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ORIGINAL ARTICLES

Effectiveness of Different Methods to Eliminate Interference by Thyroglobulin Antibodies in the ELISA for Thyroid Microsomal Autoantibodies

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SUMMARY **Thyroid microsomal autoantibodies (TMA) have been mostly detected by means of either immunofluorescence (IF), tanned red cell haemagglutination (TRCH), or radioimmunoassay (RIA) until the recent development of ELISA. False positives in the ELISA for the detection of TMA due to interference by thyroglobulin antibodies (TGA) present in some test sera reacting with thyroglobulin (Tg) present as a contaminant in the thyroid microsomal preparation (TMP) appears to be common. In this study we tried various ways of removing any Tg contaminant in the TMP by further gel filtration, affinity chromatography of the microsomal preparation or preincubation of the test sera with either Tg or Tg-sepharose 4B immunoadsorbent to absorb out TGA present in the sera.**

Further gel filtration and affinity purification of the TMP failed to totally remove all the contaminating Tg. Preincubation with Tg effectively removed any TGA present in the test sera but resulted in inhibition of the TMA-thyroid microsomal antigen reaction in the test sera including those without TGA. Preincubation with Tg-immunoadsorbent equally effectively absorbed out any TGA present in the test sera but without significant inhibition of the assay reaction in TGA-free sera.

The preincubation of the TMP with Tg-immunoadsorbent is an effective way of removing TGA present in sera without inhibiting the test reaction and thus resulting in false negatives especially in low-titre sera as occurs with the presence of free Tg in the test system.

An autoantibody to a thyroid microsomal component is frequently detected in the sera of patients with autoimmune thyroid disease and has been implicated in the aetiology and severity of the autoimmune process.[1-3] Techniques for the detection of thyroid microsomal autoantibody (TMA) include immunofluorescence (IF), tanned red cell haemagglutination (TRCH), radioimmunoassay (RIA),[4-6] and more recently enzyme-linked immunosorbent assay (ELISA).[7] The haemagglutination technique using tanned red cells or other particles[2,5] is the most widely used technique. However, the TRCH technique is subjectively and semi-quantitatively read and has been shown to be affected sometimes by haemagglutination inhibition factors.[1,8] The isotopic methods are more sensitive than the TRCH techniques and are also objectively scored,[2,6,9] but are beset with the common problems of using radioisotopes.

ELISA techniques for the detection of TMA have only been fairly recently described.[7,10,11] Some of these reports have shown that the TMA-ELISA has a poor correlation with the more widely used TRCH but others have reported the TMA-ELISA to be either as sensitive as or more so than TRCH.[7,10,11] The main problem with TMA-ELISA appears to be how to get rid of interference from any thyroglobulin autoantibodies (TGA) present in the test serum and/or thyroglobulin (Tg) which frequently contaminates the thyroid microsomal antigen preparation (TMP).[2] While some reports have indicated the procedure for the preparation of pure TMP to be straightforward and free of significant Tg contamination,[7,10] others

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have noted that almost invariably there is Tg contamination of the TMP. [2]

In this study we evaluated the effectiveness of various methods to free the TMP of Tg contamination or remove thyroglobulin antibodies in the test serum in the ELISA for TMA.

MATERIALS AND METHODS

Thyroglobulin and Thyroid microsomal antigen component preparation.

Thyroid tissue was obtained at thyroidectomy from a patient with Graves' disease. The tissue was cut into small pieces, flash frozen and then stored in liquid nitrogen until thyroglobulin (Tg) and thyroid microsomal antigen preparation (TMP) were extracted using the method described by Schardt *et al.* [7] After the differential ultracentrifugation, the microsomal antigen preparation was sonicated at 20 KHZ for 2 min at 0° C and the suspension was then centrifuged at 4° C at 1000× g for 30 min. The supernatent was then kept at -20° C. The TMP so prepared (TMP-1) was either used as such to coat the microtitre plates or subjected to further purification in order to remove possibly contaminating Tg in one of the following ways.

Gel filtration (TMP-2).

