Characterization of Monoclonal Thyroid-Stimulating and Thyrotropin Binding-Inhibiting Autoantibodies from a Hashimoto’s Patient Whose Children Had Intrauterine and Neonatal Thyroid Disease

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ABSTRACT

A multiplicity of TSH receptor autoantibodies (TSHRAbs) have been characterized after subcloning heterohybridomas produced from the lymphocytes of a patient who has Hashimoto’s thyroiditis and had three children with intrauterine or neonatal hyperthyroidism. Twelve clones produced stimulating TSHRAbs that increased cAMP levels and iodide uptake in rat FRTL-5 thyroid cells and increased cAMP levels in Chinese hamster ovary (CHO) cells transfected with the human TSHR; like 95% of Graves’ stimulating TSHRAbs, all 12 have their functional epitope on the N-terminus of the TSHR extracellular domain, requiring residues 90–165 for activity. All 12 bind to human TSHR; like 95% of Graves’ stimulating TSHRAbs, all 12 have their functional epitope on the N-terminus of the TSHR extracellular domain, requiring residues 90–165 for activity. All 12 bind to human thyroid membranes in the absence, but not the presence, of TSH, but are only weak inhibitors of TSH binding in assays measuring TSH binding-inhibiting Igs (TBII). In contrast, 8 different clones produced TSHRAbs that did not increase cAMP levels, but, instead, exhibited significant TBII activity. Four inhibited the ability of TSH or a stimulating TSHRAb to increase cAMP levels and had their functional epitope on the C-terminal portion of the TSHR external domain, residues 261–370, mimicking the properties of blocking TSHRAbs that cause hypothyroidism in patients with idiopathic myxedema. The 4 other TBII’s inhibited the ability of TSH, but not that of a stimulating TSHRAb, to increase cAMP levels, like TBII’s in Graves’ patients. The functional epitope for 3 of these Graves’-like TBII’s was residues 90–165; the functional epitope for the fourth was residues 24–89. The fourth also increased arachidonic acid release and inositol phosphate levels in FRTL-5 thyroid cells and exhibited conversion activity, i.e. the ability to increase cAMP levels in the presence of an anti-human IgG. Thus, this TBII exhibited signal transduction activity, unlike the other 3 Graves’-like TBII’s. The patient, therefore, has stimulating TSHRAbs and 3 different types of TBII’s, each with different functional properties and different epitopes on the TSHR.

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obtained myxedema, and normal subjects from whom serum was obtained. These studies showed that Graves’ TBIIs and myxedema sera had blocking TSHRAb activity measured with both TSH and a Graves’ stimulating TSHRAb using FRTL-5 cell assays or assays in human (h) TSHR-transfected CHO cells. The Graves’ and myxedema patients all exhibited positive TBI activity in a commercial assay. Normal subjects had no goiter, no personal or family history of thyroid disease and/or ophthalmopathy, normal circulating iodothyronine and TSH levels, and absent TSHR, microsomal, or thyroglobulin antibodies. In this study, we used sera from 20 normal individuals, 7 Graves’ patients, and 4 subjects with myxedema; there was no significant difference in results when IgG from individual Graves’ or myxedema sera were substituted within individual assays. Sera from these individuals were stored at −20 °C until IgG preparations were made. All sera were obtained under institution guidelines, with appropriate approval and consent. IgGs were extracted from the sera of patients by affinity chromatography with protein A-Sepharose CL-4B columns; the resulting eluent was dialyzed against distilled water, lyophilized, and stored at −20 °C as previously described (31). Normal IgG was a pool of sera from 20 normal individuals. Purified IgG was dissolved in assay buffer to the desired concentration immediately before use.

Production of lymphocyte heterohybridoma clones
Peripherally lymphocytes from the mother were harvested by Ficoll-Hypaque gradient centrifugation of 100 mL blood within 30 min of venipuncture and were washed twice with Hanks’ Balanced Salt Solution (HBSS; Biofluids, Rockville, MD). Half of the cells were taken for immediate fusion; half were fused 1 day later after being maintained overnight in glucose-supplemented DMEM (Biofluids) containing 10% FCS. Before use in fusion experiments, cell number was adjusted to 1 × 10^7/mL in DMEM. Polyethylene glycol glycol fusion with 5 × 10^5 P3-NS1/1-AG4–1 mouse myeloma cells was performed as previously described (17, 18, 36). The fusion products were dispersed in hypoxan-thine-aminopterin-thymidine selection medium; additional medium was added every 4 days until visible hybridoma colonies had reached the 100- to 200-cell stage (17, 18, 36).

Screening assays
Heterohybridoma clones producing human IgG were detected by incubating the medium from each well in a solid phase RIA system previously described (37). Hybridoma antibodies binding to thyroid membranes were assayed using human thyroid membranes in suspension in both the presence and absence of 1 × 10^{-7} mol/L/L purified bovine (b) TSH (17, 18). In brief, culture medium was incubated with a suspension of human thyroid membranes in the wells of 96-well microtiter plates for 1 h at 4 C. The plates were centrifuged at 4 C for 10 min, the supernatant solutions were discarded, and the pellets were washed with phosphate-buffered saline, pH 7.4, containing 0.1% gelatin and 0.02% sodium azide. ^125 I-Labeled antihuman F(ab)’s in phosphate-buffered saline containing 1% gelatin and 1% crystalline BSA was added and incubated for 45 min at 4 C; unbound radioactivity was removed by centrifugation, pellets were washed as described above, plates were dried under an infrared lamp, and individual wells were cut and assayed for bound radioactivity.

