

Partial characterization and clinical correlation of circulating human immunoglobulins directed against thyrotrophin binding sites in guinea pig fat cell membranes. Development of a direct enzyme immunoassay.

J R Baker Jr, ... , M Berger, K D Burman

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Research Article

To obviate several problems inherent in indirect thyroid-stimulating hormone (TSH) receptor antibody assays, we developed an enzyme-linked immunosorbent assay (ELISA) that measures antibodies binding to guinea pig fat cell membrane, which contain high concentrations of TSH receptors. Solubilized guinea pig fat cell membranes were adsorbed to plastic microtiter plates and served as the solid-phase antigen. Test sera and affinity-purified alkaline phosphatase-conjugated anti-human IgG were co-incubated with membranes, after which p-nitrophenyl phosphate was added. Results were read when a positive control reached a standard color change (OD_{405nm}). Specificity of this assay was demonstrated by the inability of albumin, insulin, TSH subunits, propranolol, or dexamethasone to block binding. 30 normal subjects had a mean OD value of 0.080 +/- 0.050 (SD). 23 of 25 untreated Graves' patients had OD values at least 2 SD above the normal mean (Grave's mean +/- SD; 0.46 +/- 0.33, P less than 0.001) and in each case 10(-6) M TSH inhibited the binding by at least 60%, suggesting that the immunoglobulins were directed at the TSH receptor. Seven of 25 serum samples from patients with Hashimoto's disease, seven of 23 serum samples from patients with transient hyperthyroidism (subacute thyroiditis or painless thyrotoxic thyroiditis), and two of 10 samples from patients with thyroid carcinoma had significant elevations in the titers of membrane-directed immunoglobulins. Graves' patients who were [...]

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Partial Characterization and Clinical Correlation of Circulating Human Immunoglobulins Directed against Thyrotrophin Binding Sites in Guinea Pig Fat Cell Membranes

DEVELOPMENT OF A DIRECT ENZYME IMMUNOASSAY

JAMES R. BAKER, JR., YVONNE G. LUKES, ROBERT C. SMALLRIDGE, MELVIN BERGER, and KENNETH D. BURMAN, *Kyle Metabolic Unit, Endocrine-Metabolic and Allergy-Immunology Services, Departments of Clinical Investigation and Medicine, Walter Reed Army Medical Center, and Department of Clinical Physiology, Walter Reed Army Institute of Research, Washington, DC 20307*

ABSTRACT To obviate several problems inherent in indirect thyroid-stimulating hormone (TSH) receptor antibody assays, we developed an enzyme-linked immunosorbent assay (ELISA) that measures antibodies binding to guinea pig fat cell membrane, which contain high concentrations of TSH receptors. Solubilized guinea pig fat cell membranes were adsorbed to plastic microtiter plates and served as the solid-phase antigen. Test sera and affinity-purified alkaline phosphatase-conjugated anti-human IgG were co-incubated with membranes, after which *p*-nitrophenyl phosphate was added. Results were read when a positive control reached a standard color change (OD_{405nm}). Specificity of this assay was demonstrated by the inability of albumin, insulin, TSH subunits, propranolol, or dexamethasone to block binding. 30 normal subjects had a mean OD value of 0.080 ± 0.050 (SD). 23 of 25

untreated Graves' patients had OD values at least 2 SD above the normal mean (Graves' mean \pm SD; 0.46 ± 0.33 , $P < 0.001$) and in each case 10^{-6} M TSH inhibited the binding by at least 60%, suggesting that the immunoglobulins were directed at the TSH receptor. Seven of 25 serum samples from patients with Hashimoto's disease, seven of 23 serum samples from patients with transient hyperthyroidism (subacute thyroiditis or painless thyrotoxic thyroiditis), and two of 10 samples from patients with thyroid carcinoma had significant elevations in the titers of membrane-directed immunoglobulins. Graves' patients who were treated with ablative therapy at least 6 mo earlier and who were euthyroid when restudied continued to have abnormally elevated membrane-directed immunoglobulins in six of eight samples studied. Further studies involved the substitution of affinity-purified alkaline phosphatase anti-IgM antisera for the anti-IgG antisera routinely used. Seven of 12 serum samples from patients with Graves' disease had significant elevations in binding which in every instance was inhibited by $>60\%$ by 10^{-6} M TSH.

In sum, the present results indicate that (a) we have developed a sensitive, specific, reproducible, convenient ELISA for the measurement both of the total amount of circulating membrane-directed antibodies and of TSH-displaceable membrane-directed immunoglobulins. (b) This ELISA detected significant elevations in TSH-displaceable guinea pig membrane binding in 23 of 25 untreated Graves' patients as well as in $\sim 30\%$ of patients with Hashimoto's thyroiditis

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Address requests for reprints to Dr. Kenneth D. Burman (Kyle Metabolic Unit, Endocrine-Metabolic Service, Walter Reed Army Medical Center).

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and subacute thyroiditis. (c) Elevated membrane directed antibodies may continue to be present many months or years after restoration of the euthyroid state. (d) Circulating membrane binding IgM immunoglobulins have been detected in patients with Graves' disease. Further studies using this ELISA should prove useful in a variety of investigative and clinical studies.

INTRODUCTION

Graves' disease is associated with the production and circulation of heterogenous antibodies, some of which are directed against thyroid-stimulating hormone (TSH)¹ receptors located in thyroid gland membranes (1-9). Recent investigations have proposed that a subset of these antibodies may bind to certain epitopes on TSH receptors and cause unabated stimulation, thereby resulting in thyroid hyperplasia and hypersecretion, the end result of which is detected as an elevated serum 3,5,3',5'-tetraiodothyronine (T₄) and 3,5,3'-triiodothyronine (T₃) and clinical thyrotoxicosis (1-9).

Currently, most assays developed to detect these heterogenous antibodies measure either the inhibition of ¹²⁵I-TSH binding or the stimulation of thyroidal adenylate cyclase (1-9). Studies measuring inhibition of ¹²⁵I-TSH binding to thyroid gland membranes have greatly advanced our understanding of the pathophysiology of Graves' disease (1-3, 5, 6, 8, 9), but suffer, in general, from several major disadvantages: (a) They require fractionation of serum with extraction of the immunoglobulins; (b) ¹²⁵I-TSH binding inhibition represents an indirect method of assessing receptor-antibody interactions and it is not certain that direct binding assays would result in comparable findings; (c) ¹²⁵I-labeled bovine TSH is routinely used rather than a system that directly assesses the relationship between human TSH and its receptor; and (d) these assays, which frequently use protein A-Sepharose columns for serum fractionation, would detect immunoglobulins of only some IgG subclasses and would probably not determine whether other classes (e.g., IgM) play an important role in the receptor-immunoglobulin interactions in Graves' disease.

