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IMMUNODETECTION WITH A MONOCLONAL ANTIBODY OF GLUTATHIONE *S*-TRANSFERASE MU IN PATIENTS WITH AND WITHOUT CARCINOMAS

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Abstract—Several monoclonal antibodies against human liver glutathione *S*-transferase mu were developed. One of these monoclonal antibodies, called GST-3H_4 was further characterized and used in this study. In hepatic tissue, after immunoblotting, GST-3H_4 stains a 27 kDa protein with a pI value of 6.2. GST-3H_4 recognizes other human class-mu glutathione *S*-transferases, but does not detect acidic or basic glutathione *S*-transferases. By immunodetection with this monoclonal antibody, glutathione *S*-transferase mu can be demonstrated in human breast, stomach, liver, small and large intestine, mononuclear blood cells, kidney and placenta. A 100% correlation is found in the distribution of glutathione *S*-transferase mu when different tissues or mononuclear blood cells from the same individuals are investigated. In 62.5% of the mononuclear blood cells from controls, glutathione *S*-transferase mu is present. In patients with polyposis coli, breast cancer or colon cancer a similar distribution is found. Therefore no important role for glutathione *S*-transferase mu deficiencies in the aetiology of these diseases is suggested.

Glutathione *S*-transferases are a class of enzymes, which have an important function in the conjugation, storage and transport within the cell of potentially toxic or carcinogenic compounds [1–4]. Human and rat glutathione *S*-transferases can be divided into three classes: class alpha, basic proteins with intermediate subunit molecular mass; class mu, near-neutral proteins with high subunit molecular mass and class pi, acidic proteins with low subunit molecular mass [5]. Within each class, several isoenzymes may exist.

Monoclonal antibodies, due to their high specificity for a single epitope, might be useful tools for the rapid screening on the presence of these various isoenzymes. To our knowledge, no monoclonal antibodies against human glutathione *S*-transferases are available yet. Several monoclonal antibodies against rat class alpha glutathione *S*-transferases have been described [6–8].

Glutathione *S*-transferase mu is an isoenzyme which very specifically seems to catalyse the conjugation of epoxides and may thus protect individuals against chemical mutagens or carcinogens [9]. In humans, this isoenzyme is absent in hepatic tissue in approximately 40% of the specimens investigated [10, 11]. Recently it was shown that the hepatic isoform mu is similar to the trans-stilbene oxide-active glutathione *S*-transferase of human mononuclear leucocytes [12]. Here again in about 40% of the individuals investigated this isoenzyme was missing [12, 13]. Evidence suggests that individuals who express the glutathione *S*-transferase mu have better protection against chemical carcinogens from cigarette smoke [14]. In this respect it is relevant to investigate the distribution of glutathione *S*-transferase mu in tissues which may be exposed to harmful compounds, such as the gastrointestinal tract, from a control population and from several groups of cancer patients. With the monoclonal antibody against glutathione *S*-transferase mu it is possible to rapidly screen small amounts of human tissue such as colonic biopsies, and possible correlations between isoenzyme deficiency and tissue pathology may be revealed.

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MATERIALS AND METHODS

Tissue. Human tissue was obtained at autopsy, from biopsies and from surgical resections. Intestinal and breast tissue used in this study was from patients with histologically proven polyposis coli, colon carcinoma or breast cancer.

Tissue handling, preparation of 150,000 *g* supernatants and isolation of glutathione–agarose purified glutathione *S*-transferases was performed as described before [15–17].

Heparinized blood was collected from 64 patients, visiting the out-patient department of internal medicine with various cardiovascular, gastrointestinal and nephrological diseases, but with no manifest malignancies. Mononuclear cells were isolated from 3 mL of fresh blood, by centrifugation on Histopaque-1077 (Sigma Diagnostics, St Louis, MO) according to the manufacturers instruction manual. Cells were counted and suspended in 20–40 μL of 1.4 mM dithiothreitol solution and lysed by ultrasonic disturbance during 20 min in a Branson 2200 ultrasonic bath (Branson Comp., Shelton, CT). After centrifugation at 6000 *g* for 15 min, 10–20 μL supernatant equivalent to at least 1 million cells was loaded on a SDS–polyacrylamide gel.

