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Induction of Graves-like disease in mice by immunization with fibroblasts transfected with the thyrotropin receptor and a class II molecule

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ABSTRACT **Graves disease is an autoimmune thyroid disease characterized by the presence of antibodies against the thyrotropin receptor (TSHR), which stimulate the thyroid to cause hyperthyroidism and/or goiter. By immunizing mice with fibroblasts transfected with both the human TSHR and a major histocompatibility complex class II molecule, but not by either alone, we have induced immune hyperthyroidism that has the major humoral and histological features of Graves disease: stimulating TSHR antibodies, thyrotropin binding inhibiting immunoglobulins, which are different from the stimulating TSHR antibodies, increased thyroid hormone levels, thyroid enlargement, thyrocyte hypercellularity, and thyrocyte intrusion into the follicular lumen. The results suggest that the aberrant expression of major histocompatibility complex class II molecules on cells that express a native form of the TSHR can result in the induction of functional anti-TSHR antibodies that stimulate the thyroid. They additionally suggest that the acquisition of antigen-presenting ability on a target cell containing the TSHR can activate T and B cells normally present in an animal and induce a disease with the major features of autoimmune Graves.**

Numerous attempts (1–10) to develop a Graves disease (GD) model by immunizing animals with the extracellular domain of the thyrotropin receptor (TSHR) have largely failed. In most cases, antibodies to the TSHR that could inhibit thyrotropin (TSH) binding and, in some cases, thyroiditis with a large lymphocytic infiltration were produced (1–10). However, in no case did the immunization produce thyroid-stimulating TSHR antibodies (TSHRAbs), which increase thyroid hormone levels, the hallmark of Graves, nor were the morphologic or histologic features of the disease induced: glandular enlargement, thyrocyte hypercellularity, and thyrocyte intrusion into the follicular lumen. Further, in most studies (1–10), the antibodies that inhibited TSH binding were not shown to inhibit TSH activity mediated specifically by the TSH receptor, a feature characteristic of TSH binding inhibitory immunoglobulins (TBIIs) in GD (11–13).

These studies depended on the ability of the animal to process the TSHR as an extracellular antigen, rather than as a receptor in a functional state on a cell. They did not take into account the possibility that the TSHR might be presented to the immune system as a result of abnormal major histocompatibility complex (MHC) class I or class II expression on thyrocytes, thereby allowing normal immune tolerance to be broken. Thus, several studies have implicated class I as an important component in the development of autoimmune thyroid disease and in the action of methimazole, a drug used to treat GD (14–18). In addition, aberrant class II expression, as well as abnormal expression of class I molecules, is evident on thyrocytes in autoimmune thyroid diseases (19–21), although the cause and role of aberrant class II in disease expression remains controversial (22). The sum of these observations (1–22) raised the possibility that immunization with full-length TSHR, in a functional conformation but in the context of abnormal MHC class I or class II expression, might lead to the development of GD.

To test the possibility that abnormal MHC expression, as well as a functional, full-length TSHR, might result in a Graves-like disease, we transfected full-length human TSHR (hTSHR) into murine fibroblasts with or without aberrantly expressed class II antigen. We report here that mice immunized with fibroblasts expressing a class II molecule and holo-TSHR, but not either alone, can develop the major features characteristic of Graves (1–10): thyroid-stimulating antibodies directed against the TSHR, increased thyroid hormone levels, an enlarged thyroid, and thyrocyte hypercellularity with intrusion into the follicular lumen. The mice additionally develop TBIIs, which inhibit TSH-increased cAMP levels in Chinese hamster ovary (CHO) cells stably transfected with the TSHR and appear to be different from the stimulating TSHRAbs, another feature of the humoral immunity in GD.

