PREPARATION OF MONOCLONAL ANTIBODY TO RABBIT
KIDNEY GAMMA-GLUTAMYL TRANSPEPTIDASE AND
ITS CROSS-REACTIVITY ANALYSIS
IN IMMUNOHISTOCHEMISTRY

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A monoclonal antibody against rabbit kidney, γ -GTP, was prepared. The subclass of the antibody was IgG_{2a} and the light chain was λ . The antibody specifically recognized the H chain, the site involved in anchoring part of this enzyme. The immunohistochemical assay detected reaction in the brush border of the proximal convoluted tubule of the rabbit kidney, particularly on the distal part. Cross-reaction between homologous and heterologous organs was immunohistochemically examined using this antibody. In the homologous organs, reaction was absorbed in the intercalated duct of the rabbit pancreas and along the stereocilia of the ductus epididymidis. No cross-reaction was detected in the kidney, pancreas or epididymis of a rat, or in the human kidney.

γ -GTP is a glycoprotein localized in abundance primarily in the brush border of the kidney of mammals. It is present as a hetero-dimer consisting of heavy and light chains, with the enzyme active site in the latter and the membrane binding site in the N-terminal of the heavy chain. Biochemically, the enzyme catalyzes the synthesis and hydrolysis of glutathione (3, 14, 15, 17, 31, 40).

Though the properties of the enzyme have been well elucidated, physiological function of γ -GTP in normal metabolism and the mechanism of its appearance as an onco-fetal protein (5, 10, 18), in liver cancer cells remain unknown. Only a few hypotheses have been proposed as to the mechanism of its physiological function at the cell level; possible involvement in amino acid resorption through the γ -glutamyl cycle (32, 34), in detoxication metabolism (33, 40) and a possible relation to the metabolism of DNA (2).

Most previous studies on the localization of γ -GTP used enzyme histochemical methods (1, 12, 24, 36, 37). Though these histochemical methods are well established, some papers have reported the possible demonstration of a γ -GTP pseudo-enzyme in addition to γ -GTP with these techniques (7, 20). Except for one study in which a monoclonal antibody was used (42), immunohistochemical studies on the distribution of γ -GTP in kidney have been performed with polyclonal antibodies against the enzyme, mostly using rats as the experimental animal (26, 35, 38, 39). Although these enzyme histochemical and immunohistochemical studies revealed

similar distribution patterns of γ -GTP in the rat kidney, detailed studies revealed slightly different localizations of the enzyme, therefore, methods with higher specificity are being awaited. In the present study, a monoclonal antibody against rabbit kidney γ -GTP was prepared and its specificity was examined by immunochemical and immunohistochemical techniques.

MATERIALS AND METHODS

Preparation of the antigen

Rabbit kidney γ -GTP was prepared by the method described by Kozac and Tate (21). The procedure used in the preparation is almost the same as that described in the previous report (43). It is summarized as follows: 1) a microvilli fraction was prepared from the renal cortex, and solubilized by treatment with papain (27); 2) after centrifugation, the supernatant was brought to 65% saturation and thereafter to 95% saturation with solid ammonium sulfate; 3) the crude enzyme preparation was further purified by column chromatography with Sephadex G-150.

A γ -GTP assay kit (Wako Pure Chemical Industries, Ltd., Japan) was used for the assay of γ -GTP activity.

Analysis and purification of the antigen

The enzyme sample was analyzed by polyacrylamide gel electrophoresis (PAGE) (8) and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (23). Protein staining was carried out by Fairbank's technique (9), and the enzyme histochemical staining of γ -GTP was performed by Rutenburg's method (36). Cytochrome C-450 was used as a reference standard. After PAGE, the band of gel with specific activity was removed, and γ -GTP was directly extracted from the gel by a protein recovery device (ATTO AE 3590 ATTO Co., Japan). The extract was concentrated and used as an antigen during the course of the study.

Immunization procedure

In the initial immunization, 100 μ g of the purified antigen was mixed with Freund's complete adjuvant and injected subcutaneously into Balb/c mice. Subsequently, 10 μ g of the antigen was emulsified with Freund's incomplete adjuvant and injected at intervals of two weeks as boosters. After the antibody titer had risen sufficiently two months later, 10 μ g of the antigen was injected intravenously. The spleens of the sensitized mice were removed three days after the final injection.

