Induction of Experimental Autoimmune Graves' Disease in BALB/c Mice

Shashi Kaithamana, Jilao Fan, Yutaka Osuga, Shan-Guang Liang and Bellur S. Prabhakar

J Immunol 1999; 163:5157-5164; http://www.jimmunol.org/content/163/9/5157

References
This article cites 30 articles, 6 of which you can access for free at: http://www.jimmunol.org/content/163/9/5157.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Induction of Experimental Autoimmune Graves’ Disease in BALB/c Mice

Shashi Kaithamana,* Jilao Fan,* Yutaka Osuga,† Shan-Guang Liang,† and Bellur S. Prabhakar‡*‡

We immunized BALB/c mice with M12 cells (H-2d) expressing either mouse (mM12 cells) or human thyrotropin receptor (TSHR) (HM12 cells). Immunized mice developed autoantibodies to native TSHR by day 90 and, by day 180, showed considerable stimulatory Ab activity as measured by their ability to enhance cAMP production (ranging from 6.52 to 20.83 pmol/ml in different treatment groups relative to 1.83 pmol/ml for controls) by TSHR-expressing Chinese hamster ovary cells. These mice developed severe hyperthyroidism with significant elevations in both tetraiodothyronine and triiodothyronine hormones. Tetraiodothyronine levels in different experimental groups ranged from a mean of 8.66 –12.4 µg/dl, relative to 4.8 µg/dl in controls. Similarly, mean triiodothyronine values ranged from 156.18 to 195.13 ng/dl, relative to 34.99 ng/dl for controls. Next, we immunized BALB/c mice with a soluble extracellular domain of human TSHR (TBP), or TBP expressed on human embryonic kidney cells (293 cells) (293-TBP cells). These mice showed severe hyperthyroidism in a manner very similar to that described above for mice immunized with the mouse TSHR or human TSHR, and exhibited significant weight loss, with average weight for treatment groups ranging from 20.6 to 21.67 g, while controls weighed 24.2 g. Early after onset of the disease, histopathological examination of thyroids showed enlargement of colloid and thinning of epithelial cells without inflammation. However, later during disease, focal necrosis and lymphocytic infiltration were apparent. Our results showed that conformationally intact ectodomain of TSHR is sufficient for disease induction. Availability of a reproducible model in which 100% of the animals develop disease should facilitate studies aimed at understanding the molecular pathogenesis of Graves’ disease. The Journal of Immunology, 1999, 163: 5157–5164.

Graves’ disease (GD) is an autoimmune thyroid disease characterized by the presence of autoantibodies directed against the thyrotropin receptor (TSHR) (1, 2). Autoantibodies bind to the TSHR and cause enhanced production of thyroid hormone, resulting in hyperthyroidism.

Since cloning of TSHR, many laboratories have attempted to develop an animal model for GD. Our earlier attempts to induce GD using thyroid membranes as a source of native TSHR was unsuccessful. This, perhaps, was due to very low levels of TSHR expressed on thyroid membranes, thus resulting in inoculation of suboptimal doses of Ag. To overcome this limitation, we expressed large quantities of nonglycosylated and glycosylated extracellular domains of TSHR (ETSHR and hETGP, respectively) in insect cells and showed that they bind TSH and patient autoantibody, respectively (3, 4). We used the ETSHR protein to immunize different strains of mice and showed that only BALB/c mice developed mild hyperthyroidism (5). Subsequently, to enhance the severity of the disease, we primed BALB/c mice with ETSRH and then challenged them with soluble thyroid membranes (5). These mice showed significant elevation in Abs against native TSHR, with concomitant increase in tetraiodothyronine (T4) hormone levels. However, hormonal levels returned to normal within a relatively short time after cessation of Ag administration, suggesting that tolerance to endogenous TSHR had not been overcome. Similarly, a number of different laboratories have attempted to induce GD in mice using various preparations of TSHR, but with only limited success (6–12). Inability to induce GD using only recombinant TSHR proteins is most likely due to inappropriate folding of the protein. Thus, availability of large quantities of TSHR with native conformation remained a major impediment to developing an appropriate animal model.

To overcome this, Shimojo et al. used RT4.15HP cells, doubly transfected with cDNAs encoding human TSHR and MHC class-II molecules, to immunize AKR mice (13). Approximately 20% of mice immunized with cells expressing both TSHR and class II, but not with cells expressing either class II or TSHR alone, developed hyperthyroidism. Based on these results, the authors suggested that coexpression of MHC class II and TSHR is essential to induce GD. Recently, Kita et al. (14) have confirmed these findings using similarly transfected RT4.15HP cells.