MATERIALS AND METHODS

Fibroblasts and Transfection of the TSHR Gene. A murine L cell fibroblast line, which expresses a hybrid gene containing A^k_β and A^d_β of murine MHC class II (RT4.15HP¶), was kindly provided by R. N. Germain (National Institute of Allergy and Infectious Diseases, National Institutes of Health). The A^d_β determinant is membrane proximal and was shown not to be associated with antigen presentation (23)—i.e., this shuffled I-A^k molecule is not different from I-A^k in antigen presenting activity. The cloning and characterization of the hTSHR was reported previously (24). After subcloning into a pSG5 vector (Stratagene), the hTSHR was transfected into RT4.15HP cells together with pMAMneo (CLONTECH) using Lipofectin (GIBCO/BRL), as described by the company. Class II-untransfected fibroblasts (DAP.3) were also transfected with hTSHR and pMAMneo. Cells were selected for neomycin resistance using 500 μg of G418 per ml (GIBCO/BRL); stable transfectants were selected by their ability to increase cAMP

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Abbreviations: GD, Graves disease; TSH, thyrotropin; TSHR, TSH receptor; TSHRAb, TSHR antibody; hTSHR, human TSHR; TBII, TSH binding inhibitory immunoglobulin; MHC, major histocompatibility complex; CHO, Chinese hamster ovary; T4, thyroxine.

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¶There were no qualitative differences in either T-cell or antibody recognition of I-A^k molecules containing either hybrid or wild-type A^k_β (23).

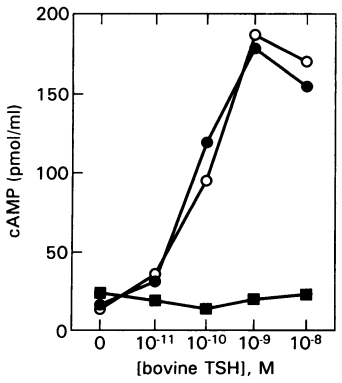

FIG. 1. Bovine TSH-induced cAMP response of hTSHR-transfected fibroblasts. hTSHR-transfected RT4.15HP cells (●), hTSHR-transfected DAP.3 control cells (○), and vector-transfected RT4.15HP cells (■) were stimulated with the indicated concentrations of bovine TSH for 1 hr and the supernatants were collected. cAMP in the supernatant was measured by a commercial radioimmunoassay kit.

levels in the presence of TSH (25). Positive cells were cloned by limiting dilution. Control RT4.15HP cells transfected with the pSG5 vector alone were similarly established.

Immunization of Mice with Transfectants and Assay of TSHRAbs. Seven-week-old female AKR/N (H-2^k) mice were intraperitoneally immunized six times every 2 weeks with 10^7 fibroblasts, which had been pretreated with mitomycin C (Kyowa Hakko Kogyo, Tokyo). These mice were chosen because they have the same class I molecules and a homologous class II I-A molecule to that of the fibroblasts containing the transfected class II and TSHR cDNAs. Preliminary experiments indicated that one immunization and 10^5 cells per immunization were not sufficient to produce the results described below; the time period chosen was the point at which hyperthyroidism first developed in a significant number of animals. Two weeks after final immunization, mice were sacrificed and bled. Mouse thyroids were histologically examined by hematoxylin and eosin staining.

Commercial radioimmunoassay kits were used to measure the ability of antibodies in the serum to inhibit [^{125}I]TSH binding (PSR, Cardiff, U.K.)—i.e TBII activity—and to measure serum thyroxine (T4) levels (Dai-ichi Radioisotopes, Tokyo). Stimulating TSHRAb activity was measured using hTSHR-transfected CHO cells (25). In brief, 4000 hTSHR-transfected CHO cells were plated in 96-well flat-bottomed plates and cultured for 48 hr in growth medium. Cells were washed with Hanks' balanced salt solution (HBSS) and incubated with 25 μl of protein A-purified IgG (1 mg/ml) and 175 μl of low sodium isotonic HBSS (8 mM Na_2PO_4/1.5 mM KH_2PO_4/0.9 mM $CaCl_2$/222 mM sucrose) containing 0.5 mM 3-isobutyl-1-methylxanthine, 0.1% glucose, and 1% bovine serum albumin. After a 3-hr incubation at 37°C, supernatants were collected and cAMP was measured with a commercial radioimmunoassay kit (Yamasa, Chiba, Japan). The ability of the IgG to inhibit TSH activity was measured using the same incubation conditions and hTSHR-transfected CHO cells, except that 10 microunits of bovine TSH per ml was present with each IgG. IgG was obtained from the sera of pairs of animals within each experimental group to overcome technical problems related to obtaining sufficient serum for all measurements in this report.