To study directly the binding of immunoglobulins

¹ *Abbreviations used in this paper:* ANS, 8-anilino-1-naphthalene sulfonic acid; β TSH, beta subunit of TSH; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; EMBA, enzyme assay-determined guinea pig membrane TSH displaceable binding assay; GFP, guinea pig fat cell membranes; HCG, human chorionic gonadotrophin; HTM, human thyroid membrane; MCTD, mixed connective tissue disease; PBS-T, phosphate-buffered saline with 0.05% Tween 20, pH 7.2; PTU, propylthiouracil; SLE, systemic lupus erythematosus; T₃, 3,5,3'-triiodothyronine; T₄, 3,5,3',5'-L-tetraiodothyronine; TSH, thyroid-stimulating hormone.

to TSH receptor in membranes and to increase the sensitivity of previous methodology, we developed a sensitive, simple, reproducible, enzyme-linked immunosorbent assay (ELISA) that can be performed on dilutions of whole serum (10).² Furthermore, this ELISA can be used to assess binding of IgG, IgM, or IgA.

METHODS

Hormones and reagents. Highly purified bovine TSH was a generous gift of Dr. J. G. Pierce, University of California, Los Angeles. Na¹²⁵I was purchased from New England Nuclear (Boston, MA) and Protein-A-Sepharose CL-4B was purchased from Pharmacia Fine Chemicals, (Uppsala, Sweden). Microtiter plates (Immulon II) were obtained from Dynatech Laboratories, Inc., Dynatech Corp. (Alexandria, VA). Affinity-purified alkaline phosphatase-conjugated goat anti-human IgG, IgM, IgA; antisera; and *p*-nitrophenyl-phosphate were purchased from Sigma Chemical Company, St. Louis, MO. Purified human TSH (alpha and beta subunit (β TSH) and intact molecule) were kindly supplied by the Hormone Distribution Program of the National Pituitary Agency (Baltimore, MD) or from New England Nuclear. Human chorionic gonadotropin (HCG) was purchased from Ayerst Laboratories Inc., New York. Purified thyroglobulin was purchased from Pharmacia Diagnostics. Lithium 3,5-diiodosalicylate was purchased from Eastman Kodak Co. (Rochester, NY) and Tween 20 was purchased from Fisher Scientific Co., Fair Lawn, NJ.

² At present the state of nomenclature of the circulating immunoglobulins is confusing and complex. In an attempt to give order to this system, it has been suggested that the acronym TSAB (thyroid-stimulating antibodies) be used to describe assays that measure the ability of circulating immunoglobulins or antibodies to increase cyclic AMP formation and that TBII (thyroid-binding inhibitory immunoglobulins) be used when immunoglobulins are demonstrated to inhibit ¹²⁵I-TSH-receptor binding. It is especially important to note the test procedure used, since it has become obvious that Graves' disease can be associated with a wide variety of circulating immunoglobulins. In an effort to continue this logical practice of denoting the method by which circulating immunoglobulins are measured we have proposed the acronym EMBA to denote immunoglobulins that are determined by an enzyme-linked fat cell membrane binding assay and are TSH displaceable.

Throughout this paper putative TSH receptor binding is defined as sample binding that is >0.18 OD units and that is displaced by >60% by 10⁻⁶ M TSH. Because cross-reactivity studies indicate that there is relative specificity, because TSH, but not other agents tested (except thyroglobulin), can inhibit the binding of circulating immunoglobulins to the crude membrane preparation, and because TSH displaceability was observed in the serum of all Graves' patients, it appears at this time that this type of binding may well be directed against the TSH receptor. It is possible, however, that these immunoglobulins in reality are not directed against the TSH receptor, but against other closely related antigenic determinates which have not yet been recognized. Thus, we cannot at present call this binding TSH receptor specific; further studies are obviously warranted.

Patients. All 25 Graves' disease patients were diagnosed by clinical criteria including elevated serum T₄ (mean±SD; 18.4±5.4 µg/dl) and radioactive iodine uptake and an enlarged thyroid gland. T₄ and TSH were determined by radioimmunoassay and the normal range (mean±2 SD) for T₄ is 4–12 µg/dl and for TSH is <2–5 µU/ml. 25 Hashimoto's thyroiditis patients were diagnosed clinically and each also had titers of either antimicrosomal or antithyroglobulin antibodies that were greater than 1 to 100. (T₄ mean±SD, 9.5±2.8 µg/dl; TSH mean±SD, 7.5±6.1 µU/ml.) Patients with other autoimmune diseases and normal control samples were obtained from clinic patients who had no clinical or laboratory evidence of thyroid disease. 23 patients with subacute thyroiditis in the early phases of their disease (T₄ mean±SD, 10.8±4.5 µg/dl) were studied. 12 of the patients had painful thyroiditis, seven had the painless variant, three had postpartum disease, and one case was indeterminate. 10 thyroid cancer patients were studied after thyroidectomy and, in most cases, after attempted ¹³¹I ablation. In these patients with carcinoma, both surgery and ¹³¹I treatment had been performed at least 6 mo before study; all patients were undergoing evaluation when studied and had T₄ values <1 µg/dl. Serum was collected in all subjects after obtaining informed consent.

Long-term clinical studies. Hyperthyroid patients with Graves' disease were repeatedly studied during the course of their disease by obtaining serial samples and analyzing them in the standard. The patients in this study formed a subset of the original 25 untreated Graves' disease patients.

Preparation of ¹²⁵I-bovine TSH. The chloramine T method of iodination was used as published previously (11). The specific activities of these preparations were ~45–60 µCi/µg. Aliquots were maintained at 4°C until used within 2–3 wk.

Preparation of IgG fractions. IgG fractions were prepared by separation of whole serum on columns of Protein-A-sepharose CL-4B by methods previously reported (9, 12, 13). IgG fractions prepared in this manner were subdivided into aliquots and kept frozen at -70°C until used.

