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A Novel Murine Model of Graves’ Hyperthyroidism with Intramuscular Injection of Adenovirus Expressing the Thyrotropin Receptor

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In this work we report a novel method to efficiently induce a murine model of Graves’ hyperthyroidism. Inbred mice of different strains were immunized by i.m. injection with adenovirus expressing thyrotropin receptor (TSHR) or β-galactosidase (1 × 1011 particles/mouse, three times at 3-wk intervals) and followed up to 8 wk after the third immunization. Fifty-five percent of female and 33% of male BALB/c (H-2b) and 25% of female C57BL/6 (H-2b) mice developed Graves’-like hyperthyroidism with elevated serum thyroxine (T4) levels and positive anti-TSHR autoantibodies with thyroid-stimulating Ig (TSI) and TSH-binding inhibiting Ig (TBII) activities. In contrast, none of female CBA/J (H-2k), DBA/1J (H-2q), or SJL/J (H-2s) mice developed Graves’ hyperthyroidism or anti-TSHR autoantibodies except SJL/J, which showed strong TBII activities. There was a significant positive correlation between TSI and TBII levels, and the correlations between T4 and TBII and between TSI and TBII were very weak. TSI activities in sera from hyperthyroid mice measured with some chimeric TSH/lutropin receptors suggested that their epitope(s) on TSHR appeared similar to those in patients with Graves’ disease. The thyroid glands from hyperthyroid mice displayed diffuse enlargement with hypertrophy and hypercellularity of follicular epithelia with occasional protrusion into the follicular lumen, characteristics of Graves’ hyperthyroidism. Decreased amounts of colloid were also observed. However, there was no inflammatory cell infiltration. Furthermore, extraocular muscles from hyperthyroid mice were normal. Thus, the highly efficient immunization protocols have failed to establish a disease model (3, 7, 8). However, some new approaches for generating animal models of Graves’ hyperthyroidism have recently been demonstrated. First, a novel, pioneering immunization protocol using transfected fibroblasts (a L cell line) coexpressing TSHR and MHC class II Ag has been described by Shimojo et al. (9) to induce hyperthyroidism in a small proportion (~20%) of immunized syngeneic AKR mice. This model was later confirmed by two other groups (10, 11).

Animal models are very useful tools to study the pathophysiology of autoimmune thyroid disease. There are some spontaneous animal models of autoimmune thyroiditis in which the autoimmune response is directed to Tg or TPO (4). Immunization of certain animals with Tg or TPO in combination with classical immunological adjuvants has also been reported to successfully induce thyroiditis (5, 6). In contrast, there are no spontaneous animal models of Graves’ hyperthyroidism, and numerous attempts using TSHR protein expressed in bacteria or insect cells and classical immunization protocols have failed to establish a disease model (3, 7, 8). However, some new approaches for generating animal models of Graves’ hyperthyroidism have recently been demonstrated. First, a novel, pioneering immunization protocol using transfected fibroblasts (a L cell line) coexpressing TSHR and MHC class II Ag has been described by Shimojo et al. (9) to induce hyperthyroidism in a small proportion (~20%) of immunized syngeneic AKR mice. This model was later confirmed by two other groups (10, 11).

Later, B lymphoblastoid cells (a M12 cell line) expressing TSHR have also been used in a similar protocol with a high proportion of the syngeneic BALB/c mice becoming hyperthyroid (12). Furthermore, genetic immunization with an eukaryotic expression vector containing TSHR cDNA has proved to be useful in inducing hyperthyroidism in a small proportion (~20%) of outbred NMRI (13), not inbred BALB/c, mice (14). Although these methods opened new ways to investigate the autoimmune reaction to TSHR, they have some drawbacks, such as use of cell lines available only for certain strains of mice (9, 12) and a low rate of disease induction (9, 14).

Therefore, the present studies were designed to establish a better means for inducing disease. Our first attempt was the genetic immunization protocol mentioned above modified by combining the TSHR expression plasmid and the cytokine expression plasmids (GM-CSF or M-CSF). However, no significant immune response was observed. We then used recombinant adenovirus to achieve higher transgene expression in the muscle. We show in this study that repeated administration of recombinant adenovirus vector expressing TSHR into the muscle efficiently generates anti-TSHR autoantibodies and induces Graves’-like hyperthyroidism in BALB/c (H-2b), and to a lesser extent in C57BL/6 (H-2b), mice. Our data also indicate the similarity of epitope(s) on

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‡ Abbreviations used in this paper: TSHR, thyrotropin receptor; Tg, thyroglobulin; TPO, thyroid peroxidase; MOL, multiplicity of infection; TBII, TSH-binding inhibiting Ig; TSI, thyroid-stimulating Ig; LHR, lutropin receptor; wt, wild type.
TSHR ectodomain recognized by TSI from Graves’ patients and those from hyperthyroid mice.

