Identification of thyroid hormone residues on serum thyroglobulin: a clue to the source of circulating thyroglobulin in thyroid diseases

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Abstract

Thyroglobulin (Tg) present in the serum of normal individuals and patients with thyroid disorders could be partly newly synthesized non-iodinated Tg and partly Tg containing iodine and hormone residues originating from the lumen of thyroid follicles. With the aim of examining the contribution of the latter source of Tg to the elevation of serum Tg concentration in thyroid pathophysiological situations, we devised a procedure to identify thyroxine (T4) and tri-iodothyronine (T3) residues on Tg from unfractionated serum. A two-step method, based on (i) adsorption of Tg on an immobilized anti-human Tg (hTg) monoclonal antibody (mAb) and (ii) recognition of hormone residues on adsorbed Tg by binding of radioiodinated anti-T4 mAb and anti-T3 mAb, was used to analyze serum Tg from patients with either Graves' disease (GD), subacute thyroiditis (ST) or metastatic differentiated thyroid cancer (DTC). Purified hTg preparations with different iodine and hormone contents were used as reference. Adsorption of purified Tg and serum Tg on immobilized anti-hTg mAb ranged between 85 and 90% over a wide concentration range. Labeled anti-T4 and anti-T3 mAbs bound to adsorbed purified Tg in amounts related to its iodine content. Tg adsorbed from six out of six sera from ST exhibited anti-T4 and anti-T3 mAb binding activities. In contrast, significant mAb binding was only observed in one out of eight sera from untreated GD patients and in 1 out of 13 sera from patients with DTC. The patient with DTC, whose serum Tg contained T4 and T3, represented a case of hyperthyroidism caused by a metastatic follicular carcinoma. In conclusion, we have identified, for the first time, T4 and T3 residues on circulating Tg. The presence of Tg with hormone residues in serum is occasional in GD and DTC but is a common and probably distinctive feature of ST.

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Introduction

Thyroglobulin (Tg), the thyroid hormone precursor protein, is normally stored in the lumen of thyroid follicles. In this compartment, Tg undergoes chemical reactions (iodination and coupling of tyrosyl residues) leading to the formation of thyroid hormone residues (thyroxine (T4) and tri-iodothyronine (T3)) within its polypeptide backbone. In the normal human thyroid gland, the hormone content of Tg ranges from one to three T4 or T3 residues per molecule (1), T3 amounting to from one-fifth to one-twentieth of T4. In normal individuals, the thyroid gland secretes low amounts of Tg into the circulation, but in diverse pathophysiological situations serum Tg concentration markedly increases. Tg molecules appearing in the circulation of normal subjects or patients with thyroid disorders probably originate from two distinct thyroid compartments. First,

newly synthesized Tg molecules (devoid of hormone residues), instead of being secreted into the lumen of follicles, could be released at the basolateral membrane of thyrocytes (2) into the extrafollicular or interstitial fluid. Secondly, iodinated Tg molecules (with hormones residues) internalized at the apical pole of thyrocytes from the follicle lumen could undergo a transcellular vesicular transport or transcytosis (3) to be released into the extrafollicular fluid. The elevation of serum Tg concentration in response to thyroid stimulation by thyrotropin (TSH) (4, 5), or by anti-TSH receptor stimulating antibodies (6) in patients with Graves' disease (GD), could result from the activation of either of these pathways, or both of them. In pathological situations leading to morphological alterations of the thyroid parenchyma, as seems to be the case in subacute thyroiditis (ST), the appearance of Tg in the blood stream might result from the leakage of Tg from

disrupted thyroid follicles. The mode of release of Tg, sometimes in very large amounts, into the circulation of patients with metastatic differentiated thyroid cancer (DTC) is more hypothetical. It certainly depends on the organization of thyrocytes within the tumor. When present, follicular structures are probably subjected to intense remodeling due to cytolysis and/or cell proliferation that could lead to the release of their Tg content. If tumor cells are not organized in follicles, newly synthesized Tg would be released as a consequence of the lack of a storage compartment. Thus, according to the nature of the thyroid disease and the structural and functional alterations of thyroid tissue, the compartment from which Tg is released and the release process are likely to be distinct. To identify serum Tg molecules originating from the lumen of thyroid follicles that have undergone hormone synthesis reactions, we devised a procedure of detection of T4 and T3 residues on low amounts of Tg in complete serum. A two-step method based on (i) the adsorption of Tg on an immobilized anti-human Tg (hTg) monoclonal antibody (mAb) and (ii) the recognition of T4 and T3 residues on adsorbed Tg by binding of radioiodinated anti-T4 and anti-T3 mAbs, was used to analyze serum Tg from patients with different thyroid disorders (GD, ST or DTC).

