Enzyme-Linked Immunosorbent Microassay and Hemagglutination Compared for Detection of Thyroglobulin and Thyroid Microsomal Autoantibodies

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We have evaluated for their potential use in the routine clinical laboratory enzyme-linked immunosorbent assays (ELISA) for human thyroglobulin antibodies (hTg-Ab) and microsomal antibodies (M-Ab). Results are expressed in terms of an "ELISA Index," based on comparison with a laboratory standard. The specificity of both ELISA assays is shown by dose-dependent inhibition of the hTg-Ab and M-Ab activities of the laboratory standards by the appropriate specific antigens. Similar concentrations of ovalbumin had no significant effect on the standard activity in both assays. For consecutive samples evaluated for hTg-Ab (n = 113) and M-Ab (n = 106) by ELISA and hemagglutination, rank order analysis of the results showed a highly significant correlation between the methods (r = 0.81, p = <0.001 for hTg-Ab; r = 0.82, p = <0.001 for M-Ab). However, 8/47 (17%) of samples positive in the hTg-Ab ELISA were negative by hemagglutination, and 7/69 (12%) of samples positive in the M-Ab ELISA were negative by hemagglutination. We effectively excluded the possibility of false positivity of these specimens by ELISA by blocking specimen positivity with the specific antigens in 12 of 14 specimens investigated. We conclude that ELISA techniques for human thyroid autoantibodies are sensitive and specific, easy to initiate, objective, and capable of use in large-scale screening. They are superior to standard hemagglutination techniques by having an increased detection rate for hTg-Ab and M-Ab.

Additional Keyphrases: thyroid status · screening · cutoff value

Circulating thyroid autoantibodies have been widely implicated in the etiology of autoimmune thyroid disease (7), and both human thyroglobulin antibodies (hTg-Ab) and microsomal antibodies (M-Ab) are measured routinely in clinical practice.1 Many techniques have been described for their detection, and newer methodologies have evolved. Immunofluorescent detection of thyroid autoantibodies is sensitive and specific (2), but the evaluation is usually nonquantitative, subjective, and not suitable for automation. Hemagglutination with tanned erythrocytes (3), being sensitive, specific, and suitable for commercial use, has replaced earlier techniques such as precipitin reactions (4) and latex fixation (5); however, it is also highly subjective, only semi-quantitative, and generally unsuitable for large-scale screening. Furthermore, hemagglutination-inhibition factors have been described in thyrotoxic patients (6). Highly sensitive and objective radioimmunoassays, both classical (7) and immunoradiometric (8, 9), have been developed, but these are technically difficult and may require isolation and radioiodination of antibody as tracer.

Recently, enzyme-linked immunosorbent assays (ELISA) for measuring thyroid autoantibodies have been developed (10-14). The ELISA technique offers several important advantages for antibody assessment, including lack of radioiodination; stable, widely available reagents; objective results; rapidity of measurement in automated multiphotometers; and suitability for large-scale screening. Here we report our development of ELISA methods for hTg-Ab and M-Ab assessment and compare these results with those obtained by the hemagglutination method.

Materials and Methods

Antigens. Human thyroglobulin (hTg) was isolated by standard procedures (15) from normal thyroid tissue obtained at autopsy. Tissue was homogenized in phosphate-buffered saline (PBS; pH 7.0, 150 mmol of NaCl and 10 mmol of K2HPO4 per liter) and centrifuged at 100 000 × g. The supernate was then eluted from a 90 cm × 15 mm column of Sephadex G-200 (Pharmacia Inc., Piscataway, NJ) with PBS, pH 7.0. The portion of eluate corresponding to the first protein peak was rechromatographed on Sepha-rose 6B (Pharmacia); the hTg peak was stored in aliquots at -70 °C. Microsomal antigen was isolated as described elsewhere (16). Thyroid tissue from patients with Graves' disease was homogenized in pH 7.4 PBS (150 mmol of NaCl, 8 mmol of KH2PO4 · 7H2O, 1.5 mmol of K2HPO4, and 3 mmol of KCl per liter), centrifuged at 100 × g for 10 min, then at 10 000 × g for 20 min in a Sorvall centrifuge at 4 °C. The resulting supernate was ultracentrifuged at 100 000 × g for 60 min and the pellet resuspended in PBS (pH 7.4). This process of recentrifugation at 100 000 × g was repeated four more times. The final pellet was solubilized in a 1 ml/L solution of Triton X-100 and stored in aliquots at -70 °C. Final protein concentrations were determined by the method of Lowry et al. (17) with human serum albumin as standard.