To optimize conditions for disease induction in a higher proportion of mice, we compared the efficacy of full-length TSHR vs soluble ectodomain; mouse TSHR (mTSHR) vs human TSHR (hTSHR); soluble purified protein (TBP) vs cell associated protein (293-TBP cells); and syngeneic (M12 cells) vs xenogeneic cells (293 cells) expressing TSHR. Our current studies clearly demonstrate that immunization of BALB/c mice using either the

Copyright © 1999 by The American Association of Immunologists
ectodomain of TSHR with native conformation (TBP), or full-length TSHR protein expressed on cell surface leads to disease induction in almost 100% of mice, with significant elevation in stimulatory Abs and thyroid hormones in their circulation.

Materials and Methods

Transfection of M12 cells

M12 cells were transfected with pSRo puro vector in serum-free RPMI 1640 containing either a cDNA-encoding hTSHR (hM12 cells) or cDNA encoding mTSHR (mM12 cells). The pSRo vector (15) consists of a Simian virus (SV40) early promoter and part of the R-U5 segment of the long terminal repeat (LTR) from human T cell leukemia virus type I. M12 cells were transfected with the plasmids using Lipofectamine (Life Technologies, Gaithersburg, MD) following the manufacturer’s protocol and cultured in RPMI 1640 medium. Transfected cells were selected for puromycin resistance. TSHR expression on stable transfectants was confirmed by flow cytometry using TSHR-specific Abs and radiolabeled bovine TSH (bTSH) binding, and by their ability to produce cAMP in the presence of bTSH. These permanently transfected hM12 and mM12 cells were used throughout the study.

Flow cytometry to detect TSHR expression

Human embryonic kidney cells (293 cells) expressing extracellular domain of hTSHR (293-TBP cells) (16, 17) and hM12 and mM12 cells, and untransfected control cells were detached from culture plates using PBS-based enzyme-free dissociation solution (Nozyme, Specialty Media, Lalvallette, NJ) and transferred into tubes as described earlier (18). Cells were pelleted at 500 × g at 4°C for 5 min, and washed once in PBS containing 1% BSA and 0.1% sodium azide (FACS buffer). Cells were incubated for 30 min at 4°C with 100 μl FACs buffer containing either 1 μl of control rabbit serum, polyclonal rabbit anti-hTSHR, or anti-mTSHR serum (10). Cells were washed three times with 1 ml FACs buffer and centrifuged as above. Cells were subsequently incubated for 30 min on ice in dark with fluorescein-conjugated goat anti-rabbit IgG, then washed three times and suspended in 400 μl of FACs buffer. Propidium iodide (10 μg/ml) was used to detect dead cells. Cells were analyzed using a flow cytometer (Coulter, Hialeah, CA).

cAMP assay

Control M12, hM12, and mM12 cells were plated into 96-well plates in RPMI 1640 supplemented with 10% FBS. Fresh medium (RPMI 1640) was added to these wells 24 h later. When cells grew to confluence, medium was removed, and cells were washed once using RPMI 1640 medium. To these wells, a serial dilution of bTSH in a hypotonic medium (HBSS) containing 0.5 mM 3-isobutyl methyl-xanthine was added and incubated for 3 h at 37°C. Culture supernatants were collected, and the cAMP released into the medium was measured by RIA (1:100 dilution) using a commercial kit (NEN Life Science Products Incorporated, Boston, MA) following the manufacturer’s protocol.

To test the ability of mouse sera to stimulate cAMP production, Chinese hamster ovary (CHO) cells expressing hTSHR were used (19, 20). Cells were grown to confluence, medium was removed, and cells were washed once using RPMI 1640 medium. To these wells, a serial dilution of bTSH in a hypotonic medium (HBSS) containing 0.5 mM 3-isobutyl methyl-xanthine was added and incubated for 3 h at 37°C. Culture supernatants were collected and assayed for cAMP production as described above.

TBII assay to detect TSHR-specific Abs

A commercial radioreceptor (Kronus, Dana Point, CA) assay is routinely used to measure autoantibodies to TSHR in the sera of patients with thyroid disorders. In this assay, the ability of Abs to bind to TSHR on porcine thyroid membranes and block the binding of 125I-labeled TSH is measured (i.e., thyrotropin-binding inhibitory index) (TBII). This assay was used to detect autoantibodies to TSHR according to the manufacturer’s protocol, and results were expressed as TBII activity.