Cell fusion

SP 2/0-Ag 14 mouse myeloma cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μ g/ml). Mouse spleen cells (2×10^8) and myeloma cells (3×10^7) were fused by using polyethylene glycol 1000 (13) according to the technique of Galfré and Milstein (11). The hybridoma cells were then selected in hypoxanthine-aminopterin-thymidine (HAT) medium.

Screening

The first screening was performed by ELISA and the second by immunohistochemical reaction on paraffin sections.

Cloning and antibody production

Hybridomas rated positive in screening were initially cloned in soft agarose (6). Re-cloning was carried out in soft agarose as well as with the limiting dilution

method.

Analysis of antibody typing

The class and subclass of the monoclonal antibodies were determined by ELISA with a MONO AB ID KIT (Zymed Lab. Inc. USA).

Electrophoresis: PAGE and SDS-PAGE with Western blotting

The crude antigen, the kidney microvillus fraction before digestion with papain, was subjected to PAGE and SDS-PAGE.

For PAGE, plate gel containing 7.5% acrylamide was used, according to the technique of Davis (8). For SDS-PAGE, plate gel containing 10% acrylamide was used, according to Laemmli's technique (23). After PAGE and SDS-PAGE, the protein in the gel was transferred to a nitrocellulose membrane by the procedure described by Bowen (4) and Lubahn (25). The blots were stained by Rutenburg's technique (36) to demonstrate the bands with enzyme activities, treated with monoclonal antibodies and then treated with the ABC technique (16) to detect the bands bearing antigen.

Inhibition of γ -GTP activity by monoclonal antibody

Inhibition of γ -GTP activity by the monoclonal antibody was performed in the following manner.

To the aliquots of purified antigen, the increasing amount of hybridoma supernatant was added in such combinations as to make each concentration of the primary antibody 0%, 20%, 40%, 60%, 80% and 100%. PBS was added to make the whole volume equal. The mixtures are incubated for 3 hr at 37°C. To each of the series of antigen-monoclonal antibody mixtures, 100 μ l of the second antibody was added. Then, the mixtures were allowed to stand overnight at 4°C. After centrifugation at 2,000 rpm for 20 min, γ -GTP activity in each supernatant was measured. The inhibition test was repeated three times, and the average value was plotted on a graph. As a control experiment, the culture medium was used in place of the hybridoma supernatant.

Enzyme histochemistry and immunohistochemistry

a) Tissue preparation: Adult rabbits and Wistar rats were perfused with PBS for 10 min and then with periodate-lysine-paraformaldehyde (PLP) solution (30) for 1 hr. Kidney, pancreas and epididymis were taken from the rabbits and rats. Small blocks of the perfusion-fixed tissues of the rabbits and rats and those of autopsy specimens of human kidney were fixed in PLP solution overnight and then in 4% paraformaldehyde for 4 hr. The specimens were then routinely embedded in paraffin, and sections were prepared.

b) Enzyme histochemistry: The paraffin sections were subjected to Rutenburg's technique (36).

c) Immunohistochemistry: The paraffin sections were allowed to react with the hybridoma culture supernatant and stained with the ABC technique. The sections were visualized with DAB-H₂O₂ solution.

Control experiment for immunohistochemical reaction

The following control staining was performed for the immunohistochemical reaction both on the nitrocellulose membrane and paraffin sections.

The following six conditions were used for the control experiments:

- a) myeloma cell culture supernatant was used instead of the primary antibody,
- b) 1% BSA was used in place of the primary antibody,

- c) mouse anti-human CRP monoclonal antibody (Cosmo Bio Co., Ltd., Japan) was used instead of the primary antibody,
- d) mouse anti-rat γ -GTP monoclonal antibody was used instead of the primary antibody,
- e) biotin-labelled secondary antibody was omitted in the ABC procedure,
- f) ABC complex was omitted.

RESULTS

Antibody production

Out of the 96 wells, 18 had two positive responses twice on ELISA. Using the supernatants of the 18 positive wells, the paraffin sections of rabbit kidney were stained immunohistochemically to check whether the positive sites coincided with those demonstrated by the enzyme histochemical technique. The hybridoma from the two wells which responded positive on both ELISA and immunohistochemical assay were cloned twice in the soft agarose and by limiting dilution methods, and one hybridoma was ultimately obtained.

Characterization and specificity of the monoclonal antibody

- 1) Ig-subclass determination

FIG. 1. Inhibition of γ -GTP activity by monoclonal antibody.

