Biochemical Pharmacology, Vol. 45, No. 10, pp. 2166–2169, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

Interindividual differences in the *in vitro* conjugation of methylene chloride with glutathione by cytosolic glutathione *S*-transferase in 22 human liver samples

(*Received* 28 *November* 1992; *accepted* 25 *February* 1993)

Abstract—The interindividual variation in the *in vitro* conjugation of methylene chloride with glutathione by cytosolic glutathione *S*-transferase (GST) was investigated with 22 human liver samples. In three of the samples no activity towards methylene chloride was observed. Eleven samples showed an activity ranging from 0.20 to 0.41 (0.31 ± 0.08) nmol/min/mg protein, and eight samples an activity of 0.82–1.23 (1.03 ± 0.14) nmol/min/mg protein. The activities towards 1-chloro-2,4-dinitrobenzene (CDNB) of these three groups were 1.17 ± 0.25, 1.12 ± 0.35 and 1.20 ± 0.53 μmol/min/mg protein, respectively. In nine of the liver samples, the α-, μ- and π-clas GST subunits were quantified. In two of these samples, no activity was observed towards methylene chloride, while α-, μ- and π-class subunits were expressed in these human liver cytosolic samples. As the highest activity towards methylene chloride was still 1.4 times lower than the activity in rat cytosol, the existence of the three populations seems to be of little importance for human risk assessment.

Methylene chloride is used as a commercial solvent in paint strippers, refrigerants, cleaning agents and in the decaffeination of coffee. Methylene chloride was found to be carcinogenic in mice exposed to high doses, but not in rats and hamsters [1, 2]. Methylene chloride is metabolized via two pathways. The first pathway is a high affinity, low capacity pathway catalysed by the microsomal cytochrome P450 mixed-function oxidase system [1–5]. The second pathway is a low affinity, high capacity pathway catalysed by cytosolic glutathione *S*-transferase (GST*) [1, 2, 6, 7]. Studies with different species revealed that the GST pathway is a major pathway for methylene chloride in the mouse, but not in rat, hamster or humans. As methylene chloride is not carcinogenic to rat or hamster, it was concluded that humans are not susceptible to the carcinogenic effect of methylene chloride [1, 2].

Methylene chloride is conjugated by GST with glutathione to *S*-chloromethyl glutathione, which is rapidly hydrolysed and degraded to glutathione and formaldehyde [1, 7]. *S*-Chloromethyl glutathione is a potential alkylating agent and probably responsible for the intitiation event in mutagenesis and carcinogenesis [1, 7, 8].

Interindividual differences in metabolism are related to the presence and amount of the various isoenzymes. The most pronounced interindividual variation of GST in humans is found in the closely related μ-class enzymes GST1a-1a and GST1b-1b, which are genetically deficient in about 40% of the Caucasian population [9–11]. μ-Class GSTs show a relatively high activity towards epoxides [11, 12] and have been reported as a marker for the susceptibility to lung cancer [10]. Besides the μ-class enzymes, interindividual differences have also been reported for the α-class subunits A1 and A2 [13].

In the present study, the interindividual variation in the *in vitro* conjugation of methylene chloride with glutathione by cytosolic GST was investigated with 22 human liver samples.

Materials and Methods

Chemicals and reagents. 1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and methylene chloride was purchased from Baker (Deventer, The Netherlands).

Origin of human liver tissue. Liver tissue was obtained at autopsy from kidney donors or from surgical biopsies. Blood circulation was maintained until the moment of removal of the organ. After removal, tissues were stored immediately on ice and frozen at −80° within 8 hr of clinical death.

Preparation of cytosol. Cytosol was prepared by homogenizing liver with 3 vol. of 0.01 M Tris–HCl/0.14 M KCl pH 7.4 with a Potter–Elvehjem tissue homogenizer and centrifuging for 75 min at 105,000 *g*. The fat layer was removed and the cytosol was frozen at −30°.

Protein assay. Protein content of the different enzyme fractions was determined using the Lowry method [14], using bovine serum albumin as standard.

Enzymatic assays. The enzymatic activities towards CDNB were assayed at 25° according to Habig *et al.* [15]. Activity towards methylene chloride was determined as follows: 500 μL 0.1 M potassium phosphate pH 7.4, 100 μL 0.1 M glutathione, 100–200 μL cytosol and 25 μL 1.6 M methylene chloride in ethanol in a total volume of 1 mL were incubated at 37° for 30 min in 1 mL glass vials with teflon-coated rubber septa. The enzymatic reaction was terminated by cooling the vials with ice water, subsequent addition of the incubation mixture to a tube already containing 250 μL ice-cold 25% zinc sulphate solution, followed by the addition of 250 μL saturated barium hydroxide solution. After centrifugation for 5 min at 2500 *g*, the supernatant was decanted, and the amount of formaldehyde, which was formed during the enzymatic reaction, quantified with Nash reagent (4.6 g ammonium acetate, 62 μL acetylacetone and 86 μL acetic acid in a volume of 10 mL): 700 μL of the supernatant and 350 μL of Nash reagent were incubated for 30 min at 60°, cooled in icewater for 5 min and centrifuged at 6000 *g*. If necessary, the samples were filtrated. Colour development was measured at 415 nm. Calibration standards were solutions of 0–0.1 mM formaldehyde in water. Chemical blanks consisted of incubations without sample: after incubation, the blank was transferred into a tube containing both the cytosolic sample and the zinc sulphate solution. The detection limit of the method was 0.04 nmol/min/mg protein.