Materials and methods

Materials

Anti-T4 and anti-T3 mAbs and the immobilized anti-hTg mAb were obtained from Cis bio international (Gif-sur-Yvette, France). The anti-T4 mAb and anti-T3 mAb were designated R41 and R30 respectively. The anti-hTg mAb, immobilized on plastic tubes with gills, was the antibody used in the ELSA-HTG Kit commercialized by Cis bio international. [125 I]T4 and [125 I]T3 (specific radioactivity $>1200 \mu\text{Ci}/\mu\text{g}$) were purchased from Amersham Pharmacia Biotech, Orsay, France. Sheep anti-mouse IgG immobilized on activated silica beads was obtained from Biogenesis (Poole, Dorset, UK).

hTg preparations

Tg was purified from fresh human thyroid tissue from euthyroid or hypothyroid patients with goiter. To minimize potential Tg degradation, thyroid tissue was minced into small pieces and Tg leaking from open follicles was collected in 0.01 mol/l sodium phosphate, 0.15 mol/l NaCl, pH 7.4 (PBS) for 16 h at 4°C. Tg in solution was precipitated by 1.4–1.8 mol/l ammonium sulfate. Precipitated protein, collected by centrifugation at 55 000 g for 20 min at 4°C, was 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, Uppsala, Sweden). The Tg concentration was assayed by

absorbance measurement at 280 nm ($\epsilon = 660\,000$ per mol/l per cm) and by the Bradford method using BSA as standard. Three hTg preparations, hTgRP3, hTgRP4 and hTgRP8 were used in this study. The structural integrity of purified Tg was checked by SDS-PAGE. In non-reducing conditions, the three Tg preparations gave a single band representing Tg monomers. In reducing conditions, two bands were observed; they correspond to the 'S' and 'F' bands described by Gentile *et al.* (7). The iodine content of hTgRP3, hTgRP4 and hTgRP8, determined by the method of Sandell & Kolthoff (8) using a Technicon AutoAnalyser (Technicon, Palo Alto, CA, USA), was respectively 8, 2 and 20 iodine atoms per Tg molecule. The T4 content of hTgRP3 and hTgRP8 (determined by T4 RIA after pronase hydrolysis) was about 1.0 and 2.1 residues per Tg molecule respectively. hTgRP4 was available in too low amounts to allow measurements of its T4 content.

Serum samples

Sera were obtained from eight patients with GD prior to any treatment. GD was diagnosed on the basis of clinical symptoms of hyperthyroidism and measurements of serum TSH and free T4 concentrations. Immunoassayable serum Tg concentrations ranged from 120 to 1230 ng/ml (mean 542 ng/ml). Serum Tg concentrations in normal subjects ranged from 2 to 40 ng/ml. Six serum samples were collected from patients with ST diagnosed on clinical and serological grounds including elevated levels of circulating Tg (65–3000 ng/ml, mean 732 ng/ml). Thirteen sera from patients with DTC were selected for their high serum Tg concentration (60 to 30 000 ng/ml, mean 6593 ng/ml). All patients had undergone total thyroidectomy and had received radioiodine for ablation of thyroid remnants. At the time of serum sampling, there was scintigraphic evidence for metastasis in all patients. None of the sera from patients with either GD, ST or DTC contained detectable anti-Tg autoantibodies assessed as previously described (9).

Sera from thyroidectomized patients shown to be devoid of immunoreactive Tg and detectable anti-Tg autoantibodies were pooled and this pool, designated 'serum So', was used as control or as diluent for patient serum and/or hTgRP samples.

All serum samples were taken from blood specimens sent for routine biochemical analyses; in no case was blood sampling solely for the purpose of the study.

Case report – patient DTC 89

Patient DTC 89, a 63-year-old man, presented in 1989 with a cervical and endothoracic compressive goiter containing a large zone without radioiodine uptake activity with the association of hyperthyroidism. An extensive cervicomedialastinal surgical procedure was performed with removal of a poorly differentiated follicular thyroid cancer exhibiting intravascular

emboli. Post-operatively, serum free T4 (105 pmol/l) and Tg levels (31 000 ng/ml) were extremely elevated. A whole body scan (WBS), after administration of 100 mCi ^{131}I , disclosed many sites of radioiodine uptake in lungs and liver while there was no uptake in the thyroid bed. These metastases were confirmed by a computerized tomography (CT) scan. Treatment with methimazole was undertaken over 4 months and was withdrawn after the second 100 mCi dose, since euthyroidism was attained. Then the patient received suppressive T4 treatment. Following repeated ^{131}I treatments, radioiodine uptake sites progressively declined in number and intensity, and serum Tg concentration markedly decreased. After administration of a total dose of 700 mCi, there was no more radioactive iodine uptake. When serum Tg concentration increased again, an exploration with Octreoscan (Mallinckrodt, Petten, the Netherlands) was performed, showing multiple mediastinal metastases, confirmed by CT scan. Finally, the patient died due to extensive metastatic diffusion. In this patient, production of thyroid hormones by hepatic and lung metastases of a follicular cancer is demonstrated by persistence of hyperthyroidism after total thyroidectomy.

