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Insight into Graves' Hyperthyroidism from Animal Models

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Graves' hyperthyroidism can be induced in mice or hamsters by novel approaches, namely injecting cells expressing the TSH receptor (TSHR) or vaccination with TSHR-DNA in plasmid or adenoviral vectors. These models provide unique insight into several aspects of Graves' disease: 1) manipulating immunity toward Th1 or Th2 cytokines enhances or suppresses hyperthyroidism in different models, perhaps reflecting human disease heterogeneity; 2) the role of TSHR cleavage and A subunit shedding in immunity leading to thyroid-stimulating antibodies (TSAbs); and 3) epitope spreading away from TSAbs and toward TSH-blocking antibodies in association with increased TSHR antibody titers (as in rare hypothyroid patients). Major developments from the models include the isolation of high-affinity monoclonal TSAbs and analysis of antigen presentation, T cells, and immune tolerance to the TSHR. Studies of inbred mouse strains emphasize the contribution of non-MHC *vs.* MHC genes, as in humans, supporting the relevance of the models to human disease. Moreover, other findings suggest that the development of Graves' disease is affected by environmental factors, including infectious pathogens, regardless of modifications in the Th1/Th2 balance. Finally, developing immunospecific forms of therapy for Graves' disease will require painstaking dissection of immune recognition and responses to the TSHR. (*Endocrine Reviews* 26: 800–832, 2005)

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Abbreviations: AchR, Acetylcholine receptor; APC, antigen-presenting cell; CFA, complete Freund's adjuvant; CHO, Chinese hamster ovary; GO, Graves' ophthalmopathy; HEK, human embryonic kidney; IDR, immunodominant region; IFN, interferon; LAMP, lysosome-associated membrane protein; LHR, LH receptor; *M. bovis* BCG, *Mycobacterium bovis Bacillus* Calmette-Guerin; MHC, major histocompatibility complex; mTSAb, monoclonal TSAb; NOD, nonobese diabetic; PTM, pretibial myxedema; SCID, severe combined immunodeficient; Stat, signal transducer and activator of transcription; TBAb, TSH-blocking antibody; TBI, TSH binding inhibition; Tg, thyroglobulin; Th1, T helper 1; TPO, thyroid peroxidase; TSAb, thyroid-stimulating antibody; TSHR, TSH receptor.

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I. Introduction

GRAVES' HYPERTHYROIDISM IS a common autoimmune disorder, affecting primarily women, with an incidence of approximately 4/10,000 per annum (1, 2). During their lifetimes, approximately 1% of the population is affected. The hyperthyroidism is directly caused by autoantibodies to the TSH receptor (TSHR) that mimic the stimulatory effects of TSH (reviewed in Refs. 3–7). None of the

currently available therapeutic options cure Graves' disease. Furthermore, the consequence of ^{131}I-induced thyroid ablation (the most widely used therapy in the United States) is frequently hypothyroidism, requiring life-long T_4 replacement in conjunction with monitoring thyroid function.

Recently, models have been developed in which a proportion of animals have some of the immunological and endocrinological hallmarks of Graves' hyperthyroidism. These models have opened the way to investigating critical issues involved in Graves' disease, as well as to exploring approaches for immune intervention in the future. Before the models are analyzed, background information is provided on the characteristics of Graves' disease and the structure of the TSHR, as well as a brief overview of the interactions between immune cells leading to production of antibodies.

A. Clinical and immunological characteristics of Graves' disease

The clinical features of Graves' disease include weight loss, hyperkinesis, tachycardia, diffuse goiter, increased levels of serum T_4 and/or T_3, and suppressed TSH. Other common manifestations are heat intolerance and anxiety and, in women, oligomenorrhea. The immunological hallmark of Graves' disease (as already mentioned) is the presence of IgG class TSHR autoantibodies [thyroid-stimulating antibodies (TSAbs)] that stimulate thyroid hormone production (reviewed in Refs. 3–7). Two assays are currently in clinical use for TSHR autoantibodies: 1) inhibition of TSH binding to the receptor [TSH binding inhibition (TBI)]; and 2) a bioassay for TSAb activity measured by a functional response [usually cAMP production by TSHR-expressing cells (thyroid cells or transfected eukaryotic cells)].

In addition to TSHR autoantibodies, approximately 75% of Graves' patients have autoantibodies to thyroid peroxidase (TPO) (8) and, depending on the assay, 25–55% have autoantibodies to thyroglobulin (Tg) (9). These autoantibodies are more prevalent in Hashimoto's thyroiditis (reviewed in Ref. 10) and, at least for TPO autoantibodies, reflect the underlying thyroid lymphocytic infiltration (*e.g.*, Refs. 11 and 12). Consistent with TPO and Tg autoantibodies, thyroid inflammation in Graves' disease, indicated by infiltrating T and B lymphocytes and plasma cells, is much less extensive than in Hashimoto's disease (reviewed in Ref. 4). Although recent data confirm the dominant role of T cells rather than antibodies in mediating thyroid destruction and hypothyroidism (13), TPO (and to a lesser extent Tg) autoantibodies remain excellent clinical markers of this process. The typical coexistence of low level thyroiditis with Graves' hyperthyroidism indicates that thyroid stimulation by TSHR autoantibodies overcomes any thyroid damage associated with thyroid inflammation. In a minority of patients, TSHR autoantibodies that block the stimulatory effects of TSH [TSH blocking antibodies (TBAbs)] give rise to hypothyroidism (*e.g.*, Ref. 14).

B. Extrathyroidal manifestations of Graves' disease

Graves' ophthalmopathy (GO) develops in 25–50% of Graves' patients with symptoms including conjunctival injection, chemosis, proptosis, and diplopia (reviewed in Refs. 15 and 16). Although fortunately mild in most cases, in severe cases of GO sight loss may occur consequent to corneal ulceration or optic nerve compression. A subset of Graves' patients (1–4%), almost always with concomitant GO, develop skin induration. Dermopathy typically affects the pretibial areas [pretibial myxedema (PTM)] and, even more rarely, thickening of the distal phalanges of the hand (acropachy) (reviewed in Refs. 16 and 17). Both GO and PTM are characterized by lymphocytic infiltration of the target tissues, activation of fibroblasts/preadipocytes, glycosaminoglycan accumulation, expansion of fat and, in the orbit, thickening of the extraocular muscles.

The etiology of these distressing extrathyroidal conditions is gradually being elucidated. Because of their association with Graves' disease, GO and PTM have long been considered to develop as a consequence of autoimmunity to cross-reacting thyroid and orbital autoantigens. Candidate autoantigens included Tg and novel muscle proteins G2 s and D1 (reviewed in Ref. 16). However, increasing evidence supports a role for the TSHR: 1) TSHR antibody levels correlate with clinical GO (18); 2) the rare occurrence of GO and PTM in patients with autoimmune hypothyroidism has been associated with extremely high TSHR antibody levels, probably with TBAb activity (19); and 3) multiple studies (many ongoing) regarding expression and function of the TSHR in Graves' and normal orbital and dermal tissues (*e.g.*, Refs. 20–25)

Against this background, it seems increasingly likely that TSHR-specific T cells are recruited to the orbit (or skin) and activated to secrete cytokines that induce preadipocyte differentiation, fibroblast proliferation, and glycosaminoglycan production (reviewed in Refs. 15 and 16). Additional local factors, such as dependency, skin trauma or pressure, cigarette smoking, and the anatomical constraints of the bony orbit, play important roles in the manifestations of PTM (26) and GO (reviewed in Refs. 15 and 16). Animal models provide the opportunity to test the role of the TSHR as the major autoantigen in these diseases. Moreover, because of limited available therapeutic options, such models would be invaluable for developing novel immunospecific therapies for GO and PTM.

C. Structural features of the TSHR

The TSHR is a member of the G protein-coupled receptor family with seven transmembrane regions. Like the related gonadotropin (LH/choriogonadotropin and FSH) receptors, the TSHR has a large N-terminal ectodomain (397 amino acid residues). However, the TSHR differs from the gonadotropin receptors in that some of the single-chain polypeptide expressed on the cell surface undergoes intramolecular cleavage to form two subunits (A and B) linked by disulfide bonds (Fig. 1, *left panel*) (27, 28). Remarkably, the process involves the removal of a looped segment between the A and B subunits (C peptide region; approximately amino acid residues 317–366) (29, 30). The C peptide region is not removed as an intact fragment but by a process of progressive degradation, starting at the upstream cleavage site and continuing downstream (30, 31). Moreover, the cleaved receptor is susceptible to loss of the A subunit by shedding (Fig. 1, *right panel*), at least *in vitro*. Suggested mechanisms for shedding include

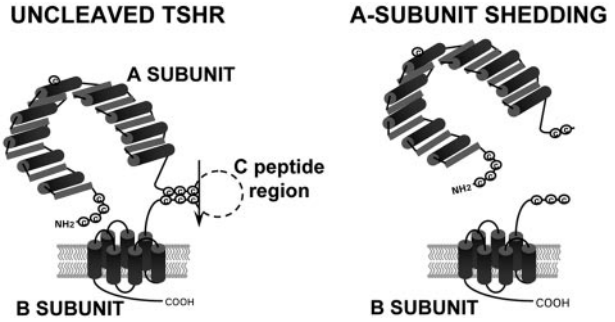

FIG. 1. TSHR structure and A subunit shedding. *Left panel*, The uncleaved TSHR with its large ectodomain, seven-transmembrane region, and short cytoplasmic tail. The horseshoe-shaped leucine-rich repeat region was modeled from the three-dimensional structure of the ribonuclease A inhibitor (280). After expression on the cell surface, the receptor cleaves into A and B subunits that are tethered by disulfide bonds (27, 28). In the cleavage process, a C-peptide region is excised (*arrow*) (29, 30). *Right panel*, The A subunit is shed if the disulfide bonds are broken (281) or if the cleavage process continues downstream to the membrane (33). [Adapted with permission from C.-R. Chen *et al.*: *J Clin Invest* 111:1897–1904, 2003 (97). © The American Society for Clinical Investigation.]

reduction of disulfide bonds linking the A and B subunits (32) as well as continued proteolytic erosion at the N terminus of the B subunit (33). In addition to receptor shedding, another important feature of the TSHR A-subunit is its high degree of glycosylation, about 45% of its mass (34, 35).

D. Interactions between immune cells leading to antibody production

Production of TSHR autoantibodies by B lymphocytes, as for most antibodies of the IgG class, requires help from T lymphocytes of the CD4+ subset. This process involves interactions between B cells, T cells, and antigen-presenting cells (APCs). There is a major difference in the way that T and B cells recognize antigen (Fig. 2A). Most antibodies to protein antigens recognize conformational epitopes comprising nonlinear amino acid segments that are contiguous with each other in the folded protein. In contrast, T cells interact with antigen that has been degraded into short linear peptides. Cells that present antigen to activate T cells, macrophages, and dendritic cells express major histocompatibility complex (MHC) molecules of class II. APCs internalize antigens (particles or proteins) by phagocytosis or pinocytosis. After internalization, the antigens are processed by proteolysis to produce peptides, 16–20 amino acids long, which bind to the MHC class II groove for presentation to T cells.

However, the engagement of a T cell with a peptide/MHC complex on an APC is insufficient to trigger T cell activation. The dialogue requires interactions between other receptor/counter receptor pairs of costimulatory molecules (Fig. 2B), some of which are not expressed on resting APCs or T cells (reviewed in Ref. 36). Briefly, unprimed APCs express MHC and CD40; naive T cells express the T cell receptor complex and CD28. Peptide presentation by APCs to a T cell (signal 1) up-regulates CD40 ligand (CD40-L) expression on T cells. The interaction between CD40-L (T cell) and CD40 (APC) induces expression of B7 molecules (B7–1/B7–2) on the APC. Binding of B7 with CD28 (T cell) delivers signal 2. CD28 also shares its B7 ligands with CTLA-4 (usually associated with down-regulation). The combination of signal 1 and signal 2 leads to T cell activation. Immune responses can be blocked by inhibition of second signal using antibodies or soluble receptors, or by the absence of costimulatory molecules in knockout mice (reviewed in Ref. 37). Other costimulatory molecules, such as inducible costimulator (ICOS) and its ligand, are involved in T cell activation (38). The interaction between T cells and APCs, termed the "immunological synapse," determines the outcome (magnitude and nature) of the T cell response (reviewed in Ref. 39).

