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Monoclonal antibodies to the thyrotropin receptor: Implications for receptor structure and the action of autoantibodies in Graves disease

(ganglioside/cholera toxin/hybridoma/adenylate cyclase/thyroid)

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ABSTRACT **Hybridoma cells have been obtained by fusing P3-NS1/1-Ag4-1 mouse myeloma cells with spleen cells from mice immunized with solubilized preparations of the thyrotropin receptor. Five clones were produced that secrete a monoclonal antibody whose binding to thyroid membranes is specifically inhibited by unlabeled thyrotropin. The antibody interacts with functioning thyroid cells in culture but not with nonfunctioning cells; this interaction is prevented by thyrotropin. The antibodies are capable of competitively blocking thyrotropin binding to bovine thyroid membrane preparations; they prevent ^{125}I-labeled thyrotropin binding to a solubilized preparation of the glycoprotein component of the bovine thyrotropin receptor but are unable to inhibit ^{125}I-labeled thyrotropin binding to liposomes containing gangliosides at comparable concentrations. They prevent ^{125}I-labeled thyrotropin binding to rat, bovine, or human (Graves disease) thyroid membrane preparations. They do not stimulate adenylate cyclase activity in thyroid membrane preparations but can inhibit thyrotropin-stimulated iodide uptake by functioning thyroid cells in culture.**

Recent studies have suggested that the thyrotropin (TSH) receptor—i.e., its surface interaction site—is composed of a glycoprotein component and a ganglioside component (1–3). An immune approach to ascertain the physiologic relevance of these receptor components and to further characterize their structure and function was unfortunately limited when it was observed that, despite their ability to precipitate solubilized receptor activity (4, 5), rabbit antibodies to the glycoprotein component of the receptor did not block TSH binding to cells or membranes (unpublished observation). This problem has also been noted in studies of antibodies to acetylcholine (6) and insulin receptors (7, 8). Nevertheless, the immune approach remained extremely attractive because it has been shown that patients who have Graves disease have autoantibodies capable of inhibiting TSH binding to membrane preparations (9–13).

In an effort to resolve questions concerning the structure and function of the TSH receptor, we adopted the immunologic technique first described by Köhler and Milstein (14) to generate homogeneous antibodies (monoclonal antibodies), each directed at a single antigenic site. In this report, we describe the production and properties of several monoclonal antibodies that have characteristics of an antibody to the binding determinant of the TSH receptor. These antibodies appear to be directed against the glycoprotein component of the TSH receptor and are inactive as thyroid stimulators. We discuss the relevance of these data to the nature of autoantibodies in patients with Graves disease.

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MATERIALS AND METHODS

Immunization and Fusion Procedures. Three-month-old BALB/c mice received subcutaneous injections of lithium diiodosalicylate-solubilized thyroid membrane preparations, 250 μg of protein each, in complete Freund's adjuvant and, on days 8 and 27, two intraperitoneal injections without adjuvant. On day 30, spleens were aseptically removed and dissociated mechanically. The single spleen cells (1×10^8) were then fused with P3-NS1/1-Ag4-1 mouse myeloma cells (2×10^7) in the presence of polyethylene glycol (15). After fusion, cells were dispersed, incubated, and fed by using selective medium as detailed by Trisler *et al.*§

Expansion of Hybridomas. Wells that contained visible colonies (10–14 days after fusion) were assayed for antibody activity by using the ^{125}I-labeled affinity-purified F(ab')$_2$ fragment of rabbit anti-mouse IgG (heavy and light chains) [^{125}I-F(ab')$_2$] indirect radioimmunoassay technique described below. The selected cell lines were then subcloned, and single clusters of cells were expanded in tissue culture in selective medium and, after reaching the desired quantities, stored at −90°C for future use. Hybrid cells were also grown as ascites tumors in BALB/c mice to yield antibody-containing ascites fluids. A partially purified IgG fraction was prepared from the culture medium and the ascites fluids of each line by two precipitations from ammonium sulfate at 0–45% of saturation. The IgG fractions were dialyzed for 48 hr at 4°C against excess 20 mM Tris·acetate, pH 7.5/20 mM NaCl.