Stability of enzymatic activity towards methylene chloride.

* Abbreviations: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene.

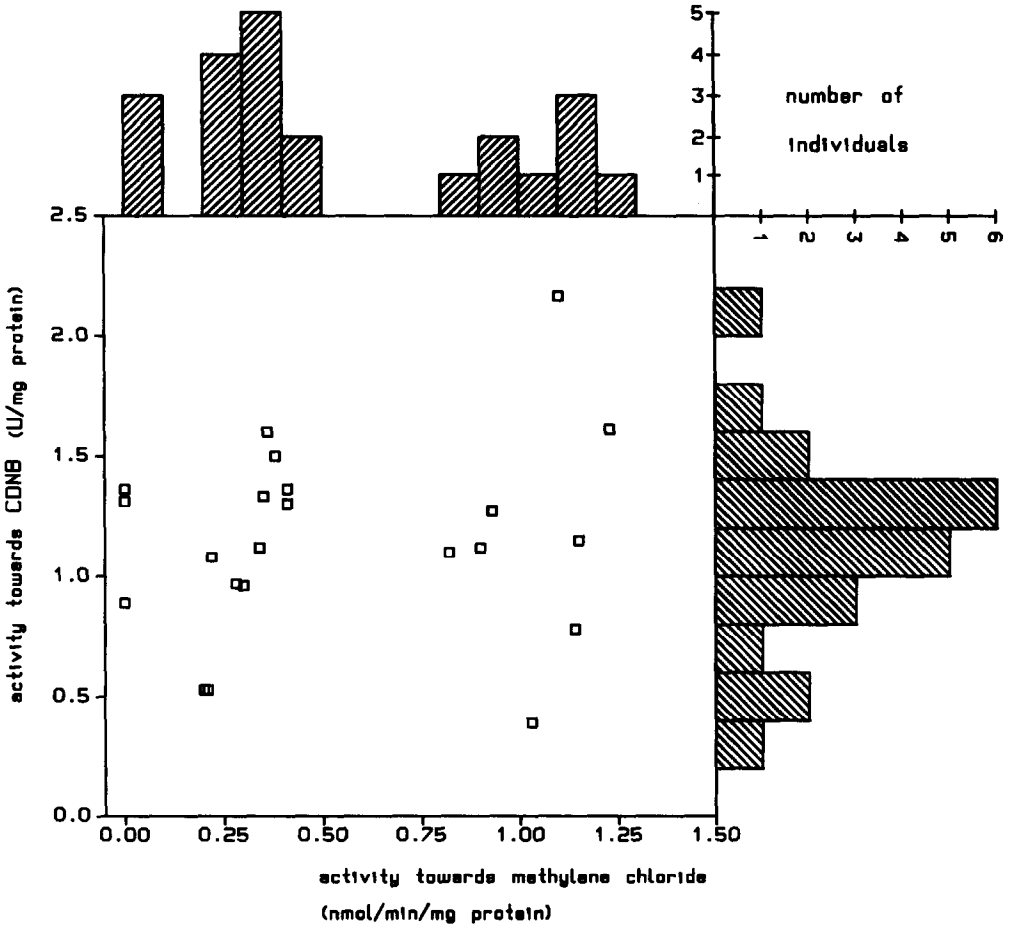

Fig. 1. Scatter diagram of the enzymatic activities of cytosolic GST towards methylene chloride and CDNB. Enzymatic activities were determined as described in Materials and Methods. The distributions of enzymatic activity towards methylene chloride and CDNB are represented by bars. The height of each bar represents the number of individuals within each activity range. The variability of the enzymatic activities towards CDNB and methylene chloride was 5% and 4%, respectively.

Human cytosolic samples were pooled and stored at room temperature and 4°. The activity was measured after 0, 1, 4 and 24 hr (room temperature, 4°) and 3, 7 and 14 days (4°).

