Monoclonal antibodies to the thyrotropin receptor: Stimulating and blocking antibodies derived from the lymphocytes of patients with Graves disease

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ABSTRACT Human monoclonal antibodies have been generated from hybridomas obtained by fusing mouse myeloma cells with peripheral lymphocytes from patients with active Graves disease. This report characterizes four antibodies as presumptive thyrotropin receptor antibodies because they specifically inhibit thyrotropin binding and competitively inhibit thyrotropin-induced cAMP levels in human thyroid cells. Two of these antibodies, 208F7 and 206H3, are representative of autoimmune stimulators in Graves disease sera because they stimulate thyroid function in all assays, including the mouse bioassay; their ability to inhibit thyrotropin-induced cAMP increases in thyroid cells competitively is complemented by more than additive agonism at (10 pM) thyrotropin concentrations. These stimulating antibodies interact more potently with human thyroid ganglioside preparations than with bovine thyroid or brain gangliosides; in contrast, they are poor inhibitors of 125I-labeled thyrotropin binding to liposomes containing the glycoprotein component of the human thyrotropin receptor. Antibodies 129H8 and 122G3 appear to be representative of inhibiting or “blocking” antibodies in Graves disease sera. Thus they have no intrinsic stimulatory action in assays of thyroid function but rather inhibit thyrotropin activity in the assays tested. These two antibodies do not react with human thyroid gangliosides but are strong inhibitors of thyrotropin binding to liposomes containing the high-affinity glycoprotein component from human, bovine, and rat thyroid membranes. The data unequivocally establish the pluritopic nature of the immunoglobulins in Graves disease and relate individual components or determinants of the thyrotropin receptor structure with specific autoimmune immunoglobulins.

Multiple experiments have suggested that immunoglobulins in the sera of patients with Graves disease serve as the effector of thyroid overactivity and that this action is mediated by the thyrotropin (TSH) receptor (1–3). The variable correlation between the ability of immunoglobulin preparations from Graves disease patients (GD Ig) to inhibit TSH binding or adenylate cyclase activity and to act as intrinsic stimulating antibodies (1–3) and the presence of growth activators and depressors in GD Ig (4) suggest that a spectrum of antibodies might exist with distinct antigenic determinants.

To clarify specific effector roles of individual immunoglobulins and their relation to TSH receptor structure, monoclonal antibodies have been obtained by fusing mouse myeloma cells with peripheral lymphocytes from patients with active Graves disease. These antibodies represent clonal idiotypes of naturally determined immunoglobulins in patients with Graves disease.

In the present report we detail the properties of four such hybridoma antibodies and compare these properties with those of monoclonal antibodies derived from experiments with solubilized bovine and human TSH receptor immunizations and fusions (5–7).

MATERIALS AND METHODS

Patients. Six patients with active Graves disease served as lymphocyte donors. The diagnosis of Graves disease was based on clinical thyrotoxicosis with diffuse toxic goiter, exophthalmos, increased blood levels of thyroid hormone, and increased 131I uptake.

Production of Lymphocyte Hybridoma Clones. Peripheral lymphocytes were harvested by Ficoll-Hypaque gradient centrifugation of blood within 30 min of venipuncture. Lymphocytes were washed twice with Hanks balanced salt solution, and cell number was adjusted to 1 × 10⁷/mL in Dulbecco modified Eagle medium. Polyethylene glycol fusion with 5 × 10⁵ P3-NS1/1-AG4-1 mouse myeloma cells was performed as described (5, 6, 8). The fusion products were dispersed in “super” hypoxanthine/aminopterin/thymidine selection medium (5, 6, 8); additional medium was added every 4 days until visible hybridoma colonies had reached the 100- to 200-cell stage. The successful fusion rate consistently approximated 1.5% per plate.

Screening. A two-stage screening procedure using bovine thyroid membranes was utilized as detailed (5–7). In the first stage, hybridomas producing antibody reactive with thyroid membranes were identified by using 125I-labeled staphylococcal protein A or a 125I-labeled goat anti-human IgG F(ab')₂, fragment-specific antibody. In the second stage, the assay was repeated with competing 1 μM TSH added during the initial incubation of membranes and hybridoma medium. Hybridoma antibodies reactive with thyroid membranes in the first screening assay but blocked by unlabeled TSH in the second were chosen as potential TSH receptor antibodies.