Preparation of monoclonal antibodies. Female Balb/c mice were immunized with 40 μg glutathione–

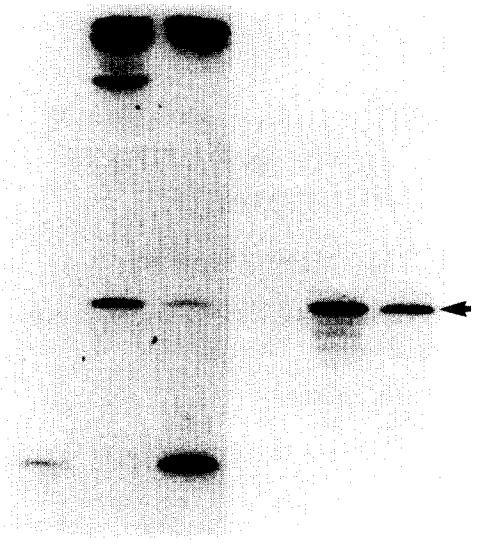


Fig. 1. Specificity of the monoclonal antibody GST-3H_4 for glutathione *S*-transferase mu. Left part (slots 1–3), Coomassie brilliant blue stained isoelectric focusing gel: after isoelectric focusing (pH range 3 to 9; basic proteins are on top of the figure) a Western blot was made by contact blotting at 35° for 5 min. Thereafter the remaining proteins were stained with Coomassie brilliant blue. Glutathione–agarose purified glutathione *S*-transferases were applied to slot 1 (colon, 5 μg), slot 2 (liver, 30 μg) and slot 3 (small intestine, 30 μg). Right part (slots 4–6): Western blot of the isoelectric focusing gel, incubated with the monoclonal antibody GST-3H_4 against glutathione *S*-transferase mu. Slots 4–6 correspond with slots 1–3, respectively. The most intensely stained protein (arrow) has a pI value of approximately 6.2 (see Ref. 16). Hepatic and small intestinal tissue are from the same individual, an 18-year-old male kidney donor [17].

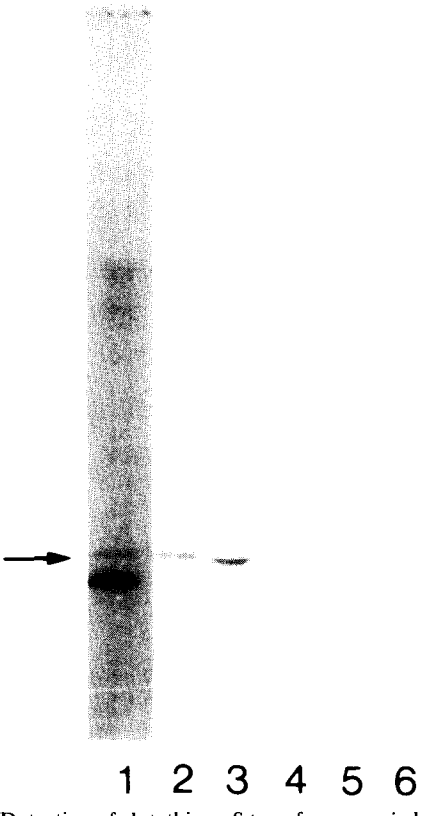


Fig. 2. Detection of glutathione *S*-transferase mu in human intestine and liver. Glutathione *S*-transferases from human liver and small intestine were subjected to SDS–polyacrylamide gel electrophoresis (10% acrylamide, w/v) and subsequently electroblotted to nitrocellulose. The left part of the blot (slot 1) was stained with amido black, and contains 1.4 μg of purified hepatic glutathione *S*-transferase. The right part of the blot (slots 2–6) was incubated with the monoclonal antibody GST-3H_4. In slot 2 purified glutathione *S*-transferase from liver (0.7 μg) is shown. Supernatants (150,000 *g*) from liver (25 μg), duodenum (70 μg), jejunum (70 μg) and ileum (70 μg) are shown in slots 3–6, respectively. Hepatic and small intestinal tissue are identical to that of Fig. 1. The arrow indicates the 27 kDa protein band representative for glutathione *S*-transferase mu.

