Co-variation of glutathione transferase expression and cytostatic drug resistance in HeLa cells: establishment of class Mu glutathione transferase M3–3 as the dominating isoenzyme

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Qualitative and quantitative analyses of glutathione, glutathione transferases (GSTs) and other glutathione-linked enzymes in HeLa cells have been made in order to study their significance in cellular resistance to electrophilic cytotoxic agents. The cytosolic concentrations of three GSTs, GST M1–1 (53 ± 9 ng/mg of cytosolic protein), GST P1–1 (11 ± 3 ng/mg) and GST A1–1 (1.1 ± 0.4 ng/mg) were quantified by isoenzyme-specific enzyme-linked immunoassays. Electrophoretic analysis and immunoblotting demonstrated another component, GST M3–3, which was identified by amino acid sequence analysis. GST M3–3 was quantified (1550 ± 250 ng/mg) by slot-blot immunoanalysis and was the most abundant GST in HeLa cells. An additional cytosolic 13 kDa protein with high affinity for immobilized

glutathione or *S*-hexylglutathione was found to be identical with a macrophage migration-inhibitory factor, previously identified as a lymphokine. Cells grown in roller bottles (HR) rather than in ordinary culture flasks contain a significantly lower concentration of all the GSTs and were found to be more sensitive to the cytostatic agents doxorubicin (2.3-fold), cisplatin (1.7-fold) and melphalan (1.4-fold). The cytosolic concentrations of glutathione reductase and glyoxalase I were also lower in HR cells, whereas the total glutathione concentration was unchanged and the glutathione peroxidase activity was increased. The results indicate that GSTs contribute to the cellular resistance phenotype.

INTRODUCTION

Unsuccessful clinical tumour chemotherapy may often be ascribed to intrinsic or acquired drug resistance. The drug-resistance phenotype of tumours is the resultant of the expressions of membrane-bound drug transporters (e.g. P-glycoprotein), drug-metabolizing enzymes, repair systems and their associated cofactors. The acquired resistance is associated with increased activities of the proteins involved in the protection of the tumour cells, which could be induced by a variety of tumour promoters, mutagens as well as genotoxic alkylating agents.

Cellular glutathione and glutathione-linked enzymes are components of an important drug-metabolizing system, known to protect living organisms against cellular oxidative stress and toxic effects caused by a wide range of electrophilic compounds of exogenous or endogenous origins (Mannervik et al., 1989). Direct as well as indirect evidence indicates that the conjugation of therapeutic alkylating agents with reduced glutathione, catalysed by glutathione transferases (GSTs), contributes to drug resistance (Dulik and Fenselau, 1987; Black and Wolf, 1991). The reduction of organic hydroperoxides as well as H_2O_2 , catalysed by selenium-dependent glutathione peroxidase and GSTs [for review see Mannervik and Danielson (1988) and Armstrong (1991)], is another glutathione-linked detoxication reaction linked to cellular drug metabolism. Buthionine sulfoximine, an inhibitor of glutathione biosynthesis, as well as ethacrynic acid, piroprost and triphenyltin chloride, inhibitors of GSTs, have been found to counteract cellular resistance to

bifunctional alkylating agents (Tew et al., 1988; Smith et al., 1989; Hansson et al., 1991).

GSTs occur in multiple forms with distinct but partly overlapping substrate specificities (Mannervik et al., 1985; Mannervik and Danielson, 1988). Rat GSTs have been denoted by Arabic numerals according to their subunit composition (Jakoby et al., 1984) and a similar novel nomenclature has recently been adopted for the human enzymes (Mannervik et al., 1992). The phenotypic expression of GST isoenzymes has been found to vary in a cell-dependent manner in both normal tissues and tumours. A high intracellular concentration of class Pi GST is a characteristic of most, but not all, tumour cells (Shea et al., 1988; Castro et al., 1990). The cytosolic concentration of various isoenzymes of GST classes Alpha and Mu varies considerably among tumour cell lines and tissues (Castro et al., 1990). However, little is known about the relationship of the expression pattern of GST isoenzymes in human tumour cells to the tumour resistance phenotype.

The HeLa cell line, derived from a cervical carcinoma, is a tumour cell line commonly used in experimental cancer research. The cells do not express the P-glycoprotein that is responsible for conventional multidrug resistance in tumour cells (Epand et al., 1991; Chao et al., 1992). Chao et al. (1991) established a cisplatin-resistant HeLa cell subline that had acquired cross-resistance to melphalan and mitomycin C. Further study failed to demonstrate any significant change in cellular glutathione levels, but amplification of the GST P1 gene and a 6-fold increase in glutathione peroxidase activity as measured with cumene

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione transferase; HF, HeLa cells grown in flat-bottomed flasks; HR, HeLa cells grown in roller bottles; LC_{50} , drug concentration causing 50% cell death as measured by level of protein synthesis; MEM, minimum essential medium with Earle's salt; $20 \times$ SSC, 17.35% (w/v) NaCl + 8.82% (w/v) sodium citrate, pH 7.0.

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hydroperoxide were found in the multidrug-resistant HeLa cells as compared with the parental cells (Chao et al., 1992). However, little is known about the roles of GST isoenzymes and other glutathione-dependent enzymes in HeLa cells. The present work was aimed at determining the levels of glutathione, GST isoenzymes and other glutathione-linked enzymes in HeLa cells in order to clarify their role in cellular resistance to cytostatic drugs.

MATERIALS AND METHODS

Cell culture and cytosol preparation

HeLa cells were seeded into flat-bottomed culture flasks (175 cm²) or roller bottles (850 cm², rotated at 2.5 rev./min.) (Becton Dickinson, Cowley, Oxford, U.K.) at an equal density of about 2000 viable cells/cm² and grown under a humidified atmosphere containing 5% CO₂ in minimum essential medium with Earle's salts (MEM) (Gibco, Renfrewshire, U.K.) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and 0.2 mg/ml gentamicin. Cell viability was examined microscopically by suspending cells in MEM containing 0.05% Trypan Blue. Cells excluding the dye were scored as viable cells. Growth rate was calculated by the following formula:

$$\frac{(\text{Number of harvested cells} - \text{number of seeded cells})}{\text{Surface area of culture vessels} \times \text{days of culture}}$$

Cell collection and preparation of microsome-free cytosolic fractions were carried out as previously described (Castro et al., 1990). Cytosolic protein concentration was determined by the modified Lowry method of Peterson (1977).

