Clearance of Different Multiple Forms of Human \( \gamma \)-Glutamyltransferase

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Several multiple forms of \( \gamma \)-glutamyltransferase (EC 2.3.2.2) have been described in serum. Most of these are large complexes between the enzyme and circulating lipoproteins. One dominating form is complexed with high-density lipoprotein; a small, hydrophilic form is present in minor amounts. We purified the two forms by immunoaffinity chromatography, injected the purified forms into rabbits, and studied the clearance of the two forms by measuring the change in enzyme activity and in enzyme protein concentration with an enzyme-linked immunoabsorbent assay. The half-life of the hydrophilic enzyme was 9 h; that of the lipoprotein–enzyme complex was 20 h. This indicates that the lipoprotein–enzyme complex accumulates in serum relative to the hydrophilic enzyme, suggesting in part an explanation of the dominance of the larger form in disease.

Measurement of \( \gamma \)-glutamyltransferase (GT; EC 2.3.2.2) in serum is frequently used as a marker of liver diseases. The enzyme originates from hepatobiliary membranes, and appears in serum in several forms.\(^1\) The major part is bound to circulating lipoproteins, particularly high-density lipoprotein (HDL) but also to larger, low-density lipoprotein complexes (1–4). A smaller, hydrophilic form is also present (1, 3), apparently identical to the form obtained by purifying the enzyme from liver membranes by protease digestion (5–8).

Several suggestions have been made for the clinical utility of these multiple forms (for a recent review, see ref. 9). The HDL–GT complex dominates in nonicteric diseases, whereas the larger molecular forms reportedly increase in cholestasis (1, 4, 10–12). However, the smaller form, presumably released from its membrane-bound state in liver by proteases, seldom amounts to >15–20% of the total activity in patients with various liver diseases (1, 6, 10).

To evaluate potential clinical applications of various multiple forms of an enzyme, one must assess their elimination from the circulation. If certain forms, such as the hydrophilic GT, are cleared rapidly from the circulation, this could lead to limitations of their effective use as a marker of disease.

We purified the small, hydrophilic form of GT from human liver and isolated the HDL–GT complex from pooled human serum. The clearance of these two forms of GT was monitored after intravenous injection into rabbits. To estimate their turnover rate, we measured both activity and GT protein concentrations, using an enzyme-linked immunoabsorbent assay (ELISA) technique.

We chose to study the rabbit because this animal has a metabolism of HDL that is more like that of humans than is that of, e.g., the rat. Furthermore, studies of experimental liver diseases performed in rabbits have shown similar alterations in GT as in human liver diseases.

Materials and Methods

Animals. Male Chinchilla rabbits (3–6 kg) were used for antisemur production, and female Himalayan rabbits (1.0–1.3 kg) were used for the clearance studies. The animals were purchased from the National Institute of Public Health, Oslo, Norway.

Enzyme purification. Human liver was obtained at autotpey within 36 h after death. GT was purified in minor amounts as previously described (5) and used for immunization. Rabbits were injected subcutaneously in multiple sites with 60–75 \( \mu g \) of enzyme in Freund's complete adjuvant, followed four weeks later with a booster injection of 40 \( \mu g \) of enzyme in Freund's incomplete adjuvant. Blood was then collected after seven or eight days. The IgG fraction was precipitated with ammonium sulfate and then bound to CNBr-activated Sepharose (Pharmacia, Uppeala, Sweden), as described by the manufacturer.

We used this immunoaffinity column as a main step in the following modified purification scheme: Liver was homogenized and solubilized with 20 g/L deoxycholate reagent (5); polyethylene glycol (PEG 6000; Merck, Darmstadt, F.R.G.) was added to give a final concentration of 20% g/L, and the solution was left on ice for 45 min. After centrifugation for 10 min at 25,000 \( \times g \), we precipitated protein in the supernate with acetone (5), then chromato-graphed the precipitate on a column of diethylaminoethyl Sepharose Fast-Flow (Pharmacia). The column was equilibrated with 0.1 mol/L Tris · HCl buffer (pH 8.0) and enzyme was eluted with NaCl, 0.3 mol/L, in this buffer. The fractions containing the major enzyme activity were pooled and digested with papain (5).

We then dialyzed this solution against 25 mmol/L sodium phosphate buffer (pH 7.5), and applied it to the IgG-coupled Sepharose, eluting with a flow rate of 0.25 mL/min. The capacity of the column was 100 U/mL of gel. We washed the column with 3 mL of dialysis buffer per milliliter of gel and eluted the enzyme with 65 mmol/L \( \text{NH}_4\text{OH} \) solution. The eluates (1.0 mL) were mixed with 0.1 mL of 1.0 mol/L Tris buffer (pH 7). The GT-containing fractions were pooled and applied to a Mono Q ion-exchange column (FPLC; Pharmacia) that was previously equilibrated with 0.1 mol/L Tris · HCl buffer (pH 7.5). We eluted the enzyme by using a linear gradient of NaCl from 0 to 0.5 mol/L in the pH 7.5 Tris buffer.