Flow Cytometry Analysis of Transfectants. Fibroblasts (10^6 cells) were incubated with 1 μg of monoclonal anti-I-A^k (MHC class II-specific) or anti-D^k (MHC class I-specific) antibodies obtained from the American Tissue Culture Collection (10.2.16 or 15-5-S, respectively) or an isotype-specific control monoclonal antibody (Becton Dickinson). After 30 min on ice, cells were washed with phosphate-buffered saline at pH 7.4 and incubated for 30 min with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Kirkegaard & Perry Labo-

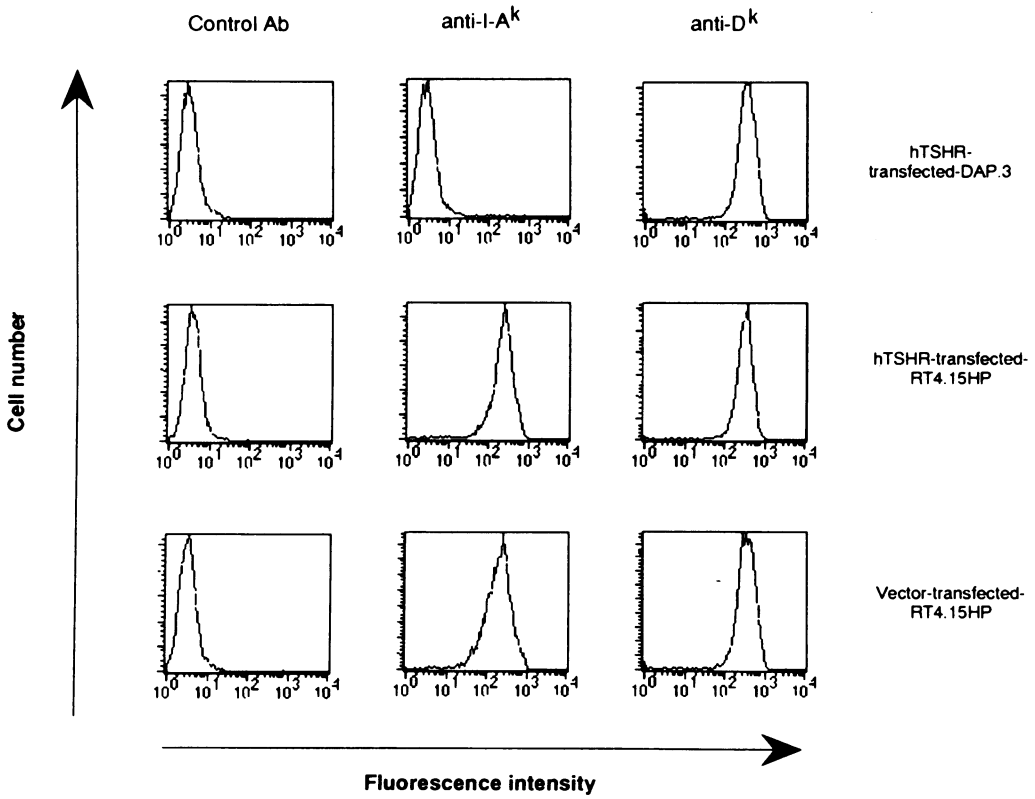

FIG. 2. Surface expression of MHC class II (middle column) and class I (right column) molecules on the surface of murine fibroblasts used for immunization. Procedures were performed as described; experiments in the left column 1 were performed using a control antibody.