^{125}I-F(ab')$_2$ Indirect Radioimmunoassay. Hybridoma antibody binding to thyroid membranes was assayed by an ^{125}I-F(ab')$_2$ radioimmunoassay using membranes in suspension or adsorbed to microtiter wells. In the suspension assays, 50 μl of each culture medium was placed in the wells of a 96-well microtiter plate (Dynatech No. 1-220-24) in the presence or absence of 1 μM unlabeled TSH and mixed with 400 μg of a bovine thyroid membrane preparation in 20 μl of 0.02 M Tris·acetate, pH 7.0. After 1 hr at 4°C, the plate was centrifuged for 5 min at 2000 rpm, and the supernatant was discarded. The pellets were then washed twice with 0.15 ml of phosphate-buffered saline P_i/NaCl/0.1% gelatin/0.02% sodium azide. ^{125}I-F(ab')$_2$ (Cappel Laboratories, Cochranville, PA), diluted in 1% crystalline bovine serum albumin/0.1% gelatin in P_i/NaCl, was

Abbreviations: TSH, thyrotropin; ^{125}I-F(ab')$_2$, ^{125}I-labeled affinity-purified F(ab')$_2$ fragment of rabbit anti-mouse IgG (heavy and light chains); P_i/NaCl, phosphate-buffered saline; Con A, concanavalin A.

§ Trisler, D. G., Schneider, M. D. & Nirenberg, M. (1981) in *Proceedings of the 5th Annual Symposium on Occular and Visual Development:* "Regulation of Occular Size and Shape during Development," Philadelphia, PA, eds. Hilfer, S. R. & Sheffield, J. B. (Springer, New York), in press.

added to each well (20,000 cpm/50 μl) and incubated for 45 min at 4°C. At the end of the incubation, the unbound radioactivity was removed; the plate was washed twice as above, heat dried for 5 min under an IR lamp, and cut into individual wells; and the bound radioactivity was assayed in a Beckman 9000 gamma counter.

In other experiments, membranes (0.5–10 μg) were adsorbed to polylysine-coated microtiter plates as detailed below in the description of the solid-surface ^{125}I-labeled TSH binding assay. After removing the nonattached membrane suspension and washing the wells, 50 μl of each culture medium was placed in the well in the presence or absence of 1 μM unlabeled TSH. After 2–12 hr at 4°C, the plates were centrifuged, the supernatant was removed, the wells were washed twice with 0.15 ml of P_i/NaCl containing 0.1% gelatin/0.02% sodium azide, and the ^{125}I-F(ab')$_2$ radioimmunoassay was continued as described above.

Solid-Surface ^{125}I-Labeled TSH Binding Assay. A solid-surface radioreceptor assay to detect binding of ^{125}I-labeled TSH to exceedingly low quantities of TSH receptor sites on thyroid membranes has been developed; its properties will be described in detail elsewhere. Briefly, Dynatech microtiter plates were precoated with 0.1 ml of poly-L-lysine (M_r 70,000; Sigma; 20 μg/ml of water) for 1 hr at room temperature. The poly-L-lysine was then removed, and 0.1 ml of thyroid membranes diluted to the desired concentrations in 20 mM Tris·acetate (pH 7.0) was pipetted to each well. Controls consisted of poly-L-lysine coated or uncoated wells incubated with 0.5% bovine serum albumin in the above buffer. After 4 hr or more at 4°C, unadsorbed membranes were removed and each well was washed with buffer (0.5% bovine serum albumin/0.02 M Tris·acetate, pH 6.7) for 30 min at room temperature. The wash buffer was removed, and each well was then incubated with ^{125}I-labeled TSH (40,000 cpm) in 50 μl of the same buffer. After 2 hr at 4°C, the microtiter plate was centrifuged, the unbound radiolabeled ligand was decanted, and the radioactivity in each well was assayed.