Human thyroid membrane (HTM) preparations. Human thyroid glands were obtained from Graves' patients undergoing thyroidectomy, each of whom had given prior informed consent. The thyroid glands were frozen at -70°C within 30 min of their removal. On a separate day, the glands were thawed and membranes were prepared as described previously (9, 12–14). In brief, minced thyroid gland in ice-cold 1 mM NaHCO₃, pH 7.4 (resuspended in a volume 10 times the original wet weight), was homogenized in a Brinkmann polytron (Brinkmann Instruments, Inc., Westbury, NY) at setting 6 for 5–10 s. This homogenate was then filtered through cheese cloth and centrifuged at 500 g for 8 min at 4°C, the supernatant of which was centrifuged at 15,000 g for 15 min. The pellet formed was washed with Tris-HCl, pH 7.4, buffer. This pellet was resuspended in buffer and separate aliquots were frozen at -70°C until used. Protein concentrations were determined by the dye-binding technique with bovine serum albumin (BSA) as a standard (Bio-Rad Laboratories, Rockville Centre, NY). An aliquot of HTM was further processed to obtain solubilized receptor as described below (14).

Guinea Pig Fat Cell Membrane (GPF) Preparation. GPF containing TSH receptors were prepared by the method of Endo et al. (12). In brief, epididymal and subcutaneous fat was obtained from ~10 guinea pigs. This fat tissue (~100–200 g) was diluted 10-fold (wt/vol) and then minced and homogenized in a polytron homogenizer for 4 s at setting 6 in 20 mM Tris-HCl buffer, pH 7.45. The homogenate

formed was filtered through cheesecloth (two layers) and then was centrifuged at 500 g at 4°C for 5 min. The resulting supernatant was filtered again through cheesecloth and centrifuged at 15,000 g at 4°C for 15 min; the pellet was washed and resuspended in 200 µl of 20 mM Tris-HCl, pH 7.4, at a final concentration of ~3 mg protein, an aliquot of which was further processed to obtain solubilized receptor as described (14). Briefly, the receptor preparation pellet just obtained was solubilized with 0.1 M lithium 3,5-diiodosalicylate (2 ml/10 mg membrane protein) in 20 mM NaHCO₃, pH 9.4. This mixture was homogenized for 3 s with a polytron at a setting of 6 and then incubated 1 h at room temperature and centrifuged at 38,000 g for 30 min. The supernatant was dialyzed at 4°C for 18 h against 20 mM NaHCO₃, pH 9.4, and stored at -70°C in aliquots containing concentrations of ~250 µg/100 µl. The yield of membrane protein was ~1–3 mg/g of wet weight of fat. Further dilutions were prepared as appropriate before use in the ELISA.

EMBA. Sera were assayed for membrane binding activity by a modified ELISA technique (10). 100 µl (~2 µg protein) of solubilized membrane was placed in flat bottomed, polystyrene microtiter plates in 10 mM phosphate and 150 mM NaCl buffer (PBS), pH 7.2, and allowed to incubate overnight at 4°C. The plates were then washed three times with PBS (pH 7.2) and filled with 100 µl of 1% human serum albumin in PBS and incubated for 1 h at 37°C. This washing and blocking procedure with 1% albumin solution was performed to decrease nonspecific binding. The plates were then washed three times with PBS, pH 7.2, containing 0.1% human serum albumin and 0.05% Tween 20 (PBS-T). 100 µl sera (diluted 1:5 with PBS-T) was added to each well and the plates were incubated for 1 h at 37°C and washed three times with PBS-T; 100 µl affinity-purified alkaline phosphatase-conjugated anti-human IgG diluted 1:1,000 with PBS-T was then added to each well and incubated for 1 h at 37°C. After three final washes with PBS-T, 150 µl of a solution of *p*-nitrophenyl phosphate (1 mg/ml) in diethanolamine buffer, pH 9.8, 0.01 M MgCl₂ was incubated in each well until a standard serum reached an OD_{405nm} of 1.10, as read by an automated spectrophotometer (Dynatech Laboratories, Inc.). In selected experiments anti-human IgM or IgA was substituted for anti-human IgG. All sera were assayed in duplicate and values are expressed as average OD_{405nm}. An aliquot of a single high-titer patient's serum was used as a standard throughout the study. The inter- and intraassay coefficients of variation of a given sample were <10%. Each plate always contained control wells to include (a) antibody conjugate alone, (b) normal sera plus antibody conjugate without membrane, and (c) membrane plus antibody conjugate. In all cases, these controls read <0.05 OD units. All samples and controls were analyzed in duplicate and expressed as the mean optical density of each sample.

ELISA (EMBA) competitive binding studies. Competitive binding studies were conducted by the same techniques as the binding assay (see above), with the following modifications. Agents to be studied as competitors for putative TSH receptor binding were diluted to appropriate concentrations in PBS-T. This solution was used to dilute test sera, which then were incubated in the membrane-coated plates for 1 h at 37°C. The assay was otherwise performed in the routine manner. Results were expressed as average optical density units of duplicate samples.

Drug interactions with membrane binding activity. Several sera with high specific binding activity were used to test the *in vitro* effect of drugs by two techniques: (a) They were performed in the same manner as the blocking studies, mak-

ing appropriate dilutions of the drugs in PBS-T and co-incubating all reagents (drugs, sera, and membranes) simultaneously. (b) Preincubation of drug and membrane were performed in the plates for 1 h at 37°C; after washing with PBS-T, sera were then added and the assay was completed in the routine manner. Results are expressed as percentage change from control OD reading.

Column fractionation of HTM and antigenic analysis. To determine whether solubilized HTM preparations could also be used as the solid phase for the assay of TSH receptor specific antibodies, we sought to evaluate the possibility that such preparations contained other antigens, such as microsomes or thyroglobulin, to which antibodies might be found in patients' sera. Thus, 1 mg solubilized HTM was passed over a G100 Sephadex column (0.9 × 60 cm) equilibrated in Tris-NaCl and 30 1-ml sequential fractions were collected over a 90-min period. This column had been calibrated with ribonuclease (mol wt 13,700), chymotrypsinogen A (mol wt 25,000), BSA (mol wt 67,000), and aldolase (mol wt 158,000).

