Analyses of the molecular forms of serum thyroglobulin from patients with Graves' disease, subacute thyroiditis or differentiated thyroid cancer by velocity sedimentation on sucrose gradient and Western blot

Laure Druetta 1, Karine Croizet 1, Hubert Bornet 1,2 and Bernard Rousset 1,3

1 Institut National de la Santé et de la Recherche Médicale, Unité 369, Faculté de Médecine Lyon-RTH Laennec, 69372 Lyon Cedex 08, France
2 Laboratoire de Biophysique, Faculté de Médecine Lyon-Grange Blanche, 8, avenue Rockefeller, 69008 Lyon, France and 3 Unité Fonctionnelle de Biologie Cellulaire, Fédération de Biochimie, Hôpital Edouard-Herriot, 69437 Lyon, France

(Correspondence should be addressed to B Rousset, INSERM U 369, Faculté de Médecine Lyon-RTH Laennec, 69372 Lyon Cedex 08, France)

Abstract

Serum thyroglobulin (Tg) concentration increases in diverse thyroid pathophysiological situations. We examined whether Tg molecules appearing in the serum of patients with Graves’ disease (GD), subacute thyroiditis (ST) or differentiated thyroid cancer (DTC) have distinctive biochemical properties. We used ultracentrifugation on sucrose gradient and Western blot to analyze structural parameters of immunoreactive Tg in complete serum from 40 patients with GD, ST or DTC. Purified human Tg was used as reference.

Immunoreactive Tg from ST or DTC sera sedimented in a single, rather symmetrical peak as purified 19S Tg. In GD sera without detectable anti-Tg autoantibodies (TgaAb), about 80% of immunoreactive Tg was recovered in a Tg dimer peak that often split into two components; the remaining Tg immunoreactivity (10–30%) sedimented faster and was polydispersed. In GD sera containing TgaAb, immunoreactive Tg was recovered in a peak sedimenting faster than 19S Tg corresponding to immune complexes identified by protein A adsorption. Using a Western blot procedure, optimized to detect 0.1 ng Tg in serum, a single band of Tg, migrating as the intact Tg subunit, was always found in non-reducing conditions; the intensity of the band correlated with the immunoassayable Tg concentration. In reducing conditions, the Tg band obtained with GD or ST sera was decreased by up to 70% compared with that of purified Tg or serum Tg from patients with DTC.

In conclusion, serum Tg from DTC is remarkably homogeneous and in the form of dimers dissociable into uncleaved monomers. In contrast, serum Tg from GD or ST is heterogeneous with respect to its sedimentation properties and/or the structural integrity of its polypeptide chains. These data provide information on the processes whereby Tg is released into the circulation.

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Introduction

From the pioneering work of Hjort (1) and Assem (2) and then of Daniel et al. (3), Roitt and Torrigiani (4) and Torrigiani et al. (5), it is known that thyroglobulin (Tg) is not restricted to the thyroid gland but is also detected in the blood of normal subjects. The development of an RIA for the measurement of Tg in human serum (6) helped to demonstrate that Tg is a normal secretory product of the thyroid gland (7) and that its release is controlled by thyrotropin (TSH) (8, 9). In normal individuals, the thyroid gland secretes low amounts of Tg and consequently serum Tg concentration is barely detectable, but in pathological situations the release of Tg by thyroid tissue can be markedly increased. Van Herle and Uller (10) originally reported that serum Tg levels are increased in patients with differentiated thyroid carcinoma (DTC). This led to the development of a large number of studies (for example 11–13) that established serum Tg as a marker for the follow-up of patients with thyroid cancer. Elevated levels of circulating Tg are also found in hyperthyroid patients (14) including patients with Graves’ disease (GD). In that case, the rise in serum Tg concentration is probably the result of the overstimulation of the gland. An increase in serum Tg concentration is also observed in patients with subacute thyroiditis (ST) (6) and in patients with congenital hypothyroidism (15).

