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Genetic Immunization Against the Human Thyrotropin Receptor Causes Thyroiditis and Allows Production of Monoclonal Antibodies Recognizing the Native Receptor

S. Costagliola, P. Rodien, M.-C. Many, M. Ludgate, and G. Vassart

The generation of Abs recognizing the native structure of the human thyrotropin receptor (hTSHR) has been difficult because there is currently no method allowing the purification of correctly folded Ag in the amounts required by classical immunization protocols. The majority of Abs made against the hTSHR react preferentially with denatured molecules. We report that a humoral response against the native hTSHR, compatible with mAb production, is elicited in mice by immunization with a DNA construct encoding the receptor. BALB/c mice were inoculated in the anterior tibialis muscle with 100 μg of plasmid DNA harboring the hTSHR cDNA. Eleven weeks after the first injection, 10 mice of 14 showed by FACS analysis a strong IgG response against the hTSHR expressed at the surface of Chinese hamster ovary cells. A clear TSH-binding inhibiting Ig and thyrotropin-blocking Ab activity (competition with TSH binding and TSH activity, respectively) was demonstrated in the majority of sera tested. One serum exhibited a clear stimulating activity. Despite the maintenance of normal circulating free T4 levels in all mice, these bioactivities persisted until 18 wk, in which mice were sacrificed, their thyroids were examined histologically, and spleens from two animals were used for mAb production. All mice displayed a severe lymphocytic infiltration of their thyroids, composed mostly of activated B cells. Three mAbs were produced against conformational epitopes of the hTSHR. We conclude that genetic immunization is an efficient method of generating Abs recognizing the native structure of the hTSHR and a new way of inducing thyroiditis in mice murine.


The growth and differentiated functions of the thyroid are controlled by thyrotropin (TSH), which, on binding to its receptor, activates primarily the adenyl cyclase-cAMP cascade (1). The TSHR, like those of other glycoprotein hormones, luteinizing hormone (LH) and follicle-stimulating hormone, is a G protein-coupled receptor that is predicted to span the cell membrane seven times (2–4). The TSHR thus has three extracellular loops and an extensive extracellular amino terminus that together constitute the ligand binding site; the membrane-spanning domains with the three intracellular loops and the carboxyl terminus are responsible for G protein coupling and signal transduction (5, 6). Recently, our group proposed a structural model for the leucine-rich domain of the extracellular portion, based on the similarity with the ribonuclease inhibitor (7).

In addition to its natural ligand, the TSHR is the target of thyrotoxic autoantibodies (TSAb) in Graves’ disease (8, 9), or TSH-blocking Abs (TBAb) in some cases of idiopathic myxoedema (10, 11). Up until now, the protein domains and mechanisms implicated in the activation of the receptor by its physiologic agonist TSH or autoantibodies have been approached mainly by site-directed mutagenesis or the study of chimeric receptors (12–16). This stems from the lack of data or tools allowing direct probing of the three-dimensional structure of the receptor. In particular, most (monoclonal) Abs produced to date recognize preferentially the denatured receptor or sequential epitopes harbored by the native protein (17–19). The large quantities of the extracellular domain (ECD) of the hTSHR produced in insect cells (20–22) or in bacteria as recombinant fusion protein (23–25) were antigenically active (18, 19, 21, 25, 26) and were indeed useful in establishing animal models of thyroiditis (27, 28). However, the immunogens consisted mainly of incorrectly folded proteins that do not bind TSH (22–24), yielding Abs recognizing preferentially the denatured molecules. These Abs are useful in some analyses, such as Western blotting, but they contribute little to studies of the native functional receptor.

Recently, one group adopted a promising approach consisting of hyperimmunization of mice with fibroblasts coexpressing MHC class II Ag and the hTSHR (29). Twenty percent of the mice presented high T4 level, TBII Abs, and histologic signs of hyperthyroidism, although thyroiditis was not induced. In the present study, we have explored a third approach called genetic immunization. In this case, induction of an immune response is achieved by immunization with an expression DNA vector encoding the protein Ag (30, 31). Mice immunized by intramuscular injection of an expression construct for the hTSHR cDNA construct developed high levels of circulating Abs recognizing the receptor and displaying TBI, TBAb, or TSAb activities; mAbs recognizing the native TSHR were isolated from splenocytes, after fusion with SP2/0...
myeloma cells. All immunized mice displayed a severe lymphocytic infiltration of their thyroids, composed mostly of activated B cells.

