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An Enzyme-Linked Immunosorbent Assay (ELISA) for Antibodies to Thyroglobulin (40786)

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Demonstration of antibodies to thyroglobulin is an important step in the diagnosis of autoimmune thyroid disorders. The presence of circulating thyroglobulin antibodies can also be a useful marker for studies on the familial transmission of autoimmune conditions as well as identifying possible high-risk family members who, while having antibodies, are as yet clinically asymptomatic.

Most of the classical serological tests have been used to measure antibodies to thyroglobulin, but only indirect immunofluorescence (IIF) and indirect hemagglutination (IHA) have found general acceptance, IHA being more sensitive. A radioimmunoassay has been developed for thyroglobulin antibodies and was stated to be useful for measuring low levels of antibody (1). There are, however, few laboratories performing this procedure.

In view of the increasing use of enzyme immunoassays for measuring antibodies, we considered it worthwhile to design and evaluate an enzyme-linked immunosorbent assay (ELISA) (2) for antibody to thyroglobulin. It is the purpose of this report to present the methodology of an ELISA for thyroglobulin antibodies, comparing the results to an established IHA method. The relationship between actual thyroid disease states and ELISA values will be the subject of a future communication.

Materials and methods. Antigen. Thyroglobulin was isolated from human thyroid tissue obtained from surgical and autopsy material. Saline extracts of thyroid tissue were fractionated by ammonium sulfate precipitation, dialyzed, then further purified with Sephadex G-200 column chromatography (3, 4). Protein determination was done by spectrophotometry at 280 nm.

Indirect hemagglutination for thyroglobulin antibodies. Antibodies to thyroglobulin were measured by IHA. Thyroglobulin was coupled to human group O erythrocytes with chromic chloride according to the method described by Bigazzi and Rose (5). The microtitration system for twofold serial dilutions was employed. The titer was considered to be the reciprocal of the last dilution to give agglutination. There were no agglutination reactions when control cells treated with chromic chloride only were used, even at low dilutions of serum.

Enzyme-linked immunosorbent assay. The indirect microplate ELISA procedure (6) was used with the modifications outlined below:

(i) Thyroglobulin was dissolved in carbonate/bicarbonate buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, made up to 1 liter with distilled water and adjusted to pH 9.6). The solution (200 μl) was added to each well of a polyvinyl microtitration plate (Dynatech M220A) and incubated in a humid chamber overnight at 4°C to allow passive adsorption of the thyroglobulin to the polyvinyl surface.

(ii) Unreacted material was removed by washing. This was achieved by emptying the plates, refilling the wells with PBS Tween (phosphate-buffered saline, 0.15 M, pH 7.4, containing 0.05% Tween 20) and leaving for 3 min. The procedure was repeated three times and then the plate was shaken dry before the next reagent was added.

(iii) The test serum or plasma

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specimens were diluted 1:100 in PBS Tween, and 200-μl samples were added to two wells in the plate, permitting duplicate tests on each sample. The plates were then incubated 4 hr at 37°C in a humid chamber.

(iv) Washing (step 2) was repeated.

(v) Conjugate (200 μl) consisting of sheep anti-human globulin labeled with alkaline phosphatase standardized and diluted in PBS Tween (6) was added to each well and the plate incubated overnight at 4°C. The preparation of the conjugate and determination of the working strength was done according to the method described by Voller *et al.*, 1976.

(vi) Washing (step 2) was repeated.

(vii) The enzyme substrate (200 μl) consisting of p-nitrophenyl phosphate, 1 mg/ml in 10% diethanolamine buffer (97 ml diethanolamine, 800 ml H_2O, 1 *M* HCl, added to give pH 9.8, made up to 1 liter with H_2O), was added to each well and incubated for 20 min at room temperature.

(viii) NaOH (50 μl of 3 *M* NaOH) was added to each well to stop the enzyme substrate reaction.

(ix) The contents of each well were removed and the absorbance read in a spectrophotometer at 405 nm.

Checkerboard titrations. It was necessary to determine the optimal concentration of thyroglobulin for coating the plates and the optimal dilution of the serum/plasma to be used in the tests. This was done by a checkerboard titration of positive and negative sera on plates coated with various concentrations of thyroglobulin. The coating solution containing 1 μg/ml of thyroglobulin gave the best separation of values between the reference positive and negative sera and was used in subsequent tests. A titration curve of the positive and negative reference sera tested on plates coated with 1 μg/ml of thyroglobulin is shown in Fig. 1. It was concluded that a serum/plasma dilution of 1:100 was satisfactory.

Results. Normal range of ELISA values. To establish the normal range of ELISA values, 237 sera negative for thyroglobulin-hemagglutinating antibodies

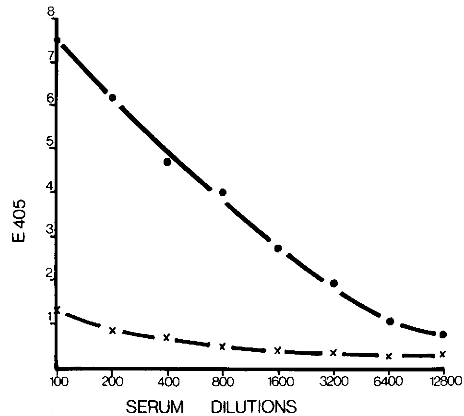

FIG. 1. The titration curves of a positive serum (●) and a negative serum (x) tested by indirect ELISA for antibodies to thyroglobulin.

were tested with the ELISA system. The sera were obtained from medical students, laboratory personnel, euthyroid hospital patients, and relatives of clinic patients. The distribution of the ELISA values for their sera is presented in Fig. 2a.

