A

bstract. Previous studies have shown that anti-idiotype antibodies can be developed in vivo through animal immunization with idioype, and that these antibodies can be isolated from other anti-immunoglobulin antibodies by affinity purification. These techniques have relied on large amounts of idioype, which were produced either by hyperimmunization or by monoclonal antibodies, to serve as the affinity adsorbent. In the present study, we produced anti-idiotype antibodies to human anti-thyroid-stimulating hormone (TSH) receptor antibodies by first injecting rabbits with (TSH receptor purified) IgG from Graves' patients. The resulting antiserum was then adsorbed with Sepharose-coupled TSH in an attempt to specifically bind and isolate the anti-idioype. The antibody obtained from this process was shown to bind specifically to TSH receptor-binding antibodies from Graves' patients, and this binding could be inhibited by 56% with the addition of $10^{-4}$ M TSH but not by HCG ($10^{-2}$ M). The anti-idioype also bound to TSH, and this binding could be specifically inhibited by receptor-purified Graves' IgG (60% inhibition at 10 $\mu$g/ml IgG), but not by IgG from normal subjects (no inhibition at 50 $\mu$g/ml IgG). In a TSH receptor binding assay, the anti-idioype could inhibit TSH receptor binding in Graves' sera at a 1,000-fold lower concentration than could anti-kappa/lambda antiserum; the anti-idioype antiserum also inhibited in vitro TSH-mediated adenylate cyclase stimulation at an IgG concentration of 5 $\mu$g/ml, while heterologous anti-TSH antisera and normal IgG at similar concentrations had no effect. Finally, despite being generated against a single patient's TSH receptor binding antibody, the anti-idioype was able to block TSH receptor binding in the serum of six other Graves' patients, thus suggesting that there may be conformational conservation in the antigen that is recognized by different individuals' TSH receptor-binding immunoglobulins.

Introduction

Graves' disease is an autoimmune disorder that is characterized by thyroid gland stimulation which is due to antibodies that bind to the thyroid-stimulating hormone (TSH) receptor (1–3). While the mechanism by which these TSH receptor-binding antibodies (Rbabs) mimic TSH action is unknown, these antibodies have been shown to bind by their F(ab) region (4), and probably recognize the receptor as an antigen through their hypervariable, antigen-combining site.

The mechanism of autoantibody-receptor interaction, such as that noted in Graves' disease, has been of interest in studying the development of autoimmunity. Recent studies that used anti-idioype (anti-id) antibodies as probes (5–7) have shown that a small fraction of anti-ids appear to be internal images of the idioype, combining sites and as a result having conformational similarity to antigen, and binding to idioype competitively with antigen. When the antigen is a hormone receptor, as in the case of beta receptor antibodies (8, 9), the anti-ids, by

1. Abbreviations used in this paper: anti-id, anti-idiotype antibody; GPF, guinea pig fat cell membranes; HCG, human chorionic gonadotropin; PNP, P-nitrophenylphosphate; PBST, phosphate-buffered saline with 0.05% Tween 20, pH 7.2; Rbabs, TSH receptor-binding antibodies; TSH, thyroid-stimulating hormone.
sharing conformational similarity with the beta receptor, will bind beta ligands. However, the extension of this work to other antigens has been limited by the need for large amounts of idiotype, which is produced either by hyperimmunization or monoclonal antibody.

The relevance of anti-idiotypic antibodies in the regulation of antibody-mediated autoimmune disorders also has recently been emphasized. Disease activity in systemic lupus erythematosus has been reported to be inversely related to the presence of anti-id antibodies against anti-DNA antibodies (10). In an attempt to apply similar concepts to Graves' disease, it has been suggested that the antibodies directed against TSH receptors that are observed in Graves' patients may actually be anti-id antibodies to anti-TSH antibodies (11). This implies that an initial step in development of Graves' disease is the formation of antibodies against TSH, and that a small fraction of anti-id is bound to the TSH receptor fortuitously due to the anti-id's conformational similarity to the TSH. While it is logical to assume that both antibodies against the TSH receptor and TSH have conformational similarities, it seems equally likely that the development of antibodies against the TSH receptor is the primary event, and that the anti-TSH antibodies that have been described may actually be anti-id antibodies to the TSH receptor antibodies. Demonstration of such an anti-id might have therapeutic significance (10, 12), especially if it bound to many different patients' TSH receptor-binding immunoglobulins. However, previous attempts to demonstrate an antibody to TSH that crossreacts with Graves' IgG have been unsuccessful (13, 14).