Heterohybridoma subcloning and antibody purification
The heterohybridomas were subcloned by three rounds of limiting dilution over a 6-month period using 96-well plates. In each round, cells were plated to 3 and 1 cells/well (17, 18). This procedure is similar to that used recently (25), which is accepted to produce cells that are clonal in origin. Single clusters of cells were expanded in 24-well, then in 100-mm tissue culture dishes using the selective medium described above. IgG was prepared from the medium of overgrown cultures from 10 750-mL flasks that had been harvested by centrifugation at 500 × g for 10 min. After ammonium sulfate precipitation (0–45% saturation), the IgG were dialyzed for 24 h with three changes of 0.1 mol/L Tris-

Materials and Methods
Clinical state of the patient at the time of these studies
The patient was 31 yr of age when both serum and cells were obtained. She had a history of Hashimoto’s thyroiditis, with hypothyroidism diagnosed at age 17 yr and had been taking 0.2 mg/day levothyroxine ever since. While receiving replacement therapy, she delivered three children with intrauterine and/or neonatal hyperthyroidism (7, 11–13), then defining their properties by combining a monoclonal approach with assays not only in functioning FRTL-5 thyroid cells, but also in CHO cells transfected with the human TSH or TSHR chimeras with residues 9–165 (Mc1+2), 90–165 (Mc2), or 261–370 (Mc4) substituted by equivalent residues of the LH/CGR. We used assays measuring cAMP, arachidonic acid release, inositol phosphate production, and TBI activity. We identify clones producing Graves’ stimulating TSHRAbs, two different types of Graves’ TBIIs (one functional and the other not), and a TBI-blocking type TSHRAb characteristic of TSHRAbs causing hypothyroidism. The data establish the potential diversity of TSHRAbs in Hashimoto’s mothers and the potential variability of anti-TSH autoimmune disease in their progeny, consistent with conclusions from studies using TSHR-LH/CGR chimeras to characterize heterogeneous populations of TSHRAbs in patient IgG preparations (31, 32). They support the conclusion that disease diversity and clinical course are caused by the differences in functional epitopes and properties of TSHRAbs.

Graves’ patients, patients with hypothyroid-idiopathic myxedema, and normal subjects from whom serum was obtained
The diagnosis of hyperthyroidism due to Graves’ disease was based on clinical findings, including diffuse goiter, diffuse ^125 I uptake on thyroid scans, elevated serum levels of free and/or total iodothyronines, and an absent serum TSH response to TRH. Each had stimulating TSHRAb activity in their serum measured in FRTL-5 cell assays. Patients with primary myxedema had clinical features of hypothyroidism, low serum levels of free and/or total iodothyronines, and no palpable goiter. Each had blocking TSHRAb activity measured with both TSH and a Graves’ stimulating TSHRAb using FRTL-5 cell assays or assays in human (h) TSHR-transfected CHO cells. The Graves’ and myxedema patients all exhibited positive TBI activity in a commercial assay. Normal subjects had no goiter, no personal or family history of thyroid disease and/or ophthalmopathy, normal circulating iodothyronine and TSH levels, and absent TSHR, microsomal, or thyroglobulin antibodies. In this study, we used sera from 20 normal individuals, 7 Graves’ patients, and 4 subjects with myxedema; there was no significant difference in results when IgG from individual Graves’ or myxedema sera were substituted within individual assays. Sera from these individuals were stored at −20 °C until IgG preparations were made. All sera were obtained under institution guidelines, with appropriate approval and consent.

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chloride, pH 7.5, subjected to high pressure liquid chromatography (38) to prepare purified IgG, and dialyzed in 50 mmol/L Tris-chloride, pH 7.4, or NaCl-free HBSS (5.4 mmol/L KCl, 1.3 mmol/L CaCl₂, 0.5 mmol/L MgSO₄, 0.3 mmol/L Na₂HPO₄, 0.4 mmol/L KH₂PO₄, and 0.1% glucose) containing 20 mmol/L HEPES, pH 7.4, and 222 mmol/L Li sucrose. After centrifugation to remove any precipitate, IgG was distributed in 100-μL aliquots and stored at −70°C. Alternatively, IgG was subjected to affinity chromatography with protein A-Sepharose CL-4B columns as described above. The method of preparation had no significant difference in any results obtained; yield varied from 0.45–1.4 mg purified IgG/10 flasks.

The IgG concentration was measured in an enzyme-linked immunosorbent assay as previously described (39), using a β-galactosidase-coupled affinity-purified antihuman IgG (Zymed Laboratories, Burlingame, CA). Reactions were stopped with 0.05 mL 8 mol/L H₂SO₄/10 flasks. In some experiments, total inositol phosphate as well as cAMP stimulated by Graves’ IgG with stimulating TSHRAb activity was included in the assay. In some experiments, total inositol phosphate as well as cAMP stimulated by Graves’ IgG with stimulating TSHRAb activity was included in the assay.

**Determinations of heavy and light chains**

Subclasses of IgG antibodies were determined using an enzyme-linked immunosorbent assay as previously described (40). Mouse monoclonal subclass-specific antibodies were obtained from Boehringer Mannheim (Indianapolis, IN) as was the peroxidase-conjugated antihuman light chain-specific antibody. Light chains were determined by an immunodot procedure (41); antihuman κ light chain-specific and antihuman λ light chain-specific mouse monoclonal antibodies were obtained from Dianova (Hamburg, Germany).