Expansion of Antibody-Producing Hybridomas and Antibody Purification. Hybridomas were subcloned for 2 months to stabilize cell morphology and antibody production. Medium from overgrown cultures was harvested by centrifugation at 500 × g for 10 min; IgG preparations of monoclonal antibodies were prepared by protein A-Sepharose column chromatography (7) or ammonium sulfate precipitation (5, 6) with no significant difference in results. After dialysis against 50 mM Tris-Cl (pH 7.4), they were stored at −90°C. Polycrylamide gel electrophoresis of the protein A-purified fractions revealed homoge-

Abbreviations: TSH, thyrotropin; GD Ig, immunoglobulin preparation from Graves disease sera; LATS, long-acting thyroid stimulator.

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neous immunoglobulin preparations with a heavy chain of 50,000 daltons, consistent with an IgG.

**Assays and Materials.** Direct measurement of 125I-labeled TSH (125I-TSH) binding inhibition by the monoclonal antibodies was accomplished with normal human thyroid membranes and the solid-phase assay described (5–7). 125I-TSH binding was measured in 10 mM Tris-HCl, pH 7.4/50 mM NaCl/0.5% bovine serum albumin; incubations were at 37°C for 2 hr. The wells were again doubly washed and residual bound radioactivity was measured. Maximal counts bound to adsorbed membranes without antibody present averaged 6% of total counts added; nonspecific binding was 0.5%. Assays of monoclonal antibody stimulation of the adenylate cyclase system in human cells and cultured rat (FRTL) cells were performed as described (9, 10), as were measurements of thymidine uptake in FRTL cells. All experiments were performed in quadruplicate and included controls with no antibody or with normal human immunoglobulin preparations. The in vitro mouse bioassay was that described by McKenzie (11).

Protein was measured by using a colorimetric method (12). TSH (26 ± 4 international units/mg) and 125I-TSH were identical to preparations used previously (5–7, 13). Human thyroid membrane preparations were prepared as described (13–15) from the normal tissue surrounding nodular goiters or from normal thyroids obtained from accident victims autopsied within 30 min of death.

Studies of the antibody effect on 125I-TSH binding to the glycoprotein component of the TSH receptor from human, bovine, and FRTL cell membranes used procedures as detailed (5–7, 16) with liposomes containing the glycoprotein component obtained by solubilization of membranes with detergent (17). Human and bovine thyroid ganglioside preparations were prepared as described (18, 19); mixed brain gangliosides were from Supelco. Assays measuring antibody interactions with gangliosides used a solid-phase technique (20) with the following modifications: the buffer was 50 mM Tris-HCl, pH 7.4/1% delipidated bovine serum albumin (Sigma), and 125I-labeled protein A (Amersham) was used to measure antibody binding to adsorbed ganglioside preparations. This assay effectively measures the ability of the monoclonal antibody to bind to ganglioside-coated plastic wells by using an iodinated second ligand and appropriate wash conditions to ensure that specific interactions are measured.

**RESULTS**

**Identification of Hybridomas That Secrete Monoclonal Antibodies Reacting with Thyroid Membranes in the Absence but Not in the Presence of TSH.** Of 5,420 wells seeded from six separate fusions, 90 antibody-producing hybridoma colonies were noted after 4 weeks in culture. Four clones (206H3, 208F7, 122G3, and 129H8) produced antibodies that were reactive with bovine thyroid membranes, were inhibited in this interaction by 1 μM TSH but not by 15% serum, control medium, or 1 μM human chorionic gonadotropin, and were stable in their antibody production throughout the course of these studies. Some clones either did not react with bovine thyroid membranes or reacted with bovine membranes but were not inhibited by 1 μM TSH ( monoclonal antibody control in Table 1). The screening procedure used bovine thyroid membranes rather than human membranes because 125I-labeled protein A and 125I-labeled goat anti-human IgG exhibit significant basal binding to human membranes. (Binding of anti-human IgG or protein A averaged 5–20% of total with human membranes, compared to 0.2–2% with bovine membranes—i.e., blanks were too high for reliable measurement of inhibition by unlabeled TSH.)

![Graph showing inhibition of 125I-TSH binding by monoclonal antibodies from human Graves disease lymphocyte fusions in comparison to a normal IgG control.](image)

**Fig. 1.** Inhibition of 125I-TSH binding by monoclonal antibodies from human Graves disease lymphocyte fusions in comparison to a normal IgG control (●). The 122G3 antibody was assayed at 37°C in 10 mM Tris-HCl (pH 7.4) containing 50 mM NaCl (▼) and at 0–4°C in 20 mM Tris-acetate (pH 7.0) with no salts (○). All other antibodies, including the normal IgG control, were tested under both conditions and gave similar results, but only data from the 37°C assay with salts are presented (●, ▼, ●).

