[CANCER RESEARCH 48, 3595-3602, July 1, 1988]

Multifactorial Resistance to Adriamycin: Relationship of DNA Repair, Glutathione Transferase Activity, Drug Efflux, and P-Glycoprotein in Cloned Cell Lines of Adriamycin-sensitive and -resistant P388 Leukemia[1]

Abdul M. Deffie, Tawfiq Alam, Charita Seneviratne, Samuel W. Beenken,[2] Janendra K. Batra, Thomas C. Shea, William D. Henner, and Gerald J. Goldenberg[3]

The Manitoba Institute of Cell Biology [A. M. D., T. A., C. S., S. W. B., J. K. B., G. J. G.] and Department of Medicine, University of Manitoba, [G. J. G.], Winnipeg, Manitoba, Canada; and The Laboratory of Cancer Pharmacology, Dana-Farber Cancer Institute [T. C. S., W. D. H.] and the Department of Medicine, Harvard Medical School [T. C. S., W. D. H.], Boston, Massachusetts 02115

ABSTRACT

Cloned lines of Adriamycin (ADR)-sensitive and -resistant P388 leukemia have been established, including P388/ADR/3 and P388/ADR/7 that are 5- and 10-fold more resistant than the cloned sensitive cell line P388/4 (Cancer Res., *46:* 2978, 1986). A time course of ADR-induced DNA double-strand breaks revealed that in sensitive P388/4 cells, evidence of DNA repair was noted 4 h after removal of drug, whereas in resistant clone 3 and 7 cells repair was observed 1 h after drug removal. The earlier onset of DNA repair was statistically significant ($p = 0.0154$ for clone 3 cells, and $p = 0.0009$ for clone 7 cells). By contrast, once the repair process was initiated, the rate of repair was similar for all three cell lines.

The level of glutathione transferase activity was determined in whole cell extracts. Enzyme activity (mean ± SE) in sensitive cells was 9.49 ± 1.00 nmol/min/mg protein, that in resistant clone 3 cells was 13.36 ± 1.03 nmol/min/mg, and that in clone 7 cells was 13.96 ± 1.44 nmol/min/mg; the 1.44-fold increase in enzyme activity in resistant cells was statistically significant ($p = 0.01$). Further evidence of induction of glutathione transferase was provided by Northern blot analysis using a ^{32}P-labeled cDNA for an anionic glutathione transferase, which demonstrated approximately a twofold increase in mRNA in resistant clone 7 cells. Western blot analysis with a polyvalent antibody against anionic glutathione transferase also revealed a proportionate increase in gene product in resistant cells.

Dose-survival studies showed that ADR-resistant cells were cross-resistant to actinomycin D, daunorubicin, mitoxantrone, colchicine, and etoposide, but not to the alkylating agent melphalan; this finding provided evidence that these cells are multidrug resistant. Using a cDNA probe for P-glycoprotein, a phenotypic marker for multidrug resistance, Northern blot analysis showed an increase in the steady state level of mRNA of approximately twofold in resistant clone 3 and 7 cells. Southern analysis with the same cDNA probe showed no evidence of gene amplification or rearrangement. Western blot analysis with monoclonal C219 antibody demonstrated a distinct increase in P-glycoprotein in resistant cells.

Efflux of Adriamycin as measured by the efflux rate constant was identical in all three cell lines. Furthermore, the metabolic inhibitors azide and dinitrophenol did not augment drug uptake in either sensitive or resistant cells. These findings suggest that despite the increase in P-glycoprotein, an active extrusion pump was not operational in these cells.

This and previous studies provide unequivocal evidence that resistance to Adriamycin is multifactorial. Decreased drug uptake, decreased formation of DNA single- and double-strand breaks, increased glutathione transferase activity, earlier onset of DNA repair, as well as elevated P-glycoprotein are all characteristic of multifactorial drug resistance.

Received 9/1/87; revised 12/2/87; accepted 3/7/88.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by a grant from the National Cancer Institute of Canada.

[2] Terry Fox Cancer Research Fellow of the National Cancer Institute of Canada.

[3] To whom requests for reprints should be addressed, at Manitoba Institute of Cell Biology, 100 Olivia Street, Winnipeg R3E 0V9, Canada.

INTRODUCTION

Resistance to ADR[4] has been attributed to the presence of an energy-dependent active extrusion pump in heterogeneous populations of ADR-resistant P388 leukemic cells (1–3) and in other anthracycline-resistant cells (4–5). In cloned P388 leukemic cells, resistance to ADR was found to be multifactorial; reduced drug uptake as well as reduced susceptibility of cells to DNA single- and double-strand breaks was observed in resistant cells (6).

GST has also been implicated in resistance of tumor cells to chemotherapeutic agents (7, 8). Elevation of an anionic GST has been reported in human MCF-7 breast cancer cells resistant to ADR (7), and human SCC-25 squamous carcinoma cells resistant to cis-diamminedichloroplatinum(II) (9). By conjugating glutathione with various xenobiotics, GST may play a role in the mechanism of resistance to antitumor agents (7–9).

Resistance to ADR is also associated with MDR or pleiotropic drug resistance (10–13). The MDR phenotype may appear as a result of amplification of the *mdr* or *gp170* gene (14, 15), which encodes for a plasma membrane glycoprotein of M_r 170,000 called P-glycoprotein. The MDR phenotype may also be found without evidence of gene amplification but with overexpression of mRNA of the *mdr* gene (14). Using DNA transfection experiments, it has been shown that a full length cDNA clone from MDR cells confers multidrug resistance on drug-sensitive cells (14). P-Glycoprotein apparently acts to facilitate drug efflux from cells (16) and a striking homology has been reported between P-glycoprotein and a bacterial hemolysin transport protein (17).

In this report, further evidence is provided in support of the proposition that resistance to ADR in cloned P388 leukemic cells is multifactorial. Evidence is also provided to indicate that increase in expression of the P-glycoprotein gene without evidence of gene amplification or rearrangement is associated with the MDR phenotype expressed in these cells. However, alteration of drug efflux could not be demonstrated in resistant cells, using two independent tests of drug efflux. The significance of these findings is discussed relative to the role of P-glycoprotein in multifactorial drug resistance.

MATERIALS AND METHODS

Cell Lines and Cultures. Cloned lines of ADR-sensitive and -resistant P388 leukemia cells have been established previously (6); the cloned sensitive line was designated P388/4 whereas the cloned resistant lines were designated P388/ADR/3 (clone 3) and P388/ADR/7 (clone 7). The cell lines were maintained *in vivo* by weekly transplantation of an

[4] The abbreviations used are: ADR, Adriamycin; GST, glutathione *S*-transferase; MDR, multidrug resistance; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DSB, double-strand breaks; CHO, Chinese hamster ovary; SSC, standard saline citrate (0.15 M sodium chloride:0.015 M sodium citrate, pH 7.4).

inoculum of 10^6 cells i.p. in 6–8-week-old female DBA/2 mice. Suspension cultures *in vitro* were maintained by growing the cells in RPMI medium supplemented with 15% fetal bovine serum (GIBCO, Grand Island, NY). Cells used in all the experiments grew exponentially with a doubling time of 11 to 12 h.