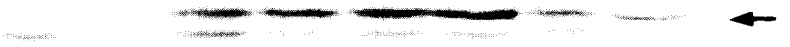
agarose purified human liver glutathione *S*-transferase, in a similar way as previously [18]. After 3 weeks the mice were tested by an enzyme linked immunosorbent assay (ELISA) as follows: purified glutathione *S*-transferases (400 ng/well) were coated to ELISA plates (Greiner, Nürtingen, F.R.G.) by overnight evaporation of a 50 μL solution in phosphate buffered saline (PBS). After blocking for 2 hr with a 1% gelatine (Merck, Darmstadt, F.R.G.) solution in PBS, the plates were washed five times with PBS/Tween 20 (0.05%, w/v; Baker Chemicals, Deventer, The Netherlands). The plates were then incubated with diluted mouse sera for 1 hr at room temperature, washed five times with PBS/Tween 20 and subsequently incubated for 1 hr with peroxidase conjugated rabbit anti mouse immunoglobulins (Dakopatts, Denmark). After washing five times with PBS/Tween 20, the wells were incubated with 5-aminosalicylic acid (80 mg/100 mL; Sigma) and H_2O_2 (80 μL of a 30% solution/100 mL) in 50 mM phosphate buffer, pH 6.0. Usually very low titers were found. Only mice with high titers were used for isolation of spleen cells and subsequent fusion with myeloma cells [18]. Before isolation of spleen cells mice were boostered for 3 subsequent days by intravenous injections with 25 μg purified glutathione *S*-transferases. Testing of the hybridoma culture media for glutathione *S*-transferase antibodies was done by ELISA, essentially as described above. Cells from ELISA positive wells were recloned by diluting to 0.5 cell per well.

Miscellaneous. Isoelectric focusing (pH gradient 3–9) and SDS–polyacrylamide gel electrophoresis (10% acrylamide; w/v) were performed as described earlier [16, 18].

Immunoblotting was done by a recently published method [19].

RESULTS

Ten mice were immunized with glutathione–agarose purified human liver glutathione *S*-transferases. Two mice developed a sufficient titer, as tested by ELISA. The spleen of one mouse was used for the production of hybridomas. One clone, called GST-3H_4 did produce antibodies against glutathione *S*-transferase which strongly reacted after ELISA and



1 2 3 4 5 6 7 8

Fig. 3. Correlation between glutathione *S*-transferase mu in mononuclear blood cells and colonic mucosa. The cytosols from both lysed mononuclear blood cells (from 1.0 mL blood) and colon mucosa (75 μg protein) from the same individual were subjected to SDS–polyacrylamide gel electrophoresis and subsequent Western blotting. Glutathione *S*-transferase mu was then detected by the monoclonal antibody GST-$3H_4$. Slots 1, 3, 5 and 7 contain mononuclear blood cells, and slots 2, 4, 6 and 8 contain the corresponding colonic mucosa from four different individuals, respectively. The arrow indicates the 27 kDa protein band representative for glutathione *S*-transferase mu.

on immunoblot, and this monoclonal antibody was studied in more detail. GST-$3H_4$ is directed against a near-neutral glutathione *S*-transferase isoenzyme with a pI value of 6.2 (Fig. 1). This isoform, glutathione *S*-transferase mu, is present in human liver and small intestine but is not detectable in the colon preparation shown in Fig. 1. No reaction of GST-$3H_4$ with basic (Fig. 1, top) or acidic isoenzymes (bottom) is detectable. Hepatic glutathione–agarose purified glutathione *S*-transferases, after SDS–polyacrylamide gel electrophoresis, Western blotting and subsequent treatment with GST-$3H_4$, show staining of a 27 kDa band (Fig. 2, slot 2). The bulk of the hepatic glutathione *S*-transferases, with lower molecular mass (25 kDa), are not recognized by GST-$3H_4$ (Fig. 2, slot 1). When 150,000 *g* supernatant from the same liver specimen is applied to the gel, only the 27 kDa band is detected by this antibody (Fig. 2, slot 3).