Three microtiter plates were prepared; each plate contained 100 μl from each of the 30 fractions (in duplicate) as well as duplicate samples of crude HTM diluted 1:100 in PBS and crude GPF diluted 1:100 in PBS. These plates were incubated overnight at 4°C and washed three times in PBS-T. To one plate, 100 μl of rabbit anti-human thyroglobulin antisera (thyroglobulin antibody test, Ames Co., Miles Laboratories Inc., Research Products Div., Elkhart, IN) diluted 1:100 with PBS-T was added to each well. To the second plate, a similar dilution of rabbit antimicrosomal antisera (thyroid microsomal antibody test, Ames Co.) was added. To the third plate, human sera with high-titer anti-TSH receptor antibody diluted 1:100 was added. All the plates were washed with PBS-T and to the first two plates affinity-purified alkaline phosphatase-conjugated anti-rabbit IgG (Miles Yeda Laboratories, Rehovot, Israel) diluted 1:1,000 with PBS-T was added. To the third, the affinity-purified anti-human IgG used in the routine ELISA assay was added. The plates were incubated 1 h at 37°C, then washed three times with PBS-T. 150 μl *p*-nitrophenyl phosphate (1 mg/ml) was added to the plates and after incubation at room temperature for 20 min, the optical density readings were determined.

IgA and IgM class studies. To determine whether there was significant TSH receptor binding by immunoglobulin of classes other than IgG, EMBA were conducted with affinity-purified alkaline phosphatase-labeled anti-IgA and anti-IgM antisera diluted 1:1,000. Results were compared with a group of normal controls analyzed at the same time.

Statistical analysis. Unpaired *t* tests were used to determine whether values were different in two comparison populations, and paired *t* tests were used when TSH receptor directed immunoglobulins were analyzed over time in a single patient (15).

RESULTS

Preliminary studies on membrane antigens. The preliminary study to determine the suitability of HTM as antigen substrate revealed binding of antithyroglobulin and antimicrosomal specific antisera in fractions corresponding to a mol wt >67,000. This binding was >100-fold increased over background. These same fractions also showed activity when incubated with Graves' patient's sera. These results were interpreted as indicating that antigenic thyroglobulin and thyroid

microsomes were present in the solubilized HTM fraction, thus making it unsuitable for assaying specifically for Graves' specific IgG. GPF did not show any specific binding to antithyroglobulin or antimicrosomal antibodies and thus was used in the assay throughout the study.

Reaction conditions. Preliminary studies determining optimal activity at different pH levels were performed. Comparison of ELISA activity with carbonate buffer, pH 9.6, or PBS, pH 7.2, revealed maximal antigen coating in PBS at a membrane protein concentration of ~20 μg/ml. Higher membrane protein concentrations resulted in small increments in specific TSH receptor binding activity, but in marked enhancement of nonspecific activity. The membrane protein could be maximally adsorbed to the microtiter wells after incubation at 4°C overnight or at 37°C for 2 h. Throughout this study, unless noted, we used PBS buffer, pH 7.2, at a guinea pig membrane protein concentration of 20 μg/ml and adsorbed the membrane at 4°C overnight.

Positive serum samples maintained full activity after being kept frozen (-4°C) for as long as 8 mo; other positive samples also maintained their original activity after being thawed as many as four separate times.

Binding activity in clinical samples from Graves' disease patients. 25 Graves' patients sera obtained before initiation of treatment were assayed for EMBA, and 23 were found to have significant elevations of their titers (defined as >2 SD above a set of 30 normal controls (Fig. 1). These values ranged from 0.19 to

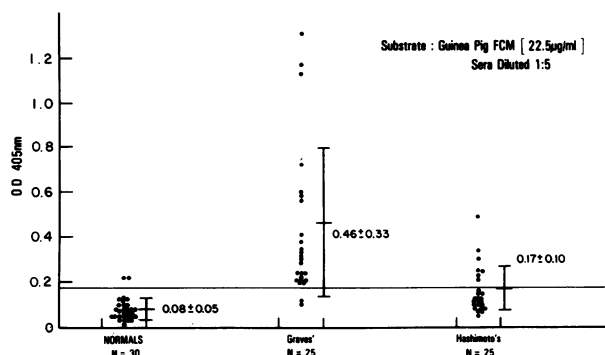


FIGURE 1 Standard EMBA determinations in sera from normal subjects and untreated patients with Graves' and Hashimoto's disease. Values represent the OD_{405nm} of the samples 25 min after addition of substrate, at which time the standard serum had an OD reading of 1.1. All OD values >0.18 (above the line) are >2 SD above the normal mean. The mean of Graves' samples is significantly elevated over controls ($P < 0.001$), and 23 of 25 values are >2 SD above the normal mean. The mean of Hashimoto's samples is also significantly elevated in comparison with normals ($P < 0.05$), but only seven of 25 samples are >2 SD above the normal mean. FCM, fat cell membrane.

1.13, and had a mean value of 0.46 ± 0.33 SD ($P < 0.001$ compared with the mean in normal serum). All positive values in the sera of patients with Graves' disease were displaceable by TSH. The correlation coefficient between serum T_4 and optical density values in the Graves' patients sera was not statistically significant.

TSH competition inhibition. The specificity of the binding assay was shown by its competitive inhibition by TSH. The first five serum samples found to be positive in the screening EMBA were tested with varying dilutions of both human TSH and thyroglobulin, and a displacement curve was developed (Fig. 2). Since all curves were nearly identical in their binding constant and shape, only one example is shown. Significantly elevated binding was judged to be binding in or near the TSH receptor if it was $\leq 60\%$ inhibited by 10^{-6} M TSH. Antisera to Forssman antigen present on guinea pig tissues did not show binding inhibition by TSH. Thus its binding activity could not be accounted for by binding on or near the TSH receptor on the membrane.

Other inhibitors. Equimolar (10^{-6} M) concentrations of alpha subunit and β TSH and high molar concentrations of insulin, and HCG did not possess the ability to inhibit the membrane binding (Fig. 3). 10^{-6} M Thyroglobulin, which was shown not to be contaminated with TSH by high-performance liquid chromatography, did show an inhibition pattern similar to that of TSH, but at higher molar concentrations (Figs.

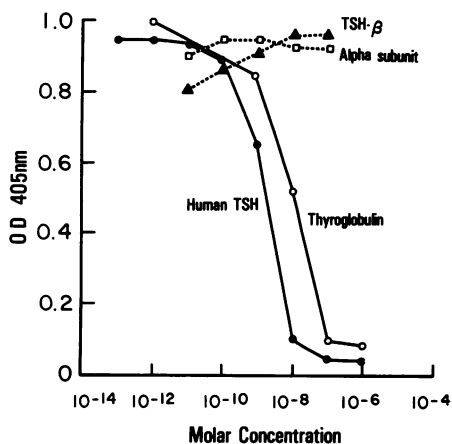


FIGURE 2 Specificity of inhibition of the binding of circulating immunoglobulins to GPF. Increasing concentrations of TSH revealed inhibition curves compatible with the displacement of one binding site. The concentration at which the optical density reading is reduced 50% by TSH is between 10^{-9} and 10^{-8} M. Thyroglobulin had a similar appearing curve, with 50% inhibition occurring at $\sim 10^{-8}$ to 10^{-7} M. Alpha subunit and β TSH showed no appreciable inhibition of binding.