The literature regarding the biochemical nature of serum Tg is scanty, mainly because the amount of Tg in the circulation is too low to allow purification of sufficient quantities to perform direct physico-chemical analyses. Most of the present knowledge comes from studies performed by Schneider’s group of serum Tg
from patients with thyroid carcinoma with metastases (16–18). At present, there is no biochemical data for serum Tg in other pathological situations. In the present study, we have tried to get new information on circulating Tg by analyzing and comparing molecular characteristics of serum Tg from patients with GD, ST or DTC. Two parameters have been studied on native Tg serum (without concentration or purification steps): (a) the degree of homogeneity or dispersion of immunoreactive Tg species after fractionation by ultracentrifugation on sucrose gradient and (b) the structural integrity of the polypeptide chains of circulating Tg molecules by identifying immunoreactive species by Western blot in non-reducing or reducing conditions. We developed a Western blot procedure that led us to visualize for the first time Tg species in the serum of patients with GD, ST or DTC containing immunoassayable Tg concentrations as low as 100 ng/ml. We report that Tg molecules present in the serum of patients with GD, ST or DTC exhibit distinctive biochemical characteristics.

Materials and methods

Human Tg preparation

Tg was purified from human euthyroid goiter tissue in conditions minimizing its breakdown. Fresh thyroid tissue was minced in small pieces from which Tg was extracted by a 16h period of treatment in 0.01 mol/l sodium phosphate and 0.15 mol/l NaCl, pH 7.4. Tg in solution was precipitated by 1.4–1.8 mol/l ammonium sulfate. Precipitated protein was collected by centrifugation at 55,000 g for 20 min at 4°C and then solubilized and dialyzed against the same buffer. Tg was purified by anion-exchange chromatography on DEAE-Sepharose and gel filtration on Sepharose CL-4B (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Tg concentration was assayed by absorbance measurement at 280 nm ($\varepsilon = 660,000$ l/mol per cm) and by the method of Bradford (35) with BSA as standard. The two purified Tg preparations used as references in this study were denominated hTgRP3 and hTgRP8. They contained 8 and 20 iodine atoms per molecule respectively. The structural integrity of purified Tg was controlled by SDS-PAGE. In non-reducing conditions, hTgRP3 and hTgRP8 gave a single band representing Tg monomers. In reducing conditions, two bands were observed; they corresponded to the S and F bands described by Gentile et al. (19). The S band corresponds to the complete Tg polypeptide chain and the F band to a natural cleavage product shortened by about 500 amino acids at the NH$_2$-terminus.

Serum samples

Sera were obtained from 21 patients with Graves’ disease prior to any treatment. Graves’ disease was diagnosed on the basis of clinical symptoms of
Preparation of anti-hTg polyclonal antibodies

Antisera were raised in rabbits by multipoint injections of purified human Tg emulsified in complete Freund’s adjuvant. After 2, 4 and 6 weeks, rabbits were given a booster injection and blood was collected 1 week after the last injection. Three immune sera pAb R1, R2 and R3 were obtained; their antibody titers (expressed as the highest dilution which gives a significant response in the radioimmunoassay) were higher than 1:4 × 10^{6} for R1 and R3 and higher than 1:10^{7} for R2. Each of the three immune sera was depleted in antibodies reacting or cross-reacting with human serum proteins by filtration on an insoluble matrix on which human serum proteins had been immobilized. At a 1:10^{6} dilution, the pAb R2 immune serum immunoprecipitated about 20% of 125I-hTg. This immune serum dilution was used for Tg RIA.

Detection of anti-Tg autoantibodies (TgaAb)

125I-hTg (40 000 c.p.m.) was incubated with 10 μl serum in a total volume of 400 μl PBS containing 2 mg/ml BSA for 1 h at room temperature. Then, 125I-hTg (40 000 c.p.m.) was added and the incubation was carried on for 6 h. Immune complexes were collected using sheep anti-rabbit immunoglobulins immobilized on activated silica beads (Biogenesis, Poole, Dorset, UK). After 30 min incubation at room temperature, beads were pelleted by centrifugation at 1500 g for 30 min at 4 °C, washed in PBS-BSA and the radioactivity was measured using a gamma counter from Packard Instruments. Each determination was made in duplicate. The sensitivity of the assay was about 0.4 ng/ml. The interassay coefficient of variation was 11%. Comparison of reference Tg with the international hTg reference material (CRM 457) (25) showed a 1.2 factor of difference.