Results and Discussion

The activities towards CDNB and methylene chloride are summarized in Fig. 1. The activities towards methylene chloride ranged from 0 to 1.23 nmol/min/mg protein. Three of the samples showed a non-detectable enzymatic activity, which was confirmed by repeated measurements. As no loss of activity of the pooled cytosolic sample towards methylene chloride was observed during storage for 24 hr at room temperature and 14 days at 4°, it is not likely that inactivation of the involved GST isoenzyme had taken place during storage at −80°. Our results confirm the data of Reitz *et al.* [16], who described the absence of activity in one of the four human cytosolic samples. From the other samples, 11 showed an activity ranging from 0.20 to 0.41 (0.31 ± 0.08) nmol/min/mg protein, while the remaining eight liver samples showed an activity of 0.82–1.23 (1.03 ± 0.14) nmol/min/mg protein. There was a significant difference (Student's *t*-test: $P < 0.001$) between the low and high activity group towards methylene chloride. The activities towards CDNB of the samples not converting methylene chloride, and of the low and high activity group were 1.17 ± 0.25, 1.12 ± 0.35 and 1.20 ± 0.53 μmol/min/mg protein, respectively. No correlation ($r = 0.171$) was found between activities towards CDNB and methylene chloride. In addition, no correlation was observed between the time of storage of the liver sample and activity towards methylene chloride.

In Table 1, the enzymatic activities of nine of the investigated liver samples towards CDNB and methylene chloride are presented, and compared to the levels of GST subunits, as determined in a previous study [13]. In all samples showing activity towards CDNB, subunits of the α- and/or μ- and π-class were quantified. However, in two of the liver samples which showed expression of α-, μ- and π-class subunits, no activity towards methylene chloride was observed. Therefore, it is concluded that the conjugation of methylene chloride is not related to any of the activities of the α-, μ- and π-class isoenzymes.

Partial purification of mouse, rat and human GST isoenzymes with catalytic activity towards methylene chloride in our institute revealed that these isoenzymes were not retained by a *S*-hexylglutathione sepharose matrix. They also showed relatively high activity towards 1,2-

Table 1. GST α-, μ- and π-class subunit levels, and enzymatic activities towards CDNB and methylene chloride in nine human liver samples

Sample	Amount of subunit (μg/mg)					Enzymatic activity	
	P1	M1a	M1b	A1	A2	CDNB (μmol/min/mg)	Methylene chloride (nmol/min/mg)
1	1.36	6.05	5.88	14.61	12.29	1.33	0.35
2	5.07	1.30	ND	37.70	40.53	2.17	1.10
3	0.93	9.92	ND	17.21	10.43	1.22	ND
4	3.28	18.45	ND	19.33	14.10	1.31	ND
5	0.52	ND	5.39	20.31	1.94	1.36	0.41
6	1.75	5.72	ND	23.48	12.37	0.39	1.03
7	4.09	ND	ND	20.76	5.55	0.53	0.21
8	0.91	ND	ND	20.99	12.16	1.12	0.90
9	2.14	ND	ND	19.26	9.47	1.50	0.38

ND, not detectable.

The GST subunits were quantified after purification with affinity chromatography and separation of subunits by HPLC with peak area integration at 214 nm [13]. Enzymatic activities towards CDNB and methylene chloride were performed according to Habig *et al.* [15] and as described in Materials and Methods, respectively.

Subunit levels and enzymatic activities were expressed per mg of cytosolic protein.

epoxy-3-(*p*-nitrophenoxy)propane in addition to low activity towards CDNB. However, the GSTs of mouse, rat and humans which were retained by the *S*-hexylglutathione sepharose matrix (α-, μ, and π-class GSTs) showed no detectable activity towards methylene chloride. Therefore, the GST isoenzymes responsible for the metabolism of methylene chloride are most likely θ-class GSTs. θ-Class GSTs show relatively high activity towards 1,2-epoxy-3-(*p*-nitrophenoxy)propane and methylene chloride, and a lack of activity towards CDNB. In addition, θ-class GSTs do not bind to *S*-hexylglutathione and glutathione affinity matrices [17]. The bacterial dichloromethane dehalogenase shows some similarity in primary structure with the rat θ-class subunits 5 and 12, and the human subunit θ [17, 18].

Although the number of investigated liver samples was limited, the data obtained indicate the existence of three distinct populations differing in activity towards methylene chloride. Whether the non-activity group is the result of enzyme deficiency or due to very low activity has yet to be clarified. Although rather large variations in conjugation of methylene chloride were observed, the highest activity was still 1.4 times lower than the activity in rat liver cytosol (1.74 nmol/min/mg protein). Therefore, the existence of the three populations seems to be of little importance for human risk assessment.

Acknowledgements—We thank Dr C. R. Wolf (Imperial Cancer Research Fund, Molecular Pharmacology Group, Edinburgh, U.K.) and Dr W. H. M. Peters (St Radboud University Hospital, Division of Gastrointestinal and Liver Diseases, Nijmegen, The Netherlands) for providing the liver samples.

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