Unless otherwise noted, the inhibition of ^{125}I-labeled TSH binding by the monoclonal antibodies, culture media, ascites fluid, or their partially purified IgG fractions from selected hybridomas, in 0.02 M Tris·acetate, pH 7.0/0.5% bovine serum albumin was evaluated by their addition to adsorbed membranes for 2 or more hr. After removing the unbound antibody, binding buffer containing ^{125}I-labeled TSH was added, and the radioactivity associated with the surface was determined.

Unless otherwise stated, all values are averages of triplicate determinations. Controls included incubations with all components except membranes and substituting medium or ascites fluid produced by hybridomas, which interact with thyroid membranes but whose interaction was not affected by TSH (see Table 1, Group I). These values are subtracted from the data presented and never exceeded 5–8% of experimental values.

Other Materials and Methods. Cholera toxin was from Schwarz/Mann. TSH, ^{125}I-labeled TSH, thyroid membrane preparations, and solubilized preparations of the glycoprotein component of the TSH receptor were obtained as described (4, 5, 16–18). The preparation of liposomes containing the glycoprotein component of the TSH receptor or mixed brain gangliosides has also been described as have the methods to measure their ability to bind ^{125}I-labeled TSH (19). Protein was measured by a colorimetric procedure (20). Adenylate cyclase was measured by using the method of Salomon (21). Assay conditions and incubations were as described (unpublished results).

Cultured rat thyroid cells were kindly provided by F. S. Ambesi-Impiombato and G. Lee (National Institutes of Health, Bethesda, MD). Functional (FRTL) cells are those described in ref. 22. Nonfunctioning thyroid cells (FRT) are a derivative line that does not concentrate iodide or synthesize thyroglobulin; they are similar in most properties to primary thyroid cell cultures grown in the absence of TSH (23, 24).

RESULTS

Identification of Hybridomas Secreting Monoclonal Antibodies Inhibiting TSH Binding. Of the 2268 wells seeded from two separate fusion experiments, 346 hybridoma colonies were detected after 2 weeks in culture. Fifty-five colonies secreted antibodies into the culture medium that were reactive with thyroid membranes as assayed by ^{125}I-F(ab')$_2$ binding. These could be divided into five groups by including unlabeled TSH or cholera toxin in the ^{125}I-F(ab')$_2$ binding assay (Table 1); group II includes the five colonies that produced antibodies whose binding to thyroid membranes was completely inhibited by unlabeled TSH. The antibodies produced by groups II–V were not inhibited in their membrane interaction by comparable (1 μM) concentrations of insulin or human chorionic gonadotropin. In addition to prevention membrane interaction by unlabeled TSH, antibodies developed by group II clones were also able to inhibit ^{125}I-labeled TSH binding to thyroid membranes. The group II clones did appear to secrete antibodies with different inhibitory capacities, but no significant differences in inhibitory activity could be detected when antibody preparations from culture supernatants or ascites fluids were interchanged.

Characterization of Monoclonal Antibodies to the TSH Receptor. Inhibition of ^{125}I-labeled TSH binding by antibodies produced by group II clones was dependent on both the concentration of antibodies added to the binding assay (Fig. 1*A*) and the concentration of membranes attached to the plate and present in the assay (Fig. 1*B*). Inhibition also varied according to the expression of TSH binding activity as modulated by concanavalin A (Con A). Thus, pretreatment of membranes with increasing concentrations of Con A causes ^{125}I-labeled TSH binding to increase in comparison with that of untreated control membranes (Fig. 2; unpublished results); concomitant with the increased receptor expression, antibody effectiveness as an inhibitor decreased. The presence of α-methyl mannoside during ^{125}I-labeled TSH binding ensures that this is a specific interaction with a membrane receptor and not simply TSH binding to membrane-bound Con A (unpublished results).