In the present studies, we have endeavored to demonstrate that (a) anti-id antibodies to human TSH receptor antibodies can be developed in animals, and affinity purified, to be used as a probe for investigations into the function of idiotype; (b) the anti-id antibodies appear to be conformationally similar to TSH receptor, by competitively binding both TSH and Rbabs; and (c) the binding of the anti-id to TSH receptor antibodies from several patients suggests that a similar antigenic determinant or conformation may be involved.

Methods

Hormones and reagents. Highly purified bovine TSH was a generous gift of Dr. J. G. Pierce, University of California, Los Angeles. Microtiter plates (Immulon 2) were purchased from Dynatech Laboratories, Inc., Dynatech Corp. (Alexandria, VA). Affinity purified, alkaline phosphatase-conjugated anti-human IgG (Fc specific) antiserum and P-nitrophenylphosphate (PNP) were purchased from Sigma Chemical Co. (St. Louis, MO). Purified human TSH was kindly supplied by the Hormone Distribution Program of the National Pituitary Agency (Baltimore, MD). Affinity-purified goat anti-rabbit IgG, both unconjugated antiserum and alkaline phosphatase conjugate, anti-human kappa, lambda, and (Fab)2 antisera, purified human IgG, and purified rabbit IgG were purchased from Cappel Laboratories Inc. (Cochraville, PA). Tween 20 was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Human chorionic gonadotropin (HCG) was purchased from Ayerst Laboratories, Inc. (New York). Heterologous anti-human TSH was obtained from Hyland Laboratories (Costa Mesa, CA).

Patients. The serum from seven patients with classic laboratory and clinical findings of Graves' disease (15) were chosen for this study. After informed consent was obtained, a 4-ml serum sample was collected from each patient and screened in an enzyme-linked immunosorbent assay for direct, TSH receptor, membrane binding (16), and selected patient sera were also analyzed in a bioassay for thyroid-stimulating capacity (courtesy of Dr. James Peter, Specialty Labs Inc., Los Angeles, CA). The patient who had the highest activity (>8 SD above the normal range in both assays) had 60 cm of blood drawn after informed consent. The serum was separated, aliquoted, and frozen for use in the rabbit immunization and for screening the rabbit antisera. The other patients' sera were also frozen (~4°C) and aliquoted for use in screening the binding capability of the purified anti-id.

Guinea pig fat cell membrane (GPF) preparation and "receptor purification" of Graves' IgG. Preparation of GPF and TSH receptor-purified immunoglobulin were performed according to the method of Endo et al. (17).

Rabbit immunization procedure. Graves' patient IgG was first pepsin digested to make F(ab)2 fragments. This digest was placed over a staphylococcus protein A Sepharose column to remove nondigested IgG, and the nonadherent fraction was collected. The F(ab)2 fragments were then receptor purified (16, 17). This fraction, which contained no detectable Fc fragments by Ouchterlony gel diffusion, was then shown by high performance liquid chromatography not to contain detectable TSH. From this fraction, a 2.5-ml aliquot which contained ~1 mg of protein was removed and mixed with 2.5 ml complete Freund's adjuvant. After obtaining serum samples, New Zealand white rabbits were injected with the Freund's F(ab)2 mixture at four locations on their hindquarters. Rabbits were immunized with 2.5 ml of the F(ab)2 concentrate at 1 and 4 wk after the initial injection, and 20-ml blood samples were obtained at 2, 4, and 6 wk after the initial injection. Control rabbits were injected with human myosin under the same protocol.