Removal of TBII activity by hM12 and mM12 cells

Cells (M12, hM12, and mM12) were grown to confluency in RPMI 1640 medium supplemented with 10% FBS in a 24-well plate. Before assay, cells were washed three times with buffer containing 10 mM Tris-HCl, pH (7.4), 50 mM sodium chloride, and 0.1% BSA. Fifty microliters of Graves’ patient sera was added to triplicate wells. After incubation for 2 h at room temperature, sera were collected and tested for their TBII activity as described above. Ability of transfected cells to react with anti-TSHR Ab and adsorb the TBII activity of the sera was calculated and reported as percentage blocking.

TSH binding assay

M12, mM12, and hM12 cells were grown to confluency in RPMI 1640, and 293 and 293-TBP cells (16, 17) were grown in DMEM-F12 supplemented with 10% FBS in a 24-well plate (0.5 × 104 cells). On the day of assay, cells were washed three times with buffer containing 10 mM Tris-HCL (pH 7.4), 50 mM sodium chloride, and 0.1% BSA. To each well, 100 μl of (8000 cpm) 125I-labeled bTSH (Kronus) was added and incubated for 2 h at room temperature. Cells were washed with ice-cold buffer, and then 500 μl of 0.1 M sodium hydroxide was added to lyse the cells. Contents of the wells were carefully transferred to individual test tubes and counted in a gamma counter to detect 125I. Results are expressed as cpm bound.

Purification of TBP protein

293-TBP cells (16, 17) were grown in DMEM/F12 with 10% FBS and 200 μg/ml of G418. Soluble TBP was purified using a previously described procedure (17). Briefly, the ectodomain of hTSHR (aa 1–390) was fused to transmembrane and cytoplasmic regions of CD8 through aa 36–66 of thyroid receptor. To express the ectodomain of human TSHR in human embryonic kidney 293 cells, the chimeric cDNA was subcloned into pcDNA3 (Invitrogen, San Diego, CA) and transfected into 293 cells and grown in DMEM/F12 with 10% FBS. 293-TBP cells were grown to confluence in T-150 tissue culture flasks. The medium was replaced with 20 ml serum-free DMEM/F12 containing 300 U α-thrombin (Enzyme Research Laboratories, South Bend, IN) and incubated overnight at 37°C. Medium containing released TBP was collected, centrifuged, and incubated at 4°C with Sepharose Fast-flow resin covalently linked with nickel. The protein was eluted with 200 mM imidazole. The eluant was concentrated using a centricon-30 (Amicon, Bedford, MA) and stored at −70°C until use. Unlike ectodomains produced in insect cells, the TBP produced in 293 cells is readily soluble.

Animals

Six- to 8-week-old female BALB/c mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Experiments were approved and conducted according to established institutional review board guidelines.

Immunization of BALB/c mice with M12 cells expressing TSHR

BALB/c mice (five mice per group) were immunized i.p. six times as per the following schedule: group 1, 1 × 10^7 M12 cells (1); group 2, hM12 cells along with cholera toxin B subunit (CTB) (2); group 3, mM12 cells along with CTB (3); group 4, 293-TBP cells along with CTB (3); group 5, mice primed once with hETGP and then challenged with hM12 along with CTB (4); group 6, mice primed once with mETGP (glycosylated ectodomain of mTSHR expressed in insect cells) and then challenged with mM12 cells along with CTB (5); group 7, mM12 cells alone (6); and group 8, hM12 cells alone (7). Before immunization, the cells were treated with mitomycin C. Blood was collected from these mice on days indicated in the legends to Figs. 4 and 5, and serum was tested for Abs and thyroid hormones.

Immunization of BALB/c mice with TBP

BALB/c mice were immunized i.p. six times as per the following schedule: group 1, 2 × 10^7 293 cells; group 2, purified TBP protein given along with CTB; group 3, primed once with purified TBP emulsified in CFA and then challenged with 293-TBP cells along with CTB; group 4, 293-TBP cells along with CTB; and group 5, primed once with hETGP in CFA and then challenged with 293-TBP cells along with CTB. Before inoculation, cells were treated with mitomycin C. Blood was collected periodically on days indicated in the legends to Figs. 4 and 5 and was tested for autoantibodies and thyroid hormone levels.

ELISA to detect mouse Ig binding to recombinant TBP protein

ELISA plates were coated with soluble TBP protein overnight at 4°C. The antisera obtained from different groups of mice were diluted serially and assayed by ELISA as described earlier (21).