Materials and Methods

Animals used, immunization schedules, and sampling

Four groups of five 6- to 8-week-old BALB/c mice were used. They were injected in the anterior tibialis muscle on day 0 with 100 μg of pcDNAII plasmid (control group), pcDNAIII-hTSHR in PBS, pcDNAIII-hTSHR in 25% sucrose, or pcDNAIII-hTSHR in PBS, but 5 days after pretreatment of the muscle with cardiotonic purified from venom of Naja nigricollis (10 mM, 100 μl/injection; Calbiochem, La Jolla, CA). Injections were repeated 3 and 6 wk thereafter. Blood samples were obtained from retro-ocular capillaries 8 and 11 wk after the initial immunization and at sacrifice, which was after 18 wk, when the spleens and thyroids were also removed. For all determinations, sera obtained at the various time points were tested individually.

FACS analysis

CHO cells expressing the hTSHR (JP09) (32) or CHO cells expressing the hLHR (FC11) (33) were detached from the plates with PBS containing EDTA and EGTA (5 mM each) and transferred into Falcon 2052 tubes (200,000 cells/tube). Cells were centrifuged at 500 × g, at 4°C for 3 min, and the supernatant was removed by inversion. They were incubated for 30 min at room temperature with 100 μl PBS/0.1% BSA containing 2 μl serum or 10 μl lysis supernatant from immunized mice were added for 2 h at room temperature. The plates were washed, and alkaline phosphatase-ples (1/500 in blocking buffer) from immunized mice were added for 2 h at room temperature. The plates were washed, and the supernatant was removed by inversion. They were incubated for 30 min on ice in the dark with fluorescein-conjugated γ-chain-specific goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) in the same buffer. Propidium iodide (10 μg/ml) was used for detection of damaged cells, which were excluded from the analysis. Cells were washed once again and resuspended in 250 μl PBS/0.1% BSA. The fluorescence of 5000 cells/tube was assayed by a FACSscan flow cytometer (Becton Dickinson, Erembodegem, Belgium).

Measurements of TBI activity

TSH-binding inhibiting activity was measured on intact CHO cells expressing the hTSHR, JP09, as previously described, with minor modifications (27). Briefly, 5 × 10⁴ cells/well in 96-well plates were incubated in 0.1 ml of modified Hanks’ buffer without NaCl (isotonicity maintained with 280 mM sucrose), supplemented with 2.5% low fat milk, 125 I-labeled TSH (30,000 cpm), and mouse serum, for 4 h at room temperature. At the end of the incubation period, the cells were rapidly rinsed with the same ice-cold buffer and solubilized with 0.2 ml 1 N NaOH, and radioactivity was measured in a gamma counter.

The 125I-labeled TSH tracer (TRAK) was the kind gift of BRAHMS Diagnostica, GmbH (Berlin, Germany). TBI activity was measured using μl of serum/well. All experiments were done in triplicate, and results are expressed as cpm bound.

Measurement of TAb and TBA b

Thyroid-stimulating and blocking activities were measured using CHO cells expressing the hTSHR, JP26 (34). Briefly, 5 × 10⁴ cells/well in 96-well plates were incubated in 5 mM KCl, 0.25 mM KH₂PO₄, 0.5 mM MgSO₄, 0.4 mM Na₂HPO₄, 1 mM CaCl₂, 0.1% glucose, 2 mM isobutylmethylxanthine, 20 mM HEPES, and 0.3% BSA containing 3 μl of serum (total volume, 100 μl/well). Incubation was for 4 h at 37°C. cAMP released into the medium was measured using a competitive binding assay (Amer sham, Bucks, U.K.). TAb were measured under the basal conditions described above and TBAb in identical conditions, but with the addition of 200 μM I/μl final concentration bovine TSH (Sigma Chemical Co.). Duplicate samples were assayed in all experiments; results are expressed as pmol CAMP/μl.

