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Expression of recombinant glutathione *S*-transferase π, Ya, or Yb_1 confers resistance to alkylating agents

(flow cytometry/multidrug resistance/COS cell/monochlorobimane/chlorambucil)

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ABSTRACT **Increased levels of glutathione *S*-transferase (GST; RX:glutathione R-transferase; EC 2.5.1.18) mRNA, protein, and activity in tumor biopsy samples and in drug-resistant cultured cells are associated with resistance to anticancer drugs. We report that each of three full-length cloned GST cDNAs, that for π (acidic), Ya (basic), and Yb_1 (neutral), can confer drug resistance when expressed in cultured mammalian cells. In one approach, stably transfected mouse C3H/10T½ cells that express GST π, Ya, or Yb_1 were cloned and analyzed for drug resistance in colony-forming assays. Transiently transfected COS cells that were sorted on a fluorescence-activated cell sorter were used in the second approach to avoid interclonal variation in factors other than the recombinant GST and to show that reversion of transient GST expression correlated with loss of drug resistance. A sorting technique, developed to separate the 20% of the electroporated COS cell population that transiently expressed GST π, Ya, or Yb_1 from the nonexpressing population, was based on a GST-catalyzed intracellular conjugation of glutathione to the fluorescent labeling reagent monochlorobimane. GST Ya conferred the greatest increase in resistance to chlorambucil and melphalan (1.3- to 2.9-fold), Yb_1 conferred the greatest increase in resistance to cisplatin (1.5-fold), and π conferred the greatest increase in resistance to a racemic mixture of 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene and 7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene and doxorubicin (1.5- and 1.3-fold) relative to controls. These resistance values to alkylating agents are commensurate with values observed clinically. Cytotoxicity curves representing recombinant GST^+ populations were significantly different from their controls with *P* values ranging from 0.005 to 0.0001. No resistance to vinblastine was detected. Conferred drug resistance was proportional to the magnitude of GST Ya expression, and reversion of transient expression in GST Ya^+ COS cell clones to a GST Ya^- phenotype was associated with total loss of drug resistance.**

A major obstacle to the effective treatment of cancer is the inability to eliminate tumor cell subpopulations that have an intrinsic or acquired resistance to anticancer drugs. Various cellular defense mechanisms have developed that may be important in conferring protection against cytotoxicity induced by anticancer drugs. Decreased intracellular drug accumulation, elevated DNA repair rates, altered topoisomerase activity, and enhanced drug detoxification are associated with development of the drug-resistant phenotype (1–3).

The glutathione *S*-transferases (GST; RX:glutathione R-transferase; EC 2.5.1.18) are a family of dimeric isozymes that can confer resistance to (detoxify) xenobiotic molecules by several mechanisms (1–3). In one mechanism, GSTs catalyze the covalent addition of the tripeptide glutathione to electrophilic molecules, including products of the cytochrome P-450 mixed-function oxidases, yielding conjugates that are generally less reactive and more readily excreted (1). As demonstrated with *in vitro* kinetic analyses, GSTs can catalyze the conjugation of glutathione to the anticancer drugs melphalan (4–6), chlorambucil (4), cyclophosphamide (4), 1,3-bis(2-chloroethyl)-1-nitrosourea (7), and mitoxantrone (8); the diuretic drug ethacrynic acid (9); and the activated metabolite (*anti*-BPDE, a racemic mixture of 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene and 7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene) of the environmental pollutant benzo[*a*]pyrene (10, 11). Additionally, GSTs can sequester hydrophobic compounds by noncovalent and covalent binding (2), and by their intrinsic peroxidase activity, some GSTs can detoxify lipid and DNA hydroperoxides (12).