**FRTL-5 rat thyroid cells and assays**

FRTL-5 cells (Interthyr Research Foundation, Baltimore, MD; American Type Culture Collection CRL 8305) are a continuous line of functioning epithelial cells whose properties have been detailed (42, 43); they were grown in Coon’s modified Ham’s F-12 medium, supplemented with 5% calf serum (Life Technologies, Grand Island, NY), 1 mmol/L nonessential amino acids (Microbiological Associates, Bethesda, MD), and a six-hormone mixture, including bTSH (1 × 10⁻¹⁰ mol/L), insulin (10 mg/mL), hydrocortisone (1 mmol/L), human transferrin (5 mg/mL), somatostatin (10 μg/mL), and glycyt-l-histidyl-l-lysine acetate (10 μg/mL). Cells were fed every other day and split every 4–5 days with trypsin-ethylenediamine tetraacetate. After harvesting, cells were plated in 24-well plates (3–4 × 10⁶ cells/well) and fed fresh medium 48 h later. They were used in assays when they were at 100% confluency, usually 60–72 h after plating. In a typical assay, there were 5–6 × 10⁶ cells/well. The cAMP response to a standard amount of TSH or a standard pool of Graves’ IgG was stable for over 3 months of continuous culture (~30 passages).

Studies of the Mc4 chimera used transient transfection procedures, as previously described (33–35, 44), wherein COS-7 cells were transfected by electroporation with 25 μg purified plasmid DNA. Cells transfected with the pSG5 vector alone were negative controls. Cells transfected with wild-type TSHR were positive controls.

Stimulating TSHRAb activity was measured as previously detailed (31, 32, 37, 44). Incubations with IgG alone or with either 1 × 10⁻¹⁰ mol/L TSH or a standard Graves’ IgG was performed for 3 h at 37°C in with NaCl-free HBSS containing 20 mmol/L HEPES (pH 7.4), 222 mmol/L sucrose, 1% BSA, and 0.5 mmol/L IBMX. cAMP was measured in the supernatants by RIA. All experiments included normal human IgG as a negative control.

**Stable transfectants containing CHO-hTSHR and CHO-hTSHR-LH/CGR chimeras and measurement of stimulating or blocking TSHRAb activity in hTSHR-transfected CHO cells**

hTSHR cloning, amplification of rat LH/CGR complementary DNA fragments by PCR, construction of chimeric receptors, subcloning into pSG5 expression vectors, and plasmid purification have been described previously (29–32). The three chimeric receptors used in this report are designated to indicate the substituted segment of the TSHR, numbered from the N-terminus start site. Mc1-gal, residues 9–165 of the TSHR were replaced by residues 10–166 of the LH/CGR. In Mc2, residues 90–165 of the TSHR were replaced by residues 91–166 of the LH/CGR; in Mc4, TSHR residues 261–570 were replaced by residues 261–329 of the LH/CGR.

Stable CHO cell transfectants were made with lipofectin (Life Technologies) by cotransfection of the wild-type hTSHR or the hTSHR-LH/CGR chimeras in pSG5 with a pMAMneo selection marker (31, 32). Cells were fed every other day and split every 4–5 days with trypsin-ethylenediamine tetraacetate. After harvesting, cells were plated in 24-well plates (3–4 × 10⁶ cells/well) and fed fresh medium 48 h later. They were used in assays when they were at 100% confluency, usually 60–72 h after plating. In a typical assay, there were 5–6 × 10⁶ cells/well. The cAMP response to a standard amount of TSH or a standard pool of Graves’ IgG was stable for over 3 months of continuous culture (~30 passages).

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**[125I]TSH binding to thyroid membrane preparations and measurement of TBI activity**

Highly purified bTSH (NIDDK bTSH I-1; 30 U/mg) was radioiodinated as previously described (47). The ability of an IgG to inhibit [125I]TSH binding to human thyroid membranes was measured in a solid phase assay, previously described (17, 18). In brief, [125I]TSH binding was measured in 10 mmol/L Tris-HCl, pH 7.4, containing 50 mmol/L NaCl and 0.5% BSA after incubations at 37°C for 2 h. Wells were doubly washed, and residual bound radioactivity was measured. Maximal binding averaged 2–15% of the total counts added; control values are subtracted from the data presented and never exceeded 1% of the radioactivity added. Controls included incubations with all components except membranes or incubations where normal human IgG substituted for the experimental IgG.

TBI assays were performed using a commercially available kit (TRAK kit, Henning, Berlin, Germany) that uses solubilized porcine thyroid membranes. Additionally, TBI values were measured in an assay using solubilized membranes from the CHO cells transfected with the wild-type TSHR, the Mc2, or the Mc1-2 chimera (32). In brief, 50 μL each of serum and solubilized membranes were mixed and incubated for 15 min at room temperature. [125I]TSH (30,000 cpm) was added and incubated for 16 h at 4°C before 300 μL 10 mmol/L Tris-chloride, pH 7.4, containing 50 mmol/L NaCl and 0.1% BSA were added together with 500 μL 30% polyethylene glycol in 1 mol/L NaCl. Tubes were centrifuged, pellets were washed with 20% polyethylene glycol in an identical
buffer, and radioactivity was measured. TBII activity is expressed as the percent inhibition of [125I]TSH binding to the TSH receptor by comparison to pooled normal IgG; a TBII value exceeding 15%, which is greater than 2 sd above the mean value from 20 normal samples, was considered positive.

Other assays and statistics

The protein concentration was determined by the method of Lowry et al. (48) or using Bio-Rad reagents (Bio-Rad, Richmond, CA); bovine IgG was the standard. When protein in membrane preparations was measured, the membranes were first solubilized by treatment with 1 N NaOH for 1 h.

In all cases, experiments were repeated on at least three occasions using different batches of cells. All incubations were performed at least in duplicate, and all wells were assayed in duplicate. Results are expressed as the mean ± sd of the results of an individual experiment unless otherwise noted. Statistical analysis was performed by one-way ANOVA to determine the P value between different groups and by Spearman’s rank correlation coefficient to validate the correlation between two series of data.

