Absence of circulating desialylated thyroxine-binding globulin in patients with hepatobiliary disease

Giuseppe Costante, Georges Sand, Pierre Reding and Daniel Glinoer

Departments of Internal Medicine and Radioisotopes, University Hospital Saint-Pierre, Université Libre de Bruxelles, Belgium

Abstract. Thyroxine-binding globulin (TBG) is the major thyroid hormone carrier protein. The molecule contains approximately 10 sialic acid residues which play a key role in the peripheral metabolism of TBG. Since the serum of patients with liver disease often contains large amounts of several desialylated glycoproteins, the aim of the present studies was to characterize circulating TBG and to examine the possible presence of desialylated TBG (dTBG) in 24 patients with a variety of hepatobiliary diseases and selected on the basis of elevated serum levels of desialylated glycoproteins. Using 4 immunochemical techniques applied for the measurement of TBG, for the detection of dTBG and for the characterization of TBG microheterogeneity, the results indicated: a) a wide scatter of serum TBG levels between 4 and 23 mg/l; b) the absence of detectable amounts of dTBG in any of the sera tested; and c) a close similarity between the microheterogeneity of TBG in patients with liver disease with that of control sera or of purified TBG. In conclusion, in patients with acute and chronic liver disease, TBG, although quantitatively modified, remains qualitatively unaltered, suggesting that diseased liver produces fully sialylated TBG and that its catabolism is not impaired.

The catabolism of circulating glycoproteins is regulated by modifications occurring at the level of their carbohydrate moiety. Following the removal of the terminal sialic acid residues, the exposure of the penultimate galactosyl residue constitutes a signal for recognition and binding of desialylated glycoproteins by specific liver cell plasma membrane binding sites. In physiological conditions, the serum is very rapidly cleared and, hence, contains only minute amounts of desialylated glycoproteins (Ashwell & Morell 1974). Thyroxine-binding globulin (TBG), the main thyroid hormone transport protein in man (Gershengorn et al. 1980), is a glycoprotein which contains approximately 10 sialic acid residues per molecule and follows this same metabolic pathway (Refetoff et al. 1975). In patients with a variety of acute and chronic liver diseases, the presence of large quantities of circulating desialylated glycoproteins has been reported (Marshall et al. 1974, 1978). Likewise the presence of a T₄ binding protein with characteristics similar to those of desialylated TBG (dTBG) was occasionally found in patients with hepatic cirrhosis (Marshall et al. 1972; Gärtnert et al. 1981). The present study was undertaken to investigate the nature of circulating TBG in a group of patients with severe hepatobiliary disease, using 3 immunochemical techniques applied to the detection of dTBG and isoelectric focusing to characterize TBG microheterogeneity.

Requests for reprints: Dr. D. Glinoer, Department of Internal Medicine, Hospital Saint-Pierre, 322, rue Haute, B-1000 Bruxelles, Belgium.

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Material and Methods

A. Patients

Twenty-four patients (13 males and 11 females, aged 34 to 78 years) were investigated. All were hospitalized for severe acute or chronic hepatobiliary disease. Clinical diagnoses were assessed by standard laboratory liver tests and by liver biopsy in the cases with cirrhosis or fatty liver. The patients were selected on the basis of elevated serum levels of desialylated glycoproteins, as determined by an in vitro inhibition assay (Van Lenten & Ashwell 1972). The patients were divided into 4 groups according to the diagnosis. Group A comprised patients with various degrees of compensated or decompensated cirrhosis. Two patients with fulminant hepatitis (group B) presented extremely severe hepatic failure; they died shortly after the study. Group C consisted of chronic alcoholic patients without histological evidence of cirrhosis. Two patients with cholestasis (group D) presented extrahepatic biliary obstruction. All patients were clinically euthyroid, without goitre or past history of thyroid disease.