Cellular mechanisms of drug resistance have been studied by analysis of altered levels of mRNA and activity of drug-metabolizing enzymes, including that of GST, in tumor biopsy samples and in cell lines that have been chronically exposed to anticancer drugs. Recent studies have shown that human GST π (acidic class GST) RNA levels were increased 2- to 4-fold in tumors of the colon, bladder, ovary, lung, and stomach relative to that in normal tissue (13). Also, GST Ya and Yc (basic class GSTs) enzyme activity levels were increased about 3-fold in cell lines selected for resistance to the nitrogen mustard alkylating agent chlorambucil (14, 15), paralleling a 4- to 8-fold amplification in the Ya and Yc genes in one case (14). However, the increased levels of GST activity were accompanied by 2- to 4-fold increases in glutathione content, γ-glutamyl transpeptidase activity, and γ-glutamylcysteine synthetase activity (14), potentially confounding evaluation of the role GST alone may play in conferring resistance to anticancer drugs. Thus, it cannot be determined whether the observed enzyme activity changes are primary or secondary to the drug resistance based on observations of such studies, which are generally descriptive in nature.

Our objective, consisting of two approaches, was to selectively modify one cellular factor only, that of GST activity, to identify those GSTs that confer drug resistance. In the first approach, mouse C3H/10T½ cells that express recombinant cytosolic GSTs were cloned and analyzed in colony-forming assays. In the second approach, fluorescence-activated cell-sorted COS monkey cells were used to avoid interclonal variation in secondary factors and to show that reversion of GST expression is correlated with loss of drug resistance. With these approaches we show that full-length cloned cDNAs for each of the π, Ya, and Yb_1 cytosolic GSTs

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Abbreviations: GST, glutathione *S*-transferase; *anti*-BPDE, racemic mixture of 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene and 7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; FACS, fluorescence-activated cell sorter; SV40, simian virus 40.

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can confer drug resistance when they alone are expressed in these mammalian cells. Furthermore, conferred drug resistance is proportional to the magnitude of recombinant GST expression.

MATERIALS AND METHODS

Cell Culture and Cytotoxicity Assays. The conditions for the growth of COS-M6 monkey kidney cells and C3H/10T½-CL8 (hereafter referred to as 10T½) mouse fibroblasts were described earlier (16). The day before cytotoxicity assays, in which three or four drugs would be tested, GST^+ and control sorted COS cells or 10T½ clones were seeded at 100, 200, 1000, 5000, and 25,000 cells per 60-mm dish. Each cytotoxicity curve represented a control (solvent alone) and six drug concentrations. Generally, at the end of the experiment, six dishes of each drug concentration were scored to assess survival. Within 45 min before each drug treatment, the standard medium (Dulbecco's modified Eagle medium containing 10% fetal bovine serum) was changed to serum-free L-15 medium (25°C), and the agents to be tested were solvated and diluted under yellow light. The cells were exposed to chlorambucil, melphalan, cisplatin, or *anti*-BPDE for 1 hr (25°C, room atmosphere). The drug-containing L-15 medium was aspirated and replaced with standard medium, which was not changed prior to staining of the dishes with crystal violet 11 days later. Doxorubicin or vinblastine was diluted in fresh standard medium and 5-ml samples were added directly to dishes after the medium into which the cells had been seeded the previous day was aspirated. The drug-containing medium was not changed prior to staining dishes at 11 days. Stained colonies that contained >35 cells were scored by machine (Biotran III, New Brunswick Scientific) or by visual and/or microscopic inspection. Colony counts were normalized to the plating efficiencies of control cultures, dishes that received only the solvent vehicle. The best-fit, second-order equation for each survival curve was determined with a computer program, and each curve of a set of curves (GST^+ and control cells) was determined to be or not to be significantly different from the other with an analysis of covariance program. Doxorubicin (Adriamycin), vinblastine, chlorambucil, melphalan (L-phenylalanine mustard) and cisplatin (*cis*-diamminedichloroplatinum) were obtained from Sigma. *anti*-BPDE was obtained from the National Cancer Institute Chemical Repository.

