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# MDR-1 gene expression, anthracycline retention and cytotoxicity in human lung-tumor cells from refractory patients*

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**Summary.** Lung-tumor cells from pleural effusion of four refractory patients and in cell lines established from them were analyzed for anthracycline retention, cytotoxicity, and MDR-1 gene and P-glycoprotein expression. Murine leukemic P388 and doxorubicin-resistant P388/R84 lines were used as controls. The 50% growth-inhibitory concentration ($IC_{50}$) for doxorubicin among lung-tumor lines varied from 0.16 to 0.31 μM in soft agar. Heterogeneity in doxorubicin or daunorubicin retention and response to the efflux-blocking action of 25 μM prochlorperazine was noted in pleural effusion of FCCL-1, -4, and -8. Among the cell lines established, an efflux-blocking effect in a subpopulation was noticed only in FCCL-1 and -4. Although the MDR-1 gene was present in all cell lines, including P388, its expression was pronounced only in P388/R84 and FCCL-1. In situ hybridization of antisense RNA probe to tumor cells showed high heterogeneity for MDR-1 message in the human lung-tumor cells as compared with the murine cells. Northern and slot blot hybridization confirmed in situ hybridization in lines with high levels of MDR-1 expression. The synthesis of MDR-1 mRNA and P-glycoprotein in tumor lines was correlated. The results suggest that because of extensive tumor-cell heterogeneity in human tumors, monitoring of MDR expression by in situ hybridization, quantitation of P-glycoprotein content by laser flow cytometry (and/or immunohistochemical methods), and drug efflux (by laser flow cytometry) may be the best ways to monitor multidrug resistance in human tumors.

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## Introduction

The presence of inherent or acquired multidrug resistance (MDR) may be one of the major obstacles in achieving long-term remissions in patients with malignant disease. One of the major mechanisms for cellular resistance to a variety of natural products (e.g., anthracyclines, vinca alkaloids) is expression of the MDR-1 gene encoding a 170-kDa membrane glycoprotein (P-glycoprotein) [44, 53, 55]. Amplification and increased expression of the MDR-1 gene in multidrug-resistant cells has been reported in both cell lines and clinical specimens [2, 10, 17, 43, 46, 53]. Transfection of the MDR-1 cDNA into drug-sensitive cells has been shown to confer resistance in transfectants [8, 9, 32, 48, 54]. The function of P-glycoprotein, although not fully understood, is believed to be an energy-dependent efflux pump for various hydrophobic drugs, thus leading to MDR [3, 15, 18]. The presence of detectable levels of P-glycoprotein in normal tissues such as the large intestine, adrenal glands, kidney, liver, and brain has led to the speculation that this highly conserved protein is involved in normal detoxification and transport of lipophilic molecules, secretory products, and other xenobiotics [6, 12, 52].

Although amplification/expression of the MDR-1 gene and the putative gene product P-glycoprotein has been amply documented in a variety of cell lines, human tumor cells, and normal tissues, the relevance of these markers in the clinical response to therapy needs to be elucidated. Several studies suggest that the presence of P-glycoprotein may indeed correlate with the emergence of resistance. However, it is not clear if amplification/expression of the MDR-1 gene leading to the presence of P-glycoprotein alone will determine the clinical response of a tumor to a drug or to agents that may block the efflux of a drug and enhance its retention and, thus, the tumor's sensitivity.

Most of the information regarding MDR is derived from studies on cells selected in vitro for their high degree of resistance. However, the level of in vitro drug resistance of tumor cells from clinical tumor specimens is generally very low, and the detection and quantitation of low-level

MDR-1 transcripts by standard techniques of blot hybridization are often cumbersome and inconclusive. Moreover, each tumor sample may be a heterogeneous mix of cells with varying levels of MDR-1 expression. Such heterogeneity cannot be determined by the standard protocols of RNA extraction and Northern and slot blot hybridization. In the present investigation, we used an in situ hybridization technique in addition to Northern blot and slot blot hybridization to study the intracellular distribution of MDR-1 mRNA in tumor cells.

We analyzed tumor cells and cell lines established from four different lung-carcinoma patients who by clinical standards would be considered as refractory. They either had rapidly growing malignant disease that did not respond to therapy or had relapsed after initially responding to a multidrug protocol. This study was undertaken to analyze MDR-1 gene expression by various analytical methods and to correlate the presence of mRNA, P-glycoprotein, and drug efflux with chemosensitivity in soft agar.

## Patients and methods

### *Patients*

*FCCL-1 (FM).* An 80-year-old woman was diagnosed with adenocarcinoma of the lung, stage IIIB, and malignant pleural effusion in February 1987. Pleural effusion (pretherapy) was layered over a Percoll gradient and tumor cells (94%) were retrieved after centrifugation. The fraction of tumor cells in pleural effusion was determined by microscopic examination of stained cytospin slides and also by flow cytometry (after gating for normal diploid cells). A monolayer cell line was established from this specimen and maintained in continuous culture. The cell line is not tumorigenic in nude mice. This patient was put on two cycles of a protocol combining doxorubicin (60 mg/m$^2$) and prochlorperazine (15 mg/m$^2$). The pleural effusion increased and the patient died in October 1987. Several samples of pleural fluid were collected for laboratory studies.

*FCCL-4 (GI).* A 55-year-old woman diagnosed with small-cell lung carcinoma (vena caval obstruction and malignant pleural effusion) in June 1986 was treated with three cycles of combination chemotherapy (cyclophosphamide, doxorubicin, vincristine, and etoposide). A complete remission lasting for nearly 1 year was followed by the reappearance of pleural effusion. Reinduction therapy with six cycles of cyclophosphamide, doxorubicin, and vincristine resulted in a second complete remission that lasted for 16 months and was again followed by a relapse. Later, a partial remission induced by bleomycin, cisplatin, etoposide, and vincristine lasted for 11 months and was followed by a relapse, brain metastasis, and death in October 1989. A cell line was established from pleural effusion obtained in November 1988, 29 months after the initial diagnosis and 11 months before the patient's death. The cell line is not tumorigenic in athymic mice.

*FCCL-8 (MR).* A 60-year-old man diagnosed with stage IV adenocarcinoma of the lung in June 1987 failed to respond to cisplatin, vinblastine, and palliative radiation therapy and expired in December 1987. Pleural fluid was collected 5 days before the patient's death and cells retrieved on a Percoll gradient were used for establishment of a monolayer cell line. The established cell line is aggressively tumorigenic in athymic mice and the histopathology resembles that of the original clinical material.

*FCCL-10 (DD).* A 43-year-old man was diagnosed with stage IIIB adenocarcinoma of the lung in November 1989. Hodgkin's disease had been diagnosed 15 years earlier and cured with nitrogen mustard, vincristine, procarbazine, prednisone, doxorubicin, bleomycin, and dicarbazine. Tumor cells retrieved from pleural effusion were used for laboratory studies as well as for establishment of a monolayer cell line. The tumor cells required Matrigel basement membrane (Becton Dickinson Lab. ware, Bedford, Mass.) to generate tumors in athymic mice. This patient died in June 1990 with advanced malignant disease.

