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Correlation between *trans*-stilbene oxide–glutathione conjugation activity and the deletion mutation in the glutathione *S*-transferase class Mu gene detected by polymerase chain reaction

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Abstract—Glutathione *S*-transferase (GST) class Mu activity was determined in 145 unrelated hospital patients in Berlin by measuring their conjugation activity towards the specific substrate *trans*-stilbene oxide (TSO) with two substrate concentrations (50 and 250 μM) in homogenates prepared from lymphocytes. Eighty individuals (55.2%) had an activity lower than 10 pmol/min/10^6 lymphocytes and were classified as GST class Mu deficient. In 142 of 145 cases, phenotype was confirmed by the results of a genotyping procedure using the polymerase chain reaction technique. Two fragments of 273 and about 650 bp including one and two introns, respectively, could always be amplified from genomic DNA in individuals with high GST class Mu activity and could not be amplified in persons with impaired glutathione–TSO conjugation activity. This indicates that persons with low activity carry a large deletion mutation within the GST class Mu gene. The enzymatically determined antimode between low and high activity determined as 10 pmol/min/1 million lymphocytes in the assay with 50 μM TSO could be clearly confirmed by genotyping.

The glutathione *S*-transferases (GSTs*) take a prominent place among phase II drug metabolizing and detoxifying enzymes [1–3]. Among the three different classes of cytosolic GSTs (Alpha, Mu and Pi), the near-neutral isoenzymes μ and ψ of class Mu are of special interest in molecular-epidemiological studies since, in Caucasian populations, GST Mu activity can only be detected in about half of the individuals tested. GST isoenzyme μ was originally purified from human liver by virtue of its high conjugation activity towards benzo(*a*)pyrene-4,5-oxide [4]. Subsequently, it was discovered that these enzymes, also present in high activity in human lymphocytes, can be specifically assayed by their unique conjugation activity towards the substrate TSO [5].

Since GST class Mu isoenzymes are capable of detoxifying mutagenic electrophilic environmental toxins and their reactive intermediates generated by metabolic activation via cytochrome P450 isoenzymes, such as the benzo(*a*)pyrene-4,5-oxide mentioned above, persons with heritably high activity of these enzymes are assumed to be better protected against certain carcinogens than persons lacking these enzymes. A recent epidemiological study [6] revealed an over-representation of phenotypically GST class Mu deficient individuals among lung cancer patients, as tested *ex vivo* with TSO.

Isolation of the cDNA of a human GST class Mu has allowed the performance of restriction fragment length polymorphism analysis [7]. Sequence analysis of this cDNA and addition of knowledge about gene structure of the highly homologous rat genes [8] enabled the design of primers for PCR diagnosis. Using the PCR technique, a complete correlation between immunologically detectable GST class Mu protein and the genetically detected deletion mutation has been described in a limited number of individuals [9]. Thus, to date, carriers of the deficiency of GST Mu can be identified by three different means, namely enzymatically, genetically, and by specific radioimmunoassays [10]. Although the correspondence between GST class Mu specific immunoreactivity and the detection of a deletion mutation by PCR has been shown casuistically, a strict correlation between the PCR results and enzyme activity requires experimental confirmation in a large population. Such a sample size is necessary to detect rare mutations.

This study describes the distribution of the TSO conjugation activity among hospital patients recruited in Berlin and its correlation with the diagnosis of a deletion mutation obtained by use of the PCR.

Materials and Methods

Reagents. Non-radioactive TSO and 2-chloro-1,2-diphenylethanone (desylchloride)were from Aldrich (Steinheim, F.R.G.). GSH was from Boehringer (Mannheim, F.R.G.). Tritiated sodium borohydride was from Amersham (Amersham, U.K.). All other chemicals were analytical grade from Merck (Darmstadt, F.R.G.). All syntheses were performed in a closed vial-system in an appropriate fumehood. The tritiated substrate TSO was synthesized [11] by [^{3}H]sodium borohydride (100 mCi, 9.76 Ci/mmol) reduction of 2-chloro-1,2-diphenylethanone (55.9 μmol) to chlorohydrin and subsequent alkaline epoxide formation. The resulting mixture of *cis*- and *trans*-

* Abbreviations: GST, glutathione *S*-transferase; TSO, *trans*-stilbene oxide; PCR, polymerase chain reaction; GSH, reduced glutathione.

stilbene oxide was separated by TLC and purity of *trans*-stilbene oxide was tested by HPLC. More than 98% of the radioactivity was eluted as a single sharp peak with exactly the same retention time as commercially available non-radioactive TSO. Prior to the assays, the purified TSO was diluted with non-radioactive TSO to give a specific activity of 91 nCi/nmol (=3.4 kBq/nmol). Primers for the PCR reaction were synthesized in the 0.2 mM scale in a Millipore (Bedford, MA, U.S.A.) DNA synthesizer using the phosphoamidide chemistry. *Thermus aquaticus* (*Taq*) DNA polymerase was from Gibco-BRL (Bethesda, MD, U.S.A.).