Ultracentrifugation on sucrose gradients

Samples of 10 μl to 300 μl human serum containing at least 30 ng immunonoasayable Tg were placed on the top of 8–25% linear sucrose gradients in PBS and centrifuged in a Beckman SW41 rotor at 100 000 g for 18 h at 4 °C using a L8–80 Beckman ultracentrifuge.
Fractions of 500 μl were collected from the top to the bottom of the gradient. Protein was detected in 200, 20 or 10 μl of each fraction (depending on the amount of serum applied to the gradient) by the method of Bradford (35) and the result was expressed by the absorbance at 595 nm. Each fraction was assayed for Tg by RIA as described above. To determine whether immunoassayable Tg was in a free form or bound to anti-Tg autoantibodies, Tg was assayed before and after treatment of the gradient fractions with protein A adsorbent (Pansorbin; Calbiochem Corp., La Jolla, CA, USA) for 30 min at room temperature and centrifugation at 1500 g for 15 min.

**SDS-PAGE and Western blot analyses**

Purified Tg and serum samples subjected or not to reduction by 5% (v/v) β-mercaptoethanol were analyzed on 5% acrylamide slab mini-gels. Proteins were either stained with Coomassie Blue or electrotransferred onto Immobilon-P membrane (Millipore Corp., Belford, MA, USA). After a 20 min treatment in PBS containing 0.2% Tween 20, membranes were incubated with polyclonal anti-hTg immune serum at a 1/30 000 dilution in PBS-Tween 20 (0.2%)-milk (2%) for 1 h at room temperature. After washings, membranes were further incubated with sheep anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase and then with NBT-BCIP (Boehringer Mannheim, Meylan, France) according to the manufacturer’s instructions. The apparent molecular mass of the identified species was determined by comparison with that of standard proteins of known molecular mass from Bio-Rad Laboratories, Richmond, CA, USA. Immunolabeled Tg spots were quantified by video-image analysis.

**Results**

Validation of the experimental conditions used to analyze the sedimentation characteristics of Tg at low concentration in serum

Although a sensitive RIA was used (with delayed addition of tracer), analyses of the distribution of Tg at concentrations ranging from 60 to 300 ng/ml in
complete serum required a rather large volume of serum (up to 300 μl on 10 ml gradient) to be loaded onto the sucrose gradient; thus a large quantity of proteins (up to 20 mg) were present that could affect Tg sedimentation. Therefore, we analyzed the changes in the sedimentation profile of purified Tg (from human and pig) and serum Tg (from a patient with DTC with a Tg concentration of 8000 ng/ml) as a function of the volume of serum introduced onto the gradient. Whatever the source of Tg, a single peak corresponding to Tg dimers or 19S Tg was observed; however, the peak was shifted towards the bottom of the gradient when the volume of serum increased from 10 to 300 μl (Fig. 1). The displacement of the peak was proportional to the serum input. The shift of the Tg peak was accompanied by a broadening and flattening without major loss of symmetry. Furthermore, the area of the peak remained constant whatever the serum input indicating that there was no loss of material at high serum loading. Of note, whatever the source of Tg, and whatever the volume of serum analyzed on the gradients, there was neither artefactual dissociation of Tg into subunits nor formation of Tg oligomers. These preliminary data indicated that experiments aimed at characterizing the molecular forms of immunoreactive Tg in serum by comparison with purified Tg must be conducted in controlled and matched conditions.