Infectious organisms induce APCs to secrete proinflammatory cytokines that potentiate antigen uptake, processing, and presentation to T cells. T cells respond by producing IL-2, a growth factor required for T cell survival and proliferation. Multiple cytokines are involved in T and B cell maturation and differentiation. Interferon (IFN)γ and IL-12, on the one hand, and IL-4, on the other hand, have mutually exclusive effects on the development of two major T cell subsets, T helper 1 (Th1) and T helper 2 (Th2) (40). Th1 and Th2 cytokines were initially considered to be exclusively associated with cellular and humoral immune responses, respectively. However, cytokines from both subsets are involved in antibody production and are responsible for switching between subclasses of IgG subclass secreted by B cells. For example,

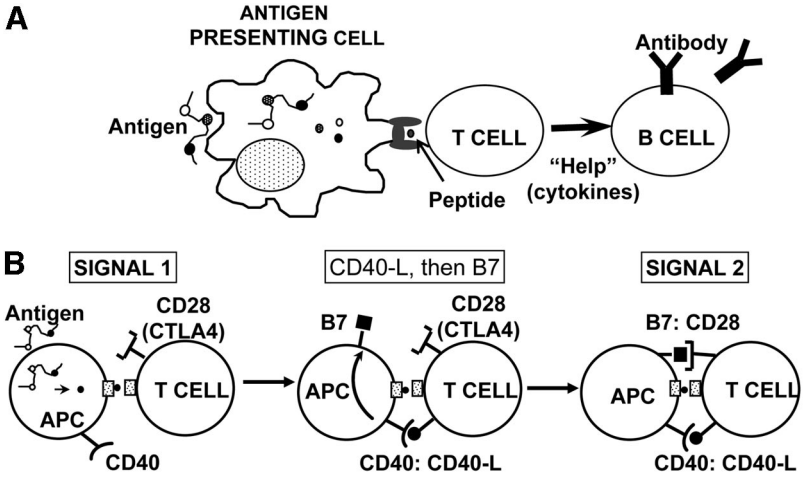

FIG. 2. Interactions between immune cells involved in antibody production. A, Protein antigen is taken up by an APC (macrophage or dendritic cell), processed to produce peptides that bind to MHC for presentation to T cells. In turn, T cells provide help (cytokines) to permit B cells specific for the same antigen to differentiate into plasma cells secreting antibody. B, Costimulatory molecules involved in T cell activation (reviewed in Ref. 36). Unprimed APCs express MHC and CD40; naive T cells express the T cell receptor complex and CD28. Peptide presentation by APCs to a T cell (signal 1) up-regulates expression of CD40 ligand (CD40-L) on T cells. Binding of CD40-L (T cell) to CD40 (APC) induces APC expression of B7. The interaction between B7 and CD28 delivers signal 2. CD28 also shares its B7 ligands with CTLA-4, and this interaction is usually associated with down-regulation of the response (36).

in humans, the Th1 cytokine IFNγ is associated with secretion of IgG1, whereas the Th2 cytokine IL-4 is required for production of IgG4 and IgE (reviewed in Ref. 41).

II. Perspective on Other Models of Thyroid Autoimmunity

Animals that develop diseases resembling those in humans have provided a major impetus to studies of human autoimmunity. Thyroiditis develops spontaneously in Obese Strain chickens, BioBreeding (BB) rats, and a substrain of nonobese diabetic (NOD) mice, and these models are valuable for understanding Hashimoto's disease (reviewed in Refs. 42 and 43). However, TSHR autoantibodies that stimulate the thyroid and cause hyperthyroidism arise spontaneously only in humans.

The lack of a spontaneous Graves' disease model can be appreciated when considering the insights NOD mice have provided into the process leading to type I diabetes (reviewed in Ref. 44). Nevertheless, there are discrepancies between autoimmune diabetes in humans and NOD mice. For example, unlike the lack of a gender difference in humans, type I diabetes is more prevalent in female than male NOD mice. Moreover, whereas numerous approaches prevent and cure diabetes in NOD mice, reversing islet inflammation and destruction is extremely difficult in humans. Consequently, for understanding disease pathogenesis and for developing novel immunotherapies in Graves' hyperthyroidism, appropriate animal models should exhibit the distinctive endocrinological and immunological features of spontaneous disease in humans.

A. Induction of thyroiditis

The conventional approach for inducing autoimmunity involves immunizing animals with protein (antigen) and adjuvant. The classic example is thyroiditis induced in rabbits by injecting rabbit thyroid extracts together with complete Freund's adjuvant (CFA) (45). Studies of mice immunized with human or murine Tg demonstrated a role for MHC antigens and cytotoxic T cells (but not antibodies) in the development of thyroiditis (reviewed in Ref. 46). Conventional immunization with TPO induces thyroiditis in some mouse strains (47), particularly using murine TPO (48). However, unlike spontaneous thyroiditis in chickens (reviewed in Ref. 49), hypothyroidism does not develop in induced thyroiditis models. Recently, transgenic mice were generated that express the T cell receptor genes of a TPO-specific human T cell clone; these mice spontaneously develop severe thyroiditis leading to hypothyroidism and weight gain (13).

B. Conventional immunization with TSHR protein and adjuvant

After the cloning of the TSHR, numerous attempts were made to induce Graves' hyperthyroidism by conventional approaches. Rabbits and mice immunized with human TSHR expressed in bacteria and/or in insect cells developed antibodies that reacted with receptor preparations (*e.g.*, Refs. 50–53). Serum antibodies and murine monoclonal antibodies from these immunized animals provided invaluable reagents for immunohistochemistry, Western blotting, and immunoprecipitation. Nevertheless, despite using different TSHR preparations, a variety of mouse strains and different adjuvants, none of these approaches induced antibodies with TSAb activity as in Graves' patients (summarized in Table 1). Because of the possibility that the human TSHR may be unsuitable for inducing stimulatory autoantibodies and hyperthyroidism in mice, the murine TSHR ectodomain was cloned and expressed in insect cells (54, 55). Again, even when using purified murine TSHR ectodomain and adjuvant, conventional immunization failed to induce Graves'-like disease in mice.

C. Engrafting human tissues in mice lacking an intact immune system

Mice with defective immune cells, nude mice, and severe combined immunodeficient (SCID) mice have been xenografted with human tissues to investigate several aspects of thyroid autoimmunity (reviewed in Ref. 56). Nude (athymic) mice accept human thyroid explants, but the hyperfunctioning characteristics, which are maintained in toxic adenoma, are lost in Graves' explants. These characteristics

TABLE 1. Conventional immunization of mice with TSHR protein and adjuvant before 1996

Antigen	MHC (H2)	TBI (% inhibition)[a]	TSAb	Serum T_3/T_4	Thyroid histology	Ref.
TSHR ectodomain	d	35 ± 16%	ND	Slight increase	No infiltrate	146, 283
	s	33 ± 13% (8% in controls)	ND		No infiltrate	
GEJ-TSHR cells (detergent solubilized)	s d, k q, b	~20%	ND	Transient, variable changes	Infiltrates (strain and sex dependent)	284
TSHR ectodomain (bacterial)	d	~50%	Absent	Low T_4	Thyroiditis	52, 285, 286
	g	Absent	Absent	Low T_4	Thyroiditis	
	k, b	Absent	Absent	T_4 unchanged	No change	
TSHR ectodomain (insect cell)	s	~40%	ND	T_4 unchanged	No infiltrate	287
	d	~20%	ND	T_4 unchanged	No infiltrate	
TSHR 43-282*	d	ND	ND	T_4 variable	Thyroiditis	288
TSHR-43-366*	d	ND	ND	T_4 variable	No thyroiditis	

Thyroid antibodies with TBI activity were induced by some protocols, but stimulating activity (TSAb), when measured, was absent. Serum thyroid hormone concentrations were variable (unchanged, slightly increased, or reduced), and thyroid lymphocytic infiltrates were induced in some mouse strains. ND, Not determined.

[a] TBI values are shown as the mean ± SEM % inhibition of TSH binding (when available) or as the average TBI value.

can be restored to Graves' tissue by injecting Graves' IgG (containing TSAb activity) (57) or, in the short term, by injecting lymphocytes from Graves' patients (*e.g.*, Ref. 58). The question of whether aberrant HLA-DR expression in autoimmune thyroid tissue (59) is primary or secondary has been explored by injecting cytokines into nude mouse recipients of Graves' *vs.* normal thyroid xenografts (60).

Because SCID mice lack mature T and B cells, both human tissue xenografts and infiltrating lymphocytes survive in these recipients (reviewed in Refs. 56 and 61). Autoantibodies to Tg and TPO develop in SCID mice engrafted with Graves' or Hashimoto blood lymphocytes, thyroid lymphocytes, or thyroid tissue (62–66). Pitfalls with SCID mouse models include the loss of thyroid autoantibodies 8–10 wk after engraftment, probably because of a rapid decline in T cell function (61), as well as variability between individual animals (62, 67). Nevertheless, SCID mice opened the way to studying several aspects of thyroid autoimmunity such as lymphocyte homing (68), regulatory (CD8+) cells (69), and T cell characterization in thyroid organoids (70).

Some mice xenografted with Graves' thyroid tissue developed TSAb activity and transient hyperthyroxinemia (66). Moreover, mice that received TSHR-specific T cell lines together with thyroid grafts developed TSAb activity, and the thyroid grafts increased in size, but serum T_3 levels were unchanged (71). Simultaneous xenotransplantation of Graves' thyroid tissue and autologous bone marrow cells induced higher TSAb titers and elevated T_4 levels (72). However, wider application of these approaches is restricted by the need to develop T cell lines before surgery, MHC matching of T cell lines and grafts (71), and the difficulty of obtaining bone marrow cells.

III. Novel Approaches to Induce Graves' Disease

Over the last 10 yr, new approaches have been developed to induce many of the features of Graves' disease in animals. The unifying characteristic of all models (with one exception) is stimulation of the immune system by *in vivo* expression of the TSHR. Animals are injected with transfected cells stably expressing the TSHR or, alternatively, with a plasmid or adenovirus for transient *in vivo* TSHR expression. Another shared feature is the need for multiple (two to six) injections of cells or DNA (plasmid or adenovirus). The characteristics of these models, as well as their advantages and limitations, are described below.

A. Cells stably expressing the TSHR

Graves' disease models using this approach have used different cell types (Fig. 3).

1. "Shimojo" model (transfected fibroblasts). The first animal model of Graves' disease was induced by injecting AKR/N mice on six occasions with fibroblasts stably transfected with MHC class II molecules (RT4.15HP cells) as well as the cDNA for human TSHR (73). Two weeks after the final injection, 90% of female AKR/N mice developed TSHR antibodies with TBI activity. Moreover, 25% of these mice became thyrotoxic, with elevated serum T_4 and T_3 levels as well as

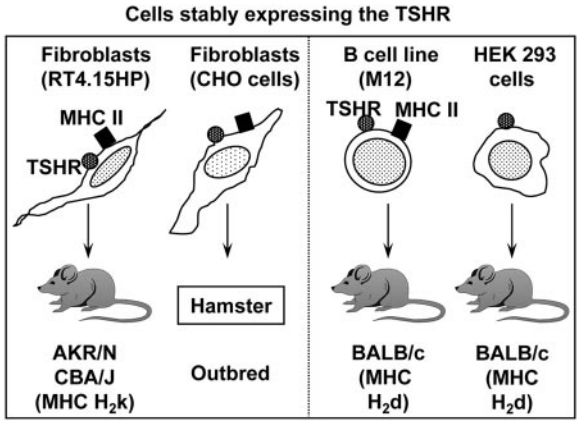

FIG. 3. Inducing Graves' disease in animals using MHC class II positive cells stably expressing the TSHR:RT4.15HP fibroblasts in AKR/N or other strains with MHC class II IA-k (73, 74); B cells (M12 cells) or HEK293 cells in BALB/c mice (79); and CHO cells in hamsters (78).

detectable TSAb activity (73). In contrast, injecting cells expressing the TSHR without MHC class II did not elicit TSAb responses (73, 74). Hyperthyroid mice had marked goiters with thyrocyte hypertrophy but no lymphocytic infiltration. The Shimojo model has been reproduced and extended by other groups (75–77). In particular, the inclusion of adjuvant in the immunization protocol modulated the induction of TSHR antibodies and disease; CFA reduced, whereas alum and pertussis toxin enhanced, these modalities (75). No gender bias was observed in this model (75, 76). An important aspect to the Shimojo approach is that it is restricted to H2-k mouse strains (AKR/N and H-2k congenics) (74) because the fibroblasts were transfected with this MHC class II molecule (73).

2. Hamster Shimojo model. Outbred hamsters repeatedly injected with TSHR-expressing Chinese hamster ovary (CHO) cells together with the adjuvants alum and pertussis toxin developed TSHR antibodies including TBI activity. Moreover, 30% of animals had elevated T_4 levels and goiters as well as thyroid lymphocytic infiltration (78). Of interest, mRNA for MHC class II was detectable in the CHO cells by PCR. Unlike inbred mice, however, the hamsters resemble human populations in being genetically different from one another. Consequently, it is possible that MHC differences between individual hamsters and the injected TSHR-expressing CHO cell line play a role in stimulating allogeneic responses.

3. B cells and human embryonic kidney cells. Murine B cells (M12 cells) stably expressing the TSH holoreceptor (human or mouse) were injected on multiple occasions into BALB/c mice, which have the same MHC (H2-d) as the M12 line (79). In a second approach, mice of the same strain were injected with xenogeneic human embryonic kidney (HEK) 293 cells expressing the TSHR ectodomain, alone or together with soluble TSHR ectodomain protein and a Th2 adjuvant, cholera toxin B. In both approaches, mice had TSHR antibodies detectable by ELISA after 1 month and TBI activity after 4 months. TSAbs, hyperthyroidism, and goiter developed by 5–6 months, followed later by focal necrosis and thyroid

lymphocytic inflammation. Human TSHR and mouse TSHR were equally effective at inducing disease. However, as discussed later, the thyroid histology in these models (79) does not correspond to that in Graves' disease. Using TSHR-expressing B cells or HEK293 cells, disease incidence was first reported to be 100% (79). However, in a subsequent study, only 50% of mice became hyperthyroid using TSHR-HEK293 cells (80). Of interest, immunization with TSHR-ectodomain protein and cholera toxin B was equally effective (79). These findings represent the sole report of Graves' disease induction not involving *in vivo* expression of the TSHR.

4. Insight into the Shimojo approach. The success of the Shimojo model for inducing TSAbs poses an intriguing question: How does the immune response differ when the antigen is presented as a cell-associated molecule *vs.* a soluble, purified protein? This question was addressed for TPO because of the availability of native human TPO and a panel of human monoclonal TPO autoantibodies that define the immunodominant region (IDR) recognized by patients' autoantibodies (81).