Enzyme activity assays

Activity assays for glutathione-linked enzymes were carried out by published procedures as follows: GST [measured with 1-chloro-2,4-dinitrobenzene (CDNB)] (Habig and Jakoby, 1981), GSSG reductase (Carlberg and Mannervik, 1975), glyoxalase I (Racker, 1951) and glutathione peroxidase (Lawrence and Burk, 1976). The cyclic glutathione reductase assay (Tietze, 1969) was used for quantification of total glutathione. All measurements were performed at 30 °C using a Hewlett-Packard 8452A diode array spectrophotometer.

GST isoenzyme e.l.i.s.a.

Human recombinant GST A1-1 (Board and Pierce, 1987), M1-1 (Widersten et al., 1991) and P1-1 (Kolm et al., 1992) were prepared as previously described. The purified enzymes were used for immunization of New Zealand White rabbits by subcutaneous injection of 100 µg of protein mixed with complete Freund's adjuvant. Polyclonal rabbit anti-(human GST) antibodies were purified by immunoaffinity chromatography on the corresponding recombinant protein immobilized on Sepharose 4B. Alkaline phosphatase-antibody conjugates were synthesized as described (Voller et al., 1980). Double-antibody-sandwich e.l.i.s.a. for individual GST isoenzymes A1-1, M1-1 and P1-1 were developed essentially as outlined by Harlow and Lane (1988). The assays were performed by procedures described elsewhere (Berhane et al., 1993). The concentrations of cytosolic proteins applied in the assays were between 0.5 and 2.0 mg/ml for GST A1-1 and 0.05 and 0.2 mg/ml for GSTs M1-1 and P1-1.

Affinity purification of GSTs

S-Hexylglutathione-Sepharose (Mannervik and Guthenberg, 1981) and glutathione-Sepharose (Simons and Vander Jagt, 1977) were synthesized by published procedures. The two affinity gels were mixed at a ratio of 1:1. After incubation with the cytosolic proteins at 4 °C overnight, the gel was washed with 20 mM Tris/HCl, pH 7.8, containing 0.2 M NaCl, and eluted successively with (a) 10 mM S-hexylglutathione in 20 mM Tris/HCl, pH 7.8 (buffer A), (b) buffer A containing 0.2 M NaCl, pH 7.8 (buffer B), (c) buffer A containing 1 M NaCl, 1 mM EDTA and 0.1% (v/v) Triton X-100, pH 7.8 (buffer C). The fraction eluted with buffer C was concentrated about 10-fold with a microcentrifuge concentration unit (Nihon Millipore, Yonezawa, Japan) for SDS/PAGE.

SDS/PAGE

SDS/PAGE was carried out using 12.5% polyacrylamide gel on a 16 cm × 20 cm Protean II system (Bio-Rad, Richmond, CA, U.S.A.) by the method of Laemmli (1970). After electrophoresis, the separated protein bands were visualized by silver staining (Blum et al., 1987).

Two-dimensional electrophoresis

Isoelectric focusing in the first dimension was carried out in the presence of 9 M urea by the protocol of Celis et al. (1990) on a disc gel electrophoresis adaptor to Protean II. After focusing, the gel rods were fixed in 20% (w/v) trichloroacetic acid for 30 min, washed with 45% (v/v) methanol and subsequently equilibrated at 50 °C for 1 h with the sample buffer [65 mM Tris/HCl, pH 6.7, 100 mM DL-dithiothreitol, 2% (w/v) SDS, 30% (v/v) glycerol, 0.001% (w/v) Bromophenol Blue] for SDS/PAGE. Second-dimension SDS/PAGE was performed with Protean II (Celis et al., 1990).

Western-blot analysis

For antigen identification and *in situ* protein digestion with trypsin, proteins were transferred on to a 0.45 µm pore diam. Hybond C Extra supported nitrocellulose membrane (Amersham International, Bucks., U.K.) by the method of Erickson et al. (1982). The primary antibodies used in immunodetection were the same as those prepared for e.l.i.s.a., and were diluted to 4 µg/ml. The secondary antibodies were goat anti-(rabbit IgG) antibodies conjugated with horseradish peroxidase. The immunocomplexes were visualized by using the enhanced chemiluminescence system of Amersham. Transfer of protein on to poly(vinylidene difluoride) membrane was performed in 25 mM Tris/glycine, pH 8.2, containing 0.01% (w/v) SDS and 20% (v/v) methanol.

Protein microsequencing

Proteins blotted on to poly(vinylidene difluoride) membrane were stained for 1 min with 0.1% (w/v) Coomassie Brilliant Blue R250 in 40% (v/v) methanol/1% (v/v) acetic acid, destained with 50% (v/v) methanol and directly applied on the sequencer for N-terminal sequencing. Proteins blotted on to nitrocellulose membrane were digested with modified trypsin (Promega Corp., Madison, WI, U.S.A.) (Aebersold et al., 1987). Thereafter, the resulting peptides were directly separated by narrow-bore reversed-phase h.p.l.c. on a µ reversed-phase C2/C18 column (2.1 mm × 100 mm) (Pharmacia-LKB, Uppsala, Sweden) with a gradient of water/acetonitrile in 0.12% (v/v) trifluoroacetic acid

at a flow rate of 100 μ l/min. The effluent was monitored at 215 nm and peak fractions were collected manually. N-Terminal microsequencing of blotted proteins was based on automated Edman degradation with an Applied Biosystems (Foster City, CA, U.S.A.) model 470A sequencer, and tryptic peptides were analysed with a model 477A sequencer. Each of the instruments was equipped with an on-line model 120A phenylthiohydantoin analyser.

Preparation of first-strand cDNA

Total RNA was prepared from a pellet of about 10^8 HeLa cells grown in flasks by a procedure designed by L. Franzén, (Department of Medical Genetics, Uppsala University, Uppsala, Sweden) (unpublished work). Oligonucleotides were synthesized by Operon Technologies (Alameda, CA, U.S.A.). OLIGODTEP, 5'-AAGAATTCTGCAGT₂₀-3' (40 nmol) was annealed to 15 μ g of total RNA and the first-strand synthesis was carried out with 200 units of murine mammary leukaemia virus H-reverse transcriptase Superscript (Bethesda Research Laboratories Life Technologies, Gaithersburg, MD, U.S.A.) following the manufacturer's instructions. The first-strand cDNA was dissolved in 20 μ l of water before PCR amplification.

