Monoclonal antibodies against rat kidney γ -glutamyl transpeptidase show species and tissue specificity

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Monoclonal antibodies have been raised against rat kidney γ -glutamyl transpeptidase (GGT). All five antibodies immunoprecipitate enzyme activity from solubilized kidney brush-border membranes, but not from hepatocellular carcinoma membranes. Three of the antibodies react immunohistochemically with brush-border membranes in sections of adult rat kidney, but none of the antibodies cross-react with sections of guinea-pig, mouse or marmoset kidney or with untreated or carcinogen-treated rat liver. The antibodies do not recognize GGT in foetal-rat kidney and react poorly with kidney from 2-year-old rat. They do react with tubules trapped within mesenchymal kidney tumours induced by dimethylnitrosamine, but epithelial tumours, which are GGT-positive (although much less so than normal kidney), are not immunoreactive.

INTRODUCTION

γ -Glutamyl transpeptidase (EC 2.3.2.2), an enzyme which removes the γ -glutamyl group from glutathione and its conjugates (Meister, 1984; Moss *et al.*, 1984), is a membrane-bound glycoprotein widely distributed among tissues and existing in a number of isoenzymic forms (Tate & Meister, 1976). So far the only evidence that the protein part of the molecule may differ between isoenzymes is the slight difference in the M_r of the unglycosylated preprotein immunoprecipitated from translations *in vitro* of mRNA from rat kidney and cultured hepatoma cells (Barouki *et al.*, 1983; Finidori *et al.*, 1984). However, there is substantial evidence for variations in carbohydrate side chains (Matsuda *et al.*, 1980; Yokosawa *et al.*, 1981; Yamashita *et al.*, 1983*a,b,c,d,e*) showing inter-organ and inter-species differences. GGT is also of interest as a marker for cells, particularly hepatocytes, altered early in the carcinogenic process (Hanigan & Pitot, 1985). There are reports suggesting that the isoenzymes of GGT induced during carcinogenesis differ from those in normal tissues, particularly with respect to the degree of sialylation (Jaken & Mason, 1978; Ding *et al.*, 1981). The activity of this enzyme is also measured clinically in serum, but the relevance of raised levels is often difficult to interpret (Penn & Worthington, 1983).

Thus in theory it should be possible to raise monoclonal antibodies against individual isoenzymes of GGT that would be tissue-, or even tumour-, specific, enabling them to be used diagnostically or to sort particular populations of GGT-positive cells such as the foci of altered hepatocytes which occur early in carcinogenesis. We describe here five monoclonal antibodies raised against rat kidney GGT that appear to be species- and tissue-specific.

A preliminary report of this work has appeared in abstract form (Manson & Green, 1986).

MATERIALS AND METHODS

Immunization and preparation of monoclonal antibodies

Female BALB/c mice were immunized three times at weekly intervals with 50 μ g of purified rat kidney GGT (Cook & Peters, 1985) mixed with an equal volume of complete Freund's adjuvant. Animals were boosted 24 weeks later with three daily i.p. (intraperitoneal) injections of GGT (30 μ g) in saline. On the day after the last injection, 1×10^8 donor spleen cells were fused with 5×10^7 NSO myeloma cells (Galfré & Milstein, 1981) by using poly(ethylene glycol) as described by Herzenberg & Oi (1980).

E.I.I.s.a. screening of hybrids

Microtitre plates were sensitized with 50 μ l of rat kidney GGT (1 μ g/ml) in 0.1 M-bicarbonate buffer, pH 9.6, overnight at 4 °C. Plates were washed twice with Dulbecco's A phosphate-buffered saline, pH 7.3, containing 0.5% Tween 20 (PBS-Tween). Non-specific binding sites were blocked with PBS-Tween containing 0.25% gelatin for 30 min at room temperature, which was then replaced by 50 μ l of culture supernatant. The plates were incubated with shaking at 37 °C for 1 h. After washing three times with PBS-Tween, 50 μ l of biotinylated anti-mouse Ig (Amersham International, Aylesbury, Bucks., U.K.), diluted 1:1500 in PBS-Tween+0.25% gelatin, was added to each well and incubated as described above for 2 h. Plates were washed three times, followed by addition of 50 μ l of streptavidin-peroxidase complex (Amersham International) diluted 1:2000 and incubated with shaking at 37 °C for 30 min. Finally the plates were washed five times with PBS-Tween and bound peroxidase was detected by addition of 50 μ l of 3,3',5,5'-tetramethylbenzidine (Sigma Chemical Co., Poole, Dorset, U.K.) (0.1 mg/ml) in 0.1 M-sodium acetate buffer, pH 6.0, containing 0.002% (v/v) H_2O_2 and incubation at room temperature for 2–5 min. The