Materials

TSH was a purified bovine preparation (49) homogeneous in the ultracentrifuge (27,500 Da), with an average specific activity of 29 ± 3 IU/mg in a mouse bioassay. Alternatively, it was a gift from the NIH Hormone Distribution Program (NIDDK bTSH I-1; 30 U/mg). bLH was obtained from the National Hormone and Pituitary Program, NIDDK; highly purified hCG was a gift from Dr. R. Canfield (Columbia University, New York, NY) or was obtained from the National Hormone and Pituitary Program, NIDDK (NIH CR-127; 14,900 IU/mg). Normal human tissue was obtained from accident victims autopsied within 30 min of demise or from the tissue surrounding nodular goiters removed during surgical procedures using institution-approved protocols. Myo-[2-N-3H]inositol (SA, −20 Ci/mmol) and cAMP RIA kits were obtained from DuPont-New England Nuclear (Boston, MA). All other materials were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Activity of the IgG in the patient’s serum at the time of this study

In FRTL-5 rat thyroid cells, the IgG from the patient exhibited a biphasic ability to increase, then decrease, cAMP levels as a function of IgG concentration (Fig. 1A). The IgG was also able to inhibit the ability of an IgG from a Graves’ patient to increase cAMP levels (Fig. 1B). These results established that the patient’s lymphocytes were still likely to be producing at least two types of TSHRAbs: stimulating and inhibiting. Additionally, they showed that the FRTL-5 rat thyroid cell assay system could be used as an assay tool during cloning, contrary to previous results (12); the basis for the discrepancy is not clear.

The sequence of addition affected the ability of the patient’s IgG to inhibit Graves’ IgG. Thus, better inhibition was evident if the patient IgG was preincubated with FRTL-5 thyroid cells before addition of Graves’ IgG during the screening studies (data not shown).
Isolation of individual autoantibodies from the lymphocytes of the patient

Fusions with NIS-1-AG4–1 mouse myeloma cells resulted in 3516 seeded wells. Human IgG was detected in 592; cells in 310 of these survived for a 6-week period and were subcloned or frozen. Supernatants from 3 colonies were reactive with a monoclonal mouse stimulating TSHRAb (17, 18) or TSH, 5 with thyroglobulin, 5 with thyroid peroxidase, and 54 with thyroid membranes. Clones producing antibodies reactive with the thyroid membranes were screened to identify clonal populations producing IgG able to bind to the membranes in the absence, but not presence, of TSH; values ranged from 470-1030 cpm bound without TSH and 200–300 cpm with TSH (data for IgG from some subclones are presented in Table 2). IgG from 20 of these clones were further characterized; each of these produced a G1 IgG with a κ light chain.

Thyroid-Stimulating TSHRAbs

IgG produced by 12 clones were stimulating TSHRAbs (Table 1, bold). Thus, each significantly (P < 0.01) increased cAMP levels and iodide uptake in rat FRTL-5 thyroid cells (Table 1, columns 2 and 3, respectively). Each increased cAMP levels in CHO cells transfected with wild-type human TSHR (Table 1, column 4), but not the parental CHO cells or CHO cells transfected with vector alone (data not shown).

Each IgG increased cAMP levels as a function of IgG concentration, as illustrated for three clones in Fig. 2. In no case was there a biphasic increase, then decrease, evident, even when concentrations as high as 1000 μg/mL were tested (data not shown), nor did they inhibit TSH- or Graves’ IgG-stimulated cAMP levels (see Fig. 6). They did not, therefore, exhibit the inhibiting activity evident in the patient’s IgG (Fig. 1B).

Like the IgG in 95% of Graves’ patients (31), the stimulating TSHRAb activity of these 12 clones was lost in CHO cells transfected with the TSHR/LH-CGR chimeras, Mc2 and Mc1+2, which substitute residues 90–165 and 9–165, respectively, of the TSHR with equivalent residues of the LH/CGR (Table 1, columns 5 and 6). Thus, the functional epitope of each of these stimulating TSHRAbs exists on the N-terminal portion of the extracellular domain of the TSHR and requires residues 90–165.

The binding of each IgG to FRTL-5 (data not shown) or human thyroid membranes (Table 2) was inhibited by 1 × 10^{-7} mol/L TSH, but not by an equivalent concentration of LH or hCG. Despite this, none of the clonal stimulating TSHRAbs exhibited significant ability to inhibit TSH binding measured in assays using solubilized porcine membranes (Table 3, column 2), solubilized membranes from CHO cells containing the wild-type human TSHR (Table 3, column 3), or intact human thyroid membranes, even as a function of the IgG concentration (Fig. 3).

TBIIs

Eight clones produced TBIIs that inhibited TSH binding to either solubilized porcine thyroid membranes (the commercial TRAK assay) or solubilized membranes from human TSHR-transfected CHO cells (Table 3, columns 2 and 3, re-
FIG. 2. Ability of representative IgGs from the noted heterohybridoma clones to increase cAMP levels in FRTL-5 thyroid cells as a function of the IgG concentration. Representative clones producing stimulating TSHRAbs (Table 2, bold; DT12B4, CM2A7, and DT1FB) are compared with those producing TBIIs of different types (Table 2, bold italics; LK7G1 (○), DT11E1 (△), LK3D21 (▼), and WH7F2 (□)). The data are the mean ± SD of quadruplicate values from one of three experiments yielding similar results. Incubations were performed for 3 h.

respectively). None of these increased cAMP levels or iodide uptake in FRTL-5 thyroid cells (Table 1, columns 2 and 3) or cAMP levels in TSHR-transfected CHO cells (Table 1, column 4). The TBIIs could, however, be grouped into TBIIs that mimicked the activity of blocking TSHRAbs prevalent in hypothyroid patients with idiopathic myxedema or Hashimoto’s disease or TBIIs similar to those found in Graves’ patients.