Enzyme-labeled conjugate and substrate. Anti-human IgG (gamma-chain specific)–alkaline phosphatase conjugate was obtained from Sigma Chemical Co., St. Louis, MO, and p-nitrophenyl phosphate was used as enzyme substrate ("Sigma 104" phosphatase substrate).

hTg-Ab ELISA. We incubated 96-well microtitration plates (no. 76-381-04, Flow Laboratories, McLean, VA) overnight at 4 °C with 200 μL of 10 mg/L hTg solution in carbonate buffer (1.5 mmol of Na2CO3 and 35 mmol of NaHCO3 per liter), pH 9.6. After washing the wells with PBS (pH 7.4) containing 0.5 mL of Tween 20 (polyoxyethylene-(20)-sorbitan monolaureate; ICI Americas, Inc., Wilmington, DE) per liter, we added bovine serum albumin ("RIA" grade, Sigma), 50 g/L in buffered saline (pH 7.4), and let the plates sit for 20 min at 37 °C. The wells were again washed, 200 μL of test
serum (diluted 100-fold in PBS, pH 7.4) or laboratory standard was added, and left to incubate for 3 h at 37 °C. After three further washes, 100 μL of alkaline phosphatase-labeled anti-human IgG (diluted 500-fold) was added and incubated for 30 min at 37 °C. After a final wash, we added to the wells 200 μL of p-nitrophenyl phosphate (1 g/L) in diethanolamine buffer (97 mL/L of water), pH 9.8. The substrate reaction was stopped after 30 min at room temperature by adding 50 μL of 3 mol/L NaOH, at which time we measured the absorbance at 405 nm with an automatic Titertek Multiskan spectrophotometer (Flow Laboratories).

M-Ab ELISA. Microtiter plates were incubated for 5 h at room temperature with 500 μL of microsomal antigen, 5 mg/L in PBS (pH 7.4), then incubated overnight at 4 °C. Subsequent washing and serial exposure to known and unknown samples, enzyme-labeled antibody, and substrate were identical to the above procedure.

Human immunoglobulin G (hIgG) ELISA. This assay was as described by Volkman et al. (18). We incubated overnight, at 4 °C in microtiter plates, 200 μL of goat anti-human IgG (Fab-fragment specific; Cappel Labs, Cochranville, PA), at a concentration of 10 mg/L in carbonate buffer. We used a 50 g/L gelatin solution in PBS (pH 7.4) to block the remaining binding sites on the plastic wells. Subsequent washing and exposure to hIgG standard or unknown were similar to the assays described above.

Hemagglutination assays. We made semi-quantitative measurements of hTg-Ab and M-Ab by using tanned turkey erythrocyte hemagglutination assays in commercially available kits (Burroughs Wellcome Co., Research Triangle Park, NC).

hTg radioimmunoassay. We used a double-antibody radioimmunoassay (15) and the hTg purified as described above. hTg-Ab for the immunoassay was kindly supplied by Dr. C. Von Westharp (University of Alberta).

Nonthyroidal autoantibodies. Antibodies to mitochondria, smooth muscle, nuclear components, and parietal cells were assessed by standard indirect immunofluorescent techniques (19).