Drugs and Chemicals. [^{14}C]ADR labeled at the 14-C position (specific activity, 28 mCi/mmol) was synthesized by M. Leaffer of the Stanford Research Institute, Menlo Park, CA and was kindly provided by Dr. Robert R. Engle, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. The radiochemical purity was 98% as determined by thin-layer chromatography on silica gel in chloroform:methanol:acetic acid:water (40:10:3:1). Nonradioactive ADR was obtained from Adria Laboratories, Columbus, OH. [^{14}C]Thymidine (specific activity, 50 mCi/mmol) and [^{3}H]thymidine (specific activity, 50–80 Ci/mmol) were obtained from New England Nuclear, Boston, MA. Nonradioactive thymidine, glutathione, and 1-chloro-2,4-dinitrobenzene were purchased from Sigma Chemical Co., St. Louis, MO. Proteinase K was purchased from E. Merck, Damstadt, Germany, and tetrapropylammonium hydroxide was from the Eastman Kodak Company, Rochester, NY.

Drug Efflux Studies. Efflux studies were performed in suspension cultures incubated *in vitro* at cell concentrations of 2 to 4 × 10^6 cells/ml in Dulbecco's phosphate buffered saline. Cells were loaded with [^{14}C]ADR by incubation for 10 min at 37°C. Incubations were terminated by rapid chilling to 4°C and the cell suspension centrifuged through a layer of SF120 oil in Hopkin's vaccine tubes to remove extracellular radioactivity. Washed cells were resuspended at 37°C in a sufficient volume of prewarmed Dubecco's phosphate-buffered saline to minimize the problem of drug reentry; aliquots were removed, chilled, and centrifuged through SF120 oil in Eppendorf tubes, the cells were solubilized in 0.5 N NaOH, and radioactivity was determined by liquid scintillation spectrometry. Cell aliquots were removed prior to efflux in order to obtain the initial intracellular concentration of labelled drug. [^{3}H]inulin was used as a marker of extracellular water to correct for extracellular drug carried through the oil with the cells. The first-order rate constant (K) for ADR efflux was obtained by linear regression analysis of a semilogarithmic plot of a time course of drug efflux over 5 min (18, 19). K is equal to the negative slope of the linear regression line; the half time ($T_{½}$) of ADR efflux was calculated using the equation $T_{½} = \ln 2/K$.

Estimation of DNA Double-Strand Breaks by Neutral Elution. To determine the time course of repair of DNA double-strand breaks, cells prelabeled with [^{14}C]thymidine were treated with Adriamycin for 1 h in RPMI medium without serum and then incubated at 37°C for 24 h in RPMI containing 15% fetal bovine serum; aliquots of the cell suspension were taken at various time points following drug treatment to determine the number of DNA double-strand breaks.

To determine DNA double-strand breaks, 1 × 10^6 drug treated cells labeled with [^{14}C]thymidine were combined with an internal standard, consisting of 1 × 10^6 cells labeled with [^{3}H]thymidine and irradiated with 3000 rads. The combined cell suspension was placed on a 2.0-μm polycarbonate filter (Nucleopore Corp.) and lysed for 1 h with a solution containing 0.5 mg/ml proteinase K. DNA double-strand breaks were detected by the neutral elution technique using a buffer consisting of 0.02 M EDTA and a sufficient quantity of 10% tetrapropylammonium hydroxide to give a final pH of 9.6 as described previously (20). Elution of [^{14}C]DNA was plotted against the simultaneous elution of [^{3}H]DNA as described previously (21, 22). The rate of elution of DNA, which is a function of the number of DNA double-strand breaks, was converted into rad equivalents using standard calibration curves obtained by treating each cell line with a dose range of radiation. The concentration of drug used for treatment of each cell line was selected to reduce the surviving cell fraction to between 0.1 and 0.15, as previously determined by clonogenic assay (6).

Assay of Cytotoxic Activity. The sensitivity of drug-sensitive and -resistant cells to a variety of chemotherapeutic drugs was determined by the clonogenic assay of Chu and Fisher (23). Exponential phase cells at a concentration of 2 to 3 × 10^5 cells/ml were treated with drug for 1 h at 37°C in RPMI 1640 medium. The cloning efficiency of treated cells was determined at each drug concentration and surviving cell fraction was calculated. Linear regression analysis of the dose-survival curves was performed, the regression equation being in the form $\log_e y = mx + b$ where y is surviving cell fraction, x is dose of drug, m is slope of the regression line, and b is the y-intercept. D_0, the dose of drug reducing survival to $1/e$, *i.e.*, 37% of the initial cell population, was derived from the negative reciprocal of the slope of the regression line as described previously (24–26).

Glutathione-*S*-Transferase Assay. Glutathione-*S*-transferase enzyme activity was measured according to the method described by Habig *et al.* (27). Exponentially growing cells were lysed by sonication three times for 10 s in distilled water. The cell lysate was centrifuged at 10,000 rpm for 15 min and the supernatant used for the enzyme assay. To a 1-ml cuvette was added 0.1 ml of 10 mM GSH in 1 M KPO_4 (pH 6.5), 20 μl of 50 mM 1-chloro-2,4-dinitrobenzene in absolute ethanol, and the appropriate amount of sample (as g protein); distilled water was then added to make up the volume to 1 ml. The absorbance at 340 nm was recorded for 5 min at 25°C in a DU-8 spectrophotometer. Enzyme activity was calculated in nmol/min/mg protein or nmol/min/cell.

Drug Uptake Studies. Drug uptake studies were performed as described previously (28, 29) by addition of [^{14}C]ADR to drug-sensitive and -resistant P388 leukemia cells suspended in Dulbecco's phosphate-buffered saline. Incubations were terminated by rapid chilling to 4°C and centrifugation through a layer of SF120 oil in Eppendorf tubes. The washed cells were solubilized in 0.5 N NaOH and radioactivity was determined by liquid scintillation spectrometry.

RNA Extraction and Northern Blot Analysis. RNA was isolated from logarithmically growing cultured cells by the guanidinium isothiocyanate method (30). Poly(A)$^+$ RNA was extracted after two cycles of absorption and elution from an oligodeoxythymidylate-cellulose column (31). Five μg of each mRNA preparation was size fractionated in a 1% formaldehyde-agarose gel and transferred onto nitrocellulose (Schleicher and Schuell, Inc., Keene, NH). The filter was prehybridized at 42°C in 50% formamide, 5× Denhardt's reagent, 5× SSPE (1 × SSPE is 0.15 M NaCl, 0.01 M sodium phosphate, and 0.001 M EDTA) and 100 μg ml^{-1} denatured salmon sperm DNA. Hybridization was performed overnight in the same solution containing 10^6 cpm of either an *Eco*R1-generated 640-base pair pCHP1 insert or a 332-base pair fragment of plasmid PTSS 1–2 that contains nucleotides 110 to 436 of the cDNA sequence for human GSTπ. After hybridization, the filter was washed in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS (sodium dodecyl sulfate) with a final wash in 0.1× SSC and 0.1% SDS at 60°C. Autoradiograms were obtained at −70°C on Kodak XAR film with DuPont Cronex Lightning-Plus screens.

DNA Isolation and Southern Blot Analysis. Genomic DNA from cultured cells was prepared by phenol-chloroform extraction (32). After digestion with *Eco*R1, *Pst*1, or *Hind*III (Boehringer-Mannheim, FRG), equivalent amounts of DNA were electrophoresed in 0.7% agarose gel and blotted onto nitrocellulose. Hybridization conditions were as described above.