Measurement of total T4 and T3

Total T4 and triiodothyronine (T3) in serum samples were determined using a commercially available assay kit, Coat-A-Count (Diagnostic Products, Los Angeles, CA). Briefly, test tubes coated with anti-T4 or anti-T3 Abs were incubated with sera and 1 ml of 125I-labeled T4/T3 for 1 h at 37°C. The contents of the tubes were decanted, and radioactivity bound to...
the tubes was quantitated in a gamma counter. The amount of T4 or T3 in the test sample was calculated by plotting the results against a reference curve generated using standards supplied with the kit.

Thyroid histology

Mouse thyroids were removed and placed in 5 ml of 10% formalin. Tissues were embedded in paraffin, and 5-micron thick sections were prepared and stained with hematoxylin and eosin. Multiple sections from each thyroid were examined.

Results

Immunization of BALB/c with mM12, hM12 cells

To test the ability of full-length mTSHR and hTSHR proteins to induce GD in mice, we developed permanently transfected M12 cells (21), a B lymphoblastoid cell line derived from BALB/c, expressing either mTSHR (mM12 cells) or hTSHR (hM12 cells) cDNA, and stable cell lines were established. 293 cells were transfected with pCDNA3 vector containing ectodomain of hTSHR (293-TBP cells) and were selected using G418 (16). The cells were analyzed by flow cytometry after staining with either an anti-mTSHR (10) or an anti-hTSHR (10) Ab, followed by FITC-conjugated goat anti-rabbit IgG, for the expression of mTSHR and hTSHR, respectively (18). Propidium iodide was used to separate live from dead cells. A, mM12; B, hM12; and C, 293-TBP.

Preincubation of Graves’ sera with hM12 or mM12 cells almost completely neutralized the Abs (reduction of TBII by 98% and 85%, respectively). Together, these experiments clearly demonstrated that M12 cells were expressing functional TSHRs.

These cells were subsequently used to immunize BALB/c mice. As shown in Fig. 4 and Table II, we immunized seven groups of mice with various permutations and combinations as described under Materials and Methods. Mice immunized with and without CTB, or primed with mETGP/hETGP protein, before immunization with hM12 or mM12 cells, respectively, developed high titers of Abs as measured in an ELISA (Fig. 4A) with concomitant increase in the TBII activity (Fig. 4B). Subsequently, we tested their sera for circulating thyroid stimulatory Abs. As shown in Fig. 4C, sera from mice in the experimental groups, and not from the control group, were able to induce high levels of cAMP production (mean ranging from 6.52 to 20.83 pmol/ml relative to 1.83 pmol/ml for controls, Table II) in CHO cells permanently transfected with a full-length human TSHR.

All mice in the experimental groups, irrespective of Ag used, developed hyperthyroidism, as evidenced by significantly higher levels of serum T4 levels (means ranging from 8.66 to 12.4 mg/dl in test groups vs 4.8 mg/dl for the controls) relative to the control group (Fig. 4D). Serum T3 levels were also significantly elevated in all the experimental groups, but not in the control group (mean

![FIGURE 1. Reactivity of anti-TSHR Abs with cells expressing TSHR. M12 cells were transfected with pSRα puro vector containing either a full-length mTSHR (mM12 cells) or hTSHR (hM12 cells) cDNA, and stable cells lines were established. 293 cells were transfected with pCDNA3 vector containing ectodomain of hTSHR (293-TBP cells) and were selected using G418 (16). The cells were analyzed by flow cytometry after staining with either an anti-mTSHR (10) or an anti-hTSHR (10) Ab, followed by FITC-conjugated goat anti-rabbit IgG, for the expression of mTSHR and hTSHR, respectively (18). Propidium iodide was used to separate live from dead cells. A, mM12; B, hM12; and C, 293-TBP.]()

![FIGURE 2. TSH binding to cells expressing TSHR. Control or transfected M12 and 293 cells (0.5 × 10^6 cells) were incubated with 8000 cpm of [125I]TSH for 2 h. Subsequently, cells were washed 3× with PBS and counted in a gamma counter to determine the amount of bound TSH. The results are expressed as means of duplicates. Preincubation of Graves’ sera with hM12 or mM12 cells almost completely neutralized the Abs (reduction of TBII by 98% and 85%, respectively). Together, these experiments clearly demonstrated that M12 cells were expressing functional TSHRs. These cells were subsequently used to immunize BALB/c mice. As shown in Fig. 4 and Table II, we immunized seven groups of mice with various permutations and combinations as described under Materials and Methods. Mice immunized with and without CTB, or primed with mETGP/hETGP protein, before immunization with hM12 or mM12 cells, respectively, developed high titers of Abs as measured in an ELISA (Fig. 4A) with concomitant increase in the TBII activity (Fig. 4B). Subsequently, we tested their sera for circulating thyroid stimulatory Abs. As shown in Fig. 4C, sera from mice in the experimental groups, and not from the control group, were able to induce high levels of cAMP production (mean ranging from 6.52 to 20.83 pmol/ml relative to 1.83 pmol/ml for controls, Table II) in CHO cells permanently transfected with a full-length human TSHR.

