA solution. The cells thus obtained were grown in 95% air and 5% CO2, the colonies formed were harvested by treatments with a 0.5% trypsin-EDTA solution. The cells thus obtained were grown as a monolayer culture until becoming confluent and then were subplanted to another pulse treatment by the method described above. We tested the AdR sensitivity of the cells from the clones selected/induced by each pulse treatment. The SRA1.2-resistant subclone was established 9 months after the treatment was initiated. Once the resistant phenotype developed, the SRA1.2 cells were grown in AdR-free growth medium.

**Drugs.** AdR was obtained from Adria Laboratories, Columbus, OH. Vincristine, vindesine, and vinblastine were purchased from Eli Lilly & Co., Indianapolis, IN. VP-16, mitomycin C, and cisplatin were obtained from Bristol Co., Syracuse, NY. Fluorouracil was obtained from the SoloPak Lab, Franklin Park, IL, and amsacrine from Warner Lambert Co., Ann Arbor, MI.

**Dose-dependent Survival Analysis for Drug Cytotoxicity.** The colony formation technique has been described previously and was used exclusively in all studies concerning the survival response to antitumor agents (20). Briefly, cells growing exponentially in 60-mm Petri dishes were treated with antitumor agents for 1 h, washed with saline, harvested as a single-cell suspension, counted in an electronic particle counter, and replated in Petri dishes using various inocula titers to obtain 50–100 colonies after a 3-week incubation at 37°C. Three replicate dishes were inoculated for each dosage. The resulting colonies were stained and counted under a stereomicroscope. Only the colonies that were composed of more than 50 cells were evaluated. Six replicate dishes served as controls in each experiment; these dishes contained cells that were exposed to the same mechanical manipulations undergone by the treated cells but that did not receive the antitumor agent. The degree of resistance was expressed as the ratio of the IC50 of the resistant cells to that of the parental LoVo line.

**Stability of Resistant Phenotype.** The cells that were resistant to the antitumor drug were maintained and passaged in the drug-free medium. At monthly intervals, the stability of the drug resistance was evaluated by the use of a dose-dependent survival analysis described above.

**Growth Curves.** To construct the growth curves for the cells at exponential growth, 1-week-old stock cultures were harvested by treatment with a 0.5% trypsin-EDTA solution for 10 min at 37°C. Monodispersed cells were then obtained by forcing the cell suspension through an 18-gauge needle. The number of cells was determined as controls in each experiment; these dishes contained cells that were exposed to the same mechanical manipulations undergone by the treated cells but that did not receive the antitumor agent. The degree of resistance was expressed as the ratio of the IC50 of the resistant cells to that of the parental LoVo line.

**Preparation of Cytosolic Extract.** Cells were washed three times with ice-cold Puck's solution A (g/liter: NaCl, 8; KCl, 0.4; glucose, 1.0; NaHCO3, 0.35), centrifuged at 700 x g for 2 min, and resuspended in the lysis solution (1 mM Tris-HCl, pH 7.2, containing 10 μg/ml phenylmethylsulfonyl fluoride) to obtain a density of 2.5 x 106 cells/ml. Cell suspensions were gently stirred at room temperature until greater than 90% of cells were lysed (about 30 min). Nuclei and intact cells were pelleted by centrifugation at 400 x g for 10 min at 4°C. The enucleated cell homogenate was centrifuged again at 22,000 x g for 30 min, and the supernatant was used as the cytoplasmic extract.