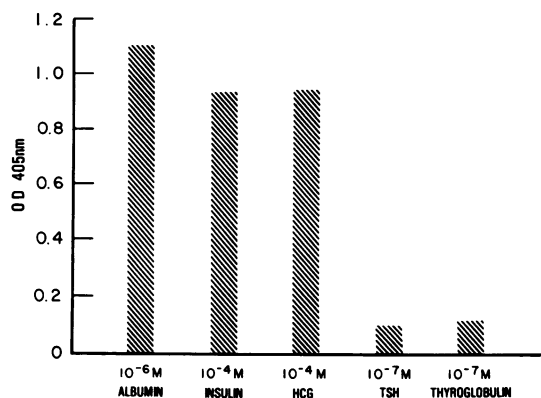


FIGURE 3 Hormone inhibitors of EMBA activity. Hormones were incubated simultaneously with positive sera and adsorbed membranes to determine whether there was competitive inhibition of binding activity. Only inhibition of $>20\%$ was considered significant in comparison with normal variation. Human TSH and thyroglobulin showed evidence of inhibition, whereas insulin, albumin, HCG, and alpha subunits and β TSH did not.

2 and 4). T_3 and T_4 did not cause inhibition in concentrations as high as 1×10^{-5} M (data not shown).

Drug studies. Various agents known to be effective in the clinical treatment of Graves' disease or are known to affect TSH receptor binding in vitro or in animal studies were investigated to determine their effect upon TSH receptor binding immunoglobulins in this ELISA (Table I). Only propylthiouracil (PTU) had an effect on binding in physiologic concentrations of 5×10^{-3} M. 8-Anilino-1-naphthalene sulfonic acid (ANS) and beta mercaptoethanol both inhibited binding (by $\sim 50\%$ at 5×10^{-4} M), probably because of a direct effect upon either circulating immunoglobulins or the receptor itself. Dithiothreitol (DTT) caused inhibition only when premixed with the immunoglobulin, and was thus thought to exert its effect by disrupting the structure of the immunoglobulin, rather than by directly interfering with receptor binding. Propranolol, methimazole, dexamethasone, oxidized glutathione, and methiamide had no effect on binding at concentrations as high as 5×10^{-2} M.

Comparison of EMBA with ^{125}I -TSH displacement. Of eight Graves' patients positive in EMBA with enough sera available to isolate IgG, all were positive by ^{125}I -TSH displacement, with GPF as the source of TSH receptor. Of 18 normals who did not have elevation of EMBA titers, none was positive for ^{125}I -TSH displacement. Two normal subjects who had significant EMBA titers were negative for ^{125}I -TSH displacement. In every instance, serum displayed concordance in activity with respective IgG fractions. Furthermore, all whole serum samples and immunoglobulin frac-

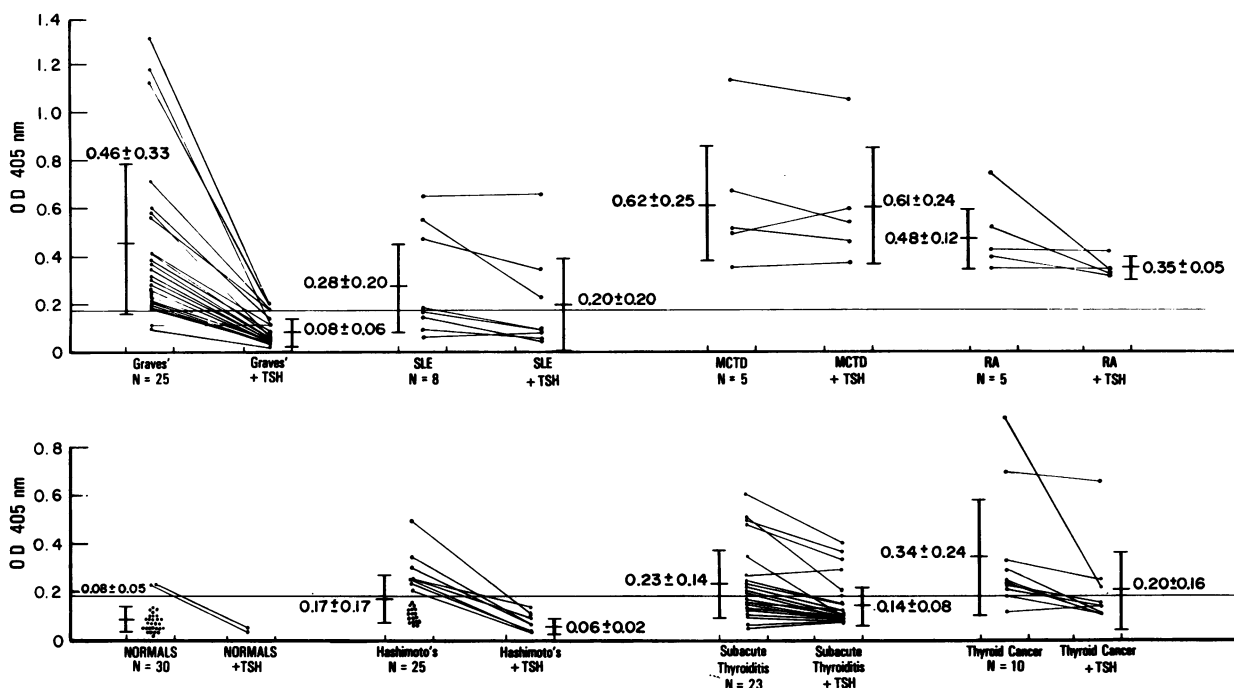


FIGURE 4 Determination of EMBA binding specificity by competitive TSH inhibition. The eight different patient groups were analyzed as to the specificity of the EMBA activity by displacement with purified human TSH at a concentration of 10^{-6} M. Inhibition of activity of $\geq 60\%$ was defined as the criterion for the presence of TSH-displaceable antibodies. The sera from all Graves' patients and from seven of seven Hashimoto's patients with significantly elevated activity showed TSH displacement, whereas only two of four SLE patients, none of five MCTD patients and one of five patients with rheumatoid arthritis (RA) showed activity that was TSH displaceable. In patients with subacute thyroiditis, seven of 13 samples with elevated activity were displaceable with TSH, whereas two of nine samples from patients with thyroid carcinoma (with elevated readings) were TSH displaceable. The two normal patients who had elevated readings also showed TSH displacement.

tions that resulted in positive activity were displaceable by TSH.