Transfection and Electroporation. 10T½ cells were transfected by the calcium phosphate coprecipitation method (17). COS cells were electroporated with supercoiled plasmids as described (16). The optimized final concentrations in each electroporation cuvette were 10 μg of plasmid DNA per 2×10^6 cells per 0.5 ml of phosphate-buffered saline ($CaCl_2$, 0.1 g/liter; KCl, 0.2 g/liter; KH_2PO_4, 0.2 g/liter; $MgCl_2 \cdot 6H_2O$, 0.1 g/liter; NaCl, 8.0 g/liter; $Na_2HPO_4 \cdot 7H_2O$, 2.16 g/liter) containing 5% fetal bovine serum; 1250 V and 50 μF were used in the discharge of the pulse. Electroporated cells (6×10^6) were pooled and seeded in a 100-mm dish in standard medium, and the medium was changed 1 day later. Approximately 30% of the cells survived electroporation.

Fluorescence-Activated Cell Sorting. Two days after electroporation, COS cells were trypsinized, rinsed, and suspended in phosphated-buffered saline supplemented with 10% fetal bovine serum to decrease clumping during the sort. Recombinant GST^+ COS cells (20% of the population) were separated (sorted) from negative control cells by a FACStar^{PLUS} (Becton Dickinson) that was equipped with a krypton laser. On-line addition of the fluorescent labeling reagent monochlorobimane during the sort ensured that all cells were exposed to the GST substrate for the same length of time. Details of the sorting technique will be presented in a separate manuscript.

Immunostaining, Western Blotting, Enzyme and Glutathione Assays. The percentage of COS cells observed to be expressing recombinant GST 2 days after electroporation was determined to be 20% by immunostaining analyses done essentially as described (16). Postmitochondrial supernatants ($15,600 \times g$, 20 min) of sonicated COS or 10T½ cells were electrophoresed and immunoblotted as described (16). The GST activity present in the postmitochondrial supernatants was measured with the substrate 1-chloro-2,4-dinitrobenzene according to published procedures (18). Protein content was determined with a Coomassie protein assay reagent (Pierce). Total cellular glutathione was assayed by an enzymatic recycling procedure based on glutathione reductase (19).

RESULTS

Sorting of GST^+ π, Ya, or Yb_1 COS Cells. Recombinant GST-expressing COS cells that were sorted on a fluorescence-activated cell sorter (FACS) were used in one of two approaches to the study of drug resistance. By testing sorted populations of transiently transfected cells, we were able to (*i*) avoid interclonal variation in secondary factors (e.g., glutathione content, topoisomerases, etc.) that could obscure the results one observes using single, stably transfected GST^+ clones and (*ii*) test whether reversion of the transient GST expression is correlated with loss of drug resistance. In order to generate transferase-expressing populations, we electroporated COS cells with a transient expression vector that contains the simian virus 40 (SV40) early promoter-enhancer, the SV40 late poly(A) signal, and a full-length cDNA for π (pSMπ), Ya (pGTA12cDV), or Yb_1 (pSVGT44-cDV), each about 750 base pairs in length (16). Approximately 20% of the surviving cells expressed the recombinant GST, as determined by immunostaining analyses. In a pilot cytotoxicity experiment, an unsorted, mixed population of π-expressing COS cells was exposed to the alkylating molecule *anti*-BPDE, a known substrate of this isozyme (10). Although the GST π^+ population had a low observed resistance to the carcinogen, 1.1-fold increase relative to the control, the kill curve representing the GST π^+ population was significantly different from that of the control ($P < 0.001$) (unpublished data). This demonstrated that expression of GST π in mammalian cells conferred resistance to the cytotoxic effects induced by *anti*-BPDE. We estimated that the sensitivity of the cytotoxicity assays or the observed relative resistance could be enhanced 5-fold by using a sorted population of GST-expressing cells rather than a mixed population. This led us to develop a means to sort out the recombinant GST^+ cells from the mixed population, providing a 5-fold enrichment (from 20% to 100% GST^+ cells) in those cells that expressed the recombinant GST.