Table 1. Anti-TSHR antibodies and thyroid function of mice immunized with TSHR-transfected cells

Immunized transfectants	Mouse no.	TBII, %	T4, μg/dl
hTSHR-transfected RT4.15HP	1	**66.1**	**12.1**
	2	**70.4**	**13.6**
	3	**52.4**	**11.0**
	4	**79.8**	**18.3**
	5	**87.4**	**9.6**
	6	**64.2**	2.1
	7	**68.9**	3.3
	8	**50.3**	3.7
	9	**77.3**	2.1
	10	**85.8**	0.8
	11	**86.3**	1.8
	12	**81.9**	1.8
	13	**87.6**	2.5
	14	**76.6**	1.4
	15	**88.8**	1.7
	16	**82.8**	1.6
	17	**51.5**	2.5
	18	**42.2**	1.4
	19	**75.8**	1.7
	20	**30.6**	2.9
	21	**24.6**	2.5
	22	**22.4**	1.9
	23	18.5	1.2
	24	15.6	2.3
hTSHR-transfected DAP.3	25	2.8	1.9
	26	2.4	2.5
	27	6.7	1.7
	28	7.5	1.9
	29	2.8	1.9
	30	2.4	2.6
	31	6.7	1.7
	32	7.5	1.9
Vector-transfected RT4.15HP	33	0	3.2
	34	0	2.4
	35	5.3	2.4
	36	11.6	1.7
	37	7.7	3.4
	38	10.8	2.4
	39	15.2	2.2
	40	8.1	2.6

TBII activity more than mean + 3 SD of that in mice immunized with vector-transfected RT4.15HP (20%) was considered positive and such activity is shown in boldface type. Similarly, serum T4 was considered high when more than mean + 3 SD of that in mice immunized with vector-transfected RT4.15HP (4 μg/ml) was observed and such activity is shown in boldface type.

ratories), then analyzed by flow cytometry on a FACScan Cytometer using CELLQUEST software (Becton Dickinson).

RESULTS

When a murine MHC class II-transfected fibroblast cell line, RT 4.15HP, or its class II-untransfected control counterpart, DAP.3, was transfected with human TSHR, both expressed the receptor in a functional array, exhibiting similar TSH-increased stimulation of the cAMP signal system (Fig. 1). Flow cytometry analysis showed that hTSHR- and control vector-transfected RT 4.15HP cells expressed comparable levels of class II molecules on their cell surface, whereas hTSHR-transfected DAP.3 control cells exhibited no surface expression of class II antigen (Fig. 2). Moreover, flow cytometry analysis showed that the transfection procedures did not result in different levels of class I expression, which were similar in all cells (Fig. 2). The hTSHR-transfected RT4.15HP cells, hTSHR-transfected DAP.3 cells, or vector-transfected RT4.15HP cells were used to immunize AKR/N mice.

Table 2. Anti-TSHR antibodies and thyroid function of mice from replicate experiments immunized with TSHR-transfected RT4.15HP cells (experimental) compared to control mice immunized with RT4.15HP cells with no transfected TSHR or with DAP.3 cells with transfected TSHR but no aberrant class II expression (control)

Experiment	Positive TBII values, % in group	Elevated T4 values, % in group	Mean T4 value, μg/dl	Mean T3 value, ng/dl
Experimental, no. 1	**83**	**17**	**12.1**	**260**
Control, no. 1	0	0	2.3 ± 0.4*	60 ± 10*
Experimental, no. 2	**92**	**25**	**12.3 ± 2.6***	ND
Control, no. 2	0	0	2.3 ± 0.8*	ND
Experimental, no. 3	**80**	**20**	**13.9 ± 3.7***	ND
Control, no. 3	0	0	2.3 ± 0.9*	ND

Experiments 1, 2, and 3 were replicates in which 6, 12, or 10 mice were immunized with RT4.15HP cells transfected with the TSHR, and 10, mice were immunized with either RT4.15HP cells with no TSHR (experiments 1 and 3) or DAP.3 cells transfected with the TSHR but without expressed class II antigens on their cell surface (experiment 2). Values in boldface type represent a significant increase in the experimental animals, compared to the control group. ND, not determined.
*Values represent mean ± 2 SD.