Antibodies could inhibit ^{125}I-labeled TSH binding when included with membranes during attachment (data not shown) or when immobilized on membranes before hormone addition (see Fig. 1*A*, preincubation) and could inhibit TSH binding if di-

Table 1. Effect of unlabeled TSH and cholera toxin on monoclonal antibody-dependent ^{125}I-F(ab')$_2$ binding to thyroid membranes

Group	Clones in group, no.	Representative hybridoma clone	Binding, % of control: Thyrotropin (1 μM)	Binding, % of control: Cholera toxin (1 μM)
I	46	T-18A3	100	94
II*	5	T-21D10	0	50
III	1	T-28E4	20	10
IV	1	T-25E2	80	0
V	2	T-17D8	150	220

The ^{125}I-F(ab')$_2$ screening procedure is detailed in *Materials and Methods*. Each well contained 50 μl of culture media from the appropriate colonies.

* Antibodies from hybridoma clones T-13D11, T22A6, T11E8, and T29C7 yielded the same results.

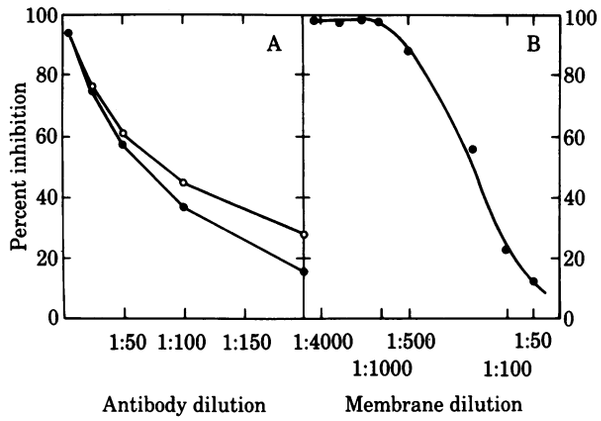

FIG. 1. (*A*) Ability of hybridoma antibodies from clone T-11E8 to compete with (●) or prevent (○) ^{125}I-labeled TSH binding to bovine thyroid membranes. Thyroid membrane-coated wells were either preincubated with the noted dilutions of a 0–45% ammonium sulfate fraction from T-11E8 ascites fluid (12 mg of protein/ml) before the ^{125}I-labeled TSH binding assay was initiated (preincubation) or the noted dilutions were present with the ^{125}I-labeled TSH during the binding assay itself (competition). TSH binding was otherwise determined as described in *Materials and Methods*. (*B*) ^{125}I-labeled TSH binding to different amounts of thyroid membranes on solid surfaces in the presence of a fixed concentration of monoclonal antibody. In both *A* and *B*, the control 100% value is the amount of ^{125}I-labeled TSH binding to the same dilution of membranes in the absence of an ascites fluid preparation or in the presence of a group I-type ascites preparation (see Table 1).

rectly included in the assay (Fig. 1*A*, competition) with ^{125}I-labeled TSH. Unlabeled TSH could also prevent or reverse the binding of antibody to thyroid membranes as measured by ^{125}I-labeled protein A binding (25). Binding of TSH to the membranes and binding of the antibody was competitive. Thus, when binding was evaluated at two different concentrations of antisera in the presence of different concentrations of unlabeled