Radioiodination of proteins

Purified Tg, anti-T4 mAb and anti-T3 mAb were labeled with ^{125}I using Iodogen (Sigma Chemical Co., St Louis, MO, USA) as a solid phase iodinating reagent. Fifty microliters of Iodogen (100 $\mu\text{g}/\text{ml}$) in chloroform were used to coat plastic tubes. After evaporation of chloroform, 10 μg Tg or 50 μg mAb were mixed with 300 μCi Na^{125}I (ICN Biomedicals, Orsay, France) in 50 μl PBS, pH 7.4. After 15 min at 20 °C, the labeling mixture was fractionated on a G-25 M Sephadex column to separate the ^{125}I -labeled protein from free radioiodide. The specific radioactivity was about 25 $\mu\text{Ci}/\mu\text{g}$ for hTg and 3–5 $\mu\text{Ci}/\mu\text{g}$ for anti-T4 mAb and anti-T3 mAb.

Tg RIA

A rabbit anti-hTg immune serum at a $1:10^6$ final dilution was mixed with 0.1–50 ng purified Tg (hTgRP8) or 0.1–100 μl human serum and 100 μl ^{125}I -hTgRP8 (40 000 c.p.m.) in a total volume of 400 μl PBS containing 2 mg/ml BSA. Incubation was carried out for 16 h at room temperature. Immune complexes were collected using sheep anti-rabbit immunoglobulins immobilized on activated silica beads (Biogenesis). 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 measured using a gamma counter. Each serum sample was assayed at two or three dilutions made in duplicate. The sensitivity of the assay was about 2 ng/ml. The interassay coefficient of variation was 10–15%. Comparison of reference Tg

with the international hTg reference material (CRM 457) (10) showed a 1.2 factor of difference.

Procedure to detect thyroid hormone residues on Tg

Adsorption of Tg Serum samples (from 30 μl to 1 ml, supplemented to 1 ml with serum So) from patients with either GD, ST or DTC, or purified Tg (hTgRP3, hTgRP4 or hTgRP8: 10–1000 ng diluted in 1 ml serum So) were introduced into anti-hTg mAb-coated tubes. After incubation for 6 h at 20 °C and overnight at 4 °C, serum was removed and its Tg content was assayed by RIA. The difference between the amount of immunoassayable serum Tg before and after adsorption gave the amount of bound or adsorbed Tg.

Hormone residue recognition on adsorbed Tg by anti-T4 mAb or anti-T3 mAb binding assay

After two washings with PBS-Tween 0.05% to remove any residual serum components, adsorbed Tg was allowed to react with ^{125}I -labeled anti-T4 mAb or ^{125}I -labeled anti-T3 mAb (about 100 000 c.p.m., i.e. 15 ng or 0.1 pmol) in a total volume of 1 ml PBS-BSA. After incubation overnight at 20 °C, unbound radioactivity was removed and tubes were washed three times with PBS-Tween 0.05%. Radioactivity was measured using a gamma counter. Each serum sample was analyzed at least in duplicate.

Results

Characterization of the anti-T4 and anti-T3 mAbs and analysis of their capacity to react with hormone residues on Tg in solution

Figure 1A shows the hormone binding activity of increasing amounts of the anti-T4 mAb and anti-T3 mAb. The amount of mAb that bound 30% of the tracer was 30 ng for anti-T4 mAb and 10 ng for anti-T3 mAb. These amounts of mAb were used to analyze the T4, T3 and reverse tri-iodothyronine (rT3) binding specificity in competitive binding experiments (Fig. 1B and C). The T4 concentration that produced a 50% decrease of the binding of [^{125}I]T4 to the anti-T4 mAb was about 8 nmol/l. T3, up to 1 $\mu\text{mol}/\text{l}$, had a very limited or no displacing activity. rT3 inhibited labeled T4 binding but at concentrations 100-fold higher than those of T4. The T3 concentration that produced a 50% decrease of the binding of [^{125}I]T3 to the anti-T3 mAb was about 3 nmol/l. rT3 did not displace labeled T3 binding. T4 competed with labeled T3 for binding to the anti-T3 mAb but at concentrations more than 100-fold higher than those of T3. Thus, each of the two mAbs exhibited a rather high apparent affinity and a good specificity for its antigen, either T4 or T3.