Measurement of anti-TSHR Abs by ELISA

Purified ECD of the TSHR, produced as a fusion protein with maltose-binding protein (MBP-ECD) in bacteria, or the fusion partner associated with β-galactosidase as control (MBP-βGal) (23) was diluted in 0.1 M of sodium carbonate-bicarbonate buffer, pH 9.6 (5 μg/ml, 100 μl/well) and incubated in microwell plates overnight at 4°C. After washing and saturation with 10 mM sodium phosphate, 150 mM NaCl, pH 7.3 (PBS), containing 0.1% Tween and 5% BSA (blocking buffer), 100 μl of serum samples (1/500 in blocking buffer) from immunized mice were added for 2 h at room temperature. The plates were washed, and alkaline phosphatase-labeled goat anti-mouse Abs were added. After 1-h incubation at room temperature, wells were washed and the substrate was added (1 mg/ml p-nitrophenyl phosphate in 10% diethanolamine buffer, 1 mM MgCl₂, pH 9.8). All determinations were done in triplicate, and the absorbance was read at 405 nm in a spectrophotometer.

SDS-PAGE and Western blotting

Purified MBP-ECD or MBP-βGal in 5% sucrose (approximately 2 μg/lane) were loaded onto 10% SDS polyacrylamide gels (35) at reduction with β-mercaptoethanol at 100°C for 2 min. After electrophoresis, proteins were stained with Coomassie blue or electrophotochemically transferred onto nitrocellulose membranes (36). Blots were incubated for 30 min with a blocking solution (TBSN + milk: 10 mM Tris, pH 8, 150 mM NaCl, 0.05% Nonidet P-40, and 5% milk) at room temperature, and then stained with a pool of sera from mice (1/200) immunized with pcDNAIII-hTSHR. Thereafter, the blots were incubated with alkaline phosphatase-labeled anti-mouse IgG and, finally, NBT-BCIP (nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl-phosphate) as substrate (both from Promega Corp., Madison, WI). The serum from a mouse immunized with the recombinant protein MBP-ECD was used as positive control.

Light microscopy and immunohistochemistry

The thyroid glands were removed and carefully dissected in saline from the surrounding connective tissue under a dissecting microscope. One lobe was processed for light microscopy and the other for immunohistochemistry. The lobe designed for light microscopy was immersed for 2 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer postfixed for 1 h in 1% osmium tetroxide and embedded in Lx112 resin. Sections (0.5 μm thick) were stained with toluidine blue.

The lobe for immunohistochemistry was inserted into a liver fragment, embedded in Tissue-Tek, and rapidly frozen in isopentane cooled in liquid nitrogen to generate cryostat sections. The frozen sections were subjected to immunoperoxidase staining using different rat mAbs specific for CD4⁺ T cells and B cells, as previously described (27, 28, 37, 38).

Biopsies of human thyroid glands were obtained at surgery and also designed for immunohistochemistry to detect the location of the TSHR using the mouse mAb BA8 generated by genetic immunization (see Results). This Ab was used at 1/100 or 1/250 dilutions.

Generation of mAbs

For mAb isolation, mice were boosted by i.v. injection, with K562 cells (2 × 10⁵ cells) expressing high level of TSHR (about 1.10⁶ receptors/cell), recently developed in our laboratory. Three days after this boost, splenocytes or lymphocytes from popliteal or inguinal lymph nodes from BALB/c mice immunized with pcDNAII hTSHR were fused with myeloma cells SP2/0 at a 5:1 ratio using 50% polyethylene glycol (39). After fusion, cells were seeded into 96-well plates at 2 × 10⁴ cells/well in selective medium (DMEM containing 10% PBS, 2% Nutridoma (Boehringer Mannheim Corp., Brussels, Belgium), 10 mM sodium pyruvate, 2 mM l-glutamine, 5 mM β-mercaptoethanol, 2.5 μM amphotericin B, 100 μM penicillin, 100 μM streptomycin, 100 μM hypoxanthine, 400 μM aminopterin, and 16 μM thymidine). Ten days after the fusion, when the cells were actively growing, culture supernatants were tested for anti-hTSHR Abs by FACS analysis on JP09 cells. Selected hybridomas were cloned by limiting dilution. Cloned hybridomas were grown as ascites in pristane-primed BALB/c mice. Sepharose-protein A affinity chromatography was used to purify IgG from mouse ascites fluid. The Ig class of mAb was determined with a mouse mAb isotyping kit (Becton-Dickinson, Erembodegem, Belgium). Ab BA8 was iodinated by the chloramine-T method to a specific radioactivity of 80 μCi/μg.