The qualitative nature of induced antibodies was assessed by comparison of AKR/N mice injected with fibroblasts coexpressing TPO and MHC class II with mice immunized with purified TPO and adjuvant. Only TPO-fibroblast-injected mice developed antibodies that resembled human TPO autoantibodies in terms of their high affinity [dissociation constant (K_d) $\sim 10^{-10}$ M] and restricted epitopic recognition of the TPO IDR (82). To date, no similar comparison has been made for TSHR antibodies, namely animals injected with TSHR and MHC class II-positive fibroblasts *vs.* the same mouse strain immunized with TSHR protein plus adjuvant. Nevertheless, these findings for TPO suggest that TSHR antibodies generated by the Shimojo protocol may have restricted epitopes and higher affinities compared with TSHR antibodies induced by conventional immunization that do not induce Graves'-like hyperthyroidism.

B. Transient TSHR expression

In addition to injection of stably transfected cells, there has been much recent interest in inducing Graves' hyperthyroidism by immunization of plasmid or adenovirus vectors with transient *in vivo* TSHR expression (Fig. 4).

1. "Naked" DNA vaccination of BALB/c mice. Injecting a plasmid im induces transient expression of the encoded protein by myoblasts or inflammatory cells at the injection site (83). In the first report using this approach, three injections of DNA encoding the human TSHR induced TSHR antibodies (detected by TBI and binding to TSHR-expressing cells) in virtually all female BALB/c mice (84). TSAb activity was detectable in the serum from some animals, but no mice were hyperthyroid. In addition, vaccinated mice developed thyroid lymphocytic infiltrates characterized by B cells and IL-4 producing T cells (84).

Subsequently, three groups were unable to reproduce these findings: TSHR antibody levels were low and thyroiditis was not observed in female BALB/c mice vaccinated im with TSHR-DNA (77, 85, 86). The lack of antibody responses and thyroiditis could be explained by one or more of the following factors: different immunization protocols (single *vs.* multiple immunization sites, with or without cardiotoxin pretreatment); subtle differences between substrains bred separately for many years (BALB/cJ *vs.* BALB/cAnCrlBR); and differences in animal housing (conventional *vs.* specific pathogen free). A recent study emphasizes that the same protocol used to vaccinate BALB/cAnCrlBR mice had variable outcomes in animals maintained in different conventional housing units (presumably exposed to different microorganisms) and fed different diets. Thus, genetic immunization in Brussels induced thyroiditis and orbital changes but no TSAbs (87), whereas the same TSHR-DNA vaccination approach in Cardiff induced TSHR antibodies and TSAbs but no thyroiditis (88).

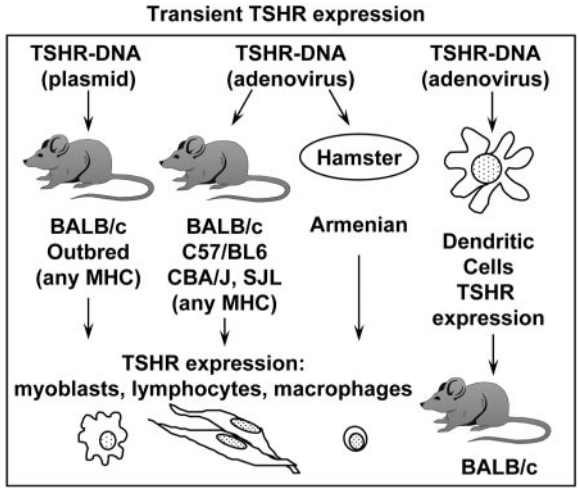

FIG. 4. Graves' disease induced by transient TSHR expression: TSHR-DNA in a plasmid vector in outbred mice (91); in an adenovirus vector in BALB/c mice (86); or by injecting dendritic cells infected with TSHR-adenovirus in BALB/c mice (100).

Modified DNA vaccination protocols induce TSHR antibodies and, to a lesser extent, hyperthyroidism in female BALB/c mice. Intradermal, rather than im, injection of TSHR plasmid DNA induced TSHR antibodies in 70% of mice, and 30% had elevated T_4 levels (89). Moreover, as described in more detail later (*Section V*), by diverting plasmid TSHR expression to the lysosome, the majority of BALB/c mice developed TSHR antibodies and about 25% became hyperthyroid (90).

2. DNA vaccination of other strains. In outbred mice housed in a conventional facility, 25% of females developed TSAbs, hyperthyroidism, and goiter 2–4 wk after the third TSHR-DNA vaccination (91). Among 30 male mice, only one developed subclinical hyperthyroidism, and one was hypothyroid. As in BALB/c mice (see above), thyroid lymphocytic infiltrates (that were phenotyped) developed in genetically immunized outbred mice (91).

In addition to outbred mice, im genetic immunization induces TSHR antibodies in other non-BALB/c strains. C57BL/6 mice maintained in pathogen-free conditions developed TSHR antibodies (but not hyperthyroidism) after vaccination with TSHR-DNA (92). Of particular interest are studies performed in mice lacking murine MHC class II and, instead, expressing the allele HLA-DR3 (DRB1*0301) asso-

ciated with Graves' disease in Caucasians (reviewed in Ref. 93). Some HLA-DR3 transgenic mice on a mixed background (50% C57BL/10 and 50% CBA, C57BL/6 and 129) developed TSHR antibodies, but all were euthyroid (94). However, TSHR-DNA vaccination induced TSHR antibodies and hyperthyroidism in 30% of HLA-DR3 transgenics on the NOD background (95). These observations, together with those described for BALB/c mice above, indicate the importance of genetic and environmental factors in the outcome of TSHR-DNA vaccination.

3. Adenovirus encoding the TSHR. Intramuscular injection of a replication-deficient adenovirus vector encoding the human TSHR-cDNA is an efficient approach for inducing Graves'-like hyperthyroidism in mice. In the original report, after three injections of TSHR-adenovirus, most BALB/c mice developed TSHR antibodies detectable by TBI, and 50% had TSAb activity and became thyrotoxic (86). In contrast, only 25% of C57BL/6 mice became hyperthyroid and DBA/1J, DBA/2J, CBA/J, and SJL/J mice remained euthyroid (86, 96). Importantly, as determined using chimeric TSH-LH receptors for detection, TSHR antibodies in BALB/c mice recognized epitopes similar to autoantibodies in Graves' sera. Thyroid glands from hyperthyroid animals were hyperplastic, but there was no lymphocytic infiltration. This adenovirus model has been applied to hamsters (78). Moreover, unlike the marked interlaboratory variability of TSHR-DNA vaccination, the TSHR-adenovirus approach has been confirmed in two laboratories (97, 98). Finally, as will be described later (*Section IV*), the TSHR-adenovirus model has been modified and optimized (97, 99).

4. Dendritic cells transfected with TSHR-adenovirus. Dendritic cells are the most potent APCs and are a prerequisite for the initiation of immune responses. Instead of injecting the adenovirus im, dendritic cells were isolated from mouse bone marrow, expanded using the cytokines IL-4 and granulocyte macrophage colony stimulating factor, and then infected with TSHR-adenovirus. After two to three injections of TSHR-expressing dendritic cells, 70% of BALB/c mice produced TSHR antibodies detectable by TBI, and 35% had TSAb activity, elevated serum T_4, and goiter (100). Again, no thyroid lymphocytic infiltration was detected. This immunization protocol demonstrated the efficacy of dendritic cell presentation of TSHR to naive T cells.

C. Mice transgenic for a monoclonal TSAb (mTSAb)

Two human monoclonal antibodies (B6B7 and 101–2) isolated by Epstein-Barr virus transformation of Graves' peripheral blood lymphocytes had weak TSAb activity when used at high concentrations (23 μg IgG/ml of B6B7) (101). The Ig genes encoding the heavy and light variable regions of these antibodies were determined (102), expressed in eukaryotic vectors (101), and their epitopes have been analyzed (103).

Recently, transgenic mice were generated with the variable region genes of antibody B6B7 expressed together with the constant region of human IgM (104). Hyperthyroidism developed in 68% of these transgenic mice, as reflected in elevated serum levels of T_4 and reduced TSH, as well as increased thyroid uptake of technetium pertechnetate. Thyroid tissue in these mice was hyperplastic but lymphocytic infiltration was absent. T_4 levels correlated positively with the level of human IgM in serum, demonstrating that hyperthyroidism was determined by the TSAb concentration. It was assumed that the isotype change (IgG to IgM) would not influence the antibody activity. However, the IgM pentamer of B6B7 likely converts the low-affinity monomeric parent IgG to a high-avidity antibody that, at high concentrations, induces hyperthyroidism. These transgenic mice provide a valuable model for studying B cell tolerance to the TSHR (*Section VI*).

D. Models of Graves' ophthalmopathy

A model of GO was developed involving the adoptive transfer of splenocytes from immunized mice (87). This protocol was based on the previous induction of thyroiditis in naive BALB/c mice by transfer of splenocytes (activated *in vitro* with antigen) from BALB/c mice immunized conventionally with TSHR plus fusion protein (105). Building on the hypothesis that GO is caused by TSHR-specific T cells, BALB/c and NOD mice were genetically immunized with TSHR-DNA or (as before) with a TSHR fusion protein and adjuvant. Splenocytes *ex vivo* were cultured with the TSHR-fusion protein and injected into unimmunized recipients. These studies confirmed that thyroiditis could be transferred to both mouse strains. Moreover, orbital pathology comparable to that in humans, developed in BALB/c (but not in NOD) mice. These orbital changes included accumulation of adipose tissue, edema, dissociation of muscle fibers, TSHR immunoreactivity, and infiltration by lymphocytes and mast cells (87). Similar findings were reported in BALB/c and outbred (CD-1) mice genetically immunized against the TSHR or the eye muscle protein G2 s (106). In contrast to these studies, no extraocular muscle abnormalities have been detected in other models of Graves' hyperthyroidism, *e.g.*, the TSHR-adenovirus model (86).

The splenocyte adoptive-transfer model appeared to open the way to further studies of GO. However, despite using the same protocol, these findings have not been reproduced in the same BALB/c substrain in a different location (Cardiff *vs.* Brussels) (88). Unexpectedly, no thyroiditis or orbital changes were observed, even using the same water, bedding, and food obtained from Brussels. Moreover, misleading artifacts were noted in extraocular muscles as well as misinterpretation of ectopic thymus as thyroid lymphocytic infiltration (88). These findings emphasize the difficulty of reproducing models in conventional (non-pathogen-free) housing.

An unexpected observation was made in transgenic mice overexpressing adiponectin, a protein secreted by adipocytes. Transgenic animals were generated to investigate the long-term effects of elevated adiponectin on insulin sensitivity. Remodeling of fat depots in older mice (12 months) led to selective enlargement of the interscapular and orbital fat pads. Proliferation of orbital fat pushed the orbit away from its bony structure and created marked proptosis, keratopathy, and, ultimately, corneal ulceration. Individual orbital muscles were separated by the expansion of adipose tissue.

Despite the potential implications for GO, orbital tissue in these animals had no lymphocytic infiltration, and TSHR antibodies were undetectable in multiple assays (binding to TSHR-expressing cells, TBI and TSAbs) (107). Although not a definitive model of autoimmune ophthalmopathy, these transgenic mice provide the opportunity to study noninflammatory orbital fat proliferation that may be a component of GO in humans.

E. Overview of mouse models for Graves' hyperthyroidism

1. Summary. In 1952, Rose and Witebsky (108) suggested criteria for establishing the autoimmune etiology of an autoimmune disease. The presence of autoantibodies to a defined antigen (the TSHR) have long been recognized to fulfill two of the Rose-Witebsky postulates, and direct proof was provided by transplacental transfer of TSHR antibodies leading to neonatal Graves' disease (*e.g.*, Refs. 109 and 110). However, the final hurdle to the Rose-Witebsky criteria, namely that "an analogous autoimmune response be identified in an experimental animal" (111), was only overcome in 1996 when the Shimojo model of Graves' hyperthyroidism (73) was described.

Since that time, a number of protocols have been tested to induce Graves' disease in animals (summarized in Table 2). These studies have generated highly diverse observations, ranging from the detection of TSAbs without alteration in thyroid hormone levels to TSAbs associated with increased serum T_4 and T_3 levels with reciprocal suppression of TSH. Essential clinical and immunological features for a useful Graves' disease model are thyrotoxicosis, goiter, and TSAbs. Moreover, as in human disease, chronically overstimulated thyroid epithelial cells are cuboidal or columnar with increased intracellular vacuolation. These findings, consistent with high secretory activity, contrast with flattened thyroid epithelium in euthyroid animals (Fig. 5, B *vs.* A). Of note, in the approach using TSHR-expressing B cells or HEK293 cells, thyroid histology involves "hypertrophy and enlargement of colloid with thinning of the thyroid epithelium" (79), an appearance inconsistent with other mouse models of Graves' disease, as well as with human Graves' disease.

2. Thyroiditis. In contrast to the clear-cut clinical features of goiter and hyperthyroidism, the extent of thyroiditis varies markedly among different models of Graves' disease, depending on the immunization protocol and mouse strain used. Moreover, even within the same strain and using the same approach, in different laboratories thyroiditis does or does not develop.