Abbreviations used: GGT, γ -glutamyl transpeptidase; e.i.i.s.a., enzyme-linked immunosorbent assay.

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reaction was stopped with 50 μ l of 2 M-H₂SO₄ and the A_{450} determined on a Titertek MC plate reader.

Positive immunoglobulin-secreting cultures were transferred to 24-well plates, rescreened, and persisting positive cultures were chosen for cloning twice in soft agar (Galfré & Milstein, 1981).

Class and subclass determination

Immunoglobulin class and subclass were also determined by e.l.i.s.a. as described above with the following modifications. Portions (50 μ l) of sheep anti-mouse class- and subclass-specific antisera (Serotec, Bicester, Oxon., U.K.) diluted 1:50 with 0.1 M-NaHCO₃ buffer, pH 9.6, were used to sensitize the plate. Wells were incubated with culture supernatant for 90 min; biotinylated anti-mouse Ig was diluted 1:1000 and streptavidin-peroxidase complex 1:1500.

Immunohistochemistry

Acetone-fixed paraffin-embedded sections of tissue were used. Some sections were treated with 0.1% (w/v) proteinase (protease type V; Sigma, Poole, Dorset, U.K.) in PBS for 15 min over a 37 °C water bath to unmask antigenic sites. Antigen-antibody reaction was detected as described previously (Manson *et al.*, 1984).

Preparation of membrane fractions and immunoprecipitation

Brush-border-membrane vesicles were prepared from rat kidney as described by Stewart & Kenny (1984) and resuspended in PBS. A crude plasma-membrane fraction was prepared (Armstrong & Neuman, 1985) from an aflatoxin-B₁-induced hepatoma. The GGT activity of the membrane fractions was measured as described previously (Manson, 1983). Portions of each membrane preparation containing equivalent amounts of GGT activity were centrifuged at 11500 *g* for 10 min and the pellet solubilized for 1 h in the same volume of 10 mM-Tris/HCl, pH 8.0, containing 0.15 M-NaCl, 1 mM-MgCl₂, 1 mM-CaCl₂ and 1% (v/v) Triton X-100. Unsolubilized material was removed by centrifugation as described above. Portions (50 μ l) of the extracts were diluted to 500 μ l with PBS, and 75 μ l of the appropriate culture supernatant (10 $\times$ concentrated) were added. One portion was treated with a non-specific monoclonal antibody as a control.

After 2 h at room temperature, 50 μ l of rabbit anti-mouse IgG (IgG fraction) were added and incubation continued at 4 °C overnight. Precipitates were collected at 11500 *g* and washed by brief sonication in solubilization buffer and repelleted. Pellets were suspended by sonication in sample buffer, incubated at 42 °C for 1 h, spun briefly to remove any precipitate and the supernatant electrophoresed in 10% non-denaturing polyacrylamide gels containing 0.5% Triton X-100 and 0.25% sodium deoxycholate (Bordier *et al.*, 1978). Proteins were then electrophoretically transferred to nitrocellulose (Khyse-Andersen, 1984) (80 mA, room temperature, 1 h). GGT activity on the nitrocellulose sheet was detected *in situ* by using the slightly modified histochemical method of Rutenburg *et al.* (Manson *et al.*, 1981).