PCR amplification of GST cDNAs from HeLa cells

Sequences of oligonucleotides used for priming PCRs, as well as for probing amplified DNA, were chosen such that they were able to discriminate between related subunit cDNAs. Their sequences were as follows. For GST subunit A1 (Board and Webb, 1987): A1-i, 5'-TTCTGAATTCAGGAGACTGCTATCATG-3'; A1-ii, 5'-TTGGTACCAAACCTGAAAATCTTCCT-3'; A1-iii, 5'-ATTTACCCAGATCTGCTATA-3'; for GST subunit A2 (Rhoads et al., 1987): A2-i, 5'-CCCATATGGCAGAGAAGCCGAAG-3'; A2-ii, 5'-CCTCTAGAGAGCTCGTTCATTAGCTTCACAC-3'; for GST subunit M1 (DeJong et al., 1988; Seidegård et al., 1988): M1-i, 5'-CCATGATACTGGGGTACTGG-3'; M1-ii, 5'-CTCAAAGCGGGAGATGAAGT-3'; M1-iii, 5'-CGTCCAAGCACTTGGGCTCAA-3'; for GST subunit M3 (Campbell et al., 1990): M3-i, 5'-CCGAATTCATGTCGTGCGAGTCTGCTATG-3'; M3-ii, 5'-GGCTGCAGCACCAGTAACATAAGTGCTAT-3'; for GST subunit P1 (Kano et al., 1987): P1-i, 5'-TACACCGTGGTCTATTTCCC-3'; P1-ii, 5'-CTGTTTCCCGTTGCCATTGAT-3'; P1-iii, 5'-CCTCCTCCTTCCAGCTCTGGCCC-3'. The sequences for class Mu subunit M1 discriminated against subunit M2 (Vorachek et al., 1991) but would not distinguish subunit M1 from subunits M4 (Comstock et al., 1993) and M5 (Takahashi et al., 1993). (The sequences for subunits M4 and M5 had not been reported at the time of the present experiment.) The primers chosen for subunit M3 cDNA were subunit-specific and further designed to accommodate the possibility of subcloning for expression of the recombinant protein. Underlined bases indicate endonuclease-recognition sites. Five PCRs were performed, each in a total volume of 100 μ l containing 2 μ l of first-strand cDNA (about 60 ng of cDNA/ μ l) described above as template, 1 μ M each primer, 1.5 mM MgCl₂, 0.2 mM deoxynucleotides and 2.5 units of *Taq* DNA polymerase (Boehringer-Mannheim, Mannheim, Germany). The primer pairs used for priming were A1-i + A1-ii, A2-i + A2-ii, M1-i + M1-ii, M3-i + M3-ii and P1-i + P1-ii (see Figure 4). The reaction mixtures were incubated in a Thermal Cycler (Perkin-Elmer-Cetus, Emeryville, CA, U.S.A.) using a cycle profile of 1 min denaturation (95 °C), 2 min annealing (55 °C) and 1 min extension (72 °C), repeated 30 times. A 10 μ l portion of each completed PCR was electrophoresed in

1.5% (w/v) agarose gel, and the amplified DNA was subsequently stained with 0.5 μ g/ml ethidium bromide.

Southern-blot analysis of PCR-amplified cDNA

PCR-amplified DNA (10 μ l) was separated in a 1.5% agarose gel and subsequently transferred to nitrocellulose essentially as described by Maniatis et al. (1982) using capillary transfer in $20 \times$ SSC. The immobilized DNA was probed with three distinct [γ -³²P]ATP end-labelled oligonucleotides (Maniatis et al., 1982): A1-iii (specific for GST A1 cDNA), M1-iii (specific for GST M1a cDNA) and P1-iii (specific for GST P1 cDNA). Prehybridization was carried out at 68 °C in a solution containing 2% (w/v) Blocking Reagent (Boehringer-Mannheim), $5 \times$ SSC, 0.1% (w/v) sodium *N*-lauroylsarcosine and 0.02% (v/v) SDS for 1 h. Hybridization to the specific probes was performed at 68 °C for 30 min, followed by incubation at room temperature for 30 min in $6 \times$ SSC. Membranes were washed in $6 \times$ SSC and 5 °C below the calculated melting temperature (T_m) before autoradiography.

Heterologous expression of GST M3-3 in *Escherichia coli*

The amplified fragment from the PCR primed by M3-i and M3-ii was digested with *Eco*RI and *Pst*I and subsequently subcloned into the polylinker of pGEM3Zf(-) (Promega Corp.). Recombinant plasmid DNA was prepared by the method of He et al. (1990) and sequenced (Sanger et al., 1977). The insert was subsequently transferred into plasmid pKK223-3 (Pharmacia-LKB Technologies) and the encoded GST subunit M3 was expressed in *E. coli* XL1-Blue (Stratagene, La Jolla, CA, U.S.A.).

Immunoblot determination of cytosolic GST M3-3 concentration in HeLa cells

Portions of cytosolic fractions containing 20–25 μ g of protein in 100 μ l of PBS were transferred on to nitrocellulose membrane by using a Minifold II Slot-Blotter (Schleicher & Schuell GmbH, Dassel, Germany). Recombinant GST M3-3 purified by glutathione-Sepharose affinity chromatography was used as a standard. After transfer, the membrane was treated as described under 'Western-blot analysis'. The primary antibodies used were polyclonal rabbit anti-(rat GST 3-3) antibodies, which had been purified by immunoaffinity chromatography on immobilized rat GST 3-3 on Sepharose 4B, and which have been found to cross-react considerably more strongly with GST M3-3 than with GST M1-1 in immunoblot analysis (X.-Y. Hao and B. Mannervik, unpublished work). After detection with secondary antibodies, the visualized bands were scanned for quantification by using a model 620 CCD densitometer (Bio-Rad).

Cytotoxicity assay

Cytotoxicity assays based on the measurement of protein synthesis of surviving cells were carried out as described by Wilson (1986). The drugs were diluted with serum-free MEM at concentrations of 0, 0.39, 1.56, 6.25, 25 and 100 μ g/ml. The cells were incubated with the drugs for 2 h. After a recovery period of 16 h, the surviving cells were incubated with 5 μ Ci/ml L-[³H]leucine (Amersham International) in leucine-free MEM for 3 h. L-[³H]leucine radioactivity incorporated into proteins was determined in 2.4 ml of OptiPhase 'HiSafe' 3 (FSA Laboratory Supplies, Loughborough, Leics., U.K.) with an LKB 1209 liquid-scintillation counter (LKB-Wallac, Bromma, Sweden). Cytotoxicity was expressed as LC₅₀, the drug concentration causing 50% cell death, as measured by protein synthesis.