Our experimental details for the establishment of lung-tumor cell lines have been described elsewhere [39]. The phenotype of cultured tumor cells in the established cell lines was confirmed by pathological examination of cytospin preparations.

### *Murine leukemic cell lines*

The murine leukemic P388 cell line and its doxorubicin-resistant (approximately 100-fold) subline P388/R84 were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

### *Cytotoxicity studies*

The 50% growth-inhibitory concentration ($IC_{50}$) for doxorubicin (Adriamycin hydrochloride, NSC-123127; Adria Labs, Columbus, Ohio) in each cell line was determined in soft-agar assays as previously described [19].

### *Flow cytometry*

Cells incubated for 1 or 2 h at 37° C in a humidified atmosphere containing 5% $CO_2$ and 95% air with 3.5 μM daunorubicin hydrochloride (Cerubidine, NSC-821151; Wyeth Labs, Philadelphia, Pa.) or doxorubicin with or without the addition of prochlorperazine edysilate (25 μM, Compazine; Smith Kline and Beecham Labs, Philadelphia, Pa.) were analyzed for cellular drug fluorescence in an Epics 753 cell sorter interfaced with an MDADS data-acquisition and -analysis system (Coulter Electronics, Hialeah, Fla.). Details of our flow-cytometric method for monitoring anthracycline fluorescence have been reported earlier [26]. The propidium iodide/hypotonic citrate method was used for monitoring of cellular DNA content (DI).

### *Preparation of slides and of RNA and DNA probes*

Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, washed twice with PBS, and resuspended at $10 \times 10^6$ cells/ml in RPMI 1640 medium [31]. Cells were then cytocentrifuged onto acid-washed and precleaned slides, air-dried for 5 min, and stored at 4° C until used for hybridization.

The recombinant plasmid p683 carrying an Hae III fragment of the human MDR gene (MDR-1) was a generous gift from Dr. A. T. Fojo (National Cancer Institute, Bethesda, Md.). The MDR-1 cDNA is a 683-bp fragment covering the residues from –27 up to 656 bp of the coding region cloned onto the Sma I site of pGEM 3Z (Promega) plasmid vector. The *Escherichia coli* carrying the recombinant plasmid was cultured and plasmid DNA was isolated by banding in a cesium chloride density gradient [45]. Purification by digestion with 20 μg RNase/ml at 37° C for 30 min was followed by proteinase K digestion (50 μg/ml, 37° C, 1 h), phenol: chloroform extraction, and ethanol precipitation. The orientation of the insert relative to SP6 and T7 promoters on the vector was established by restriction analysis using Bam HI and EcoRI. Linear templates encoding in vitro transcripts complementary to MDR-1 mRNA (antisense or noncoding strand) and opposite insert orientation (sense or coding strand) were obtained by truncation of purified plasmid DNA at the EcoRI (18 bp downstream) and Bam HI (about 3 bp downstream) sites, respectively. The digested samples were extracted sequentially with phenol: chloroform and chloroform and were passed over a Sephadex G-50 column.

Antisense and sense RNA probes were transcribed from 2.0 μg linearized plasmids in 20 μl reaction mixtures containing 1.0 μl RNase inhibitor; 2 μl 10X transcription buffer; 1.0 μl each of 10 μM adenosine triphosphate (ATP), cytidine triphosphate (CTP), and guanosine triphosphate (GTP) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.); and [$^{35}$S]-uridine triphosphate (UTP, 80 μCi; sp. act., 1000 Ci/mM; New England Nuclear, Boston, Mass.). Transcription was initiated by the addition of 0.5 μl SP6 or T7 RNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as appropriate. Following incubation at 40° C for 30 min, an additional 0.5 μl polymerase was added and the incubation was continued for another 30 min [21]. The probe was purified by phenol:chloroform extraction followed by Sephadex G50 column chromatography. The length of labeled sense and antisense probes was reduced to approximately 100 nucleotides by hydrolyzing the samples in a solution containing 40 mM $NaHCO_3$ and 60 mM $Na_2CO_3$ (pH 10.2) at 60° C [7]. The RNA probes were later neutralized by the addition of sodium acetate and glacial acetic acid to 0.1 M and 0.5%, respectively, and were ethanol-precipitated overnight at –20° C in the presence of 10 μg glycogen as the carrier (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Following centrifugation, the precipitate was dissolved in Denhart's solution [0.02% each of bovine albumin serum (Sigma Chemical Co.), Ficoll (Pharmacia, Piscataway, N.J.) and polyvinylpyrollidine, and 10% dextran sulfate (both from Sigma Chemical Co.)] containing 300 mM NaCl, 20 mM TRIS HCl (pH 8.0), 5.0 mM ethylenediaminetetraacetate (EDTA), and 50% recrystallized formamide to a concentration of about $7 \times 10^4$ cpm/μl.

For preparation of the $^{32}$P-labeled DNA probe, plasmid p683 was restricted with EcoRI and Bam H1 and electrophoresed on a 1% agarose gel. The inserted gene was electro-eluted from the gel, purified by passage through an Elutip-D column (Schlecher and Schuell, Keene, N.H.), and concentrated by ethanol precipitation. DNA fragments were labeled with [$^{32}$P]-CTP (3000 Ci/mol; New England Nuclear, Boston, Mass.) by the random prime method [11] using hexadeoxyribonucleotides (Pharmacia) and Klenow fragment enzyme (Bethesda Research Laboratories, Bethesda, Md.).

## *In situ hybridization*

$^{35}$S-labeled antisense and sense transcripts were hybridized to preparations according to the procedure described elsewhere [41]. Slide preparations were first incubated in a solution containing 100 mM TRIS HCl (pH 8.0), 50 mM EDTA, and 1.0 μg proteinase K/ml for 10 min at 37° C and were then washed briefly in water and air-dried. For annealing, 15 μl $^{35}$S-labeled probe (total, $1 \times 10^6$ cpm) was placed on the slide under a 22- × 22-mm coverslip. The edges of the coverslip were sealed with rubber cement and the slides were incubated in a moist chamber at 45°–47° C for 24 h. The antisense MDR-1 RNA probe primed by SP6 promoter was hybridized to slide preparations of all cell lines. Duplicate slides hybridized to a T7 promoter-primed sense probe of the same specific activity served as controls. Cytological preparations treated with RNase A [50 μg/ml in 100 mM TRIS and 50 mM EDTA (pH 8.0), 37° C, 1 h] were also hybridized for further determination of the specificity of the antisense probe. The preparations treated with DNase I (20 μg/ml, 30 min) were hybridized with antisense probe to check for the possible binding of the latter with homologous DNA sequences. After hybridization, the rubber cement was removed and the slides were washed in five changes of 2 × SSC (SSC: 150 mM sodium chloride, 15 mM sodium citrate) at room temperature for 10 min each except that the third wash was done at 50° C. The nonspecifically bound radioactivity was removed by incubating the slides in RNase A (20 μg/ml in 2 × SSC) at 25° C for 20 min. This was followed by five more washes in 0.1 × SSC at room temperature for 10 min each and dehydration in an ascending series (30%, 50%, 70%, and 90%) to 100% ethanol. Slides were air-dried and coated with Kodak NTB3 liquid emulsion diluted with an equal volume of water for autoradiography. Following exposure in a dark environment for 7 days at 4° C, the slides were developed in Kodak D-19 developer, fixed, washed, stained with toluidine blue, and mounted in Pro-texx. Autoradiographic slides were examined under an Olympus microscope. For determination of the extent of hybridization in different cell lines, the number of silver grains were counted in individual cells. Silver grains were also counted in slides annealed with sense strand to determine the nonspecific background, and the mean values were subtracted from the corresponding antisense-hybridization data. A minimum of 30 cells were counted from each cell line.