All mice in the experimental groups, irrespective of Ag used, developed hyperthyroidism, as evidenced by significantly higher levels of serum T4 levels (means ranging from 8.66 to 12.4 mg/dl in test groups vs 4.8 μg/dl for the controls) relative to the control group (Fig. 4D). Serum T3 levels were also significantly elevated in all the experimental groups, but not in the control group (mean

![FIGURE 3. TSH induces cAMP response in mM12 and hM12. Cells were stimulated with the indicated amount of bTSH for 2 h. Culture supernatants were collected, and the amount of cAMP produced was determined as described under Materials and Methods. Bars represent the means of triplicate determinations along with SD.]()
of 156.18–195.13 ng/dl, for test groups vs 34.99 ng/dl for controls) as shown in Fig. 4E. Moreover, mice in several experimental groups showed a tendency toward lower body weight, with mice in group 4 showing significantly lower weights (Fig. 4F), than the control mice (mean body weight of 19.5–23.24 g for the test group vs 23.9 g for controls). Together, these studies clearly showed that mice immunized with TSHR had developed pathogenic Ab responses to TSHR and showed perturbation of thyroid function (Table II).

Immunization of BALB/c mice with TBP

Mice were immunized with either a soluble TBP or TBP-293 cells (Fig. 1C). Native conformation of TBP was confirmed by its ability to bind both TSH (Fig. 2) and patient autoantibodies (Table I). Sera from immunized and control mice were tested against purified TBP in an ELISA (Fig. 5A). Normal mouse sera showed little or no binding (<1:100) to TBP, whereas, mice immunized with either purified TBP or 293-TBP cells (groups 2 and 4, respectively) showed considerable Ab response (1:12800). Similarly, mice primed with either TBP or hETGP, and subsequently challenged with 293-TBP cells (groups 3 and 5, respectively) also showed significant Ab response (>1:12800). Sera from these mice showed considerable TBII activity (mean of 34.8–44.3%, relative to 3.84% for controls) (Fig. 5B and Table III). Moreover, sera from affected mice induced enhanced levels of cAMP production in CHO cells expressing human TSHR (Fig. 5C), with mean values ranging from 16.1–24.2 pmol/ml for the test groups, compared with a mean of 1.75 pmol/ml for controls (Table III). All mice in experimental groups showed significant elevations in their T4 (Fig. 5D) and T3 (Fig. 5E) levels. The mean T4 values for the experimental groups ranged from 9.53 to 12.56 μg/dl, whereas the mean for controls was 4.58 μg/dl (Table III). Mean T3 values ranged from 140.18–162.35 ng/dl, relative to a mean of 37.37 ng/dl in

**FIGURE 4.** Induction of Graves’ disease using hM12 and mM12 cells. Different groups of mice (five mice/group) were immunized as described under Materials and Methods. Mice in groups 1, 2, 3, 6, and 7 received $2 \times 10^7$ mitomycin C-treated cells on days 0, 14, 28, 48, 65, and 91. Groups 4 and 5 were injected s.c. with 50 μg of protein along with CFA on day 0 and with mitomycin C-treated cells ($2 \times 10^7$ along with CTB) on days 14, 28, 48, 65, and 91. For monitoring the immune response, sera from blood obtained on day 120 were tested for ELISA and TBII activity. Sera obtained on day 180 was used for T4 and T3 measurements. Two microliters of serum obtained on day 180 was diluted to 100 μl in assay buffer and used in cAMP assay. Weights of mice on day 240 of the experiments are shown. The bars indicate mean ± SD. Filled circles indicate mean value for the group and each of the other symbols indicates value for individual mouse within each group.
controls (Table III). In addition, mice from experimental groups showed considerably lower total body weight (Fig. 5), with average ranging from 20.6 to 21.67 g, whereas the controls weighed 24.2 g. Together, these results showed that immunization of BALB/c mice with the ectodomain of TSHR, either in a soluble form or expressed on xenogeneic cells, can induce severe hyperthyroidism (Table III).