EMBA in patients with rheumatic disorders. In the serum from eight patients with FANA-positive systemic lupus erythematosus (SLE), four had elevated readings; two out of these four were displaced by TSH. Five of five patients with mixed connective tissue disease (MCTD) had elevated optical density readings, but none showed $>60\%$ TSH suppression. Five of five rheumatoid arthritis patients had elevated readings, but only one of these had binding values decreased by $>60\%$ with TSH.

EMBA in patients with other thyroid disorders. In contrast to the Graves' patients, only seven of 25 patients with Hashimoto's thyroiditis had optical density readings >0.18 and all were displaced by TSH (Figs. 1 and 4). Patients with subacute thyroiditis showed significant elevations of binding activity in 13 of 23 tested; seven of these 13 positive values were TSH displaceable. The mean (\pm SD) OD reading of

0.188 ± 0.12 in the painful variant was not significantly different from that of 0.29 ± 0.19 in the painless variant. Patients with thyroid carcinoma had elevated titers in nine of 10 samples assayed (mean 0.34 ± 0.24 SD); two of these nine positive samples were displaced by TSH.

Clinical correlations. Results from Graves' patients were divided into two groups, allowing a retrospective analysis of EMBA titers before treatment, and again at least 6 mo after normalization of thyroid status and restoration of the euthyroid state. Of the group that received only medical treatment, mean pretreatment titers were 0.18 ± 0.06 and did not significantly drop after thyroid function tests had normalized. Patients who had surgery or received ^{131}I therapy had a significantly higher ($P < 0.01$) mean initial titer of 0.5 ± 0.35 than medically treated patients. Six of eight samples from patients treated with surgery or ^{131}I were still abnormally elevated when they were restudied in the euthyroid state, but mean values dropped significantly to 0.32 ± 0.28 ($P < 0.05$). When the medically

TABLE I
*In Vitro Effect of Various Agents on the Binding of
 Circulating Immunoglobulin to GPF*

Agent	Concentration above which an alteration of binding activity is noted	Change in OD Reading
		%
PTU	5×10^{-3} M	36
Methimazole	None (5×10^{-2} M)	None
Dexamethasone	None (5×10^{-2} M)	None
Glutathione (oxidized)	None (5×10^{-3} M)	None
Methiamine	None (5×10^{-3} M)	None
Propranolol	None (5×10^{-3} M)	None
ANS	5×10^{-4} M	50
Beta mercaptoethanol	5×10^{-4} M	60
Na Iodate	5×10^{-4} M	None
DTT	5×10^{-3} M*	90

Results in all studies (except with DTT) were concordant when agent was preincubated with membranes before the addition of a positive serum or when agent was co-incubated with membrane and positive serum.

* DTT showed inhibition only when incubated with antibody.

treated patients were restudied when they were euthyroid, the highest sample decreased from 0.3 to 0.28 and the remaining five pretreatment borderline elevated samples did not generally change greatly as the posttreatment range for these five samples was 0.100 to 0.220, mean 0.16 ± 0.06 (NS).

IgM and IgA studies. 12 Graves' patients sera and 20 sera from normal subjects were analyzed with affinity-purified alkaline phosphatase-conjugated goat anti-human IgM or IgA antiserum, instead of IgG antisera. When either the IgA or IgM antiserum was incubated with membrane alone or patients serum alone (Graves' and normals) the optical density reading was < 0.05 . Furthermore, this IgM antiserum was shown not to cross-react with IgG in whole serum or IgG after fractionation on columns of Protein-A-Sepharose CL-4B. All normal and Graves' patients sera had optical density readings less than controls when IgA antiserum was used, suggesting they did not have immunoglobulins of the IgA class (Fig. 5) directed against GPF. In contrast, when the IgM antiserum was used, the mean reading of the Graves' sera (0.43 ± 0.24) was significantly higher ($P < 0.05$) than that of 0.23 ± 0.07 noted in normal patients' sera, suggesting the presence of membrane binding antibodies. Further, seven of seven positive Graves' sera tested with anti IgM had $> 60\%$ suppression by TSH, suggesting that these circulating membrane-directed immunoglobulins were directed specifically at or near the TSH receptor (Fig. 6).

DISCUSSION

We developed a rapid ELISA capable of detecting circulating GPF antibodies (putative TSH receptor antibodies) in dilutions of whole serum. This assay can be easily performed within one working day and has the further advantages that isotope is not required and that direct interactions of the circulating antibodies with the putative TSH receptor are assessed. This ELISA has inherent advantages over the ^{125}I -TSH binding assays used to date (1-3, 5, 6, 8, 9). ^{125}I -TSH binding assays require the preparation of IgG fractions from sera, generally performed with protein A-Sepharose Cl-4B columns. These column procedures are quite time consuming and usually only about five to 10 samples can be processed daily. In contrast to the more difficult ^{125}I -TSH binding assay, the ELISA assay presented herein is sensitive enough to detect circulating antibodies in 23 of 25 untreated Graves' patients. In the majority of reports with the ^{125}I -TSH binding assay, it has been noted to be positive in only ~ 70 -80% of Graves' patients (5, 16-19), although two recent studies have indicated this percentage to be 86% (8) and 94% (9). Borges et al. (9) modified the ^{125}I -TSH binding inhibition assay so that whole serum could be analyzed; ^{125}I -bovine TSH binding was $86.3 \pm 7\%$ with control normal serum samples and decreased to $61 \pm 15\%$ in serum from Graves' disease patients. There was, however, considerable overlap between the two sets of samples as the normal serum samples ranged from 74 to 104% of serum-free controls. These authors report (9) that 27 of 35 samples (77%) from Graves' disease patients were > 2 S.D. below the mean average in normals.