Routinely, 2 days after electroporation of COS cells, a FACS was used to viably sort out the 20% of the COS population that expressed the recombinant GST (Fig. 1). Monochlorobimane, a hydrophobic fluorescent labeling reagent and substrate of GSTs (20), was used to enable the FACS to identify recombinant GST-expressing COS cells. GST present in COS cells catalyzed the conjugation of glutathione to monochlorobimane, generating a highly fluorescent product. The degree of a cell's fluorescence was, therefore, proportional to cellular GST activity. The catalytic period, concentration of monochlorobimane, and kinetic parameters of the expressed isozyme determine the degree to which the positive cells (Fig. 1*B*) fluoresced more intensely than the controls (Fig. 1*A*). On-line reagent addition during the sort ensured that all cells were exposed to monochlorobimane for the same length of time. Purity of the sorted populations was verified by immunostaining. Hydrophilic glutathione-monochlorobimane conjugates were released from the cells in <12 hr. Assays of total glutathione content

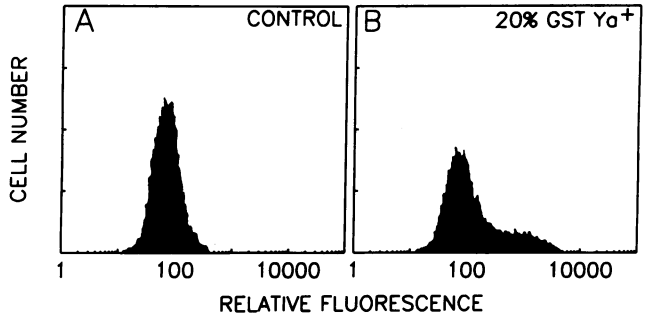

FIG. 1. FACS histogram of GST Ya$^+$ COS cells. Cells were electroporated with control vector pGTB38 (16) (*A*) or with expression vector pGTA12cDV (*B*). Cells were harvested 48 hr later and then sorted on a FACStar^{PLUS} after on-line exposure to 25 μM monochlorobimane for 45 sec. The shoulder in *B* comprises 20% of the total peak area and corresponds to GST Ya$^+$ cells as verified by immunostaining analysis.

demonstrated that both of the sorted populations, the negative control and the GST$^+$ cells, had equivalent glutathione contents the day after the sort—that is, the day of the cytotoxicity assay.

As anticipated, there was an approximate 5-fold enrichment (by densitometry) of recombinant GST$^+$ COS cells upon sorting as evidenced by Western blot results (Fig. 2, lanes 3 and 4 vs. lanes 5 and 6) and GST immunostaining analyses of sorted cells (not shown). The level of GST activity for 1-chloro-2,4-dinitrobenzene, measured in a sorted population of Yb$_1$-expressing cells, was 1.8-fold greater than that of the control. In the reversion experiment, control and GST Ya-expressing COS cells were sorted and seeded, and individual cell clones, each of which was derived from a single GST Ya$^+$ or Ya$^-$ progenitor, were picked and expanded. Western blots of cytosolic protein prepared from each of the 20 originally GST Ya$^+$ clones and 20 GST Ya$^-$ clones demonstrated that there was no immunodetectable level of Ya expression 1 month after sorting (Fig. 2, lanes 11–14).