**Isolation of Plasma Membrane.** The plasma membranes were prepared by the method of Atkinson and Summers (25), with minor modifications. The cells were lysed and enucleated as described above. The supernatant was collected and centrifuged at 37,500 rpm for 1 h using the rotor T150. The pellets were resuspended in 0.5 ml buffer H (10 mM Tris-HCl, pH 8.0, containing 75 mM sucrose, 25 mM MgCl2, 1.5 mM EDTA; 5 mM dithiothreitol; 0.15 mM NaCl, 0.15 mM KCl, and 10 μg/ml each of following protease inhibitors: phenylmethylsulfonyl fluoride, leupeptin, benzamidine, aprotinin, and pepstatin) and mixed with an equal volume of 81% sucrose in buffer A (5 mM MgCl2, 0.5 mM EDTA, 1 mM dithiothreitol, 0.15 mM KCl) to obtain a final sucrose density of 40.5%. A 1.5- to 2.0-ml aliquot of the mixture was layered at the bottom of a discontinuous sucrose gradient consisting of equal volumes of 20, 34, and 40% sucrose in buffer A (3.2 ml each). The gradient was then centrifuged at 37,500 rpm for 3.5 h at 4°C using the rotor SW41. Membrane-rich fractions were collected from the 20/34% and 34/40% interfaces. Each fraction was diluted with 10 mM Tris-HCl, pH 7.6, into a final volume of 10 ml and centrifuged at 27,000 rpm for 1 h using the rotor SW41. The pellets were resuspended in a minimal volume of 10 mM Tris-HCl, pH 7.6. The protein in each fraction was determined by the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA). The purification of the plasma membrane was monitored by the activity of the plasma membrane marker 5'-nucleotidase. The activity of the enzyme was assayed according to the method of Aronson and Touster (26). Briefly, 10 μg of plasma membrane protein or 40 μg cell homogenate in a volume of 0.05 ml were added to 0.45 ml of a reagent mixture containing 50 mM NaAMP (pH 7.0), 0.5 mM MgCl2, 0.65 mM Na2ATP, 0.15 mM dithiothreitol, and 0.5 mM MgCl2. The mixture was incubated at 37°C for 30 min in a water bath with shaking. The reaction was stopped by adding 2.5 ml of 8% trichloroacetic acid to the incubate, which was then immediately centrifuged at 5000 x g for 5 min at 4°C. An aliquot of the supernatant (2 ml) was diluted with 2.3 ml H2O. The inorganic phosphate content was determined by adding 0.5 ml of a 2.5% (w/v) solution of ammonium molybdate in 5 N H2SO4 and 0.2 ml of 1-amin-2-naphthol-4-sulfonic acid reagent (Fisher Scientific, Pittsburgh, PA) to the diluted supernatant. Ten min after trichloroacetic
acid was added, the optical density of the sample was determined by a spectrophotometer (Gillford, Oberlin, OH) at 660 nm. A purified preparation of 5'-nucleotidase (Sigma) was used to construct a standard curve. A known quantity of the membrane-rich protein preparation was analyzed by SDS-PAGE according to the method of Laemmli (27).

Western Blot Analysis. Plasma membrane-rich subcellular fractions were isolated from LoVo, SRA1.2, AdR1.2, AdR1.2 revertant, and MDR Chinese hamster CH3C5. CH3C5 cells were a gift from Dr. Victor Ling (Ontario Cancer Institute, Toronto, Ontario, Canada) and served here as a positive control for P-glycoprotein. Fifty µg of the membrane-rich subcellular fraction from each line was subjected to SDS-PAGE. The gels were soaked in the electrotransfer buffer (5 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, 2 mM sodium acetate, and 20% ethanol) for 1 h and then placed in a gel membrane transfer electrophoresis cell (Hofer Scientific Instruments, San Francisco, CA). Transfer of proteins to a nitrocellulose membrane was achieved by a second electrophoresis in the electrotransfer buffer at 4°C for 18 h at a constant voltage of 40 V. The nitrocellulose was then immunoblotted with tritiated monoclonal antibody C219 against p170. The C219 was a gift from Dr. V. Ling and was radiolabeled according to the method of Fraker and Speck (28), using the Iodo-Gen iodination reagent (Pierce, Rockford, IL).

In Vitro Phosphorylation of Plasma Membrane. In vitro phosphorylation was performed according to the method of Marsh et al. (29). Briefly, an aliquot (25 µg) of the plasma membrane preparations (a mixture of materials from the 20/34% and 34/40% interfaces) was incubated with a reaction mixture containing 2 µCi γ-[^32]P]ATP (3000 Ci/mmol; Amersham, Arlington Heights, IL), 0.05 M Tris-HCl (pH 7.6), 2 mM β-mercaptoethanol, and 5 mM MgCl2 in a total volume of 25 µL. The reaction was carried out at room temperature for 20 min and stopped by the addition of 10 mM EDTA. The proteins in the mixture were separated by SDS-PAGE (7.5% gel), and the phosphorylation in proteins was analyzed by autoradiography.

