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Differential expression of glutathione transferases by native and cultured human lymphocytes

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The glutathione transferases (GSTs) are a unique group of dimeric proteins which catalyse the conjugation of reduced glutathione (GSH) with a variety of compounds possessing an electrophilic centre [1]. Many chemical carcinogens such as the polycyclic aromatic hydrocarbons are thought to exert their effect through the formation of highly reactive epoxide intermediates which can be detoxified by GST-catalysed formation of GSH conjugates [2] or may lead to the initiation of carcinogenesis by covalent interaction with nucleic acids or critical proteins. Interindividual differences in the expression of cytochromes P-450 and GSTs could therefore have important implications in the susceptibility of individuals to some forms of cancer.

The human GSTs are subject to a tissue-specific distribution, and on the basis of differing immunological and physical properties, substrate specificities and protein structure, these isoenzymes can be assigned to one of three classes: alpha, mu or pi [3]. Class alpha forms, for example, GSTs α–ε were originally described in human liver [4] as the predominant forms present whilst the presence of up to thirteen immunochemically identical basic hepatic forms has now been reported [5]. Two basic forms have been isolated from human skin, one of which (pI 8.5) was found to be immunologically related to GST α–ε and the other (pI 9.9) showed greater similarity to rat GST 2-2 than other human class alpha forms [6]. However, it appears that the predominant GSTs of extrahepatic tissues such as placenta [7], lung [8], uterus [9] or erythrocytes [10] are generally members of class pi. It has been reported that as much as 97% of GST activity in lung [8] and 85–90% activity of uterus [9] can be attributed to the presence of acidic forms.

Human GST-μ, a member of class mu with a "near-neutral" isoelectric point, has been isolated from adult liver [11] and more recently a protein having immunological identity with human hepatic GST-μ has been detected in peripheral blood mononuclear leukocytes [12]. Hepatic GST-μ shows a higher efficiency for conjugating epoxides such as benzo[*a*]pyrene 4,5-oxide (BPO) and styrene 7,8-oxide than either class alpha or pi forms and the expression of this form differs markedly between individuals [11]. The conjugation of *trans*-stilbene oxide with GSH in peripheral mononuclear leukocytes also displays a polymorphic distribution [13] where the GST form responsible is reported to be identical with hepatic GST-μ [12]. In addition, a recent study using a specific RIA method to measure class mu GST forms μ and γ in mononuclear leukocytes found that only 55% individuals expressed these forms [14]. There is now evidence to suggest that interindividual variations in the expression of class mu GSTs may be a factor in the susceptibility of cigarette-smokers to lung cancer [15].

We have been assessing the suitability of cultured lymphocytes such as Epstein–Barr virus (EBV) transformed B-cell lines and interleukin-2- (IL-2) dependent T-cells for use in investigations of GST activity with special regard to the polymorphism of class mu GSTs. We have previously reported marked differences in the rate of formation of GSH conjugates with BPO and 1-chloro-2,4-dinitrobenzene (CDNB) between freshly isolated and cultured lymphocytes [16, 17]. In this paper we have investigated the expression of GSTs by native and cultured human lymphocytes using Western immunoblotting.

Materials and methods

Cell culture. Venous blood (40–60 ml) from normal volunteers was collected into sterile containers (10 units preservative-free heparin per ml blood) and lymphocytes were separated by density gradient centrifugation as previously described [17]. T-cells were separated from freshly-isolated lymphocytes using the washed nylon wool column method where the eluate enriched for T-lymphocytes was collected [18]. IL-2- dependent T-cells and phytohaemagglutinin (PHA)-stimulated cultures [19] and EBV-transformed B-cell lines [20] were grown up from freshly-isolated lymphocytes with modifications as detailed elsewhere [17].