Observations can be categorized into three groups. 1) Thyroid lymphocytic infiltrates are absent in mice made hyperthyroid using the Shimojo approach (73, 75), following intradermal vaccination with TSHR-DNA (89), injection of TSHR-adenovirus-transfected dendritic cells (100), and im injection of TSHR-adenovirus (86, 97). 2) Thyroiditis without hyperthyroidism has been reported in TSHR-DNA-vaccinated BALB/c mice in one study (84) [but not in three other studies (77, 88, 112)], as well as in HLA-DR3 transgenic mice on a mixed C57BL/10 background (94) (Fig. 5, panel D *vs.* panel C). 3) Both thyroiditis and hyperthyroidism are reported after im TSHR-DNA vaccination of outbred mice (91) and HLA-DR3 transgenics on the NOD background (95), as well as after injection of TSHR-expressing B cells or HEK293 cells in BALB/c mice (79). It should be noted that assessing thyroid lymphocytic infiltration in mice is complicated by the frequent occurrence of ectopic thymus, and the proximity of parathyroid glands, which may be mistaken for dense lymphocytic infiltrates (113). Indeed, as noted above, a recent

TABLE 2. Approaches for inducing Graves' disease in animals

Approach	Strain	TSAb	% Hyperthyroid	Thyroid function[a]	Goiter	Thyrocyte hypertrophy	Infiltrate	Ref.
Cells stably expressing the TSHR								
Fibroblasts (RT)	AKR/N	Yes	25	T_4, T_3	Yes	Yes	No	75, 75
Fibroblasts (CHO)	Hamster	Yes		T_4	Yes	Yes	Yes	78
B cells (M12)	BALB/c	Yes	~100	T_4, T_3	Yes	Yes	Yes	79
HEK293	BALB/c	Yes	~100	T_4, T_3	Yes	Yes	Yes	79
TSHR-DNA vaccination								
im	Outbred	Yes	17	T_4, TSH	Yes	Yes	Yes	91
im	BALB/c	Yes	0	No change	No	No	Yes	84
im	BALB/c	No	0	No change	No	No	No	77, 86, 112
im	BALB/c	Yes	20	T_4			No	88
Intradermal	BALB/c	Yes	27	T_4	Yes	Yes	No	89
LAMP-TSHR im	BALB/c	Yes	20	T_4	Yes	Yes	No	90
TSHR-adenovirus								
im WT TSHR	BALB/c	Yes	50	T_4	Yes	Yes	No	86
	C57BL6		25	T_4	Yes	Yes	No	
im A subunit	BALB/c	Yes	70	T_4	Yes	Yes	No	97, 99
	C57BL6	Yes	5	T_4	Yes	Yes	No	262
Infected dendritic cells	BALB/c	Yes	35	T_4	Yes	Yes	No	100
Use of mTSAbs								
Transgenic for B6B7 genes	C57BL6	Yes	68	T_4 TSH	Yes	Yes	No	104
MS-1 (acute)	CBA/J	Yes	100	T_4		Yes	No	177
MS-1 (chronic)	Nude	Yes	Variable	T_4, T_3, TSH		Yes	No	177
IRI-SAb3 IgG	NMRI	Yes	100	T_4, TSH		Yes	Yes	161

WT, Wild type.
[a] T_4 and/or T_3 elevated; TSH suppressed.

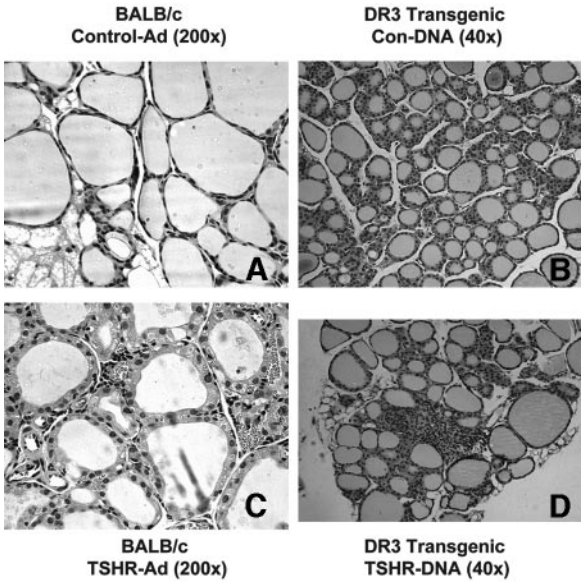

FIG. 5. Thyroid histology in murine Graves' disease. A and B, Normal thyroid *vs.* hyperthyroid tissue in BALB/c mice immunized with control-adenovirus (control-Ad, panel A) and TSHR-adenovirus (TSHR-Ad, panel B). No lymphocytic infiltrates were present in control- or TSHR-adenovirus-immunized mice. C and D, Small thyroid lymphocytic infiltrates in HLA-DR3 transgenic mice (mixed C57BL/6 background) vaccinated with TSHR-DNA (D) are not present in transgenic littermates vaccinated with control-DNA (Con DNA, panel C). [Panels A and B reproduced with permission from C.-R. Chen *et al.*: *J Clin Invest* 111:1897–1904, 2003 (97). © The American Society for Clinical Investigation. Panels C and D reproduced with permission from P. Pichurin *et al.*: *Thyroid* 13:911–917, 2003 (94). © Mary Ann Liebert, Inc.]

study points to possible misidentification of thyroid lymphocytic infiltrates in some previous reports (88).

In Graves' disease, focal thyroiditis is often present, but lymphocytic infiltration is less extensive than in Hashimoto's thyroiditis (114, 115). As discussed above, autoantibodies to other thyroid antigens, particularly TPO, are also present in many Graves' patients. An unanswered question, therefore, is whether thyroiditis in human Graves' disease is more closely associated with TPO (and perhaps Tg) autoantibodies than with autoimmunity to the TSHR. The clinical syndrome of Graves' disease is dependent on a humoral immune response and not thyroid lymphocytic infiltration. Transient neonatal Graves' disease arises from transplacental transfer of maternal TSAbs to the fetus (*e.g.*, Ref. 109) without the requirement for lymphocytic involvement in the thyroid. Also, in thyroiditis-prone NOD.H-2h4 mice, TSHR-adenovirus immunization induced TSHR antibodies and hyperthyroidism without enhancing thyroiditis or autoantibodies to Tg (116). These data support the possibility that the variable thyroiditis component in Graves' patients involves autoimmunity to TPO (and perhaps also to Tg) rather than to the TSHR. On the other hand, a cell-mediated immune response to the TSHR may occur at a later stage of the disease process. Such a late event would be compatible with the long time course required for development of thyroiditis, TSAbs, and hyperthyroidism in the B cell/HEK293 cell model for Graves' disease (79).

3. Advantages and disadvantages of different mouse models. The Shimojo approach provides an *in vivo* model with many features of Graves' disease, including TSHR antibodies with TSAb activity and elevated T_4 in 25% of mice (73, 75, 76), TSAb epitopes like those in Graves' patients (117), and a role for genetic factors other than MHC class II (74) (see below). One limitation is restriction to a particular MHC (H2-k). More important, because RT4.15HP fibroblasts constitutively express a costimulatory molecule (B7–1; Fig. 2B), they induce a potent, nonspecific activation of antibodies, T cells, and cytokine production (118). These responses preclude detailed *in vitro* dissection of the cellular immune mechanisms in these animals. On the positive side, however, TSAbs and hyperthyroidism develop within approximately 3 months, a shorter time frame than the 6 months required for Graves' disease induction with TSHR-expressing B cells (79).

The reason for the greater efficacy of TSHR-expressing B cells *vs.* TSHR-expressing fibroblasts (incidence of hyperthyroidism ~100% *vs.* 25%, respectively) is not known but may relate to different mouse strains, BALB/c *vs.* AKR/N, respectively. Both B cells and fibroblasts express MHC class II as well as the B-7 molecule required for T cell activation (79, 118). However, other molecules present on B cells (but not fibroblasts) may provide additional activation signals for T cells. Because of the differences between human and mouse MHC, it is not clear why injecting mice with TSHR-expressing human HEK293 cells is as effective as injecting TSHR-expressing murine B cells for inducing Graves' hyperthyroidism. Of interest, these studies with HEK293 cells showed that the TSHR ectodomain alone, without the serpentine region of the receptor, is sufficient to induce TSHR antibodies resembling those in Graves' disease (79).

Intramuscular vaccination with TSHR-DNA in a plasmid is less effective than injecting TSHR-expressing fibroblasts or B cells for inducing TSHR antibodies and hyperthyroidism, at least in the inbred BALB/c strain. The problems associated with TSHR-DNA vaccination include lack of reproducibility in different laboratories (Ref. 84 *vs.* Refs. 77, 85, 86, and 88) and the likely effects of conventional *vs.* pathogen-free housing facilities. However, DNA vaccination has greater power than the Shimojo model for studying a variety of mouse strains and, as described later (*Section VI*), permits *in vitro* analysis of memory T cell responses to the TSHR (85).

TSHR-adenovirus immunization combines the advantages of naked DNA vaccination without the disadvantages of the Shimojo approach. Mice of any strain can be immunized with TSHR-adenovirus to investigate induction of TSHR antibodies and hyperthyroidism. Moreover, the induction of TSHR antibodies, goiter, hyperthyroidism, and thyroid hyperplasia in BALB/c mice has been reproduced in three different laboratories (86, 97, 98). Also, unlike the Shimojo approach, splenocytes from TSHR-adenovirus-immunized mice can be used to analyze memory T cell responses (119) (*Section VI*).

IV. TSHR Structure and Antibody Epitopes

Animal models of Graves' disease have advanced our understanding of the relationship between TSHR structure

and the pathogenesis of Graves' disease, as well as the method by which TSAbs activate the TSHR to cause hyperthyroidism.

A. TSHR shedding and induction of Graves' disease

As mentioned above, some TSHRs on the cell surface undergo intramolecular cleavage to form disulfide-linked A and B subunits, a process that may be followed by A subunit shedding (Fig. 1, *right panel*). In addition, the epitope for TSAbs in Graves' disease is partially obscured in the wild-type TSHR but is exposed on the same TSHR ectodomain tethered to the cell surface by a glycosylphosphatidylinositol anchor (120). These observations raised the possibility that the shed A subunit, rather than the full-length, cell surface TSHR, initiates or enhances immune responses to the TSHR that lead to hyperthyroidism.

This hypothesis was tested in the TSHR-adenovirus Graves' model utilizing two different forms of the TSHR. If correct, immunization with the free A subunit, but not a TSHR engineered to prevent cleavage and A subunit shedding (Fig. 6A), should preferentially generate TSAbs with consequent hyperthyroidism. Indeed, this hypothesis was confirmed. BALB/c mice immunized with A subunit adenovirus, unlike animals injected with noncleaving TSHR-adenovirus, developed elevated T_4 levels (Fig. 6B) (97). Remarkably, however, TBI levels were not significantly different between the two groups (Fig. 6C). On the other hand, TSAb levels were significantly higher, and TBAb activities were significantly lower, in A subunit adenovirus-injected mice than in animals immunized with adenovirus expressing the noncleaving TSHR (Fig. 6, D and E). These findings were confirmed recently: adenovirus expressing the TSHR A subunit was superior to adenovirus encoding the holoreceptor in inducing hyperthyroidism in BALB/c mice (98).

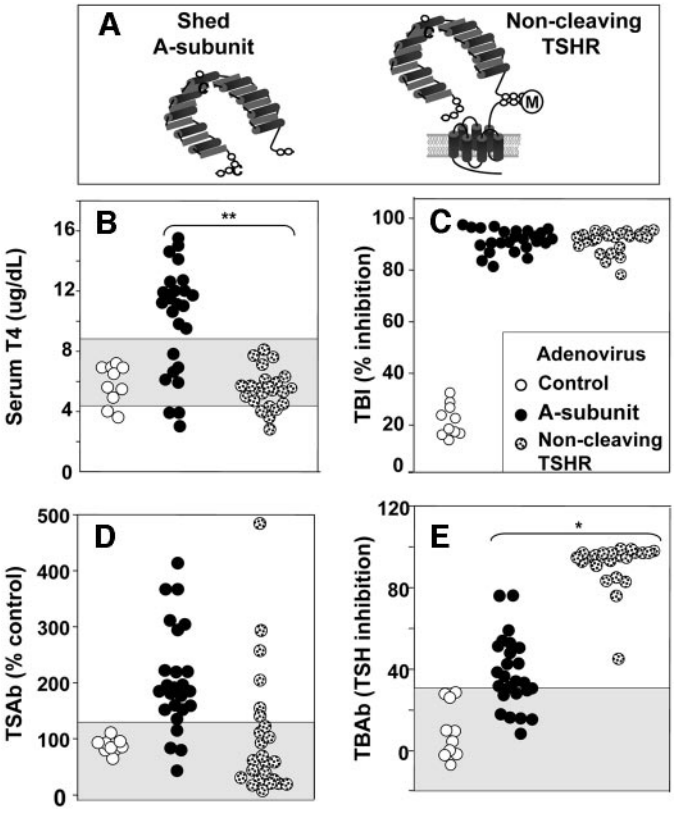

FIG. 6. Role of A subunit shedding in the induction of TSAb. Adenoviruses were constructed encoding the A subunit and a mutated noncleaving TSHR (A). After three immunizations with adenovirus for the A subunit, the noncleaving TSHR, or control adenovirus, sera from BALB/c mice were studied for serum T_4 (B), TBI (C), TSAb (D), and TBAb (E). [Derived from data in Ref. 97.]