Thyroid histology

As indicated in Fig. 6, a majority of hyperthyroid mice, irrespective of TSHR preparation used for immunization, showed enlargement of the thyroid gland relative to thyroids from control mice due to hyperactivity (Fig. 6A). Hematoxylin and eosin-stained sections from different regions of the thyroid, harvested on day 120

![Graphs showing titers, cAMP levels, T4 levels, T3 levels, and weights over different groups and days.](http://www.jimmunol.org/)

**FIGURE 5.** Induction of hyperthyroidism using ectodomain of human TSHR. Different groups of mice (five mice/group) were immunized as described under Materials and Methods. Groups 1 and 4 were i.p. injected with $2 \times 10^7$ mitomycin C-treated cells along with CTB on days 0, 14, 28, 65, and 91. Groups 2 and 3 and group 5 mice were injected either with 50 μg of TBP or hETGP protein, respectively, along with CFA on day 0. Then these mice received $2 \times 10^7$ mitomycin C-treated 293-TBP cells (groups 3 and 5) or soluble TBP protein (group 2) along with CTB on days 14, 28, 48, 65, and 91. For monitoring the immune response, serum from blood obtained on day 120 was tested for ELISA and TBII activity. Sera obtained on day 180 was used for T4 and T3 measurements and for cAMP induction. Two microliters of serum was diluted to 100 μl in assay buffer and used in the cAMP assay. Weights of mice on day 240 of the experiments are shown. The bars indicate mean ± SD. Filled circles indicate mean value for the group, and each of the other symbols indicates value for individual mouse within each group.

---

**Table II. Summary of results from mice immunized with hM12 and mM12 cells***

<table>
<thead>
<tr>
<th>Group</th>
<th>TBII (%)</th>
<th>cAMP (pmol/ml)</th>
<th>T4 Level (μg/dl)</th>
<th>T3 Level (ng/dl)</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.6 ± 3.68</td>
<td>1.83 ± 0.22</td>
<td>4.8 ± 0.67</td>
<td>34.99 ± 1.34</td>
<td>23.9 ± 0.79</td>
</tr>
<tr>
<td>2</td>
<td>70.0 ± 9.77</td>
<td>19.44 ± 5.18</td>
<td>8.66 ± 3.72</td>
<td>173.74 ± 26.15</td>
<td>23.24 ± 0.90</td>
</tr>
<tr>
<td>3</td>
<td>49.4 ± 27.4</td>
<td>20.83 ± 4.83</td>
<td>12.4 ± 0.72</td>
<td>156.18 ± 35.61</td>
<td>22.23 ± 1.10</td>
</tr>
<tr>
<td>4</td>
<td>65.9 ± 6.12</td>
<td>18.25 ± 11.84</td>
<td>11.02 ± 1.39</td>
<td>180.13 ± 27.94</td>
<td>19.5 ± 1.10</td>
</tr>
<tr>
<td>5</td>
<td>49 ± 22</td>
<td>16.22 ± 6.83</td>
<td>11.21 ± 1.32</td>
<td>195.13 ± 26.38</td>
<td>22.9 ± 0.93</td>
</tr>
<tr>
<td>6</td>
<td>50.9 ± 12.27</td>
<td>6.52 ± 2.56</td>
<td>10.9 ± 2.02</td>
<td>158.73 ± 16.17</td>
<td>23.92 ± 0.98</td>
</tr>
<tr>
<td>7</td>
<td>41.5 ± 25.89</td>
<td>10.8 ± 0.54</td>
<td>11.4 ± 0.88</td>
<td>180.32 ± 30.32</td>
<td>21.1 ± 1.75</td>
</tr>
</tbody>
</table>

*The details of protocol used for immunization of mice in different groups are described under Materials and Methods and in legend to Fig. 4: group 1, 2 × 10^7, M12 cells; group 2, hM12 cells along with CTB; group 3, mM12 cells along with CTB; group 4, mice were primed once with hETGP and then challenged with hM12 along with CTB; group 5, mice were primed once with mETGP and then challenged with mM12 cells along with CTB; group 6, mM12 cells alone; and group 7, hM12 cells alone. Prior to immunization, the cells were treated with mitomycin C. Each group consisted of five mice. The results represent mean ± SD.*
after initiation of immunization, showed hypertrophy and enlargement of colloids with thinning of the thyroid epithelium (Fig. 6C). However, when thyroids collected from affected mice on day 240 were examined for histopathology, some of them showed focal necrosis and inflammation characterized by lymphocytic infiltration (Fig. 6D). These observations are consistent with features of Graves’ disease. Thyroids from control mice showed normal histology (Fig. 6B).