Isolation of Membrane Proteins and Western Blot Analysis. Cells were homogenized by sonication and plasma membrane fractions were isolated by ultracentrifugation as described previously (33). Protein was assayed by the method of Lowry *et al.* (34). Membrane protein, 50 μg per lane, was resolved by SDS-PAGE using the method of Debenham *et al.* (35) and transferred to nitrocellulose by the method of Towbin *et al.* (36). The blots were incubated at 37°C overnight in blocking buffer (3% bovine serum albumin, 10.5 mM Tris hydrochloride at pH 7.4, 0.175 M NaCl and 15 mM sodium azide) followed by incubation with C219 monoclonal antibody in fresh blocking buffer at 4°C for 16 h. The filters were washed with Tris-saline buffer, incubated with alkaline-phosphatase conjugated goat anti-mouse IgG in blocking buffer at room temperature, rewashed with Tris-saline buffer and developed using substrate for alkaline phosphatase as described by the supplier (Bethesda Research Lab, Gaithersburg, MD).

Isolation of Cytosolic Proteins and Western Blot Analysis. Exponentially growing cells were sonicated and centrifuged at 11,000 × g for 20 min at 4°C. The supernatant was ultracentrifuged at 105,000 × g for 1 h at 4°C and 100 μg of the cytosolic protein was separated by 12%

sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (37) and transferred by electroblotting to nitrocellulose (36). The nitrocellulose sheet was blocked with 3% bovine serum albumin/0.05% Tween 80 in phosphate-buffered saline, followed by incubation with rabbit-anti-human GSTπ and subsequently alkaline phosphatase conjugated goat anti-rabbit antibody. After further washing, the blot was developed as described above.

RESULTS

Cytotoxic Activity of Various Chemotherapeutic Agents against Adriamycin-sensitive and -resistant P388 Leukemic Cells. Dose-survival curves of ADR-sensitive and -resistant P388 leukemic cells were determined following treatment with a variety of chemotherapeutic agents (Table 1). At least a 50-fold variation was noted in the D_0 for sensitive P388/4 cells ranging approximately from a D_0 of 9 to 13 nM for daunorubicin and actinomycin D, respectively, the most potent agents, to a D_0 of 601 nM for etoposide and of 684 nM for melphalan, the least active compounds. P388/ADR/3 cells were 5-fold more resistant and P388/ADR/7 cells 10-fold more resistant to Adriamycin than the drug-sensitive P388/4 cells (6). Resistant clone 3 and clone 7 cells were cross-resistant to all compounds studied with the exception of melphalan and the relative resistance profile was actinomycin D > daunorubicin ≅ mitoxantrone ≅ colchicine > etoposide. Although clone 7 cells were twofold more resistant to ADR than clone 3 cells, the level of cross-resistance between clone 3 and 7 cells towards the other agents studied appeared less marked.

Time Course of Repair of DNA Double-Strand Breaks Induced by Adriamycin in Adriamycin-sensitive and -resistant P388 Leukemic Cells. DNA double-strand breaks induced in ADR-sensitive and -resistant P388 cells by treatment with ADR for 1 h were determined by the neutral elution procedure of Kohn (20). Evidence of repair of DNA double-strand breaks was observed in all three cell lines as shown in a time course of DSB extending 24 h after removal of drug (Fig. 1). A split-plot analysis of variance was calculated for the data and least squares means comparisons were made between the peak values of DNA damage and DSB remaining at 24 h: these differences were highly significant for all three cell lines ($p = 0.0016$ for sensitive cells, $p = 0.0035$ for resistant clone 3 cells, and $p = 0.0001$ for clone 7). Furthermore, onset of DNA repair and/or reversal of DNA damage in drug-resistant cells (clones 3 and 7) was apparent 1 h after removal of drug, whereas in sensitive cells this did not occur until 4 h. The time course of repair of DNA DSB from peak damage onward was plotted as a double-log relationship (Fig. 1, *inset*). Analysis of covariance comparing the intercepts of these curves, which is an index of the time of onset of repair, showed that there was a significant difference between the sensitive cells and either clone 3 cells ($p = 0.0154$) or clone 7 cells ($p = 0.0009$), but there was no significant difference between the two resistant cell lines. Once the repair process was initiated, the rates of repair, as reflected by the slopes of the repair curves, showed no significant difference between the three cell lines as determined by analysis of covariance (Fig. 1, *inset*).

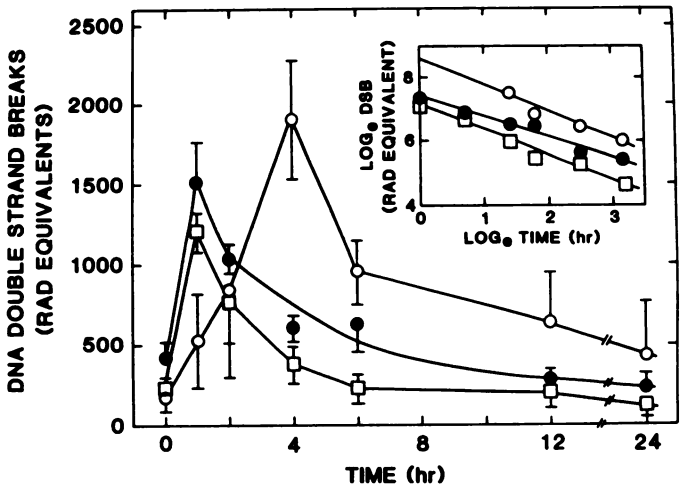

Fig. 1. Time course of repair of DNA double-strand breaks induced by Adriamycin in P388 leukemia cells. Cells were treated with Adriamycin for 1 h at a concentration of 0.2 μM for sensitive P388/4 cells (○), at 0.35 μM for clone 3 cells (●) and 0.5 μM for clone 7 cells (□). The number of double-strand breaks, expressed as rad-equivalents (dose of radiation producing an equivalent number of breaks), is plotted against time after removal of drug. *Curves*, mean of five experiments; *bars*, SE; *inset*, time course of repair of DNA double-strand breaks of the same data from peak DNA damage induced in the resistant clones onward, plotted as a double-log relationship. The linear regression equation for the double log plot for sensitive P388/4 cells was $\log_e y = -0.7881 \cdot \log_e x + 8.464$ with a correlation coefficient of −0.9659, that for resistant P388/ADR/3 cells was $\log_e y = 0.6132 \log_e x + 7.344$ with a correlation coefficient of −0.9845, and that for resistant P388/ADR/7 cells was $\log_e y = 0.7701 \log_e x + 7.071$ with a correlation coefficient of 0.9889.

Glutathione Transferase Activity in Adriamycin-sensitive and -resistant P388 Leukemia Cells. The level of glutathione transferase activity was determined in whole cell extracts of ADR-sensitive and -resistant cells. Enzyme activity (mean ± SE) in sensitive P388/4 cells was 9.49 ± 1.00 nmol/min/mg protein, that in resistant clone 3 cells was 13.36 ± 1.03 nmol/min/mg protein, and that in clone 7 cells was 13.96 ± 1.44 nmol/min/mg protein. Thus the level of glutathione transferase activity was 1.44-fold greater in resistant cells compared to sensitive cells and this difference was statistically significant ($p = 0.01$).