TBIIs that are blocking TSHRAbs associated with hypothyroidism. Four TBIIs in this group inhibited TSH binding to human thyroid membranes as a function of the IgG concentration, without exhibiting the initial increase in binding caused by the patient IgG (Fig. 4). All four inhibited both TSH- and Graves’ IgG-stimulated cAMP levels in FRTL-5 cells (Table 4B, columns 2 and 3) or TSHR-transfected CHO cells (Table 4B, columns 4 and 5). These four activities mimicked those of a blocking TSHRAb from a patient with hypothyroidism and idiopathic myxedema (Tables 3 and 4B, rows 6 and 1, respectively).

Inhibition of Graves’ IgG-stimulated cAMP levels was concentration dependent, as evidenced in FRTL-5 thyroid cells (Fig. 5), and none of these four TBIIs inhibited forskolin-stimulated cAMP levels. For example, 4 × 10⁻⁶ mol/L forskolin increased cAMP levels in FRTL-5 cells from 0.1 ± 0.1 to 4.9 ± 0.3 pmol cAMP/μg DNA in the absence or presence of 200 μg/mL IgG from clones CM6F4, DT11E1, LK3D21, or DT4D2.

The Mc2 chimera responds to TSH, but not to a stimulating TSHRAb, whereas the converse is true in Mc4 cells (Table 4A) (29–32). The ability of these TBIIs to inhibit TSH binding or to inhibit TSH activity was not lost in assays using the Mc2 chimera (Table 3, column 4, and Table 4B, column 6, respectively), whereas the ability to inhibit stimulating TSHRAb activity was lost in the Mc4 chimera (Table 4B, column 7).

Again, this mimicked the actions of an IgG from a patient with hypothyroidism and idiopathic myxedema (Tables 3 and 4B, rows 6 and 3, respectively).

In summary, these data indicated that TBIIs from four independent clones, DT11E1, LK3D21, CM6F4, and DT4D2, had the characteristics of TBII-blocking TSHRAbs found in some patients with idiopathic myxedema or Hashimoto’s disease and hypothyroidism (1–4, 26–28, 32, 50). Thus, they inhibited the activity of both TSH as well as a stimulating TSHRAb (27), and their functional epitope was on the C- rather than the N-terminal portion of the TSHR extracellular domain, as evidenced by their ability to retain activity in the Mc2, but not the Mc4, chimeras.

TSHRAbs with characteristics of Graves TBIIs. The other four TBIIs (LK7G1, WH7F2, DT2F1, and CM5C3) also inhibited TSH binding to human thyroid membranes as a function of the IgG concentration (Fig. 3) and did not increase, then decrease TSH binding (Fig. 3) as did the patients total IgG (Fig. 4). Unlike the CM6F4, DT11E1, LK3D21, and DT4D2 TBIIs, the LK7G1, WH7F2, DT2F1, and CM5C3 TBIIs inhibited TSH-stimulated, but not Graves’ IgG-stimulated, cAMP levels in FRTL-5 (Table 4B, columns 2 and 3, respectively) or TSHR-transfected CHO cells (Table 4B, columns 4 and 5, respectively). The ability of monoclonal antibodies LK7G1, WH7F2, and DT2F1 to inhibit TSH binding or TSH activity was lost in assays using the Mc2 chimera (Table 4B, column 6, and Table 3, column 4, respectively).

The ability of CM5C3 to inhibit TSH binding was lost in the Mc1+2 chimera (Table 3, column 5) as was its ability to inhibit TSH increased activity. Thus, TSH-stimulated cAMP levels in the Mc1+2 chimera were 156 ± 12-fold higher than control activity in both the presence and absence of the CM5C3 IgG.

None of the IgGs inhibited forskolin-increased cAMP levels in FRTL-5 or TSHR-transfected CHO cells. Thus, for example, 4 × 10⁻⁶ mol/L forskolin increased cAMP levels in FRTL-5 cells from 0.1 ± 0.1 to 4.9 ± 0.3 pmol cAMP/μg DNA in the absence or presence of 200 μg/mL IgG from clones LK7G1, WH7F2, DT2F1, and CM5C3.

In summary, these data indicated that TBIIs, LK7G1, WH7F2, DT2F1, and CM5C3, had their functional epitope on the N- rather than the C-terminal portion of the extracellular domain and did not behave like an IgG from a patient with hypothyroidism and idiopathic myxedema in all assays (Tables 3 and 4B, rows 6 and 1, respectively), but, rather, behaved like Graves’ TBIIs. The functional epitope of three, LK7G1, WH7F2, and DT2F1, was between residues 90–165, and the functional epitope of the fourth, CM5C3, was between residues 24–89, presuming the absence of the signal peptide.

Early studies of monoclonal TSHRAbs reported that some TBIIs increased thyroid cell growth in the absence of increased cAMP levels (18, 19, 51) and that this activity was inhibited by indomethacin (19). Subsequently, IgGs were described in Graves’ patients that increased arachidonic acid release and growth, but not cAMP levels (52, 53). The IgG produced by clone CM5C3, but not that produced by clone...
TABLE 2. Ability of normal human IgG or the clonal stimulating TSHRAbs from the patient (see Table 1) to bind to human thyroid membranes in the presence of TSH, LH, or hCG