Discussion

We immunized BALB/c mice with either hM12 cells or mM12 cells. As evident from our results (Fig. 4 and Table II), there was no difference in the ability of either TSHR protein to induce GD. The disease was characterized by the appearance of Abs as detected in an ELISA followed by the appearance of TBII and then TSAb activities. Subsequently, the thyroid hormone levels were elevated. Consistent with these observations, mice from most treatment groups showed a tendency toward weight loss, with one group (group 4) showing a significant reduction in weight. To our knowledge, this is the first report of induction of Graves’ disease in nearly 100% of mice. Moreover, in the present study, for the first time, the mouse TSHR protein was used to successfully induce Graves’ disease in mice.

Our present study confirms and extends two earlier studies in which RT4.15HP cells transfected with human TSHR were used for immunization (13, 14). In our study, we were able to induce disease in 100% of mice, whereas in earlier studies (13, 14, 22, 23) only 20% of the mice developed the disease. This, we believe, could be due to several factors. In previous studies, RT4.15HP cells, which are murine fibroblasts expressing MHC class-II and TSHR proteins, but devoid of B-7 molecules, were used for immunization. On the other hand, in the current study, M12 cells which are B cells that have been extensively characterized as APCs and are known to express class-II and B-7, were used for immunization. In the present study, M12 cells which are B cells that have been extensively characterized as APCs and are known to express class-II and B-7, were used for immunization. Prior to inoculation, cells were treated with mitomycin C. Each group consisted of five mice. The results represent mean ± SD.

Table III. Summary of results from mice immunized with hTSHR.

<table>
<thead>
<tr>
<th>Group</th>
<th>TBII (%)</th>
<th>cAMP (pmol/ml)</th>
<th>T4 Level (μg/dl)</th>
<th>T3 Level (ng/dl)</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.84 ± 5.2</td>
<td>1.75 ± 0.22</td>
<td>4.58 ± 0.75</td>
<td>37.37 ± 5.69</td>
<td>24.2 ± 1.3</td>
</tr>
<tr>
<td>2</td>
<td>43.4 ± 16.7</td>
<td>16.1 ± 4.2</td>
<td>12.56 ± 2.53</td>
<td>142.86 ± 23.44</td>
<td>21.6 ± 0.54</td>
</tr>
<tr>
<td>3</td>
<td>34.8 ± 16.48</td>
<td>22.80 ± 1.84</td>
<td>9.53 ± 2.61</td>
<td>154.46 ± 17.86</td>
<td>20.6 ± 0.89</td>
</tr>
<tr>
<td>4</td>
<td>44.3 ± 16.9</td>
<td>24.2 ± 4.19</td>
<td>12.05 ± 1.12</td>
<td>162.35 ± 10.55</td>
<td>21.67 ± 0.57</td>
</tr>
<tr>
<td>5</td>
<td>35.14 ± 16.48</td>
<td>19.58 ± 4.45</td>
<td>11.86 ± 1.77</td>
<td>140.18 ± 44.36</td>
<td>21 ± 0.70</td>
</tr>
</tbody>
</table>

The details of protocol used for immunization of mice in different groups are described under Materials and Methods and in legend to Fig. 5: group 1, 2 × 105, 293 cells; group 2, purified TBP protein given along with CTB; group 3, primed once with purified TBP emulsified in CFA and then challenged with 293-TBP cells with CTB; group 4, 293-TBP cells along with CTB; and group 5, primed once with hETGP with CFA and then challenged with 293-TBP cells along with CTB. Prior to inoculation, cells were treated with mitomycin C. Each group consisted of five mice. The results represent mean ± SD.
derived from H-2k, and β1 domain derived from H-2d (25). These cells were originally constructed to identify functional domains of class II molecule and had been used in vitro as APCs in T cell proliferation assays to measure recall responses of T cells primed in vivo by direct Ag inoculation (25). Expression of β1 domain of H-2d did not affect H-2d-restricted APC function and suggested that β1 domain was not important for either peptide binding or for recognition by T cells. RT4.15HP cells transfected with TSHR were used to immunize AKR (H-2k) mice, and it is not clear whether allosereactive responses against the chimeric class II molecule rather than optimal TSHR-specific T cell responses were induced since alloantigens are more potent immunogens than self Ags. Third, it is likely that BALB/c (H-2d) mice used in this study, relative to AKR mice used in the earlier studies (13, 14), might be more susceptible to GD. In fact, several earlier studies have shown strain-specific differences in immune response to TSHR (5, 23, 26). Fourth, it is well known that BALB/c mice most often mount Th2 type of CD4+ T cell responses (27) and that Th2 cells provide appropriate help to B cells for high affinity Ab production. In this context, it is interesting to note that CTB promotes activation of Th2 cells; therefore, we used it as an adjuvant to see whether disease can be induced in a higher proportion of mice. Since we were able to induce GD in 100% of the mice without CTB (Table II, groups 6 and 7), the effects of CTB, if any, could not be evaluated in this study. In fact, Kita et al. (14) have reported that immunization of AKR mice with RT4.15HP cells expressing TSHR along with the pertussis toxin, a Th2 response-inducing adjuvant, caused disease in a higher proportion (~50%) of animals. Since GD is an Ab-mediated disease, at the present time it is not clear whether differential activation of Th1- or Th2-type of CD4+ T cells in different strains of mice contributed to differential susceptibilities. Currently, efforts are underway in our laboratory to induce GD in different strains of mice using CTB as an adjuvant.