Partial purification of cytosols. Cytosols were prepared as previously described [17]. Samples pooled from four newly-established EBV-transformed B-cell lines and IL-2 dependent T-cells generated from six individuals were loaded onto a 200 μl GSH-agarose (Sigma Chemical Co., Dorset, U.K.) affinity column equilibrated with ice-cold 10 mM potassium phosphate buffer pH 7.0 with 0.025 mM PMSF and 2 mM EDTA (buffer A). GSTs were eluted by flushing the column with ice-cold 50 mM Tris–HCl pH 9.1 containing 7 mM GSH. Four volumes of ice-cold acetone were added to the column eluate and the resulting protein precipitate was resuspended in 500 μl of buffer A. Estimated protein recovery was marginally higher for the IL-2-dependent T-cell samples (1.7%) than for the EBV-transformed B-cell samples (1.4%).

Western immunoblotting. Cytosolic fractions were prepared [17] from freshly-isolated lymphocytes pooled from four individuals, from T-cells pooled from three individuals, from PHA-stimulated cells pooled from two individuals and from four individual, newly-established, B-cell lines. Cytosolic samples (generally 25 μg protein per track) were analysed by SDS–polyacrylamide gel electrophoresis essentially according to the method of Towbin *et al.* [21] as previously described [22]. Proteins were transferred onto nitrocellulose paper by electroblotting overnight and the filters were "blocked" in Tris-buffered saline pH 7.9 containing 3% dried low fat milk for 1 hr. Filters were extensively washed and then probed with antiserum raised as previously described [23, 24] against human hepatic GST-μ, hepatic GST-ε and lung GST-λ. All antisera and purified protein standards were kindly provided by Dr. J. D. Hayes, University Dept. Clinical Chemistry, The Royal Infirmary, Edinburgh, U.K. Filters were incubated with a second antibody, anti-rabbit IgG conjugated with horseradish peroxidase for 1 hr. Visualisation of antibody-reactive proteins was achieved using 1-chloro-4-naphthol as substrate and blots were further incubated with ^{125}I protein A labelled with Bolton and Hunters reagent (Amersham Int. plc., Bucks., U.K.) followed by autoradiography with exposure times of 12 hr to 3 weeks as noted.

Enzyme assays. 1-Chloro-2,4-dinitrobenzene (CDNB) conjugating activity was measured as described previously [1, 17]. Protein concentrations were estimated according to the method of Lowry *et al.* [25].

ELISA assay for human GST-μ. A "Mukit" sandwich enzyme-linked immunosorbent assay using the biotin–streptavidin–biotinylated peroxidase assay (Medlabs, Dublin, Eire) was used for the detection of GST-μ in cytosol samples. Samples were assayed for GST-μ according to the manufacturers instructions. In brief, 75–100 μg cytosolic protein derived from IL-2-dependent T-cells or EBV-transformed B-cells or 200 μl whole blood lysed with 10% Triton X100 was added in 100 mM potassium phosphate buffer pH 7.0 to microwells coated with affinity-purified rabbit polyclonal antibody to human GST-μ. Incubations with biotinylated rabbit anti-human GST-μ followed by biotinylated–streptavidin–peroxidase complex and addition of substrates (stabilised hydrogen peroxide solution and stabilised chromagen) solution were performed as per the manufacturers instructions. A bright blue colour in the microwell 5 min after addition of substrates indicated the presence of GST-μ in the test sample. No colour was observed in negative samples. Positive and negative controls in the form of lyophilised whole blood samples supplied with the kit were assayed simultaneously. The manufacturer claims that the limit of detection of the "Mukit" is 5 ng antigen and that the assay is highly specific for GST-μ since the primary antibody employed does not cross-react with basic GST [26], GST-π [27] or any other blood component. The "Mukit" is therefore sensitive enough to detect the presence or absence of GST-μ in 200 μl of whole blood.

Results and discussion

Antibodies raised against human hepatic GST-ε gave no detectable immunocomplex with any of the lymphocyte samples tested even after prolonged (3 week) autoradiography. Thus, native and cultured lymphocytes were not found to express GSTs having immunochemical identity to class alpha human hepatic GSTs α–ε at levels that could be detected using the Western blotting techniques described. Our data suggest that class alpha GSTs may be expressed at very low levels, if at all, by human lymphocytes in common with other cells of the erythropoietic system such as platelets and erythrocytes which have not been reported to express basic forms [28].