Measurements of TBII activity showed that most mice immunized with hTSHR-transfected RT4.15HP cells, for example, 22 of 24 mice in Table 1, developed serum TBII activity. This was not true of the 16 mice in the same experiment that were immunized with vector-transfected RT4.15HP cells or with DAP.3 cells expressing hTSHR alone (Table 1). Five of 24 mice in the experiment noted in Table 1 also developed hyperthyroidism as evidenced by significantly ($P < 0.01$) elevated serum T4 levels. This was again not true of mice immunized with vector-transfected RT4.15HP cells or with DAP.3 cells expressing hTSHR alone (Table 1). Three independent experiments yielded the same results: most mice immunized with hTSHR-transfected RT4.15HP cells, but not with control cells, developed serum TBII activity and a reasonably constant proportion of mice, 17–25%, developed elevated T4 values.

Serum triiodothyronine values in mice have been suggested to be highly variable (26); nevertheless, we measured these in some experiments. When measured, serum triiodothyronine levels were significantly increased ($P < 0.01$) in parallel with the increased T4 levels. For example, triiodothyronine was 280 ng/dl in a mouse with high serum T4 levels in experiment 1 (Table 2); this was significantly higher ($P < 0.01$) than the mean level of 60 ± 10 ng/dl in normal animals or in animals who were immunized but did not exhibit increased T4 levels in the same experiment. We additionally evaluated the mean increase in T4 levels by comparison to preimmune values in one experiment (experiment 2; Table 2). T4 values in mice immunized with TSHR-transfected RT4.15HP fibroblasts were significantly increased ($P < 0.01$) in comparison to preimmune measurements in the same animals: 12.3 ± 2.6 versus 2.4 ± 1.0 (mean ± 2 SD). The development of increased thyroid hormone levels did not correlate with the development of TBII activity nor the TBII level (Table 1).

The thyroid glands of mice with high serum T4 showed marked hypertrophy (Fig. 3*A*) and exhibited thyrocyte hyper-