The hybridoma supernatant (open circles) or the culture medium (filled circles) was added to aliquots of γ -GTP. After incubation for 3 hr at 37°C, 100 μ l of the secondary antibody (rabbit anti-mouse IgG antiserum) was added and allowed to stand overnight at 4°C. After centrifugation, the enzyme activity of the supernatant was determined. Filled circles: Enzyme activity of the supernatant of the mixture of the enzyme, the primary antibody and secondary antibody. Open circles: Enzyme activity of the supernatant of the mixture of the enzyme and the secondary antibody. When the γ -GTP activity in the supernatant, which contained the secondary antibody but no hybridoma supernatant was set at 100%, maximum inhibition reached 50%.

FIGS. 2A–D. Electrophoretic analysis of γ -GTP

A. After PAGE, the antigen was transferred to a nitrocellulose membrane and stained by the enzyme histochemical technique. Activity is observed in the region indicated by the arrowhead.

B. After transfer to the nitrocellulose membrane, as in (A), the membrane was stained by the immunohistochemical technique using anti-rabbit γ -GTP monoclonal antibody. The positive band (arrowhead) corresponds to that of the band showing enzyme activity in (A). The range of migration of the band is narrower than that of the band in (A).

C. The antigen blotted onto the nitrocellulose membrane was stained by the immunohistochemical technique using an irrelevant monoclonal antibody, anti-rat γ -GTP monoclonal antibody. No cross-reaction with rabbit crude antigen is observed.

D. The blot was stained by the immunohistochemical technique using the myeloma culture supernatant containing no antibody, in place of the primary antibody. No reaction is observed.

E. SDS-PAGE was performed on the purified antigen. After SDS-PAGE, the gel was stained with Coomassie brilliant blue. The purified antigen consists of a heavy subunit (54.6 kd) and light subunit (24.6 kd).

F. SDS-PAGE was performed on the crude rabbit antigen protein. After SDS-PAGE, the gel was stained with Coomassie brilliant blue. The crude protein definitely contains γ -GTP protein.

G. After SDS-PAGE, the crude rabbit antigen protein was transferred to the nitrocellulose membrane. Immunohistochemical staining with anti-rabbit γ -GTP monoclonal antibody was performed on the nitrocellulose membrane. The antibody responded specifically to the heavy subunit of γ -GTP.

H. Immunohistochemical staining with anti-rat γ -GTP monoclonal antibody was performed on a similar nitrocellulose membrane. No cross-reaction with rabbit γ -GTP is observed.

I. Immunohistochemical assay with the myeloma culture supernatant as the primary antibody was carried out on a similar nitrocellulose membrane. No reaction is observed.

ELISA examination revealed that the monoclonal antibody prepared in this study belonged to the IgG_{2a} class and to λ light chain.

2) Inhibition of enzyme activity by the antibody

When the hybridoma culture supernatant alone was added to the purified γ -GTP, enzyme activity was not affected. When the secondary antibody was

added to the mixture of hybridoma supernatant and purified antigen, enzyme activity in the supernatant decreased to 46.7% (Fig. 1). Thus, the prepared antibody was found to bind specifically to the antigen.

3) Analysis by PAGE and SDS-PAGE

After PAGE, the crude antigenic protein was transferred from the acrylamide gel to a nitrocellulose membrane which was then stained by Rutenburg's enzyme histochemical procedure. Another membrane was stained by the immunohistochemical procedure with the monoclonal antibody. As a result, the broad band positive for enzyme histochemical reaction (Fig. 2A) corresponded to that stained by the immunohistochemical technique (Fig. 2B).

Analysis by SDS-PAGE showed that the purified γ -GTP was composed of a heavy subunit of a 54.6-kilodalton polypeptide and a light subunit of 24.6 kilodaltons (Fig. 2E). When the crude antigen protein was applied to SDS-PAGE, transferred to the nitrocellulose membrane and stained by the immunohistochemical technique with the monoclonal antibody, the antibody reacted exclusively to the heavy subunit (Fig. 2G).

Immunohistochemistry and enzyme histochemistry

1) Localization in rabbit kidney

Enzyme histochemistry: The proximal convoluted tubule showed a marked reaction, which gradually increased toward the distal portion (S₃) (19) of the tubule. In some specimens, a weak reaction was noticed along the luminal mem-

FIG. 3. Enzyme histochemical staining of γ -GTP in the rabbit kidney. $\times 625$

Paraffin sections, 4–5 μ m in thickness, were stained according to the method of Rutenburg (36). Nuclei are counterstained with hematoxylin. γ -GTP activity is observed in the proximal convoluted tubule of the rabbit kidney (arrow). The reaction is negative in the glomerulus (arrowhead) and in the distal convoluted tubule.

brane of the thin portion of Henle's loop. Glomerulus, Bowman's capsule, distal convoluted tubule and collecting duct were negative for the reaction (Fig. 3).