Affinity purification of the rabbit antiserum. Rabbit antiserum was adsorbed against a column of normal IgG (Cohn fraction II, Hyland Laboratories), and then affinity purified using a bovine TSH affinity column. Both columns were made by coupling the respective proteins to Aff-Gel 10 (Bio-Rad Laboratories, Richmond, CA). 200 mg of normal IgG was added to 20 ml of packed gel for the IgG column and 50 mg of bovine TSH was added to 10 ml of gel for the TSH column. Both were allowed to react for 1 h at 27°C in 0.1 M NaHCO3, pH 8.5. The gels were then blocked with 1 M ethanolamine (pH 8.0) using 0.1 ml/ml of gel; coupling efficiency was estimated to be ~90% for both columns. 50 mg of an IgG fraction from an immunized rabbit were concentrated to a 5 ml vol, and adjusted to a 150 mM NaCl concentration. This fraction was then placed on the IgG column (20-ml bed vol) which was kept at 4°C. The sample was allowed to equilibrate for 15 min, then a 5-ml aliquot was collected and reapplied to the column. This was repeated three additional times before the column was washed with phosphate-buffered saline (PBS) and 30 1-ml fractions were collected. These 30 fractions, which contained a total of 25 mg of protein, were stepwise concentrated to 1 ml and dialyzed extensively (overnight, 4°C) against PBS to adjust the NaCl concentration to 150 mM. This 1-ml fraction was then placed on the TSH affinity column (10-ml total bed vol), and flushed through three times with PBS. The column was then washed extensively with PBS to remove nonadherent protein; the IgG was eluted with 3.5 M NaSCN, and a 50-ml fraction was collected. This fraction was dialyzed extensively (overnight, 4°C) against PBS until no evidence of thiocyanate was present on analysis by high performance liquid chro-
matography. This fraction was concentrated to a rabbit IgG concentration of 20 μg/ml and frozen in aliquots at −20°C until used.

Rabbit IgG assay. The affinity-purified rabbit IgG was assayed for IgG concentration using an adaptation of the double antibody capture enzyme-linked immunosorbent assay technique, as previously published (18).

Human IgG binding assay. Microtiter plate wells were covered with 100 μl of a 50-μg/ml solution of Cohn fraction II, normal IgG (Hyland Laboratories) was diluted in PBS, and it was incubated overnight at 4°C. The plates were then washed once with PBS and were coated with triplicate dilutions of rabbit antiserum. After a 2-h incubation at 37°C, the plates were washed three times with phosphate-buffered saline with 0.05% Tween 20, pH 7.2 (PBST) (0.1%), and 100 μl of a 1:1,000 dilution of alkaline phosphatase-conjugated anti-rabbit IgG was added to all wells. That was diluted in PBS and incubated overnight at 4°C. The plates were again washed three times with 0.1% PBST and substrate (150 μl of PNP in diethanolamine buffer, pH 9.6) was added. The color change was read with an automated spectrophotometer when control samples reached a predetermined value of optical density at 405 nm. Values from each triplicate were then averaged, and a standard deviation was calculated. Inhibitor studies were done in the same manner except that inhibitors were added to the diluted of rabbit antiserum and co-incubated for 30 min before being added to the microtiter plates.

TSH binding assay. Microtiter plate wells were coated with human TSH at 10^{-7} I U/ml (100 μl/well) and incubated overnight at 4°C. They were then washed once with PBS, and 100 μl of PBS with 1% human serum albumin was added. After incubating for 1 h at 37°C, the plates were washed three times with PBST, and samples to be evaluated for TSH binding antibodies were diluted in PBST and placed in the wells in triplicate wells. After a second 1-h incubation, the plates were washed three times with 0.1% PBST and a 1:1,000 dilution of affinity purified, alkaline phosphatase-labeled, anti-rabbit IgG was added. The plates were then incubated for a final 1-h period at 37°C, and then washed three times with PBST. Enzyme substrate (PNP in diethanolamine buffer, pH 9.6) was added, and color change was read when control wells reached a predetermined optical density (405 nm) reading. Inhibitor studies were done in the same manner, except that inhibitors were added to the dilutions of rabbit antiserum and co-incubated for 30 min before being added to the microtiter plates.

Enzyme-linked membrane (TSH receptor) binding assay and the competitive inhibition of enzyme-linked membrane (TSH receptor) binding assay. These assays were performed as previously described (16). The inhibitor studies were performed by adding the inhibitor to the human sera dilutions immediately before placing them in microtiter plates.