ELISA values on IHA positive sera. A total of 194 serum samples positive for thyroglobulin antibodies by hemagglutination was obtained from referred hospital patients, and from children with thyroid disease and their relatives. Of these, 41 had very low titers of IHA antibody (2, 4, 8), 66 had low titers (16–128), 56 had medium titers (256–2048), 19 had high titers (4096–16,384), and 12 had very high titers (32,768 and above). The distribution of the ELISA values in each of these groups is shown in Figs. 2b, 2c, 2d, 2e, and 2f, respectively.

The overall correlation of ELISA and IHA titers is presented in Fig. 3. In samples where IHA was negative 95% of the sera gave ELISA values below 1.4 (Fig. 2a). Values over 1.4 were considered as positive. Only three of the IHA negative individuals had values considerably greater than 1.4. All were hospital patients. One person (ELISA 4.0) had sustained a traumatic injury to the thyroid, the other two (ELISA values 2.2 and 2.4) had antibodies to the microsomal fraction of the thyroid as measured by indirect immunofluorescence.

Many (45%) of the sera with very low

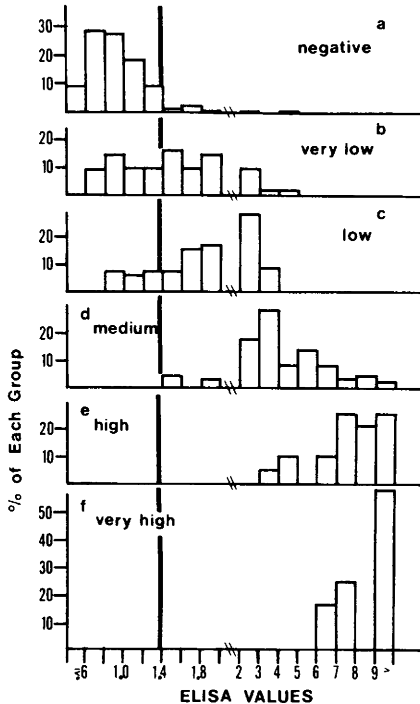

FIG. 2. Distribution (%) of ELISA values for groups of sera tested for IHA antibodies to thyroglobulin: (a) IHA negative sera, (b) very low titers (4–8), (c) low titers (16–128), (d) medium titers (256–2048), (e) high titers (4096–16,384), (f) very high titers (32,768 and greater).

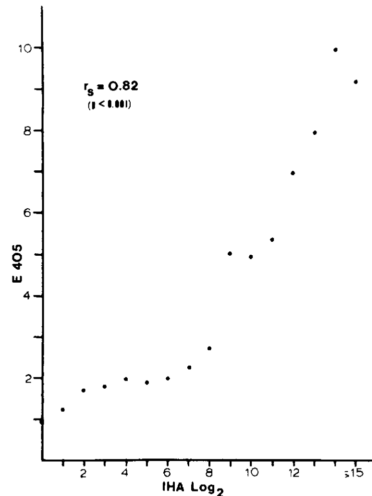

FIG. 3. Overall correlation between the IHA and ELISA tests. Each dot represents the mean ELISA value for the group of sera with the individual IHA titers.

IHA titers (2, 4, or 8) had ELISA values under 1.4 as did 22% of those sera with low IHA titers (16, 32, 64, 128). All sera with medium (256–2048) or high IHA (4096–16,384) titers were positive by ELISA. In fact, 90% of these latter groups had values over 2.0. The Spearman rank correlation between ELISA values and IHA titers (presented in Fig. 3) is 0.82 ($P < 0.001$).

Discussion. The titration curve (Fig. 1) shows that antibody to thyroglobulin can be detected in the reference positive serum sample even when the latter is diluted 1:12,800. However, serial dilution is tedious, and the remainder of the ELISA tests were performed on sera diluted 1:100, which gave the best separation between the reference positives and negatives.

It is apparent that ELISA readily detects antibody to thyroglobulin in those sera which have medium to high IHA titers, but is not very efficient at discriminating between negatives and those samples with low IHA titers. The reason for this is not clear, especially since ELISA has proved to be as sensitive as isotopic assay in other antibody detection systems (7). Possibly the ELISA as set up here is less sensitive than IHA to particular subclasses of antibody to thyroglobulin.

Although it is disappointing that the ELISA exhibited this lower sensitivity in serum samples with lower titers of hemagglutinating antibody, it is convenient for large-scale screening, especially as all the reagents can be provided in a stable form with a long shelf life. However, until ELISA is improved, IHA will remain the test of choice for the screening of thyroglobulin antibodies.

Summary. An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of thyroglobulin antibodies. The ELISA was compared with an indirect hemagglutination technique using thyroglobulin coupled to human group O erythrocytes with chromic chloride. The overall correlation between the two techniques was 0.82 ($P < 0.001$). ELISA proved to be suitable for large-scale screening, as

all reagents are stable, with a long shelf life. However, because of its lower sensitivity, it will probably not as yet replace the chromic chloride IHA test.

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