Table 1 Glutathione level and activities of glutathione-linked enzymes in HeLa cells grown in flat-bottomed culture flasks (HF) and roller bottles (HR)Results are means $\pm$ S.D. ($n = 4$).

HeLa cells	$10^{-3} \times$ growth rate (cells/day per cm^2)	GSH equivalents (nmol/mg)	Specific enzyme activity (nmol/min per mg of cytosolic protein)			
			GSSG reductase	Glyoxalase I	GSH peroxidase	
					Cumene hydroperoxide	H_2O_2
HF	16.1	31.4 ± 3.4	71.2 ± 10.7	755 ± 55	49.6 ± 5.1	49.3 ± 4.4
HR	5.1	30.7 ± 3.2	59.5 ± 4.3	522 ± 81	61.4 ± 1.7	56.0 ± 7.2

RESULTS

Cellular glutathione level and specific activities of glutathione-linked enzymes

The level of total GSH (including GSSG) in HeLa cells cultured in flat-bottomed flasks (HF) was equivalent to about 30 nmol of GSH/mg of cytosolic protein determined with a cyclic glutathione reductase assay (Table 1). Cytosolic specific activities of glutathione-linked enzymes were also determined (Tables 1 and 2). In comparison with earlier measurements in other tumour cell lines (Castro et al., 1990), HF cells showed relatively low GST activity with CDNB, moderate activity of GSSG reductase and high activity of glyoxalase I. Glutathione peroxidase activity, measured with cumene hydroperoxide, was also higher than that in other tumour cells.

Quantitative analyses of GST isoenzymes by e.l.i.s.a.

Measurement of enzymic conjugation of GSH and CDNB does not discriminate between the contributions of class Alpha, Mu and Pi GST isoenzymes. Representatives of these three major GST classes were therefore further quantified in the HeLa-cell cytosolic fraction by using double-antibody e.l.i.s.a. (Table 2). Significant differences in the expression levels of the enzymes were noted. GST M1-1 was the most abundant isoenzyme of those measurable by the e.l.i.s.a., and GST A1-1 was the least abundant enzyme.

Identification of GST subunit M3

Cytosolic GSTs from about 1.5×10^8 HeLa cells grown in flasks were further studied by using affinity purification on a mixture of *S*-hexylglutathione-Sepharose 4B and glutathione-Sepharose 4B. The components bound to the column were eluted stepwise

Table 2 GST activity and isoenzyme composition of HeLa cells

GST activity was measured with the substrate CDNB and expressed as mean $\pm$ S.D. ($n = 4$); GST M3-3 content was densitometrically estimated by immunodetection using slot-blot and expressed as mean $\pm$ S.D. ($n = 4$); the concentrations of GSTs A1-1, M1-1 and P1-1 were quantified with e.l.i.s.a. and expressed as means $\pm$ S.D. ($n = 4$).

HeLa cells	GST activity (nmol/min per mg)	GST isoenzymes (ng/mg)			
		M3-3	A1-1	M1-1	P1-1
HF	51.1 ± 5.6	1550 ± 250	1.1 ± 0.4	53 ± 9	11 ± 3
HR	34.2 ± 5.4	1290 ± 330	0.4 ± 0.1	32 ± 4	9 ± 5

Figure 1 SDS/PAGE (a) and Western-blot (b) analyses of affinity-chromatography-purified GSTs from HeLa cells

The mixed *S*-hexylglutathione and glutathione-Sepharose affinity chromatography, SDS/PAGE and Western blot are described in the Materials and methods section. (a) Silver-stained SDS/polyacrylamide gel: lanes 1 and 5, 200 ng of recombinant GST M2 subunit (marker); lane 2, fraction A eluted with 10 mM *S*-hexylglutathione in 20 mM Tris/HCl, pH 7.8 (buffer A); lane 3, fraction B eluted with buffer A in the presence of 0.2 M NaCl; lane 4, fraction C, 10 $\times$ concentrated, buffer A containing 1 M NaCl, 1 mM EDTA and 0.1% (v/v) Triton X-100; lane 6, recombinant GST subunits M1a and P1 as indicated. The molecular-mass markers were purchased from Pharmacia. (b) Western blot, detected by using rabbit anti-(human GST M1-1) antibodies as probe and horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibodies as tracer: 100 ng of recombinant GST M1b subunits as indicated; lane A, fraction A; lane B, fraction B; lane C, fraction C. The visualization was based on Amersham's enhanced chemiluminescence detection system of horseradish peroxidase, with a prolonged exposure time of 6 h at room temperature.

in three fractions, A, B and C, as described in the Materials and methods section. In the silver-stained SDS/polyacrylamide gel (Figure 1a), fraction A (lane 2) eluted with 10 mM *S*-hexylglutathione contained the main fraction of the bound proteins. The protein bands between 20 and 30 kDa were

Figure 2 Two-dimensional PAGE identification of affinity-chromatography-purified GST isoenzymes from HeLa cells

The pH gradient of the denaturing urea isoelectric focusing gel was estimated by cutting a blank isoelectric focusing gel into 0.4 cm intervals, soaking each of the slices in 0.5 ml of water for 30 min, followed by pH determination. The dashes mark molecular masses corresponding to the mobilities of recombinant GST subunits M1a and P1 in the same second-dimension SDS/polyacrylamide gel. The identifications of GST subunits M1a, M3, P1 and glyoxalase I (Glyl) are discussed in the Results section. (a) Fraction A (see the legend to Figure 1); (b) fraction B.

Figure 3 Reversed-phase h.p.l.c. resolution of peptides obtained by trypsin cleavage of GST subunit M3 from HeLa cells

The GST subunit M3 was purified by two-dimensional PAGE, transferred on to nitrocellulose membrane, and digested with trypsin. The resolved peptides were collected from reversed-phase h.p.l.c. separation for microsequencing as described in the Materials and methods section. The peptide sequences and their absorptions monitored at 215 nm and 280 nm are indicated. In the peptide sequences, each X represents an unidentified residue which is probably a blocked cysteine (see the Results section).

identified as glyoxalase I and GST subunit P1 (23 kDa) by immunoblot analysis (results not shown). The strongly silver-stained protein band at about 27 kDa cross-reacted weakly with anti-(human GST M1-1) antibodies on Western blots (Figure 1b).