## *Southern blot hybridization*

Genomic DNA was extracted from cell lines and restricted with EcoRI (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) [45]. The digested DNA (20 μg) was fractionated on 1% agarose gel, stained with ethidium bromide, and photographed. DNA in the gel was denatured with a solution containing 1.5 M NaCl and 0.5 M NaOH for 1 h and then neutralized with buffer containing 1.5 M ammonium acetate and 0.02 M NaOH for 1 h. The denatured DNA from the gel was transferred to a Nytran filter using neutralizing solution as the solvent during blotting [51]. After baking for 2 h at 80° C, the filters were prehybridized and hybridized with the $^{32}$P-labeled random prime probe of MDR-1. The blots were washed and autoradiographed using Kodak X-omat film with intensifying screen at –70° C.

## *RNA extraction and Northern and slot blot hybridization*

Total RNA was extracted from the tumor cells according to the procedure described by Chirgwin et al. [4]. Polyadenylated RNA [poly $(A)^+$ RNA] was isolated from total RNA by chromatography on an oligo (dT) cellulose column (Bethesda Research Laboratories, Bethesda, Md.) using standard procedures [50].

For Northern blots, poly $(A)^+$ RNA (2 μg) was electrophoresed on a 1% agarose gel containing 6% formaldehyde and transferred to a Nytran filter (Schleicher and Schuell, Keene, N.H.) using 10 × SSC as the solvent for 12- to 16-h blotting. The filter was dried and baked in a vacuum oven at 80° C for 2 h and prehybridized in 6 ml hybridization buffer [50% formamide, 0.75 M NaCl, 0.15 mM TRIS HCl (pH 8.0), 10 mM phosphate buffer (pH 6.8), 1 × Denhart's solution, 10% dextran sulfate, 0.1% sodium dodecyl sulfate (SDS)] for 4–6 h. The buffer was drained and replaced with fresh hybridization buffer containing $3 \times 10^7$ cpm $^{32}$P-labeled MDR-1 gene. After incubation at 42° C for 24 h, the filter was washed twice with 2 × SSC containing 0.1% SDS at 25° C for 15 min, followed by three more washes with 2 × SSC at 50° C for 2 h and another wash at 25° C for 10 min. The washed filter was air-dried and exposed to Kodak X-omat film at –70° C for 24 h using an intensifying screen. The film was developed using Kodak GBX developer.

For RNA slot blot analysis, 2 μg total RNA from different cell lines was spotted onto the Nytran filter in a Bio-Dot slot-format apparatus (Bio-Rad Laboratories). The blot was baked, prehybridized, hybridized, washed, and autoradiographed as described above. After autoradiography, RNA slots were cut out from the filter and the radioactivity in each slot was determined by liquid scintillation spectrometry.

## *Immunoprecipitation*

For metabolic labeling of P-glycoprotein, $10^7$ cells were grown in culture medium containing 50 μCi [$^{35}$S]-methionine (New England Nuclear, Boston, Mass.) for 16 h [47]. The [$^{35}$S]-methionine-labeled cells were washed twice with PBS and lysed and proteins were extracted in 1 ml buffer containing 0.2 M TRIS (pH 8.0), 0.1 M EDTA (pH 8.0), 0.1% mercaptoethanol, and 1% polyvinylpyrollidine. The lysate was centrifuged, the supernatant was carefully separated, and a small aliquot was used for determination of trichloroacetic acid (TCA)-precipitable counts.

Immunoprecipitation was carried out by incubating aliquots of labeled extracts containing equal amounts of TCA-precipitable counts with 50 μl (10 μg) C219 monoclonal antibody (Centocor Diagnostic, Inc., Malvern, Pa.) for 16 h at 4° C. Immune complexes were purified with a Protein A-Sepharose Cl-4B (Pharmacia, Piscataway, N.J.) suspension for 1 h at room temperature. The immunoprecipitates were collected by centrifugation and washed twice with NET gel buffer (50 mM TRIS HCl, pH 7.5; 150 mM NaCl; 0.1% Nonidet P-40; 1 mM EDTA, pH 8.0; 0.25% gelatin; and 0.02% sodium azide) and once with buffer containing 10 mM