Expression of GST π, Ya, or Yb$_1$ cDNAs in 10T½ Clones. In our second experimental approach, 10T½ mouse cells were cotransfected with a neomycin-resistance plasmid (pSV2neo) and an expression vector that contains the mouse α(2)I-collagen promoter, the SV40 late poly(A) signal, and a full-length cDNA for π (pαcolπcDV), Ya (pαcol12GTcDV), or Yb$_1$ (pαcolb1cDV), each approximately 750 base pairs in length (16). The collagen promoter was subcloned from plasmid pAZ1003 (21). This promoter was chosen for the cytotoxicity studies because it was the most efficient one of five eukaryotic promoters tested for recombinant GST expression in mouse fibroblasts. Controls were transfected with pSV2neo only. G418-resistant colonies were cloned and screened by Western blot for levels of recombinant GST expression. Clone A5 expressed subunit Ya to a slightly greater extent than did clones 12 and A18, as shown in the Western blot (Fig. 2, lanes 7–10). The degree of Ya expression in the 10T½ clones and in the sorted COS cells was approximately equal (Fig. 2, lane 6 vs. lane 7). Preliminary results indicated that the degree of transient Ya expression in COS cells was greater when a second SV40 early region fragment was located upstream and adjacent to the SV40 early promoter that drove the expression of the Ya cDNA (Fig. 2, lane 2). The level of GST activity for 1-chloro-2,4-dinitrobenzene in clone A5 was 1.3-fold greater than that found in the 10T½ control group; this is less than the 1.8-fold increase that was measured in sorted Yb$_1$-expressing COS cells.

Drug Resistance Is Conferred by GST cDNA Expression. Recombinant GST-expressing 10T½ clones or purified populations of GST-expressing COS cells were exposed to several structurally diverse anticancer drugs in colony-forming assays. These cytotoxicity assays were done in triplicate, and the degree of protection observed in recombinant GST$^+$ cells was measured as a relative resistance (increase in resistance of GST$^+$ cells relative to recombinant GST$^-$ cells) at an LD$_{90}$ drug concentration. The LD$_{90}$ is the concentration of drug that is required to kill 90% of the cells in a population as measured by a colony-forming assay. Resistance values derived in this way were approximately the same across each set of curves from an LD$_{90}$ to an LD$_{99.9}$ within a 10% variation. An analysis of covariance was used to determine whether the kill curves of each set were significantly different from each other.

When sorted GST Ya$^+$ and Ya$^-$ COS cells were exposed to chlorambucil in a colony-forming assay, the Ya-expressing population had a 1.4-fold increase in relative resistance to this nitrogen mustard (Fig. 3). The two curves were significantly different from each other ($P < 0.001$). Therefore, to induce equivalent cytotoxic effects in both populations, the GST$^+$ cells required 1.4 times as much chlorambucil as the control cells. The reverted Ya-expressing COS clone (Fig. 2, + D), displayed a total loss of resistance to chlorambucil (Fig. 3 *Inset*). The curves were not significantly different from each

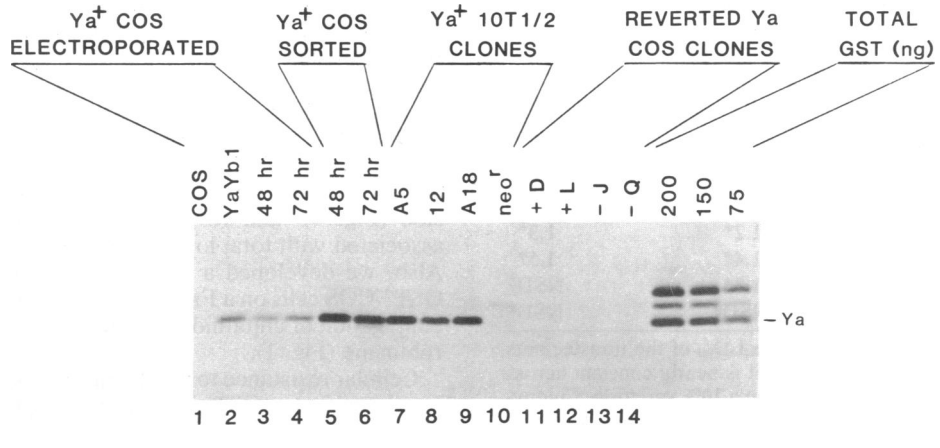

FIG. 2. Western blot analysis of GST Ya expression in sorted COS cells and 10T½ clones. COS cells were electroporated with pGTA12cDV and sorted 48 hr later. Postmitochondrial supernatants were prepared from COS cells that had or had not been sorted 48 hr after electroporation and that had or had not been sorted but had been seeded and harvested 72 hr after electroporation (time of cytotoxicity assay). Postmitochondrial supernatants were also prepared from GST Ya$^+$ 10T½ clones and reverted Ya COS clones. Ten micrograms of supernatant protein was electrophoresed per lane. GST Ya expression was detected with polyclonal antiserum raised in rabbits against purified rat liver GST.