As judged by the relative amounts of the protein and GST M1 markers in Figures 1(a) and 1(b), the 27 kDa band contained protein in addition to the GST subunit M1. As revealed on a silver-stained two-dimensional polyacrylamide gel (Figure 2a), the major component of the protein band was an acidic protein with a pI between pH 4 and 5 on the denaturing isoelectric focusing gel. The GST M1-1 spot at pH 6 was stained much more weakly and was identified by its position, which corresponded to that of the allelic variant GST M1a-1a. For comparison, the recombinant allelic GSTs M1a-1a and M1b-1b

were run on a parallel isoelectric focusing gel under the same experimental conditions (results not shown). To identify the major acidic protein as a GST isoenzyme, it was first transferred on to a poly(vinylidene difluoride) membrane for Edman N-terminal protein sequencing. However, no phenylthiohydantoin derivatives were obtained, indicating a blocked N-terminus. The protein spots were then transferred to a nitrocellulose membrane and digested with trypsin. The reversed-phase h.p.l.c. separation of the resulting peptides and the sequences of the two tryptic peptides analysed are shown in Figure 3. There was one unidentified residue in each peptide sequence determined, which was probably a modified cysteine, as the thiol groups were not derivatized before sequencing. It is therefore reasonable to conclude that the peptide sequences are identical with the corresponding sequences deduced from cDNA encoding GST subunit M3 (Campbell et al., 1990). The appearance of multiple spots of GST M3-3 in the two-dimensional system has also been observed with the recombinant protein, suggesting that it reflects an inherent property of the protein.

On the Western blot, protein bands cross-reacting with anti-(human GST M1-1) antibodies were also detected in fractions B and C. The protein in fraction B was shown to have the same mobility as GST subunit M3 on two-dimensional PAGE, and is probably identical with GST M3-3. The protein in fraction C was not studied further.

A major protein band of about 13 kDa in fraction B (Figure 1a) was subjected to N-terminal amino acid sequence analysis. The structure was determined to be Pro-Met-Phe-Ile-Val-Asn-Thr-Asn-Val-Pro-Arg-Ala-Ser-Val-Pro, which is identical with the deduced N-terminus of a macrophage migration-inhibitory factor discovered by Weiser et al. (1989).

PCR analysis of GST expression

To study further the expression of GST isoenzymes at the RNA level, first-strand HeLa-cell cDNA was synthesized and probed by PCR. Primers specific for GST subunits A1, A2, M1, M3 and P1 were used and were expected to yield amplification products of the following sizes: 690 bp (A1); 799 bp (A2); 564 bp (M1); 780 bp (M3); and 621 bp (P1). This methodology has the advantage of a high sensitivity as well as providing further evidence for the identification of forms observed at the protein level. After agarose-gel electrophoresis and staining with ethidium bromide, the fragments generated with the GST subunit M1-, M3- and P1-specific PCRs were found in the expected size ranges, whereas the PCR supposedly specific for GST subunit A1 cDNA resulted in multiple bands and the A2 PCR failed to yield amounts of DNA detectable with ethidium bromide staining (gel not shown). To determine the identities of the PCR products further, the samples that yielded amplified products were subjected to Southern blotting using internal oligonucleotide probes for subunits A1, M1 and P1 (Figure 4). The [γ - 32 P]ATP-labelled probes specific for subunits M1 and P1 hybridized to the corresponding PCR-amplified products, and the probe for subunit A1 cDNA hybridized to a corresponding PCR product of appropriate size in the mixture of amplified material (Figure 4).

Identification of GST subunit M3 cDNA, heterologous expression of GST M3-3 in *E. coli* and estimation of the cytosolic GST M3-3 concentration

The PCR-derived fragment obtained by using GST subunit M3-specific primers (Figure 4) was subcloned into a plasmid vector and its nucleotide sequence determined. The cDNA sequence was identical with the previously published sequences isolated from human brain and testis libraries (Campbell et al., 1990)

Figure 4 Southern-blot analysis of DNA fragments amplified by PCR on first-strand-synthesized cDNA from HeLa cells grown in flat-bottomed tissue culture flasks

(a) Positions of primers used for PCR: A1-i, A1-ii, M1-i, M1-ii, M3-i, M3-ii, P1-i and P1-ii, and for oligonucleotides used for probing amplified DNA: A1-iii, M1-iii and P1-iii. Also indicated are the expected fragment sizes of amplified cDNAs. (b) Autoradiogram of Southern-blot analysis. Panel A1-iii, PCR products probed using GST A1-specific oligonucleotide; panel M1-iii, PCR products probed using GST M1-specific primer; panel P1-iii, PCR products probed using GST P1-specific primer. Arrows indicate migration of size-marker DNAs (bp).

except for one base in codon 147, which was G rather than the previously reported T (TGG→GGG), causing a replacement of Trp¹⁴⁷ by Gly in the deduced amino acid sequence. The expression of protein from the cDNA was made in *E. coli* XL1-Blue. The recombinant protein, after purification on a glutathione–Sepharose column, exhibited the same mobility as the native GST M3–3 in two-dimensional PAGE (results not shown). Using the recombinant protein as a standard in immunoblot analysis, the concentration of GST M3–3 in the HeLa cells was estimated to be 30-, 150- and 1000-fold higher than the values for GSTs M1–1, P1–1 and A1–1 respectively (Table 2).

Changes in growth rate and expression level of glutathione-linked enzymes in HeLa cells grown in different culture vessels

A comparison based on cell counting showed that the proliferation rate of HeLa cells grown in roller bottles (HR) was about one-third of those grown in ordinary flat-bottomed culture flasks (HF) within a 6-day period of culture (Table 1).

As shown in Table 2, the GST activity with the substrate CDNB was lower in HR than in HF cells. The lower activity may partially reflect the decreases in the different GSTs as quantified by e.l.i.s.a. (Table 2). The concentration of the predominant form (GST M3–3) was also found to be lower in the HR cells, as determined by immunoblot analysis (Table 2).