Furthermore, a radiolabeled anionic glutathione transferase cDNA probe demonstrated on Northern blot analysis an increased level of GST mRNA in the resistant cells (Fig. 2). This cDNA probe was cloned from human lung tissue (38), and is identical except for guanine at position 313 and thymine at 341 to the cDNA for human placental GSTπ recently reported by Kano *et al.* (39). However, no changes were detected in the genomic DNA by Southern analysis (data not shown). Densitometric analysis of the Northern blot indicated a progressive increase in mRNA in resistant cells reaching 1.9-fold in clone 7 cells, with reference to signals obtained by hybridization of a similar blot with a control β-actin probe. Western blot analysis with a polyclonal antibody prepared against anionic GSTπ from human mesothelioma (40), demonstrated a proportionate in-

Table 1 *Cross-resistance patterns of cloned P388 leukemic cells to various antitumor agents*

Drug	D_0 for sensitive P388/4 cells[a] (nM)	Relative resistance[b] P388/ADR/3	Relative resistance[b] P388/ADR/7
Adriamycin	33 ± 5	5[c]	10[c]
Daunorubicin	9 ± 1	11[c]	16[c]
Actinomycin D	13 ± 1	18[c]	18[c]
Mitoxantrone	168 ± 4	13[c]	11[c]
Etoposide	601 ± 107	4[c]	6[c]
Colchicine	152 ± 4	ND[d]	12[e]
Melphalan	684 ± 57	1.1[f]	1.2[f]

[a] D_0 values, derived from dose-survival curves of sensitive P388/4 leukemic cells and resistant P388/ADR/3 and P388/ADR/7 cells following treatment with appropriate drug for 1 h at 37°C, as described in the text. Each value is a mean of at least two experiments, ±SE.

[b] Relative resistance is the ratio of D_0 for resistant cells treated with a particular drug to the D_0 for sensitive cells treated with the same agent. A series of t tests were performed comparing the significance of the difference in slopes of the dose survival curves of drug-sensitive and -resistant cells.

[c] $P < 0.001$.

[d] ND, not determined.

[e] $p < 0.01$.

[f] Not significant.

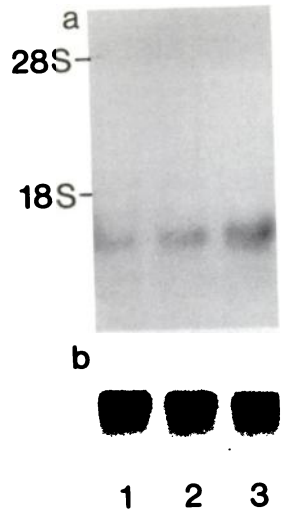

Fig. 2. Northern blot analysis of GST. *A*, RNA blot hybridization with a ^{32}P-labeled GSTπ cDNA fragment showing the relative expression of anionic glutathione transferase. *Lane 1*, drug sensitive P388/4 cells; *lane 2*, drug-resistant P388/ADR/3 cells; *lane 3*, drug-resistant P388/ADR/7 cells. Hybridization and electrophoresis conditions are described in the text. *B*, blot hybridized with a β-actin probe.

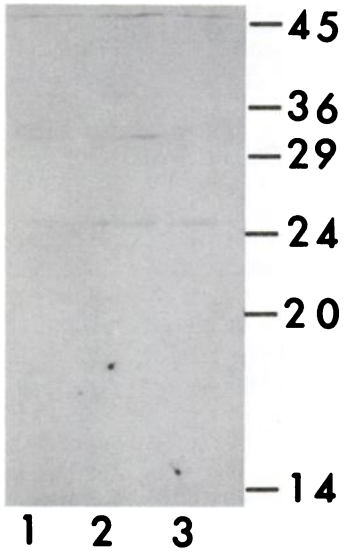

Fig. 3. Western blot analysis showing the relative level of anionic GST in drug sensitive and resistant P388 leukemia cells. Each lane was loaded with 100 μg of cytosolic protein; details of separation and blotting are described in the text. *Lane 1*, sensitive P388/4 cells; *lane 2*, drug-resistant P388/ADR/3 cells; *lane 3*, drug-resistant P388/ADR/7 cells. Positions of molecular weight markers ($M_r \times 10^{-3}$) are indicated on the right.

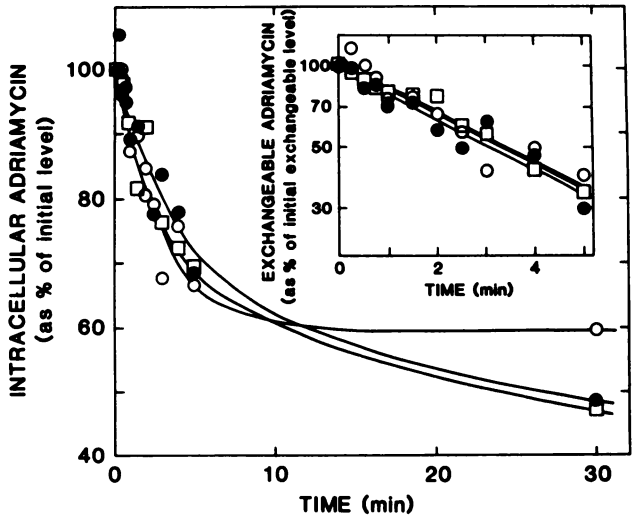

Fig. 4. Efflux of [^{14}C]Adriamycin from P388 leukemia cells. Cells were preincubated for 10 min with ADR at a concentration of 0.1 μM for P388/4 cells (○), 0.15 μM for clone 3 cells (●), and 2.0 μM for clone 7 cells (□). The intracellular drug level (mean ± SE) at the onset of efflux was 2.9 ± 0.6 attomol in sensitive cells, 4.3 ± 0.5 attomol in clone 3 cells, and 5.0 ± 1.7 attomol in clone 7 cells. The cells were washed through a layer of SF120 oil to remove extracellular drug, resuspended in drug-free transport medium at 37°C, and efflux measured as indicated. The intracellular drug concentration was determined and the data expressed as a percentage of initial intracellular drug concentration plotted against efflux time. *Points*, mean of at least four experiments. *Inset*, decay time analysis of drug efflux over the first 5 min presented as a semilogarithmic plot of exchangeable intracellular Adriamycin concentration (expressed as a percentage of the initial exchangeable intracellular drug level) plotted against efflux time. The exchangeable intracellular drug concentration was obtained by subtracting nonexchangeable drug from total intracellular Adriamycin; nonexchangeable drug was derived from the plateau region of the time course of drug efflux. The linear regression equation of the plot for drug efflux from sensitive P388/4 cells was $\log_e y = -0.00345x + 4.6022$ with a correlation coefficient of −0.9480; that for P388/ADR/3 cells was $\log_e y = -0.00351x + 4.5621$, with a correlation coefficient of −0.9480; and for P388/ADR/7 cells $\log_e y = -0.00350x + 4.6110$, with a correlation coefficient of −0.9853.

crease of a protein band with a molecular weight of about 25,000 in the resistant lines (Fig. 3). A control experiment with preimmune antisera detected no comparable band. Parallel studies with cDNA probes for cationic GST isozymes from rat liver provided by Dr. C. B. Pickett and a polyclonal anti-rat liver GST antibody from Dr. K. Tew were negative (data not shown).

Thus, the enzyme activity data and the findings by Northern and Western blot analysis indicate that although the changes are small, taken together the results are consistent with alteration of anionic but not cationic GST in resistant cells.

Time Course of Adriamycin Efflux. A time course of efflux of [^{14}C]ADR from P388 cells is shown in Fig. 4. Drug efflux was relatively rapid for 3 to 5 min, thereafter reaching a plateau level at 30 min with approximately 45 to 60% of the initial radioactivity remaining in the cells. A semilogarithmic plot of exchangeable intracellular ADR against time over the first 5 min of efflux was linear, suggesting that initial drug efflux followed first-order kinetics (Fig. 4, *inset*). The slope of the linear regression equation for drug efflux was used to calculate the $T_{½}$ for efflux, which was virtually identical for all 3 cell lines; the $T_{½}$ was 3.35 min in sensitive cells, 3.29 min in clone 3 cells and 3.30 min in clone 7 cells.