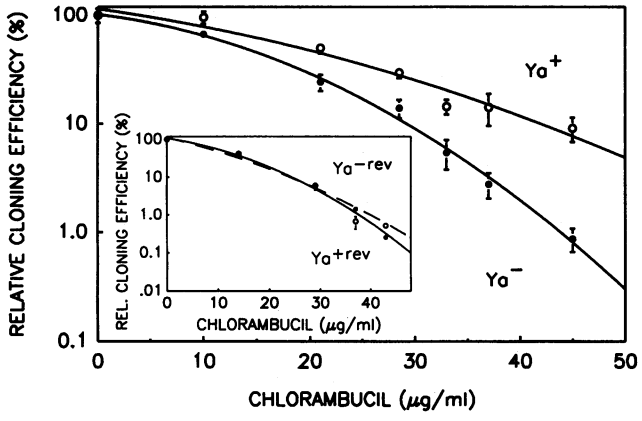

FIG. 3. Survival of sorted GST Ya$^+$ COS cells after exposure to chlorambucil. Cells were electroporated with pGTA12cDV, sorted 48 hr later, and then seeded. The day after seeding, sorted cells were exposed to chlorambucil for 1 hr in a colony-forming assay done in triplicate. Colonies were stained at 11 days and scored. The sorted GST Ya$^+$ population had a 1.4-fold increase in resistance to chlorambucil relative to the GST Ya$^-$ control. The curves are significantly different from each other ($P < 0.001$). (*Inset*) Survival of reverted COS cells after exposure to chlorambucil. Cells were electroporated with pGTA12cDV, sorted 48 hr later, and then seeded at cloning density. Clones were picked, expanded, and screened by immunoblot analysis (Fig. 2). Reverted clones were exposed to chlorambucil for 1 hr in a colony-forming assay done in triplicate. The curves are not significantly different from each other ($P > 0.9$).

other ($P > 0.9$). In a follow-up to the pilot experiment in which 20% of the mixed COS population contained GST π^+ cells and showed a 1.1-fold increase in relative resistance to *anti*-BPDE (unpublished data), sorted populations of GST π^+ and π^- cells were likewise tested in a colony-forming assay. The relative resistance of the π^+ population to *anti*-BPDE was increased 5-fold to 1.5 ($P < 0.001$) (Table 1). This 5-fold enhancement corresponds to the 5-fold enrichment of the GST π^+ cells in the population upon sorting. Sorted populations of GST π-, Ya-, or Yb_1-expressing COS cells were independently exposed to the nitrogen mustards chlorambucil and melphalan, the pollutant *anti*-BPDE, the agents cisplatin and doxorubicin, and the natural product vinblastine. For experiments in which drug resistance was observed, the relative resistance values ranged from 1.1 to 1.5, and these values were derived from curves that were significantly different from their controls, with P values that ranged from

Table 1. Relative resistance of GST$^+$ electroporated/sorted COS cells and transfected 10T½ clones that express π, Ya, or Yb_1

	Relative resistance				
	π		Ya		Yb_1
Agent	COS	10T½	COS	10T½	COS
Chlorambucil	1.2*	1.7*	1.4*	1.5–2.9*	1.3*
Melphalan	1.1*	1.5*	1.3*		1.1†
anti-BPDE	1.5*	1.1–1.5*	1.2*		1.3*
Cisplatin	1.3*		1.4*		1.5*
Doxorubicin	1.3*		1.3*		NSD‡
Vinblastine	NSD§		NSD§		NSD‡

Relative resistance is expressed as the LD_{90} of the transfectants divided by the LD_{90} of the control, and it is nearly constant across the two curves from LD_{90} to $LD_{99.9}$ within a 10% variation. Colony-forming assays were done in triplicate. Statistical significance was determined by analyses of covariance. NSD, not significantly different.
*$P < 0.001$.
†$P = 0.005$.
‡$P > 0.05$.
§$P > 0.4$.