Binding of 125I-labeled Ab to hTSHR

JP09 cells were seeded in 96-well plates (50,000 cells/well) and incubated at room temperature in PBS/2.5% milk with 125I-labeled BA8 mAb (100 000 cpm/well) in presence of various concentrations of the same cold Ab. One hour later, the cells were washed twice with the same buffer and solubilized with 1 M of NaOH. Bound radioactivity was determined in a gamma counter. The competition-binding curves have been fitted by non-linear regression (40), using the fitting module of Sigma Plot for Windows (Jandel Scientific GmbH, Erkrath, Germany).

Results

Evaluation of Ab titers by ELISA

Eight weeks after the first injection, all mice immunized with pcDNAIII-hTSHR had high titers of IgG Abs, recognizing the.
cardiotxin pretreatment clearly recognized the MBP-ECD fusion protein on Western blots (Fig. 3a), without cross-reaction with the fusion partner MBP. This confirms the presence within the antisera of Abs recognizing (most likely linear) epitopes present on the amino-terminal portion of the hTSHR produced in Escherichia coli.

Measurement of TBI/TBAb/TSAb bioactivities

Eleven weeks after the first injection, sera from all mice were tested individually for TBI activity on JP09 cells expressing stably the hTSHR (Table I). No TBI activity was observed in the sera of mice immunized with the empty vector. In contrast, the sera from one mouse immunized with hTSHR cDNA in PBS (serum B5), from four mice immunized with hTSHR cDNA in sucrose (sera C1, C2, C3, and C4), and from all mice immunized with hTSHR cDNA after cardiotxin pretreatment, exhibited TBI activity. Inhibition of $^{125}$I-labeled TSH binding ranged from 40 to 80%.

All sera were tested for TBAb activity on JP26 cells expressing the hTSHR and exposed to 200 μIU/ml bovine TSH (Table I). No TBAb activity was measured in sera of mice immunized with the vector alone. Serum from one mouse immunized with hTSHR cDNA in PBS, which was positive for TBI activity (serum B5), exhibited an inhibition of TSH-mediated cAMP production of 50%. With mice immunized with hTSHR cDNA in sucrose, one serum (serum C2) caused an inhibition of cAMP production of 60%. Finally, all four sera with TBI activity from mice immunized with hTSHR cDNA after cardiotxin pretreatment showed a TBAb activity; inhibition of cAMP production ranged from 60% (mouse D1) to 85% (mouse D2).

All sera were tested individually for TSAb activity on JP26 cells. Only one serum, devoid of TBI activity, from a mouse immunized with hTSHR cDNA in sucrose (mouse C5) exhibited a clear TSAb activity, with a stimulation of cAMP production about 800%. This stimulating activity was specific for the hTSHR, as the serum failed to stimulate cAMP production in JP02 cells, which do not express the hTSHR, or in FC11 cells, which express the hLHR (Fig. 4).

Production and characterization of mAbs

Two mice immunized with the hTSHR cDNA (mouse C5, TSAb positive from the protocol with cardiotxin) were boosted with K562 cells expressing the hTSHR and killed 3 days later for mAb production. Three mAbs were isolated after screening by FACS on JP09 cells (see Materials and Methods); two were obtained from the spleens (BA8 and BH10), and one from lymph nodes (AB1). These Abs, of IgG2a isotypes, clearly recognized the native hTSHR expressed at the surface of CHO cells (Fig. 5), but failed to recognize the dog TSHR, rat TSHR, and the hLHR (not illustrated). These Abs, of IgG2a isotypes, clearly recognized the native hTSHR expressed at the surface of CHO cells (Fig. 5), but failed to recognize the dog TSHR, rat TSHR, and the hLHR (not illustrated). In contrast with the 2C11 Ab kindly provided by Dr. A. P. Johnstone (19), they do not recognize a mutant hTSHR with an amino acid substitution (I167N) (41), which is expected to destroy the three-dimensional structure of the ECD (7) and hinders expression of the mutant at the cell surface (to be published).