Laboratory standards. A laboratory standard for each of the assays was established with sera found by hemagglutination to be high titers. A single serum positive for M-Ab at 1:102 000 titer was used as standard in the M-Ab ELISA. A pool of sera positive for hTg-Ab at >1:5000 titer was used as standard for the hTg-Ab ELISA. The standards were diluted 100-fold in PBS (pH 7.4) and stored in frozen aliquots at -20 °C. The same standards were used throughout the experiments reported here.

ELISA Index. Patients’ results were expressed in terms of an ELISA Index, derived by dividing the absorbance of the unknown sample by the absorbance of a 10 000-fold dilution of appropriate standard. We subtracted background absorbance, as measured for 50 g of bovine serum albumin per liter of PBS (pH 7.4), from both unknown and standard results. Use of the Index allowed comparison of data between assays. We used a 10 000-fold dilution of the standards because this value was on the sensitive part of the dose–response curves (see below).

Results

“Checkerboard” analysis. The optimal antigen concentration for coating the microtiter plates was determined for both assays by analysis of the appropriate laboratory standard at a single dilution on a plate coated with various concentrations of either hTg or microsomal antigen. The maximum difference in absorbance between the laboratory standard and a reference serum negative for thyroid autoantibodies by hemagglutination was at 5 mg/L for microsomal antigen and at 10 mg/L for hTg. The microsomal antigens were incubated at room temperature for 5 h to ensure adequate adsorption to the plastic. To determine the optimal dilution of sera to be used routinely in the assays, we examined dose–response curves of six specimens positive for hTg-Ab by hemagglutination. The closest separation of positive from negative sera occurred at 100-fold dilution, where the absorbances of positive samples were two- to 4.5-fold greater than that of the negative. We used this dilution of sera in both the hTg-Ab and the M-Ab ELISA assays.

Kinetic analysis. Incubation periods for both ELISA systems were evaluated at 4, 22, and 37 °C for 24 h. Absorbance of positive and negative sera was maximum and stable after 3 h at 37 °C for the incubation of the sample, and after 30 min at 37 °C for the incubation of the enzyme-conjugated anti-hlgG.

Standard dose–response curves. Dose–response curves for the hTg-Ab and M-Ab laboratory standards were sigmoidal (Figure 1). The absorbance of the laboratory standard remained greater than the background absorbance of bovine

![Figure 1](https://via.placeholder.com/150)

Fig. 1. Dose–response relationship of hTg-Ab standard in the hTg-Ab ELISA (top) and of M-Ab standard in the M-Ab ELISA (bottom)

Inset A illustrates the SEM for increasing dilutions of standard sera expressed as percent of the 1000-fold dilution absorbance for multiple assays (n = 17) of hTg-Ab, or of the 100-fold dilution absorbance for multiple assays (n = 10) of M-Ab. Inset B demonstrates specific antigen inhibition of the activities of the respective standards (5000-fold diluted) by overnight incubation at 4 °C with increasing concentrations of antigen or ovalbumin.
albumin at a 100 000-fold dilution in both assays. Insets A to Figure 1 express the data from multiple assays (±SEM) as the percentage change from the maximum absorbance obtained for the standard curve. The coefficient of interassay variation, derived from eight consecutive response curves for a 1000-fold diluted standard over an eight-week interval, was 10.1% for the hTg-Ab ELISA and 10.4% for the M-Ab ELISA: the intra-assay CV, derived from eight replicates of 10 000-fold diluted standard, was 5.7% for the hTg-Ab ELISA and 3.4% for the M-Ab ELISA.

Analysis of specificity. Insets B (Figure 1) illustrate the inhibition of antibody activities in 5000-fold diluted standard after overnight incubation with the appropriate antigen. Absorbance decreased with prior incubation with increasing concentrations of antigen. The absorbance of the hTg-Ab and M-Ab standard activities was nearly completely, and specifically, inhibited with antigen concentrations of 10 mg/L (inhibition was 96% for hTg-Ab, 95% for M-Ab). With the ovalbumin control at 10 mg/L, however, the inhibition of absorbance was insignificant (inhibition was 3% for hTg-Ab, 7% for M-Ab).