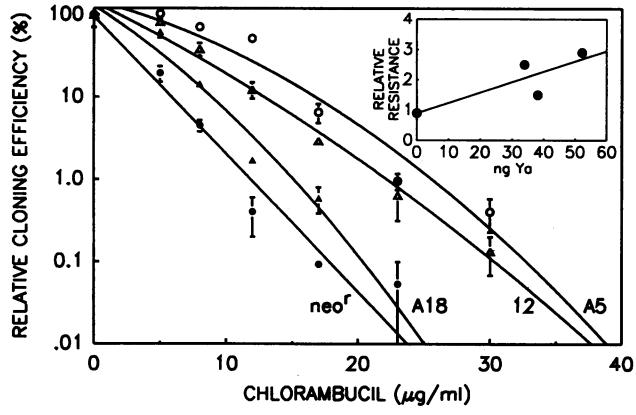

FIG. 4. Survival of GST Ya$^+$ 10T½ clones after exposure to chlorambucil for 1 hr in colony-forming assays done in triplicate. Clones A18, 12, and A5 showed a 1.5-, 2.5-, and 2.9-fold increase in resistance, respectively, to chlorambucil relative to neo^r control clone. The curves are significantly different from neo^r control ($P < 0.001$). For the points on the survival curves where error bars are not visible, the error bars were shorter than the diameter of the point. (*Inset*) Degree of relative resistance of these clones is related to the degree of their Ya expression as determined by immunoblot analysis (Fig. 2).

0.005 to 0.0001. Those curves of a set that were not significantly different from each other ($P > 0.05$) are referred to as NSD (Table 1).

Three recombinant GST Ya$^+$ 10T½ clones (A18, 12, A5) showed a 1.5-, 2.5-, and 2.9-fold increase in relative resistance, respectively, to the cytotoxic effects induced by chlorambucil ($P < 0.001$) (Fig. 4). These resistance values were compared with the Ya content of each clone (Fig. 2, lanes 7–10), and it was determined that the degree of GST Ya expression was related to the degree of drug resistance (Fig. 4 *Inset*). When GST π-expressing 10T½ clones were exposed to chlorambucil, melphalan, or *anti*-BPDE, the relative resistance values ranged from 1.1 to 1.7 ($P < 0.001$) (Table 1). Drug resistance values for recombinant GST-expressing 10T½ clones tended to be somewhat greater than those for sorted COS cells.

DISCUSSION

We demonstrated that transient or stable expression of full-length cloned GST cDNAs for π, Ya, and Yb_1 confers cellular resistance to alkylating agents. GST π conferred the greatest increase in resistance to *anti*-BPDE (1.5-fold), Ya conferred the greatest increase in resistance to chlorambucil and melphalan (1.3- to 2.9-fold), and Yb_1 conferred the greatest increase in resistance to cisplatin (1.5-fold) relative to controls (Table 1). These resistance values are statistically significant ($P < 0.001$) and are commensurate with values observed clinically (22–25). Conferred resistance to chlorambucil was proportional to the magnitude of GST Ya expression (Fig. 4), and reversion of transient expression was associated with total loss of drug resistance (Figs. 2 and 3). Also, we developed a technique to enrich in recombinant GST$^+$ COS cells on a FACS that is based on a GST-catalyzed conjugation of glutathione to the labeling reagent monochlorobimane (Fig. 1).