None of the monoclonals exhibited any TBI/TBAb or TSAb activity (data not shown). After production in ascites and purification, the BA8 Ab was labeled with $^{125}$I and its affinity was shown to be in the high nanomolar range by competitive binding on JP09 cells (Kd about 4.5 $\times$ 10$^{-11}$ M) (Fig. 5). The binding of labeled BA8 was not displaced by an excess of cold bovine TSH, nor by autoimmune human sera with a strong TBI activity (Table II). The labeled BA8 was displaced by the other two mAbs isolated in this study, but not by the 2C11 Ab (Table II), which recognizes a linear epitope in the carboxyl-terminal portion of the ECD of the
receptor (354-VFFEEQ-359). Contrary to the antisera from the immunized mice, the three monoclonals failed to recognize the hTSHR in Western blots containing the MBP-ECD fusion protein (data not illustrated).

Immunohistochemistry of human thyroid with BA8 Ab

Human thyroid follicles showed a positive labeling after immunostaining with the BA8 mAb. The labeling concentrated at the basal pole of the thyroid cells (Fig. 6).

Table I. Biologic activity of the immune sera

<table>
<thead>
<tr>
<th></th>
<th>TBII (cpm bound)</th>
<th>TBAb (pmol/well)</th>
<th>TSAb (pmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (pcDNA III)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>2947 ± 55</td>
<td>87 ± 0.86</td>
<td>1.00 ± 0.016</td>
</tr>
<tr>
<td>A2</td>
<td>2828 ± 277</td>
<td>96 ± 6.5</td>
<td>1.30 ± 0.03</td>
</tr>
<tr>
<td>A3</td>
<td>2799 ± 29</td>
<td>93 ± 5.35</td>
<td>1.33 ± 0.07</td>
</tr>
<tr>
<td>A4</td>
<td>2918 ± 62</td>
<td>86 ± 5.9</td>
<td>1.22 ± 0.009</td>
</tr>
<tr>
<td>A5</td>
<td>2820 ± 3</td>
<td>91 ± 6.53</td>
<td>1.32 ± 0.044</td>
</tr>
<tr>
<td>pcDNA III-TSHR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−PBS</td>
<td>2226 ± 113</td>
<td>64 ± 5.1</td>
<td>0.70 ± 0.22</td>
</tr>
<tr>
<td>B1</td>
<td>2290 ± 36</td>
<td>108 ± 8.38</td>
<td>1.96 ± 0.09</td>
</tr>
<tr>
<td>B2</td>
<td>2914 ± 175</td>
<td>80 ± 2.7</td>
<td>0.81 ± 0.046</td>
</tr>
<tr>
<td>B3</td>
<td>2734 ± 56</td>
<td>70 ± 1.6</td>
<td>0.74 ± 0.006</td>
</tr>
<tr>
<td>B4</td>
<td>677 ± 4</td>
<td>44 ± 1.6</td>
<td>0.77 ± 0</td>
</tr>
<tr>
<td>−Sucrose</td>
<td>C1</td>
<td>1562 ± 2</td>
<td>68 ± 10</td>
</tr>
<tr>
<td>C2</td>
<td>1035 ± 41</td>
<td>41 ± 2.4</td>
<td>0.60 ± 0.006</td>
</tr>
<tr>
<td>C3</td>
<td>1098 ± 11</td>
<td>73 ± 2.5</td>
<td>0.59 ± 0.018</td>
</tr>
<tr>
<td>C4</td>
<td>1861 ± 49</td>
<td>71 ± 0.6</td>
<td>0.75 ± 0.054</td>
</tr>
<tr>
<td>C5</td>
<td>2605 ± 220</td>
<td>88 ± 15</td>
<td>8.58 ± 0.15</td>
</tr>
<tr>
<td>−Cardiotx</td>
<td>D1</td>
<td>959 ± 10</td>
<td>38 ± 0.17</td>
</tr>
<tr>
<td>D2</td>
<td>608 ± 8</td>
<td>14 ± 3</td>
<td>0.48 ± 0.05</td>
</tr>
<tr>
<td>D3</td>
<td>390 ± 11</td>
<td>18 ± 0.71</td>
<td>0.56 ± 0.03</td>
</tr>
<tr>
<td>D4</td>
<td>577 ± 35</td>
<td>24 ± 0.61</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td>Buffer</td>
<td>3244 ± 149</td>
<td>3 ± 0.002</td>
<td>2.7 ± 0.04</td>
</tr>
<tr>
<td>Cold TSH</td>
<td>203 ± 26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>121 ± 2.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>153 ± 4.11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> 10 mU/ml of cold bovine TSH.

