INTERFERENCE OF ANTI-T4 AUTOANTIBODIES IN THE SINGLE STEP FREE T4 RADIOIMMUNOASSAYS

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ABSTRACT

The thyroid hormone profile of a hypothyroid patient, with moderate sized diffuse goiter and history of exogenous thyroid hormone therapy, was found to be highly ambiguous. The total as well as free T4 levels were consistently high (out of range) with suppressed T3 (total and free) and elevated thyrotropin levels. We incubated the serum with the radio-iodine labelled T4 and observed that the patient's serum bound about 50.5% of the total radioactivity resulting in 85.3% interference in the radioimmunoassay. On resolving the serum proteins by cellulose acetate electrophoresis, the radioactivity was detected in the gamma globulin band. The protein profile showed mildly raised levels of IgG. Therefore it was established that the patient's serum carried T4 reactive antibodies (IgG) which were interfering in the immunoassays. Elevated anti-thyroid microsomal antibodies were also present in the patient's serum. The anti-T4 antibodies were highly specific for T4 and did not show any cross reactivity with the T3 or its analogues used in the estimation of free T3. We stripped the serum by precipitating the immunoglobulins and were successful in estimating the levels of free T4 in the stripped serum by single step radioimmunoassay.

KEY WORDS: Thyroid antibodies, anti-T4 antibodies, Free T4 RIA

INTRODUCTION

Autoimmunity plays an important role in the pathogenesis of thyroid disease. Circulating antibodies to various thyroid antigens like thyroperoxidase, thyroglobulin, thyrotropin receptors and microsomal proteins may be found in various autoimmune thyroid disorders (1). Sometimes the autoantibodies may be generated against the thyroid hormones themselves (2,3,4). The incidence of anti-T3 or anti-T4 antibodies with varied specificity and dissociation constants in a euthyroid healthy population is about 1.8% (5). The presence of these antibodies interferes with the estimation of total and free hormones by single step immuno assay (3,4,5,6) and may result in very low (undetectable) values (in competitive RIA/EIA that uses precipitation for the separation of bound and free ligand) or spuriously high values (in immunoassays using antibody coated tubes) of either or both T3 and T4 (7,8). The antibodies in

most of the cases are specific for either of the two hormones although concomitant occurrence of anti-thyroperoxidase or antimicrosomal antibodies has been reported (8,9).

The case of a hypothyroid patient having circulating anti-T4 autoantibodies is discussed here and the interference of this antibody in radioimmunoassays of free T4 has been investigated.

Case Report : The serum sample of a 48 year old hypothyroid male on exogenous thyroid hormone replacement therapy was referred to our laboratory for thyroid function evaluation. He had been on levo-thyroxine for about eleven years, for hypothyroidism diagnosed by low Radio-iodine uptake and extremely low total T4. Clinically, the patient appeared to be doing well with no obvious symptoms of over or under dosage. His thyroid hormone profile showed exceedingly high free T4 levels (out of range) whereas free T3 level was slightly below normal despite continuous exogenous replacement, which the patient did not stop at the time of sampling. The thyrotropin levels were

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found to be elevated. The unconformity of high free T4 with the thyrotropin levels as well as with the clinical condition remained an unanswered question.

We investigated the case in our laboratory, suspecting the presence of some interfering agent in the patient's serum, in the form of anti-T4 autoantibodies or high affinity albumin (familial dys-albuminemia).

MATERIALS AND METHODS

Assay of Hormones: Free T3, T4 and thyrotropin concentrations were assayed in unextracted serum by RIA using the commercial kits supplied by Diagnostic Products Corporation (DPC) USA; Incstar, USA as well as those supplied by Immunotech, France. The DPC kits use polyclonal anti-T3 (or T4) antibodies coated on the polypropylene tubes and are based on competitive type of RIA; Immunotech kits use labelled-antibody and the Incstar kit is a double step RIA kit. The titre of anti-thyroid microsomal antibodies was measured with the help of an EIA kit from Clark Inc., USA.

Demonstration of Anti-T4 antibodies

- a) **Binding of the Tracer:** The method described by Allan *et. al.* (10) was used with slight modifications to detect the binder in the patient's serum. The serum (100 µl) was incubated with 1.0 ml (approx, 20,000 to 40,000 CPM)^{125I}-labelled T3-analogue or T4-analogue from the DPC kits. The incubation was carried out for 2 hours in uncoated glass tubes followed by precipitation with 15% polyethylene glycol (PEG). The precipitates were separated by centrifugation at 1500 x g for 15 minutes followed by a washing with 15% PEG. The precipitates were counted for radioactivity and the binding was calculated as percent of total added radioactivity. The sera from thirty patients with no evidence of any interference in radioimmunoassays of thyroid function tests were processed as above to determine the normal range of binding.