The membrane-bound TSHR is (of course) required for thyroid stimulation by TSAb. If the TSAb-binding site is partially obscured on the full-length TSHR, how does the antibody activate the receptor and cause hyperthyroidism? Possible explanations include a flexible or 'plastic' interaction between an antibody and its epitope (121). Such plasticity in the case of a TSAb, the epitope of which is only partially accessible, could exert an allosteric, torsional effect on the TSHR ectodomain, thereby activating the receptor. Alternatively, partial steric hindrance to TSAb binding could involve direct impingement by the IgG molecule on a critical segment in the TSHR ectodomain or extracellular loops (120). Regardless of the precise mechanism involved in TSHR stimulation by TSAbs, observations in the TSHR-adenovirus model of Graves' disease (97) support the hypothesis that the shed A subunit plays an important role as the form of autoantigen responsible for initiation or amplification of the immune response leading to Graves' hyperthyroidism.

B. Antibody titer and TSAbs vs. TBAbs

Other than TSAbs, autoantibodies that activate rather than inhibit antigen function are a rare phenomenon, no doubt requiring a highly specific epitope(s). The relatively common occurrence of TSAbs may also be related to the susceptibility of the TSHR to be activated by mutations (largely within the membrane-spanning regions) (122). TSAb titers in Graves' patients' sera are typically very low (34, 123–126). Moreover, the functional balance between TSAb and TBAb activities is known to be related to antibody titer (109). The relationship between TSAb epitope(s) and titer is therefore a highly relevant issue.

1. Genetic immunization with TPO. Comparing TSHR and TPO antibodies induced by different immunization protocols is potentially informative for the pathogenesis of Graves' disease because of the frequent concurrence of TPO antibodies in this condition. BALB/c mice were studied for antibodies induced by injecting TPO-plasmid, TPO-adenovirus, or dendritic cells infected with TPO-adenovirus (127). TPO antibody levels were highest after TPO-adenovirus immunization, intermediate after TPO-transfected dendritic cells transfer, and lowest by TPO-plasmid vaccination (Fig. 7A).

In humans, TPO autoantibody epitopes are restricted to a small facet on the surface of the antigen, the TPO IDR (81). The high titer TPO antibodies induced using adenovirus interacted predominantly with non-IDR epitopes, whereas low-titer antibodies induced by DNA-plasmid recognized epitopes largely restricted to the IDR. The inverse relationship between antibody titer and IDR restriction is likely due

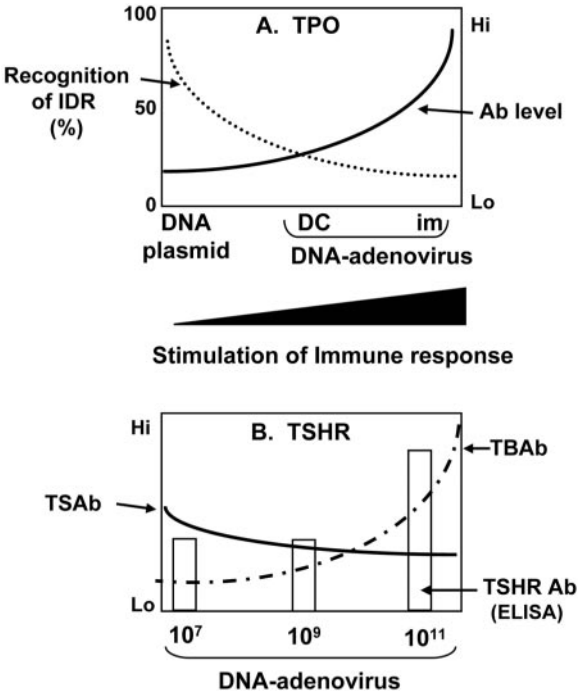

FIG. 7. Antibody epitope spreading in relation to immune stimulation. A, TPO antibody epitopes in BALB/c mice are lowest after vaccination with TPO-DNA in a plasmid, intermediate in mice injected twice with TPO-adenovirus-injected dendritic cells (DC), and highest in mice immunized three times with TPO-adenovirus. Increasing levels of TPO antibodies (Ab) are associated with decreased recognition of IDR epitopes, indicating epitope spreading. B, TSHR antibodies measured by ELISA increase as BALB/c mice are immunized with increasing doses of TSHR A subunit adenovirus. TSAb levels fall with increasing TSHR Abs in ELISA and, conversely, TBAb levels rise (99). [Panel A adapted with permission from J. Guo *et al.*: *Clin Exp Immunol* 132:408–415, 2003 (127). © Blackwell Publishing.]

to epitope spreading induced by the strong antigenic stimulation of the TPO-adenovirus vector. Remarkably, TPO antibody epitope spreading does not occur in Hashimoto's thyroiditis, despite high autoantibody levels (128, 129). In humans, TPO autoantibody titers likely rise gradually over time, and persistent, low-level antigenic stimulation does not change the spectrum of antibody epitopes.

2. Shimojo model. The dynamics of TSHR antibodies have been studied in the Shimojo model of Graves' disease. AKR/N mice injected with fibroblasts, coexpressing the TSHR and MHC class II, developed TSHR antibodies by 7–8 wk. The majority of individual animals had either TSAbs or TBAbs, and these patterns were maintained throughout the 17–24 wk of the study (130). In a small proportion of mice, TSAbs and TBAbs appeared to cycle over time.

3. Low-dose TSHR-adenovirus. Immunization with TSHR A subunit-adenovirus induces hyperthyroidism in 65–85% of BALB/c mice (97) (Fig. 6B) and is more effective than adenovirus expressing the full-length TSHR (50% of mice) (86). Nevertheless, antibodies in these mice have higher TBAb levels than most Graves' patients. A possible explanation for this difference between the Graves' mouse model and human disease was a greater degree of antigenic stimulation in the model after immunization with a high dose of TSHR-adenovirus. To address this possibility, BALB/c mice immunized with the standard high (10^{11}) viral particle dose were compared with animals receiving injections of medium (10^9) and low (10^7) doses of viral particles. Not surprisingly, mice receiving lower viral doses generated lower TSHR antibody titers on ELISA as well as (more importantly) lower TBAb activity (Fig. 7B). Remarkably, however, and consistent with the hypothesis, TSAb levels and the incidence of hyperthyroidism (~80%) remained comparable regardless of viral dose. Thus, low-dose TSHR A subunit adenovirus immunization provides an induced model with a high prevalence of hyperthyroidism and a TSHR antibody profile more closely resembling autoantibodies in human Graves' disease.

Together, the studies on TPO and TSHR provide important insight into human disease. Higher TPO antibody levels are accompanied by decreased recognition of IDR epitopes (Fig. 7A). Similarly, increasing TSHR antibody levels diverts the balance away from TSAbs and toward TBAbs (Fig. 7B). Despite different endpoints, these observations indicate that increased immune stimulation leads to higher antibody titers and epitopic spreading. Unlike the very low concentrations of TSHR antibodies in Graves' disease (34, 123–126), patients with hypothyroidism caused by TBAbs have much higher TSHR antibody titers (*e.g.*, Refs. 120 and 131) as well as epitopic differences (described below).

C. TSHR antibody epitopes in polyclonal sera

1. Human autoantibodies. Patients' TSHR autoantibodies are polyclonal, although commonly restricted in terms of IgG subclass and light chain type (132, 133). There are several reasons for the difficulty of identifying the precise TSHR amino acids involved in the epitopes of these functional autoantibodies. First, TSH and TSHR autoantibody epitopes comprise discontinuous sequences of the polypeptide chain that are contiguous in the folded protein under native conditions (134, 135). Second, autoantibodies preferentially recognize the glycosylated TSHR (136, 137), although it is likely that the glycan component is not part of their epitopes. Rather, complex glycan is acquired after correct TSHR folding and trafficking to the cell surface. Incidentally, a complication for some assays is nonspecific serum IgG binding to the heavily glycosylated A subunit (138). A third reason, mentioned above, is the extremely low serum concentration of TSHR autoantibodies (34, 123–126), typically nanograms per milliliter (139) compared with micrograms per milliliter of TPO autoantibodies.

Two approaches have been used to analyze the epitopes of TSHR autoantibodies in humans (Fig. 8A), namely synthetic peptides (typically 20 amino acids long) encompassing the receptor ectodomain (residues 22–417) and chimeric receptors with sections of the TSHR replaced by homologous regions of the LH receptor (LHR). In studies from different laboratories, Graves' sera bound to different peptides without consistent recognition of particular linear epitopes (reviewed in Ref. 5). In contrast, investigations using chimeric receptors expressed in eukaryotic cells provided more reproducible insight into the binding sites of TSHR autoantibodies (Fig. 8B). In general, despite some overlap, TSAbs interact mainly with N-terminal components of the ectodo-

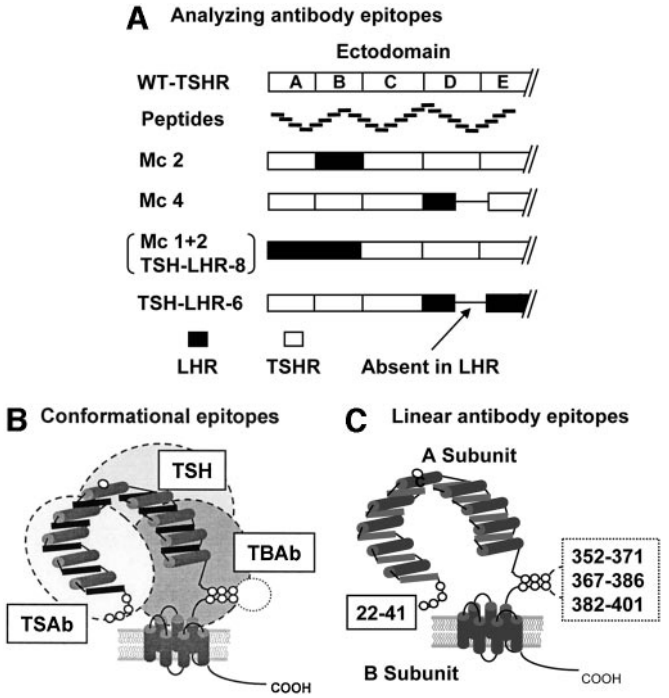

FIG. 8. Epitopes recognized by TSHR autoantibodies and animal models of Graves' disease. A, Approaches for analyzing TSHR antibody epitopes. A panel of 26 overlapping TSHR synthetic peptides (20-mers) encompasses the TSHR ectodomain (282). Chimeric TSH-LHRs previously generated to characterize human autoantibodies and used to study immunized mice: ectodomain segments A through D are in *white* for the TSHR and in *black* for the homologous LHR regions. Mc 1 + 2 (140) and TSH-LHR-8 (134) are similar but not identical. B, Overview of conformational epitopes recognized by TSAbs, TSH, and TBAbs in humans (based on Ref. 135 and 140). C, Linear antibody epitopes recognized by immunized animals. Peptide 22–41 is the immunodominant epitope in TSHR-DNA- and TSHR-adenovirus-vaccinated mice (92) and animals immunized with TSHR-protein and adjuvant (144–147). Peptides overlapping the TSHR cleavage region and C-terminal region of the ectodomain (amino acids 352–401) are recognized by TSHR-fibroblast-immunized mice (Shimojo model) (75), TSHR-adenovirus-immunized hamsters (78) and, to a moderate extent, by TSHR-adenovirus-immunized mice (92). WT, Wild type. [Panel C reproduced with permission from L. Schwarz-Lauer *et al.*: *Endocrinology* 144:1718–1725, 2003 (92). © The Endocrine Society.].

main (*e.g.*, Refs. 135 and 140). Moreover, the TSHR A subunit neutralizes TSAb activity in all sera tested (141) and can be used to affinity-enrich the TSAb component from Graves' sera (138). Chimeric receptor studies indicated that TBAbs interacted primarily with C-terminal components of the TSHR ectodomain (*e.g.*, Refs. 135 and 140). However, unlike TSAbs, the epitopic range of some TBAbs can be much broader, also extending into the TSHR N-terminal region (141). Thus, TBAb activity in some patients' sera can be adsorbed, at least in part, by the purified A subunit. The TSH binding site is primarily composed of components in the leucine-rich repeats in the midregion of the TSHR (142, 143). Nevertheless, there is epitopic overlap between TSH and TSAbs or TBAbs (Fig. 8B). For this reason, and taking into account the larger size of an antibody (150 kDa) *vs.* TSH (~30 kDa) and the TSHR ectodomain (~60 kDa), it is not surprising that both TSAbs and TBAbs are measured by the TBI assay.

2. Sera from animals immunized to induce Graves'-like hyperthyroidism. Unlike the data for human TSHR autoantibodies, linear antibody epitopes have been readily identified in TSHR-immunized animals using panels of synthetic TSHR peptides. In immunized mice and hamsters, serum antibodies bound to two regions of the TSHR ectodomain: the extreme amino terminus (residues 22–41 immediately downstream of the deleted signal peptide) and residues 352–401, which include part of the deleted C peptide region (Fig. 8C). The N-terminal cysteine-rich peptide is the immunodominant epitope in mice vaccinated with TSHR-DNA or TSHR-adenovirus (92), as well as in rabbits and mice conventionally immunized with TSHR-protein and adjuvant (144–147). Moreover, the folding of this N-terminal peptide is crucial for recognition by human TSHR autoantibodies (35, 92, 148). In other Graves' models, serum antibodies interact primarily with peptides in the vicinity of the TSHR cleavage region; such epitope recognition occurs with TSHR-fibroblast-immunized mice (Shimojo model) (75), TSHR-adenovirus-immunized hamsters (149), and (although less well recognized than N-terminal peptide 22–41) TSHR-adenovirus-immunized mice (92). It is of interest that immunodominant epitopes of antibodies to other antigens are located at the amino terminus (150, 151) and the carboxy terminus (152). Incidentally, peptide 97–116 recognition by some Shimojo mice (75) is likely to be nonspecific because of similar observations in sera of mice injected with control fibroblasts or vaccinated with control DNA or control adenovirus (92). The likely relationship between linear epitope recognition and TSAb activity in these sera could only be determined subsequent to mTSAb isolation (see below).