RESULTS

Sensitivities of Derivative Sublines to AdR. The IC50 values for LoVo and AdR1.2 were 0.30 and 29.0 µg/mL, respectively, in the 1-h AdR treatment and were 0.13 and 12.2 µg/mL, respectively, in the 24-h treatment (Fig. 1). The IC50 values indicate that AdR1.2 is 94 to 97 times more resistant to AdR than its parental line (Table 1). Fig. 2 shows the survival curves for SRA1.2 at each AdR pulse treatment. The first six pulse treatments each seemed to enhance the magnitude of resistance in the subclones selected/induced; the magnitude of resistance peaked at the sixth pulse treatment, and the degree of the resistance remained unchanged thereafter in at least the next three treatments.

Methods that apply intermittent exposures to cisplatin (30, 31) and mitomycin C (32) in selecting drug-resistant cell lines have been reported. However, we described here a similar approach but used AdR. In an attempt to validate the AdR pulse selecting method by demonstrating its reproducibility, we created four additional SRA1.2 subclones from LoVo by the same procedure. The survival curves for these subclones at each pulse treatment were constructed in a similar manner. The survival curves were similar to those of an independent SRA1.2 subclone established earlier: the degree of drug resistance within the selected subclones increased as a function of the number of the pulse treatments, and the magnitude of resistance peaked at the sixth pulse treatment (data not shown). The IC50 values indicated that SRA1.2 was about 3 to 4 times more resistant to AdR than was the LoVo line (Fig. 2).

MDR Phenotype. To verify the MDR phenotype of AdR1.2 and SRA1.2, we assessed the collateral resistances of these two subclones to eight selected antitumor agents. Table 1 shows that although the subclones had different magnitudes of resistance to each agent tested, they had identical spectra of cross-resistance (to vincristine, vinblastine, vindesine, VP-16, mitomycin C, and amssacrine) and of cross-sensitivity (to cisplatin and fluorouracil). However, the degrees of resistance to each agent tested differed between the sublines: AdR1.2 was most resistant to AdR, whereas SRA1.2 was more resistance to the Vinca alkaloids than to the other agents, including AdR. To test the reproducibility of the collateral resistance of SRA1.2, we measured the magnitudes of resistance to AdR and vincristine in two of the four additional SRA1.2 subclones described above. These SRA1.2 subclones were also more resistant to vincristine than to AdR (data not shown).

Stability of Resistance. We examined the stability of the resistance in the two subclones. AdR1.2 was grown continuously in medium containing 1.2 µg/mL AdR for more than 6 months before it was transferred to a drug-free medium to test its stability. In the AdR-free medium, the loss of resistance in AdR1.2 appeared to occur in two steps. In the first phase, the magnitude of the AdR resistance decreased rapidly; in the second phase, the resistance decreased at a slower rate. During
the first 5 months of growth in drug-free medium, AdR1.2 lost 89% of its magnitude of resistance, at a rate of 18%/month; the rate of loss decreased to 1.8%/month after that. Overall, the magnitude of resistance in AdR1.2 decreased from 95 to 3 times normal resistance within 10 months. The resistance levels in SRA1.2 however, were rather stable: the magnitude of resistance remained unchanged throughout the entire 10-month testing period (Fig. 3).