| Clone        | Antibody bound (cpm) |
|--------------|----------------------|
|              | Alone | +TSH (1 x 10^-2 mol/L) | +LH (1 x 10^-2 mol/L) | +hCG (1 x 10^-2 mol/L) |
| N1 IgG       | 214 ± 56 | 310 ± 48 | 230 ± 73 | 198 ± 67 |
| CM2A7        | 975 ± 95 | 310 ± 63* | 945 ± 110 | 1043 ± 61 |
| CM2D11       | 1240 ± 104 | 248 ± 89* | 1098 ± 94 | 1290 ± 110 |
| CM3E4        | 810 ± 61 | 198 ± 43* | 936 ± 90 | 840 ± 52 |
| CM4A10       | 2415 ± 178 | 310 ± 90* | 2310 ± 122 | 2490 ± 130 |
| CM4F1        | 1118 ± 101 | 211 ± 68* | 1098 ± 98 | 1070 ± 49 |
| CM8C6        | 784 ± 74 | 208 ± 39* | 822 ± 62 | 810 ± 92 |
| CM11E5       | 1412 ± 94 | 267 ± 86* | 1339 ± 102 | 1365 ± 93 |
| DT1F8        | 958 ± 73 | 310 ± 64* | 1021 ± 84 | 860 ± 63 |
| DT3A3        | 1444 ± 65 | 248 ± 43* | 1551 ± 73 | 1450 ± 123 |
| DT3A9        | 1940 ± 167 | 303 ± 56* | 1820 ± 104 | 2110 ± 134 |
| DT7G6        | 2304 ± 154 | 340 ± 74* | 2310 ± 130 | 2360 ± 135 |
| DT12B4       | 1249 ± 94 | 209 ± 90* | 1351 ± 101 | 1340 ± 91 |

Values were determined as described in Materials and Methods using 125I-labeled antihuman IgG. Values are the mean ± SD of quadruplicate values from three separate experiments. Normal IgG was a pool from 20 normal individuals.

* Statistically significant decrease in binding (P < 0.01).

Fig. 3. Ability of representative IgGs from the heterohybridoma clones to inhibit TSH binding to human thyroid membrane preparations as a function of IgG concentration. IgG from representative clones producing stimulating TSHRAbs [Table 2, bold (♂); CM11E5, CM2A7, CM2D11, CM3E4, DT1F8, DT3A3, DT3A9, and DT12B4] are compared with those producing TBIIs [Table 2, bold italics; LK7G1 (♀), WH7F2 (△), DT2F1 (■), and CM5C3 (◇)] and with human IgG from a pool of 20 normal individuals (○) in their ability to inhibit 125I-TSH binding to human thyroid membranes (see Materials and Methods). The data are the mean ± SD of quadruplicate values from three different experiments; the hashed bar area represents the range of activity for the stimulating TSHRAbs.

In summary, the IgG produced by clone CM5C3 was different from the other TBIIs with their functional epitope on the N-terminal portion of the TSHR. Its functional epitope appeared to be within residues 24–89, rather than 90–165; it activated the inositol phosphate signal transduction system, whereas the other TBIIs did not; and it could be converted to a stimulating TSHRAb that increased cAMP levels in the presence of antihuman IgG.

Discussion

The present report adapted monoclonal antibody techniques and TSHR-LH/CGR chimera assays (42) to characterize the TSHRAbs in a patient with Hashimoto’s disease and hypothyroidism who had occult stimulating autoantibodies in her serum that caused intrauterine and/or neonatal hyperthyroidism, compatible with the diagnosis of Graves’ disease, in all her children. Early studies of the patient indicated that her IgG had TSAb activity but that this activity was masked at high IgG concentrations by the presence of an inhibitor of TSAb activity (11–13). The hypothesis was that she had a mixture of stimulating and inhibiting TSHRAbs in her serum and that these had both caused and influenced, respectively, the expression of hyperthyroidism in the children.

The nature of the TSHRAbs in the patient was uncertain. For example, the TSAb activity appeared to be unlike the TSAb activity in Graves’ patients; the activity was not retained by the F(ab) fragment, was not evident in FRTL-5 cells, and was absorbed by thyroid, but not fat, cell membranes. They did find that the TSAb inhibitory activity was not directly related to TSAb activity, consistent with the accumulation of evidence in monoclonal TSHRAb studies that a Graves’ TBI and a Graves’ stimulating TSHRAb were distinct antibody populations (15–27). However, as Graves’ TBIIs had been reported not to inhibit stimulating TSHRAb activity in monoclonal TSHRAb studies (26, 27), despite their ability to inhibit TSH-increased cAMP levels, it was unclear how a Graves’ TBI and a Graves’ stimulating TSHRAb could account for alternating hyper- and hypothyroid states during the neonatal period. Rather, one had to presume that these
patients might have a TBII seen in patients with primary myxedema or Hashimoto’s disease with hypothyroidism that inhibits stimulating TSHR Abs as well as TSH (28). It was, therefore, unclear whether two types of TBII exist in the patient, whether the TSAb inhibitor in the patient of this study was only one of these, and why they functioned differently. The monoclonal approach was used to isolate the individual TSHR Abs; our recently described chimera assay system (29–32) categorized the different TSHR Abs and identified their epitopes.

Using TSHR-LH/CGR chimera assays, we had made the observation that there might be three types of TBII as well as multiple types of stimulating TSHR Abs (29–32). Two types of TBII were in Graves’ patients, one that completely lost TBII activity when assayed using chimera receptors in which the N-terminus of the TSHR, residues 90–165, was replaced by the LH/CGR, and the other that did not (32). This second Graves’ TBII, which retained activity with the Mc2 chimera, was associated with conversion activity, i.e., the ability of antihuman IgG to convert a TBII to a stimulating TSHR Ab (32, 54). The third TBII was present in the serum of a patient with hypothyroid patients with idiopathic myxedema or Hashimoto’s disease and was associated with blocking TSHR activity, but, like the second type of Graves’ TBII, retained binding activity using Mc2 chimeras and exhibited conversion activity (32). We suggested that this was different from the Graves’ TBII based on site-directed mutagenesis studies that identified the functional epitope for TBII-blocking TSHR Abs on the C-terminal portion of the TSHR extracellular domain (34–36, 50). Definitive proof of this, however, required the characterization of monoclonal TBII from patients, as in the present studies.

The data in the present report show unequivocally that the patients might have a TBII seen in patients with primary myxedema or Hashimoto’s disease with hypothyroidism that inhibits stimulating TSHR Abs as well as TSH (28). It was, therefore, unclear whether two types of TBII exist in the patient, whether the TSAb inhibitor in the patient of this study was only one of these, and why they functioned differently. The monoclonal approach was used to isolate the individual TSHR Abs; our recently described chimera assay system (29–32) categorized the different TSHR Abs and identified their epitopes.