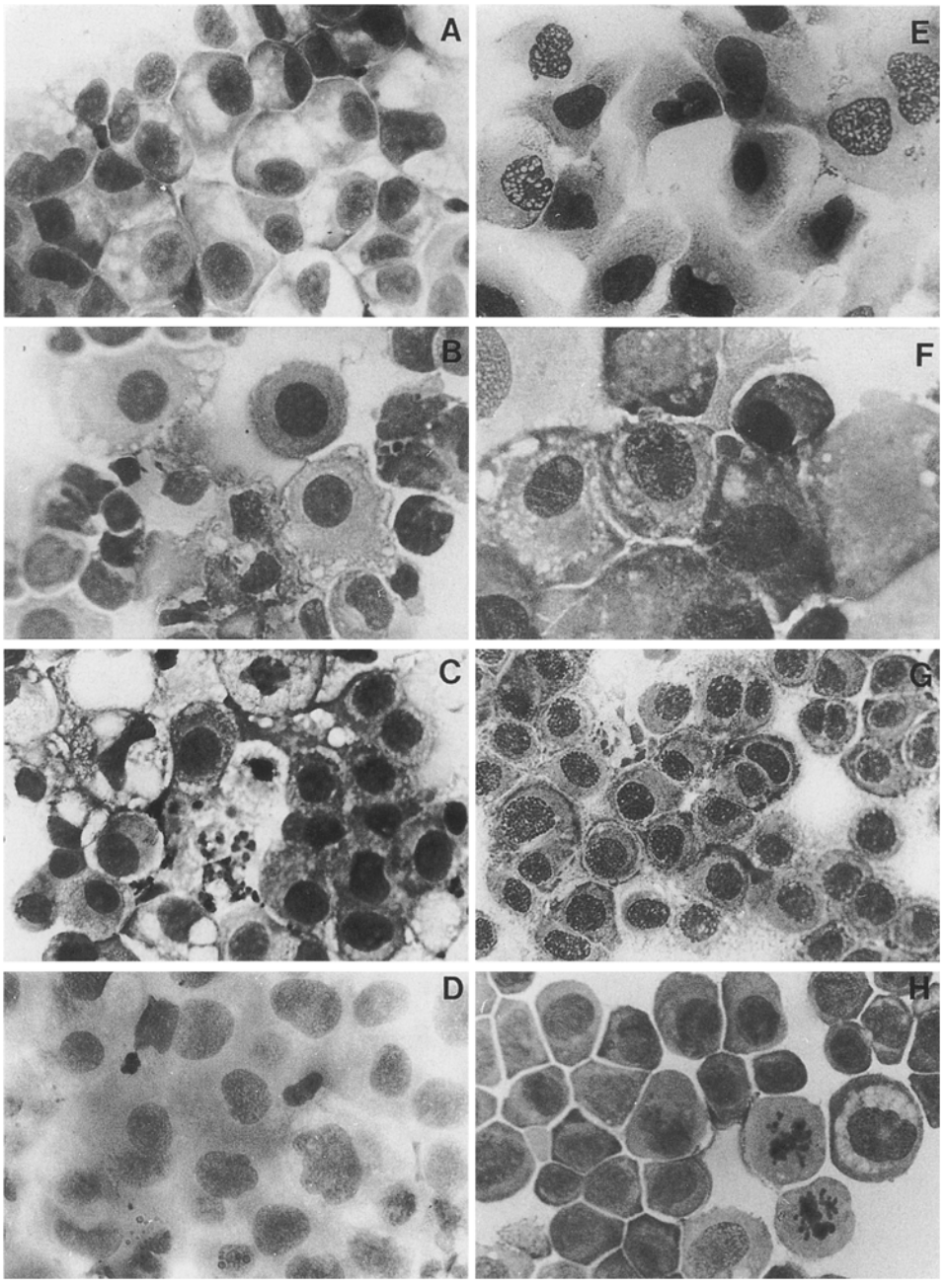


**Fig. 1 A–H.** Morphology of lung-tumor cells from pleural-effusion samples (PE) of patients and from cell lines (TC) established from pleural effusion. **A** PE of FCCL-1. **B** PE of FCCL-4. **C** PE of FCCL-8. **D** PE of FCCL-10. **E** TC of FCCL-1. **F** TC of FCCL-4. **G** TC of FCCL-8. **H** TC of FCCL-10

TRIS HCl (pH 7.5) and 0.1% Nonidet P-40. During each washing, pellets were vortexed vigorously and collected by centrifugation. The radioactivity in the immunoprecipitated protein was then determined by scintillation spectrometry and the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% gel. After drying under vacuum, the gels were exposed for 2 weeks at –70° C for autoradiography [29, 47].

### *Flow-cytometric determination of P-glycoprotein*

Tumor cells ($2.0 \times 10^7$) were analyzed flow cytometrically for P-glycoprotein by our published protocol [27] using P-glycoprotein-specific C219 monoclonal antibody (Centocor Diagnostic Inc., Malvern, Pa.).

## Results

### *Doxorubicin cytotoxicity and MDR-1 expression*

Photomicrographs A–D and E–H in Fig. 1 are of cytospins prepared from pleural effusion of patients and of cell lines established from these specimens, respectively. In general, the cellular morphology of long-term cell lines established from the clinical specimens was similar to that of the tumor cells recovered from pleural effusion.

Human cell lines established from tumor cells isolated from pleural effusion of four patients in the present study had an $IC_{50}$ (1 h exposure) of 0.16–0.31 μM doxorubicin

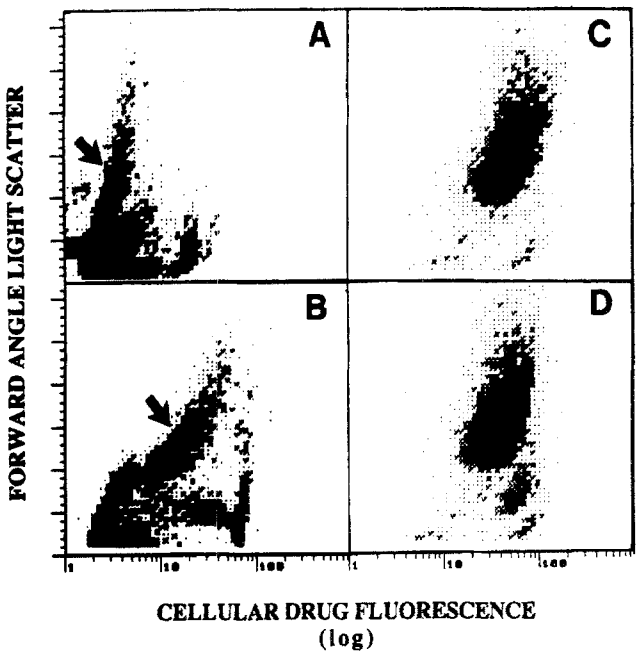


**Fig. 2A–D.** Cellular drug retention **A, B** in the pleural fluid of patient FCCL-1 (FM) and **C, D** in a cell line (FCCL-1) established from this specimen. The *vertical axis* records forward-angle light scatter, which approximates cell size, whereas the horizontal axis records on a 3-decade log scale cellular drug fluorescence. The figure shows drug retention in FCCL-1 (FM) cells (**A**) and in a second sample obtained after 1 month of therapy on a protocol using prochlorperazine (**B**) for blocking of doxorubicin efflux. The *arrow* in **B** shows the presence of cells with enhanced drug retention. Drug retention in FCCL-1 tissue culture cells alone (**C**) or in the presence of 25 μM prochlorperazine (**D**) is also illustrated

in soft-agar assays (Table 1). In contrast, P388 and its drug-resistant subline P388/R84 had an $IC_{50}$ of 0.14 and 7.44 μM doxorubicin, respectively. Thus, the most sensitive lung-tumor cell lines (FCCL-1 and 8) were as sensitive as the murine P388 cells, whereas the most resistant cell line (FCCL-4) was approximately 2 times more resistant.

*Doxorubicin retention*

In cells retrieved from pleural effusion and analyzed by laser flow cytometry for determination of doxorubicin retention, heterogeneity was often observed. Tumor cells concentrated on a Percoll gradient from a pretherapy pleural-effusion sample from a patient (FCCL-1) showed extensive heterogeneity in both forward-angle light scatter (approximate cell size) and drug retention (Fig. 2A). Two smaller populations with high retention but a lower light-scatter signal are visible on the right-hand side of this major population. A second sample obtained after 1 month of therapy on a protocol using prochlorperazine (15 mg/m$^2$ for 2 h) for blocking of doxorubicin efflux showed enhanced doxorubicin retention in the major tumor-cell population (Fig. 2B, arrow). A third pleural-effusion sample obtained after 12 weeks of therapy yielded a similar profile, and in vitro incubation with prochlorperazine (25 μM) did not enhance drug retention or affect heterogeneity. A cell line established from the pretherapy pleural effusion showed high doxorubicin retention and homogeneous doxorubicin retention (Fig. 2C). Coincubation of these cells with prochlorperazine (25 μM) did not enhance doxorubicin retention (Fig. 2D).