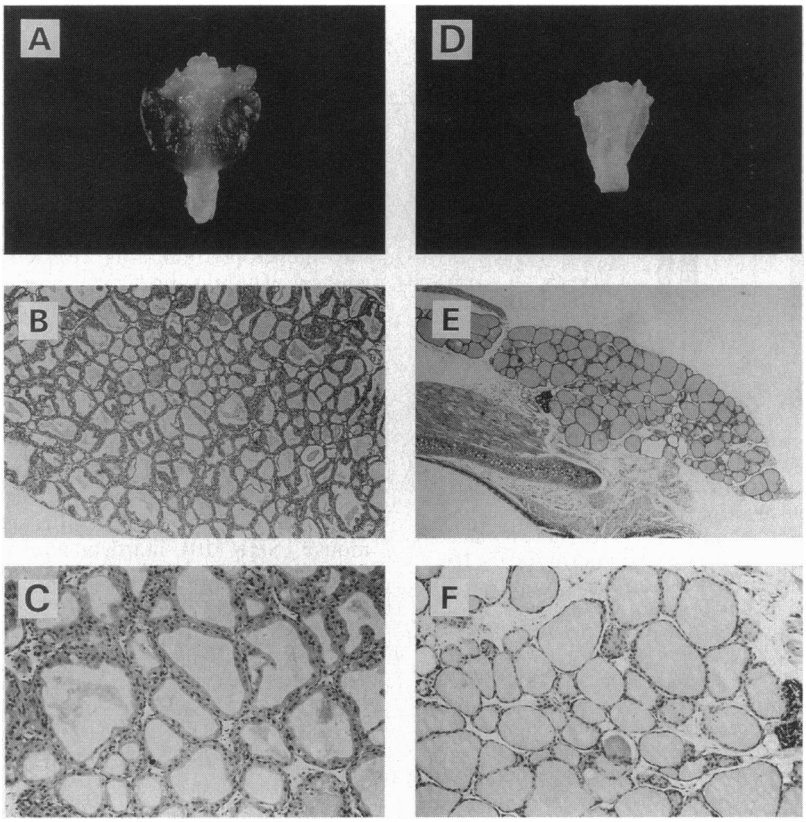

FIG. 3. Thyroids of mice immunized with hTSHR- or vector-transfected RT4.15HP cells. Representative pictures of thyroid glands are shown. (*A*) Thyroid gland of hTSHR-transfected RT4.15HP-immunized mouse no. 2 in Table 1. *B* (magnification, ×13) and *C* (magnification, ×40) show histology of the thyroid gland shown in *A*. (*D*) Thyroid gland of vector-transfected RT4.15HP-immunized mouse no. 35 in Table 1. *E* (magnification, ×13) and *F* (magnification, ×40) show histology of the thyroid gland shown in *D*. Thyroid glands were fixed in formalin for histological examination before hematoxylin and eosin staining. Note that the magnification is same for *A* and *D*, *B* and *E*, and *C* and *F*.

cellularity with intrusion into the follicular lumen (Fig. 3 *B* and *C*). There was minimal immune cell infiltration, typical of GD rather than thyroiditis (27). All mice immunized with vector-transfected RT4.15HP cells expressing a class II antigen or DAP.3 cells transfected with hTSHR showed normal thyroid gland size and morphology (Fig. 3 *D–F*). Similarly, in all hTSHR-transfected RT4.15HP-immunized mice whose thyroid hormone levels were not elevated, thyroid size and morphology were normal and no immune cell infiltrates were noted (data not shown).

Protein A-purified IgG from mice immunized with hTSHR-transfected RT4.15HP cells, who showed high serum thyroid hormone levels, had significant levels of thyroid-stimulating activity in cAMP assays, measured using CHO cells transfected with hTSHR (ref. 25; Fig. 4*A*, column c). In contrast, IgG from mice immunized with hTSHR-transfected RT4.15HP cells, whose thyroid hormone levels were not elevated (Tables 1 and 2), exhibited no thyroid-stimulating activity (Fig. 4*A*, group b), despite the presence of TBII activity in their sera (Tables 1 and 2). The development of increased thyroid hormone levels correlates, therefore, with the development of stimulating antibodies directed against the TSHR not TBII activity. IgG from mice immunized with vector-transfected RT4.15HP cells also had no thyroid-stimulating activity (Fig. 4*A*, group a).

Animals with TBII activity (Tables 1 and 2) showed inhibition of TSH-induced cAMP levels in CHO cells transfected against the TSHR (Fig. 4*B*, column b as well as a), a feature of TBIIs in Graves patients (11–13). The ability to inhibit TSH-induced increases in cAMP levels correlated with TBII rather than stimulating TSHRAb activity—i.e., TSHRAb with thyroid-stimulating and TBII activities are functionally distinct, consistent with studies of monoclonal TSHRAbs from Graves patients (28, 29).

DISCUSSION

In these studies, ≈20% of all mice immunized with fibroblasts containing the hTSHR in the context of aberrant class II expression developed features characteristic of Graves disease: stimulating TSHRAbs, increased thyroid hormone levels, TBIIs directed at the TSHR, and enlarged thyroids with thyrocyte hypercellularity and thyrocyte intrusion into the follicular lumen. The incidence is statistically significant ($P < 0.05$) in comparison to controls, and was replicated in multiple experiments (Tables 1 and 2). Most of the remaining mice developed TSHRAbs characteristic of Graves TBIIs—i.e., having the ability to inhibit TSH-increased cAMP levels; this incidence is statistically significant by comparison to the control group at $P < 0.01$. These features were not duplicated in mice immunized with control fibroblasts expressing the TSHR alone or expressing aberrant MHC class II alone.