Immunohistochemistry: The reaction in the distal segments of the proximal convoluted tubule was stronger than that in the proximal segments. Reaction product was seen along the luminal membrane. In the thin portion of Henle's loop, a weak reaction product was sometimes found along the luminal membrane.

Enzyme histochemical and immunohistochemical findings were nearly consistent with each other (Figs. 3, 4).

2) Reaction in organs other than the kidney of the rabbit

Pancreas: Enzyme histochemical staining showed a weak reaction along the intercalated duct of the acinus. The luminal membrane of the intra-lobular excretory ducts showed a very weak reaction (Fig. 6). The immunohistochemical technique revealed a reaction product along the intercalated duct of the acinus as well as the luminal surface of the intra-lobular excretory duct (Fig. 7). Cells in the Langerhans' islets were negative in both enzyme histochemical and immunohistochemical stainings.

Epididymal duct: With both enzyme histochemical (Fig. 8) and immunohistochemical (Fig. 9) stainings, positive reactions were observed along the luminal border, mostly stereocilia, of the epithelium of the duct in the head portion of the epididymis (Fig. 9).

3) Reaction in organs of species other than the rabbit

FIG. 4. Immunohistochemical reaction in the rabbit kidney with monoclonal anti-rabbit kidney γ -GTP antibody. $\times 625$

Reaction is seen in the proximal convoluted tubule. Reaction in the distal segment of the proximal tubule is particularly strong. Reaction product is observed along the luminal border, but not in the cytoplasm.

FIG. 5. Control specimen for immunohistochemical staining. $\times 625$

Paraffin sections of rabbit kidney were stained by ABC technique, using an irrelevant antibody, monoclonal anti-rat kidney γ -GTP antibody, as the primary antibody. No reaction is recognized.

FIG. 6. Findings in the rabbit pancreas stained by enzyme histochemical method for γ -GTP. $\times 125$
Reaction is observed along the intralobular duct and cytoplasm of some excretory cells. The reaction in the intercalated duct is not clearly defined.

FIG. 7. Findings in rabbit pancreas by immunohistochemical staining with monoclonal anti-rabbit kidney γ -GTP antibody. $\times 125$
Reaction product is observed along the intercalated duct (arrowhead) and intralobular duct (arrow). Reaction is slight in the luminal surface of the intralobular excretory duct.

FIG. 8. Rabbit epididymal duct stained by enzyme histochemical method for γ -GTP. $\times 1,250$
The luminal surface, mostly composed of stereocilia, is positive in reaction.

FIG. 9. Rabbit epididymal duct stained with immunohistochemical technique, using monoclonal anti-rabbit kidney γ -GTP antibody as the first antibody. $\times 1,250$
Reaction products are observed along the stereocilia of the epididymal duct.

FIG. 10. Rat kidney stained with immunohistochemical method, using monoclonal anti-rabbit kidney γ -GTP antibody as the first antibody. $\times 1,250$
No cross-reaction is observed.

Rat kidney, pancreas, epididymal duct and human kidney: The positive reaction sites with enzyme histochemical staining coincided with those produced with immunohistochemical staining in rabbits. No cross-reaction was observed in the rat kidney in immunohistochemical staining with the monoclonal antibody against the rabbit kidney γ -GTP (Fig. 10).

Controls for immunohistochemical staining

All control specimens were negative for reaction under the present conditions (Figs. 2-C, D, H, I, Fig. 5).

DISCUSSION

1. Properties of the Prepared Antibody

A hybridoma secreting monoclonal antibody against rabbit kidney γ -GTP was obtained. The monoclonal antibody was specific for rabbit kidney γ -GTP on the basis of the results of four assays: (1) ELISA, (2) enzyme inhibition assay with the addition of the secondary antibody, (3) immuno-blotting and (4) immunohistochemical assay on paraffin sections. The monoclonal antibody was found to recognize 54.5 Kd polypeptide, the heavy subunit of the γ -GTP molecule. In addition, the absence of direct inhibition of enzyme activity by the monoclonal antibody in the inhibition test suggests that the antibody does not bind to the active site of the enzyme. Immuno-blotting following SDS-PAGE revealed that the antibody was specific for the heavy subunit of γ -GTP. It has been reported that the

site of activity of this enzyme is in the light subunit (15). Therefore, if the prepared antibody was specific for the light subunit, it would have directly bound the active site of the enzyme and inhibited enzyme activity. Since the monoclonal antibody prepared in this study was specific for the heavy subunit, 54.5 Kd, the antibody did not inhibit enzyme activity by simply being mixed with the enzyme.