In one of the initial pleural-effusion samples obtained from a patient (FCCL-4), a predominant cell population with a DNA index of 1.0 revealed homogeneous daunorubicin retention. In a subsequent sample, extensive heterogeneity in cellular daunorubicin retention was noted. As shown in Fig. 3A, this pleural-effusion sample (after gradient purification) had at least four distinct populations with very low to very high daunorubicin fluorescence. On coincubation with 25 μM prochlorperazine (in vitro), an increase in daunorubicin fluorescence was seen (Fig. 3B). A comparison of the peak fluorescence channel numbers between cells incubated with daunorubicin alone (Fig. 3A) and those incubated in the presence of prochlorperazine (Fig. 3B) showed an increase in peak channel fluorescence from 4 to 6 (log scale), indicating that in the presence of the efflux blocker, some of the cells in this population displayed enhanced daunorubicin retention. However, no major change in the fluorescence of the subpopulations with the lowest and the highest daunorubicin retention was noted. A cell line established from this pleural effusion displayed heterogeneity in light scatter and high daunorubicin retention (Fig. 3C). In the presence of 25 μM prochlorperazine, enhanced daunorubicin retention was evi-

**Table 1.** Doxorubicin cytotoxicity, retention, and modulation

| Cells | | DNA index | $IC_{50}$ (μM DOX) | Retention | DOX heterogeneity | Efflux-blocker effect |
|---|---|---|---|---|---|---|
| FCCL-1 | PE | ND | ND | +++ | +++ | ++ |
| | TC | 1.7 | 0.16 ± 0.02 | +++ | + | – |
| FCCL-4 | PE | ND | ND | +++ | + | ++ |
| | TC | 1.0 | 0.31 ± 0.09 | +++ | + | ++ |
| FCCL-8 | PE | ND | ND | +++ | +++ | ++ |
| | TC | 1.5 | 0.17 ± 0.02 | +++ | + | – |
| FCCL-10 | PE | ND | ND | +++ | ++ | – |
| | TC | 1.2 | 0.28 ± 0.04 | +++ | ++ | – |
| P388 | TC | 1.0 | 0.14 ± 0.03 | +++ | – | – |
| P388/R84 | TC | 1.0 | 7.44 ± 1.36 | + | – | +++ |

PE, Pleural effusion; TC, tissue culture; DOX, doxorubicin; ND, not determined

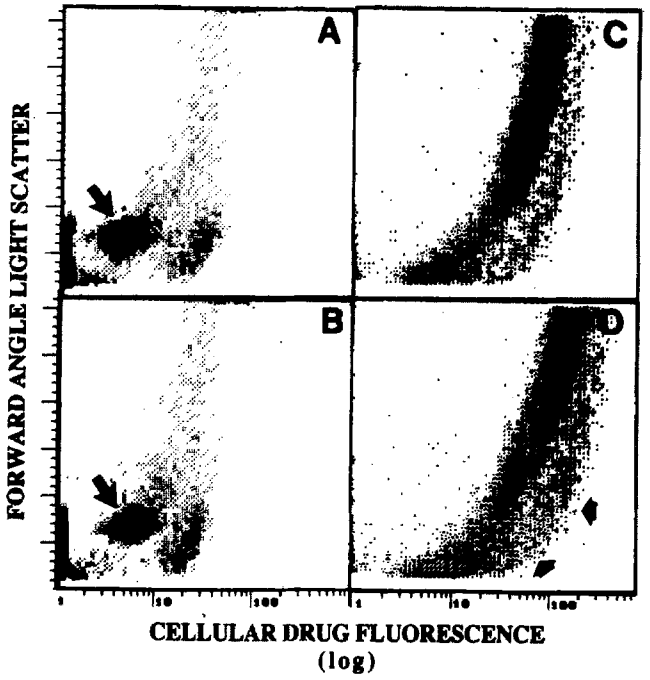


**Fig. 3 A – D.** Cellular drug retention **A, B** in the pleural fluid of patient FCCL-4 (GI) and **C, D** in a cell line (FCCL-4) established from this specimen. The figure shows drug retention in cells incubated with daunorubicin alone (**A, C**) or in the presence of the efflux blocker prochlorperazine (25 μM; **B, D**). The *arrows* in **B** and **D** show the presence of cells with enhanced retention

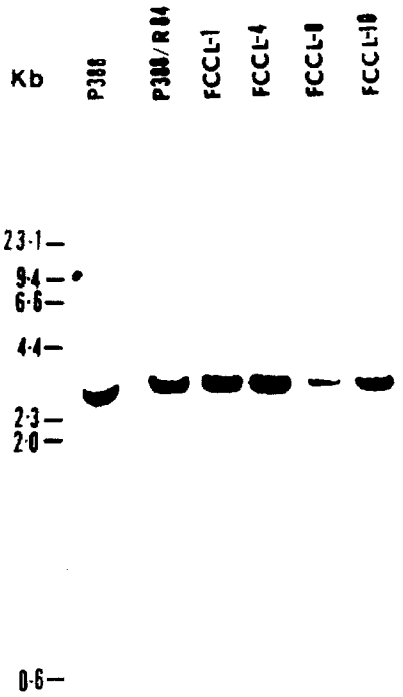


**Fig. 4.** Southern blot analysis of genomic DNA of cell lines. DNA (20 μg) was digested with EcoRI, electrophoresed, and hybridized with the $^{32}$P-labeled MDR-1 probe

dent and the peak fluorescence channel shifted from 80 to 110 (log scale; Fig. 3C, D).

In tumor cells from gradients of pleural effusion (FCCL-8), heterogeneity in doxorubicin retention and enhancement of doxorubicin fluorescence in a subpopulation on coincubation with prochlorperazine was seen (data not shown). However, in the cell line established from this tumor, high doxorubicin retention was not further enhanced by coincubation with the efflux blocker (data not shown). Tumor cells retrieved from pleural effusion (FCCL-10) and the cell line established from it showed high doxorubicin retention (similar to that of P388 cells), which was not further enhanced by coincubation with prochlorperazine (data not shown).

*Southern blot hybridization*

When annealed with the MDR-1 gene probe, EcoRI-digested genomic DNA showed intense hybridization to the 3.1-kb fragment in all murine leukemic and human lung-tumor cell lines (Fig. 4). No amplication of the MDR-1 gene was observed in any cell line. However, only a thin band was observed in FCCL-8, which may indicate a low copy number, probably due to deletion.

*Specificity of antisense probe*

To determine the specificity of the antisense probe, cytospin preparations of cell lines were also hybridized with the sense RNA probe under conditions similar to those employed for incubation with the antisense probe. The silver-grain density in P388/R84 cells annealed with the sense probe was low (Fig. 5A). However, when cells were annealed with antisense transcripts, silver grains were detected well above the background level (Fig. 5B). The label was distributed uniformly in the cytoplasm of cells hybridized with the antisense probe and no concentration in any particular region was noticed. Also, treatment of cells with DNase prior to annealing with the antisense probe did not decrease the number of silver grains. P388/R84 cells depleted of RNA by RNase A treatment showed little hybridization with the antisense probe (Fig. 5C).