Previous studies in which mice were immunized with the soluble extracellular domain of TSHR, either baculovirus-produced and glycosylated or prokaryotic in origin, largely failed in their intent to produce a model of Graves-disease amenable to study the pathophysiology of this disease process (1–10). Thus, even if TBII activity was detected in these studies, in most cases, the activity was not shown to reflect the existence of an antibody against the TSHR in TSHR-transfected cells (1–10). Similarly, there were no histological findings of thyrocyte hypertrophy together with increased serum thyroid hormone levels in any of these studies, only thyroiditis in some. Most important, in no case were stimulating TSHRAbs produced that could cause hyperthyroidism, thyroid enlargement, or thyrocyte hypercellularity. The present results thus show that a functional TSHR within the cell membrane, if presented to the immune system in the

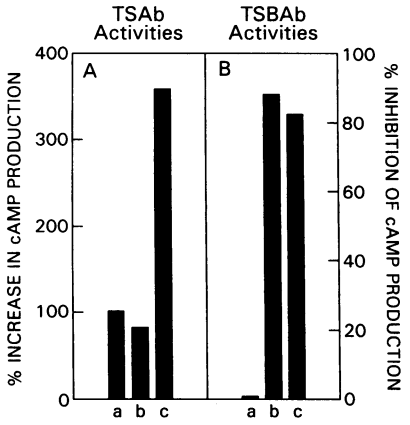

FIG. 4. Ability of IgG from immunized mice to increase cAMP levels (TSAb activity; left column) or to inhibit TSH-increased cAMP levels (thyrotropin stimulation blocking antibody: TSBAb activity; right column). Because only small amounts of serum could be obtained from individual mice, the sera from two mice were pooled and the IgG was purified on a protein A-Sepharose column. The data presented were obtained from mice an IgG pool from the following mice: (column a) mice nos. 37 and 38, vector-transfected RT4.15HP-immunized mice whose TBII and T4 levels were within normal limits; (column b) mice nos. 11 and 13, also immunized with hTSHR-transfected RT4.15HP cells but with normal serum T4 levels despite positive TBII activity; (column c) mice nos. 2 and 3, immunized with hTSHR-transfected RT4.15HP cells showing elevated serum T4 levels and positive TBII activity. Data are shown as mean of duplicate determinations. These data were duplicated by multiple different pools (>10) of IgG from comparable animals in the groups in columns a and b. Comparable positive levels of stimulating TSHRAb activity were measured in mice nos. 4 and 5 from Table 1 and in two mice with high T4 levels in each of the experiments in Table 2.

context of an aberrantly expressed MHC antigen, can induce an immune disease with major features of GD: stimulating TSHRAbs, TSHRAbs that inhibit TSH binding and activity, increased thyroid hormone levels, thyroid enlargement, and thyrocyte hypercellularity.

Increased iodide uptake is an important component of Graves disease, as are suppressed TSH levels. These measurements should be made, along with free triiodothyronine and T4 levels, basal and after disease development, in future studies that would complete the characterization of the model. Analysis of skin, hair, and pulse changes as a function of time should also be pursued. Monoclonal stimulating autoantibodies isolated from immune cells of the afflicted animals must be shown to cause the hyperthyroxinemia and thyroidal enlargement to make the model incontrovertible. Nevertheless, the present study represents an advance that should help investigators better understand the pathophysiology of Graves disease and plan future studies.