The crude TMP (TMP-1) as prepared above was passed through a small column, using a modification of the procedure described by Goodburn *et al.* [12] Sepharose-2B (Pharmacia, Uppsala, Sweden) was packed into a small column (2.5cm × 22cm) and eluted with PBS at a flow rate of 30 ml per hour. Two ml portions were collected until the eluate contained no appreciable amount of protein spectrophotometrically. The eluates (fractions 16-18) with high protein concentration and with high thyroid microsomal antigen activity on testing with the standard serum were pooled, dialysed against PBS and concentrated.

Affinity purification using TGA-Sepharose 4B immunoadsorbent, (TMP-3).

Human thyroglobulin antibody prepared in the rabbit (Dakopatt's, High Wycombe, U.K.) was coupled to cyanogen bromide activated sepharose 4B (10mg/ml of gel) (Pharmacia, Uppsala, Sweden) using the instructions on the pack. The crude TMP-1 was mixed with the immunoadsorbent at 4° C overnight and then the mixture was centrifuged at 4° C. The supernatent was then stored frozen at -20° C until utilized.

Thyroglobulin-sepharose 4B immuno adsorbent.

Human thyroglobulin was coupled to cyanogen bromide activated sepharose 4B at a concentration of 15 mg per ml of the gel. This preparation was used to remove thyroglobulin antibodies present in the serum being assayed by preincubating the test sera with Tg-immunoadsorbent.

Thyroid microsomal preparations : TMP-1, TMP-2, TMP-3, and TMP-4 (TMP purified by adsorbing the TMP onto antimicrosomal IgG-sepharose 4B and eluting the IMP), were used to coat the wells of the microtitre plate. In another set of experiments the test serum was preincubated with Tg-immunoadsorbent or Tg to remove any thyroglobulin antibodies present in the test serum. When the test sera were preincubated with Tg, Tg or TGA-Tg complexes were present in the TMP-TMA reaction system. In the Tg-immunoadsorbent system, after overnight incubation, the Tg-immunoadsorbent was separated from the test serum by centrifugation, before introducing the test sera (supernatant) onto the wells of the microtitre plate.

TRCH.

The Thymune-M Wellcome-Burrough kit for the semi-quantification of TMA was employed in the haemagglutination technique. A titre of above 1 in 40 was accepted as positive.

ELISA Procedure

The test was essentially as desribed previously [13,14] except that the coating antigen was one of the microsomal antigen preparations.

ELISA Index

A single serum strongly positive for TMA by TRCH and negative for thyroglobulin antibodies by TRCH and ELISA was used as the standard for the ELISA. The standard serum was diluted in PBS, aliquotted and stored frozen at -20° C. Results are expressed in terms of an ELISA index derived by the following formula :

$$\text{ELISA Index} = \frac{\text{Mean absorbance of test serum - Absorbance of PBST.}}{\text{Mean absorbance of standard serum - Absorbance of PBST}}$$

Sera.

Five sera initially tested for TMA and TGA by both TRCH and IF and with the following results were used: serum U moderately positive for TMA but negative for TGA, serum V strongly positive for both TMA and TGA, serum X was positive for TGA but negative for TMA, serum Y was positive for TMA but negativa for TGA and serum Z was negative for all autoantibodies in an autoimmune profile run. Each serum was tested for TMA by five ELISA procedures using further purified TMP or absorption of TGA in test serum by preincubation with Tg or Tg-immunoadsorbent.

Checkerboard analysis showed the optimum thyroid microsomal preparation (TMP) to be 0.5-2 μg per well, the least concentration of which was chosen. Optimum serum dilution was taken as 1 in 500. Serum samples were run in duplicates and serial dilutions of the standard serum were run in triplicates. On each

plate were included a negative and a positive control sera, aliquots of which were kept frozen.

RESULTS

Precision of ELISA system.

Two sera A and B, respectively positive and negative for TMA were run in ten duplicates in one assay and were also run in each assay as controls. The intra-assay and inter-assay coefficients of variation (%CV) were satisfactory and are summarized in Table 1.

Effect of TMP purification or absorption of TGA on ELISA values of TMA.

The results of ten determinations of each serum by each of the ELISA procedures are summarized in Table 2.