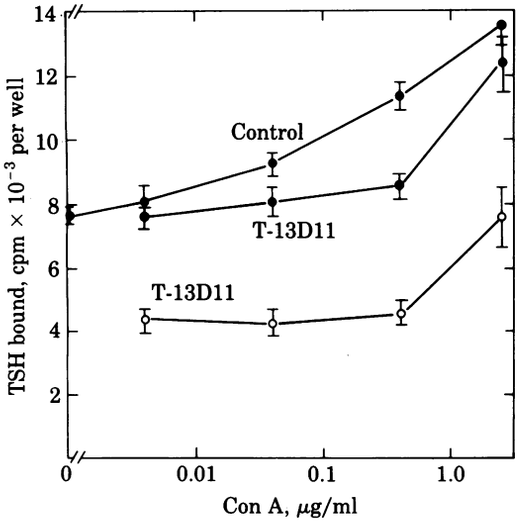

FIG. 2. Ability of the T-13D11 monoclonal antibody to inhibit ^{125}I-labeled TSH binding to thyroid membranes whose TSH binding ability was enhanced by pretreatment with Con A. Con A treatment was performed on the adsorbed membranes by adding 0.1 ml at the noted concentration in 0.02 M Tris·acetate, pH 6.7/0.5% bovine serum albumin. After 1 hr at 4°C, the Con A solution was removed, and the normal washing procedure with 0.5% bovine serum albumin/0.02 M Tris·acetate, pH 6.7, proceeded. An ascites fluid antibody diluted to 0.11 mg/ml (○) or 0.011 mg/ml (●) was added for 2 hr prior to the addition of ^{125}I-labeled TSH.

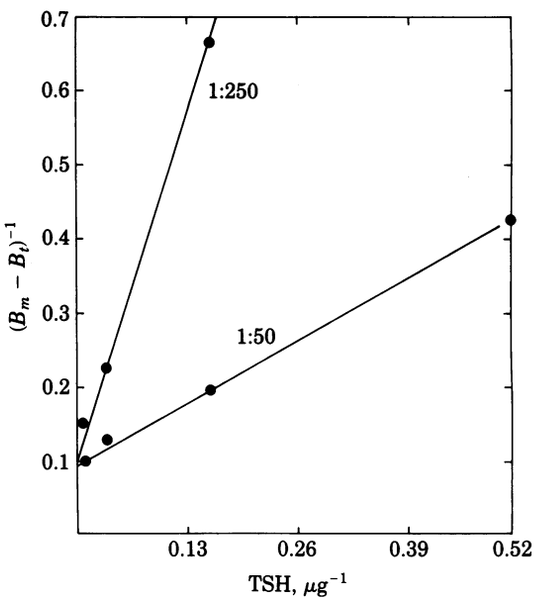

FIG. 3. Ability of unlabeled TSH to competitively inhibit a preparation of the T-13D11 monoclonal antibody binding to thyroid membranes as measured by using ^{125}I-labeled protein A. Bovine thyroid membranes (0.2 mg/ml) mixed with various concentations of TSH were added in 50-μl aliquots in microtiter wells and incubated for 30 min at room temperature. Antibody from T-13D11 ascites fluid at a final concentration of 0.2 mg/ml (1:50) or 0.04 mg/ml (1:250) was added, and the mixture was incubated for 3 hr at 4°C. After centrifugation and washing, 20,000 cpm of ^{125}I-labeled protein A was added to the wells, and the radioactivity was assayed. B_m, reciprocal of the maximum ^{125}I-labeled protein A bound in the absence of TSH; B_t, percent of protein A bound at given TSH concentration. Values are the average of triplicate determinations. Identical results were obtained when T-11E8 antibody preparations were used.

TSH, ^{125}I-labeled protein A binding (the measure of antibody binding) exhibited competitive curves when expressed as a double reciprocal analysis (26)—i.e., as the inverse of displaceable ^{125}I-labeled protein A binding versus the inverse of unlabeled TSH added to the assay (Fig. 3).

Antibodies are able to bind to the surface of FRTL thyroid cells in culture but not to nonfunctional thyroid cells in culture (Fig. 4). With appropriate focusing, rim fluorescence is evident; unlabeled TSH can prevent antibody binding to the functional thyroid cells.

Antibody preparations were able to inhibit TSH binding to human membrane preparations (Table 2) as well as to rat thyroid membranes (see Fig. 4) and bovine thyroid membranes (see Figs. 1–3). The antibodies inhibited ^{125}I-labeled TSH binding to the glycoprotein component of the bovine thyroid membrane TSH receptor alone or in liposomes but not to gangliosides embedded in liposomes (see Table 2). The antibodies were unable to stimulate adenylate cyclase activity in thyroid membrane preparations even at 10- and 100-fold higher concentrations than used to completely inhibit TSH binding (Table 3).