B. Methods

1. Inhibition assay

For this assay, desialylated TBG, radiolabelled with the lactoperoxidase method (Glinoer et al. 1978) was used as ligand (specific activity: 50 mCi/mg). Rat liver cell plasma membranes were prepared by a previously described method, using differential ultracentrifugation in discontinuous sucrose gradient (Ray 1970). Aliquots were stored in liquid nitrogen until used. The protein content of the membrane preparations was determined by the method of Lowry et al. (1951). A binding assay was performed using 50 µg of membrane preparation, incubated in the presence of 0.1 µCi [125I]dTBG and increasing amounts of non-radioactive dTBG (from 10--1000 ng dTBG/assay tube), for 1 h at 37°C in a shaking bath. After incubation, the unbound radioactivity was separated by Millipore filtration and the bound radioactivity retained on the filter was counted. Non-specific binding of [125I]dTBG to liver membranes was less than 1% of the total radioactivity added. This binding assay was used to determine the amount of dTBG necessary to saturate the membrane binding sites (80 ng). The inhibition assay was performed in the same conditions as the binding assay, except that membranes were incubated first for 1 h with either purified non-radioactive dTBG (to establish the standard curve) or with 10 µl of serum to be tested. Immediately thereafter, a saturating amount of [125I]dTBG was added and the incubation was continued for one additional hour. Thereafter, the incubation mixture was filtered. Radioactivity bound for each serum sample was compared to the curve obtained with purified non-radioactive dTBG and results were expressed as µg equivalents of dTBG per ml of serum.

2. Detection of dTBG

Human TBG used in the present studies was purified by previously reported methods (Sand & Glinoer 1983). The sialic acid content measured by high pressure liquid chromatography (HPLC) was 9--11 moles per mole TBG. In vitro desialylation was carried out by incubating 300 µg of TBG with 600 mU of neuraminidase from Vibrio Cholerae (Worthington, Millipore Corporation, Freehold, N.J.), in acetate buffer (50 mM, pH 5.5) containing calcium chloride (9 mM), at 37°C. After 24 h of incubation, TBG was shown to contain less than 0.5 mole sialic acid/mole TBG. In other experiments in which partially desialylated TBG was required, the same conditions were used with a 100-fold lower neuraminidase/TBG ratio. Aliquots were sampled at various incubation intervals (0.5, 3, 8 and 24 h). The desialylation was stopped by increasing the pH to 8.6 and by freezing the protein at -20°C.

Radioimmunoassay (RIA) of TBG was performed as previously described (Glinoer et al. 1978). Standard curves ranged from 1 to 15 ng TBG per assay tube. Standards or serum samples were measured in triplicate, in the same run. Coefficient of intra-assay variation was 4.5%.

Electroimmunodiffusion (EID) or 'rocket' immuno-electrophoresis was performed using the technique of Laurell (1966). Standard solutions of TBG or serum samples (5 µl) were submitted to electrophoresis (20 V/cm for 3 h) in 1% agarose gels (agarose M. LKB, Bromma, Sweden) in Tris-barbital buffer (30 mM, pH 8.6) containing 0.2% monospecific rabbit antihuman TBG serum. After washing and drying, the gels were stained with 1% Coomasie Brilliant Blue R-250. Serial dilutions of purified TBG, ranging from 25 to 150 ng/well, were used as standards. The coefficient of inter-assay variation was 5%.

Crossed immunoelectrophoresis (CEI) was performed according to the method of Laurell (1965). After electrophoresis in the first dimension for 50 min, the gels were cut and carefully applied onto a second gel containing 0.2% human TBG antiserum. The second electrophoresis, in perpendicular direction, was carried out as described above for EID.

3. Characterization of TBG microheterogeneity

The microheterogeneity of TBG in whole serum was assessed by isoelectric focusing (IEF) followed by immunofixation. Serum samples were submitted to IEF in preformed 1 mm thin layer 5% acrylamide gels (Ampholine PAG plates, LKB), in a pH gradient between 4 and 6.5 using the LKB multiphor No. 2117 chamber and the LKB power supply No. 2103. Running conditions used a constant power of 25 W, for 2.5 h, 8--10°C. The pH gradient, measured with a surface electrode, was linear between pH 4.2 and 6. After focusing, a 1% agarose gel (Agarose L, LKB) containing 2.5% antihuman TBG was layered on the IEF gel and kept in a humid chamber for
Liver and thyroid function tests.