Mononuclear leukocytes, B-lymphocytes and lymphoblastoid B-cell lines all expressed a protein band which reacted strongly with antiserum to human lung GST-λ (Fig. 1). In contrast, native T-cells gave no detectable immunocomplex with this acidic, class pi antibody. However, only 15 μg of cytosolic protein was available for SDS–PAGE analysis of this sample and it is possible that immunocomplex detection may require the use of increased protein levels. Stimulation of freshly isolated lymphocytes with PHA resulted in a protein band that reacted weakly with antibodies to GST-λ as was the case for PHA-stimulated cultures subsequently treated with IL-2. Therefore, Western blot analysis of cytosolic samples derived from both native and cultured lymphocytes revealed the expression of proteins that were immunochemically similar to human lung GST-λ. The strong signal seen on this blot also indicates that the predominant GST expressed by human lymphocytes is probably anionic in nature as has been reported for mononuclear leukocytes [12], platelets [28] and erythrocytes [10]. It is also of interest that EBV-transformed B-cell cytosols gave a stronger reaction with antiserum to GST-λ than either cytosols prepared from PHA-stimulated lymphocytes or IL-2-dependent T-cells. The class pi form expressed by IL-2-dependent T-cells and PHA-stimulated lymphocytes may either share less immunochemical identity with GST-λ than that expressed by EBV-transformed B-cells or alternatively, the latter may express more class pi protein per μg cytosolic protein. This second interpretation is more attractive in the light of the increased rate of conjugation of CDNB and BPO in EBV-transformed B-cells compared to IL-2-dependent T-cells [10]. The relative difference in expression of class pi GSTs by these cultured lymphocyte subsets may be even greater than that suggested by the blot in that 25 μg protein represents approximately 5×10^6 EBV-transformed B-cells compared to approximately 8×10^6 IL-2-dependent T-cells.

The reaction of lymphocyte samples with antibodies to human hepatic GST-μ after extended autoradiography showed weakly positive bands corresponding to immunocomplex formation were apparent in three out of four cytosols derived from EBV-transformed B-cell lines although interpretation was hampered by the presence of multiple non-specific bands. Expression of the class mu GSTs was further investigated using partially purified fractions derived from the cytosolic pools of IL-2-dependent T-cells from six individuals and lymphoblastoid B-cell lines generated from four individuals. B-cell line fractions were

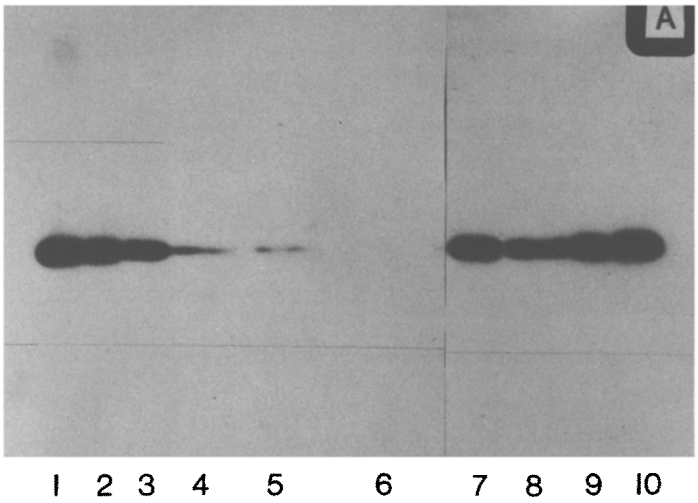

Fig. 1. Western immunoblot analysis of human GSTs in cytosol samples of human lymphocytes using antiserum against human GST-λ. Samples (from left to right), standards (1, 2, approx. 0.4 μg and 0.2 μg purified protein respectively), mononuclear leukocytes pooled from four individuals (3), PHA-stimulated lymphocytes pooled with two individuals (4), IL-2-dependent T-cells pooled from two individuals (5), T-lymphocytes (6, 15.4 μg protein) pooled from three individuals, lymphoblastoid B-cell lines (7, 8) and standards (9, 10, approx. 0.2 μg and 0.4 μg purified protein respectively). Immunocomplexes were detected by overnight autoradiography. Immunoblot analysis was repeated using samples pooled from different individuals and gave results identical to those shown above.