We examined whether anti-T4 mAb and anti-T3 mAb were capable of detecting hormone residues on Tg

Figure 1 Characterization of the anti-T4 and anti-T3 mAbs. (A) Hormone binding activity of mAbs. [^{125}I]T4 or [^{125}I]T3 ($\approx 40\,000$ c.p.m.) was incubated with variable amounts of anti-T4 mAb or anti-T3 mAb (0–300 ng) in a total volume of 400 μl PBS–BSA. Incubation was carried out for 1 h at 37 °C and immune complexes were collected using sheep anti-mouse IgG immobilized on activated silica beads. After 20 min incubation at room temperature, beads were pelleted by centrifugation at 1500 g for 30 min at 4 °C and washed in PBS–BSA. Bound [^{125}I]T4 or [^{125}I]T3 was expressed as a percentage of the total radioactivity introduced into the assay. Each symbol represents the mean of duplicates. (B) and (C) Hormone binding specificity of mAbs. Competitive binding between labeled T4 or T3 and unlabeled T4, T3 or rT3. [^{125}I]T4 or [^{125}I]T3 ($\approx 40\,000$ c.p.m.) was incubated with 30 ng anti-T4 mAb (B) or 10 ng anti-T3 mAb (C) and increasing concentrations of unlabeled hormone (0–1000 nmol/l) using the experimental conditions described above. Binding values, 'B', obtained for each concentration of competitor were expressed as a percentage of maximum binding, 'Bo', obtained in the absence of competitor. Symbols are the means of duplicates in a representative experiment.

in solution. Three purified hTgRP, with different iodine content, were used as competitor in the RIA; results are reported in Fig. 2. Each of the three purified Tg induced a concentration-dependent displacement of the binding of (i) labeled T4 to the anti-T4 mAb and (ii) labeled T3 to the anti-T3 mAb. As the competition curves were roughly parallel to the curves generated with unlabeled T4 or T3, it was possible to estimate the number of immunoreactive T4 or T3 residues on each purified Tg. Values are reported in Table 1. The higher the iodine content of Tg, the higher the number of immunoreactive T4 residues. As far as T3 is concerned, the low-iodinated Tg, hTgRP4 (two iodine atoms/molecule), exhibited about the same immunoassayable T3 content as the most iodinated Tg, hTgRP8 (20 iodine atoms/molecule).

Adsorption of Tg onto immobilized anti-hTg mAb

Data in Fig. 3 show that purified Tg (from the three reference preparations), diluted in serum So, and serum

Figure 2 Capacity of the anti-T4 and anti-T3 mAbs to detect hormone residues on Tg in solution. Displacement of the binding of labeled T4 to the anti-T4 mAb (A) and of labeled T3 to the anti-T3 mAb (B) by purified Tg. [^{125}I]T4 or [^{125}I]T3 was incubated with 30 ng anti-T4 mAb or 10 ng anti-T3 mAb and increasing concentrations of unlabeled hormone (T4 or T3) or purified Tg (hTgRP3, hTgRP4 and hTgRP8). Incubation and measurements were performed as indicated in the legend of Fig. 1A.

Table 1 Detection of thyroid hormone residues on purified Tg using anti-T4 and anti-T3 mAbs. The T4 and T3 content of three purified Tg preparations, hTgRP3, hTgRP4 and hTgRP8 was evaluated by (i) competitive binding between T4 or T3 and soluble Tg in RIA using the anti-T4 mAb or the anti-T3 mAb (Fig. 2) and (ii) mAb binding assays on adsorbed Tg (Fig. 4). Results are expressed in moles T4 or moles T3 per mole Tg and presented as the mean $\pm$ S.E.M. of the values obtained with three quantities of Tg in at least two different assays. T4 and T3 values obtained by mAb binding assays were calculated from the labeled anti-T4 mAb and labeled anti-T3 mAb binding values and the known specific radioactivity of the labeled mAbs; it was assumed that one mAb molecule reacts with one hormone residue.