<table>
<thead>
<tr>
<th>Groups</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
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<tr>
<td>Clinical diagnosis</td>
<td>Cirrhosis</td>
<td>Fulminant hepatitis</td>
<td>Fatty liver</td>
<td>Cholestasis</td>
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<tr>
<td>Number of patients</td>
<td>15</td>
<td>2</td>
<td>5</td>
<td>2</td>
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<tr>
<td>Albumin (35–50 g/l)*</td>
<td>28–( 38–) 45**</td>
<td>29–43</td>
<td>37–( 42–) 54</td>
<td>33–39</td>
</tr>
<tr>
<td>PTT (80–100%)</td>
<td>29–( 68–) 100</td>
<td>16–29</td>
<td>60–(100)–100</td>
<td>100</td>
</tr>
<tr>
<td>Bilirubin (&lt;17 µmol/l)</td>
<td>12–( 22–) 44</td>
<td>87–172</td>
<td>&lt;17</td>
<td>65–177</td>
</tr>
<tr>
<td>ALT (&lt;35 IU)</td>
<td>30–( 59–) 558</td>
<td>1283–2930</td>
<td>28–( 38–) 71</td>
<td>109–375</td>
</tr>
<tr>
<td>AST (&lt;35 IU)</td>
<td>17–( 44–) 177</td>
<td>1326–2000</td>
<td>15–( 34–) 59</td>
<td>245–642</td>
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<tr>
<td>Alkaline phosphatase (&lt;270 IU)</td>
<td>152–(303–) 906</td>
<td>152–248</td>
<td>184–(232–) 344</td>
<td>336–913</td>
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<tr>
<td>Total T4 (70–160 nmol/l)</td>
<td>63–(107–) 140</td>
<td>31–84</td>
<td>86–(127–) 182</td>
<td>109–133</td>
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<tr>
<td>Total T3 (1.4–3.2 nmol/l)</td>
<td>0.7–( 1.6–) 3.1</td>
<td>0.5–1.0</td>
<td>1.6–( 2.2–) 2.9</td>
<td>1.5–1.7</td>
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<td>TBG (10–21 mg/l)</td>
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<td>4–12</td>
<td>15–( 18–) 23</td>
<td>17–21</td>
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<tr>
<td>FT4 index (0.9–2.1)***</td>
<td>0.9–( 1.5–) 2.0</td>
<td>1.4–1.5</td>
<td>1.3–( 1.5–) 1.9</td>
<td>1.4–1.6</td>
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<tr>
<td>TSH (&lt; 8 mU/l)</td>
<td>2.8–( 5.9–) 7.2</td>
<td>6.7–7.7</td>
<td>4.2–( 6.1–) 7.5</td>
<td>3.4–4.9</td>
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</table>

* Normal range for the laboratory.
** Extreme individual values observed in each group; in group A and C, median values are also given (in parentheses).
*** Free T4 index was derived from T4/TBG ratio.

Results

Liver and thyroid function parameters are presented in Table 1. Low serum T4 and T3 levels were found in groups A and B. A striking feature was the scatter of serum TBG levels: widely distributed within the normal range in 19 patients, below 10 mg/l in 3 and slightly above the normal range in 2 patients (Fig. 1). Total T4 values were significantly correlated with serum TBG (r = 0.91; P < 0.001). Basal TSH and free T4 index were normal in all patients. Fig. 2 illustrates the results of the inhibition assay performed with rat liver cell membrane. All sera exhibited elevated levels, ranging from 1.6 to 8 µg equivalent dTBG/ml. The highest serum ‘inhibitory activity’ was found in cirrhosis and in fulminant hepatitis (mean values of 4.8 and 4.3 µg, respectively).

Fig. 1.
Comparison of serum TBG levels measured by radioimmunoassay and by electroimmunodiffusion in 24 patients with hepatobiliary disease (r = 0.91; P < 0.001).
In order to assess the suitability of RIA for the detection of dTBG, serial dilutions of purified standard TBG and of enzymatically desialylated TBG were compared in RIA using an antiserum raised against native TBG. Fig. 3 illustrates the parallelism of dTBG serial dilutions to the standard curve and shows that dTBG was quantitatively recovered. These results indicate that TBG and dTBG were not distinguishable by standard RIA procedures.