*Intracellular variation in the expression of MDR-1*

Doxorubicin-sensitive P388 cells annealed with the antisense probe showed almost the same level of low grain density as did cells annealed with sense transcripts (Fig. 5D). However, in doxorubicin-resistant P388/R84 cells, high expression of the MDR-1 gene was observed uniformly in all cells. When hybridized with the antisense probe, almost all P388/R84 cells showed silver grains varying in number from 18 to 53, with the average being 32.07 grains/cell (Table 2). In contrast, MDR-1 expression was heterogeneous in cells from the four human lung-tumor cell lines studied. Heterogeneity was quite pronounced in FCCL-1 cells, with autoradiographs showing from 1 to 28 grains/cell (Fig. 5E). Among the four lung-

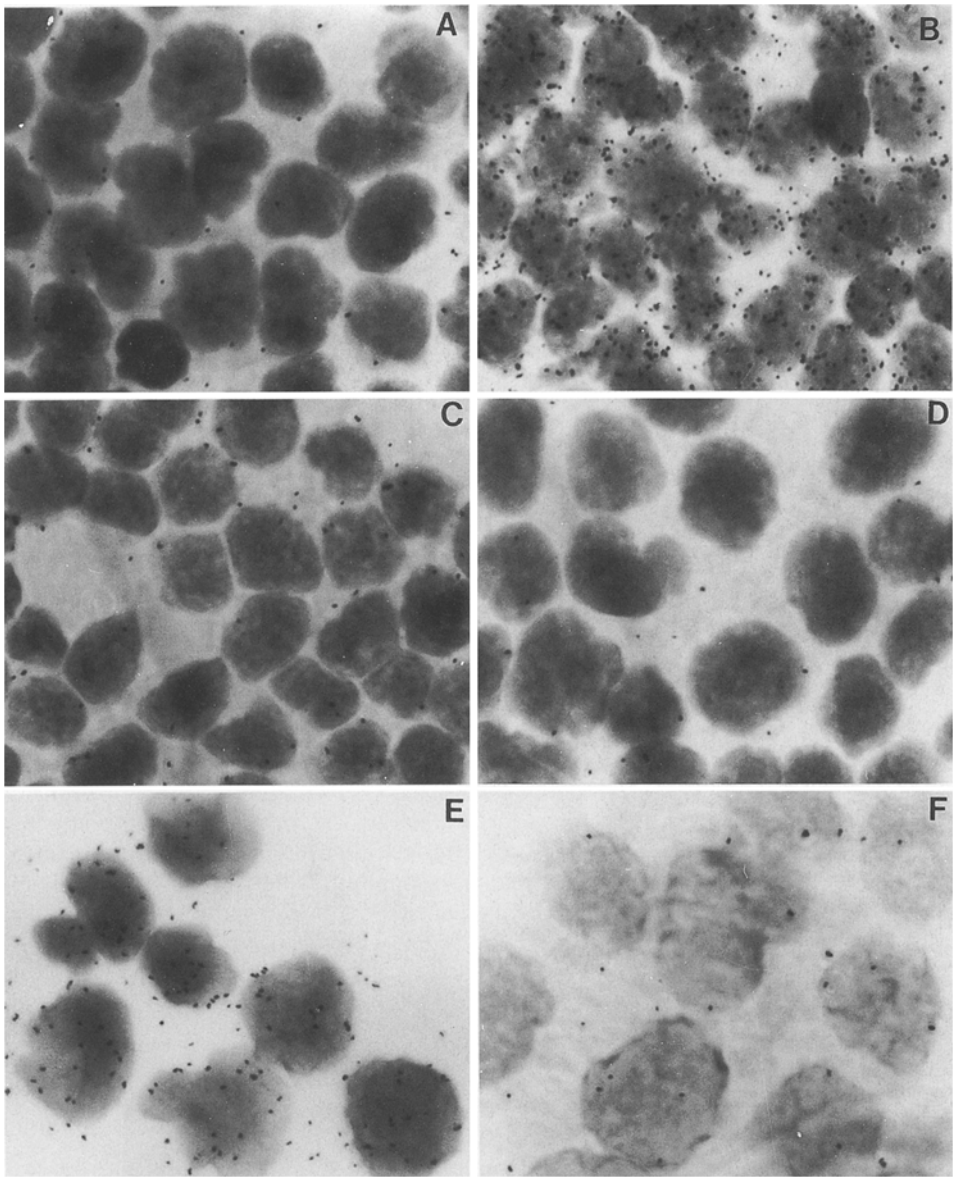


**Fig. 5 A – F.** Autoradiographs of murine leukemic and human lung-tumor cell lines in situ hybridized with $^{35}S$-labeled RNA probes of MDR-1. **A** Doxorubicin-resistant P388/R84 cells annealed with the sense probe. **B** P388/R84 cells in situ hybridized with the antisense probe. **C** P388/R84 cells treated with RNase A and annealed with the antisense probe. **D** Doxorubicin-sensitive P388 cells annealed with the antisense probe. **E** Human lung-tumor cells from FCCL-1 in situ hybridized with the antisense probe. **F** Human lung-tumor cells from FCCL-4 in situ hybridized with the antisense probe

**Table 2.** Distribution of autoradiographic silver grains in tumor-cell lines following in situ hybridization with sense and antisense RNA probes of MDR-1

| Cell line | Probe | Number of silver grains/cell | |
|---|---|---|---|
| | | Range | Mean ± SD |
| FCCL-1 | Antisense | 1–28 | 10.75 ± 6.64 |
| FCCL-4 | Antisense | 0–10 | 3.50 ± 2.50 |
| FCCL-8 | Antisense | 1– 7 | 2.64 ± 1.44 |
| FCCL-10 | Antisense | 3–11 | 6.39 ± 2.66 |
| P388 | Sense | 0– 1 | 0.43 ± 0.57 |
| P388 | Antisense | 0– 3 | 2.07 ± 1.14 |
| P388/R84 | Sense | 0– 2 | 1.17 ± 0.53 |
| P388/R84 | Antisense | 18–53 | 32.07 ± 9.18 |

tumor cell lines, the expression of MDR-1 was highest in FCCL-1 cells followed by FCCL-10 cells. The other two lung-tumor cell lines, FCCL-4 (Fig. 5F) and FCCL-8, displayed low MDR-1 transcript expression.