The *in vitro* studies reported here demonstrated that the ELISA described is relatively specific, as only TSH (and thyroglobulin) displaced antibody binding, whereas the alpha TSH subunit, β subunit, insulin, HCG, and albumin did not interact. This inhibition was not observed in most antisera that were thought to be against antigens on the GPF not involved with TSH binding. Thyroglobulin has previously been noted to inhibit ^{125}I -TSH binding to TSH receptors (16). The pathophysiologic meaning of this thyroglobulin interaction is unknown. We have also performed various *in vitro* studies directly assessing whether various agents influenced immunoglobulin-membrane binding. We observed that 5×10^{-3} M PTU decreased the binding of serum immunoglobulin to the TSH receptor. This level of PTU is higher than that observed in serum (17), but may be comparable to that achieved intrathyroidally. Our results suggesting that PTU may inhibit immunoglobulin binding to TSH receptors on membranes represents another mechanism involving immunoglobulin formation or action by which PTU

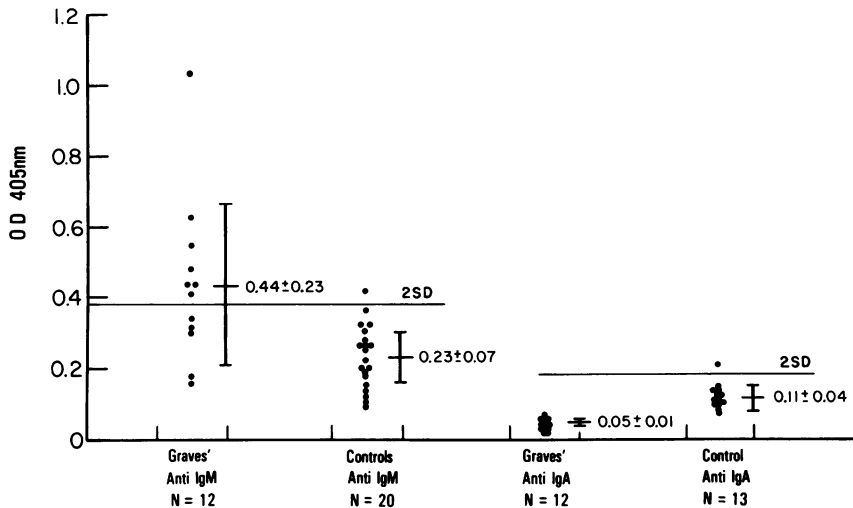


FIGURE 5 Assay for IgM and IgA antimembrane antibodies was conducted in the same manner as the routine EMBA, except that a 1:1,000 dilution of affinity-purified alkaline phosphatase-conjugated anti-IgM or IgA was substituted for the anti-IgG. Results are optical density values determined at 15 min after the addition of substrate, when a control positive serum read ~ 1.0 . Graves' patients had significantly higher mean values than normals ($P < 0.05$) in the IgM assay, but not in the IgA assay. Seven of 12 Graves' samples had significant elevations (>2 SD above the normal mean) in their optical density readings.

could be effective in the treatment of Graves' disease; recent studies have already indicated that carbimazole and PTU may decrease total immunoglobulin produc-

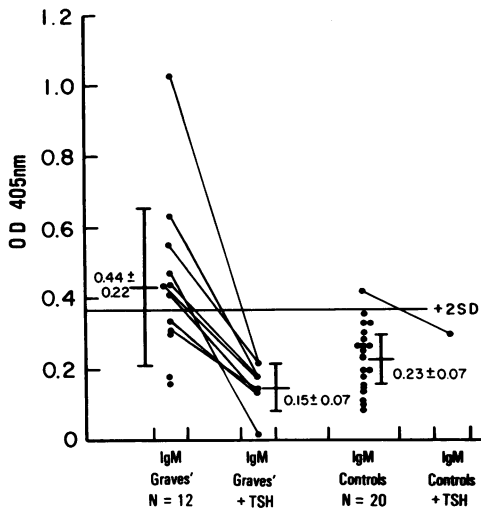


FIGURE 6 IgM antimembrane antibodies in serum are shown to be relatively specific in their binding by inhibition with TSH. Six of seven serum samples with IgM activity >2 SD above the normal mean suppressed $>60\%$ with the addition of TSH, and the seventh was just under the cutoff, at 56%. The serum from the one normal individual with an elevated reading suppressed only 33%.

tion in cultured lymphocytes (18, 19). We noted that other agents used clinically to treat thyrotoxic subjects (e.g., propranolol, dexamethasone, and ipodate) had no direct effect on decreasing TSH displaceable binding in our assay. Propranolol and hydrocortisone have both been reported (20) to inhibit TSH receptor release into the incubation medium, presumably by their membrane stabilizing effect. No direct effect on ^{125}I -bovine TSH binding to the TSH receptor was observed (20), findings consistent with those we observed with the ELISA. It also appears from our studies that DTT, a sulfhydryl reducing agent, altered the immunoglobulin structure and thus decreased binding. Ozawa et al. (21) had previously observed that sulfhydryl oxidizing agents increased and reducing agents decreased TSH receptor-mediated adenylyl cyclase activity. The mechanism of the ANS-mediated inhibition of ELISA activity is unknown, although ANS is capable of inhibiting thyroxine binding to serum binding proteins and of decreasing T_3 binding to its receptor (22).

GPF have been noted to be a particularly rich source of TSH receptors (9, 12, 13). We used this source of receptor exclusively in the present studies, mainly because their use abrogates the potential artifactual positive binding that may be present in thyroid gland membranes. Indeed, we demonstrated that crude human thyroid membranes are unsuitable for the assay of TSH receptor-specific antibodies, since they contain antigens that react with antithyroglobulin and anti-

crossosomal antiodies (23). These antigens probably represent contamination due to the relative impurity of the membrane preparations used.