Electroimmunodiffusion is a technique in which the height of the rockets is a direct function of the concentration of the antigen to be tested. It has, however, recently been shown that desialylated glycoproteins yielded, by this technique, smaller rockets when compared to identical amounts of the native protein. Therefore, the degree of sialylation of a serum glycoprotein can be measured by comparing EID results to a reference method in which the sialic acid content plays no role (Bordas et al. 1981). Fig. 4 shows the results of preliminary experiments using purified TBG before and after enzymatic desialylation: a 24% decrease in the rocket height was found after 0.5 h and a 45% maximal decrease after 24 h desialylation. In the 24 patients with liver disease, serum TBG levels, measured by EID, were almost identical to those obtained by RIA (see Fig. 1), with no systematic underestimation of TBG measurements. Since the sera of patients with liver disease could contain only small amounts of partially desialylated TBG – not detectable by EID – we next used crossed immunoelectrophoresis. In this technique the serum proteins are first separated according to electrophoretic mobility, then identified by precipitation with a specific antiserum. As shown in Fig. 5, the peaks obtained by CIE using purified TBG without or after desialylation were clearly separated, indicating that even slightly desialylated TBG could be identified. Fig. 6 illustrates typical CIE patterns of serum TBG from patients with liver disease. In all cases the electrophoretic mobility was similar to that of purified TBG and single peaks with nearly identical shapes were found.

Finally, the microheterogeneity of circulating TBG was analyzed by isoelectric focusing. Fig. 7 illustrates the IFF patterns of 9 patients' sera. Four major protein bands with isoelectric pH of 4.2 (I),

Fig. 2.
Serum 'inhibitory' activity in the 24 patients subdivided into groups A, B, C and D. The dots represent the individual measurements and the horizontal bars the mean of each group. The results are expressed as microgram equivalents of dTBG/ml serum.

Fig. 3.
Logit-log representation of a RIA standard curve obtained with purified TBG (○) and compared to serial dilutions of dTBG (×). Each experimental point is the mean of triplicate determinations.
**Fig. 4.**
Electroimmunodiffusion of identical amounts (0.1 µg/well; duplicate determinations) of purified TBG before or after mild enzymatic desialylation.

**Fig. 5.**
Crossed electroimmunodiffusion of identical amounts of purified TBG before (a) and after 0.5 h (b) or 8 h (c) of mild enzymatic desialylation. Samples a, b and c were submitted to CIE, in the same run, on separate gels. The stained glass plates were superimposed for illustration.
4.30 (II), 4.35 (III) and 4.45 (IV) were observed with purified TBG. However, some individual variation was noted, as indicated by the ranges in the relative distribution of the protein bands: 7–22% of the total circulating TBG for band I, 14–24% for band II, 26–43% for band III, and 23–47% for band IV, respectively. In some sera the IEF profiles showed the presence of doublets which were more frequently evidenced in the less acidic protein bands.

Discussion

During the early studies on human TBG purification, Marshall et al. (1972) demonstrated that the removal of sialic acid residues from purified TBG resulted in a slower electrophoretic mobility of the glycoprotein. The authors presented evidence that this 'slow' TBG was analogous to the slow-moving T₄ binding protein occasionally observed with human sera submitted to electrophoresis (Premachandra et al. 1970). It is clearly established today that TBG is a glycoprotein consisting of a single polypeptide chain with 4 carbohydrate units, and containing an average of 10 sialic acid residues/molecule in the terminal position (Zinn et al. 1978). Sialic acid residues play a key role in the regulation
of the metabolism of several serum glycoproteins: the removal of sialic acid, with the consequent exposure of the penultimate galactosyl residues, is followed by a rapid clearance of the desialylated glycoproteins from the circulation, after binding to specific hepatocyte plasma membrane receptors (Ashwell & Morell 1974). Human TBG has been shown to follow a similar pathway (Refetoff et al. 1975).

TBG displays a typical charge microheterogeneity when analyzed by isoelectric focusing techniques (Marshall et al. 1973; Sand & Glinoer 1983). The microheterogeneity of TBG was attributed to differences in the sialic acid content of the molecule subspecies (Gärtner et al. 1981). It can, however, not be accounted for entirely by sialic acid variation, since the enzymatic desialylation of TBG does not result in the total disappearance of the charge differences observed in IEF (Sand et al. 1982). Furthermore, interindividual polymorphism of TBG – genetically determined – was recently described, mainly in black Americans (Daiger et al. 1981). Additional studies of TBG purified from single black or white normal donors suggested also that differences in the aminoacid composition of the molecule were involved in this polymorphism (Grimaldi et al. 1983).