- b) **Fractionation of the binder by electrophoresis:** The patient's serum proteins, along with two control sera were fractionated on cellulose acetate strips by electrophoresis. After visualization with the help of Poncaeu-S stain, the strips were scanned on a densitometer (Beckman, USA). The protein bands, were cut and incubated overnight at room temperature with the radiolabelled (^{125I}) T4 from DPC FT4 kit. The excess of the tracer was removed by two washings with 0.5% tween-20. The radioactivity bound with the protein bands was counted in a multicrystal gamma counter (Berthold, W. Germany).
- c) **Percent Interference:** The percent interference was calculated from the difference in CPM observed in the patient's serum and the expected CPM without the interference of auto-antibodies. An artificial sample with FT4 concentration equivalent to true FT4 level in patient's serum (determined by Incstar assays) was prepared and run in the assay to get the expected CPM and the interference calculated as:

Percent Interference:

$$\frac{\text{Expected CPM} - \text{observed CPM}}{\text{Expected CPM}} \times 100$$

- d) **Protein Profile :** Total protein and albumin in patient's serum was estimated by Biuret and BCG methods, respectively, using commercial kits supplied by Ranbaxy, India. Serum IgG, IgA and IgM were determined by immunoturbidity method with the help of kits procured from Bayer, India.

RESULTS AND DISCUSSION

Thyroid Profile: The thyroid function profile of the patient is tabulated in Table 1. The FT3 was below the normal range, whereas the thyrotropin levels were elevated considerably thus suggesting a hypothyroid state of the patient and insufficient dose of the exogenous therapy. The patient's serum also had a high titer (more than the assay range of the kit used) of anti-thyroid-microsomal antibodies confirming that autoimmune disease

may be the underlying cause of hypothyroidism. The concentration of serum free thyroxine, as determined by the DPC kit, was extremely high i.e. 19.8 pM. This did not correlate with the clinical history of the case. The patient had been diagnosed eleven years back with low total T4 levels and did not have any clinical symptoms of thyrotoxicosis. The possibility of T4-thyrotoxicosis was also ruled out when the FT4 values consistently remained out of range even when the serum was diluted ten times. Therefore, the interference by a serum component seemed probable. A number of preanalytical and analytical factors can be responsible for the aberrations in thyroid function testing (6) and the anomalies in the present case appeared to be because of the presence of an inherent T4-binder in the serum. In our attempt to demonstrate the presence of a specific T4-binder in the patient's serum, we incubated the serum with the radiolabelled T4 analogue. The patient's serum exhibited very strong binding of the tracer (Table 1). The percent binding of the serum was 50.5% compared to the laboratory normal range of 0.68 - 2.02% (mean $\pm$ 2SD). The affinity of patient's serum for 125 I-T3 (0.62%) was within normal range (0.0 - 0.78). This observation confirmed that the FT3 levels as determined by RIA represented the true thyroid hormone status i.e. the exogenous replacement therapy for the patient was inadequate. The extremely high levels of serum FT4 in this patient can be explained by the presence of some interfering agent which

Table 1. Thyroid function test profile of the patient's serum

Investigation	Result	Normal
Free T3 (DPC)	2.2 ng/dl	(2.5 - 5.8)
Free T4 (DPC)	254.1 ng/dl	(0.8 - 2.5)
Free T4 (Incstar)	0.92 ng/dl	(0.9 - 2.5)
Thyrotropin	56.4 μ U/ml	(0.2 - 5.0)
Anti-thyroid-MS-Ab	>3600	(0.0 - 20.0)
Percent Binding of 125 I-FT3	60.2%	(0.0 - 0.78)
Percent Binding of 125 I-FT4	50.5%	(0.68 - 2.02)
Percent Interference in FT4 RIA	85.25 %	(-)

Table 2. Protein profile of the patient's serum

Serum Protein	Concentration	
Total Protein	8.8 g/dl	(6.0 - 8.4)
Albumin	4.8 g/dl	(3.5 - 5.0)
A : G ratio	1.2 : 1.0	
IgG	2270.8 mg/dl	(1000 - 2000)
IgA	310.3 mg/dl	(150 - 350)
IgM	130.0 mg/dl	(15 - 280)
% Serum protein fractionated by electrophoresis		
Albumin	61.3 %	(55 - 65)
Alpha 1 globulins	1.5 %	(1.0 - 5.7)
Alpha 2 globulins	2.7 %	(4.9 - 11.2)
Beta globulins	7.7 %	(7.0 - 13.0)
Gamma globulins	26.8 %	(9.8 - 18.2)

appeared to compete with the antibodies used in the radioimmunoassay. The autoantibodies against thyroid hormones interfering in the RIA have been reported (3,4,8), but the binder reacting with radiolabelled T4 resulting in its precipitation could be any other serum protein also. The increased affinity/capacity of different plasma proteins particularly albumin has been reported as in familial dys-albuminemic hyperthyroidism(11).