hTg	Iodine content (atoms/Tg molecule)	Hormone content of Tg (mole/mole Tg) determined by			
		RIA		mAb binding assay	
		T4	T3	T4	T3
RP4	2	0.14 $\pm$ 0.01	0.014 $\pm$ 0.002	0.012 $\pm$ 0.002	0.0031 $\pm$ 0.0004
RP3	8	0.52 $\pm$ 0.07	0.029 $\pm$ 0.003	0.029 $\pm$ 0.013	0.0071 $\pm$ 0.0004
RP8	20	0.66 $\pm$ 0.06	0.011 $\pm$ 0.001	0.036 $\pm$ 0.001	0.0032 $\pm$ 0.0003

Figure 3 Adsorption of purified Tg and serum Tg on immobilized anti-hTg mAb. Various amounts of purified Tg (10–800 ng of hTgRP3, hTgRP4 or hTgRP8) in 1 ml serum So (A) or serum Tg from DTC, GD or ST patients (30 μ l to 1 ml serum according to Tg concentration) supplemented to 1 ml with serum So (B) were incubated in anti-hTg mAb-coated tubes. After a 6 h incubation period at room temperature and overnight at 4 °C, serum was removed and assayed for its residual Tg content by Tg RIA as described in Materials and methods. Adsorbed Tg was calculated as the difference between the amount of Tg introduced into the tube (total Tg) and the amount of Tg recovered in the serum at the end of incubation (free Tg).

Tg from either DTC, ST or GD patients were quantitatively retained on anti-hTg mAb-coated tubes. The percentage of adsorption of Tg varied from 85 to 90% over a wide range of concentrations (10–1000 ng/ml).

Binding of labeled anti-T4 or anti-T3 mAbs to adsorbed Tg

Binding of radiolabeled anti-T4 or anti-T3 mAbs to anti-hTg mAb-coated tubes, previously incubated (adsorption step) with serum So, was lower than or equal to 0.5%. Radiolabeled mAb binding values significantly higher than the background level were obtained when 10–15 ng hTgRP3, hTgRP4 or hTgRP8 were added to serum So. To exclude a possible interference of serum thyroid hormones through the non-specific adsorption of serum proteins to the anti-hTg mAb-coated tubes, it was verified that neither 1 μ mol/l T4 nor 1 μ mol/l T3 added to serum albumin in PBS or to serum So (during the adsorption step) increased radiolabeled mAb binding over the background value.

As shown in Fig. 4, the binding of radiolabeled anti-T4 mAb to adsorbed hTgRP3, hTgRP4 and hTgRP8 increased linearly with the amount of adsorbed Tg; the slope of the line was different for the three hTgRP, indicating that each purified Tg had a different number of T4 residues accessible to the mAb (Fig. 4). Similarly, the binding of radiolabeled anti-T3 mAb was proportional to the amount of adsorbed Tg. The binding of labeled anti-T4 mAb to Tg was specific since a preincubation of the labeled mAb with 100 nmol/l T4 totally prevented its binding to adsorbed Tg. A preincubation with T3 did not modify the interaction of the labeled anti-T4 mAb with Tg. Similarly, T3 but not T4, inhibited the binding of the labeled anti-T3 mAb to adsorbed Tg. From the binding values of Fig. 4A and B and the known specific radioactivity of labeled mAbs, it was possible to calculate the number of anti-T4 mAb or anti-T3 mAb molecules bound per Tg molecule. There was a good relationship between the number of anti-T4 mAb or anti-T3 mAb molecules bound to adsorbed Tg

Figure 4 Detection of thyroid hormone residues on adsorbed Tg by mAb binding assay. Binding of labeled anti-T4 mAb (A) or labeled anti-T3 mAb (B) as a function of the amount of adsorbed purified Tg. About 100 000 c.p.m. of either ^{125}I -labeled anti-T4 mAb (67 fmol) or ^{125}I -labeled anti-T3 mAb (155 fmol) were added to tubes containing various amounts (0–1000 ng) of adsorbed hTgRP3, hTgRP4 or hTgRP8. Incubation and measurements were performed as described in the Materials and methods section. Binding of ^{125}I -labeled mAbs to adsorbed Tg was expressed as a percentage of total radioactivity. (C) and (D) Relationship between the anti-T4 mAb binding (C) or the anti-T3 mAb binding (D) to adsorbed Tg and the number of T4 or T3 residues detected on Tg in solution by RIA. The mAb binding values reported in panels A and B were expressed in moles anti-T4 mAb or moles anti-T3 mAb bound per mole Tg and plotted versus the number of T4 or T3 residues detected on each hTgRP by RIA (Table 1). Symbols represent the mean, and vertical and horizontal bars the s.e.m. of the values ($n=6-8$) obtained by mAb binding assay and RIA respectively.