Passage of the crude TMP-1 through sepharose 4B (Test-2) resulted in lowering of the thyroid microsomal antibody ELISA Index (TMA-EI) of the sera containing both TMA and TGA but also showed significantly high EI in serum X that contained TGA but no TMA. TMP-3 (TMP-1 further purified by absorption of TMP-1 of any contaminating Tg by incubation with thyroglobulin antibody-sepharose 4B immuno adsorbent) still showed considerable false positivity in the serum that contained only TGA.

In tests 4 and 5 in which any TGA present was absorbed from the test serum by a Tg-immunoadsorbent or by free Tg, cross-reactivity was minimal. However, the EI of the sera positive for TMA only (sera U and Y) were appreciably reduced when the serum was preincubated and then assayed with the Tg in the assay diluent. Preincubation with Tg-sepharose 4B and then separating the immunoadsorbent before proceeding with the assay produced very little cross-reactivity with TGA and did not significantly alter the EI of sera positive for TMA only. In using the Tg-sepharose 4B to absorb TGA before assay,a ratio of diluted serum to gel of 1:50 was found to remove over 95% of cross-reacting TGA. With low titre TGA positive serum, a serum:immunoadsorbent gel ratio of 1:10 removed most of the cross-reacting TGA. Sera preincubated with immunoadsorbent were assayed in a final dilution of

Table 1. Precision of ELISA for thyroid microsomal antibodies (TMA).

Control serum	Mean ELISA INDEX	SD.	%CV
Intra–assay precision.			
A (N=8)	0.522	0.023	4.4
B (N=8)	0.080	0.010	12.5
Inter–assay precision.			
A (N=6)	0.590	0.049	8.3
B (N =6)	0.077	0.011	14.3

Serum A was strongly positive for TMA while serum B was negative for all autoantibodies in an autoimmune profile screen.

Table 2. ELISA Indices of sera tested for TMA using TMP purified by different methods and by absorption by preincubation of test and standard sera with an immunoadsorbent or thryoglobulin. Each sample was tested ten times in the same assay run.

	Serum				
	U	V	X	Y	Z
TRCH–TMA TITRE	1 : 160	1 : 25000	< 1 : 40	1 : 102000	< 1 : 40
TRCH–TGA TITRE	< 1 : 40	1 : 25000	1 : 102000	< 1 : 40	< 1 : 40
Test system					
1. TMP–1	0.45 (0.07)*	2.52 (0.33)	1.20 (0.10)	2.51 (0.27)	0.09 (0.02)
2. TMP–2	0.41 (0.05)	1.80 (0.25)	0.65 (0.08)	2.31 (0.19)	0.06 (0.01)
3. TMP–3	0.38 (0.05)	1.75 (0.17)	0.54 (0.06)	2.45 (0.21)	0.04 (0.01)
4. Tg adsorbent	0.40 (0.06)	1.64 (0.13)	0.10 (0.01)	2.33 (0.18)	0.08 (0.02)
5. Tg	0.25 (0.04)	1.49 (0.10)	0.11 (0.01)	1.65 (0.15)	0.03 (0.01)

*Mean (SD) ELISA Index of test sera

In test 4 the diluted test serum was incubated with Tg–immunoadsorbent, centrifuged and the supernatant tested in wells coated with TMP–1. In test–5 the diluted serum was preincubated with Tg and added to wells coated with TMP–1.

Table 3. Mean (SEM) ELISA Indices of sera with varying concentrations of thyroglobulin (TGA) and antimicrosomal (TMA) antibodies.

Sera	(N)	TRCH* TGA	TRCH* TMA	Mean (SEM) ELISA index for TMA. Test–1	Test–4	Test–5
PBST	(5)	–	–	0.09 (0.01)	0.08 (0.02)	0.09 (0.02)
Normal	(12)	< 40	< 40	0.14 (0.02)	0.10 (0.01)	0.12 (0.01)
Low TGA	(3)	160	< 40	0.45 (–)	0.14 (–)	0.15 (–)
Low TMA	(7)	< 40	400	0.52 (0.06)	0.54 (0.05)	0.30 (0.09)
High TGA	(3)	6400	400	1.46 (–)	0.40 (–)	0.46 (–)
High TMA	(9)	< 40	25600	0.85 (0.12)	0.85 (0.12)	0.74 (0.11)
Low TGA/Low TMA	(5)	160	1600	0.82 (0.22)	0.61 (0.12)	0.52 (0.10)

*TRCH results expressed as the reciprocal of the titre and the mode titre is given for each group.
(N) = total number of sera tested

1:500 like the rest of the test and standard sera. In test-5, in which the Tg is added to the assay diluent, absorption of all TGA activity was also found to depend on the TGA titre of the serum. In low TGA titre sera ($\leqslant$ 1:320) all TGA activity was abolished at a Tg concentration of 2 μg/ well; whereas, the sera with very high TGA titre often required as much as 16 μg/well to abolish all TGA activity. All microsomal preparations and test systems showed no false positivity in serum (Z) negative for all autoantibodies tested for in an autoimmune profile.