Patients and sample collection. Blood samples were taken from 145 unrelated patients after informed and written consent. The patients had a variety of pulmonary, cardial, neurological and surgical diagnoses, including 49 patients suffering from lung cancer. All 145 consecutively collected samples were included in this presentation without selection. Mean age of the patients was 54 years, ranging between 32 and 84 years. Forty-six were female and 99 were male. Only persons of German extraction were included. Mononuclear cells were prepared using the Ficoll-paque™ (Pharmacia, Bromma, Sweden) technique as described by the manufacturer. Isolated lymphocytes were counted microscopically after staining with acetic acid gentian violet solution, and adjusted to a cell concentration near 10^6 cells/100 μL. The exact cell concentration was recorded, and all results were finally normalized for 1 million of lymphocytes. The cell samples were frozen at −80° pending enzymatic analysis. Genetic analysis was carried out using all white blood cells prepared by centrifugation after lysis of the erythrocytes.

Assays for glutathione TSO conjugation activity. Assays 100 μL were performed in 175 mM sodium phosphate buffer, pH 7.2, containing 4 mM GSH as described [4]. Lymphocytes suspended in this buffer in a concentration of 10^6 cells/mL were homogenized on ice by sonification prior to the assay. [^{3}H]TSO substrate dissolved in hexane was added to the borosilicate glass incubation vials prior to the assay and the solvent was allowed to evaporate off completely. Reactions were started by addition of the lymphocyte–GSH mixture and immediately placing the incubation vials in a rapidly shaking water bath (37°). The reactions were stopped by transferring the incubation vials into ice-water and further dilution with 100 μL phosphate-buffer. Fifty microlitres of each sample were withdrawn for determination of the total activity by liquid scintillation counting and the unconjugated TSO was immediately extracted from the remaining 150 μL incubate with 300 μL 1-hexanol. Fifty microlitre portions of the aqueous phase were measured for conjugated TSO by liquid scintillation counting. Two assays differing in substrate concentration and reaction time were performed with each sample. A 50 μM substrate-concentration assay was incubated for 60 min and a 250 μM substrate assay was incubated for 12 min. Non-enzymatic activity, obtained from the turnover in an incubation with heat-inactivated lymphocyte homogenate co-analysed in each assay, was subtracted. Precision was recorded by measurement of one low and one high activity control each time the assay was performed. Interassay coefficient of variation was 6.3% for the low activity assay (N = 10) and 5.5% (N = 10) for the high activity assay.

PCR-analysis. Genomic DNA was prepared by standard phenol-extraction from white blood cells. PCR assays (25 μL) were performed with 50–100 ng of DNA in 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM of each desoxynucleotide triphosphate, 1 μM of each primer (corresponding to 25 pmol of each primer per assay), 0.02% of heat sterilized gelatin, and 0.8 units of *Taq*-polymerase. Location of the amplified gene segments is illustrated in Fig. 1. Primers 1 and 2 [9] contained the described sequences of the human GST μ cDNA as published [7], namely 5′-CTGCCCTACTTGATTGATGGG-3′ for primer 1 and 5′-CTGGATTGTAGCAGATCATGC-3′ for primer 2. All samples were further tested with the combination of primers 2 and 3. Primer 3 had the sequence 5′-CTCCTGATTATGACAGAAGCC-3′ and the amplificate (covering another intron) had the expected molecular weight around 650 bp. Primer 4 with the sequence of 5′-TCCTGCAAACCATGGCCGCTT-3′ resulted in combination with primer 1 in a fragment of 1200 bp size when using DNA of persons with high TSO–GSH conjugation activity. Efficacy of this amplification was however low and this reaction was not used for routine genotyping. Amplifications were performed in a programmable water bath (Autogene™, Grant instruments, Cambridge, U.K.) under the following conditions: after a pretreatment for 4 min at 94°, 35 cycles of amplification were performed with a 1 min denaturation step at 94°, 1.5 min annealing at 53°, and 1.5 min extension at 73°. The PCR products were detected and controlled for correct size by agarose gel electrophoresis as illustrated in Fig. 2.

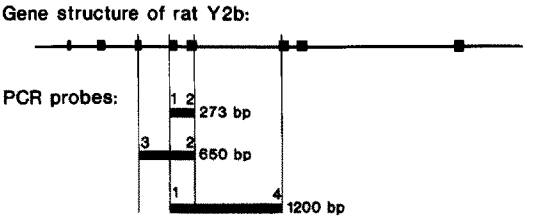

Fig. 1. Location of the gene segments flanked by the primers 1, 2, 3, and 4 and amplified by the PCRs described. Since the complete human gene is not known yet, location of the fragments is illustrated on the base of the homologous rat Yb gene [8], where the broad segments denote the exons of this gene. The sequences of the PCR primers were derived from the human glutathione *S*-transferase cDNA sequence [7, 9].