Protein Profile: The albumin and A:G ratio in the patient's serum were normal (Table 2), with marginally increased total protein level. The electrophoresis pattern on cellulose acetate paper also did not show any gross abnormality although the intensity of gamma globulin band was slightly increased as compared to the normal. Estimation of the individual gamma globulins showed increased levels of total IgG in the serum. The increased serum IgG levels indicated that the binder may be from immunoglobulins of IgG sub-class.

In order to confirm that the immunoglobulins were the T4-binder in the patient's serum, the proteins separated on cellulose acetate strips were incubated with radiolabelled T4-analogue.

Table 3 Thyroid function test profile of the patient's serum stripped of the immunoglobulins

Investigation	Result	Normal Range
Free T3 (DPC)	2.5 ng/dl	(2.5 - 5.8)
Free T4 (DPC)	0.75 ng/dl	(0.8 - 2.5)
Free T3 (Immunotech)	1.7 ng/dl	(2.0 - 5.0)
Free T4 (Immunotech)	0.77 ng/dl	(0.8 - 2.5)
Anti-thyroid-MS-Ab	28.0 units	(0.0 - 20.0)
Percent ¹²⁵ I-Binding	1.8%	(0.68 - 2.02)

The gammaglobulin fraction in the patient's serum was found to carry almost all the FT4-tracer bound with the cellulose acetate paper; whereas the immunoglobulin fraction of the control sera did not show any significant binding of the tracer (Fig 1). The albumin fraction also did not show any affinity for the tracer thus excluding the possibility of dys-

albuminemic hyperthyroidism. The T4-analogue tracer supplied with the DPC kits and used for this experiment displayed no radioactivity in the beta globulin band carrying TBG due to the presence of 8-anilino-1-naphthaleno sulphonic acid (ANS) in the preparation (12).

It was established from the above study that the patient's serum carried immunoglobulins (IgG) against T4 which were interfering in the assays resulting in falsely elevated T4 results. The effects of these immunoglobulins were eliminated by precipitating them with 15% PEG. The thyroid hormone levels were then measured in the stripped serum. Since this precipitation may disturb the equilibrium between the bound and free hormones, two controls (one hypothyroid and one hyperthyroid) were run in parallel. The results showed that the precipitation resulted in overestimation of the free hormones probably due to release of the hormones from the binding proteins. We calculated a correction factor for this effect and after applying this factor, we were able to get the results very close to that obtained by

Fig 1: Binding of ¹²⁵I-T4 tracer to the protein bands fractionated by cellulose acetate electrophoresis

using Immunotech kit, in which the effect of antithyroid hormone antibodies is known to be minimal (Table 3). Recently, Sapin *et.al.* (13) have suggested that the percent interference calculated as ratio of the radioactivity bound by patient's serum in the presence and absence of the interfering immunoglobulins correlates better with the assay results than the percent binding of tracer as such. In the present case, the interference in the assay was found to be 85.3%. It has been suggested that, in autoimmune thyroid diseases, the iodothyronines within the thyroglobulin molecule simulate the behaviour of a hapten bound to a carrier and thus elicit the immune response to generate antibodies (6). In this patient, the antibodies were specific for T4 and did not appear to have any affinity for T3 or its analogue.

The above report demonstrates the occurrence of T4 antibodies and the dilemma associated with it. The presence of such anti-T3 and/or anti-T4 antibodies, although a rare occurrence, can present

with bizarre laboratory findings. The associated presence of antithyroid microsomal and/or antithyroperoxidase titres has been found to be almost a consistent phenomenon (1,2). In the present study, anti-T4 antibodies interfered only with the estimation of total and free T4 and did not produce any aberrations in the T3 RIA.

The simple incubation of the patients serum with radiolabelled thyroid hormone followed by the precipitation of immune complexes with polyethylene glycol was found to be very effective in demonstrating the presence of these antibodies. The cellulose acetate electrophoresis of serum followed by incubation with radiolabelled tracer appears to be a powerful tool for the detection of abnormal binding proteins.

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