To further assess specificity, we incubated the 5000-fold diluted hTg-Ab standard overnight at 4 °C with increasing concentrations of microsomal antigen; similarly, the 5000-fold dilution of M-Ab standard was incubated overnight with increasing concentrations of hTg. Inhibition of M-Ab standard absorbance was minor (11%) with hTg at 10 mg/L; however, 57% of hTg-Ab standard absorbance was inhibited with 10 mg of microsomal antigen per liter, suggesting the presence of hTg in the microsomal antigen preparation. All specimens were, therefore, examined in the M-Ab ELISA with and without prior overnight incubation with hTg (10 mg/L) to block possible interaction of hTg-Ab in the sample with hTg in the microsomal antigen on the microtitration plate. The mean (±SEM) inhibition of the M-Ab ELISA indices resulting from prior incubation with hTg in 13 sera positive for both thyroid antibodies by hemagglutination was 10.7% ± 2% (Table 1).

We concluded that apparent contamination of the microsomal antigen with hTg was unimportant in the specificity of the M-Ab ELISA, a conclusion further supported by analysis of three sera negative for M-Ab but positive for hTg-Ab (at titers of 1:5000, 1:80, and 1:20) by hemagglutination, all of which were reported as negative in the M-Ab ELISA. In addition, there was no detectable hTg in a concentration of the microsomal antigen of 7.2 mg/L (greater than that used to coat the microtitration plates) by specific hTg-RIA. These data suggested that inhibition of microsomal antigen in the hTg-Ab ELISA was nonspecific rather than indicative of hTg contamination. Overnight incubation of increasing concentrations of microsomal antigen with hlgG standard (1 mg/L) resulted in nonspecific inhibition of the hlgG standard absorbance in an hlgG ELISA (inhibition was 51% with microsomal antigen of 1 mg/L). The hlgG standard absorbance was inhibited by only 18% and 15% with hTg and ovalbumin (1 mg/L each), respectively. There was no significant binding of alkaline phosphatase-labeled anti-hlgG to antigen attached to microtitration plates, obviating the possibility of hlgG contamination in the antigen preparation.

Establishment of normal range. To determine a normal reference interval for both ELISA systems, we used bovine serum albumin, 50 g/L in pH 7.4 PBS, as control because of the known frequent occurrence of thyroid autoantibodies in the "normal" population (20). An ELISA Index for bovine albumin was constructed from a series of 10 assays for each antibody, by using one of the triplicate determinations as background. The mean Index ± 2 SD gave an upper limit of 0.13 in both assays. To determine whether this range was appropriate, we assessed 16 control sera from hospital personnel with no medical or family history of thyroid disease. The mean ELISA Index ± 2 SD for these 16 controls gave an upper limit for hTg-Ab ELISA of 0.09, and for the M-Ab ELISA, 0.13. We concluded that an upper limit of 0.13 for the normal range would be suitable for both assays.

Comparison of ELISA and hemagglutination. For 113 consecutive specimens for hTg-Ab followed by 106 consecutive specimens for M-Ab received by our routine thyroid laboratory, we used both ELISA and hemagglutination to determine hTg-Ab and M-Ab (Figure 2). All but two specimens positive by hemagglutination had ELISA indices outside the designated normal ranges. Most specimens that were negative by hemagglutination had ELISA Indices within the designated normal ranges, but 15 specimens negative by hemagglutination were positive in the ELISA assays. Rank order analysis (21), in which we assigned a value of 1 to all hemagglutination-negative specimens, showed a highly significant correlation between the results by ELISA and those measured by hemagglutination (r = 0.81, p < 0.001 for hTg-Ab; and r = 0.82, p < 0.001 for M-Ab). Eight of 47 (17%) that were positive in the hTg-Ab ELISA were negative for hTg-Ab by hemagglutination, and 7/60 (12%) that were positive in the M-Ab ELISA were negative for M-Ab by hemagglutination.