Results and Discussion

Two enzymatic assays were applied for characterization of the samples. While the high substrate concentration of 250 μM TSO was appropriate for measurement of the higher enzyme activities, this condition was associated with great scatter in the low activity samples because of the significant non-enzymatic conjugation activity, which resulted in several lymphocyte samples with lower conjugation activity than in the respective boiled controls (Fig. 3). This result merely reflects variation in the non-enzymatic conjugation between samples, due to either differences in TSO binding proteins, or differences in content of thiols other than GSH. The mean conjugation rate of the GST class Mu deficient samples was similar under the two assay conditions (2 and 0.9 pmol/min/10^6 lymphocytes, respectively). However, the standard deviation was only 2.0 in the 50 μM assay, but 6.8 pmol/min/10^6 lymphocytes in the 250 μM assay. The antimodes between high and low activity corresponding in both assays were 10 and 20 pmol/min/10^6 lymphocytes in the 50 and 250 μM TSO assays, respectively (Fig. 3). These antimodes represent the point of intersection of the frequency distribution curves of the low and high activities.

These antimodes could be clearly confirmed by the PCR assay, as illustrated in Fig. 3. All persons whose DNA could serve as a template for amplification of the 273 and of the 650 bp fragments (see Fig. 1 and the examples in Fig. 2) are labelled by the dark boxes in Fig. 3. Of the 145 persons tested 80 (55.2%) had TSO–GSH conjugation activity lower than 10 pmol/min/10^6 cells (50 μM assay) and were classified as GST Mu deficient. A more detailed

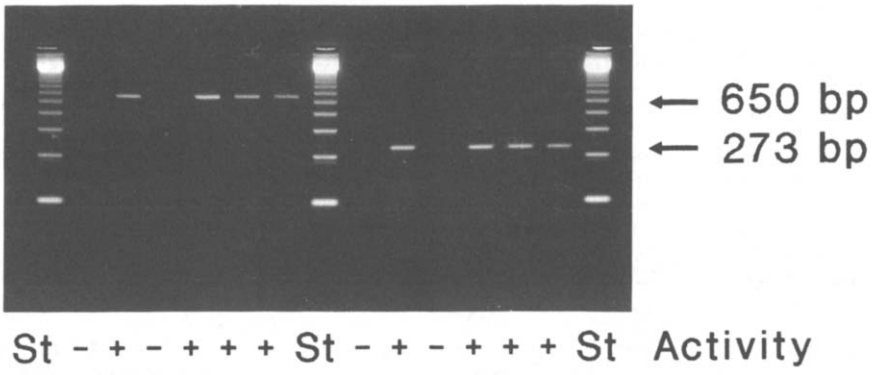

Fig. 2. Detection of the PCR amplificates by 1.4% agarose gel electrophoresis with ethidium bromide staining. For estimation of the fragment sizes, a commercial standard (St; 123 basepair ladder Gibco-BRL, Bethesda, MD, U.S.A.) was used. The same five samples shown were analysed by the two PCR primer sets. High TSO conjugation activity is indicated by the +.