Human sera binding to anti-human (Fab'). Microtiter plate wells were coated with 100 μl of 2 μg/ml of affinity-purified anti-human (Fab'), that was diluted in PBS, and incubated overnight at 37°C. Plates were then washed once with PBS, and 100 μl of 1% human serum albumin in PBS was added and incubated for 1 h at 37°C. The plates were washed three times with 0.1% PBST and human sera samples were added in triplicate. After a 2-h incubation at 37°C, the plates were again washed three times and a 1:1,000 dilution of anti-human IgG that was conjugated to alkaline phosphatase was added. After a final 2-h incubation at 37°C, the plates were washed three times with 0.1% PBST and PNP substrate was added. The plates were then read with an automated spectrophotometer. Inhibitor studies were done in the same manner except that inhibitors were incubated with the dilutions of human sera for 30 min before their addition to the microtiter plates.

Adenylate cyclase assay. This assay was performed according to the method of Kleinmann et al. (19) using human thyroid membranes and a double column purification in order to isolate radioactive cyclic-AMP. Inhibitors were added to samples immediately before addition to membrane. After informed consent, human thyroid glands were obtained at the time of surgery for Graves' disease or multinodular goiter. Membranes were isolated by previously published techniques (16, 17, 19).

Statistical analysis. Unpaired t tests were used to determine whether values were different between control and maximally inhibited samples.

Results

Binding capabilities of whole rabbit antiserum. Antiserum from rabbits injected with Graves' IgG that was isolated by receptor precipitation showed significant binding against human TSH and normal human IgG, as well as towards the receptor-purified Graves' IgG to which it had been raised (Fig. 1). The binding to normal IgG was completely suppressible with the addition of normal IgG but the binding to Graves' IgG was only partially (40%) suppressible by the addition of normal IgG (not shown). After passage over the Sepharose-bound normal IgG column, binding to TSH or Graves' IgG in the void volume was unchanged or increased, but binding to normal IgG was substantially reduced (P < 0.001, Fig. 1). After passing the void volume from the IgG column over the TSH column, the fraction which bound to TSH and was then eluted still had significant binding to TSH and Graves' IgG, but had no significant binding to normal IgG. It is this fraction which was labeled as rabbit anti-id. Control antiserum showed no difference in binding from preimmune sera (data not shown). Yield of antibody was about 1/500 of starting IgG (100 μg from 50 mg).

![Figure 1](image-url)

Figure 1. Binding of rabbit antibodies raised against TSH receptor-purified (Fab'), fragments during sequential purification. Binding against human TSH, Graves' patient IgG, and normal IgG was assayed with the rabbit IgG concentration maintained constant at 1 μg/ml. Binding is initially noted against all three antigens; however, passage over a heterologous, human IgG affinity column preferentially removes binding to normal IgG. Specific absorption and elution from a TSH column preserves binding to both TSH and Graves' IgG while completely removing binding to normal IgG. Error bars in all figures indicate ±1 SD.
Inhibition of rabbit anti-id binding to TSH by Graves' IgG. Rabbit anti-id at a concentration of 1 μg/ml consistently bound to washed microtiter plates coated with human TSH. The addition of increasing concentrations of receptor-isolated Graves' IgG resulted in significant, progressive inhibition of binding (P < 0.001, Fig. 2). This ranged from 80% of control at 500 ng/ml of receptor-purified Graves' IgG to 40% at 10 μg/ml. In comparison, the addition of receptor protein at a concentration of 2 μg/ml or normal human IgG at 500 μg/ml had no significant effect on the binding of the rabbit anti-id to TSH, which indicated that the inhibition was not due to contaminating receptor or nonspecific protein interactions. Higher concentrations of receptor (20 μg/ml, not shown) did result in inhibition, presumably through competition for TSH binding.

Inhibition of rabbit anti-id binding to Graves' receptor-purified IgG by bovine TSH. Rabbit anti-id at a concentration of 1 μg/ml, bound to receptor (purified) IgG that was coated on microtiter plates. The addition of bovine TSH (adequate quantities of human TSH could not be obtained) caused progressive inhibition of binding, up to 54% of control at a 10⁻⁴ M concentration of TSH (P < 0.001, Fig. 3). In contrast, higher concentrations of HCG, up to 10⁻² M, did not have any effect on the binding, which indicated the specificity of inhibition by TSH.