**Sedimentation characteristics of serum Tg in different thyroid diseases**

Serum samples from 10 patients with DTC, 4 patients with ST and 15 patients with GD were subjected to ultracentrifugation on 8–25% sucrose gradients. Protein and immunoreactive Tg distribution profiles of representative sera are given for each thyroid disorder in Figs 2 and 3. In all sera from patients with DTC, immunoreactive Tg was always found in a single peak sedimenting as hTgRP8 (Fig. 2). Monomeric Tg or smaller Tg fragments were never observed. The immunoreactivity recovered in the gradient fractions represented 85.0 ± 3.0% (n = 10) of the amount of Tg loaded on the gradients. As can be seen in Fig. 2, the Tg dimer peak often exhibited an extension towards the bottom part of the gradient. The ‘tail’ of the peak (fractions 16 to 22) accounted for 4.3 ± 1.6% (n = 10) of assayable Tg in the gradient. To check for the presence of heavy Tg species that could have been pelleted, empty centrifugation tubes (after gradient removal) were thoroughly washed to collect pelleted material. The resulting solutions were devoid of immunoreassayable Tg.

In the few cases of ST that were analyzed, immunoreactive Tg was also almost exclusively in the form of dimers. Two profiles are reported in Fig. 2. In one case, a shoulder to the main peak could indicate the presence of a very small proportion of the Tg oligomer usually defined as 27S Tg.

Serum Tg from patients with GD exhibited a more complex sedimentation pattern (Fig. 3). Immunoreactive Tg distribution profiles obtained with two sera without detectable TgaAb (GD 14 and GD 83) and one serum with TgaAb (GD 20) are shown in Fig. 3. The main proportion
of immunoreactive Tg from sera without detectable TgaAb sedimented as Tg dimers. However, the peak was non-homogenous; it was split into two components in 5 out of the 7 sera that were analyzed (Table 1). When these sera were subjected to a second or third analysis in separate centrifugation runs, the splitting of the peak was reproducibly observed. The proportion of the slower and faster components of the peak was somewhat variable from serum to serum. In addition to these main Tg components, up to 30% of Tg immunoreactivity was found dispersed in the bottom fractions of the gradient. The distribution of immunoreactive Tg in GD sera containing TgaAb was markedly different. A main peak much wider than that of reference Tg and shifted towards the bottom of the gradient was found in each of the 8 sera that were analyzed. Immunoreactive Tg was in the form of soluble immune complexes since it was totally removed from the gradient fraction by protein A treatment (bottom panel of Fig. 3).

**Identification of serum Tg by Western blot**

To test whether serum Tg could be visualized by Western blot using anti-hTg polyclonal antibodies (anti-Tg pAb), we selected a serum with a high Tg concentration (13,700 ng/ml) from a patient with DTC, which was analyzed in parallel with purified Tg. Results in Fig. 4A show that different anti-Tg pAbs (R1, R2 and R3) labeled a band with the same electrophoretic mobility in DTC 24 serum and hTgRP3. By reference to molecular mass standards, this band corresponded to intact Tg monomers (resulting from the SDS-induced dissociation of Tg dimers). A second more diffused labeled band was detected in lanes containing serum proteins. It was unrelated to Tg since it was also observed in serum So. Using pure human IgG, we found that this band resulted from a cross-reaction between the second antibody (sheep anti-rabbit IgG conjugated to alkaline phosphatase) and human IgG present in serum samples. As shown in panel B of Fig. 4, increasing amounts (1 to 10 ng) of purified hTg (diluted in either PBS-BSA or serum So) or Tg from DTC 24 serum gave bands with increasing intensity that could be quantified and used to generate standard curves (Fig. 5). To detect Tg in serum with a low immunoassayable Tg concentration, it was necessary to increase the sensitivity of the Western blot procedure. This was done by increasing the time of the reaction between alkaline phosphatase conjugated to the second antibody and its substrate. Although the background was significantly increased, as can be seen in Fig. 6 (top panel), the detection threshold could be shifted to 0.05–0.1 ng. It was thus possible to detect Tg in serum with an immunoassayable concentration as low as 100 ng/ml. The maximum volume of serum loaded in each lane was 0.7 ml containing 40–50 μg serum protein. Data obtained with 14 sera from patients with GD, ST or DTC are shown in Fig. 6; they derive from 4 separate experiments in which different amounts of hTgRP8, DTC 24 or DTC 54 serum were used to generate reference curves. The increase in the enzyme–substrate reaction time led to a shift of the reference curves which remained parallel. Whatever the sera, including GD sera with TgaAb, there was a good correlation between the
intensity of the Western blot signal (monomeric Tg band) and immunoassayable Tg concentration.