<sup>b</sup> 200 μU/ml of cold bovine TSH.
Histologic analysis of the thyroids

Signs of thyroiditis were observed in the 14 immunized BALB/c mice. Clusters of immune cells were often located at the periphery of the thyroid (Fig. 7A), where they comprised CD4+ T cells (Fig. 7B) and were surrounded by B cells (B220+ (Fig. 7C)). In addition to the periphery, B cells were distributed abundantly all over the thyroid interstitium (Fig. 7C).

Thyroid follicles did not present any sign of cell destruction. Those located at the periphery of the gland, near the clusters of immune cells, showed signs of stimulation, as suggested by the thickening of the epithelial layer and the irregularity of the apical poles protruding into the follicular lumina (Fig. 7D). The follicles located inside the gland and surrounded by immune cells had greatly variable sizes. There were numerous small follicles, showing an irregular basal outline, and there were frequent pictures suggestive of follicular budding, a process implicated in folliculoneogenesis (Fig. 7E).

Discussion

Generation of Abs against G protein-coupled receptors is reportedly difficult. To our knowledge, this study is the first to demonstrate that specific Abs can be generated in mice against a G protein-coupled receptor, by genetic immunization. With a single exception, all mice immunized with the expression cDNA construct developed anti-hTSHR Abs. Most of these antisera recognized both a recombinant fusion protein made in E. coli and the native TSHR expressed at the surface of CHO cells. However, there was no obvious correlation between the titers of Abs estimated by these two techniques, suggesting that some antisera contain a sizable proportion of Abs directed toward purely conformational epitopes. These epitopes must be located in the ECD of the hTSHR because the antisera failed to recognize by FACS, chimera with the ECD of the hLHR hooked to the serpentine domain of the hTSHR (binding experiments demonstrated that the chimera was well expressed at the plasma membrane; data not shown). Most of

Table II. Competition of the mAb anti-TSHR with 125I-labeled BA8

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>cpm Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>6164 ± 178</td>
</tr>
<tr>
<td>BA8a</td>
<td>956 ± 8</td>
</tr>
<tr>
<td>BH10</td>
<td>2456 ± 345</td>
</tr>
<tr>
<td>AB1</td>
<td>2520 ± 386</td>
</tr>
<tr>
<td>2C11</td>
<td>6129 ± 289</td>
</tr>
<tr>
<td>TSHb</td>
<td>5955 ± 13</td>
</tr>
</tbody>
</table>

a 10 μl of culture supernatant was used for all the mAb tested.
b 50 μl/ml of cold bovine TSH.

FIGURE 4. Analysis of serum C5 TSAb activity. TSAb activity was tested on CHO cells expressing the hTSHR (black columns), hLHR (gray columns), or control CHO cells (white columns), 2 wk and 5 wk after the last injection of cDNA encoding the hTSHR. Results are expressed as picomoles of cAMP produced per well.

FIGURE 5. Binding of mAb BA8 labeled with radioactive iodine to CHO cells expressing the hTSHR. The assay was performed in PBS with 50,000 cells/well, in presence of various concentrations of the same cold Ab. Each point was tested in triplicate, and the results are expressed as cpm bound.