markedly more efficient at conjugating CDNB than IL-2-dependent T-cell fractions where the specific activity of B-cell fractions was 4.95 μmol conjugate formed/min/mg protein and was 1.64 μmol conjugate formed/min/mg protein in the T-cell fractions. Western immunoblots of these samples using antiserum raised against human GST-μ and GST-λ showed immunocomplex formation with both antibodies in B-cell line fractions and IL-2-dependent T-cell fractions indicating expression of proteins sharing immunochemical identity with GST-μ and GST-λ by these cultured lymphocyte subsets (Fig. 2). In view of the reported polymorphism of GST-μ it is difficult to discern whether the stronger reaction observed with antiserum to GST-μ in the B-cell line fractions is due to higher levels of expression of class mu GSTs compared to IL-2-dependent T-cells or is a reflection of the mu phenotype of the individuals from whom the B-cell lines and Il-2-dependent T-cells were established. We have data from two individuals showing that the mu phenotype assigned after ELISA "Mukit" testing of whole blood, or freshly-isolated lymphocyte cytosolic fractions agrees with the mu phenotype assigned after testing of corresponding EBV-transformed B-cell lines. We have also examined eleven newly-established B-cell lines and IL-2-dependent T-cells established from sixteen individuals and a series of paired whole blood and freshly-isolated lymphocyte cytosolic fractions from eleven individuals for presence or absence of GST-μ using the ELISA "Mukit" assay (Table 1). Assignment of mu phenotype after testing of whole blood was found to correlate exactly with the phenotype assigned after analysis of the corresponding freshy-isolated lymphocyte cytosolic fraction. Four out of the eleven B-cell lines and five out of the twelve IL-2-dependent T-cell fractions tested were found to be positive for GST-μ and a positive reaction in these samples was discernible at protein concentrations down to 5 μg whereas no reaction was discernible in negative samples at protein concentrations of up to 100 μg (EBV-transformed B-cell lines) and 200 μg (IL-2-dependent T-cells). These data confirm the findings of our Western blot experiments in that expression of GST-μ can be detected in both EBV-transformed B-cell lines and IL-2-dependent T-cells. In addition, these data indicate that GST-μ may be subject to a polymorphic pattern of expression in cultured human lymphocytes in common with that found in resting human lymphocytes by assay with the ELISA "Mukit" or by RIA [4].

Table 1. Determination of GST-μ expression by ELISA assay of cytosolic fractions derived from EBV-transformed B-cell lines and IL-2-dependent T-cells and in paired samples of whole blood and cytosol fractions from freshly-isolated lymphocytes

Sample	N*	GST-μ positives (%)
Whole blood	11	36
Resting lymphocytes	11	36
IL-2-dependent T-cells	12	42
B-cell lines	11	36

* Number of samples assayed.

Mononuclear leukocytes have been extensively used for the study of human aryl hydrocarbon hydroxylase (AHH) inducibility [29] and more recently for the study of the polymorphism of class mu GSTs [13, 14]. Previous studies have detected human GST conjugating activity towards a number of substrates using PHA-stimulated lymphocytes [30], fractionated peripheral lymphocytes [31–33], IL-2-dependent T-cells [16, 17] and lymphoblastoid B-cell lines [31, 16, 17]. Some of the difficulties encountered in the study of AHH inducibility using PHA-stimulated lymphocytes may be associated with the use of a mixed cell population especially when samples have been obtained from diseased patients [34]. Our data indicate that GSTs may be differentially expressed by cultured human lymphocyte