Conformational TSHR antibody epitopes have been analyzed in two of these mouse models. In the Shimojo model, mice were injected with fibroblasts expressing chimeric TSH-LHRs (Mc 1 + 2, Mc 2 and Mc 4) (Fig. 8A) previously used to characterize TSHR autoantibodies in Graves' patients (140). Injection of fibroblasts expressing chimeric receptors with N-terminal substitutions Mc 1 + 2 (residues 9–165) or Mc 2 (residues 90–165) could not induce TSHR antibodies or hyperthyoidism. Fibroblasts expressing Mc 4 (substitution of C-terminal residues 262–370) did induce TBI antibodies, but information was not provided regarding hyperthyroidism (117).

In mice immunized with TSHR-adenovirus, TSAb activity was analyzed using different chimeric receptors, TSHR-LHR-6 and TSH-LHR-8 (the latter being similar to Mc 1 + 2) (Fig. 8A). With TSHR-LHR-6, the N-terminal region of the TSHR (residues 1–260) remains intact. This chimeric receptor responded to TSAbs in the mouse sera (86). In contrast, TSH-LHR-8 (N-terminal residues 1–160 replaced) was unresponsive to TSAbs. These findings in both mouse models are consistent with data from human autoantibodies and reinforce the importance of the TSHR N-terminal region for recognition by TSAbs in Graves' patients (Fig. 8B).

D. mTSAbs from immunized animals

1. Isolating mTSAbs. Monoclonal antibodies were initially generated by fusing a B cell line (such as a myeloma) with splenic lymphocytes from immunized mice (153). The tech-

nique has been standardized and reproduced for many antigens including the autoantigens Tg (154) and TPO (155). Many TSHR monoclonal antibodies have been generated that are invaluable for immunoprecipitation and Western blotting (*e.g.*, Refs. 156 and 157). However, the successful isolation of functional mTSAbs was only possible after novel approaches had been developed to induce Graves' hyperthyroidism in animals.

Even with these models, isolating mTSAbs has been extremely difficult and only recently accomplished (158–161). In addition to dogged persistence, the problems were overcome in two ways (reviewed in Ref. 162): first, by selecting donor animals with very high TSHR antibody (TBI) activity (158, 160) and/or hyperthyroidism (159, 161); and second, by screening for TBI, TSAb, or binding to TSHR-expressing cells rather than by ELISA (which may preclude detecting functional antibodies). mTSAbs have been isolated from TSHR-DNA-vaccinated mice (158, 160, 161) or TSHR-adenovirus-immunized hamsters (159). Moreover, despite the greater difficulties in isolating human monoclonal antibodies, an extremely potent human mTSAb has been cloned from a Graves' patient (163).

2. Stimulating activities of mTSAbs. mTSAbs can be compared based on the IgG concentration required to stimulate cAMP generation by TSHR expressing eukaryotic cells in the sensitive NaCl-free system (Table 3). Low concentrations (~200 ng/ml IgG) of some mouse or hamster mTSAbs increased basal cAMP levels 2- to 3-fold (158–160). One murine mTSAb (IRI-SAb3) is as potent as the single human monoclonal TSAb (hTSmAb1) (163), requiring only 10 ng/ml to induce maximal stimulation comparable to TSH (161). Fab prepared from mTSAbs were as active as their parent mouse IgGs (158, 161).

As mentioned earlier (*Section III*), two human monoclonal antibodies (B6B7 and 101–2) isolated from Graves' lymphocytes weakly increase cAMP levels (103). Compared with the data for the recently isolated mouse, hamster, and human TSAbs, both B6B7 and 101–2 required extremely high IgG concentrations (>16,000 ng/ml) for maximal cAMP stimulation (Table 3).

3. TBI, TBAbs, and affinities. In addition to TSAb, all mTSAbs reported to date (except IRI-SAb1) had TBI activity (161). Although some TSAbs, such as MS-1, can inhibit TSH-induced cAMP stimulation (159), this observation does not imply clinically significant TSH blocking activity, a rare cause of hypothyroidism. As discussed for TSAbs (141, 164), an elementary pharmacological principle is that a weak (or partial) agonist is also an antagonist. Therefore, it is not possible to conclude that a monoclonal antibody or polyclonal serum (*e.g.*, Ref. 165) contains functionally significant TBAb activity unless TSAb activity is absent.

Before monoclonal antibodies were available, it was difficult to evaluate TSAb affinities for the TSHR. That low concentrations (nanograms to micrograms) of TSHR (A subunits or TSHR ectodomain) neutralized TBI (34), and TSAb (125, 141) activities in patients' sera suggested a high affinity of TSHR autoantibodies for the TSHR. However, it has now been shown unequivocally that, like the single human mTSAb (163), mTSAbs from immunized animals (TSHR-DNA or TSHR-adenovirus) can be high-affinity antibodies with dissociation constants in the nanomolar or lower range (158, 160, 161, 166).

4. mTSAb epitopes. Murine mTSAbs do not bind to nonglycosylated ^{35}S-labeled TSHR transcribed *in vitro* (163). These data do not necessarily imply that TSAb epitopes contain glycan; for reasons described previously, abnormal polypeptide folding is a more likely explanation. Furthermore, unlike nonstimulating monoclonal antibodies, the epitopes of which have been identified by peptide scanning (*e.g.*, Refs. 149, 156, and 157), mTSAbs do not recognize linear epitopes (158, 159, 161). These data indicate that serum antibody components in immunized mice or hamsters that bind to TSHR peptides (Fig. 8C) are unlikely to have TSAb activity.

Competition assays can test the relationship between the epitope of a monoclonal antibody and those of polyclonal serum antibodies. Importantly, human sera containing either TSAb or TBAb activity inhibited the binding of murine mTSAbs to the receptor, namely TSMAb 1–7 (158, 167), IRI-SAb2 and 3 (but not IRI-SAb1) (161), and mTBAbs 28.1, A9, and 31.7 (168). Consequently, these murine mTSAbs and TBAbs overlap with epitopes of most, if not all, spontaneously arising TSHR autoantibodies in human disease. Similar overlap between TSAb-, TBAb-, and

TABLE 3. mTSAbs from immunized animals and Graves' patients

Species	Name	TSAb[a]		TBI	Affinity (K_d)	Graves' sera competition	Ref.
		2–3 × basal	Maximum				
Mouse	TSmAb1	200 ng/ml	2,000 ng/ml	Yes		Yes	158
	TSmAb2	200 ng/ml	20,000 ng/ml	Yes	1.4×10^{-10} M	Yes	
	TSmAb3	20,000 ng/ml	20,000 ng/ml	Yes	4.0×10^{-10} M	Yes	
Mouse	IRI-SAb1		~3,000 ng/ml	No	2.0×10^{-8} M	No	160, 161
	IRI-SAb2		100 ng/ml	Yes	7.0×10^{-11} M	Yes	161
	IRI-SAb3		10 ng/ml	Yes	2.8×10^{-10} M	Yes	161
Hamster	MS-1	~200 ng/ml	5,000 ng/ml	Yes	4.0×10^{-10} M	ND	159, 166
Human	hTSmAb1	0.3 ng/ml	10 ng/ml	Yes	2.0×10^{-11} M	Yes	163
Human	B6B7	30,000 ng/ml	30,000 ng/ml	No		ND	101
	101-2	16,000 ng/ml	16,000 ng/ml				

ND, Not determined.
[a] NaCl-free buffer.

TSH-binding sites was observed using affinity-purified autoantibodies (126). These recent data obtained with monoclonal antibodies help correct a misperception prevalent for many years that TSAb and TBAb epitopes are *restricted* to the N terminus and C terminus of the TSHR ectodomain, respectively. However, this new information is consistent with previous data obtained using TSH-LHR chimeric receptors that TSAbs, TBAbs, and TSH have overlapping but nonidentical binding sites (135).

Epitopic components for three mTSAbs have been characterized using a variety of TSHRs (including chimeric receptors). MS-1 (derived from a hamster) recognizes a receptor lacking the unique 50-amino acid segment (residues 317–366) (159) as well as a chimeric receptor (TSH-LHR-6; Fig. 8A), which retains only TSHR amino acids 1–260 in the extracellular domain (166). Thus most, or all, of the MS-1 epitope lies within the TSHR A subunit (149, 166). Selective recognition by MS-1 of the active conformation of the TSHR A subunit (35, 166) further localizes a component of the MS-1 epitope to the cysteine-rich N terminus of the TSHR. Of note, MS-1 reacts less well than serum TSAb with the purified A subunit despite similar recognition of the holoreceptor by MS-1 and TSAb autoantibodies (166). This phenomenon supports previous observations (169, 170) that not all antibodies with thyroid-stimulating activity have identical epitopes, a conclusion confirmed using other mTSAbs (161) (see below).

The most detailed epitope mapping has recently been reported for three murine mTSAbs, IRI-SAb-1, -2, and -3 (161). As is well known, polyclonal human serum autoantibodies cross-react with TSHRs from other species including guinea pigs (171), mice (172), and rats (173, 174). Indeed, with one exception (79), all approaches for inducing Graves' disease in animals involve the human TSHR. However, cross-reactivity of human autoantibodies with the TSHR of other species does not necessarily imply the converse. In particular, mTSAb IRI-SAb-1 generated against the human receptor does not bind to rat, cat, or dog TSHR. Studies with human/rat chimeric TSHR excluded the epitope of this antibody from the inner surface of the horseshoe domain of the TSHR. On the other hand, IRI-SAb2 and SAb3 recognize TSHR from other species. Using an extensive panel of TSH-LHR chimeras, the amino acids involved in the epitopes of IRI-SAb2 and SAb3 were mapped to the amino-terminal region of the concave portion of the horseshoe-shaped TSHR ectodomain (161). However, there is no information concerning the interaction of these mTSAbs with the N terminus of the TSHR, a region crucial for TSHR recognition by human serum TSAb autoantibodies (35, 92, 148). Very recently, a model for the TSHR was described (175), and the structure of the related FSHR (or its A subunit equivalent) bound to FSH was determined (176). This information is likely to provide the basis for future mutagenesis studies analyzing the interaction of mTSAbs with the N terminus of the TSHR.

E. Injecting mTSAbs into mice

In addition to studies *in vitro*, the stimulating effects of three mTSAbs have been tested *in vivo*. CBA/J mice were injected ip with increasing concentrations (0.5−10 μg) of hamster-derived MS-1 IgG. The maximum dose induced thyrocyte hyperplasia and elevated serum T_4 levels that peaked after 24 h (177). To study the chronic effect of a mTSAb, mice were injected ip with MS-1 secreting hybridoma cells. Immune responses to hamster IgG were precluded by using athymic nude mice. Unexpectedly, compared with pretreatment controls, T_4 levels were not increased in these mice 2 wk after MS-1 cell injection. Surprisingly, however, there was an inverse relationship between MS-1 serum levels and hyperthyroidism (elevated T_3 levels and thyrocyte hypertrophy). Mice with the highest serum levels of MS-1 were not thyrotoxic.

Based on these findings, the authors suggested that TSAb down-regulation and/or desensitization of the TSHR accounted for the frequent disparity between TSAb levels and the degree of hyperthyroidism in Graves' disease. In our view, interpretation of these data is difficult. The mice were pretreated with pristane before receiving MS-1 hybridoma cells (177), a procedure often performed to enhance IgG levels in ascites. Mice pretreated in the same way but injected with a non-TSAb hamster hybridoma were not studied as controls. Therefore, a role for MS-1 *in vivo* cannot be established in mice suffering from pristane pretreatment combined with the progressively adverse effects of an expanding peritoneal tumor.

A different study does not support the concept of TSAb desensitization abrogating its functional effect. BALB/c mice were injected iv with a high concentration (100 μg) of purified IRI-SAb 2, IRI-SAb-3, a nonfunctional antibody (BA8), or a monoclonal TBAb (161). In recipients of IRI-SAb 2 and IRI-SAb-3, T_4 levels were elevated and TSH levels were reduced after 48 h. These changes were sustained for 4 d without evidence of desensitization. Consistent with the half-life of the mTSAb (IgG2a), T_4 and TSH levels returned to baseline by 7 d. Consistent with thyroid stimulation, thyroid tissue in hyperthyroid mice had hypertrophic epithelial cells. Surprisingly, the tissues exhibited acute signs of toxicity (necrotic cells) and an extensive lymphocytic infiltrate, including lymphocytes and macrophages.

Does the protocol of injecting a purified mTSAb into mice provide a new model of Graves' disease? At face value, BALB/c recipients of mTSAb IRI-SAb 2 (or IRI-SAb-3) have the classic features of Graves' patients (elevated T_4, reduced TSH) combined with thyrocyte hyperplasia and thyroiditis, all achieved within a conveniently short time. However, there are potential problems and unanswered questions with this protocol. As discussed earlier (*Section III*), thyroiditis is not as prominent in Graves' hyperthyroidism as in Hashimoto's disease and may be related to TPO-associated autoimmunity. In addition, future studies are required to establish whether, as suggested previously (161), chronic overstimulation of the thyroid gland leads to generalized thyroid autoimmunity and autoantibodies to Tg and TPO. Even more crucial is an active immune response to the TSHR itself. In its absence, thyroid inflammation induced by passive mTSAb transfer does not provide a model in which TSHR-specific T and B cell responses can be modulated to elucidate disease pathogenesis or to develop novel therapies for Graves' disease.