Growth Kinetics. The $T_d$ of each of the three cell lines in exponential growth was calculated by a linear regression analysis of the data taken from the growth curve (Table 2). AdR1.2 had the longest lag phase and the longest $T_d$ among the three cell lines. AdR1.2 had a lag phase of 48 h and a $T_d$ of 55.5 h when the cells were grown in the drug-free medium. The lag phase was prolonged to 72 h if AdR1.2 cells were grown in the medium that contained AdR. SRA1.2 and LoVo had $T_d$s of 31.5 and 30.2 h, respectively; the lag phase lasted 24 h for both cell lines. In addition, results from the continuous labeling of cells with $[^3]H$ thymidine showed that AdR1.2 had a lower growth fraction than did SRA1.2 (Table 2). Thus, AdR1.2 had the longest lag phase and the smallest proliferating fraction, which probably accounts for its having the slowest growth rate of the cell lines. AdR1.2 also had a lower colony-forming ability than did the other two lines. That was indicated by a lower plating efficiency [6.1 ± 3.1% (SE)], as well as smaller colonies (about 50–100 cells in each). SRA1.2 and LoVo formed bigger colonies containing 250–500 cells and had plating efficiencies of 41 ± 8.2% (SRA1.2) and 43 ± 11% (LoVo). Table 2 also shows the cell cycle compartment distributions of the three cell lines as measured by flow-cytometric analysis of DNA contents. The percentage of AdR1.2 cells in S phase was about 50% that of the parental LoVo line (19 versus 32%), whereas the $G_{1/0}$ fraction of AdR1.2 was about 20% higher than that of LoVo. The cell cycle distributions of SRA1.2 cells, conversely, were not significantly different from those of LoVo cells.

SDS-PAGE Analysis of MDR-related Cellular Proteins. Cytosolic and plasma membrane-rich proteins from the resistant cells and parental LoVo cells were analyzed. Fig. 4 shows that there were no detectable differences in the cytosolic protein profiles of the parental LoVo and the two derivative resistant sublines, except that a band representing a $M_0$ 46,000 protein was absent only in AdR1.2 (Lanes 1 and 4) but detectable in SRA1.2 and AdR1.2 revertant cells (Lanes 2 and 6). However, the identity and the significance of the $M_0$ 46,000 protein remain unknown. The plasma membrane subcellular fractions were isolated by discontinuous sucrose gradient centrifugation. The fraction collected from the 34/40% interface contained mainly the intact sheets of the plasma membrane, while the material from the 20–34% interface contained mostly broken plasma membrane particles. The purity of the isolated plasma membrane was determined by assaying the marker enzyme 5'-nucleotidase. The enzyme specific activities in the cell homogenate and in the combined 20/34% and 34/40% interfaces were calculated as 0.56 ± 0.02 and 6.6 ± 0.3 units/mg, respectively, indicating that the combined fractions contain a 12-fold-enriched plasma membrane preparation compared with the cell homogenates. The plasma membrane-rich proteins were analyzed by 7.5% SDS-PAGE, and the protein profiles are shown in Fig. 5. Although no overproduction of the $M_0$,$^3$
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150,000 to 180,000 plasma membrane proteins was detectable by SDS-PAGE in either resistant subline by this method, an intensified band representing a $M_r$, 130,000 protein was shown in both SRA1.2 and AdR1.2 cells. Moreover, this $M_r$, 130,000 protein did not immunoreact with C219 as detected by immunoblotting in Western analysis. Lanes 2 and 3 in Fig. 5 represent two separate preparations of the plasma membrane-rich fractions isolated from AdR1.2 at different passages; the identical protein profiles of the two plasma membrane preparations demonstrate the reproducibility of the results.

Southern Blot Analysis. The amplification of the MDR gene in the resistant sublines was determined by the intensity of the hybridization signal by using a gel-purified MDR1 cDNA probe. The quantity of DNA for each sample was normalized by a $\beta$-globin gene probe. The autoradiogram showed that the 4.4-kilobase HindIII-cleaved fragments of DNA isolated from the three cell lines were all hybridizable by the MDR1 probe. No increase in the copy number of the MDR1 gene, however, was detectable in AdR1.2 or SRA1.2, while a positive control of CHC5B30 (a gift from Dr. J. Riordan) showed a high amplification of the MDR1 gene, as indicated at the 2.8- and 2.3-kilobase HindIII-cleaved fragments. The results suggest that the MDR1 gene is not amplified in either of the resistant sublines (results not shown).