FIGURE 6. Immunoperoxidase staining on frozen section of human thyroid using BA8 mAb (×450). Almost all thyroid cells show a positive labeling concentrated at the basal pole.
the antisera presented a TBII/TBAb activity, and one serum exhibited a clear and specific TSAb activity. Whereas TBII and TBAb activities can be obtained by immunization against recombinant immunogens (17, 21, 27), convincing TSAb activity has been obtained only recently by an original immunization protocol involving transfected fibroblasts coexpressing the hTSHR and MHC class II Ags (29). The results obtained with mouse C5 of the sucrose immunization group demonstrate that antisera with strong TSAb activity can be obtained by genetic immunization. Surprisingly, the antiserum does not display significant TBII activity and scores rather poorly in the FACS analysis. TSAb activity in the absence of TBII activity is also found in the human autoantibodies of some patients with Graves’ disease (42, 43). Furthermore, receptor Abs have been shown to be present at low concentration in the serum in a recent study by McLachlan et al. (44). These anomalies illustrate only limited understanding of the true nature of TSAb activities, whether experimental or from Graves’ patients.

Genetic immunization was also efficient in generating mAbs against the TSHR. Three mAbs of IgG2a-κ isotype were isolated, two from the spleen and one from lymph nodes. They recognized the native hTSHR at the surface of CHO cells, but failed to do so with the dog or the rat TSHR (data not illustrated). They did not recognize the rECD of the hTSHR in Western blot, suggesting that they are directed against conformational epitopes. When used as culture supernatant (at about 1 μg/ml IgG), these Abs did not show TBII, TBAb, or TSAb activities. One of these mAbs, BA8, isolated from a fusion with spleen cells, was characterized further. It recognized the native hTSHR with high affinity (apparent $K_d$ about $4.5 \times 10^{-11}$ M). Its binding was not displaced by TSH, neither by autoantibodies from patients, nor by the 2C11 mAb. In contrast, the two other Abs isolated, BH10 and AB1, competed with the binding of BA8, suggesting that, despite their coming from two different fusions, the three monoclonals are probably closely related. The three monoclonals are of the IgG2a isotype, suggesting presentation of Ag by dendritic cells rather than macrophages. Dendritic cells are capable of eliciting both Th1 and Th2 responses (45–47), and the IgG2a isotype reflects a switch to Th1, probably as a consequence of IFN-γ production (45). The BA8 molecule is able to recognize in FACS a series of 16 TSHR mutated in the serpentine domain, and available in our laboratory (not shown) (48, 49). For receptors mutated in the ECD, this Ab recognizes efficiently the S281T/N (48) and the P162A (41) mutants, as well as

FIGURE 7. A–C, Frozen sections of thyroids from immunized mice. A, Hematoxin-eosin staining (×130). A large cluster of immune cells is located at the periphery of the gland (arrowheads). B, Immunoperoxidase staining of CD4⁺ T cells (×130). CD4⁺ T cells are numerous in the peripheral cluster of immune cells. C, Immunoperoxidase staining of B cells (×150). B cells are numerous all over the thyroid interstitium. D–E, Semithin (0.5-μm-thick) sections of thyroids from immunized mice. Toluidine blue staining. D, ×500. Peripheral follicles located near the immune cells show signs of stimulation, with thickening of the thyrocytes, whose apical poles often protrude into the follicular lumen (arrowheads). E, ×400. Follicles located inside the gland have variable sizes. Pictures of follicular budding are frequent (arrowheads).
as the polymorphic P52T variant (50). In contrast to 2C11, BA8 did not recognize the I167N (41) mutant receptor, even after permeabilization of the cells. Together these results indicate that BA8 reacts only with the native, normally folded TSHR and recognizes conformational epitopes specific for the human receptor. For this reason, it constitutes an invaluable tool for the purification of the bioactive receptor. Its ability to recognize the TSHR on tissue sections will also make it useful in probing the structure of the receptor in samples from diseased thyroids.