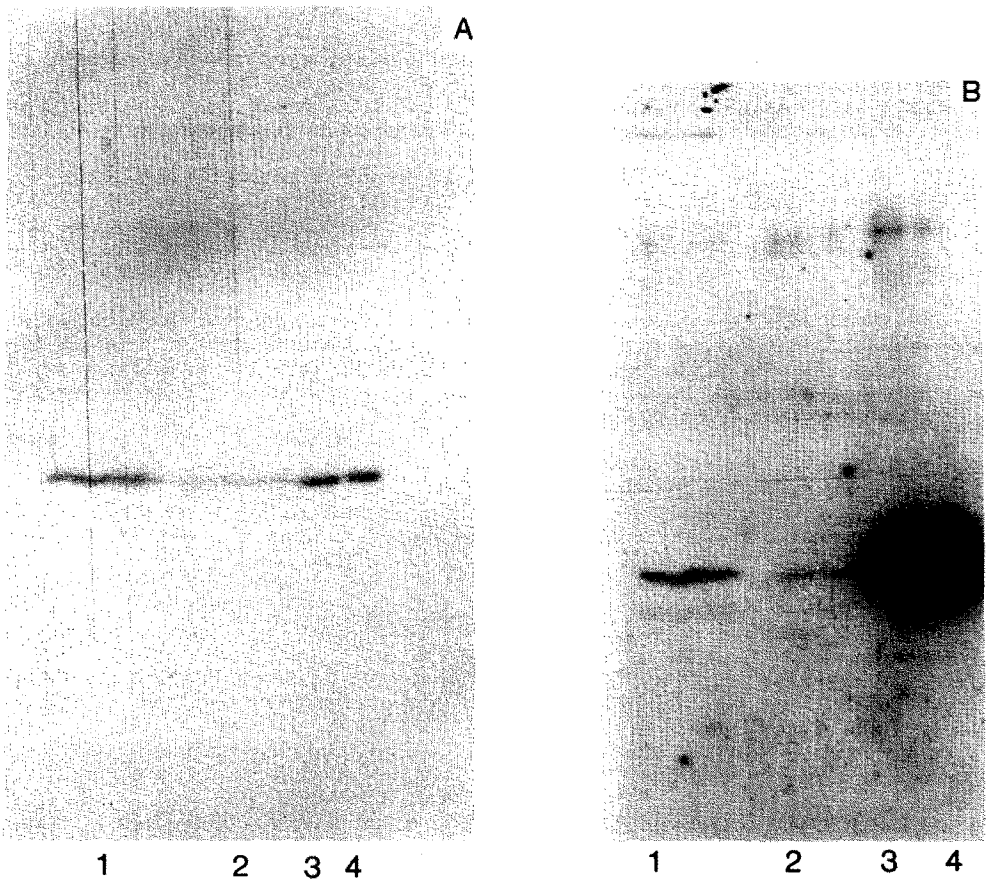

Fig. 2. Western immunoblot analysis of human GSTs in partially purified cytosol fractions of IL-2 dependent T-cells pooled from six individuals and lymphoblastoid B-cells pooled from four lines using antibodies against human GST-λ (A) and antibodies against human GST-μ (B). Samples (from left to right), lymphoblastoid B-cell fraction (1), IL-2-dependent T-cell fraction (2) standards (3, 4, approx. 0.2 μg and 0.4 μg purified protein respectively). Immunocomplexes were visualised after overnight (A) or 2 week (B) autoradiography.

subsets. Investigations of interindividual differences in expression of GSTs using mixed cell populations such as mononuclear leukocyte fractions might be confounded if the apparent differential expression of GSTs by cultured human lymphocytes is a reflection of that in native or resting lymphocytes. Lymphoblastoid B-cell lines constitute a homogeneous cell population and could provide an alternative model system in which to study certain aspects of human GST activity.

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Failure of oral gossypol to inhibit hepatic microsomal and cytosolic drug-metabolising enzymes

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Gossypol, a polyphenolic extract from the cotton seed and well known for its antifertility action, is also known to possess hypolipidemic, antitumor and antimicrobial properties [1]. Enzyme inactivation seems to be one of the major actions in bringing about its effect. Several key enzymes are known to be inhibited by gossypol, including the drug-metabolising enzymes [2, 3]. However, the inhibitory effects of gossypol on succinic acid dehydrogenase, cyto-