Iodide uptake by FRTL cells is one measure of their functional state (22). FRTL cells exposed to a 1/10th dilution of T-13D11 antibody for 1 hr decreased their iodide uptake 60%. The same antibody, but not a group I antibody (see Table 1), at 1/10th and 1/100th dilutions, prevented TSH-stimulated iodide uptake. In these experiments, TSH (1 and 10 μM) caused 2- and 3-fold increases in iodide uptake over media without TSH supplements; cells were used 15 min or 3 hr after their detachment by the trypsin-collagenase-serum mixture used in their passage (22) and assays measured iodide uptake at various times

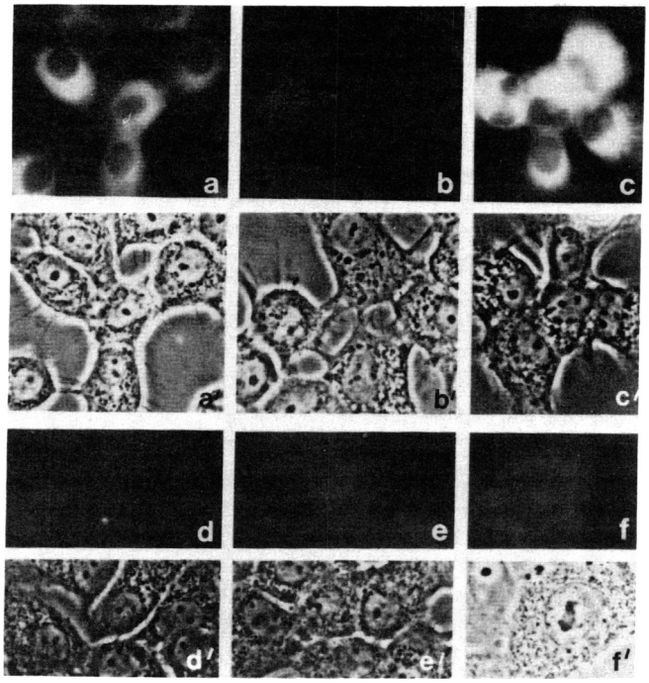

FIG. 4. Indirect immunofluorescence labeling of thyroid cells by the anti-TSH receptor monoclonal antibody. FRTL (*a–e*) and FRT (*f*) rat thyroid cells grown on poly-L-lysine-precoated glass coverslips for 3 or 4 days were rinsed in P_i/NaCl and layered with group II antibody preparations from either T-11E8 (*a*, *b*, and *f*) or T-13D11 (*c* and *d*) ascites fluids (10–12 mg/ml) diluted 1:20 in P_i/NaCl. TSH (1 mg/ml) was added to experiments shown in *b* and *d*. After 1 hr at room temperature, the coverslips were rinsed three times in P_i/NaCl and then exposed to fluorescein-conjugated goat anti-mouse IgG (Miles, Yeda) diluted 1:20 for 1 hr at room temperature. After washing, the coverslips were mounted in glycerol/0.1 M glycine, pH 8.6 (2:1 by vol) and examined with a Zeiss microscope equipped with phase-contrast and fluorescence optics. Control cells using a native ascites (type I; see Table 1) are shown in *e*. (*a'–f'*) Phase-contrast counterparts of *a–f*. (×780.)

between 1 min and 6 hr. Details of the experiments will be presented in a separate report.