V. Antigen Presentation

A. Overview of APCs

The primary (or "professional") cells that present antigen to T cells constitutively express MHC class II molecules (*Section II*). After antigen internalization by APCs, the particles or proteins are processed by proteolysis to produce peptides that bind within the MHC groove. Macrophages and dendritic cells take up whole organisms by phagocytosis, and particulate or soluble antigens gain entry by pinocytosis (Fig. 9A). Antigen uptake by macrophages or dendritic cells can also be receptor mediated, for example, by Fc receptors that capture and internalize antigen-antibody complexes. Another antigen uptake mechanism by APC involves glycoprotein binding to C-type lectin receptors including the mannose receptor (178), the dectin receptor, and DC-SIGN (reviewed in Ref. 179).

Although B cells constitutively express MHC class II, they have limited phagocytic capacity. However, membrane-bound antibodies on B cells function as antigen receptors, conferring antigen specificity and the ability to capture minute amounts of antigen (Fig. 9B). Consequently, B cells can perpetuate and amplify secondary T cell responses (reviewed in Refs. 180 and 181). An antibody (membrane bound or secreted) can shield critical amino acids during proteolytic processing and thereby influence the peptides made available to T cells. It has been hypothesized that processing of antibody/antigen complexes may expose cryptic T cell epitopes and lead to autoimmunity (182). Moreover, peptide modulation by antibody may account for a characteristic of human autoimmunity, namely restricted epitopic recognition by autoantibodies (183).

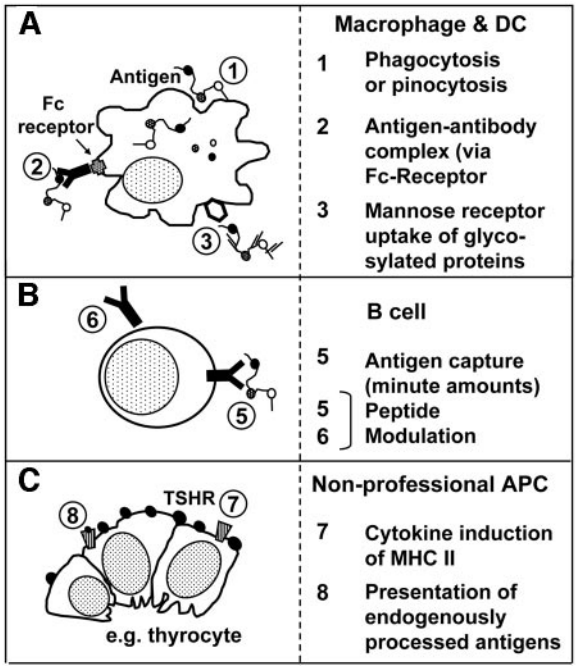

FIG. 9. Antigen uptake and processing for MHC class II presentation by macrophages or dendritic cells (DC, panel A), B cells (B), and nonprofessional APCs (C). In addition to their well-known capacity for phagocytosis and pinocytosis, macrophages and dendritic cells internalize antigens by Fc-mediated uptake of antigen/antibody complexes, or by C-type lectin receptors including the mannose receptor (178), the dectin receptor, and DC-SIGN (reviewed in Ref. 179). B cells capture minute concentrations of antigen by their antigen-specific membrane-bound Igs (180); and antibodies (membrane bound or secreted) can modulate the peptides made available to T cells (reviewed in Ref. 181). Finally, nonprofessional APCs can also be involved in antigen presentation after cytokine-induced induction of MHC class II expression. Such nonprofessional APCs include thyrocytes (59) and myoblasts (186).

Finally, cells that do not constitutively express MHC class II can also be involved in antigen presentation (Fig. 9C). A role for nonprofessional APCs was first appreciated in thyroid autoimmunity and arose from the observation that thyrocytes in Hashimoto or Graves' glands express MHC class II (59) and can function *in vitro* as APCs (184, 185). Other nonprofessional cells, such as human myoblasts, have been shown to function as APCs after cytokine induction of MHC class II (186).

B. Dendritic cells and the mannose receptor in thyroid autoimmunity

1. Dendritic cells. Early studies of the autoimmune response indicated that macrophages play an important role in the presentation of Tg, an abundant, soluble antigen. For example, in rats recovering from induced thyroiditis, injection of Tg-primed macrophages enhances Tg autoantibody levels (187). Dendritic cells are extremely potent APCs and play a crucial role in initiating immune responses (reviewed in Ref. 188). In human autoimmunity, Tg autoantibody synthesis *in vitro* is stimulated using Tg captured by dendritic cells (189).

Of particular relevance to animal models, TSAbs and Graves' hyperthyroidism can be induced in BALB/c mice by injecting dendritic cells infected with TSHR-adenovirus (100) (*Section IV*). However, whereas mature dendritic cells stimulate T cells, immature dendritic cells induce tolerance in naive T cells (190). Consequently, dendritic cells may be manipulated to regulate the induction of Graves' disease in mice and possibly, in the future, in humans.

2. Mannose receptors. Compared with pinocytosis, mannose receptor-mediated uptake of some soluble glycoproteins markedly enhanced the efficacy of T cell responses (191). This calcium-dependent lectin comprises an amino-terminal cysteine-rich domain, eight carbohydrate recognition domains, and transmembrane and intracellular domains (192). The cysteine-rich domain binds to sulfated carbohydrate side chains (193), whereas the carbohydrate recognition domains interact with sugars such as mannose, fucose, and *N*-acetylglucosamine, but not galactose (194).

Recent studies suggest that the mannose receptor plays a role in thyroid autoimmunity. In addition to its interaction with Tg (195), the mannose receptor binds to the TSHR, but not to TPO, in solid-phase binding assays (196). Moreover, blocking mannose receptor binding of TSHR A subunit protein interferes with memory T cell responses. Overall, mannose receptor binding of the heavily glycosylated TSHR protein suggests a mechanism by which the minute amounts of A subunits shed from the thyroid may be captured by APCs located in the gland or in draining lymph nodes.

C. The role of B cells in T cell responses in thyroid autoimmunity

The importance of B cells as APCs is increasingly recognized in immunity to infectious organisms and autoimmunity. Type I diabetes mellitus, the classic example of a T cell-mediated autoimmune disease, does not develop in NOD mice lacking B lymphocytes (197, 198). The explanation for this finding is that antigen-specific B cells are required to present islet cell autoantigens to T cells, which ultimately damage the islets (199, 200). On the other hand, there is also evidence of a non-antigen-specific role for B cells in T cell responses. Thus, B cells are necessary for mice to generate a diverse T cell repertoire (201) as well as for the development of memory T helper cells (202, 203). Against this background, the contribution of B cells to T cell responses in Graves' models has been examined in two ways: first, by studying memory T cells in mice with defective B cells and second, by comparing T cell epitopes recognized by TSHR-immunized mice that have, or do not have, TSHR antibodies.

1. B cell knockout and deficient mice. Three types of B cell-deficient mice (BALB/c background) have been studied after immunization with TSHR-adenovirus: J_HD mice lacking all B cells; mIgM mice with B cells expressing membrane-bound monoclonal IgM; and (m+s)IgM mice that have B cells expressing membrane-bound monoclonal IgM as well as secreted monoclonal IgM. When challenged with TSHR antigen, only splenocytes from TSHR-adenovirus-immunized wild-type and mIgM mice, but not from J_HD or (m+s)IgM mice, demonstrated a memory response as determined by IFNγ production (204). A possible explanation for the difference among mutant mouse strains was that, in contrast to the two unresponsive strains, the mIgM mice were "leaky" and produced low levels of IgG.

Overall, T cell responses to TSHR antigen developed only in mice with IgG-secreting B cells, suggesting that some normal B cells are required for the development of memory T cells in the TSHR-adenovirus Graves' model. These findings are consistent with previous studies showing that neither transfer of serum autoantibodies nor B cell reconstitution of adults could replace the early requirement of B cells for the development of spontaneous thyroiditis in NOD.H-2H4 mice (205).

2. Antigen modulation by B cells. Antigen-specific B cells or their antibodies can influence the peptides available for presentation to T cells (Fig. 9B). For example, human B cell clones specific for different tetanus toxoid epitopes enhance or suppress presentation of particular peptides to T cells (206). Likewise, different monoclonal Tg antibodies can enhance or suppress processing of a pathogenic epitope to T cells (207). Therefore, it seemed likely that the spectrum of TSHR-T cell epitopes would be different in TSHR-immunized mice with or without TSHR antibodies. However, as described later (*Section VI*), the same T cell epitopes were recognized, regardless of the presence or absence of TSHR antibodies. These data preclude a major role for TSHR-specific B cells or antibodies in peptide processing, at least in this model involving BALB/c mice. However, T cell clones or hybridomas will be required for detailed dissection of the contribution by macrophages, dendritic cells, and B cells in immune responses to the TSHR.

D. "Nonprofessional" APCs

1. Endogenous vs. exogenous antigen processing. Endogenous proteins (like intracellular viral proteins) are degraded to linear peptides in the proteasome and transported by transporters in antigen processing molecules for binding to MHC class I expressed on all cells (reviewed in Ref. 208). Infected target cells presenting viral peptides in MHC class I are recognized and killed by cytotoxic $CD8^+$ T cells. In contrast, exogenous proteins (such as bacterial products) are internalized by macrophages or dendritic cells and transported to lysosomes where they are processed into peptides for binding to MHC class II (reviewed in Ref. 209). MHC class I *vs.* MHC class II processing and presentation are not mutually exclusive. However, reduced MHC class II presentation of endogenously processed peptides translates into diminished $CD4^+$ T cell activation that, in turn, leads to reduced help for B cells.

2. Thyrocytes as APCs. Thyrocytes constitutively express MHC class I but not class II. However, thyroid cells from Hashimoto and Graves' patients aberrantly express MHC class II (210). From these and other studies, Bottazzo and colleagues (59, 211) hypothesized that thyroid cells function as APCs and present their own thyroid antigens. The Shimojo Graves' model (73), which involves injecting fibroblasts coexpressing the TSHR and MHC class II (*Section III*), is based on these early findings. Indeed, subsequent studies showed that TSHR-positive fibroblasts lacking MHC class II could not induce TSAbs or hyperthyroidism (73, 74).

Despite these findings, increasing evidence suggests that MHC class II thyrocyte expression alone is insufficient to induce thyroid autoimmunity. An important recent observation is that neither thyroid autoantibodies nor lymphocytic infiltration develop spontaneously in transgenic mice with thyrocyte-targeted MHC class II expression (212, 213). This absence of thyroid autoimmunity is consistent with inefficient antigen processing of endogenous proteins (such as the TSHR) by thyrocytes for peptide presentation by MHC class II. However, the genetic background of the MHC class II transgenics may be inappropriate, and thyroid-specific responses could be enhanced after immunization.

In summary, a major role for TSHR presentation by thyrocytes seems unlikely in Graves' disease. Although able to present peptides, thyrocytes cannot process and present exogenous antigen (214). Moreover, several lines of evidence (*Section IV*) suggest that the shed A subunit, rather than the membrane-bound TSHR, is involved in initiating or enhancing immune responses (97, 98, 120). Finally, as suggested (196), the very small amounts of shed A subunit are likely captured by mannose receptors on dendritic cells within the thyroid (215) or in draining lymph nodes.

3. Antigen targeting to lysosomes. Because the lysosome-associated membrane protein (LAMP)-1 has a sorting signal that directs it to lysosomes (216), this molecule has been used as a tool to direct proteins to the lysosome. For example, the acetylcholine receptor (AChR) α-subunit cDNA has been

substituted for that of LAMP-1 between the signal peptide and the transmembrane /cytoplasmic tail of the latter. APCs transfected with this chimeric AChR-LAMP-1 DNA were more potent T cell stimulators than the same cells transfected with the AChR α-subunit alone (217).

As mentioned previously, im vaccination with TSHR plasmid DNA is relatively ineffective at inducing TSHR antibodies in BALB/c mice (Table 2). Part of the explanation for this problem may be weak activation of CD4+ T cells arising from *endogenous* TSHR expression in muscle and or immune cells. To test this hypothesis, immunization with a chimeric LAMP-TSHR plasmid, constructed to "hijack" the TSHR to the *exogenous,* lysosomal antigen-processing pathway did, indeed, induce TSHR antibodies and hyperthyroidism in some BALB/c mice (90). Nevertheless, despite the improvement over TSHR-DNA vaccination with a conventional vector, LAMP-TSHR-DNA was still less effective than immunization with TSHR-adenovirus. Two factors, possibly acting in concert, are likely involved. First, bacterial DNA contains immunostimulatory CpG motifs (218), but adenoviruses have more powerful adjuvant properties (219). Second, membranes prepared from muscle tissue of mice injected with TSHR-adenovirus, but not with TSHR-DNA, bound labeled TSH (86), demonstrating higher expression levels of functional receptor by the adenovirus.