The heavy subunit of γ -GTP has a hydrophobic amino acid in the N terminal through which the enzyme is bound to the membrane (28, 40). By treating the crude microsomal fraction of kidney with a protease such as papain, this enzyme is solubilized and becomes hydrophilic in nature leaving the membrane-binding site of the enzyme unsolubilized (17). Papain is reported to cut off the heavy subunit of γ -GTP in the vicinity of the N-terminal (29, 41). Since papain digestion is used during the preparation of γ -GTP in the present study, γ -GTP used as an antigen is thought not to contain a membrane-binding site in its heavy subunit. Therefore, the monoclonal antibody is specific for the heavy subunit but does not recognize the membrane-binding site.

2. Cross-Reactivity Analysis

The cross-reactivity of the monoclonal antibody against the rabbit kidney was examined by the immunohistochemical method on the heterologous organs, (pancreas and epididymis), of a rabbit: and on a homologous organ, (kidney), of a human and rat. As shown in Table 1, this antibody reacted specifically with rabbit organs but not with the organs of rat and human. Compared with the results of the previous study (43) on the characterization of monoclonal antibody against the rat kidney, in which the antibody reacted with several rat organs as well as a human kidney, this antibody reacted with several rabbit organs but not with the human kidney. Thus, the antibody obtained in this study, which recognizes the 54.5-kilodalton polypeptide, is considered to be more species-specific than that against rat kidney γ -GTP which recognizes the 51-kilodalton polypeptide. Nevertheless, neither antibody is organ-specific.

In the previous study on the monoclonal antibody to rat kidney γ -GTP, the antibody was reported to recognize the 51-kilodalton protein of the heavy subunit of the antigen (43), while the monoclonal antibody to rabbit kidney γ -GTP, prepared in this study, reacted with the 54.5-kilodalton protein. Even though both antibodies recognized the protein of the heavy subunit of γ -GTP of each animal, each antibody reacted with a different determinant, although the difference in the estimated values of the molecular weight of the polypeptides of two antigens recognized by each monoclonal antibody, 51 and 54.5 kilodaltons respectively, was small. This difference may be attributable to the species difference between the two antigens. This fact also corresponds to the report that the sugar chain of this enzyme shows no racial differences (28, 41, 42).

There has been no report which dealt with the localization of γ -GTP in rabbit organs, however, the distribution of the reactive sites in the kidney was almost the same as that reported by Rutenburg *et al.* (36) on rats, Kugler (22) on rats and mice, (using the histochemical technique), Taniguchi *et al.* (39) on rats and Yasuda *et al.* (43) on rats, (using the immunohistochemical technique with monoclonal antibodies). The reaction was mostly concentrated in the brush border of the proximal convoluted tubule. The fact that the reaction became stronger toward the distal portion of the proximal convoluted tubule and was not seen in Henle's loop or in the

distal convoluted tubule suggests that the transfer of the amino acids and peptides takes place mostly in the distal portion of the proximal convolution.

The reaction was clearly concentrated in the brush border in this study, though the final reaction products were seen even in the cytoplasm of the cells of the proximal convoluted tubule in the histochemical sections. A positive reaction in the cytoplasm was also reported by Rutenburg *et al.* (36), but this must be caused by diffusion of the final reaction product.

The existence of the reaction in the intercalated and intralobular ducts of the rat pancreas was reported by Yasuda *et al.* (43). On the other hand, the results obtained by enzyme histochemical technique showed positive reaction in the acinal cells of rats (1) and guinea pigs (36). The immunohistochemical study on the rabbit pancreas with monoclonal antibody revealed that the reaction was mostly restricted to the intercalated duct and the intra-lobular excretory duct. Thus, the result of this study coincided with that on rats reported by Yasuda *et al.* (43). Even though the reaction in the centroacinal cell was not demonstrated in this study, it was found that the antigen was concentrated in the duct system, rather than in the acinal cells.

The distribution pattern of γ -GTP demonstrated by the immunohistochemical technique was more precise than that shown by the histochemical technique. The results show that γ -GTP is mostly localized in the luminal membrane of the epithelial cells which come in contact with the materials containing amino acids, polypeptides and proteins.

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