Inhibition of Graves' serum binding to TSH receptor by rabbit anti-id. The addition of rabbit anti-id to a Graves' patient's serum before the serum was incubated with solid-phase TSH receptor caused the progressive inhibition of receptor binding, to 60% of control at an anti-id concentration of 1 μg/ml (P < 0.002, Fig. 4 A). Anti-human kappa and lambda antisera had a similar effect on binding, but only at an IgG concentration of 1 mg/ml, which was 1,000-fold greater than the concentration of rabbit anti-id that was required to cause similar inhibition. Also, normal rabbit IgG at 1 mg/ml had no effect on the binding (Fig. 4 A). Control wells that were run with alkaline phosphatase-labeled anti-rabbit IgG showed no evidence of the anti-id binding to solid-phase receptor (data not shown), which ruled out anti-id receptor binding as the cause of inhibition. In comparison, preincubation of rabbit anti-id had no effect on the binding of Graves' serum to solid phase, anti-human F(ab')² (Fig. 4 B), while anti-kappa/lambda showed suppression at a lower concentration than was required to block receptor binding (40 μg/ml vs. 1 mg/ml). Preincubation of anti-id with receptor and incubation with 3.5 M sodium thiocyanate (data not shown) also had no effect on the binding of Graves' sera to TSH receptor.

Inhibition of TSH-mediated adenylate cyclase production by rabbit anti-id. Anti-id showed a progressive inhibition of cyclic-AMP production to 20% of control at a concentration of 0.5 μg/ml (Fig. 5). Commercial, heterologous rabbit anti-TSH had no effect at similar rabbit IgG concentrations. Sodium thiocyanate was measured in the dialyzed anti-id fraction and found to be <1.5 mg/l, a concentration which does not alter TSH stimulated adenylate cyclase.

Inhibition of multiple Graves' patient's sera binding to TSH receptor. Six Graves' sera (not including the patients' sera against which the anti-id was developed) were examined in the receptor-binding assay. All six showed inhibition of receptor binding upon the addition of rabbit anti-id at a concentration of 1 μg/ml (Fig. 6). This inhibition ranged from 46 to 84%. Preimmune rabbit IgG and rabbit IgG from animals that were immunized...
patients' immunoglobulin can block binding of radiolabeled TSH to receptor (2, 20, 21) and can stimulate adenyl cyclase production in a system that contains TSH receptors (3, 19, 22), which suggests action parallel to that of TSH. However, the biological activity of Graves' immunoglobulin on the thyroid gland is not identical to that of TSH, with time (23) and functional (24) differences that call into question the site and mode of action. The studies presented here give support to the theory that the antibody actually binds to the TSH receptor and causes stimulation of the gland. The finding that the Rbabs demonstrated enough conformational similarity to TSH to give rise to anti-id antibodies which bind to TSH, suggests that Rbabs recognize the receptor in the same manner as TSH. Also, anti-id antibodies not only bound TSH, but also specifically blocked the production of thyroidal adenylate cyclase, which suggests that the portion of the Rbabs resembling TSH is that portion which is involved in binding or activation of the receptor. Heterologous anti-TSH antiserum did not inhibit adenylate cyclase production in equimolar concentrations to the anti-id; this may reflect the fact that these antibodies were raised against the whole TSH molecule (including portions that were not involved in receptor binding and activation), which supports the specificity of the binding of the anti-id.

The stimulatory activity of Graves' IgG has previously been shown to reside in the F(ab')2 portion of the immunoglobulin, both by actual stimulation with F(ab')2 fragments (4) and by the inhibition of stimulatory activity with anti-kappa/lambda antisera (25, 26). Our results support this hypothesis, as both anti-kappa/lambda antisera and anti-id (raised against F(ab')2 determinants) were able to inhibit receptor binding. However, the fact that anti-id specifically inhibited binding to the TSH receptor but had no effect on the binding of the same antibody to anti-

**Figure 4.** Comparative inhibition of Graves' IgG binding to TSH receptor and anti-human F(ab')2 by anti-id (solid line) and anti-kappa/lambda (Anti-κ/λ) antiserum (dotted line). The addition of anti-id to Graves' IgG resulted in a progressive inhibition of binding to receptor, to a level 60±4% of control value (100±5% without inhibitor) at a rabbit IgG concentration of 1 μg/ml (P < 0.002). The same concentrations of anti-id had no effect on the binding of Graves' IgG to anti-human F(ab')2. Anti-κ/λ antiserum also inhibited binding of Graves' IgG to receptor, but required a concentration of 1 mg/ml for 60% inhibition (P < 0.001). It was capable of blocking binding of Graves' IgG to anti-human F(ab')2 to a level of 20±4% of control value at a concentration of only 10 μg/ml (P < 0.001), a 100-fold lower concentration than that required to block receptor binding.

with myosin in complete Freund's adjuvant had no effect (data not shown) on Graves' IgG binding to GPF.