The Western blot procedure was used to investigate the structural integrity of serum Tg after reduction of disulfide bonds. The signal obtained with non-reduced serum samples was compared with the signal obtained after treatment with β-mercaptoethanol (βM). Results are shown in Fig. 6. As expected, hTgRP8 in reduced conditions (+βM) gave two immunoreactive bands corresponding to the S and F forms of Tg (see Materials and methods). In contrast, all serum samples treated with β-mercaptoethanol yielded a single band with the same electrophoretic mobility as the S band of hTgRP8.

On close inspection of blots (as those of Fig. 6), it was observed that the intensity of the monomeric Tg band from many sera was markedly decreased after reduction of disulfide bonds. Densitometric measurements of the intensity of Tg bands were used to determine the +βM/−βM Tg immunoreactivity ratio as a quantitative estimate of these alterations. To compare data obtained on separate Western blots, +βM/−βM Tg immunoreactivity ratios were normalized to the ratio obtained with hTgRP8 to which was assigned a value of 1. The graphical representation of data (bottom part of Fig. 6) shows that disulfide bond reduction did not affect Tg immunoreactivity of serum from patients with DTC but decreased by 30 to 70% (mean: 47 ± 5%, n=8) the immunoreactivity of serum Tg from GD. The +βM/−βM Tg immunoreactivity ratio was variable in the 3 sera from ST that were studied.

Discussion

Using two analytical approaches, sucrose density gradient ultracentrifugation to identify circulating immunoreactive Tg species and Western blot to probe...
the size of Tg polypeptide chains and their structural integrity, we present evidence for definite differences between the molecular forms of Tg found in the serum of patients with GD, ST or DTC.

Immunoreactive Tg present in the serum of patients with DTC (with metastasis) corresponded to dimeric Tg which was indistinguishable by velocity sedimentation analysis on sucrose gradient from human 19S Tg. Dissociation into 12S subunits was never observed. Data from Western blot analyses also indicate that circulating Tg from patients with DTC had a high degree of homogeneity. In all DTC sera examined, serum Tg was fully dissociable into monomers that were apparently neither qualitatively nor quantitatively affected by reduction of disulfide bonds.

Serum Tg from GD patients without detectable TgaAb appears to differ from Tg detected in the serum of patients with DTC in two main points. First, it was composed of Tg dimers, that appeared non-homogeneous in most cases, and of faster sedimenting polydisperse species. Secondly, its detection by Western blot was affected by disruption of disulfide bonds. The polydispersed immunoreactive material, amounting to up to 30%, could correspond to Tg complexed to trace amounts of TgaAb (26–28). As this immunoreactive material was not precipitated by protein A, these soluble immune complexes might be formed mainly from Ig other than IgG. The splitting of the Tg dimer peak, revealing the existence of a fraction of immunoreactive Tg sedimenting slightly slower than purified 19S Tg, could indicate the presence of Tg species with an altered conformation. The loss of monomeric Tg upon disruption of disulfide bonds, as demonstrated by the decrease of the +βM/-βM Tg immunoreactivity ratio, suggests that part of the Tg polypeptide chains present structural alterations that could derive from a limited proteolytic attack. These two molecular characteristics of Tg from GD sera are very much reminiscent of the properties of Tg molecules previously identified as Tg intermediate degradation products in prelysosome-lysosome compartments of the thyroid gland (23). Tg molecules extracted from purified lysosomes had three main physico-chemical characteristics among which two are pertinent to Tg from GD sera. First, lysosomal Tg was found in the form of dimers with a sedimentation coefficient slightly lower than that of intact Tg. Secondly, lysosomal Tg, analyzed by Western blot, had the same apparent molecular mass as that of intact Tg monomers under non-reducing conditions but almost completely disappeared after reduction of disulfide bonds. Thirdly, these Tg molecules contained iodine but no hormone residue. From points 1 and 2, it was concluded that lysosomal Tg was cleaved as discrete sites, the resulting polypeptides remaining bound through disulfide bonds to yield molecules with an apparent normal size and a slightly altered structure (23). These particular features seem to apply to part of the Tg molecules present in the serum of patients with GD. Cirulating Tg in GD would be composed of intact Tg molecules and Tg molecules made of monomers cleaved at discrete sites. Given the low amount of Tg that was subjected to Western blot, it has not been possible to identify Tg fragments released upon reduction. If one refers to what was found for lysosomal Tg (23), fragments could have a variable size (from 220 kDa to less than 50 kDa).