(mole per mole Tg) and the number of T4 or T3 residues (mole per mole Tg), disclosed on Tg in solution by RIA (Fig. 4C and D). Considering that one antibody molecule reacts with one hormone residue, the number of mAb molecules bound per Tg molecule was converted into the number of T4 or T3 residues detected per Tg molecule; values are reported in Table 1. About 6% of the T4 residues and 25% of the T3 residues detected on Tg in solution by RIA did react with mAbs on adsorbed Tg.

Identification of hormone residues on serum Tg from patients with thyroid disorders

Serum Tg from 27 patients (8 GD, 13 DTC, 6 ST) was analyzed for its capacity to bind labeled mAbs after adsorption onto the immobilized anti-hTg mAb. The average amount of adsorbed Tg from GD, DTC and ST sera, subjected to the mAb binding assays, was 280 ± 73 , 465 ± 59 and 217 ± 99 ng (mean $\pm$ s.e.m., $n=6-13$) respectively; there was no statistically significant difference between the three groups. The binding of

labeled anti-T4 mAb and labeled anti-T3 mAb to each serum Tg sample is shown in Fig. 5. With one exception, serum Tg from patients with GD did not have detectable anti-T4 mAb or anti-T3 mAb binding activity. The patient whose Tg exhibited a low but significant anti-T4 mAb binding activity did not present any biological or clinical peculiarity as compared with other patients with GD. Out of 13 patients with DTC, only 1 had serum Tg molecules capable of interacting with anti-T4 and anti-T3 mAbs. This case (patient DTC 89) is presented below. In contrast, all of six sera from patients with ST contained Tg with anti-T4 mAb and anti-T3 mAb binding activities. Data in Fig. 6 allow a comparison of the apparent T4 and T3 contents of serum Tg and purified Tg. In seven of the eight serum samples that were positive in the mAb binding assay, the apparent T4 content of serum Tg was close to that of hTgRP4 characterized by a low iodine content. In one case, the apparent T4 content of serum Tg exceeded that of the most iodinated reference Tg, hTgRP8 containing 20 iodine atoms/molecule. Serum Tg samples that were positive in the anti-T3 mAb binding assay showed an

Figure 5 Detection of thyroid hormone residues on adsorbed Tg from human sera by mAb binding assays. Tg present in the serum of 8 patients with GD, 13 with DTC and 6 with ST was adsorbed on the immobilized anti-hTg mAb (see Fig. 3) and incubated with labeled anti-T4 mAb (A) or labeled anti-T3 mAb (B) in the conditions described in the legend of Fig. 4. Labeled mAb binding, expressed as a percentage of total radioactivity, was plotted versus the amount of adsorbed Tg. Symbols represent the mean of duplicate or triplicate measurements on individual sera.

apparent T3 content equal to or higher than that of the three purified Tg.

Analyses of the T4 and T3 content of serum Tg from a patient with thyroid hormone producing metastases. Effect of antithyroid drug therapy

Four serum samples from patient DTC 89 were analyzed for their anti-T4 mAb and anti-T3 mAb binding activities (Fig. 7). The first serum sample (6–89) (corresponding to the positive serum in the group of patients with DTC – Figs 5 and 6) presented a very high Tg concentration and low but significant anti-T4 mAb and anti-T3 mAb binding activities. At that time, the patient, who was hyperthyroid, was placed under methimazole treatment. Four months later (10–89), serum Tg concentration was still elevated but the

apparent T4 and T3 content of serum Tg was decreased by 90%. As the patient was euthyroid, the treatment was stopped and 2 months later (12–89), there was a marked elevation of the apparent T4 and T3 content of serum Tg. From that time on, only one serum sample could be analyzed. Serum Tg from the 4–94 sample was devoid of mAb binding activity. At that time, the patient had received a total dose of 700 mCi and there were no more sites of radioiodide uptake.

Discussion

Using mAbs, an anti-hTg mAb immobilized on a solid phase and radiolabeled anti-T4 or anti-T3 mAbs, we developed a procedure for probing the presence of thyroid hormone residues on minute amounts of Tg in unfractionated human serum. The two steps of the method, (i) quantitative adsorption or capture of Tg and (ii) hormone residue recognition by specific mAbs, have been validated using purified hTg preparations. The adsorption of Tg, through an immobilized anti-hTg mAb, was quantitative for both purified Tg and Tg from the serum of patients with different thyroid disorders. This was not unexpected since this anti-hTg mAb is currently used in a commercialized immunoradiometric Tg assay. The anti-T4 and anti-T3 mAbs, which show a good specificity for either T4 or T3, selectively recognized T4 and T3 residues on Tg in solution and on adsorbed Tg. Serum thyroid hormones, either free or bound to serum proteins, did not interfere in the assay.