**Discussion**

The central hypothesis of the etiology of the hyperthyroidism of Graves' disease is auto-antibody-induced thyroid stimulation, although the events that initiate production of such an auto-antibody are unknown. It has been previously shown that Graves'
F(ab')2 antisera suggests that the anti-id antiserum is binding to antigens on the F(ab')2 region that are not commonly found on antibodies. These antigens are most likely in the area of the hypervariable region, since adsorption of the antisera with the pooled immunoglobulin column would remove antibodies against allotypic determinants.

Recently, it has been shown that anti-id antibodies to anti-
TSH antibodies have the capability of binding to the TSH receptor and causing stimulation (27). Similar studies have been performed using acetyl-choline (5), beta adrenergic (8), insulin (28), and F-met-leu-phe (29) receptors. Islam et al. (27) have suggested that an antibody response to TSH may be the initiating event in Graves' disease, with the Rbabs being anti-id antibodies that develop in response to the anti-TSH (11). This is unlikely, in our view, for a number of reasons. Firstly, anti-TSH antibodies have only been reported in a fraction of patients with Graves' disease, and in low titer (30), while Rbabs are present in 90% of Graves' patients, and often persist in high titer throughout the course of the disease (3, 31–34). Results with other systems have indicated that the primary response that is required to produce an anti-id is substantial, and while the primary antibody response tends to persist, the anti-id of normal immune responses appears to be short lived (35). Secondly, there are multiple epitopes on the TSH molecule, and only a fraction of which are involved in receptor binding and activation. Thus, the anti-
id response to anti-TSH that resulted in the prolonged production of Rbabs would be directed against only a small fraction of the antibodies that were produced against TSH. In contrast, the ability of the receptor to produce a primary immune response has been shown (8, 28, 36), and our work indicates that the anti-TSH antibodies that is found in Graves' patients may be the result of an anti-id response to Rbabs. Although our data can support either point of view, we currently support the concept that production of Rbabs may be the primary immunological event in Graves' disease.

The inhibition of multiple Graves' patients' antibody binding to the TSH receptor with anti-id that was raised against only a single patient's Rbabs is of possible clinical importance. Recent studies have shown potential usefulness for anti-id antibodies as antigen-specific immunosuppressive agents (37–39). However, in complex antigen systems, such as tetanus toxoid, crossreactivity between idiotypes of different patients may not occur (40). This lack of crossreactivity may be the result of anti-id that recognize genetic or regulatory determinants on the immunoglobulin, and has also been implied in myasthenia gravis (41), an antigenic system which is presumptively similar to that in Graves' disease. The potential usefulness of anti-id for antigen-specific immunosuppression is limited by the need to produce specific, anti-id antisera for each patient. Our results demonstrating crossreactivity remove this limitation, and are in accordance with findings in such diverse antigen systems as Hepatitis B (42), rheumatoid factor (43), p-azophenylarsonate (44), and more recent studies with acetylcholine receptor (45). The findings of crossreactivity and an anti-id that conformationally appears to be the "internal image" of antigen places this anti-id in the second class of a recently proposed system for categorizing anti-ids (46, 47), and implies a common, antigenic determinant for the Rbabs binding site.

In conclusion, this work demonstrates that a common idiotypic exists in Graves' disease, which has an "internal image" that appears to be similar to TSH. However, despite reiterating the "internal image" concept of antibody antigen interaction that was first proposed by Jerne (48), our studies cannot determine whether there are different "internal images" of Rbabs that interact with different TSH binding sites in the TSH receptor. This differentiation is important because of the report of multiple
epitopes in the TSH receptor that have different functional activity (49). These questions might be clarified by application of the techniques we have developed to monoclonal or human anti-id systems.

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