The purified enzyme had a specific activity of 1300–1500 kU/g protein. To test the homogeneity of the eluate, we used polyacrylamide electrophoresis (PhastSystem; Pharmacia) with silver staining. The enzyme was concentrated by lyophilization after dialysis against distilled water, and then dissolved in isotonic saline.

We used the immunoaffinity column to purify the HDL–GT complex from pooled sera from nonicteric patients (GT activity 200–1200 U/L). The serum was applied directly onto the column and the enzyme eluted as described above. The enzyme-containing fractions were pooled, dialyzed against distilled water, lyophilized, and dissolved in saline. To test for the presence of intact HDL–GT complexes, we used gel filtration and incubated aliquots with antisemur against apolipoprotein A (Behring, Marburg, F.R.G.) to
precipitate the HDL–GT, as described earlier (1).

Processing of antibodies. Enzyme purified as described above was used to produce antibody for the ELISA technique, by the same immunization procedure as above. Rabbit serum was collected after two to four re-immunizations, and the IgG fraction was purified by using a Protein A–agarose column (Affi-Gel Protein A MAPS II kit; Bio-Rad, Richmond, CA). A sample of this IgG fraction was biotinylated with biotinamidoacaproate N-hydroxysuccinimide ester (Sigma Chemical Co., St. Louis, MO), as described (13).

Enzyme measurements. GT activity was measured by the Scandinavian-recommended method (14) at 37°C, with use of an LKB Ultraspec photometer (Pharmacia).

We developed an ELISA double-sandwich assay for measuring GT protein. Microtiter plates (MicroWell Module; Nunc, Roskilde, Denmark) were coated with the Protein A-purified IgG fraction: we applied to each well 100 \( \mu \)L of 50 mmol/L \( \text{Na}_2\text{CO}_3 \) solution (pH 9.6) containing 7 mg of IgG per liter. After overnight incubation at 4°C, the wells were washed three times with washing buffer (per liter: 10 mmol of sodium phosphate (pH 7.2), 345 mmol of NaCl, and 1 mL of Tween 20).

For the assay, we added to each well 100–1000 \( \mu \)L samples, diluted in washing buffer containing 5 g of bovine serum albumin per liter, and incubated this for 1 h, then washed the wells. We then added to each well 100 \( \mu \)L of biotinylated antibodies diluted in washing buffer to 1.5 mg/L. After 1 h, we washed the wells and added 100 \( \mu \)L of avidin-conjugated horseradish peroxidase (EC 1.11.1.7; Dakopatts, Glostrup, Denmark), diluted 4000-fold in dilution buffer. The plates were incubated for 30 min, washed, then developed with, per well, 100 \( \mu \)L of 3.7 mmol/L o-phenylenediamine solution (Dakopatts) and 3.7 mmol/L \( \text{H}_2\text{O}_2 \) solution in water. After adding 150 \( \mu \)L of 1.0 mol/L \( \text{H}_2\text{SO}_4 \) reagent to stop the reaction, we read the absorbance at 492 nm in a Titer Multiscan PLUS (Flow Labs., Oslo, Norway), diluted 4000-fold in dilution buffer. The assay was standardized with purified human liver GT (1500 kU/g). GT concentrations in the range of 2–3 \( \mu \)g/L to 200 \( \mu \)g/L could be determined with a coefficient of variation (CV) of <12%.

Clearance measurements. The purified enzyme samples (8–15 kU of enzyme activity, corresponding to 6–12 \( \mu \)g of enzyme protein) were injected into an ear vein after sedating the rabbits with 0.3 mL of “Hypnorm Vet” (flurisron plus fentanyl, Janssen Pharmaceutica, Oslo, Norway), followed by 0.15–0.2 mL every other hour. Blood samples were drawn from the other ear, before enzyme injection and 30 min and 1, 2, 4, 6, 8, and 10 h thereafter. The blood was allowed to clot before serum separation. GT enzyme activity was measured within 1–3 h of collection, whereas the serum samples were frozen before the measurements of GT protein. The clearance of GT was calculated by linear regression of the semilogarithmic plots of both GT activity and GT protein measurements. Mean values and standard deviations were then calculated for the two groups of animals.