**Materials and Methods**

**Construction of the recombinant adenovirus expressing hTSHR**
pBluescript-human (h)TSHR (15) was digested with EcoRI, blunt ended with T4 DNA polymerase, and digested with XhoI. The TSHR cDNA fragment was then ligated into pHMCMV6 (15), which had been digested with Nhel, blunt-ended, and digested with Xhol. The resultant plasmid, pHMCMVTSHR, was then digested with I-Ceu I/PstI/Sce I and ligated into I-Ceu I/PstI/Sce Idigested pAdHM4 (16, 17). pAdHM4CMVTSHR was linearized with FucI and transfected into 293 human embryonal kidney cells with SuperFect (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions. Recombinant adenovirus expressing TSHR (designated AdCMVTSHR) was then plaque-purified. Adenovirus was propagated in 293 human embryonal kidney cells and purified through two rounds of CsCl density gradient centrifugation (18). The multiplicity of infection (MOI) was defined as the ratio of total number of particles used in a particular infection divided by the number of cells. The viral particle concentration was determined by measuring the absorbance at 260 nm following the incubation of the virus solution in 10 mM Tris-HCl, 1 mM EDTA, and 0.1% SDS at 56°C for 10 min; an absorbance of 1 corresponds to 1.1 × 1012 particles/ml (19). Adenovirus expressing β-galactosidase (AxCALacZ) (18) was used as a negative control.

**Immunization protocols**

BALB/c (H-2d), CBA/J (H-2b), C57BL/6 (H-2b), DBA/1J (H-2b), and SJL/J (H-2b) 6-wk-old mice were purchased from Charles River Breeding Laboratories (Tokyo, Japan). All experiments were conducted in accordance with the principles and procedures outlined in the Guideline for the Care and Use of Laboratory Animals in Nagasaki University (Nagasaki, Japan). Mice were kept in a specific pathogen-free condition through the experiments. For DNA vaccination, groups of mice were injected in the leg muscle with 50 μl of pCAGTSHR alone or in combination with the expression vectors for M-CSF or GM-CSF (RIKEN DNA Bank, Saitama, Japan). Injection was repeated twice at 3-wk intervals. For immunization with adenovirus, mice were i.m. injected with 50 μl PBS containing 1 × 1011 particles of AdCMVTSHR or AxCALacZ. The same immunization schedule was repeated twice at 3-wk intervals.

**125I-TSH binding and TSH-induced cAMP synthesis in COS cells or muscle injected with adenovirus or plasmid**

A total of 1 × 10⁶ COS cells in a 24-well culture plate were infected with AdCMVTSHR or AxCALacZ at a MOI of 1,000 or 10,000 particles/cell. Two days later, 125I-TSH binding to intact cells and intracellular cAMP measurements were performed with 125I-bovine TSH (TRAb kit; RSR, Llanes, Spain) and with a cAMP radioimmunoassay kit (Yamasuka, Tokyo, Japan), respectively, as previously described (20). Unlabeled TSH used in TSH binding study was of bovine origin (Sigma-Aldrich, St. Louis, MO). 125I-TSH binding was also performed with 50 μg crude membranes prepared as previously described (20) from muscles 5 days after infection of either 1 × 10¹¹ particles of AdCMVTSHR or 100 μg of pCAGTSHR.

**Thyroid function test**

Total thyroxine (T₄) in murine sera was measured with a commercially available radioimmunoassay kit (Eiken Chemical, Osaka, Japan). The normal range was defined as the mean ± 3 SD of control mice.

**TSI and TSH measurements**

TSI activities in murine sera were measured with FRTL5 cells (20) or CHO cells stably expressing wild-type (wt)-hTSHR or chimeric TSH-lutropin receptor (LHR)-6 and TSH-LHR-8 (21). The cDNAs for wt-TSHR and chimeric TSHR/LHRs were ligated into the eukaryotic expression vector pcR3 with the constitutive CMV promoter (Invitrogen, Groningen, The Netherlands). The cells were seeded at 3 × 10⁴ cells/well in a 96-well culture plate and incubated in 50 μl hypotonic HBSS containing 1 mM isobutyl-methylxanthine, 20 mM HEPES, 0.25% BSA, and 5 μl serum for 2 h at 37°C. CAMP >150% of control mice was judged as positive.