Glyoxalase I activity in the HR cells was down-regulated whereas GSSG reductase activity was only slightly lowered (Table 1). In contrast, glutathione peroxidase activity, as

Table 3 LC₅₀ of chemotherapeutic agents to HeLa cells grown in different vessels

The LC₅₀ is defined as the drug concentration that causes the death of 50% cells, measured by L-[³H]leucine incorporation in protein synthesis and expressed as mean ± S.D. (*n* = 3)

HeLa cells	LC ₅₀ (μg/ml)		
	Doxorubicin	Cisplatin	Melphalan
HF	1.8 ± 0.2	6.5 ± 1.1	7.8 ± 0.7
HR	0.8 ± 0.3	3.8 ± 1.0	5.7 ± 0.8

measured with cumene hydroperoxide and H₂O₂, was increased in HR cells (Table 1). The total glutathione level was the same in both cell populations (Table 1).

Increased sensitivity to cytostatic agents of HeLa cells grown in roller bottles

To test for possible differences in the resistance phenotypes of HF and HR cells, cytotoxicity assays were carried out as described in the Materials and methods section, and the results are given in Table 3 and Figure 5. For the three drugs tested, the HR cells exhibited higher sensitivities than the HF cells on the basis of calculated LC₅₀ values, which were about 2.3 times higher for doxorubicin, 1.7 times higher for cisplatin and 1.4 times higher

Figure 5 Dose-response curves of HR and HF cells in cytotoxicity assays with cytostatic drugs doxorubicin, cisplatin and melphalan

The error bars representing the S.D. values were calculated from the values obtained in three independent assays. Each assay was carried out with five drug concentration points and drug-free controls, of which every point was measured in triplicate. The radioactivity (c.p.m.) of L-[³H]leucine incorporation into control cells was treated as 100% cell survival and placed on the y-axis only for the convenience of plotting. ●, HF cells; ○, HR cells.

for melphalan. At lower drug concentrations (50–90 % survival, see Figure 5), the drug sensitivities differed between the HR and HF cells even more than at the level of LC_{50} .

DISCUSSION

The present study of HeLa cells has established a distinct profile of GST isoenzyme expression by using a combination of qualitative and quantitative analyses. Four isoenzymes were identified at both the protein and the mRNA levels: GST A1-1, M1-1, M3-3 and P1-1. The cytosolic concentrations of GST A1-1, M1-1 and P1-1 were determined quantitatively by double-antibody e.l.i.s.a. (Table 2). The identification of the GST isoenzymes was confirmed by probing a HeLa-cell cDNA preparation using PCR with subunit-specific primers, followed by Southern-blot analysis (Figure 4). GST subunit M3 cDNA was subsequently subcloned, sequenced and expressed in *E. coli*. The predominance of GST M3-3 in the HeLa cells was demonstrated by the two-dimensional PAGE pattern of the purified GSTs (Figure 2a). The amount of GST M3-3 corresponded to 0.1–0.2 % of the total cytosolic protein as estimated by immunoblot analysis. Failure to detect GST subunit A2 by PCR and GST subunit M2 by Western-blot analysis suggests the absence of these proteins. A protein in affinity fraction C eluted in the presence of 1 M NaCl cross-reacted with anti-(human GST M1-1) antibodies (Figure 1b), indicating the possible existence of an additional class-Mu GST.

The oligonucleotides for GST subunit M1 used in the PCR and in the Southern-blot analysis do not distinguish subunit M1 from subunits M4 and M5, for which sequence information was not available when the experiments were carried out. Recombinant GST subunit M4 occurs in a more basic position than do GST subunits M3 and P1 after denaturing two-dimensional PAGE, and no cross-reactivity with anti-(GST M1-1) antibodies was noted i.e. 1 μg of GST M4-4 was not detectable with the e.l.i.s.a. for GST M1-1 (X.-Y. Hao and B. Mannervik, unpublished work). The calculated pI for GST subunit M5 is 7.1 (Takahashi et al., 1993) and a more basic protein than subunit M1 should consequently be expected. Protein spots in positions

expected for GST subunits M4 or M5 were not observed (Figure 2), indicating the absence or low expression of these proteins.

The present study has also demonstrated a macrophage migration-inhibitory factor in HeLa cells, a protein previously characterized as a lymphokine involved in the cellular immune response (Weiser et al., 1989). The intercellular localization of this factor and its high affinity for glutathione-based affinity matrices suggest that it may fulfil glutathione-linked intracellular functions in addition to serving as an extracellular lymphokine.

The cDNA sequence of the GST subunit M3 from HeLa cells differs in the codon for one amino acid residue as compared with the previously published sequence. The variant residue is Trp¹⁴⁷ in the brain and testis GST M3-3 (Campbell et al., 1990), whereas the HeLa-cell GST M3-3 sequence, like all other known class-Mu subunits, contains a Gly in position 147. Residue 147 is present in a loop far away from the active site, as judged by comparison with the homologous three-dimensional structure of rat class-Mu GST 3-3 (Ji et al., 1992), suggesting that a Trp→Gly replacement is of little consequence for enzymic function. The expressed recombinant GST M3-3 appears to be indistinguishable from the enzyme identified in HeLa cells.

It has previously been demonstrated that tumour cells of different origins vary markedly in their expression of GST isoenzymes. Most tumours and tumour-derived cell lines are dominated by GST P1-1, which has been demonstrated *in vitro* to inactivate acrolein, a toxic metabolite of cyclophosphamide (Berhane and Mannervik, 1990). A predominance of class-Alpha GSTs is observed in a few cases, such as the breast cancer cell lines, Zr 75-1 (Tsuchida et al., 1989) and MCF-7 (Cowan et al., 1986), a hepatoma line, Hep G2, and a Burkitt's lymphoma line, Raji (Castro et al., 1990). An unambiguous correlation between human GST A1-1 and M1-1 expression and resistance against cytostatic drugs is not yet available, but elevated levels of a class-Alpha GST were found in rat mammary tumour (Wang and Tew, 1985) and Chinese hamster ovary cells (Lewis et al., 1988) in connection with enhanced resistance to chlorambucil. The HeLa cells analysed in the present investigation represent the first case in which GST M3-3 has been identified as the dominant GST. More detailed information on, for instance, its tissue

distribution, substrate selectivity and importance in cellular detoxication is still lacking. Nevertheless, it is clear that the diverse patterns of GST isoenzyme expression in tumour tissues and cell lines will result in distinct resistance phenotypes of importance for the cellular processes that inactivate genotoxic alkylating agents.