Sodium azide and 2,4-dinitrophenol are metabolic inhibitors which have been shown to inhibit the active extrusion of ADR especially in drug-resistant cells (3, 5). A time course of [^{14}C] ADR uptake by P388 leukemia cells in the presence or absence of sodium azide or 2,4-dinitrophenol showed that these drugs did not augment uptake of ADR as would be expected in the presence of an active efflux pump (Fig. 5). On the contrary, sodium azide or 2,4-dinitrophenol actually diminished uptake of [^{14}C]ADR. The finding of an identical efflux rate constant in drug-sensitive and -resistant cells, together with the failure of metabolic inhibitors to augment ADR uptake strongly suggest that an active extrusion pump was not operational in these cells.

Enhanced Expression of P-Glycoprotein in Adriamycin-resistant Cells. The cDNA clone pCHP1, encoding a 640-base pair fragment of the M_r 170,000 P-glycoprotein, was recently isolated from a cDNA library of a colchicine-resistant CHO cell line (15). The pCHP1 insert was used to determine the relative expression of P-glycoprotein in P388 leukemia cells selected against ADR. Northern blot analysis demonstrated that the probe hybridized to a single mRNA species of 4.7 kilobases, which was relatively enhanced in the resistant lines (Fig. 6*A*).

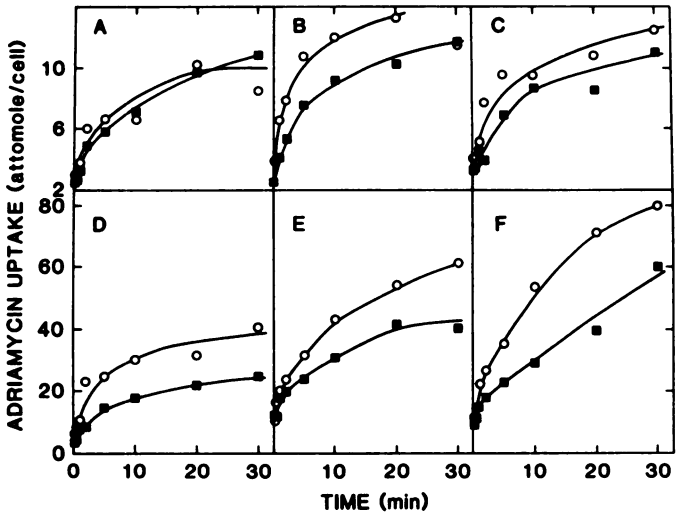

Fig. 5. Time course of uptake of [^{14}C]Adriamycin by drug-sensitive P388/4 cells (*A* and *D*), drug-resistant P388/ADR/3 cells (*B* and *E*), and P388/ADR/7 cells (*C* and *F*) suspended in Dulbecco's phosphate buffered saline at 37°C in the presence of 10 mM glucose (○) or either 1 mM sodium azide or 1 mM 2,4-dinitrophenol (■). Concentration of [^{14}C]Adriamycin used was either 0.2 μM for experiments with sodium azide (*A–C*) or 2 μM for experiments with 2,4-dinitrophenol (*D–F*). Approximately 2–4 × 10^6 cells/ml were incubated with drugs, aliquots of cell suspension were removed at the times indicated and radioactivity was determined by methods described in the text.

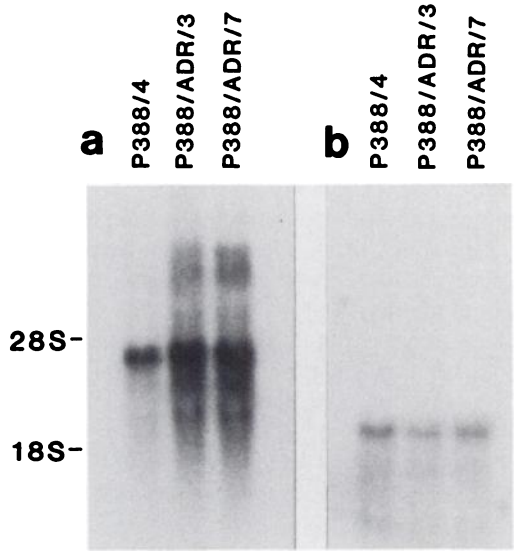

Fig. 6. Northern blot analysis of P-glycoprotein. *A*, RNA analysis showing the relative expression of P-glycoprotein in drug sensitive parental P388 cells and multidrug resistant sublines designated as clone 3 and clone 7. Five μg of each mRNA preparation was electrophoresed in a 1% formaldehyde-agarose gel, transferred onto nitrocellulose and hybridized to pCHP1, a 640-base pair cDNA fragment of P-glycoprotein, as described in "Materials and Methods." *B*, As a control, the first probe was removed by washing in a heated solution of 0.1 × SSC, 0.1% SDS and the same blot was reprobed with ^{32}P-labeled rat β-actin cDNA.

Densitometric scanning of the 4.7-kilobase transcripts, showed an increment of approximately 1.8-fold in clone 3 cells and 2.0-fold in clone 7 cells. By contrast, there was no significant difference in the level of the 2-kilobase β-actin mRNA in the three cell lines (Fig. 6*B*). Using the C219 monoclonal antibody (15), a definite increase in P-glycoprotein level was also detected by Western blot analysis (Fig. 7). The slight difference in molecular weight of P-glycoprotein in resistant cells from that of the positive control represents species variation between mouse and Chinese hamster cells.

To determine if the over expression of P-glycoprotein was due to an increase in gene copy number, DNA from all three

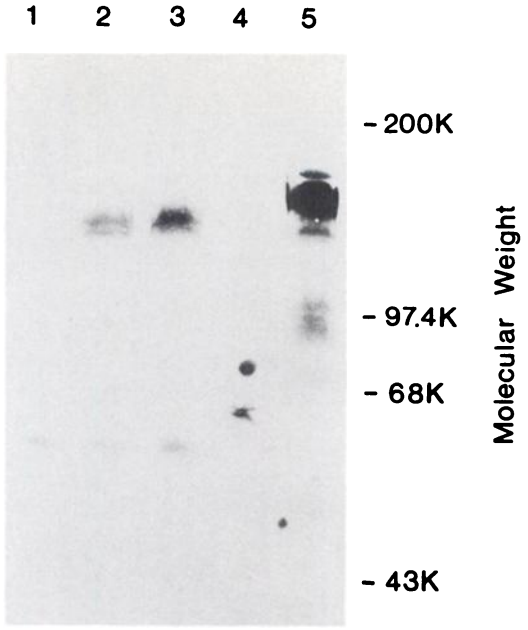

Fig. 7. Western blot analysis of membrane proteins showing increased expressions of P-glycoprotein in drug resistant cell lines. Each lane was loaded with 50 μg of membrane protein; details of separation and blotting are described in the text. A plasma membrane protein-enriched preparation was isolated from sensitive P388/4 cells (*lane 1*); drug-resistant P388/ADR/3 cells (*lane 2*); P388/ADR/7 cells (*lane 3*); Aux B_1 CHO hamster cells (*lane 4*); and CHRC5 drug-resistant CHO cells (*lane 5*), serving as a positive control.

cell lines and DNA derived from the spleen of DBA/2 mice was digested with *Eco*R1, *Hind*III, or *Pst*I (Fig. 8). The Southern blots of the digestion mixture yielded the same banding pattern and hybridization intensity in all cases, suggesting that neither gene amplification nor rearrangement had occurred.