Earlier, we and others have clearly shown that the ectodomain of TSHR is sufficient for TSH as well as stimulatory Ab binding (3, 4, 17, 19, 20, 22, 28, and 29). Moreover, earlier studies showed that immunization of mice with fibroblasts expressing either TSHR or Class II alone was incapable of inducing an effective immune response against TSHR (13). However, upon immunization with cells expressing both TSHR and Class II molecules, mice developed autoantibody response to TSHR (13). This was taken to suggest that aberrant Class II expression might be critical for disease induction (13, 23). To test whether soluble ectodomain was sufficient to induce the disease and to address the requirement of TSHR-expressing cells to function as APCs, we conducted additional studies.

For these studies we used 293-TBP cells, from which soluble functional TBP was purified. The 293 cells are human in origin and xenogeneic to mice. As shown by our results (Fig. 5 and Table III), immunization of BALB/c mice with either purified TBP or TBP-293 cells resulted in development of severe hyperthyroidism in all mice except one. These studies clearly demonstrated that soluble ectodomain of TSHR is sufficient for disease induction in BALB/c mice. Previously, we used ETSR, hETGP, or mETGP produced in insect cells (3, 4, and 10) to immunize mice. These mice developed moderate hyperthyroidism but failed to show classical symptoms often associated with human GD (5, 26, 30). This, we believe, is due to lack of native conformation resulting from aggregation of protein produced in insect cells. Although insect cell lysates contain a very small amount of appropriately folded TSHR (as demonstrated by neutralization of TBII activity in patient sera), most of the protein remains aggregated. Since aggregated proteins are more immunogenic than soluble proteins, a substantial Ab response was most likely directed against epitopes on the aggregated protein, which may or may not often be expressed on the native TSHR (30). In contrast, TBP is readily soluble (16), and the purified protein is fully functional in that it efficiently binds both TSH and autoantibodies (16, 17) (Table I and Fig. 2). Current studies further demonstrate that there is requirement neither for TSHR to be cell associated nor for aberrant syngeneic Class II expression on the cell surface. Since a typical APC requires expression of co-stimulatory and adhesion molecules on its cell surface, it is not apparent how TSHR-transfected RT4.15HP cells, which lacked co-stimulatory molecules, could have served as APCs to prime anti-TSHR responses. Moreover, RT4.15HP cells constitutively expressed TSHR; therefore, they would be expected to process TSHR as an endogenous Ag and present peptides bound to MHC class I. Since we know that TSHR is a protein Ag, the immune response to it is likely to be regulated by CD4+ T cells. These CD4+ T cells often recognize antigenic peptides bound to MHC class II, which requires exogenous Ag processing pathway. Based on these observations and our current results, it is apparent that TSHR ectodomain with native conformation is sufficient to cause GD in 100% of immunized mice.

Now that we have a fully developed animal model for GD, further studies could lead to the development of new therapeutic strategies that could either down-modulate the immune response or shift it away from the production of pathogenic Abs.

Acknowledgments

We thank Drs. Leonard D. Kohn and Kazuo Tahara, National Institutes of Health, for providing us the cDNA encoding the full-length hTSHR and transfected CHO cells. J. Larry Jameson and Peter Kopp, Northwestern University, for providing us cDNA encoding full-length mouse TSHR. We acknowledge the technical help provided by Mohammed El Azami El Idrissi, Seetharamaiah G. S., Seema Rao, James Artwohl, Mark Holtermann, and Karen Hagen, and thank Adeeb Al Zoubi and Amit Gupta for help in preparation of the manuscript.

References

ECTODOMAIN OF TSHR INDUCES AUTOIMMUNE GRAVES’ DISEASE