Figure 2 shows also 150,000 *g* supernatants from proximal and distal small intestinal mucosa, after incubation with GST-$3H_4$. In addition to the 27 kDa

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 4. Detection of glutathione *S*-transferase mu in normal and tumorous colonic tissue from seven different patients. Cytosols (150,000 *g* supernatant) from both normal and tumorous colonic tissue were analysed for the presence of glutathione *S*-transferase mu. Slot 1 contains liver cytosol (25 μg) and slot 2 small intestinal cytosol (75 μg), used as markers for the 27 kDa band (arrow), representative of glutathione *S*-transferase mu. Slots 3, 5, 7, 9, 11, 13 and 15 contain cytosols (75 μg) from normal colon mucosa, and slots 4, 6, 8, 10, 12, 14 and 16 contain cytosols from the corresponding tumors from seven patients with colonic cancer.

band, here also a 25.5 kDa band is visualized. A similar pattern is observed in most tissues (see Figs. 2–5). Supernatants from distal small intestine (slot 6) and several preparations from large intestine give some extra staining in the 40 kDa area.

We also investigated the distribution of the neutral isoenzyme in both mononuclear blood cells and colon mucosa from the same individuals. From eight different patients both colon biopsies and blood cells were available. Figure 3 is an immunoblot which shows the results obtained from part of these patients. In four patients glutathione *S*-transferase mu was present in both colon mucosa and blood cells and in the remaining four patients neither colon nor mononuclear blood cells contained the enzyme. Such a 100% correlation was also found in another five patients. From four of these patients both small and large intestinal tissue was available and from 1 patient both hepatic and small intestinal tissue was analysed. In both tissues from each individual glutathione *S*-transferase mu was either present or absent (results not shown).

An immunoblot with 150,000 *g* supernatants from normal mucosa and its corresponding tumor from seven patients with colon carcinomas is presented in Fig. 4. Besides glutathione *S*-transferase mu deficiencies, this figure also reveals that the distribution of glutathione *S*-transferase mu in normal and colon cancer tissue from the same individual has a 100% correlation. Such an uniform distribution was also found in another four patients investigated (results not shown).

The distribution of glutathione *S*-transferase mu in a control population was studied by analysing mononuclear white blood cells from 64 persons. Results from 18 samples are shown in Fig. 5. In 40 individuals (62.5%) glutathione *S*-transferase mu was detectable whereas in 24 individuals (37.5%) no 27 kDa band was observed.

Subsequently we screened human breast and large intestinal tissue from patients with polyposis coli or carcinomas for the presence of glutathione *S*-transferase mu (Table 1). Distribution of glutathione *S*-transferase mu in patients with breast or colon cancer is not different from the values found in the control population. For polyposis coli patients a similar conclusion can be drawn, however the number of patients investigated is very small.

DISCUSSION

A monoclonal antibody (GST-3H_4) against glutathione *S*-transferase mu has been developed and characterized. No cross reactivity with the quantitatively most prominent basic (liver) or acidic (intestine) isoenzymes is observed. In hepatic tissue, GST-3H_4 specifically stains a high molecular mass (27 kDa) subunit, which is typical for the near-neutral class mu enzymes [20, 21]. In rat liver cytosol a subunit with lower molecular mass (26.5 kDa) is detected with this monoclonal antibody (WHM Peters, unpublished results). This could be the Yb (GST-3 or GST-4) subunit, which is immunologically related to the human near-neutral polypeptide but has a slightly lower molecular mass [20, 22, 23]. The significance of the band with lower molecular mass (25.5 kDa) detected in addition to the 27 kDa (hepatic mu type) band in most tissues (see Figs 2–5) is not known yet. If this band represents a glutathione *S*-transferase subunit, then it may be identical with or related to the mu type GST-4 subunit isolated from muscle by Board *et al.* [24], since its physicochemical properties are very similar.