Attempts at establishing a correlation between drug resistance and GST expression have previously been made through regulation of the cellular isoenzyme profiles by transfecting cDNAs encoding individual GST subunits. For instance, in a breast cancer cell line, MCF-7, expression of recombinant GST P1-1 (Moscow et al., 1989), A2-2 or M1-1 (Townsend et al., 1992) was accompanied by 2-4-fold enhancement in resistance to ethacrynic acid, but not to doxorubicin, cisplatin or melphalan. In transfected monkey COS cells, on the other hand, the expression of rat GSTs 1-1 (class Alpha) and 3-3 (class Mu), and human GST P1-1, enhanced cellular resistance to melphalan (1.3-fold), cisplatin (1.5-fold) and doxorubicin (1.3-fold) (Puchalski and Fahl, 1990). Differences in the efficiency of transfection as well as the stability of the transfectants may be causes of the variable results, but other cellular changes resulting from manipulation of the cells may also contribute.

HR cells encounter a different milieu from HF cells. As a consequence, differences in growth rate and expression of GSH-linked proteins occur (Tables 1 and 2). The regulatory factors and mechanisms of the altered cellular phenotype have not been determined in the present work, but we have taken advantage of the differences to explore the relationship between cellular drug responses and the GSH-linked detoxication system. Changes in GST expression in response to environmental factors have previously been found in rat hepatocytes in primary culture compared with their parental cells (Vandenbergh et al., 1990) and in human Hep G2 cells grown in different media (Doorstard et al., 1988), but the effect on the cellular drug-resistance phenotype has not been investigated.

Among the GSH-linked factors, cellular GSH and GSH peroxidase provide important contributions to detoxication processes (Mannervik et al., 1989; Flohé, 1989). The unchanged total GSH level and increased GSH peroxidase activity in the HR cells suggest that the increased drug sensitivity in the HR cells depends on other parameters. The significance of differences in the glyoxalase I activity is unclear. However, the down-regulation of GST activity co-varied with the higher sensitivities (LC_{50}) of the HR cells to doxorubicin (2.3-fold), cisplatin (1.7-fold) and melphalan (1.3-fold). Calculated on the basis of the specific CDNB activities of individual GST isoenzymes (Mannervik and Danielson, 1988; Campbell et al., 1990), a major portion of the CDNB activity in both the HR and HF cells was contributed by GST M3-3, whereas the contributions of GST A1-1 and P1-1 to the measured cytosolic CDNB activity were negligible (Table 2). The difference in GST activity between the cells can therefore be attributed mainly to GST M3-3 (Table 2). This decrease in GST activity in the HR cells may in part be responsible for the increased sensitivities towards the tested cytostatic drugs. Moreover, as demonstrated in dose-response curves (Figure 5), the cellular protection in the HF cells was comparatively higher at the drug concentrations below the LC_{50} values in comparison with that of the HR cells. The peak plasma levels of melphalan (0.86 μ g/ml), cisplatin (3.8 μ g/ml) [for review see Wilson (1986)] and doxorubicin (1.6 μ g/ml) (Speth et al., 1987) after clinical drug administration fall into the ranges in which maximal differences in resistance between the HF and HR cells occur.

In summary, the present study shows that external factors such as growth conditions can alter cytosolic GST concentrations

with collateral modifications of the drug-resistance phenotype. This simple means of changing the cellular GST levels is an alternative to manipulating cells by transfection or administration of enzyme inhibitors or inducers and lends support to the notion that the enzymes contribute to drug resistance in tumour cells. The evidence for the involvement of the enzymes is only indirect, but previous work has shown that human GSTs are capable of inactivating cytostatic drugs (Ciaccio et al., 1991; Meyer et al., 1992). Therefore the conclusion about their participation in cellular drug resistance appears inevitable, even if their relative importance in relation to other resistance factors remains to be elucidated.

This work was supported by the Swedish Cancer Society and the Swedish Natural Science Research Council.