Western Blot Analysis. Although the MDR1 gene was not amplified in AdR1.2 and SRA1.2 cells, the product of the MDR1 gene was further investigated to ascertain the possibility of regulation at a secondary level. The 125I-labeled monoclonal antibody C219 against p170 was used to immunoblot the plasma membrane proteins isolated from the cell lines by using the Western blot technique. The results of the immunoblotting analysis show that C219 detects a $M_r$, 150,000 plasma membrane protein in AdR1.2 but not in LoVo or in SRA1.2 cells (Fig. 6). To determine whether the absence of the $M_r$, 150,000 protein in SRA1.2 cells could be explained solely by the lower degree of resistance of this subclone, the plasma membrane of an AdR1.2 partial revertant, which retains a 3-fold higher
resistance to AdR than LoVo and is thus comparable to SRA1.2, was analyzed for the presence of the M, 150,000 protein by the same method. Interestingly, the M, 150,000 protein was also detectable in the AdR1.2 partial revertant, although the intensity of the signal was weaker than in AdR1.2 (Fig. 6, Lane 3).

In Vitro Phosphorylation of Plasma Membrane Proteins. In *vitro* phosphorylation was carried out in the presence of 5 mM Mg"\(^{2+}\) with the plasma membrane-rich fractions isolated from LoVo, AdR1.2, SRA1.2, AdR1.2 partial revertant, and CH"\(^{+}\)C5 (a positive control) cells. Fig. 7 shows that a novel phosphorylated protein was present in AdR1.2 (Lane 3) and its partial revertant (Lane 5) migrated on gels in a position corresponding to a M, 150,000 molecule. However, the intensity of the signal for the phosphorylation in the partial revertant was much weaker than that in AdR1.2. A similar phosphoprotein was not detectable in LoVo (Lane 2) or in SRA1.2 cells (Lane 4). In CH"\(^{+}\)C5 cells, a strongly phosphorylated M, 170,000 plasma membrane protein can be clearly seen (Fig. 7, Lane 1). Together, these results suggest that p150, which immunoreacts with the C219 monoclonal antibody, is present only in AdR1.2 and the AdR1.2 revertant cells and is probably a p170-related phosphoprotein. Moreover, a hyperphosphorylation of a M, 66,000 plasma membrane protein was found in the AdR1.2 revertant only (Lane 5).

**DISCUSSION**

The MDR sublines AdR1.2 and SRA1.2 both were induced/selected from the LoVo cell line by the same agent, AdR, but with the use of different approaches. AdR1.2 was produced by the conventional method of continuous drug exposure, whereas SRA1.2 was produced by pulse drug treatments. The subclone produced by the pulse treatment was reproducible: four independently induced/selected sublines exhibited identical patterns in the development of resistance (i.e., the magnitude of resistance peaked at the sixth treatment) and had a consistent phenotypic expression (i.e., clones tested were more resistant to vincristine than to AdR). The reproducibility of the conventional stepwise exposure method has not been tested. However, that method has been used in many laboratories; the characteristics of our AdR1.2 including its unstable MDR phenotype in drug-free medium and highest resistance to AdR were similar to those of the acquired MDR cells reported by others (33-35). Therefore, our two models were validated by the reproducibility of the model generation and by the stability of the MDR phenotype.

AdR1.2 had an altered cell cycle distribution shown by its lower proportion of proliferating cells and slower growth rate than those of LoVo and SRA1.2. Thus, the altered growth kinetics in AdR1.2 (i.e., higher G"\(_{1}\) and lower S phase subpopulations, and a lower growth rate) indicate that the AdR1.2 had an increased proportion of nonproliferating cells and a decreased cell cycle progression rate. Those changes may be partly responsible for the reduced cytotoxicities of many antitumor agents to AdR1.2. Such changes did not occur in SRA1.2. Others have associated of a lower growth rate with drug resistance in several cell lines, including a human sarcoma MDR line, MES-SA (36), a small cell lung carcinoma MDR line, H69/LX4 (37), and Chinese hamster lung cells (38). However, contradictory results have also been obtained with a human leukemic MDR line, HL-60/AR (35) and a human small cell lung cancer MDR line, H69AR (39); in these studies, the growth rates of the derivative MDR cells and their parental cells did not differ. In the present study, AdR1.2 and SRA1.2 were both derived from LoVo by the same selecting agent; our data indicate that the possibility that alterations in cell growth of MDR cells solely depend on the cell origin may therefore be ruled out. Nevertheless, it remains to be answered whether the alteration in cell growth is important to the development of highly resistant MDR cells and whether these growth alterations bear any relationship to the MDR-related changes in the cell membrane. The answers to these questions may help us understand drug resistance in cancers.