**Monoclonal Antibody Effects on 125I-TSH Binding.** All four antibodies demonstrated inhibition of 125I-TSH binding to normal human thyroid membranes, although individual potencies varied (Fig. 1). Binding inhibition was evident at 37°C in a salt-containing medium claimed to be specific for the high-affinity glycoprotein component of the TSH receptor (15). Binding in isolation of the monoclonal antibodies (Ab) to stimulate adenylate cyclase activity in human thyroid cells or to perturb the TSH- or cholera toxin-stimulated activity of these cells

<table>
<thead>
<tr>
<th>Ligand or clone</th>
<th>Amt. added</th>
<th>Direct effect on cells*</th>
<th>After Ab preincubation with cells†</th>
<th>Effect of TSH</th>
<th>Effect of cholera toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal human IgG control</td>
<td>0.1 mg/ml</td>
<td>0.7</td>
<td>—</td>
<td>—</td>
<td>12.3</td>
</tr>
<tr>
<td>TSH</td>
<td>0.5 mM</td>
<td>—</td>
<td>—</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>Cholera toxin</td>
<td>0.1 μM</td>
<td>12.2</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>208F7</td>
<td>0.1 mg/ml</td>
<td>1.4†</td>
<td>2.7†</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>1.5†</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>206H3</td>
<td>0.1 mg/ml</td>
<td>1.4†</td>
<td>2.9</td>
<td>27.7†</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>1.1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>129H8</td>
<td>0.1 mg/ml</td>
<td>1.0</td>
<td>3.98†</td>
<td>15.8</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>1.05</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>122G3</td>
<td>0.1 mg/ml</td>
<td>0.8</td>
<td>2.52†</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.6</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Monoclonal Ab control†</td>
<td>0.1 mg/ml</td>
<td>0.6</td>
<td>7.1</td>
<td>12.8</td>
<td></td>
</tr>
</tbody>
</table>

* Incubated for 2 hr at 37°C in Hanks basal salt solution (9).
† Preincubation was for 2 hr in Hanks basal salt solution before addition of TSH (0.5 milliunit/ml) or cholera toxin (0.1 μM).
‡ P < 0.01 for comparison of the antibody effect with the normal human IgG control or the monoclonal antibody control within each assay system utilized (i.e., within each column of data).

A monoclonal antibody able to interact with thyroid membranes but devoid of activity in any of the assays used in this report.
Inhibition was equally evident when assays were performed at 0–4°C in a salt-free buffer (20 mM Tris acetate at pH 7.0) (refs. 13 and 14: and Fig. 1).

Effects of Monoclonal Antibodies on cAMP Levels of Thyroid Cells and in the McKenzie Mouse Bioassay. When preincubated with human thyroid cells in culture, each of the antibodies inhibited the cAMP production induced by TSH (Table 1). Antibodies 208F7 and 206H3 were also able to increase cAMP levels in the human thyroid cell cultures. Dose-dependent stimulatory activity by these two antibodies was also evident with FRTL5 thyroid cells (Fig. 2A); furthermore, their inhibition (agonism) of the TSH response could be shown to be competitive at high (10 nM) TSH concentrations (Fig. 2C) and to be associated with more than additive agonism at low (0.1 nM) TSH concentrations (Fig. 2B). Some CD Ig have also been shown to elicit more than additive stimulation (agonism) of adenylate cyclase activity when incubated with cholera toxin (21); the 206H3 antibody had this characteristic (Table 1). Both the 208F7 and 206H3 antibodies were active in the mouse bioassay, as was the 22A6 "stimulating" antibody to the bovine TSH receptor previously described (7) (Fig. 3A). The antibody activity pattern in the mouse bioassay resembled that of TSH rather than the time course of a long-acting thyroid-stimulating immunoglobulin (Fig. 3B). In sum, antibodies 208F7 and 206H3 demonstrated the stimulatory properties defined for autoimmune stimulators present in the sera of patients with Graves disease; they also exhibit agonism–competitive antagonism phenomena with respect to TSH which additionally characterize them as antibodies against a determinant associated with the TSH receptor structure.