The question that arises is: when or where are Tg molecules cleaved? Two main secretory pathways have been proposed to explain the release of Tg from the thyroid gland. Part of newly synthetized Tg escaping from the secretion into the lumen of thyroid follicles would be released at the basolateral membrane of thyrocytes (29) into the extracellular fluid. Alternatively, Tg molecules internalized at the apical pole of thyrocytes (originating from the follicle lumen) and conveyed to early endosomes (30) would escape from
these compartments and undergo a transcellular vesicular transport or transcytosis (31) to be released into the extracellular fluid. The elevation of serum Tg concentration, in response to a stimulation of the thyroid gland by TSH or by anti-TSH receptor stimulating antibodies in GD, could result from the activation of either or both of these pathways. It has also been reported that TSH stimulation could modify tight junction permeability and thus promote leakage of proteins in and out of the follicles (32). In the serum of patients with GD, the fraction of Tg sedimenting as 19S Tg and resistant to disulfide bond reduction might represent newly synthesized non-iodinated Tg directly released into the circulation. We propose that Tg molecules sedimenting slightly slower than 19S Tg and sensitive to reduction could correspond to Tg molecules released by transcytosis that have undergone discrete proteolytic cleavage during their transcellular transport. Apical to basolateral transcytosis of Tg has been studied in much detail by Herzog’s group (31, 33): however, it has never been reported whether the polypeptide chains of Tg are intact or not, i.e. sensitive or resistant to reduction of disulfide bonds, after transcytosis.

Analyses of serum Tg from GD patients with TgaAb did not provide information on Tg per se because most, if not all, immunoreactive Tg was found in the form of soluble immune complexes. Although somewhat different from serum to serum, the immune complexes exhibited a limited size compatible with a few IgG bound per Tg molecule. This is in keeping with previous reports showing that, unlike antibodies produced in animals, TgaAb are directed against a limited number of epitopes (26). Circulating Tg in the form of immune complexes was visualized on Western blot and there was a correlation between the intensity of the monomeric Tg band and immunoassayable Tg concentration. One might infer from this observation that, in GD sera containing TgaAb, Tg in the form of immune complexes was fully accessible to heterologous antibodies. Serum Tg from patients with ST, which appeared very homogeneous after fractionation on sucrose gradient, showed a variable sensitivity to reduction. Thus, some polypeptide chains of Tg from ST sera are probably cleaved at some sites. Proteolytic cleavage reactions could occur during the process of release of Tg as a consequence of the destruction of the thyroid parenchyma. Generation of Tg fragments following tissue injury and cell lysis have been clearly identified in the circulation of patients undergoing thyroid surgery (34).

In conclusion, by combining two analytical methods we have provided new information on the molecular forms and structural properties of Tg in human serum. Data deriving from biochemical analyses of Tg from the serum of patients with GD led us to postulate that Tg molecules leaving thyroid follicles by transcytosis might be subjected to proteolytic processing during their transcellular transport.

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