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TABLE 4. Ability of 200 μg/ml normal human IgG or IgG from the cloned heterohybridomas that have TBII activity to inhibit TSH- or Graves’ IgG-increased cAMP activity in FRTL-5 thyroid cells or CHO cells transfected with the wild-type TSHR or with the Mc2 TSHR-LH/CGR chimera

A: Ability to increase cAMP levels (fold over control)

| Addition               | FRTL-5 cells | TSHR CHO cells | Mc2 cells | Mc4 cells |
|------------------------|--------------|----------------|-----------|-----------|
| None                   | 1            | 1              | 1         | 1         |
| TSH                    | 142 ± 12     | 160 ± 15       | 143 ± 10  | 1 ± 0.2   |
| Normal IgG             | 1 ± 0.2      | 1 ± 0.3        | 1 ± 0.3   | 1 ± 0.2   |
| Graves’ IgG            | 64 ± 4       | 68 ± 3         | 1 ± 0.2   | 54 ± 6    |

B: % Inhibition of control stimulating activity

| Clone               | FRTL-5 cells, TSH activity | FRTL-5 cells, Graves’ IgG activity | TSHR CHO cells, TSH activity | TSHR CHO cells, Graves’ IgG activity | Mc2 cells, TSH activity | Mc4 cells, Graves’ IgG activity |
|---------------------|----------------------------|-----------------------------------|-----------------------------|-------------------------------------|------------------------|--------------------------------|
| Myxedema            | 95 ± 4                     | 96 ± 4                            | 98 ± 2                      | 99 ± 1                              | 94 ± 4                 | <15                            |
| IgG 1               |                           |                                   |                             |                                     |                        |                                |
| DT11E1              | 92 ± 6                     | 90 ± 8                            | 94 ± 5                      | 97 ± 3                              | 92 ± 8                 | <15                            |
| LK3D21              | 98 ± 2                     | 95 ± 3                            | 99 ± 1                      | 97 ± 3                              | 100                   | <15                            |
| CM6F4               | 90 ± 7                     | 92 ± 6                            | 94 ± 5                      | 89 ± 8                              | 94 ± 6                 | <15                            |
| DT4D2               | 93 ± 6                     | 94 ± 6                            | 88 ± 8                      | 90 ± 6                              | 91 ± 4                 | <15                            |
| LK7G1               | 91 ± 5                     | <15                               | 90 ± 7                      | <15                                 | <15                   | ND                             |
| WHITF2              | 88 ± 4                     | <15                               | 86 ± 8                      | <15                                 | <15                   | ND                             |
| DT9F1               | 75 ± 6                     | <15                               | 71 ± 6                      | <15                                 | <15                   | ND                             |
| CM5C3               | 68 ± 8                     | 73 ± 5                            | 36 ± 5                      | ND                                  | ND                    |                                |

A. Bold values indicate significant increases (P < 0.01) in cAMP levels caused by 1 × 10⁻¹⁰ mol/L purified bovine TSH or 200 μg/ml Graves’ IgG 1 in FRTL-5 thyroid cells and CHO cells transfected with the wild-type TSHR, the Mc2 TSHR-LH/CGR chimera, or the Mc4 chimera, measured as described in Materials or Methods. Data are the mean ± SD of 4 experiments, each performed in quadruplicate. Data using IgGs from all 6 other Graves’ patients were similar, albeit quantitatively different. Normal IgG was a pool from 20 normal individuals.

B. Bold values indicate significant inhibition (P < 0.01) of the ability of 1 × 10⁻¹⁰ mol/L purified bovine TSH or 200 μg/ml of Graves’ IgG (no. 2) to increase cAMP levels in FRTL-5 thyroid cells and CHO cells transfected with the wild-type TSHR, the Mc2 TSHR-LH/CGR chimera, or the Mc4 chimera, as measured in Materials or Methods. Data are the mean ± SD of 4 experiments, each performed in quadruplicate. Data using IgGs from all 3 other myxedema patients were similar, as were data using Graves’ IgG 1, 3, and 4. Normal IgG was a pool from 20 normal individuals. Clones noted in italics are TBIs.

Fig. 5. Ability of four TBII (DT11E1, LK3D21, CM6F4, and DT4D2) to inhibit the ability of a Graves’ stimulating TSHR Ab to increase cAMP levels when added together in FRTL-5 cell assays. FRTL-5 cells were preincubated with the noted monoclonal TBI-TSHR inhibitor or a representative stimulating TSHR Ab (DT12B4) for 30 min before Graves’ IgG no. 6 was added. After 3 h, cAMP levels were measured as described. The data are the mean ± SD of quadruplicate values from one of three experiments, each yielding similar results. A single asterisk represents a significant decrease in activity at P < 0.05, whereas two asterisks denote a significant decrease at P < 0.01. Data using four other Graves’ IgGs (no. 1, 2, 4, and 5) were similar except for the absolute values.

The patient also has a TBII with blocking activity that inhibits both stimulating TSHR Ab and TSH activity. Its functional epitope is on the C- not the N-terminus of the TSHR extracellular domain, as neither activity is not lost in Mc2 or Mc1+2 chimeras with TSHR residues 90–165 or 8–165 substituted, but both are lost in Mc4 chimeras with residues 261–370 substituted. This antibody very likely accounted for the ability of the maternal IgG to inhibit TSAb activity (11–13). Transplacental passage of this and the stimulating TSHR Ab at different times, as a result perhaps of different maternal populations of antibodies, cannot unreasonably account for variable degrees in the delayed onset of hyperthyroidism and the different clinical courses of the children in the absence of therapy. From the point of view of the management of mothers such as the patient herein, it is clear that the CHO-chimera assay system, as evidenced in recent reports (31, 32), can provide advance recognition of these two antibody populations in the IgG of a mother, thereby better enabling physicians to anticipate the clinical course of the neonate.

