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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-2d) 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 (TBI) 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 TBI activities. There was a significant positive correlation between TSI values and T4 levels, but the correlations between T4 and TBI and between TSI and TBI 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 means that we now report to induce Graves’ hyperthyroidism in mice will be very useful for studying the pathogenesis of autoimmunity in Graves’ disease. The Journal of Immunology, 2002, 168: 2789–2794.

A autoimmune thyroid diseases such as Graves’ disease and Hashimoto thyroiditis involve abnormal autoimmune reactions to thyroid-specific proteins, including the thyrotropin receptor (TSHR),2 thyroglobulin (Tg), and thyroid peroxidase (TPO) (1). In Graves’ disease, autoantibodies directed against TSHR mimic the action of TSH and are therefore called thyroid-stimulating Ig (TSI). TSI cause overstimulation of the thyroid gland and hyperthyroidism (2, 3).

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-2d), 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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2 Abbreviations used in this paper: TSHR, thyrotropin receptor; T4, thyroxine; Tg, thyroglobulin; TPO, thyroid peroxidase; MOL, multiplicity of infection; TBI, 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 XhoI. The resultant plasmid, pHMCMVTSHR, was then digested with I-Ceu I/I-PstI and ligated and into I-Ceu I/I-PstI-digested 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 (AxCA-LacZ) (18) was used as a negative control.

Immunization protocols

BALB/c (H-2d), CBA/J (H-2b), C57BL/6 (H-2b), DBA/1J (H-2q), and SJL/J (H-2s) 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 25% sucrose in PBS containing 100 μg of pCAGTSHR and 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 AxCA-LacZ. 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 AxCA-LacZ 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 10-6 M TSH used in Materials and Methods. 125I-TSH used in each experiment was ~10,000 cpm. B, 125I-TSH binding in 50 μg crude membranes from muscles injected with AdCMVTSHR, AxCA-LacZ (1 × 1011 particles), or pCAGTSHR (100 μg). C, cAMP response to TSH stimulation in COS cells infected with AdCMVTSHR or AxCA-LacZ. Cells infected with AxCA-LacZ at a MOI of 10,000 particles/cell; ■, cells infected with AdCMVTSHR at a MOI of 1,000; □, cells infected with AdCMVTSHR at a MOI of 10,000. Data are representative of two separate experiments; each point represents the mean ± SD of triplicate experiments. *, p < 0.01; **, p < 0.05 vs AxCA-LacZ infection.

FIGURE 1. 125I-TSH binding and TSH-stimulated cAMP synthesis in COS cells or muscles transduced with AdCMVTSHR, AxCA-LacZ, or pCAGTSHR. A, 125I-TSH binding in COS cells infected with AdCMVTSHR or AxCA-LacZ (MOI of 10,000 particles/cell) in the presence or absence of unlabeled 10−6 M TSH was performed as described in Materials and Methods. 125I-TSH used in each experiment was ~10,000 cpm. B, 125I-TSH binding in 50 μg crude membranes from muscles injected with AdCMVTSHR, AxCA-LacZ (1 × 1011 particles), or pCAGTSHR (100 μg). C, cAMP response to TSH stimulation in COS cells infected with AdCMVTSHR or AxCA-LacZ. Cells infected with AxCA-LacZ at a MOI of 10,000 particles/cell; ■, cells infected with AdCMVTSHR at a MOI of 1,000; □, cells infected with AdCMVTSHR at a MOI of 10,000. Data are representative of two separate experiments; each point represents the mean ± SD of triplicate experiments. *, p < 0.01; **, p < 0.05 vs AxCA-LacZ infection.
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 × 10¹¹ 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 (3.4–5.7) 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 excluding 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 mice with increased T₄ levels displayed positive TSI. No euthyroid mice 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 normal upper limits are shown by horizontal lines).
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 colloids 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
Indeed, serum T4 levels, which re
i.m. injection of adenovirus, the concentration of these Abs in the
known that expression level of transgene by adenovirus infection
genetic background as the authors have speculated (14). It is
plausible that different experimental protocols may induce the sub-
the exact reasons for this difference are presently unknown, it is
BALB/c mice by DNA vaccination may not be related to their
incidence in BALB/c mice (12). It is also suggested that the reason
for the failure to produce effective stimulating autoantibodies in
BALB/c mice by DNA vaccination are not related to their
gene expression by adenovirus infection
was indicated to be another reason for low prevalence of Graves
manifestations of Graves disease) were not observed in hyperthy-
disease in human seems more

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 domi-
nant. First, monoclonal anti-TSHR Abs established from BALB/c
mice immunized with DNA vaccination are IgG2a, an isotype pre-
dominant 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 de-
scribed that the immune response occurred in the thyroid gland is
Th2 dominant, DNA vaccination with plasmid generally leads to
Th1-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 hyperthy-
roid 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 sub-
tle distinct immune responses. It is apparently suggested, as Vas-
sart et al. (14) have mentioned, that a purely humoral immune
response is induced in our and Shimojo’s models (9), which, how-
ever, 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 find-
ungs. 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 ex-
pressing TSHR efficiently induces anti-TSHR Abs with thyroid-
stimulating activity, resembling TSI, and hyperthyroidism. Our re-
sults 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 autoim-
munity 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 develop-
ment of new therapeutic approaches.

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