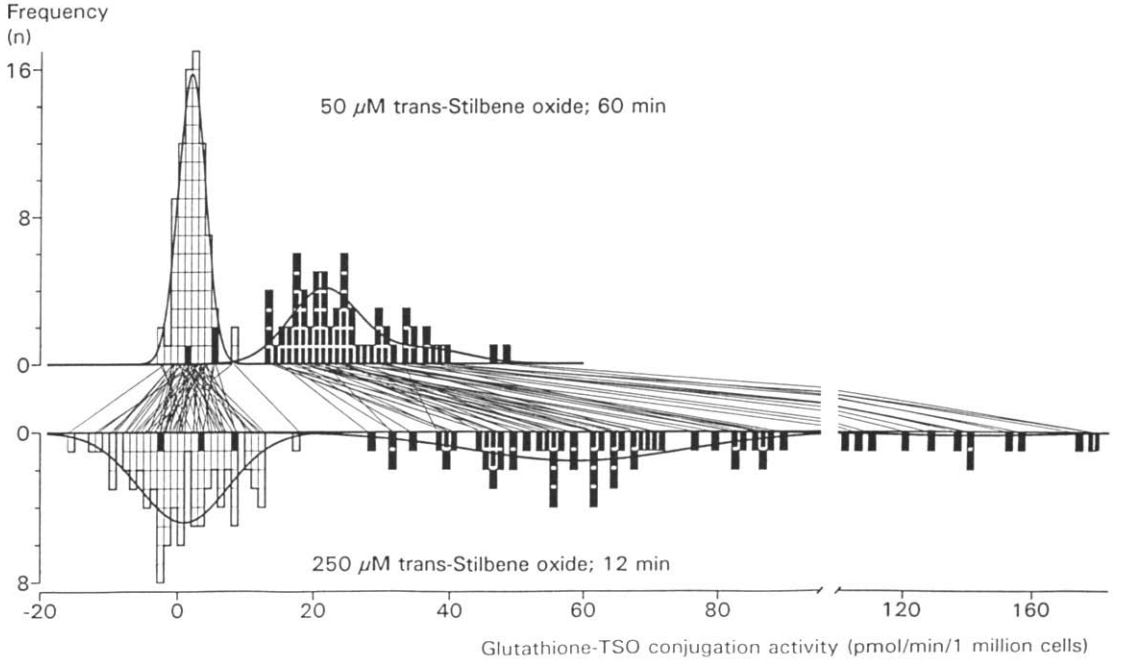

Fig. 3. GST class Mu activity in 145 samples from different unrelated hospital patients as analysed under two enzymatic conditions. Open columns indicate PCR diagnosis of a deletion mutation, while the black boxes refer to the detectability of both the 273 and 650 bp fragments after PCR amplification. The given enzymatic activities represent total activities minus the non-enzyme catalysed conjugation, which sometimes results in negative values.

analysis revealed no trend of over-representation of GST class Mu deficient individuals among the special group of 49 lung cancer patients. Three of the phenotypically GST Mu deficient individuals presented (as confirmed by repeated analysis of different samples of their DNA) 273 and 650 bp fragments with the PCR method. In all persons with high conjugation activity, presence of the amplifiable segments corresponded to and thereby confirmed the phenotypically efficient conjugation activity.

This proportion of 55% of persons as found in Berlin with impaired TSO–GSH conjugation activity corresponds to previous results of Seidegard *et al.* obtained in New York [12], who found 133 of 248 persons (54%) with low activity. Their study also differentiated 21 of the 115 active persons (8.5%) as highly active (presumed homozygotes). These persons might correspond to the 13 persons (9%) with activities higher than 100 pmol/min/10^6 cells in our study (referred to the 250 μM TSO assay), although the individual detailed composition of the μ, ψ and null alleles needs further experimental elucidation. Phenotypic detection of the homozygotes may be confounded by environmental factors, since it is known, at least in the homologous rat system, that the GST class Mu is inducible by enzyme inducers such as phenobarbital and methylcholanthrene [13].

The three persons classified as genetically positive, but

enzymatically negative were re-analysed with the same result and the two enzymatic assays gave corresponding results as shown in Fig. 3. Interestingly, two individuals had activities at the upper end of the low activity scale, especially with the 50 μM TSO assay designed to differentiate within the individuals of low activity. Thus, these three discrepant cases are probably not due to experimental error, but rather may indicate that conjugation activity may become phenotypically low e.g. due to disease or unknown environmental factors. Moreover, we cannot exclude rare mutations located at other sites of the gene that may not be detected by the PCR methods employed here.

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Carboplatin as opposed to cisplatin does not stimulate the expression of the human immunodeficiency virus long terminal repeat sequences

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Abstract—The recombinant plasmid pBHIV1 carrying the long terminal repeat (LTR) of the human immunodeficiency virus 1 (HIV-1), linked to the chloramphenicol acetyl transferase (CAT) gene, was introduced into human and rat fibroblasts. Stable transfectants resistant to geneticin expressed CAT activity from the HIV-1 LTR. It was found that the cytotoxic drug *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II) (carboplatin) at concentrations from 1×10^{-6} to 1×10^{-4} M does not stimulate the expression of CAT from the HIV-1 LTR. These results differ from previous studies with the related drug *cis*-diamminedichloroplatinum(II) which showed stimulation of gene expression from the HIV-1 LTR and suggest that carboplatin could be used in the treatment of cancer patients with Acquired Immune Deficiency Syndrome.

cis-Diamminedichloroplatinum(II) (cisplatin*) is an important cytotoxic agent used in the chemotherapy of several types of human tumor such as testicular, ovarian and lung cancer [1]. However, the severe toxicity related to this compound led to the development of second generation analogues [2]. Of these, carboplatin lacks much of the

* Abbreviations: LTR, long terminal repeat; HIV-1, human immunodeficiency virus 1; CAT, chloramphenicol acetyl transferase; carboplatin, *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum (II); cisplatin, *cis*-diammine dichloroplatinum(II); FCS, fetal calf serum.