### *Northern and slot blot hybridization*

We used Northern- and slot-blot analysis to monitor expression of the MDR-1 gene in all cell lines for corroboration with the in situ hybridization experiment. As expected, the $^{32}P$-labeled MDR-1 probe hybridized strongly to the 4.5-Kb mRNA species in P388/R84 cells but underwent

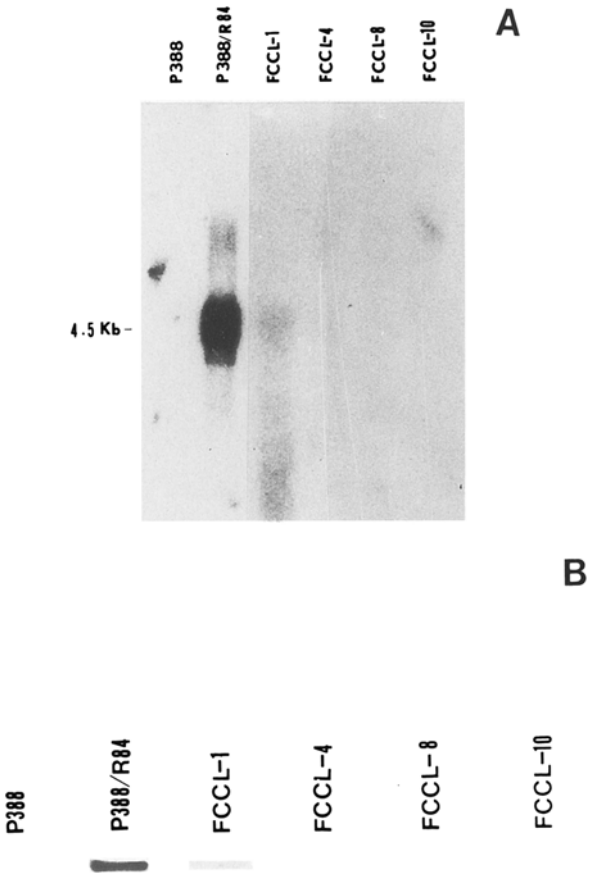


**Fig. 6. A** Northern- and **B** slot-blot analysis of MDR-1 mRNA in different cell lines. In all, 2 µg poly $(A)^+$ RNA for Northern blots and 2 µg total RNA for slot blots was electrophoresed and hybridized with the $^{32}P$-labeled MDR-1 probe

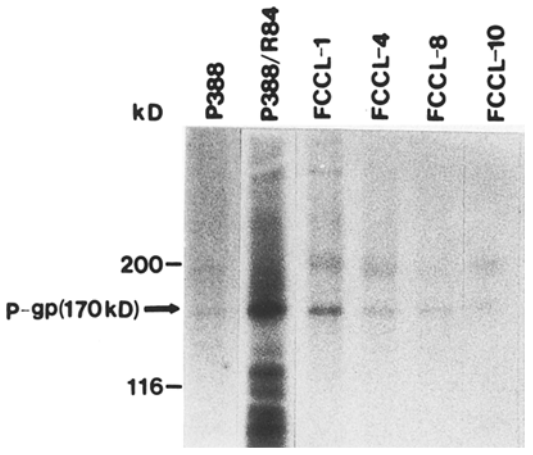


**Fig. 7.** Autoradiograph of immunoprecipitated $^{35}S$-labeled proteins separated by SDS-PAGE. The [$^{35}S$]-methionine-labeled total cellular proteins were immunoprecipitated with C219 monoclonal antibody and separated by SDS-PAGE on a 7.5% gel. The gel was treated with en$^3$Hance, dried, and exposed for 2 weeks for autoradiography

little hybridization with the RNA of sensitive P388 cells (Fig. 6A). Whereas FCCL-1 cells showed a faint band at the 4.5-kb region, all other human lung-tumor cell lines failed to give any signal in the Northern analysis. For quantitation of the MDR-1 mRNA expression, total RNA was hybridized in a slot blot with $^{32}P$-labeled probe. The pattern of hybridization was analogous to that obtained in Northern blots (Fig. 6B). The extent of hybridization as measured by determination of the radioactivity in individual slots showed an approximately 12-fold increase in MDR-1 transcript abundance in P388/R84 cells as compared with P388 cells.

*P-glycoprotein expression*

In Table 3, data on the incorporation of [$^{35}S$]-methionine into proteins precipitated with C219 antibody is presented. Figure 7 shows the separation of C219-precipitated proteins as analyzed by SDS-PAGE. The synthesis of the 170-kDa P-glycoprotein was highest in P388/R84 cells followed by FCCL-1 cells. The incorporation of [$^{35}S$]-methionine was 15–16 times higher in P388/R84 cells than in the drug-sensitive P388 cells. Among the lung-tumor cell lines, only FCCL-1 cells showed about 4 times higher labeling of [$^{35}S$]-methionine into P-glycoprotein than did P388 cells.

Figure 8 shows the flow-cytometric quantitation of P-glycoprotein expression in the four lung-tumor cell lines after staining with the C219 antibody. Single-parameter histograms (insets) and two-parameter dot plots analyzing P-glycoprotein (fluorescein isothiocyanate fluorescence) and DNA content (propidium iodide fluorescence) are presented. The percentage of P-glycoprotein-positive cells was 18%, 5%, 5%, and 12%, respectively, in FCCL-1, -4, -8, and -10 cells. In contrast, P388 and P388/R84 cells showed 13.6% and 81.4% P-glycoprotein expression, respectively (Table 3).

## Discussion

Lung cancer is the leading cause of death from malignant disease in the United States [49]. In spite of some major advances in our understanding of the biology and therapy of this malignancy, long-term cures remain elusive and recurrent refractory disease often leads to death [35]. Although several extracellular mechanisms may account for

**Table 3.** Synthesis of P-glycoprotein in tumor-cell lines[a]

| Cell line | Incorporation of [$^{35}S$]-methionine into proteins precipitated with C219 antibody ($1 \times 10^4$ cpm/mg protein) Mean ± SD | % of P-glycoprotein-positive cells[b] |
|---|---|---|
| FCCL-1 | 1.84 ± 0.23 | 18.0 |
| FCCL-4 | 0.19 ± 0.11 | 5.0 |
| FCCL-8 | 0.29 ± 0.13 | 5.0 |
| FCCL-10 | 0.17 ± 0.04 | 12.0 |
| P388 | 0.43 ± 0.10 | 13.6 |
| P388/R84 | 7.95 ± 0.86 | 81.4 |

[a] $1 \times 10^6$ cells were labeled with 50 µCi [$^{35}S$]-methionine
[b] Determined by flow cytometry

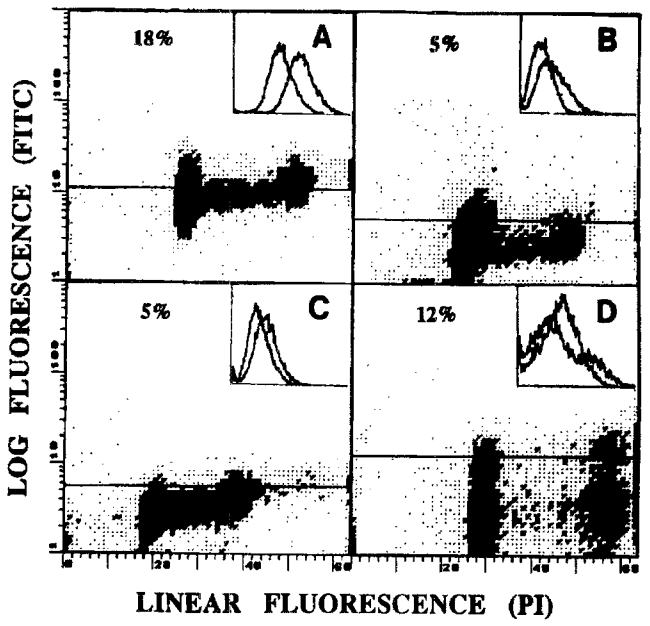


**Fig. 8 A–D.** Two-parameter dot plots and single-parameter histograms (*insets*) of **A** FCCL-1, **B** FCCL-4, **C** FCCL-8, and **D** FCCL-10 cells stained for cellular P-glycoprotein expression [log fluorescein isothiocyanate (*FITC*) fluorescence] after incubation with C219 P-glycoprotein-specific antibody. The *horizontal axis* records cellular DNA content [linear fluorescence, propidium iodide (*PI*)]. The *horizontal lines* indicate electronic gates set to exclude 99% of the fluorescent cells in the isotype control. The *single-parameter insets* show the FITC fluorescence of the isotype control and of cells incubated with C219 antibody