In five sera negative for TMA and TGA but positive for rheumatoid factor, there was no significant cross reactivity in all the test systems as well as in sera positive for antinuclear factor (data not shown). Tests 4 and 5 therefore produced the best results. Further testing was limited to comparing the results of the crude TMP (TMP-1) and tests 4 and 5.

Table 3 shows the comparative ELISA values of different types of sera tested using TMP-1 and tests 4 and 5. Test 5 produced lower results than either test-1 or test-4 False positivity with the crude preparation TMP-1 was found in all the sample groups that contained TGA. The results of test-1 were however not significantly different from those of test 4 in sera groups which did not contain antithyroglobulin antibodies. The mean ELISA index of samples with only TMA present in the sera were significantly lower in test-5 than in either test-1 or test-4 ($p < 0.001$). Three of the seven sera weakly positive for TMA by TRCH were negative by test 5 but were all clearly positive by test 4. Wells to which PBST was added instead of serum consistently yielded absorbances that were lower than the absorbances of wells containing the negative control serum. Addition of BSA 50g/l to the assay diluent resulted in higher absorbances both in wells that contained only PBST-BSA and in wells that contained sera but the EI were not significantly changed. In running the assay therefore BSA was not added to the assay buffer.

DISCUSSION

The clinical usefulness of the detection of thyroid autoantibodies particularly TMA in the sera of patients with thyroid autoimmune disease is well recognised.[2,3,15,16] Whether the presence of these autoantibodies in low titres has any prognostic significance is however uncertain.[15,16] The need to separate clearly the presence of TMA from TGA is therefore of scientific and to some extent clinical interest. Such differentiation is often hampered by the almost invariable contamination of TMP by Tg.[2] Attempts at removing the contaminating Tg have included mostly gel filtration.[2,11,12] In this study, gel filtration appeared to have made little impact in removing the contaminating Tg. Even after affinity separation there was still considerable amount of Tg in the further purified TMP as evidenced by false positivity of sera negative for TMA but positive for TGA. On the other hand, preincubation with Tg or Tg immunoadsorbent effectively removed any thyroglobulin antibodies present in the test sera but was associated with a significant drop in the absorbances of the sera positive for TMA (but contained no TGA) as has been noted by others in the ELISA[11] and in the TRCH[10] systems. Preincubating with Tg-immunoadsorbent gel did not result in any significant decrease in the absorbances of test sera containing only TMA and also effectively removed TGA in sera positive for the latter. The lower readings obtained with Tg preincubation may be because of the non-specific inhibitory effects which the large molecular weight Tg present in the wells

during incubation of sera with the TMP may have on the reaction; whereas, in the Tg-immunoadsorbent system, the physical presence of the adsorbent is not there at the time the antigen-antibody reaction occurs.

The results of this study highlight again the difficulty in preparing pure thyroid microsomal antigens for use in the ELISA of TMA, the main problem being how to effectively remove any contaminating Tg. Contaminant Tg gives rise to false positive TMA detection in sera with antithyroglobulin antibodies. None of the methods reported in the literature and/or tested in this study appears to remove completely the contaminant Tg. The non-specific inhibition associated with preincubation with Tg which would be mostly of significance in samples that are weakly positive was not evident when TGA was absorbed from test sera by preincubating the test sera with Tg-immunoadsorbent. This method is however rather laborious for routine screening of sera for TMA. A pragmatic approach would be to routinely test sera for TMA with the "crude" TMP and TGA initially and to retest those sera positive for both TMA and TGA for TMA by preincubating such test with Tg-immunoadsorbent and testing the resultant supernatant in the ELISA system.

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