DISCUSSION

Biochemical and pharmacological evidence indicates that resistance to ADR in the cloned lines of Adriamycin-sensitive and -resistant P388 leukemia cells is multifactorial. In an earlier study, it was shown that resistance to Adriamycin in these cells could be attributed to reduced levels of DNA single- and double-strand breaks induced by the drug in resistant cells; the cytotoxic activity of ADR appeared to correlate more closely with formation of DNA double-strand breaks than with single-strand breaks (6).

As a result of this relationship, we compared the time course of repair of DNA double-strand breaks induced by ADR in the drug-sensitive and -resistant cell lines in order to assess the role of DNA repair in the development of drug resistance. The drug-resistant clones 3 and 7 appeared to display an earlier onset of DNA repair compared to drug-sensitive cells (Fig. 1). An alternate interpretation is that DNA damage was more prolonged in sensitive cells and that altered drug accumulation, distribution and/or retention in resistant compared to sensitive cells might account for these findings. This study does not provide a clear choice between these two possibilities, however, we favor the first explanation since extracellular drug concentrations were selected to give comparable cell kill and intracellular drug levels in sensitive and resistant cells (6). Although evidence of repair was earlier in resistant cells, once underway the rate of repair of DNA double-strand breaks was similar in both sensitive and resistant cells. This process whereby resistant cells display an early onset of DNA repair without an increase in the actual rate of repair has also been proposed to account for

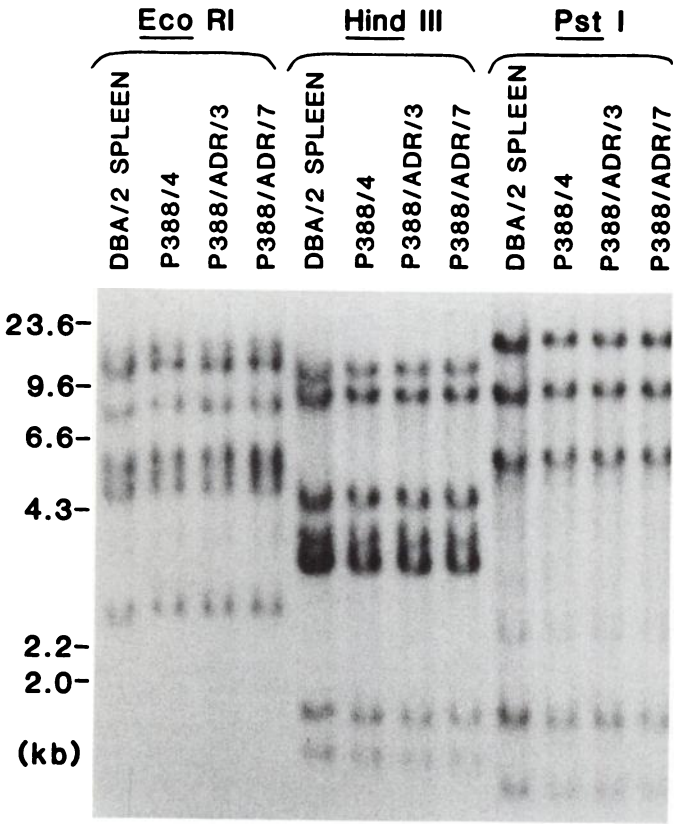

Fig. 8. Southern blot analysis of genomic DNA derived from normal DBA/2 mice, parental sensitive P388 cells, drug resistant clone 3 and clone 7 cells. Genetic DNA (10 μg) was digested to completion with *Eco*RI, *Hind*III, or *Pst*I, transferred onto nitrocellulose and hybridized to pCHP1. The DNA size markers are *Hind*III-digested λ-DNA.

resistance to *cis*-DDP in L1210 cells (41). Other investigators, using other drugs and cell culture systems, have demonstrated a correlation between the ability to repair drug-induced DNA damage and relative drug sensitivity (41–45).

An additional finding that supports the multifactorial nature of resistance to ADR in these cloned P388 leukemia cells is that the level of GST enzyme activity was elevated approximately 50% in drug-resistant cells compared to sensitive cells. Northern and Western analysis show a concomitant increase in mRNA and protein levels of an anionic GST (Fig. 2 and 3); however, we found no such alterations for cationic isozymes (data not shown). Earlier reports indicated a similar overexpression of an anionic GST, without gene amplification, in a MCF-7 cell line 200-fold resistant to ADR (7). GST is a family of enzymes that can detoxify a wide spectrum of xenobiotics via conjugation with glutathione or by reduction of organic peroxides to less reactive alcohols (7, 8, 46–48). The latter mechanism has been suggested to play an important role in the development of resistance to ADR (7). The finding of no alteration of cationic GST together with no cross resistance to melphalan is consistent with a previous report implicating cationic GST in resistance to alkylating agents (8).

Efflux of ADR as measured by the efflux rate constant was identical in sensitive and resistant clone 3 and clone 7 cells (Fig. 4). Furthermore, the metabolic inhibitors azide and dinitrophenol did not augment uptake of ADR in either sensitive or resistant cells (Fig. 5). These findings suggest that an active extrusion pump was not operational in these cells, in contrast to other ADR-resistant cell lines, in which an energy-dependent active drug efflux system has been demonstrated (1–5).

Resistance to ADR in the cloned P388 leukemic cells is also associated with multidrug resistance (MDR) or pleiotropic drug resistance. Cross-resistance is noted towards a variety of other cytotoxic drugs with the notable exception of melphalan (Table 1), a finding noted by others (49, 50). Some of the drugs used in the study of cross-resistance are chemically unrelated to ADR, the drug which was originally used to select the resistant lines. In addition, the level of resistance to actinomycin D and mitoxantrone is higher than that observed for ADR itself, indicating as others have reported that in MDR, the cells need not be most resistant to the primary agent used (16, 51–53).

Pleiotropic resistance to unrelated drugs has been strongly correlated with the presence of P-glycoprotein, a plasma membrane protein of M_r 170,000 (11, 12, 15, 54, 55). A 640-base pair cDNA clone, pCHP1, was recently used to demonstrate P-glycoprotein overexpression due to gene amplification in CHO lines resistant to colchicine (15). There is considerable evidence for gene amplification leading to enhanced P-glycoprotein expression in cells resistant to various drugs (14–17, 56–58); however, although increase in gene copy number seems to be commonly associated with altered expression for cells cultured *in vitro*, this may not hold true *in vivo*, where low level of drug exposure is encountered. Here, we report a transcriptionally upregulated model in cloned P388 leukemia cells selected with ADR. We have shown that a 1.8- and 2-fold increment in P-glycoprotein mRNA levels in clone 3 and 7 cell lines, respectively (Fig. 6), is not the result of gene amplification or rearrangement (Fig. 8). The lack of difference in β-actin gene expression in the sensitive and resistant cell lines suggests that comparable levels of RNA were loaded on the gels (Fig. 6). Moreover, Western analysis with a monoclonal antibody showed a distinct increase in the P-glycoprotein levels in the resistant cells (Fig. 7). Therefore, it would be reasonable to suggest that control of P-glycoprotein gene expression may be exerted by increasing the rate of transcription, message stability, or other posttranscriptional events necessary for maturation and delivery of the RNA to the cytoplasm (59, 60).