**Thyroid and eye histology**

Thyroid tissues and extraocular muscles were removed and fixed with 10% formalin in PBS. Tissues were embedded in paraffin and 5-μm-thick sections were prepared and stained with H&E.

**Data analysis**

Data were analyzed by unpaired Student’s t test or by the χ² test. Correlations among T₄ and autoantibodies were assessed by linear regression using StatView 4.02 software (Abacus Concepts, Berkeley, CA). Values of p < 0.05 were considered statistically significant.

**Results**

Our first attempt at a model for Graves’ disease was to immunize female BALB/c mice with the expression plasmid for TSHR (pCAGTSHR) alone or in combination with the expression vectors for M-CSF or GM-CSF. We expected that the cytokines expressed would enhance the immune reaction against TSHR and that M-CSF and GM-CSF would shift the immune response to Th1 and Th2, respectively (22). In contrast to the previous reports (13, 14), however, this approach did not elicit any significant immune response to TSHR (data not shown). Lack of the immune response to TSHR is considered a pathogenic factor in Graves’ disease.
to DNA vaccination in BALB/c mice has also been described recently (23). One reason for this difference may be housing conditions (specific pathogen-free conditions in our study and Ref. 23 vs conventional housing in Ref. 14).

Therefore, we next challenged the animals with recombinant adenovirus expressing TSHR (AdCMVTSHR) to increase transgene expression in the muscle. Integrity of AdCMVTSHR was confirmed by specific 125I-TSH binding and by TSH-induced cAMP synthesis in COS cells infected with AdCMVTSHR, not with AxCALacZ (a negative control) (Fig. 1, A and C). Furthermore, a higher expression level of TSHR was observed in muscle with AdCMVTSHR infection as compared with that with pCAGTSHR (Fig. 1B). In this experiment, TSH binding induced with pCAGTSHR was negligible.

Groups of five different mouse strains were immunized with $1 \times 10^{11}$ particles of AdCMVTSHR or AxCALacZ three times at 3-wk intervals. Between 5 and 6 wk after the last immunization, one female BALB/c mouse died spontaneously and was found to have a large diffuse goiter (see below). Two female BALB/c mice and one C57BL/6 mouse were sacrificed because they exhibited significant weight loss and exhaustion. All other mice were sacrificed 8 wk after the last immunization. Thyroid hormone and anti-TSHR autoantibody levels in sera, as well as thyroid and eye histology, were then examined. Means ± 3 SD of serum T4 levels in naive mice were 4.9 ± 0.31 μg/dL (ranging from 4.7 to 5.5) in BALB/c, 4.2 ± 0.99 (3–4.7) in C57BL/6, 3.6 ± 0.53 (3.1–4.4) in CBA/J, 1.96 ± 0.51 (1.2–2.6) in DBA/1J, and 1.2 ± 0.12 (1.1–1.3) in SJL/J mice. As shown in Fig. 2A, serum T4 levels were increased over mean ± 3 SD in 10 of 19 (53%) female BALB/c, three of nine (33%) male BALB/c, and two of eight (25%) female C57BL/6 mice, but in none of CBA/J, DBA/1J, or SJL/J mice. Thus, by including the one female BALB/c mouse, which died as mentioned above, 11 of 20 (55%) female BALB/c mice appeared to develop hyperthyroidism. There was no significant difference in the rate of disease induction between male and female BALB/c mice. No mice showed any significant decrease in T4. BALB/c mice immunized with AxCALacZ were all in a euthyroid state (T4, 4.5 ± 0.57 μg/dL).

TSI determined with FRTL5 cells are shown in Fig. 2B. Sera from all but one mouse with increased T4 levels displayed positive TSI. No euthyroid mouse of any strain showed TSI activity. As shown in Fig. 3A, there was a significant positive correlation between TSI values and T4 levels ($r = 0.89$). Thus, BALB/c and, to a lesser degree, C57BL/6 mice are susceptible to TSI generation and disease induction.