Immunoblotting of purified GGT

Purified GGT (15 μ g/lane) was separated into its subunits on a SDS/10%-(w/v)-polyacrylamide gel and

Fig. 1. Immunohistochemistry of rat kidney GGT

Immunohistochemistry of normal-adult-rat kidney with monoclonal antibody MRCTU/GGT4, showing intense staining of brush-border membranes. Magnification $\times$ 250.

blotted on to nitrocellulose. The individual lanes were incubated in 3% gelatin in TBS-Tween (0.05 M-Tris/HCl, pH 8.5, containing 150 mM-NaCl and 0.5% Tween-20) for 2 h at room temperature to block non-specific binding sites. Individual strips were then incubated overnight with 5 ml of the appropriate antibody in culture supernatant containing 0.5% Tween 20, followed by 3 $\times$ 5 min washes in TBS-Tween and incubation for 2 h with 2 ml of rabbit anti-mouse IgG (IgG fraction) (Miles Laboratories, Stoke Poges, Slough, Berks., U.K.), diluted 1:50 in TBS-Tween/1% gelatin. After three further washes, strips were incubated with 1:50 dilution of mouse alkaline phosphatase/anti-(alkaline phosphatase) (APAAP) (Dako, High Wycombe, Bucks., U.K.) for 2 h. The blots were washed and incubations in anti-mouse IgG and APAAP were repeated for 15 min each. Finally, after 5 $\times$ 5 min washes, bound phosphatase activity was detected with 5-bromo-4-chloroindoxyl phosphate.

RESULTS AND DISCUSSION

Of nine cultures which were cloned twice in soft agar, five gave positive results in the e.l.i.s.a. screen throughout. One clone from each of these five cultures, designated 'MRCTU/GGT1-5', was chosen for further characterization. All monoclonals were shown to be IgG₁, except MRCTU/GGT3, which was IgG_{2a}.

Immunohistochemistry

Only MRCTU/GGT2 and 4 (Fig. 1) showed any significant reaction with kidney sections from either Sprague-Dawley or F344 rats before proteinase treatment. Brush-border membranes of proximal tubules throughout the cortex were stained. After treatment with proteinase to unmask antigenic sites, the reaction with MRCTU/GGT2 was more intense and MRCTU/GGT3 also reacted. Sections that had been treated with proteinase were stained histochemically for GGT activity to check that the enzyme was not being degraded or removed by the treatment. In addition, cryostat sections (both air-dried and acetone-fixed) were used in

Fig. 2. GGT histochemistry of foetal kidney

Histochemical staining of 20-day-foetal-rat kidney showing enzyme activity in a number of tubules (darkly stained), mainly in the inner cortex. No immunological reaction was obtained with any of the antibodies. Magnification $\times 125$.

Fig. 3. Immunohistochemistry of kidney from a 7-day-old rat using monoclonal antibody MRCTU/GGT2

Despite the fact that most of the tubules contain high levels of GGT, only a small number of selected areas react with the antibody (shown by arrows). Magnification $\times 125$.

case some antigenic sites had been damaged by paraffin embedding. However, no significant improvement in reaction with MRCTU/GGT1, 3 or 5 was achieved. Since the original antigen was a solubilized form of GGT, it is possible that the epitopes recognized by some of the antibodies are not readily available in tissue sections.

No immunohistochemical reaction was seen with any of the antibodies on any rat liver sections, with or without proteinase treatment. Sections tested included control liver in which GGT activity is confined to bile-duct epithelial cells, an aflatoxin B₁-induced (1 p.p.m. in diet for 14 weeks) hepatocellular carcinoma with high levels of GGT activity and liver from an ethoxyquin-treated rat (0.5% in diet for 10 months)

Fig. 4. Immunohistochemistry of kidney from a 2-year-old-rat using monoclonal antibody MRCTU/GGT2

Only a weak reaction (arrows) was seen, despite high levels of GGT activity persisting in most tubules. Magnification $\times 250$.

with elevated GGT activity in periportal areas, and 20-day-foetal-rat and neonatal-rat liver, where GGT is present on hepatocyte membranes in addition to bile ducts.

In 20-day-foetal-rat kidney the most mature tubules in the inner cortex contain quite high levels of GGT (Fig. 2), but this could not be detected by any of the antibodies. By 7 days after birth GGT activity is approaching that of adult kidney, with most of the tubules strongly positive, but only a small amount of enzyme in selected tubules was immunoreactive (Fig. 3). Similarly the reaction in 2-year-old-rat kidney was much decreased compared with that in the young adult (Fig. 4).