Cellular resistance to alkylating agents was directly related to recombinant GST expression in COS cells and 10T½ clones. Kinetic analyses done *in vitro* (4–11) were predictive of the ability of GST to confer resistance to alkylating agents; specifically, as we demonstrated, of the three GST classes, GST π and Ya (acidic and basic classes of GST) would confer the greatest resistance to *anti*-BPDE (10, 11) and chlorambucil (4–6), respectively. In support of the findings that GST

Ya may play a critical role in detoxifying chlorambucil, it is the expression of the Ya and Yc basic class GSTs that is induced when cultured cells are chronically exposed to chlorambucil (14, 15). Also, Cowan and coworkers (26) recently demonstrated that GST π-expressing transfected clones were resistant to *anti*-BPDE (1.4- and 2.9-fold), cisplatin (1.1- to 1.5-fold), and melphalan (0.9- to 2.1-fold). Thus, GSTs clearly play a role in resistance to alkylating agents. No resistance to vinblastine was detected (Table 1) and this is almost certainly a reflection of the different mechanism responsible for the chemotherapeutic effect of the vinca alkaloids. Although the intrinsic peroxidase activity of GSTs may have mediated the 1.3-fold increase in relative resistance to doxorubicin detected in this study, more work is needed to determine what role these enzymes play in the detoxification of this anthracycline antibiotic.

Relative resistance to alkylating agents observed clinically is of the same magnitude as that which we have demonstrated experimentally (22–25). In an elegant study, GST activity, glutathione content, and resistance to chlorambucil and cisplatin were measured in two ovarian cell lines that were derived from the ascites of a patient before (control) and after the onset of resistance to chemotherapy involving cisplatin, chlorambucil, and 5-fluorouracil (22, 23). The increases relative to controls in total GST activity (2.1-fold), glutathione content (2.0-fold), cisplatin resistance (3.0-fold), and chlorambucil resistance (1.3- to 3.0-fold) paralleled the increases observed in our transfected cells: total GST activity (1.3- to 1.8-fold), glutathione content (no change), cisplatin resistance (1.3- to 1.5-fold), and chlorambucil resistance (1.2- to 2.9-fold). Relatedly, in a clinical setting, when the dose of the cytostatic drug is doubled, 30–40% of those patients whose tumors had become refractory to the initial dose responded favorably to the higher dose (24, 25). In aggregate, these data suggest that resistance factors of 1.5–3.0 are important in chemotherapy.

Reversion of transient GST expression in our studies is a powerful tool used to demonstrate that resistance to alkylating agents depends on the presence of recombinant GST. We used the unique characteristics of COS cells to show that reversion of transient expression in GST Ya^+ COS cell clones to a GST Ya^- phenotype (Fig. 2) was associated with total loss of resistance to chlorambucil (Fig. 3). Thus GST alone can confer resistance to alkylating agents. Interestingly, there appears to be a general belief that COS cells that transiently replicate expression vectors ultimately die (27). However, Tsui and Breitman (27) and we in this study demonstrated that single cells that replicate episomal DNA can be cloned and that the resulting population, if not undergoing biochemical selection for the presence and maintenance of a plasmid-encoded selectable marker, will lose the episomes over a 4-week period (reversion). Consequently, COS cells that replicate plasmids can form colonies as demonstrated in our drug resistance studies. It is likely however, that sorted GST^+ COS cells that have the greatest abundance of recombinant GST, owing to high levels of plasmid, may be least able to generate colonies in colony-forming assays. Inability to propagate these particular cells may contribute to the lower levels of drug resistance observed in COS cells as compared with 10T½ clones. In spite of these limitations, the use of COS cells to express specific GST cDNAs enabled us to avoid interclonal variation in factors other than the recombinant GST and to show that reversion of transient GST expression correlated with loss of drug resistance.

Once the molecular mechanism by which GST confers resistance to alkylating agents in intact cells is elucidated, specific inhibitors of GST (2) could be chosen and possibly used to inhibit tumor cells' GST activity, thereby increasing the therapeutic effectiveness of alkylating agents. Also, expression of recombinant GSTs in a cancer patient's hematopoietic cells may protect these highly sensitive cells from the toxic effects of alkylating agents and thereby increase the effectiveness of the chemotherapy that is being directed elsewhere in the body.

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