Inhibition of TSH-induced cAMP levels in human cell cultures by antibodies 129H8 or 122G3 was also associated with their ability to inhibit TSH-induced cAMP levels competitively in the FRTL5 cell culture system (data not shown, but similar to Fig. 2C). However, 129H8 and 122G3 were not agonists in the FRTL5 system when tested at lower TSH concentrations (0.01–1 nM) nor were they able to increase cAMP levels in the FRTL5 system when tested alone (Fig. 2A). The antibodies were inactive in stimulating thyroid iodine release in the mouse bioassay at all concentrations tested (0.01–2 mg/ml) but, when coincubated with TSH, the antibodies were able to decrease TSH activity in this bioassay (Fig. 3C). When stimulating antibodies 208F7 and 206H3 were coincubated with TSH (1 milliunit/ml) over the same concentration range (0.01–2 mg/ml IgG per ml), no inhibition was evident. In sum, 129H8 and 122G3 appeared to represent autoimmune "blocking" or "inhibiting" antibodies suggested to exist in Graves disease sera (1–3) and also fit criteria of being antibodies directed at a determinant associated with the TSH receptor structure.

Thyroid Membrane Components Reactive with the Mono- clonal Graves Disease Antibodies. In separate studies we have found that a stimulating monoclonal antibody [22A6 obtained from bovine TSH receptor fusions (7)] interacts primarily with gangliosides (7), whereas a blocking monoclonal antibody [13D11 and 11E8 (5, 6) or 59C9 and 60F5 (unpublished data) from bovine or human TSH receptor fusions] interacts poorly with gangliosides but is a good inhibitor of TSH binding to the glycoprotein receptor component imbedded in liposomes.

The stimulating antibodies described in this report interacted with human thyroid gangliosides; this interaction appeared to be specific with respect to bovine thyroid or bovine brain gangliosides (Table 2). The blocking antibodies (129H8 and 122G3), in contrast, were only weakly reactive with ganglioside preparations but were significantly better inhibitors of TSH binding to liposomes containing the glycoprotein receptor component derived from human, rat, or bovine membrane preparations by solubilization with lithium diiodosalicylate. Stimulating antibody 208F7, which was a better inhibitor of TSH binding to thyroid membrane preparations than was 206H3 (Fig. 1), was a better inhibitor of TSH binding to liposomes containing the glycoprotein receptor component. It is notable, however, that 208F7 was less effective in the liposome assay than 122G3 which

![Figure 2](image-url)
Fig. 3. Stimulatory activity of monoclonal antibodies in the McKenzie mouse bioassay (11). In all experiments, 0.1 ml of material was injected into each of six mice. The mean ± SEM are reported. (A) Stimulatory activity at 2 hr after injection as a function of concentration. TSH concentrations were 1.0, 0.4, and 0.15 milliunit/ml. Concentrations of 208F7, 206H3, and 22A6 (7) were 0.2, 0.08, and 0.02 mg/ml. Concentrations of normal IgG, 129H8, and 122G3 were 0.2 mg/ml. (B) Stimulatory activity of TSH (1 milliunit/ml) and 208F7 (0.2 mg/ml) as a function of time after injection. (C) Ability of monoclonal antibodies 129H8, 122G3, and 11E8 (5-7) to inhibit TSH activity; stimulating antibodies 208F7, 206H3, and 22A6 had no such activity at the same or at other concentrations tested (see text). Antibody (0.2 mg/ml) and TSH (1 milliunit/ml) were mixed and injected together; assays were at 2 hr.

was a poorer inhibitor of TSH binding to thyroid membranes. The potential significance of this last observation is discussed below.

Effect of the Antibodies on [14C]Thymidine Uptake by Rat Thyroid Cells. Recent studies have indicated that autoantibodies in patients with autoimmune thyroid disease affecting cell growth appear to be distinct from stimulating antibodies as currently defined (4). A separate study (22) has shown that TSH-stimulated thymidine uptake in FRTL-5 cells is a valid measure of TSH-dependent thyroid cell growth; the antibodies therefore were evaluated for their effect on [14C]thymidine uptake by using the same procedure (22). Compared to control uptake (1,890 ± 197 cpm with buffer alone or 2,287 ± 66 cpm in the presence of a normal IgG), antibodies 208F7 (2,776 ± 150 cpm), 129H8 (2,790 ± 157 cpm), and 206H3 (2,740 ± 180 cpm) were significant stimulators of [14C]thymidine uptake (P < 0.05 for comparison to both controls), like TSH (2,880 ± 418 cpm at 1 milliunit/ml and 6,070 ± 350 at 10 milliunits/ml). In contrast,