Genetic immunization was developed primarily to induce protective immunity. In protocols having this goal, autoimmune reactions would be an unwanted side effect, and indeed, when they have been sought they have not generally been found. One exception to this involved the generation of autoantibodies to dsDNA, analogous to those occurring in patients with systemic lupus erythematosus, in mice immunized with the cDNA for the polyoma virus large T Ag. The authors found that a particular cDNA encoding a DNA-binding protein was required and that plasmid DNA in itself was not able to induce DNA Abs. The lack of Abs to other autoantigens suggested that polyclonal activation was not responsible, but rather Ag-cognate T-B cell interaction (51). Furthermore, genetic immunization of the neonate has been shown to induce tolerance, an effect that diminishes and is absent even in mice at 2 wk of age (52). Traditional immunization schedules are often, although not always, associated with tolerance induction in the first weeks of life (53–55).

To our knowledge, this is the first report of an organ-specific autoimmune response (as evidenced by the thyroiditis present in all mice treated with the hTSHR cDNA construct) being obtained via genetic immunization and in the absence of conventional adjuvants. The human and murine TSHR display sequence similarity, both at the nucleotide and amino acid levels (86.7% identity in amino acids). In these experiments, in which tolerance has been broken, one must assume that differences between the two receptors are sufficient to trigger T cell responsiveness. After intramuscular injection, the hTSHR gene is probably expressed by the myocyte and presented at its surface in the context of HLA class I. Expression of genes in myocytes from injected cDNA constructs has been well documented (56–58). However, these cells do not have the costimulatory signal necessary to elicit an immune response and are more likely to induce tolerance or cytotoxic reactions. The myocyte is thus unlikely to be the APC responsible for the humoral response and thyroiditis, which suggests presentation by a professional APC such as a dendritic cell. If the protein is overproduced, or if a cytotoxic response is elicited first, lysis of myocytes could lead to the release of hTSHR peptides that could be recovered by APC present around the injection site and subsequently presented within the context of MHC class II. Peptides differing from the mouse TSHR would be seen as nonself, but in the course of the ensuing proliferation and hypermutation, T cells having specificities for the murine TSHR could be produced, resulting in homing to the thyroid gland and thyroiditis. Alternatively or in addition, cDNA at the injection site could possibly be endocytosed and expressed by APC. Treatment with cardiototoxin could recruit large numbers of APC, particularly dendritic cells that would explain the acute humoral response observed. The receptor Abs induced by the protocol appear to be specific for the hTSHR. They exhibit bioactivity in in vitro assays using the hTSHR, but this is not mirrored by changes in circulating T4 levels. This indicates that, despite the infiltration of activated B cells into the mouse thyroids, the receptor Abs exhibit little or no bioactivity on the endogenous host TSHR. How does this equate with human disease? It has been suggested that Graves’ disease could result from immunization against cross-reacting Ag(s) of Yersinia enterocolitica (59–61), a phenomenon known as molecular mimicry (62, 63). In our model, there is obvious close mimicry between the human Ag encoded by our immunogen and mouse TSHR. This is a likely explanation for the breakage of tolerance in our model, while it has been reported that defects in tolerance mechanisms exist in autoimmune disease (64, 65).

Future refinement of our model might build on the recent demonstration that immunization with fibroblast coexpressing the TSHR and class II Ags leads to thyroiditis with TSAb (29). Expression of the TSHR cDNA in isolated dendritic cells, followed by their infusion in mice, or direct intradermal injection of TSH cDNA, aiming at Langerhans cells, might lead to an even stronger immune stimulation, due to the simultaneous presence at the cell surface of the native TSHR, class II Ag loaded with TSHR peptide and costimulatory molecules.

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**References**


ulovirus expression system. Autoimmunity 14:315.
crinol. 115:199.
11074.
docrinol. 115:199.
36. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of pro-
41. Seetharamaiah, G. S., R. K. Desai, J. S. Dallas, K. Tahara, L. D. Kohn, and B. S. Prabhakar. 1993. Induction of TSH binding inhibitory immunoglobulins with the extracellular domain of human thyrotropin receptor produced using bac-
ulovirus expression system. Autoimmunity 14:315.
42. Wlasek, H., M. Nakashima, P. N. Graves, Y. Tomer, J. C. Morris, and T. F. Davies. 1995. Defining the major antibody epitopes on the human thyrotropin receptor in immu-
docrinol. 115:199.
11074.