However, as shown in Fig. 2C, TBII assay revealed that not only BALB/c and C57BL/6 but also SJL/J mice are susceptible to TBII generation. Besides all the hyperthyroid and some other euthyroid BALB/c and C57BL/6 mice (16 of 19 (84%) female BALB/c, five of nine (56%) male BALB/c, and six of eight (75%) C57BL/6 mice), six of eight (75%) SJL/J mice were positive for TBII, the values being the highest among five groups. There was no significant correlation between T4 levels and TBII values ($r = 0.38$) (Fig. 3B), and correlation between TSI and TBII values was significant but extremely poor ($r = 0.40$) (Fig. 3C).

To delineate the epitope(s) on TSHR for TSI in the sera of hyperthyroid mice, TSI activities were also measured with two chimeric TSH/LHRs, TSHR-LHR-6 and -8, in which the C-terminal and the N-terminal two-fifths of TSHR ectodomain were, respectively, replaced with the corresponding region of LHR (Fig. 4A). Although wt-TSHR and TSHR-LHR-6 expressed on CHO cells responded well to stimulation by TSH and TSI of both human and mouse origin, TSH-LHR-8 did so only to TSH stimulation, not to TSI (Fig. 4B). These data are essentially identical to those previously reported with human Graves’ sera (24, 25), suggesting the crucial role of the N-terminal region of TSHR ectodomain in both human and murine TSI.

In histological examination, the thyroid glands from all the hyperthyroid mice and the one that died spontaneously (see above) exhibited diffuse enlargement with hypertrophy and hypercellularity of follicular epithelia with occasional protrusion into the follicular lumen, all consistent with Graves’ hyperthyroidism in humans. These findings are consistently observed in most follicles in the thyroid glands from hyperthyroid mice with higher T4 (>11–12 μg/dL), as shown in Fig. 5, C and D (compared with the

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** T4, TSI, and TBII values in mice immunized with AdCMVTSHR or AxCALacZ. O, Female mice; □, male mice. TSI was determined with FRTL5 cells as described in Materials and Methods. The normal upper limits are shown by horizontal lines.

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Relationship between T4 and TSI (A), T4 and TBII (B), and TSI and TBII (C). Data shown in Fig. 2 were used. Correlation coefficients are 0.89 in A, 0.38 in B, and 0.40 in C.
normal thyroid in Fig. 5, A and B), but are less evident and heterogeneous in hyperthyroid mice with lower T4; papillary protrusion of the follicular epithelia was sparsely observed and the epithelia consisted of either flat cells (inactive) or columnar cells (hyperactive), as previously reported (14). Decreased amounts of colloid were also observed. However, no inflammatory cell infiltration was observed. Furthermore, extraocular muscles from hyperthyroid mice were normal (data not shown).

Discussion

In this article, we succeeded in establishing a novel method to efficiently induce Graves’ hyperthyroidism. This was achieved by repeated injection of recombinant adenovirus expressing TSHR into the muscle. Although several disease models have been described recently (9, 12, 14), our method clearly possesses at least two advantages. First, the rate of disease induction in our model is much higher than those reported by Shimojo et al. (9) and Vassart et al. (14) (55% vs ~20%). Although Prabhakar and his colleagues have reported to be able to induce Graves’-like hyperthyroidism in nearly 100% of BALB/c mice (12), thyroid histology from affected mice in their study showed “hypertrophy and enlargement of colloid with thinning of the thyroid epithelium.” This finding contradicts that in Graves’ disease, in which thyroid epithelial cells are tall and columnar and sometimes extend as papillary folds into the follicles, indications of hypertrophy and hypercellularity.

Second, in contrast to previously reported methods that are applied only to certain strains of inbred mice or to outbred mice, our method can be used for any mouse strain, which allows analysis of the genetic influence(s) on the immune response to TSHR. Our data clearly show that BALB/c (H-2d) and, to a lesser degree, C57BL/6 (H-2b) mice are susceptible to generation of TSI and induction of Graves’ hyperthyroidism. These data, together with a previous report (26), suggest the genetic backgrounds play a role in susceptibility to an induced form of Graves’ disease in mice. Indeed Graves’ disease is known to be a multigenic disease in humans (27); both HLA and non-HLA genes, such as CTLA-4, are associated with a predisposition to autoimmune thyroid disease (28, 29). In addition, immunized SJL/J mice show negative TSI and strongly positive TBII, the highest among five groups of different strains, suggesting that the genetic factor(s) crucial for Graves’ disease/TSI induction and those for TBII generation may also be different. Our results may explain a lower incidence of disease induction in Shimojo’s model (AKR with H-2k) (9) and...
Multiple dosing to the muscle (30). Thus, adenovirus infection muscle may be lower than that in the serum, allowing effective i.m. injection of adenovirus, the concentration of these Abs in the can be elicited not only following i.v. injection but also following that with plasmid. Although adenovirus-specific neutralizing Abs can be elicited not only following i.v. injection but also following i.m. injection of adenovirus, the concentration of these Abs in the muscle may be lower than that in the serum, allowing effective multiple dosing to the muscle (30). Thus, adenovirus infection seems to elicit superior immune response to DNA vaccination. Indeed, serum T₃ levels, which reflect the degree of the immune reaction induced, in our model are higher than those in Vassart’s model (9–20 vs 8–10 μg/dL) (14). Usefulness of adenovirus as a means to induce strong in vivo immune response has also been reported recently by Chen et al. (31).