Table 2. Ability of the monoclonal Graves disease antibodies to react with various gangliosides or to inhibit TSH binding to liposomes containing the glycoprotein component of TSH receptor from various thyroid membrane preparations

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Ganglioside reactivity*</th>
<th>125I-TSH bound to liposomes, cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No lipid added</td>
<td>Human thyroid</td>
</tr>
<tr>
<td>Monoclonal antibody control</td>
<td>150</td>
<td>160</td>
</tr>
<tr>
<td>Stimulators</td>
<td>208F7</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>206H3</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>(22A6)</td>
<td>137</td>
</tr>
<tr>
<td>Blockers</td>
<td>129H8</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>122G3</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>(11E8)</td>
<td>156</td>
</tr>
</tbody>
</table>

Values were ±10% in replicate assays.
* In a solid phase assay (20). Values shown are cpm of 125I-labeled protein A bound to antibody-ganglioside complex.
1 Binding to liposomes used 20 μg of antibody per ml and 55,000 cpm of 125I-TSH. The filtration procedure was as described (5-7, 16-19). The different assay conditions are noted at the heads of the columns.
122G3 was not a stimulator (2,247 ± 85 cpm) but rather inhibited TSH-induced [14C]thymidine uptake completely at 1 milliunit/ml and 80% at 10 milliunits/ml at the same IgG concentrations (P < 0.025). At the same IgG concentrations 208F7, 129H8, and 206H3 did not inhibit the TSH response.

**DISCUSSION**

Autoantibodies in patients’ sera have been implicated in the pathogenesis of Graves disease (1–3, 21); however, the antigenic determinants and characteristics of individual autoantibodies are largely unknown. In the current series of experiments we have generated human monoclonal antibodies determined by the native immune message present in individual peripheral lymphocytes from patients with active Graves disease. With respect to activities currently used to assay autoimmune-stimulating antibodies in Graves disease sera, antibodies 206H3 and 208F7 are stimulating antibodies whereas 122G3 and 129H8 can be characterized as blocking or inhibiting antibodies. Yet, based on their ability to inhibit TSH binding specifically and to inhibit TSH-stimulated increase in cAMP levels in cells competitively, all four antibodies react with determinants on or closely approximated with the functional or “physiologic” TSH receptor on the cell.

With respect to specific membrane components that interact with TSH, as well as with respect to the properties of monoclonal antibodies to solubilized bovine and human TSH receptor preparations (5–7), the following generalization appears possible. Stimulating antibodies [206H3, 208F7, 22A6 (7)] appear to be more reactive with the ganglioside component of the TSH receptor (23), whereas the blocking antibodies [129H8, 122G3, 11E8, 13D11, 59C9, and 60F5 (5, 6)] appear to be primarily reactive with the high-affinity glycoprotein receptor component (23). Within this preliminary generalization, however, several additional comments should be made.

First, the species specificity exhibited by the stimulating antibodies with respect to interactions with gangliosides may provide a physicochemical basis for the “species specificity” exhibited by some autoimmune stimulating antibodies in Graves disease sera (1–3, 21). Preliminary results show that antibodies 206H3 and 208F7 do exhibit species specificity with respect to their ability to stimulate human and rat FRTL cells in comparison to bovine thyroid membranes.

Second, the simple generalization does not preclude the existence of a wide panel of antibodies with individual bioactivity and membrane component reactivity patterns. Several possibilities could account for this. For example, the carbohydrate determinant of the ganglioside could also exist on the glycoprotein receptor component, clones could recognize a conglomerate structure composed of both receptor components which might exist in situ, or monoclonal antibodies could view the TSH receptor complex in situ from multiple three-dimensional orientations. Any of these possibilities could account for the 208F7 and 122G3 discrepancy noted above.

Third, it has been predicted that autoimmune antibodies in Graves disease sera can affect thyroid cell growth, possibly by using mechanisms analogous to those for insulin or somatomedins, rather than operate via the cAMP message system (4). The present report confirms this by showing that at least one antibody, 129H8, can increase growth without being a stimulator in other assays but establishes that antibodies perturbing growth are nevertheless related to the TSH receptor structure.