We studied further 14 of these specimens negative by hemagglutination but positive by ELISA, using specific-antigen blockade (overnight incubation at 4 °C with hTg or M-antigen, 10 mg/L).

Two samples were inhibited by less than 70% specific-antigen blockade (Figure 3). The single specimen that showed no blockade was repeatedly positive in the M-Ab ELISA and unaffected by microsomal antigen (10 mg/L). Immunofluorescent studies of this serum for autoantibodies (including mitochondrial, smooth muscle, nuclear, and parietal cell antibodies) gave negative results. The ELISA index of the second specimen was repeatedly inhibited by only 30 to 40% with microsomal antigen (10 mg/L) and was negative by the same immunofluorescence screen for antibodies.

Appropriate antigen blockade of the remaining specimens resulted in a mean inhibition of the ELISA index of 87% (SEM 2.5%). Three sera from patients with primary biliary cirrhosis, positive for mitochondrial antibody (1:40, 1:40, 1:60) and with markedly increased concentrations of serum alkaline phosphatase, were negative in the M-Ab ELISA, indicating that neither increased serum alkaline phosphatase nor mitochondrial antibodies produced false positivity in the assays.

Table 1. hTg Blockade of Specimens Positive by Hemagglutination for Both hTg-Ab and M-Ab

<table>
<thead>
<tr>
<th>hTg-Ab M-Ab ELISA Index</th>
<th>Unblocked</th>
<th>Blocked*</th>
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<tbody>
<tr>
<td>1:640 1:1600</td>
<td>0.80</td>
<td>0.86</td>
</tr>
<tr>
<td>1:320 1:1600</td>
<td>1.08</td>
<td>0.99</td>
</tr>
<tr>
<td>1:20 1:400</td>
<td>0.70</td>
<td>0.82</td>
</tr>
<tr>
<td>1:5000 1:6400</td>
<td>0.82</td>
<td>0.76</td>
</tr>
<tr>
<td>1:10 1:100</td>
<td>0.36</td>
<td>0.38</td>
</tr>
<tr>
<td>1:320 1:6400</td>
<td>0.82</td>
<td>0.89</td>
</tr>
<tr>
<td>1:10 1:6400</td>
<td>0.98</td>
<td>0.99</td>
</tr>
<tr>
<td>1:5000 1:25600</td>
<td>0.97</td>
<td>1.03</td>
</tr>
<tr>
<td>1:160 1:1600</td>
<td>0.97</td>
<td>1.03</td>
</tr>
<tr>
<td>1:160 1:6400</td>
<td>0.97</td>
<td>1.03</td>
</tr>
<tr>
<td>1:10 1:6400</td>
<td>0.95</td>
<td>1.00</td>
</tr>
<tr>
<td>1:10 1:6400</td>
<td>0.77</td>
<td>0.59</td>
</tr>
</tbody>
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*Incubated overnight with hTg, 10 mg/L. The indices were derived from closely agreeing triplicate determinations. Paired Student's t-test of unblocked vs blocked M-Ab ELISA indices gave a t-value of 0.41 (not significant).
**Discussion**

ELISA techniques for measurement of hTg-Ab and M-Ab have been developed and characterized. As described, the assays have low intra- and interassay CVs, when used with the ELISA Index based on high-titer hemagglutination sera laboratory standards. Although the derivation of an index assumes a linear rather than a sigmoidal dose–response relationship, we found it simple to apply and convenient for establishing these assays with minimal procedures and reagents. When human monoclonal antibodies to hTg and microsomal antigen become available, then data for standards expressed in mass terms will be simple to derive.