We previously reported that LoVo produced a moderate amount of CEA (19). In the present study, the CEA secretion of SRA1.2 was not different from that of parent line but CEA was undetectable in AdR1.2. The cessation of CEA synthesis and/or secretion in the development of drug resistance may have significant clinical implications because CEA has been considered a specific marker for the diagnosis and prognosis of human colorectal cancers (40, 41). Of course, whether this observation can be generalized to similar MDR human colon cancer models awaits further investigation.

Based on the fact that p170 serves as a drug efflux pump in MDR cells, the mechanisms responsible for MDR in AdR1.2 and SRA1.2 may be different, even though the cell lines were both derived from LoVo. This postulate is supported by the results of immunoblotting, which revealed that C219 detected a p150 plasma membrane protein in AdR1.2 and in its partial revertant, which was comparable to SRA1.2 in terms of the magnitude of MDR. This protein was not found in SRA1.2 or in LoVo. In addition, the enhanced energy-dependent drug efflux occurred in AdR1.2 (26%), but not in SRA1.2 when compared to LoVo.3

The p150 in AdR1.2 and the p170 in other MDR cells (1, 34, 42) may be produced from the same gene family, in view of the fact that the two proteins have similar properties: they both immunoreact with the C219 monoclonal antibody; their cellular levels correlate with the degree of resistance of the cells; and both are plasma membrane-bound phosphoproteins whose

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3 Unpublished data.
phosphorylations are Mg++ dependent and can be enhanced by verapamil (43, 44).3 Interestingly, the reversal of resistance in the AdR1.2 revertant resulted in a concomitant increase in the phosphorylation of a M, 66,000 plasma membrane protein. The phosphorylation of the M, 66,000 protein in AdR1.2 was also found to be enhanced by the presence of 10 μM verapamil.3 The relationship between the phosphorylation of the M, 66,000 protein and the reversal of resistance in human colon tumor cells is currently under investigation.

Elevated production of a M, 130,000 protein was found in AdR1.2 and SRA1.2, but the protein was not detectable in LoVo and the AdR1.2 revertant, suggesting that the M, 130,000 protein is a MDR-related plasma membrane protein. Although the M, 130,000 protein was found in the plasma membrane, its presence may not be related to drug efflux, in view of the lack of immunoreactivity of this protein with the C219 monoclonal antibody. Other supportive evidence for this notion is the presence of this protein in SRA1.2 cells which do not possess enhanced energy-dependent drug efflux.1 The function of the M, 130,000 plasma membrane protein and whether it can be considered as a marker for MDR in human colon cancer cells remain to be studied.

Although the mechanisms responsible for MDR have been extensively studied, some results of studies using cell lines in which resistance was induced/selected by the conventional method have contradicted those from studies using fresh tumor tissues. It is conceivable that the prevailing mechanisms for MDR acquisition, uncovered by observations made in the laboratory setting, may apply to only a specific type of MDR developed after continuous treatment with anti-tumor agents. However, other types of MDR may develop in a cancer, not because of drug-induced mutation but rather as a result of spontaneous mutation. In this case, the drug would merely serve as a selecting agent to allow the mutated resistant cell subpopulations to outgrow their sensitive counterparts. These two newly derived AdR-resistant sublines of LoVo human colon cancer cells had remarkable differences in: (a) the drug to which they are most resistant to; (b) P-glycoprotein expression; (c) stability of MDR phenotype; (d) CEA secretion; and (e) growth kinetics. These results imply that different MDR subclones can be derived from the same cell population using the same selecting agent but different means, suggesting that various types of MDR may coexist in a cancer. On the basis of the method of production, the stability of resistant phenotype in drug-free medium and the degree of resistance, SRA1.2 appeared to be more relevant to the properties of clinical drug resistance than AdR1.2 would be. Further studies, however, are required in order to establish the accuracy of this prediction.

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Biological Characterization of Multidrug-resistant Human Colon Carcinoma Sublines Induced/Selected by Two Methods

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