DISCUSSION

We have produced and partially characterized several hybridoma cell lines that secrete monoclonal antibodies that inhibit TSH binding to thyroid membrane preparations. The ability of these antibodies to inhibit TSH binding is specific; inhibition does not occur with all antibody preparations that can interact with thyroid membranes, and the antibody interaction with thyroid membranes is not prevented by insulin or human chorionic gonadotropin. In the case of two of the monoclonal antibodies, those from hybridoma lines T-13D11 and T-11E8, we show that these antibodies can competitively (see Fig. 3) block TSH binding to thyroid membrane preparations and that unlabeled TSH blocks their binding to thyroid cells in culture (see Fig. 4). These results plus the very fact that they are monoclonal—i.e., are antibodies to a single antigenic determinant—suggest that they are antibodies to the physiologically relevant binding site of the TSH receptor; the ability of the antibodies to block an early TSH-mediated response, iodide uptake, further supports this view.

Table 2. Effect of antibody on ^{125}I-labeled TSH binding to human membranes, to gangliosides, and to the glycoprotein component of the bovine TSH receptor

	Human (Graves disease) membranes*	Glycoprotein component of TSH		Bovine gangliosides in liposomes†
		Free*	In liposomes†	
Control	10.0	3.1	15.2	18
+ T-11E8 antibody	1.9	1.0	3.1	17.5

* Aliquots (0.1 ml) of either human thyroid membranes (50 μg/ml) or solubilized bovine thyroid membrane preparations (40 μg/ml) were used to coat the wells as described in *Materials and Methods*. Antibody preparation from T-11E8 ascites fluid was used at a dilution of 1:100 and was added along with the labeled hormone.

† These assays used a filtration procedure described in ref. 20. Binding to liposomes was in 0.01 M Tris·acetate, pH 6.0/0.5% bovine serum albumin.

Table 3. Inability of monoclonal antibodies to the TSH receptor to stimulate adenylate cyclase activity of bovine thyroid membranes

Addition	Adenylate cyclase activity
None (basal)	106
+ TSH (0.1 μM)	388
+ T-13D11 (group II)	105
+ T-18A3 (group I)	110

Values are expressed as (pmol of cyclic AMP/15 min)/mg of membrane protein. Antibodies were tested as 0–45% ammonium sulfate fractions of culture media (10–12 mg/ml). All assays presented at 1:10 dilution. No changes in adenylate cyclase activity were evident at 1:100 or 1:1000 dilutions. Antibodies secreted by clones T-21D10, T-22A6, T-11E8, and T-29C7 yielded the same results. For group classification, see Table 1.

Autoantibodies that inhibit TSH binding and stimulate adenylate cyclase activity have been implicated in the pathogenesis of Graves disease (9–13). The several experimentally produced monoclonal antibodies that inhibit TSH binding fulfill all criteria established in clinical studies (9–13) for such autoantibodies in terms of inhibition of TSH binding. The failure of these antibodies to stimulate adenylate cyclase activity in membrane preparations (see Table 3) is paralleled by their inability to stimulate adenylate cyclase activity in slice systems (unpublished observations). At the very least, therefore, we must conclude that antibodies directed against the glycoprotein component of the TSH receptor need not be thyroid stimulators.

As noted above, our current view is that the TSH receptor is composed of a glycoprotein and a ganglioside (1–3). The hybridoma antibodies in this report appear to be directed against the glycoprotein component (see Table 2). The importance of this component as the physiologically relevant binding determinant on the surface of cells is clear from Fig. 4 and supports the conclusion originally evident in studies using trypsinized thyroid cells (23, 27). The inability of monoclonal antibodies directed primarily against the glycoprotein component of the TSH receptor to stimulate the adenylate cyclase response is consistent with the suggestion (1–3) that the glycoprotein component alone is insufficient to transmit the TSH message to the cyclase system and that the functional TSH receptor may require the ganglioside.

It has been suggested that the cholera toxin receptor structure shares a number of properties with the TSH receptor (1–3). The ability of cholera toxin to partially inhibit the interaction of the monoclonal antibodies to the TSH receptor supports this hypothesis. Relationships among the structure and function of receptors for TSH, tetanus toxin, and interferon have also been suggested (1–3); the monoclonal antibodies offer a means of distinguishing between determinants common to all and determinants unique for each ligand–receptor interaction.

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