Immunohistochemistry was also carried out on kidney sections from rats that had received a single injection (40 mg/kg) of dimethylnitrosamine as weanlings. At 1 year after treatment, two types of tumour had developed. Mesenchymal tumours of the interstitial cells were GGT-negative, but contained many GGT-positive tubules trapped within them. These tubules were also immunoreactive. Epithelial tumours of the tubule cells were GGT-positive, but much less so than normal kidney tissue. These tumours were not immunoreactive.

No cross-reaction was obtained with any of the monoclonals on tissue sections from guinea-pig, mouse or marmoset kidney, even after proteinase treatment.

Immunoprecipitation of GGT activity from solubilized membranes

Membranes were prepared from two GGT-rich rat tissues, namely brush-border vesicles from kidney and an aflatoxin-B₁-induced hepatocellular carcinoma. The enzyme activities of these two preparations were 8.9 μ mol of product formed/min per mg of protein and 1.6 μ mol of product formed/min per mg of protein respectively. All five monoclonal antibodies immunoprecipitated GGT from solubilized kidney brush-border membranes (Fig. 5), although MRCTU/GGT3 consistently precipitated less enzyme than did the other four. None of the antibodies precipitated any detectable enzyme activity from solubilized hepatocellular carcin-

Fig. 5. GGT immunoprecipitated from brush-border membranes

Five monoclonal antibodies were used to immunoprecipitate GGT from solubilized membranes from kidney brush border or aflatoxin-B₁-induced hepatocellular carcinoma. The immunoprecipitated proteins were run on a non-denaturing polyacrylamide gel and blotted on to nitrocellulose, which was then stained histochemically for GGT. Lane 1, solubilized protein from kidney brush-border membranes; lane 2, solubilized protein from aflatoxin-B₁-induced hepatocellular carcinoma; lanes 3, 5, 7, 9 and 11, immunoprecipitate from brush-border membranes with monoclonal antibodies MRCTU/GGT1-5 respectively; lanes 4, 6, 8, 10 and 12, no GGT (1-5 respectively) was immunoprecipitated when hepatoma plasma membranes were used. Monoclonal antibody MRCTU/GGT3 (lane 7) consistently precipitated less activity than did the other four antibodies.

oma membranes containing equivalent amounts of enzyme activity.

Subunit specificity of antibodies

The two subunits of GGT run as broad bands on SDS/polyacrylamide gels (Fig. 6), because of the mixture of isoenzymic forms. By far the strongest reaction for all the antibodies on the Western blot was with a narrow band of the high- M_r (55000) subunit, perhaps indicating that the antibodies recognize preferentially the heavy subunit of one isoenzymic form that may have been dominant in the original preparation used to immunize the mice. The fainter-staining band in the position of the light subunit only appeared after prolonged incubation in the substrate solution. In another immunoblot using a different batch of purified GGT and a slightly different detection technique, staining of a broader band in the heavy subunit was seen.

The monoclonal antibodies described here appear to be specific for isoenzymes found predominantly in young-adult-rat kidney and do not cross-react with GGT in other tissues or with the kidney enzyme in other species. They do not even cross-react well with enzyme present very early or late in development. This may be due to the heterogeneity of carbohydrate side chains between different isoenzymes. These results suggest that it should therefore be possible to raise monoclonals to liver-specific GGT isoenzymes, in particular those found in preneoplastic lesions or tumours, which would be

Fig. 6. Immunoblot of purified GGT

Purified kidney GGT was run on a SDS/10%-polyacrylamide gel and part of the gel was stained with PAGE Blue 83 (lane 2). The remainder of the gel was blotted on to nitrocellulose and individual strips were incubated with one of the antibodies (lanes 3-7, monoclonal antibodies MRCTU/GGT 1-5). One strip was incubated with culture medium (lane 8). The major reaction for all antibodies was with part of the heavy-subunit band.

diagnostically valuable. While this paper was in preparation a report appeared (Taniguchi *et al.*, 1985) of a monoclonal antibody raised against human hepatoma GGT which, although not cross-reacting effectively with normal liver GGT, did cross-react with human kidney GGT.

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