It has been shown that P-glycoprotein exhibits sequence homology to bacterial hemolysin export protein (17). In the cloned P388 leukemia cells, although enhanced transcription and translation of P-glycoprotein was detected, no pharmacological evidence of increased pump activity was found. There are at least two possible explanations of this apparent paradox: firstly, the level of P-glycoprotein in these cells may be inadequate to result in measurable pump activity, or secondly, P-glycoprotein may serve in a nontransport fashion to protect cells from low levels of toxic lipophilic compounds; presumably in resistant cells this basic function is augmented as suggested by Gerlach *et al.* (17). If P-glycoprotein acts as a poreforming protein, the pump activity, at least in these cells, may be a relatively late contributor to multifactorial drug resistance. In cells with a higher level of resistance and more dramatic alteration of P-glycoprotein expression, functional evidence of an active extrusion pump might be expected to appear, as reported for other cell lines (1–5).

In the cloned ADR-resistant cells studied here with a relatively modest 5- to 10-fold increase in resistance, pump activity was not evident. A great deal of interest has been directed at P-glycoprotein, however, increased P-glycoprotein is but one of several changes that comprise multifactorial drug resistance, and indeed in some resistant cells enhanced expression of P-glycoprotein does not occur (53). Decreased drug uptake, decreased formation of DNA single- and double-strand breaks, increased GST activity, earlier onset of DNA repair, as well as

elevated P-glycoprotein are all characteristic of multifactorial drug resistance in these cells.

ACKNOWLEDGMENTS

We thank Dr. J. R. Riordan for the pCHP1 clone, Dr. V. Ling for the C219 monoclonal antibody, Dr. C. B. Pickett for cDNA probes against cationic GST transferase, Dr. K. Tew for a polyclonal antibody against GST, Mary Cheang for statistical analyses, and Natalia Eyolfson and Agnes Warkentin for typing the manuscript.

REFERENCES

1. Inaba, M., and Johnson, R. K. Uptake and retention of Adriamycin and daunorubicin by sensitive and anthracycline-resistant sublines of P388 leukemia. Biochem. Pharmacol., *27:* 2123–2130, 1978.
2. Inaba, M., and Sakurai, Y., Enhanced efflux of actinomycin D, vincristine and vinblastine in Adriamycin-resistant subline of P388 leukemia. Cancer Lett., *8:* 111–115, 1979.
3. Inaba, M., Kobayashi, H., Sakurai, Y., and Johnson, R. K. Active efflux of daunorubicin and Adriamycin in sensitive and resistant sublines of P388 leukemia. Cancer Res., *39:* 2200–2203, 1979.
4. Dano, K. Active outward transport of daunorubicin in resistant Ehrlich ascites tumor cells. Biochem. Biophys. Acta, *323:* 466–483, 1973.
5. Skovsgaard, T. Carrier-mediated transport of daunorubicin, Adriamycin and rubidazone in Erlich ascites tumor cells. Biochem. Pharmacol., *27:* 1221–1227, 1978.
6. Goldenberg, G. J., Wang, H., and Blair, G. W. Resistance to Adriamycin: relationship of cytotoxicity to drug uptake and DNA single- and double-strand breakage in cloned cell lines of Adriamycin-sensitive and -resistant P388 leukemia. Cancer Res., *46:* 2978–2983, 1986.
7. Batist, G., Tulpule, A., Sinha, B. K., Katki, A. G., Myers, C. E., and Cowan, K. H. Overexpression of a novel anionic glutathione transferase in multidrugresistant human breast cancer cells. J. Biol. Chem. *261:* 15544–15549, 1986.
8. Wang, A. L., and Tew, K. D. Increased glutathione-*S*-transferase activity in a cell line with acquired resistance to nitrogen mustard. Cancer Treat. Rep., *69:* 677–682, 1985.
9. Teicher, B. A., Holden, S. A., Kelley, M. J., Shea, T. C., Cucchi, C. A., Rosowsky, A., Henner, W. D., and Frei, E. Characterization of a human squamous carcinoma cell line resistant to *cis*-diamminedichloroplatinum(II). Cancer Res., *47:* 388–393, 1987.
10. Ling, V., and Thompson, L. H. Reduced permeability in Chinese hamster ovary cells as a mechanism of resistance to colchicine. J. Cell Physiol., *83:* 103–116, 1974.
11. Carlsen, S. A., Till, J. E., and Ling, V. Modulation of drug permeability in Chinese hamster ovary cells. Possible role for phosphorylation of surface glycoproteins. Biochem. Biophys. Acta 467: 238–250, 1977.
12. Elliott, E. M., and Ling, V. Selection and characterization of Chinese hamster ovary cell mutants resistant to melphalan. Cancer Res., *41:* 393–400, 1981.
13. Biedler, J. L., Chang, T., Meyers, M. B., Peterson, R. H. F., and Spengler, B. A. Drug resistance in Chinese hamster lung and mouse tumor cells. Cancer Treat. Rep., *67:* 859–867, 1983.
14. Gros, P., Croop, J., Roninson, I., Varshavsky, A., and Housman, D. E. Isolation and characterization of DNA sequences amplified in multidrug-resistant hamster cells. Proc. Natl. Acad. Sci. USA, *83:* 337–341, 1986.
15. Riordan, J. R., Deuchars, K., Kartner, N., Alon, N., Trent, J., and Ling, V. Amplification of P-glycoprotein genes in mutidrug-resistant mammalian cell lines. Nature (Lond.), *316:* 817–819, 1985.
16. Riordan, J. R., and Ling, V. Genetic and biochemical characteristics of multidrug resistance. Pharmacol. Ther., *28:* 51–75, 1985.
17. Gerlach, J. H., Endicott, J. A., Juranka, P. F., Henderson, G., Sarangi, F., Deuchers, K. L., and Ling, V. Homology between P-glycoprotein and a bacterial hemolysin transport protein suggests a mode for multidrug resistance. Nature (Lond.), *324:* 485–489, 1986.
18. Sirotnak, F. M., Moccio, D. M., and Young, C. W. Increased accumulation of methotrexate by murine tumor cells *in vitro* in the presence of probenecid which is mediated by a preferential inhibition of efflux. Cancer Res., *41:* 966–970, 1981.
19. Begleiter, A., Grover, J., and Goldenberg, G. J. Mechanism of efflux of melphalan from L5178Y lymphoblasts *in vitro*. Cancer Res., *42:* 987–991, 1982.
20. Bradley, M. O., and Kohn, K. W. X-ray induced DNA double-strand break production and repair in mammalian cells as measured by neutral filter elution. Nucleic Acids Res., *7:* 793–804, 1979.
21. Kohn, K. W., Ewig, R. A. G., Erikson, L. C., and Zwelling, L. *In:* E. C. Friedberg and P. C. Hanawalt (eds.), DNA Repair: A Laboratory Manual of Research Techniques, p. 379. New York: Marcel Dekker, 1981.
22. Goldenberg, G. J., and Froese, E. K. Induction of DNA single- and double-strand breaks by diethylstilbestrol in murine L5178Y lymphoblasts *in vitro*. Biochem. Pharmacol., *34:* 771–776, 1985.
23. Chu, M. Y., and Fischer, G. A. The incorporation of ^{3}H-cysteine arabinoside and its effect on murine leukemic cells (L5178Y). Biochem. Pharmacol., *17:* 753–767, 1968.
24. Goldenberg, G. J., and Alexander, P. The effects of nitrogen mustard and dimethyl myleran on murine leukemic cell lines of different radiosensitivity *in vitro*. Cancer Res., *25:* 1401–1409, 1965.
25. Elkind, M. M., Sutton-Gilbert, H., Moses, W. B., and Kamper, C. Sub-lethal and lethal radiation damage. Nature (Lond.), *214:* 1088–1092, 1967.
26. Goldenberg, G. J., Lyons, R. M., Lepp, J. A., and Vanstone, C. L. Sensitivity to nitrogen mustard as a function of transport activity and proliferative rate in L5178Y lymphoblasts. Cancer Res., *31:* 1616–1619, 1971.
27. Habig, W. H., Pabst, M. J., and Jakoby, W. B. Glutathione-*S*-transferase A: the first enzymatic step in mercapturic acid formation. J. Biol. Chem., *249:* 7130–7139, 1974.
28. Goldman, J. D., Lichtenstein, N. S., and Oliverio, V. T. Carrier-mediated transport of the folic acid analogue methotrexate, in the L1210 leukemia cell. J. Biol. Chem., *243:* 5007–5017, 1968.
29. Goldenberg, G. J., Vanstone, C. L., Israels, L. G., Ilse, D., and Bihler, I. Evidence for a transport carrier of nitrogen mustard in nitrogen mustard sensitive and resistant L5178Y lymphoblasts. Cancer Res., *30:* 2285–2291, 1970.
30. Chirgwin, J. M., Pryzbyla, A. E., MacDonald, R. J., and Rutter, W. J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry, 18: 5294–5299, 1979.
31. Aviv, H., and Leder, P. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA, *69:* 1408–1412, 1972.
32. Blin, N., and Stafford, D. W. Isolation of high-molecular-weight DNA. Nucleic Acids Res., *3:* 2303–2308, 1976.
33. Riordan, J. R., and Ling, V. Purification of P-glycoprotein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced cochicine permeability. J. Biol. Chem., *254:* 12701–12705, 1979.
34. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin-phenol reagent. J. Biol. Chem., *193:* 265–275, 1951.
35. Debenham, P. G., Kartner, N., Siminovich, L., Riordan, J. R., and Ling, V. DNA-mediated transfer of multiple drug resistance and plasma membrane glycoprotein expression. Mol. Cell. Biol., *2:* 881–889, 1982.
36. Towbin, H., Staehlin, T., and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci., USA, *76:* 4350–4354, 1979.
37. Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (Lond.), *227:* 680–682, 1970.
38. Shea, T., Kim, M., and Henner, W. D. DNA sequence analysis of a cDNA clone of GST. Proc. Am. Assoc. Cancer Res., *28:* 6, 1987.
39. Kano, T., Sakai, M., and Muramatsu, M. Structure and expression of a human class glutathione *S*-transferase messenger RNA. Cancer Res., *47:* 5626–5630, 1987.
40. Shea, T. C., Kelley, S. L., and Henner W. D. Identification of an anionic form of glutathione transferase present in many human tumors and human tumor cell lines. Cancer Res., *48:* 527–533, 1988.
41. Micetich, K., Zwelling, L. A., and Kohn, K. W. Quenching of DNA: platinum(II) monoadducts as a possible mechanism of resistance to *cis*-diamminedichloroplatinum(II) in L1210 cells. Cancer Res., *43:* 3609–3613, 1983.
42. Fujiwara, Y., Tatsumi, M., and Sasaki, M. S. Crosslink repair in human cells and its possible defect in Fanconis anemia cells. J. Mol. Biol., *113:* 635–649, 1977.
43. Fraval, H. N. A., and Roberts, J. J. Excision repair of *cis*-diamminedichloroplatinum(II)-induced damage to DNA of Chinese hamster cells. Cancer Res., *39:* 1793–1797, 1979.
44. Meyn, R. E., Jenkins, S. F., and Thompson, L. H. Defective removal of DNA crosslinks in a repair-deficient mutant of Chinese hamster cells. Cancer Res., *42:* 3106–3110, 1982.
45. Plooy, A. C. M., van Dijk, M., Berends, F., and Lohman, P. H. M. Formation and repair of DNA interstrand crosslinks in relation to cytotoxicity and unscheduled DNA synthesis induced in control and mutant human cells treated with *cis*-diamminedichloroplatinum(II). Cancer Res., *45:* 4178–4184, 1985.
46. Jakoby, W. B. The glutathione *S*-transferases: a group of multifunctional detoxification proteins. *In:* A. Meister (ed.), Advances in Enzymology, Vol. 46, pp. 383–414. New York: John Wiley and Sons, 1978.
47. Habig, W. H., and Jakoby, W. B. Assays for differentiation of glutathione-*S*-transferases. *In:* W. B. Jakoby (eds.), Methods in Enzymology, Detoxification and Drug Metabolism: Conjugation and Related Systems, Vol. 77, pp. 398–405. New York: Academic Press, 1981.
48. Suzukake, D., Petro, B. J., and Vistica, D. T. Reduction in glutathione content of L-PAM resistant L1210 cell confers drug sensitivity. Biochem. Pharmacol., *31:* 121–124, 1982.
49. German, D., and Center, M. S. Alterations in cell surface membranes in Chinese hamster lung cells resistant to Adriamycin. Biochem. Biophys. Res. Commun., *105:* 157–163, 1982.
50. Dalton, W. S., Durie, B. G. M., Alberts, D. S., Gerlach, J. H., and Cross, A. E. Characterization of a new drug-resistant human myeloma cell line that expresses P-glycoprotein. Cancer Res., *46:* 5125–5130, 1986.
51. Harker, W. G., and Sikic, B. I. Multidrug (pleiotropic) resistance in doxorubicin-selected variants of the human sarcoma cell line MES-SA. Cancer Res., *45:* 4091–4096, 1985.
52. Danks, M. K., Yalowich, J. C., and Beck, W. T. Atypical multiple drug resistance in a human leukemia cell line selected for resistance to teniposide (VM-26). Cancer Res., *47:* 1297–1301, 1987.
53. Mirski, S. E. L., Gerlach, J. H., and Cole, S. P. C. Multidrug resistance in a