It is generally believed that autoimmune reaction in human Graves’ disease is Th2 dominant (32). In animal models, previous studies performed by Kita et al. (10) have also demonstrated that immunization of AKR mice with L cells expressing TSHR and MHC class II with the pertussis toxin, a Th2 response-inducing adjuvant, led to Graves’ disease in a higher proportion of mice, and use of CFA, a Th1 adjuvant, delayed the disease onset. Indeed, BALB/c mice are reported to inherently elicit Th2-dominant immune response (33, 34). This could be one of the reasons for high susceptibility of BALB/c mice to Th2-dominant Graves’ hyperthyroidism.

However, recent studies indicate that the immune response induced in Shimojo’s and Vassart’s models is not simply Th2 dominant. First, monoclonal anti-TSHR Abs established from BALB/c mice immunized with DNA vaccination are IgG2a, an isotype predominant in Th1 immune response (13). Second, splenocytes from immunized mice in Shimojo’s and Vassart’s models produce IFN-γ, a Th1 cytokine, spontaneously and in response to TSHR Ag, respectively (23, 35). Although Vassart et al. (14) have described that the immune response occurred in the thyroid gland is Th2 dominant, DNA vaccination with plasmid generally leads to Th-1-biased immune response, because plasmids propagated in bacteria contain unmethylated CpG sequences that induce T cells to a Th1 proinflammatory immune response (36). Although Th1/Th2 balance in our model is at present unknown, this finding may be another reason for low prevalence of Graves’ disease in these models.

In contrast to Vassart’s model (14), female bias, intrathyroidal lymphocyte infiltration, or eye lesion (one of the extrathyroidal manifestations of Graves’ disease) were not observed in hyperthyroid mice in our study as in Shimojo’s models (9–11). Although the exact reasons for this difference are presently unknown, it is plausible that different experimental protocols may induce the subtle distinct immune responses. It is apparently suggested, as Vassart et al. (14) have mentioned, that a purely humoral immune response is induced in our and Shimojo’s models (9), which, however, does not simply indicate the Th2-dominant immune response as mentioned above. It is at present uncertain whether the adequate murine model of Graves’ disease should include these three findings. Pathophysiology of Graves’ disease in human seems more complex than that in an animal model induced with TSHR as a single immunogen, because not only TSHR but also Tg and TPO are autoantigens in the former (1). Further studies will be required to address these issues.

Our study with chimeric receptors suggests that the epitopes on TSHR recognized by TSI in our model appear very similar to those observed in human disease (24, 25), suggesting the involvement of the N terminus of TSHR ectodomain in both human and murine TSI. This is in agreement with the studies by Kikuoka et al. (37), who have shown the importance of the N terminus of TSHR ectodomain (domains A and B in our chimeras) in induction of Graves’ hyperthyroidism with Shimojo’s model. The N terminus of TSHR ectodomain contains a major portion of the B cell epitope(s) for TSI, although this region does not appear to contain T cell epitopes.

In summary, in this work we report a novel murine model of Graves’ disease in which repeated i.m. injection of adenovirus expressing TSHR efficiently induces anti-TSHR Abs with thyroid-stimulating activity, resembling TSI, and hyperthyroidism. Our results demonstrate that BALB/c (H-2b) and, to a lesser degree, C57BL/6 (H-2b) mice appear susceptible to the disease. This new model will be a useful tool to study the pathogenesis of autoimmunity in Graves’ disease; the high disease penetrance in our model is particularly advantageous over other methods for some studies, such as identification of susceptibility genes and development of new therapeutic approaches.

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**References**