Apart from the liver, GST-3H_4 reveals the presence of glutathione *S*-transferase mu (27 kDa band) in breast, stomach (not shown), small and large intestinal mucosa, mononuclear blood cells, kidney

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Fig. 5. Detection of glutathione *S*-transferase mu in mononuclear blood cells. The cytosols from lysed mononuclear blood cells originating from approximately 1.0 mL blood were subjected to SDS–polyacrylamide gel electrophoresis (10% acrylamide; w/v). After subsequent Western blotting the nitrocellulose sheet was incubated with a monoclonal antibody against glutathione *S*-transferase mu ($GST\text{-}3H_4$). In slots 1 and 20, cytosol (25 μg) from a glutathione *S*-transferase mu positive human liver is shown. Slots 2–19 contain cytosols from mononuclear blood cells from 18 different individuals. In slots 10, 11, 12 and 14 glutathione *S*-transferase mu is absent.

Table 1. Distribution of glutathione *S*-transferase mu in individuals with and without carcinomas

Pathology	Glutathione *S*-transferase mu positive (% of total)
None* (N = 64)	62.5
Polyposis coli (N = 4)	75.0
Breast cancer (N = 52)	59.6
Colon carcinoma (N = 50)	66.0

The presence of glutathione *S*-transferase mu is determined via immunoblotting, in mononuclear blood cells for controls, and in breast or colon tissue for the carcinoma and polyposis patients.

N, the number of individuals involved.

* These are patients visiting the polyclinic for various non-cancerous diseases.

(not shown) and placenta (not shown). Earlier, this enzyme has been detected in human liver [9], adrenal gland [9, 25], leucocytes [12], kidney [25, 26], spleen [25], uterus [27], muscle [24], lung [28], heart [29], diaphragm [29], prostate [30] and probably also in skin [31].

This minor but widely distributed enzyme may have a very specific function in the detoxication of epoxides [9]. Such reactive compounds are often formed intracellularly by phase I reactions, and are suspected as aetiological factors in chemical carcinogenesis [28]. In this respect deficiencies of glutathione *S*-transferase mu in human leucocytes have been related to a higher susceptibility to lung cancer [14]. Besides in leucocytes [12, 13] absence of transferase mu activity was demonstrated before in hepatic tissue from several individuals [10–12]. Seidegard *et al.* [12] found a 100% correlation with

regard to the distribution of glutathione *S*-transferase mu in liver and leucocytes from eight patients. We made the same observations when analysing mononuclear blood cells and colon tissue (eight patients), small intestinal and large intestinal mucosa (four patients), small intestinal and liver tissue (one patient) or normal colon mucosa and the corresponding colon tumor (seven patients). This strongly suggests that expression of glutathione *S*-transferase mu is not tissue specific just like class alpha and pi transferases, but is more uniformly distributed in the various tissues, or is not present at all.

Smokers, deficient of glutathione *S*-transferase mu in their leucocytes, may have an increased risk of developing lung carcinoma [14]. We now investigated whether or not the absence of glutathione *S*-transferase mu is coupled to an increased susceptibility for developing other cancers such as those from breast and colon. Since distribution of transferase mu within an individual is uniform in colon mucosa and mononuclear blood cells, we determined the normal appearance of this enzyme by analysing blood samples from a control population. Subsequently presence or absence of transferase mu was determined in biopsies or surgical resection specimens from patients with polyposis coli, colon or breast cancer. In contrast to the findings of Seidegard *et al.* [14] for lung cancer patients, we could not find any difference in the distribution of glutathione *S*-transferase mu in patients with breast or colon cancer, as compared to controls. This strongly suggests that the absence of transferase mu is not an important factor in the aetiology of breast- and colon cancer.

It should be pointed out that Seidegard *et al.* [14] screened for presence of glutathione *S*-transferase mu activity, whereas by the immunoblot analysis both active and inactive enzyme protein is detected. However, by measuring activity towards trans-stilbene oxide in several hepatic specimens, a good correlation was found between high and low activity, and presence or absence of 27 kDa protein on immunoblot, respectively (van Ommen, Bogaards, Peters and van Bladeren, unpublished results).

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