human small cell lung cancer cell line selected in Adriamycin. Cancer Res., *47:* 2594–2598, 1987.
54. Kartner, N., Shales, M., Riordan, J. R., and Ling, V. Daunorubicin-resistant Chinese hamster ovary cells expressing multidrug resistance and a cell-surface P-glycoprotein. Cancer Res., *43:* 4413–4419, 1983.
55. Roninson, I. B., Chin, J. E., Choi, K., Gros, P., Housman, D. E., Fojo, A., Shen, D. W., Gottesman, M. M., and Pastan, I. Isolation of human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells. Proc. Natl. Acad. Sci. USA, *83:* 4538–4542, 1986.
56. Martinsson, T., Dahloff, B., Wattergren, Y., Leffler, H., and Levan, G. Pleiotropic drug resistance and gene amplification in a SEWA mouse tumor cell line. Exp. Cell Res., *158:* 382–394, 1985.
57. Meyers, M. B., Spengler, B. A., Chang, T. D., Melera, P. W., and Biedler, J. L. Gene amplifications-associated cytogenetic aberrations and protein changes in vincristine-resistant chinese hamster, mouse, and human cells. J. Cell Biol., *100:* 588–597, 1985.
58. Van der Blick, A. M., Van der Velde-Koerts, T., Ling, V., and Burst, P. Overexpression and amplification of five genes in multidrug-resistant chinese hamster ovary cell line. Mol. Cell. Biol., *6:* 1671–1678, 1986.
59. Darnell, J. E., Variety in the level of gene control in eukaryotic cells. Nature (Lond.), *297:* 365–371, 1982.
60. Nevins, J. R. The pathway of eukaryotic mRNA formation. Ann. Rev. Biochem., *52:* 441–466, 1983.