Detection of thyroid hormone residues on Tg was only semi-quantitative. The yield of T4 residue detection on Tg in solution (assessed by competitive binding using the anti-T4 mAb in RIA) was about 30% for hTgRP8 (20 iodine atoms/molecule), 50% for hTgRP3 (8 iodine atoms/molecule) and probably near to 100% for hTgRP4 (2 iodine atoms/molecule), assuming that hTgRP4 had an average T4 content of 0.15 residue per molecule. This value was extrapolated from data of Rolland *et al.* (1) who reported that low-iodinated Tg (one to three iodine atoms/molecule) contains 0.1–0.2 T4 residue/molecule. It appears thus that the lower the iodine content, the higher the yield of detection of T4 residues. This finding is in keeping with previous data from Byfield *et al.* (11) who studied the capacity of polyclonal antibodies raised against a T4–albumin conjugate to react with hTg. The yield of detection of T3 on Tg in solution could not be estimated since the actual T3 content of purified Tg preparations could not be accurately measured.

Several factors may contribute to the variability and lowering of the yield of detection of hormone residues on intact Tg in solution. Individual T4 or T3 residues on Tg could vary in their accessibility and/or reactivity with mAbs. The influence of the three dimensional structure of Tg on the ability of mAbs to detect hormone residues on Tg has already been reported (12). Steric hindrance to the approach of a second immunoglobulin after the

Figure 6 Apparent T4 and T3 content of Tg from the serum of patients with DTC, GD or ST (C and D) deduced from the mAb binding assays. Comparison with the apparent thyroid hormone content of purified Tg preparations with different iodine content (A and B). Values of T4 and T3 content of hTgRP3, hTgRP4 and hTgRP8 were taken from Table 1. The apparent T4 and T3 content of serum Tg was calculated from the labeled anti-T4 mAb and labeled anti-T3 mAb binding values presented in Fig. 5 and the known specific radioactivity of labeled mAbs. It was assumed that one mAb molecule reacts with one hormone residue.

first has bound might represent another limitation for the detection of T4 residues on more iodinated Tg molecules.

The capacity of anti-T4 mAb and anti-T3 mAb to react with purified Tg, once adsorbed onto a solid phase, was markedly lowered but to about the same extent for each purified Tg preparation. This indicates that immobilization of Tg causes additional constraints in the reactivity and/or accessibility of hormone residues to mAbs; these constraints were much more pronounced for T4 than for T3. Indeed, the yield of T4 detection on adsorbed Tg was decreased about 16-fold as compared with the yield of T4 detection on Tg in solution, whereas the adsorption of Tg only led to a 4-fold reduction in the T3 detection yield.

Although not quantitative, mAb binding assays provide parameters related to the actual hormone content of Tg. First, the number of T4 residues uncovered on purified Tg by the mAb binding assay increased with the iodine content of Tg. Secondly, as expected from literature data (13, 14), more T3 (as compared with T4) was detected on Tg with a low iodine content than on Tg with a high iodine content. Despite its low yield, the method of hormone residue detection on adsorbed Tg presents a good sensitivity.

Indeed, T4 and T3 residues were readily detected from 40 ng of the low-iodinated Tg, hTgRP4. Considering that hTgRP4 (two iodine atoms/molecule) contained, at the very most, 25% of Tg molecules with one hormone residue (1), one can estimate that, starting from 200–400 ng of adsorbed Tg, the mAb binding assays could detect Tg molecules (with at least one hormone residue) accounting for not more than 2–5% of total Tg. Thus, patient sera with an immunoreactive Tg concentration equal to or higher than 200 ng/ml that were negative in the mAb binding assays, were probably devoid of or contained less than 5% of Tg molecules with thyroid hormone residues. This is the case for 16 out of 21 sera from patients with either DTC or GD. Among the four sera with a lower Tg concentration, one was clearly positive in the anti-T4 mAb binding assay; the presence of Tg with hormone residues in the three other sera cannot be excluded.