the failure of cancer chemotherapy, emergence of resistance at the cellular level may be a major factor in refractory disease. Several mechanisms and markers of cellular resistance have been reported [22, 28, 36]. MDR has recently been investigated both in vitro and in vivo, and expression of the MDR-1 gene has been indicated as a possible factor in inherent or acquired resistance [17, 43, 44, 53, 55]. Protocols have been devised to block the effect of MDR-related drug efflux with the aim of enhancing cellular drug retention and overcoming resistance [20, 34].

The amplification and/or increased expression of MDR-1 had been suggested to be responsible for imparting multidrug resistance in human tumor cells [2, 10, 42, 54]. In the present investigation, the MDR-1 gene was transcribed and translated to the highest level in P388/R84 cells. Among the human lung-tumor cell lines established from refractory patients, we did not see any major increase in the number of gene copies, and in Southern blots, the P388, P388/R84, and FCCL-1, -4, and -10 cell lines showed almost the same intensity of hybridization. Several investigators have previously reported that overexpression of MDR-1 (as determined by mRNA) in clinical tumor specimens and cell lines is not associated with gene amplification [16, 24] and that MDR-1 gene expression may be regulated at the transcriptional level [25]. The absence or low involvement of *cis*-regulatory sequences and/or their interaction with nuclear transacting factors may be one of the reasons for the absence or low expression of the MDR-1 gene in P388 and human lung-tumor cells, respectively [24, 25]. Moreover, in a recent study [33], the CpG-rich sequences marking the 5′-end of MDR-1 has been found to be hypomethylated to different extents in different cell lines, and the hypomethylation is correlated with the transcriptional activity of MDR-1 in cell lines. Hence, it is possible that methylation of DNA may also play a role in the regulation of MDR-1 gene expression.

Northern and slot blot hybridization involve the extraction of RNA from cells and, hence, cannot detect the cellular heterogeneity of MDR-1 transcripts in tumor cells. Even the recently reported polymerase-chain-reaction method for quantitation of MDR-1 message [13, 38, 40] would not be capable of providing this information. The technique of in situ hybridization used in the present study showed that FCCL-1 cells had the maximal heterogeneity in MDR-1 expression as compared with the homogeneous expression observed in the doxorubicin-resistant P388/R84 cells. The silver grains in the in situ hybridized cells were distributed throughout the cytoplasm, and no specific binding of the probe in any particular cellular compartment was evident. This observation rules out the possibility of any binding of the probe to the homologous DNA sequences. We also did not observe any decrease in the level of hybridization in DNase-treated cells. This implies that the MDR-1 transcripts are transported to the cytoplasm following their transcription in the nucleus.

Because of the small number of tumor cells available in the clinical specimens, we did not analyze the gene expression in the pleural effusions from the lung cancer patients. Moreover, the MDR-1 gene clone was not available to us at that time. Instead, the pleural effusions were used for establishing cell lines and conducting drug-uptake and -modulation experiments. The lung-tumor cell lines established were also analyzed for the expression of MDR-1 mRNA and P-glycoprotein. All lung-tumor lines examined showed a high degree of heterogeneity in the expression of MDR-1 mRNA. The expression level of MDR-1 mRNA was low in lung-tumor cells as compared with P388/R84 cells. Among the human lung-tumor lines, FCCL-1 and FCCL-10 showed a high level of heterogeneity and enhanced synthesis of MDR-1 mRNA and P-glycoprotein expression.

Moderate to high heterogeneity for doxorubicin or daunorubicin uptake and retention was the major characteristic of pleural-effusion samples from the lung cancer patients. FCCL-1 and FCCL-8 cells exhibited a high degree of heterogeneity. The cell lines established from pleural-effusion samples were also heterogeneous, although to a lower degree, for drug accumulation in the cells. Some populations of cells in the pleural-effusion samples from FCCL-1, -4, and -8 showed increased drug retention when the cells were incubated with prochlorperazine.

In a detailed study, Lai et al. [30] suggested that clinical multidrug resistance cannot be explained solely on the basis of expression of the MDR-1 gene in lung cancer. Many other workers have reported the absence of detectable levels of MDR-1 mRNA and P-glycoprotein expression in lung-tumor cells [1, 5, 37]. Recently, Ganapathi et al. [14] also failed to detect any difference in MDR-1 mRNA and P-glycoprotein levels or any alteration in the cellular uptake, retention, or cytotoxicity of vincristine between drug-sensitive and 5-fold doxorubicin-resistant

L1210 mouse leukemic cells. However, in the 10-fold and 40-fold doxorubicin-resistant sublines, expression of P-glycoprotein was correlated with the level of resistance. Similarly, Keizer et al. [23] have reported a correlation between decreased drug accumulation, altered subcellular drug distribution, and increased P-glycoprotein expression in four multidrug variants of a human squamous lung-cancer cell line, with doxorubicin resistance levels ranging from 10- to 2000-fold. In the present study, we failed to detect a direct correlation between drug resistance and P-glycoprotein expression in FCCL-4 cells. The extremely low level of resistance in the refractory tumor cells and the high heterogeneity of cell populations in drug retention and MDR-1 expression may be the factors responsible for the absence of a correlative picture between drug resistance and P-glycoprotein expression. We also did not observe any correlation between Adriamycin resistance levels and other markers of drug resistance such as glutathione levels and expression of glutathione-related enzymes or of topoisomerase II, in these lung-tumor cell lines (data not shown). However, the level of resistance of these lung-tumor cell lines very well reflects the clinical level of resistance, which is usually very low (<10-fold).

The present observations would indicate that in view of extensive tumor-cell heterogeneity, monitoring of MDR expression by the in situ hybridization, quantitation of P-glycoprotein content by laser flow cytometry (and/or immunohistochemical methods), and determination of drug efflux (by laser flow cytometry) may be the best methods to monitor MDR in human tumor cells. Our earlier studies have shown that tumor cells retrieved from patients do not uniformly respond to the efflux-blocking action of a second agent. Thus, cells that may show enhanced doxorubicin retention in the presence of prochlorperazine may be quite resistant to the efflux-blocking action of verapamil, or vice versa. Similarly, tumor-cell subpopulations in a heterogeneous and mixed population may display a differential response to efflux blockers. Thus, it is important that human tumors be screened not only for the presence of P-glycoprotein, efflux, and heterogeneity but also for sensitivity to a particular efflux blocker. Methodologies have recently been developed that can provide these results with the speed necessary for intelligent decision making based on laboratory data.

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