The present studies also establish that the prediction of two different TBII populations in Graves’ patients (32) is correct, and the patient has both. One TBII, tentatively termed type I, is directed against residues 90–165 and can block TSH, but not stimulating TSAb, activity. It is termed a type I TBII in our previous report (32). This antibody would not be likely to affect the ability of a stimulating TSHR Ab to induce hyperthyroidism, but could well influence the course of the disease during therapy. Thus, as stimulating TSHR Abs are decreased in the sera by methimazole and as TSH levels return to normal, this type of TBII could cause hypothyroidism by blocking TSH action (31). Its presence, when detected by the chimera assay, could influence the need for thyroid...
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Fig. 6. Ability of IgGs from heterohybridoma clones CM5C3, DT2F1, LK7G1, and WH7F2 to increase \([^{3}H]\)arachidonic acid release (A) or inositol phosphate production (B) in FRTL-5 thyroid cells. The activity in A is compared with the activity of two positive (10 μmol/L A23187 and 1 × 10^{-6} mol/L purified bovine TSH) as well as three negative controls (ethanol at the concentration in the A23187 solution, the DT11E1 blocking TSHRAb, and IgG from a pool of 20 normal individuals). In B, the activities of CM5C3 (△), WH7F2 (□), LK7G1 (○), and DT2F1 (▼) are compared as a function of IgG concentration, and results are expressed relative to the action of the same concentration of a pool of IgG from 20 normal individuals, whose activity is set at 1 and approximates 750 ± 100 cpm in all cases. Data are the mean ± SD of quadruplicate values from one of three experiments, each yielding similar results.

Table 5. Ability of antihuman IgG to cause TBIIs to increase cAMP levels in Mc2 chimeras

| IgG source             | cAMP level (fold over control) in Mc2 chimeras |
|------------------------|-------------------------------------------------|
|                        | No antihuman IgG                               |
|                        | Plus antihuman IgG                             |
| LK7G1                  | 1 ± 0.3                                        |
| WH7F2                  | 1 ± 0.2                                        |
| DT2F1                  | 1 ± 0.3                                        |
| CM5C3                  | 1 ± 0.3                                        |
| Myxedema IgG 1         | 12 ± 2                                         |
| DT11E1                 | 14 ± 4                                         |
| LK3D21                 | 7 ± 1                                          |
| CM6F4                  | 11 ± 2                                         |
| DT4D2                  | 14 ± 3                                         |
| Normal IgG             | 1 ± 0.2                                        |
| TSH (1 × 10^{-10} mol/L)| 143 ± 10                                       |

Bold values represent a statistically significant increase in cAMP levels, *P < 0.01. The ability of 200 μg/ml of each IgG to increase cAMP levels in the presence or absence of goat antihuman IgG (1:50 diluted Fab fragment) was measured as described in Materials and Methods. Data using IgGs from all 3 other myxedema patients were similar. Normal IgG was a pool from 20 normal individuals.

The second type of Graves’ TBI, termed tentatively type II, has a different functional epitope from the type I TBI, resides between 24–89, not residues 90–165. Thus, its binding is lost to membranes from the Mc1 + 2, but not the Mc2, chimera. The type II TBI exhibits conversion activity, as we previously suggested (32), and activates the inositol phosphate signal transduction system, as predicted in for some TBII in early monoclonal TSHRAb studies (19). This Graves’ TBI is functional and cannot be construed simply as an inhibitor of TSH activity. These antibodies could have important functional consequences in increasing iodide efflux, increasing iodination of TG by activating the peroxidase system, and increasing thyroid growth in concert with antibodies increasing CAMP levels (15, 16, 19, 51–53).

Several miscellaneous points to consider are as follows. In a previous report (55), the TSHRAb population in the patient was predicted to have a κ light chain; the present report confirms this prediction. In previous reports (11–13), the patient’s IgG enhanced TSH binding at low concentrations, but inhibited it at high concentrations. This phenomenon is not accounted for by the TSHRAb populations described herein; however, clones with enhancing properties were identified in early studies of monoclonal TSHRAbs (17). The patient does have clones producing antibodies that can enhance TSH binding and activity; the existence of such clones was noted during the screening process, but they were not further characterized. Finally, the results suggest that the term blocking TSHRAb might be best reserved for the TBI autoantibodies that inhibit both stimulating TSHRAb and TSH activities and that a Graves’ TBI should not be equated with it, because it has a different functional epitope and is not a blocker of stimulating TSHRAb activity.

In summary, the patient’s lymphocytes produce stimulating TSHRAbs, two TBIIIs characteristic of Graves’ TBIIIs, and a TBI that is a blocking TSHRAb associated with hypothyroidism in some patients with idiopathic myxedema and Hashimoto’s disease. One of the purposes of the characterization of monoclonal receptor antibodies is to define the epitopes and mechanisms of TSHRAbs; this report achieves this, but requires the chimera assays to accomplish the task. The data validate predictions in studies using the TSHR-LH/CGR chimeras to characterize the heterogeneous population of TSHRAbs in patient IgG preparations (31, 32, 56) that disease diversity is caused by the differences in functional epitopes and properties of TSHRAbs and that the chimera assay system can be used to identify individual types of TSHRAbs in the serum, and this information can be used to predict the clinical course of autoimmune thyroid disease caused by TSHRAbs. Additionally, a reasonable conclusion from the data is that Graves’ and Hashimoto’s patients have a diversity of IgGs, reinforcing conclusions from other studies (17–19, 22, 25–35, 44, 50–54, 56).
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