REFERENCES

- Aebersold, R. H., Leavitt, J., Saavedra, R. A., Hood, L. E. and Kent, B. S. H. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6970-6974
- Armstrong, R. N. (1991) *Chem. Res. Toxicol.* **4**, 131-140
- Berhane, K. and Mannervik, B. (1990) *Mol. Pharmacol.* **37**, 251-254
- Berhane, K., Hao, X.-Y., Egyházi, S., Hansson, J., Ringborg, U. and Mannervik, B. (1993) *Cancer Res.* **53**, 4257-4261
- Black, S. M. and Wolf, C. R. (1991) *Pharmacol. Ther.* **51**, 139-154
- Blum, H., Beier, H. and Gross, H. J. (1987) *Electrophoresis* **8**, 93-99
- Board, P. G. and Pierce, K. (1987) *Biochem. J.* **248**, 937-941
- Board, P. G. and Webb, G. C. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2377-2381
- Campbell, E., Takahashi, Y., Abramovitz, M., Peretz, M. and Listowsky, I. (1990) *J. Biol. Chem.* **265**, 9188-9193
- Carlberg, I. and Mannervik, B. (1975) *J. Biol. Chem.* **250**, 5475-5480
- Castro, V. M., Söderström, M., Carlberg, I., Widersten, M., Platz, A. and Mannervik, B. (1990) *Carcinogenesis* **11**, 1569-1576
- Celis, J. E., Gesser, B., Rasmussen, H. H., Madsen, P., Leffers, H., Dejgaard, K., Honore, B., Olsen, E., Ratz, G., Lauridsen, J. B., et al. (1990) *Electrophoresis* **11**, 989-1071
- Chao, C. C.-K., Lee, Y.-L., Cheng, P.-W. and Lin-Chao, S. (1991) *Cancer Res.* **51**, 601-605
- Chao, C. C.-K., Huang, Y. T., Ma, C. M., Chou, W. Y. and Lin-Chao, S. (1992) *Mol. Pharmacol.* **41**, 69-75
- Ciaccio, P. J., Tew, K. D. and LaCreta, F. P. (1991) *Biochem. Pharmacol.* **42**, 1504-1507
- Comstock, K. E., Johnson, K. J., Riftenberg, D. and Henner, W. D. (1993) *J. Biol. Chem.* **268**, 16958-16965
- Cowan, K. H., Batist, G., Tulpule, A., Sinha, B. K. and Meyers, C. E. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9328-9332
- DeJong, J. L., Chang, C.-M., Whang-Pang, J., Knutsen, T. and Tu, C.-P. D. (1988) *Nucleic Acids Res.* **16**, 8541-8554
- Doorstard, H., Duthie, S. J., Burke, M. D., Melvin, W. T. and Grant, M. H. (1988) *FEBS Lett.* **241**, 15-18
- Dulik, D. M. and Fenselau, C. (1987) *Drug Metab. Disp.* **15**, 195-199
- Epand, R. F., Epand, R. M., Gupta, R. S. and Cragoe, E. J. (1991) *Br. J. Cancer* **63**, 247-251
- Erickson, P. F., Minier, L. N. and Lasher, R. S. (1982) *J. Immunol. Methods* **51**, 241-249
- Flohé, L. (1989) In *Coenzymes and Cofactors: Glutathione* (Dolphin, D., Poulson, R. and Avramovic, O., eds.), vol. 3A, pp. 643-731. John Wiley & Sons, New York
- Habig, W. H. and Jakoby, W. B. (1981) *Methods Enzymol.* **77**, 398-405
- Hansson, J., Berhane, K., Castro, V. M., Jungnelius, U., Mannervik, B. and Ringborg, U. (1991) *Cancer Res.* **51**, 94-98
- Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, pp. 511-612. Cold Spring Harbor Laboratory, Cold Spring Harbor
- He, M., Wilder, A. and Kaderbhai, M. A. (1990) *Nucleic Acids Res.* **18**, 1660
- Jakoby, W. B., Ketterer, B. and Mannervik, B. (1984) *Biochem. Pharmacol.* **33**, 2539-2540
- Ji, X., Zhang, P., Armstrong, R. N. and Gilliland, G. L. (1992) *Biochemistry* **31**, 10169-10184
- Kano, T., Sakai, M. and Muramatsu, M. (1987) *Cancer Res.* **47**, 5626-5630
- Kolm, R. H., Sroga, G. E. and Mannervik, B. (1992) *Biochem. J.* **285**, 537-540
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
- Lawrence, R. A. and Burk, R. F. (1976) *Biochem. Biophys. Res. Commun.* **71**, 952-958
- Lewis, A. D., Hickson, I. D., Robson, C. N., Harris, A. L., Hayes, J. D., Griffiths, S. A., Manson, M. M., Hall, A. E., Moss, J. E. and Wolf, C. R. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8511-8515
- Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Mannervik, B. and Danielson, U. H. (1988) *CRC Crit. Rev. Biochem.* **23**, 283-337
- Mannervik, B. and Guthenberg, C. (1981) *Methods Enzymol.* **77**, 231-235

- Mannervik, B., Ålin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M. and Jörnvall, H. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7202–7206
- Mannervik, B., Carlberg, I. and Larson, K. (1989) in *Coenzymes and Cofactors: Glutathione* (Dolphin, D., Poulson, R. and Avramovic, O., eds.) vol. 3A, pp. 475–516, John Wiley & Sons, New York
- Mannervik, B., Awasthi, Y. C., Board, P. G., Hayes, J. D., Di Ilio, C., Ketterer, B., Listowsky, I., Morgenstern, R., Muramatsu, M., Pearson, W. R., et al. (1992) *Biochem. J.* **282**, 305–306
- Meyer, D. J., Gilmore, K. S., Harris, J. M., Hartley, J. A. and Ketterer, B. (1992) *Br. J. Cancer* **66**, 433–438
- Moscow, J. A., Townsend, A. J. and Cowan, K. H. (1989) *Mol. Pharmacol.* **36**, 22–28
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Puchalski, R. B. and Fahl, W. E. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2443–2447
- Racker, E. (1951) *J. Biol. Chem.* **190**, 685–696
- Rhoads, D. M., Zarlengo, R. P. and Tu, C.-P. D. (1987) *Biochem. Biophys. Res. Commun.* **145**, 474–481
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- Seidegård, J., Vorachek, W. R., Pero, R. W. and Pearson, W. R. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7293–7297
- Shea, T. C., Kelley, S. L. and Henner, W. D. (1988) *Cancer Res.* **48**, 527–533
- Simons, P. C. and Vander Jagt, D. L. (1977) *Anal. Biochem.* **82**, 334–341
- Smith, M. T., Evans, C. G., Doane-Setzer, P., Castro, V. M., Tahir, M. K. and Mannervik, B. (1989) *Cancer Res.* **49**, 2621–2625
- Speth, P. A., Linssen, P. C., Boezeman, J. B., Wessels, H. M. and Haanen, C. (1987) *Cancer Chemother. Pharmacol.* **20**, 305–310
- Takahashi, Y., Campbell, E. A., Hirata, Y., Takayama, T. and Listowsky, I. (1993) *J. Biol. Chem.* **268**, 8893–8898
- Tew, K. D., Bomber, A. M. and Hoffman, S. J. (1988) *Cancer Res.* **48**, 3622–3625
- Tietze, F. (1969) *Anal. Biochem.* **27**, 502–522
- Townsend, A. J., Tu, C.-P. D. and Cowan, K. H. (1992) *Mol. Pharmacol.* **41**, 230–236
- Tsuchida, S., Sekine, Y., Shineha, R., Nishihira, T. and Sato, K. (1989) *Cancer Res.* **49**, 5225–5229
- Vandenberghe, Y., Foriers, A., Rogiers, V. and Vercruysse, A. (1990) *Biochem. Pharmacol.* **39**, 685–690
- Voller, A., Birdwell, D. and Barlett, A. (1980) in *Manual of Clinical Immunology*, 2nd edn. (Rose, N. R. and Friedman, H., eds.), pp. 359–371, American Society for Microbiology, Washington D.C.
- Vorachek, W. R., Pearson, W. R. and Rule, G. S. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4443–4447
- Wang, A. L. and Tew, K. D. (1985) *Cancer Treatment Rep.* **69**, 677–682
- Weiser, W. Y., Temple, P. A., Witek-Giannotti, J. S., Remold, H. G., Clark, S. C. and David, J. R. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7522–7526
- Widersten, M., Pearson, W. R., Engström, Å. and Mannervik, B. (1991) *Biochem. J.* **276**, 519–524
- Wilson, A. P. (1986) in *Animal Cell Culture: A Practical Approach* (Freshney, R. I., ed.), pp. 183–216, IRL Press Limited, Oxford