Important variations in serum TBG levels are frequently observed in patients with liver disease. Since the liver is the site of both synthesis and catabolism of TBG, normal, elevated, or low TBG concentrations can be found, depending on the type and severity of the liver injury (Gershengorn et al. 1980). In addition to these quantitative modifications, qualitative alterations of serum TBG were also reported, particularly in patients with liver cirrhosis: an 'abnormal' TBG was evidenced and considered to correspond to partially desialylated TBG (Marshall et al. 1972). These observations were corroborated by the demonstration that serum from patients with liver disease presented the ability to compete for the binding of radioactive desialylated TBG to rat liver plasma membranes. This 'inhibitory activity' was attributed to the presence in the circulation of excessive amounts of a heterogeneous group of desialylated glycoproteins, either because of a reduced uptake by the damaged liver or because of a release of glycoproteins with incomplete carbohydrate chains from the injured hepatocytes (Marshall et al. 1974, 1978).

We studied 24 patients with various hepatobiliary diseases, selected on the basis of high serum levels of desialylated glycoproteins, as estimated by an elevated 'inhibitory activity' in the in vitro assay with rat liver plasma membranes. The aim of our study was to characterize circulating TBG in these patients. Low TBG levels were observed only in decompensated cirrhosis and in fulminant hepatitis, as a result of liver protein insufficiency. In most patients, serum TBG was normal. Free T4 index, derived from T4-TBG ratio, was normal in all patients, indicating the absence of significant alterations of the T4 binding interaction with TBG (Glinoer et al. 1978).

The RIA data indicated that removal of sialic acid residues did not affect TBG immunoreactivity. These results are in agreement with those reported by Cheng et al. (1979) for TBG after complete deglycosylation. Presence of circulating dTBG in patients was assessed by use of EID and CIE techniques. Based on the results obtained with purified TBG, it was expected that significant amounts of dTBG would result in underestimation of values found in EID as well as modifications of CIE peaks (i.e. slow-moving or 'cathodal' TBG). These features were not observed, indicating that no detectable amounts, even of slightly desialylated TBG, were present in any of the sera tested. Furthermore, the microheterogeneity of TBG showed a banding pattern similar to that of purified TBG, providing further evidence for the absence of circulating dTBG. Previous data from our laboratory have indicated that partial desialylation of TBG results in rapid disappearance of the most acidic TBG subspecies and sequential appearance of proteins bands with elevated isoelectric pH (Sand et al. 1982). A relative increase of the less acidic TBG bands was reported in patients with liver cirrhosis and elevated serum TBG levels (Gärtner et al. 1981), suggesting an accumulation of dTBG in serum. Some differences in the relative distribution of TBG subspecies were found in the present studies, but there was no correlation between TBG patterns and either TBG concentration or serum 'inhibitory activity'. It is therefore more likely that these interindividual differences correspond to TBG polymorphism, found in normal individuals (Lasne et al. 1980), rather than to the presence of circulating dTBG.

Finally, during the preparation of this manuscript, an article by Reilly & Wellby (1983) was published on the same topic. These authors used a modified CIE technique in which Concanavalin A (Con A) was added to the first dimension gel. They
reported an increase in Con A non-reactive TBG in patients with severe non-thyroidal illnesses and considered it to represent desialylated TBG. However, since Con A reactivity of glycoproteins does not primarily depend on sialic acid but on other carbohydrate units (Schwick et al. 1977), these observations cannot be ascribed to desialylated TBG. Moreover, in preliminary experiments with purified TBG, without or after partial and complete in vitro desialylation, we were unable to reproduce these findings.

In conclusion, the present data indicate that, in most patients with acute or chronic liver disease, TBG is synthesized and secreted into the circulation with a complete carbohydrate moiety. Furthermore, defects in TBG catabolic mechanisms were not observed even though the patients exhibited elevated levels of circulating desialylated glycoproteins. Therefore, in our patients, hepatic function was preserved well enough to assure a normal clearance of desialylated TBG from the circulation. It remains, however, plausible that far-advanced chronic liver disease may lead to an impairment of TBG catabolism with consequent accumulation of desialylated TBG in serum.

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