As reported by Tao and Kriss (24), we also observed that sera from patients with various autoimmune diseases have circulating immunoglobulins directed against membranes. They (24) observed that 12 of 21 patients with Hashimoto's thyroiditis, three of six patients with rheumatoid arthritis, seven of eight with SLE, and four of six with MCTD had antibodies directed against GPF, using ^{125}I -Staphylococcus Protein A as the probe. It is known that ^{125}I -Staphylococcus Protein A binds mainly to IgG (Subclasses 1, 2, 4), but it may also react with other Ig classes (25, 26); thus, this assay does not define the subclass to which these circulating antiimmunoglobulins belong. Finally, because TSH displacement was not performed, it is not possible to ascertain whether their ^{125}I -Staphylococcus Protein A binding assay detects receptor-directed immunoglobulins. The ELISA described herein confirms the findings of a high frequency of circulating immunoglobulins directed against GPF in autoimmune diseases (24). Approximately 30% of serum samples from patients with Hashimoto's disease, painless thyrotoxic thyroiditis, or subacute thyroiditis had TSH-displaceable immunoglobulins; in contrast, only 20% or less of serum samples from patients with thyroid carcinoma or a variety of rheumatic or collagen diseases had TSH-displaceable immunoglobulin binding. We interpret these findings to indicate that most membrane binding antibodies in autoimmune diseases are directed against antigens separate from the TSH receptors. The ~20% that were TSH displaceable may represent antibodies against determinants near or part of the TSH receptor itself. However, they may be different from the antigenic determinants in the receptor responsible for thyroid stimulation in Graves' disease, as these autoimmune patients had no evidence of hyperthyroidism. We also confirmed the presence of TSH-displaceable antibodies in some normal patients. We do not understand why normal subjects appear to possess these antibodies (in a slightly higher frequency than expected by statistical definition, i.e., 2.5% of the normal population), but we were intrigued to find that several of the euthyroid individuals with such antibodies had a positive family history for autoimmune thyroid disease. Perhaps the EMBA assay is detecting antibodies that antedate the initiation of clinical disease. Alternatively, it is possible that even normal individuals produce these TSH receptor antibodies, as Brown et al. (27) have recently suggested. At present, we are not certain whether these membrane-directed TSH-displaceable immunoglobulins have any physiologic implications. It has been postulated that in normal subjects these antibodies could be present as part

of the normal homeostatic regulation of receptor production and/or disposal (28, 29). Further studies in normal patients as well as in patients with collagen diseases are required to determine the role of TSH receptor-directed immunoglobulins in these conditions.

One of the more intriguing aspects of the present study is that TSH displaceable GPF-binding immunoglobulins that were initially present in the sera of Graves' patients did not, in many subjects, normalize with restoration of the euthyroid state, regardless of the treatment modality. In a retrospective analysis, patients who went into remission while receiving only antithyroid medications had a significantly lower mean EMBA titer at initial diagnosis than those patients who required definitive therapy. Despite maintaining elevated levels of TSH displaceable antibodies, it would seem unlikely that these patients would become thyrotoxic again, especially because of the long duration of follow-up at which they have maintained euthyroidism (with or without thyroid hormone treatment). Our results with ELISA techniques suggest that some Graves' patients who are restored to euthyroidism may still have detectable TSH-displaceable membrane-directed immunoglobulins and appear discrepant with several previous reports (7, 16, 30-32) indicating that after several months the levels of these immunoglobulins were restored to normal in this circumstance. Although we are uncertain why our results differ, we speculate that it could be related to the fact that our assay is more sensitive or, perhaps, is measuring a different population of circulating antibodies. In this regard, it should be emphasized we are measuring putative receptor binding, not glandular stimulation. However, our results are consistent with the recent findings by How et al. (33) that nine of 18 patients who had thyrotoxic Graves' disease (treated with PTU) 2 yr earlier were still sensitized to thyroid antigen and continued to demonstrate a suppressor-cell abnormality, even though they were clinically and biochemically euthyroid. Moreover, Wood and Ingbar (34) reported that, when 15 patients with Graves' disease who had PTU-induced remission were studied ~25 yr later, approximately nine had a demonstrable abnormality in thyroid hormone homeostasis. The types of abnormalities generally indicated hypothyroidism with a subnormal T_4 response to exogenous TSH administration, abnormal perchlorate discharge test and the presence of antimicrosomal antibodies (and recurrent thyrotoxicosis in one), supporting the concept that Graves' disease is an autoimmune process that is progressive over time and theoretically could be associated in some patients with persistent detectable levels of TSH receptor-directed immunoglobulins. Furthermore, it is consistent with present theories of

immunoglobulin regulation that treatment modalities directed at the thyroid gland might not be expected to affect the production of immunoglobulins (1), especially if the putative antigenic stimulus (i.e., TSH receptor) was physically still present.

Perhaps the most interesting data in this paper relate to the possibility that there are membrane-directed circulating IgM class immunoglobulins that may be receptor specific. Several recent studies have indicated that the levels of TSH receptor-directed immunoglobulins appear to be directly proportional to the IgG concentration and that it is considered likely that IgG immunoglobulins account for most of the antireceptor activity (35-37). However, these studies also indicate that the ability to inhibit ¹²⁵I-TSH binding to receptors also can reside in other serum fractions, including a 19S and an alpha-globulin-albumin-containing fraction. (35) To date, however, no other study has investigated the possibility of IgM-TSH receptor interaction. If our results are confirmed, then further detailed assessment of this receptor interaction and of the possible role of IgM-mediated thyroïdal actions is indicated. It is conceivable, for example, that previous reports of Graves' patients with negative circulating anti-TSH receptor IgG antibodies may be explained by the presence of IgM immunoglobulins. Indeed, the treatment of the serum in most earlier studies (e.g., separation by Staphylococcus Protein A-linked Sepharose columns) would have excluded most of the IgM class immunoglobulins, so that their presence would have been overlooked. Moreover, if further studies do confirm the finding of IgM receptor-binding antibodies in some patients with thyroid disease, additional physiologic characterization of their actions are warranted, since IgM and IgG can mediate different effects, for example, complement activation (38). The presence of several different classes of antibody against the receptor may also have implications concerning the immunoregulatory derangement involved in the production of autoantibodies and the immunoglobulins gene rearrangement involved in autoantibody production (39, 40).

Thus, in summary, the ELISA reported seems to be a very useful tool, which will allow the sensitive and rapid measurement of TSH-displaceable, membrane-binding immunoglobulins and may prove useful in a variety of investigative studies. It may prove helpful in predicting the likelihood of relapse in Graves' disease or could aid in the diagnosis of autoimmune thyroid disease either in the routine case of thyrotoxicosis or in the more unusual case of a patient with euthyroid ophthalmopathy (7, 9, 41-43). Indeed, in contrast to previously reported assays (42), this ELISA appears to meet the requirements of simplicity, rapidity, and sensitivity which are essential for extensive clinical ap-

plication. However, it should be emphasized that this assay measures only binding immunoglobulins and that it is not known, at present, what relation these immunoglobulins have to the stimulation or inhibition of thyroïdal synthesis and/or secretion.

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