Since the immunized mice have a normal complement of T and B cells, the mechanism by which this disease develops must involve the breaking of normal immune tolerance. The mechanism by which the antigen is processed by the normal immune cells, for example, the role of costimulatory molecules in the development of this immune response must be evaluated. However, recent work (30) showed that immunization of mice with fibroblasts transfected with viral protein could induce a cytotoxic T lymphocyte response in the absence of costimulatory molecules on the immunizing fibroblasts, suggesting costimulatory signals are host derived. At this time, we cannot completely exclude the possibility that the A^d_β determinant in the shuffled A_β gene in RT4.15HP cells (23) might contribute to an enhanced immune response; however, it is a membrane proximal determinant that is not involved in antigen presentation by the I-A molecule and should not, therefore, influence immune recognition or response (23). Since immunization with the same RT4.15HP fibroblasts containing a TSHR-LH/CGR chimera without the major functional stimulating TSHRAb epitopes did not result in the development of stimulating TSHRAbs and exhibited an attenuated TBII response (N.S., Y.K., K.T., and L.D.K., unpublished data), it seems unlikely that such determinants were significant enhancers of the immune response, which allow tolerance to be broken.

There are several possible explanations why only ≈20% of mice develop stimulating TSHRAbs which caused hyperthyroidism, whereas most mice produced anti-TSHR antibodies detected by the TBII assay. First, these experiments were short-term, with a total of six immunizations, 2 weeks apart, before termination of the experiment. Longer time periods of observation may result in more animals with stimulating TSHRAbs and hyperthyroidism. Second, the low frequency of stimulating TSHRAb induction may be due to the use of human TSHR, which is a xenogeneic TSHR for mice. Anti-TSHR antibodies generated in these mice may preferentially recognize portions of the human TSHR that differ from the mouse TSHR (10). Third, an alternative explanation may lie in the mouse strain we used. H-2^s and H-2^q have been reported to be high-responder strains in terms of their levels of anti-TSHR antibodies when immunized with soluble human TSHR (5). This possibility would be consistent with the prevalence of certain HLA haplotypes (B35) in GD but not Hashimoto thyroiditis (31). Immunization of high-responder strains with the fibroblasts expressing class II plus the native form of TSHR may raise the frequency of TSHRAb-positive mice. Unfortunately, fibroblast cell lines derived from those strains are not available at present, which is one rationale for the choice of animals and fibroblasts made in this attempt to develop a Graves-like model. Fourth, background genes other than H-2, such as the immunoglobulin allotype (32), may be important for the development of stimulating TSHRAbs.

Although aberrant MHC class II expression was first reported almost a decade ago (19–21), its biological significance has never been systematically examined and shown to be a primary causative factor, rather than a factor secondary to cytokine production by invading immune cells (22). A recent report by Sopedra *et al.* (33), which showed the hyperinducibility of HLA class II expression in thyroid follicular cells from patients with GD, supports a possible role for class II molecules in the development of GD; our present data directly implicate aberrant class II expression as a potential causal factor in the development of stimulating TSHRAbs. Nevertheless, studies of 5′-flanking region cis regulatory elements of the class I and TSHR genes, together with their respective trans factors, suggest the importance of abnormal class I molecules in the expression of GD or other forms of autoimmunity (15–18) and additionally indicate there are common elements in the class I and class II molecules (18). These findings (15–18) indicate that both class I and class II molecules are important in the development of GD. Variant models of the one described herein—i.e. with overexpressed class I or aberrant II, alone or both together, may clarify their respective roles. Thus, greater levels of class II expression in the fibroblasts may increase the frequency of stimulating TSHRAb-positive mice, or increased MHC class I expression, by transfection or α-interferon treatment of the fibroblasts, might enhance the frequency of stimulating TSHRAb-positive mice, since the class I molecule has been shown to be involved in the development of autoimmune diseases (15–18).

In summary, we present the novel result showing that aberrant expression of class II molecules on a cell containing a functional TSHR results in an immune response, mediated by the normal T- and B-cell population, which mimics the major features of anti-TSHR receptor autoimmunity expressed in Graves disease. This model may provide new insight into the pathogenesis of GD from the point at which immune

tolerance is broken. The basis for the conversion of a target tissue to an antigen-presenting cell, possibly by abnormal expression of class I or class II as a result of the loss or attenuation of the activity of transcription factors normally suppressing these genes, for example, Y-box proteins, which regulate MHC class II and class I expression, is under separate investigation (18).

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