Results

Purification of enzyme forms. The combined use of the immunoaffinity column and the Mono Q column yielded 100-fold purified enzyme, with a recovery of 50–60%. The specific activity of the final preparation was 1300–1500 kU/g of protein, and the enzyme showed only one band after polyacrylamide gel electrophoresis. Total recovery of enzyme in this modified purification scheme was close to 20%.

Gel filtration of the pooled serum used for the purification of HDL–GT showed three peaks of activity (Figure 1).

The major peak (peak II, Figure 1), was substantially decreased after incubation of serum with antibodies against apolipoprotein A before gel filtration. The other peaks were not affected. The materials in peaks II and III bound to the immunoaffinity column but that in peak I did not. The enzyme material obtained after elution from the column consisted of 80–85% of intact HDL–GT complexes, as shown by gel filtration and immunoprecipitation.

Clearance. Figure 2 shows the clearance of GT activity from rabbit circulation after intravenous injection of purified hydrophilic GT and HDL–GT complexes. The hydrophilic enzyme is cleared faster than the lipoprotein-bound enzyme, the half-lives being 9 and 20 h, respectively. Whether we calculated the half-lives from the enzyme activity data or the enzyme protein data, we obtained the same values (Table 1).

The HDL–GT complexes appeared to be intact in the rabbit circulation during the observation period. No transfer of GT to other forms was detected after gel filtration of serum sampled either 30 min or 8 h after injection.
Table 1. Estimated Half-Lives of GT and HDL-GT In Rabbit Circulation

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<th>GT activity</th>
<th>GT protein</th>
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<tr>
<td>Hydrophilic GT</td>
<td>9 ± 1</td>
<td>9 ± 1</td>
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<tr>
<td>HDL-GT complexes</td>
<td>19 ± 2</td>
<td>20 ± 2</td>
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The half-lives of purified hydrophilic GT and HDL-GT complexes were calculated after measuring GT activity (U/L) or GT protein (μg/L) in serum as shown in Fig. 2. The values represent the means (±SD) of data from four animals.

Discussion

The present data show that the clearance rate of the HDL-GT complexes is much slower than that of the hydrophilic form. This difference indicates that the lipoprotein-bound forms of GT will tend to accumulate in circulation, compared with the hydrophilic form, which is why these larger forms dominate in patients' sera. GT is apparently cleared from rabbit circulation as an active enzyme because identical clearance rates were obtained from measurements of either enzyme activity or enzyme protein. Further, the HDL-GT complex may also be removed intact, because no cleavage of the complex was indicated by gel filtration of serum sampled at various times after injection. The mechanisms by which these forms are cleared from the circulation, and how the clearance processes are altered by disease, remain to be studied.

There have been few reports on the clearance of serum GT. Klein et al. (15) reported a half-life of 4.1 days in patients with cholestasis, whereas Orrego et al. (16) estimated a value of 21 days in alcoholics after alcohol withdrawal. These values are apparently not corrected for continued release of GT from liver. Using an inhibition technique with acivicin, an irreversible inhibitor of GT, Rambabu et al. (17) reported a biphasic clearance curve of GT in rats after chronic ethanol administration, with half-life values of 5.2 and 13.2 h; the half-life of serum GT in control animals was 10.4 h. In their study (17) the rate of clearance was compared with the rate of synthesis and release from various organs.

A biphasic clearance of the hydrophilic enzyme is indicated in Figure 2, with a somewhat more rapid uptake in the first 2 h. This pattern may be due to several factors, one being the variation in sialic acid content of the enzyme injected. GT is rich in sialic acid, and microheterogeneity of both the serum and the liver enzyme has been reported (18, 19). Less-sialylated enzyme may be cleared more rapidly from the circulation, as asialoforms of several glycoproteins have shorter half-lives than their fully sialylated forms.

The lipid-bound and the hydrophilic forms are apparently released from liver by different mechanisms (9). The membrane-bound and amphiphilic enzyme may be released after solubilization of liver membranes by bile (1, 11) or as part of membrane fragments released during cell disruption (20). A transfer to various lipoprotein carriers can take place in the circulation. The hydrophilic form is produced by proteases that remove the hydrophobic domain of the enzyme, a process that has been reported when incubating liver homogenate at pH 7, in saline or in serum (6-8). The small form might therefore result from autolysis in liver and, as such, be of diagnostic interest. However, in patients with various liver diseases, the hydrophilic form has been detected in relatively small amounts, <15-20% of total activity. Only patients that suffered severe necrosis of the liver showed significant amounts of the hydrophilic form in serum (1, 6, 10). Because the small form is more rapidly cleared from the circulation than the lipoprotein-bound forms, and therefore is seldom detected in patients with liver disorders, more sensitive assays are needed for evaluation of its clinical significance.

References