Both assay systems were specific for their respective antibodies, as judged by appropriate antigen inhibition of the absorbance of the standard. The apparent interaction of the microsomal antigen in the hTg-Ab ELISA initially suggested contamination of this antigen with hTg, but further studies indicated that this interaction was nonspecific. This interaction produced no false positivity in the M-Ab ELISA because the M-Ab ELISA Indices of specimens positive for both hTg-Ab and M-Ab were not significantly affected by prior incubation with hTg. Moreover, three specimens positive only for hTg-Ab were negative in the M-Ab ELISA. We concluded that there was no significant cross reactivity of hTg-Ab and M-Ab in these systems. The nonspecific interactions of microsomal antigen may have been due to the detergent used to solubilize the preparation. Detergent may inhibit adsorbance of the hTg-Ab standard through steric interference with hTg-Ab attachment to the hTg-coated plates, as has been shown in other systems (22).

Comparison of ELISA and hemagglutination results revealed that 98% of all specimens determined as negative by ELISA were negative by hemagglutination. However, 17% of specimens that were positive in the hTg-Ab ELISA and 12% that were positive in the M-Ab ELISA were negative in the respective hemagglutination assays. We considered that these specimens were falsely negative in the hemagglutination assay, false positivity by ELISA having been excluded by blockade of the ELISA activity with the appropriate antigen in all but two specimens. Micelle formation enclosing the microsomal antigen may have been responsible for the poor antigen-induced blockade in these two high-titer sera; increasing the concentration of antigen may have achieved greater inhibition.

We are uncertain whether increased sensitivity or a methodological difference between the ELISA and hemagglutination techniques accounted for this increased detection of thyroid autoantibodies. Methodological differences have been previously observed (6) to increase the detection rate of hTg-Ab (double-antibody precipitation technique vs hemagglutination). One interpretation of these observations is the presence of a hemagglutination-inhibition factor, although we have no direct evidence for this. A difference in antibody properties, such as a lack of hemagglutination activity by
some antibody molecules, may be more probable. Methodological differences may account for the two specimens that were positive by hemagglutination but negative in the ELISA. If the thyroid autoantibodies in these sera were IgM rather than IgG, they would not have been detected by the enzyme-labeled anti-IgG in our ELISA methods.

Earlier reports (10, 11) suggested that ELISA techniques for hTg-Ab were not as sensitive as hemagglutination. Our studies suggested that the ELISA techniques were more effective in detecting the presence of thyroid autoantibodies. In retrospect, the hTg used in these previous reports (10, 11) lacked the second-column chromatographic purification step and may have been contaminated with immunoglobulin, which would produce nonspecific interactions with the enzyme-labeled second antibody. McLachlan et al. (12) reported a peroxidase-based ELISA technique for detection of hTg-Ab secretion in lymphocyte cultures and suggested that the ELISA method was more sensitive than hemagglutination for detecting hTg-Ab in vitro. Another ELISA technique requiring the preparation of enzyme-labeled hTg has also been reported to be sensitive but was apparently complex to initiate (13). A further report, which appeared during our studies, described a peroxidase-based M-Ab ELISA (14); these authors obtained a high correlation of results with those by hemagglutination for selected sera of known hemagglutination positivity, but all specimens required hTg blockade because the microsomal antigen was not directly investigated for hTg contamination. We have validated the specificity of our M-Ab ELISA without the necessity for hTg blockade.

Schradt et al. (14) also noted sera positive for mitochondrial antibody to be falsely positive in their M-Ab ELISA. Three such sera were negative in our experiments.

In conclusion: we describe sensitive and specific ELISA techniques for the detection of human thyroid autoantibodies. These assays are easy to initiate; the reagents used are widely available and can be stored almost indefinitely within the laboratory; the assays could be automated; and they provide objective data. In addition, they are superior to standard hemagglutination techniques as shown by their increased detection rate for both hTg-Ab and M-Ab. We conclude that the ELISA technique is suitable for the routine measurement of human thyroid autoantibodies in the clinical laboratory.

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References