The presence of thyroid hormone residues on serum Tg from patients with ST brings experimental support to previously made hypotheses about the source and the mode of release of Tg appearing in the circulation in this disease. In the acute phase of the inflammatory process, Tg molecules that have undergone hormonogenesis in the lumen of thyroid follicles are probably released into

Figure 7 Sequential measurements of serum TSH, serum free T4, serum Tg and apparent T4 and T3 content of serum Tg in patient DTC 89. Date of serum sampling is indicated by two numbers corresponding to the month and the year. Arrows indicate the time of administration of therapeutic doses of ^{131}I . The outcome of WBS after administration of 100 mCi ^{131}I is given at the bottom of the figure (M: metastases disclosed by radioiodide uptake; O: no radioiodide uptake). Oct+: metastases disclosed by Octreoscan. The 4 month period of treatment by methimazole and the period of treatment by L-T4 are indicated at the top of the figure.

the blood stream as the consequence of the rupture of the tightness of follicle lumina. Serum Tg from patients with ST was distinguishable from purified Tg by its apparent T3/T4 ratio, which was 3- to 10-fold higher than that of the three purified Tg. This could be due to particular alterations of Tg structure that would favor T3 recognition and/or minimize T4 detection. Alternately, in the course of their exit from the follicles, Tg molecules might be subjected to proteolytic reactions leading to cleavage of T4 residues. The latter hypothesis is in keeping with previous data (9) showing that immunoreactive serum Tg, identified by Western blotting, decreased after reduction of disulfide bonds.

We have been unable (except in one case) to detect hormone residues on serum Tg from patients with GD. This was unexpected considering previous studies in human (4–6) and in rat (15, 16) suggesting that Tg released by the stimulated thyroid gland would mainly correspond to iodinated Tg molecules, thus originating from the lumen of thyroid follicles. From physicochemical analyses of serum Tg by velocity sedimentation on

sucrose gradients and Western blotting (9), we recently proposed a pathway of Tg release that could explain the lack of T3 and T4 residues on serum Tg from patients with GD. Tg molecules internalized at the apical pole of thyrocytes, conveyed to early endosomes (17) and subjected to apical-basolateral transcytosis (3) could undergo, during their transcellular transport, discrete proteolytic cleavage reactions that could generate hormone-depleted molecules. It has been demonstrated (18) that removal of T4 and T3 residues from Tg can occur without major alterations of Tg structure. Tg from the GD serum which was positive in the anti-T4 mAb binding assay could represent a case of incomplete thyroid hormone removal. Studies on a larger number of GD sera are necessary to determine whether the presence of Tg with hormone residues is incidental or represents a more frequent situation.

Our inability to detect hormone residues on serum Tg from patients with DTC further supports the idea that Tg released by metastatic thyroid carcinoma mostly, if not exclusively, corresponds to non-iodinated molecules

(19). The patient DTC 89, whose serum Tg was found to contain T4 and T3 residues, is an exceptional case in many respects. According to the review of Paul & Sisson (20), less than 20 cases of metastatic thyroid cancer causing thyrotoxicosis have been reported. In the present patient, the production of thyroid hormones by distant metastases was very high; free T4 concentration was about 5-fold higher than the upper limit of the normal range. Tg production by metastases was also extremely high. Using metabolic parameters of serum Tg determined by Lo Gerfo *et al.* (21) in patients with DTC, one can estimate that the maintenance of serum Tg at a value of 30 µg/ml (serum Tg concentration of patient DTC 89) would require a daily production of about 165 mg. By comparison, the amount of Tg synthesized per day by a normal thyroid gland is about 30 mg (22). The high serum T4 and serum Tg concentrations were related to the existence of large tumor masses with differentiated thyroid activities. The presence of T4 and T3 residues on serum Tg from patient DTC 89 indicates that part of the circulating Tg was coming from follicular structures where iodination and coupling reactions take place. This is further shown by the fact that methimazole treatment that efficiently restored euthyroidism caused an almost complete disappearance of serum Tg with hormone residues.

The identification of thyroid hormone residues on circulating Tg, in diverse pathophysiological situations, supports the idea that circulating Tg may serve as substrate for extrathyroidal production of thyroid hormones. The group of Herzog (23, 24) recently reported that macrophages in culture are capable of releasing T4 and T3 from Tg, a process requiring the internalization and then the proteolytic processing of Tg. Furthermore, these authors suggest that T3 and T4 produced by Kupffer cells of the liver could directly act on neighboring hepatocytes. To have a biological significance, this production of thyroid hormones would require elevated serum Tg concentration and/or serum Tg with an elevated hormonal content. There are probably only a few pathological situations in which these conditions are fulfilled. In patients with ST, serum Tg at a concentration ≥ 1000 ng/ml (as was the case in two patients of this study) possessing one or two T4 residues per molecule, could generate an amount of T4 accounting for more than 10% of the average daily normal production of T4 (80 µg/day). It is thus reasonable to think that, in some patients with ST, the extrathyroidal production of T4 may contribute to the transient thyrotoxicosis that characterizes the acute phase of the disease (25).

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