VI. T Cells and Tolerance

A. T cell recognition of the TSHR

1. Shimojo model (fibroblasts expressing the TSHR and MHC class II). Splenocytes from mice immunized by the Shimojo approach proliferated *in vitro* when stimulated with fibroblasts coexpressing MHC class II and the TSHR but not to fibroblasts expressing only MHC class II (117). These findings demonstrate the presence of TSHR-specific memory T cells in mice immunized in this manner, potentially a very valuable observation for further study of cellular interactions in the autoimmune response to the TSHR. Indeed, *ex vivo* challenge of splenocytes with a panel of 80 synthetic peptides spanning the extracellular domain of the TSHR revealed that multiple peptides (T cell epitopes) induced responses. Maximal proliferation occurred with a peptide including TSHR amino acid residues 121–140. This region, therefore, appears to contain a major TSHR T cell epitope in AKR/N mice presented by class II molecule IA-k (220).

However, the initial promise of this model for *in vitro* cell studies to further understand the immune response to the TSHR has not been fulfilled. First, no further data on TSHR memory T cells have been reported by the Shimojo group. Second, an unexpected problem became apparent for this model. Other investigators observed that injection of MHC class II-expressing fibroblasts (RT4.15HP cells) led to extremely high background, nonspecific activation of T cells even without *in vitro* antigenic challenge (118). As mentioned before (*Section III*), the likely explanation for this activation is constitutive expression by RT4.15 HP fibroblasts of B7–1, a key costimulatory molecule in the process leading to T cell activation (reviewed in Ref. 36).

2. TSHR-DNA vaccination. Naked TSHR-DNA vaccination induces strong cellular immune responses including splenocyte proliferation and production of the cytokine IFNγ when challenged *in vitro* with TSHR-protein (85, 112). In contrast to the spontaneous, high-background lymphocyte activation in mice immunized by the Shimojo approach, proliferation and cytokine secretion are minimal or undetectable in unstimulated splenocytes from animals vaccinated with control-DNA or TSHR-DNA in a plasmid vector. Because of this low background, and using panels of TSHR synthetic peptide encompassing the TSHR ectodomain and three extracellular loop peptides (221), it has been feasible to investigate T cell epitopes. Thus, based on IFNγ production as a readout for memory T cell activation, four mouse strains with three different MHC class II genes (termed "IA" in mice) have been studied: BALB/c (IA-d), NOD.H-2h4 (IA-k), AKR/N (IA-k), and C57BL/10/129 (IA-b) (222).

In accordance with the crucial role of MHC in determining peptide binding, TSHR-DNA-vaccinated mice with different MHC genes responded to different sets of peptides. Unexpectedly, a restricted number of peptides were recognized by each strain, with limited commonality between strains. Thus, three peptides stimulated lymphocytes from BALB/c mice (amino acids 52–71, 67–86, and 157–176; annotated C, D, and J); four peptides were stimulatory in NOD.H-2h4 mice (residues 112–131, 232–251, 247–266, and 307–326; referred to as G, O, P, and T); and two peptides in C57BL/10/129 mice (residues 112–131 and 157–176; peptides G and J) (222, 223) (Fig. 10). Peptide recognition by AKR/N mice could not be established because of erratic splenocyte cytokine production in this strain. Of interest, seven of the eight peptides recognized by these three mouse strains lie within the A subunit, *upstream* of the initial TSHR cleavage site TSHR ($\sim$ amino acid 310). None of the extracellular loop peptides were recognized by any of the mouse strains.

3. TSHR-adenovirus immunization. TSHR-DNA vaccination, unlike TSHR-adenovirus immunization, rarely induces TSHR antibodies in BALB/c mice (Table 2). A possible explanation for the efficacy of adenovirus *vs.* DNA plasmid immunization is that T cells are responding to different TSHR T cell epitopes consequent to altered processing of antigen. Surprisingly, the three peptides recognized by splenocytes from TSHR-adenovirus-immunized BALB/c mice (amino acids 52–71, 67–86, and 157–176; peptides C, D, and J) (119) were also the major epitopes for TSHR-DNA plasmid-vaccinated BALB/c mice (222). Lesser responses were observed to other peptides in mice immunized more vigorously with TSHR-adenovirus (three *vs.* one injection). However, the peptide response pattern was more consistent with increased background than with spreading from primary to secondary epitopes. Most importantly, no particular T cell epitope(s) was associated with the clinical outcome in these mice, namely hyperthyroidism *vs.* euthyroidism. These studies in TSHR-adenovirus-immunized mice indicate that factors other than particular TSHR T cell epitopes, such as adenovirus-induced expression of conformationally intact

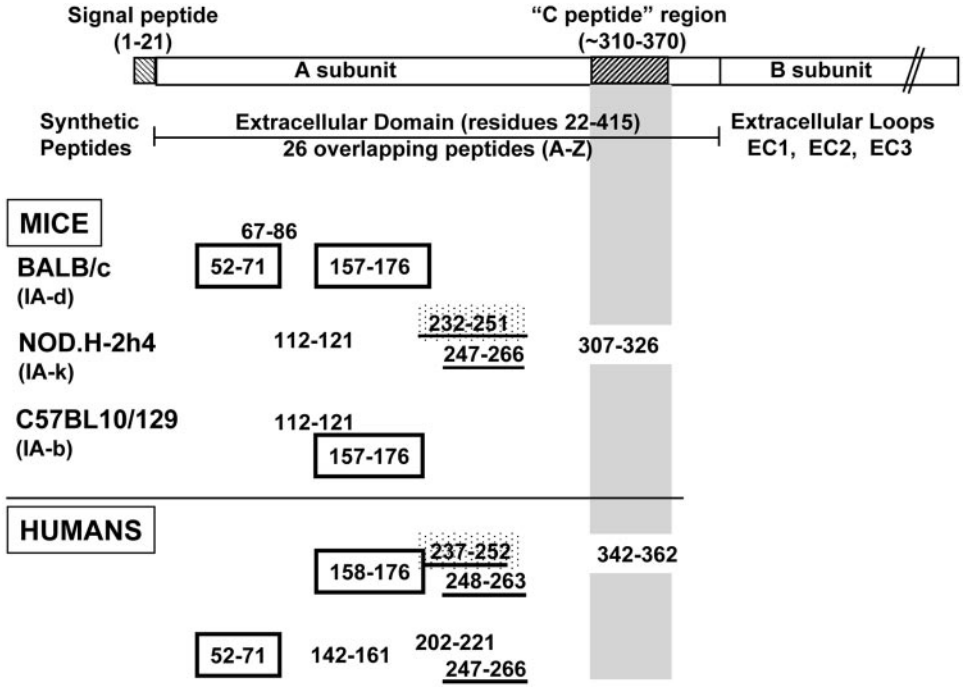

FIG. 10. T cell epitopes on the TSHR recognized by immunized mice and humans with Graves' disease. BALB/c mice were vaccinated with TSHR-DNA or TSHR-adenovirus, and NOD.H-2h4 and C57BL/10 mice were vaccinated with TSHR-DNA. [Adapted with permission from P. Pichurin *et al.*: *Thyroid* 12:755–764, 2002 (222). © Mary Ann Liebert, Inc.]

TSHR protein, contribute to the generation of TSAbs. Moreover, as mentioned earlier, these findings suggest that antigen-specific B cells and/or antibodies are unlikely to play a major role in modulating T cell responses to the TSHR.

4. Epitopes recognized by humans vs. TSHR-immunized mice. Four TSHR peptides recognized by TSHR-plasmid DNA-vaccinated mice are also recognized by T cells from Graves' patients (Fig. 10) (reviewed in Ref. 224). Peptide C (amino acids 52–71) is recognized by BALB/c mice and also corresponds to one of the four immunodominant human T cell epitopes (225). Moreover, peptide J (residues 157–176) is almost identical to a peptide recognized by T cells from Graves' patients before and after therapy (226) as well as in Graves' siblings (227). Of the four peptides to which NOD.H-2h4 mice responded, peptides O (residues 232–251) and P (residues 247–266) closely resemble T cell epitopes recognized by some Graves' patients. Peptide P is of particular interest because it has been reported to be a human TSHR T cell epitope in three independent studies (225, 228, 229).

B. Tolerance and responses to the TSHR

Tolerance represents the inability, or suppression, of the well-armed immune system to attack self. Autoimmunity implies a breakdown in tolerance to self-proteins such as the TSHR in Graves' disease. Development of tolerance is a complex process that occurs at different developmental stages of mammals and includes central and peripheral mechanisms acting in concert (reviewed in Refs. 230–232). Self-reactive T and B lymphocytes can be eliminated (clonal deletion), silenced by immature dendritic cells, regulatory cells (Treg), deprived of essential cytokine growth factors (anergy), or exposed to regulatory cytokines (such as IL-10). The new animal models now make it possible to begin to explore tolerance mechanisms in Graves' disease. Understanding how tolerance to the TSHR is established could provide insight into approaches for future immunospecific therapy for Graves' disease.

1. TSHR knockout mice. Central T cell tolerance is established by intrathymic T cell education in which immature T lymphocytes are exposed to peptides processed from proteins from diverse tissues expressed ectopically in the thymus. T cells with receptors that bind with high affinity to peptides from self-antigens undergo apoptosis and are deleted from the repertoire (233). The TSHR is expressed at low levels in human and rodent thymic tissue, as detected by TSHR mRNA and protein (234–236). Therefore, it is likely that T cells in humans and mice develop central tolerance for the TSHR by its expression in the thymus during development. However, for TSHR null mice that lack TSHR expression in all tissues (237) the TSHR would be a foreign protein.

Previous studies have shown major differences between immune responses induced by immunization of myelin-basic protein-knockout mice *vs.* wild-type mice (238). Therefore, immunization would be expected to induce TSHR antibody responses of greater magnitude and recognition of different T cell epitopes in the TSHR null *vs.* tolerant wild-type mice. Surprisingly, however, after TSHR-DNA vaccination, TSHR antibodies developed in approximately 60% of TSHR knockouts and 80% wild-type mice (on the same C57BL/6/129 genetic background) (223). Antibody levels were comparable and both strains recognized the same immunodominant linear antibody epitope at the TSHR amino terminus. Moreover, splenocytes from TSHR-knockout and wild-type mice responded to the same T cell epitopes (residues 112–131 and 157–176).

Several hypotheses can be put forward to explain these unanticipated findings. First, the DNA used for vaccination expresses human TSHR, not mouse TSHR. Despite 87% amino acid homology (239), this difference may be sufficient

for the human TSHR to be foreign in wild-type mice. However, in previous studies, immune responses were similar in mice injected with B cells expressing murine or human TSHR (79). A second possibility is that homologous gonadotropin receptors could (if expressed in the thymus) cross-tolerize T cells to the TSHR in knockout mice. Against this possibility is that the TSHR epitopes (antibody and T cell) recognized by wild-type and knockout strains have low homology with the corresponding regions of the gonadotropin receptors.

A third possible explanation for the normal response to TSHR immunization in TSHR-null mice is that intrathymic TSHR expression in wild-type mice is very low (234–236). Therefore, wild-type mice may have low or deficient central T cell tolerance to the TSHR. Support for this thesis is that resistance to induced autoimmune uveitis correlates with higher thymic antigen expression (240). Conversely, only trace amounts of myelin oligodendrocyte glycoprotein are expressed in the thymus, and mice lacking myelin oligodendrocyte glycoprotein respond to the same T and B cell epitopes as wild-type animals (241). A type I diabetes susceptibility locus in humans maps to a variable number of tandem repeats upstream of the insulin gene. These polymorphisms are associated with high *vs.* low intrathymic insulin expression, suggesting that tolerance to insulin may involve the protective effect of particular variable number of tandem repeats (242, 243). In the light of this information, it is possible that low intrathymic TSHR expression prevents the development of central tolerance, at least in mice on this genetic background. Alternatively (or in addition), peripheral tolerance toward the TSHR may also be less effective than for other autoantigens.

2. TSHR A subunit transgenic mice. Further insight into TSHR tolerance is provided by transgenic mice with the human TSHR A subunit targeted to the thyroid using the Tg promoter. In principle, these mice provide an opportunity to study the potential role of TSHR A subunit shedding by thyrocytes. Founder mice (mixed C57BL/6 and BALB/c background) had normal thyroid histology and thyroid function. Mice were backcrossed to the BALB/c strain, which is highly susceptible to developing Graves'-like hyperthyroidism on TSHR A subunit adenovirus immunization (*Section IV*) (97).

After immunization, all transgenic animals were euthyroid, and none developed TSHR antibodies (TBI). Moreover, splenocytes from the TSHR-adenovirus-immunized transgenic mice remained unresponsive to TSHR antigen *in vitro*. Wild-type littermates, immunized in parallel, all developed TSHR antibodies, approximately 50% became thyrotoxic, and their splenocytes were responsive to TSHR antigenic stimulation. The absence of T and B cell responses to the TSHR in the transgenic mice indicates that the immunizing efficiency of the TSHR-expressing adenovirus vector is insufficient to break tolerance when the human TSHR A subunit is expressed as self during ontogeny (244). Central tolerance to the TSHR is likely to be particularly effective because the heterologous Tg promoter in the transgene, which is more powerful than the natural TSHR promoter, will lead to high TSHR A subunit expression in the thyroid and also, presumably, in the thymus. As mentioned previously, a wide panoply of proteins, including Tg, are expressed in the thymus.

Another transgenic strain has been generated in which the human TSHR, under the control of the human TSHR promoter, is expressed in the thyroid and adipose tissue (245). Whether these mice respond to TSHR immunization is unknown. However, the outcome of such investigations will shed light on the potency of different promoters in inducing tolerance to the TSHR.

3. B cell tolerance in mice transgenic for TSAbs. Like central tolerance that involves T cell deletion, B cells that bind to self-proteins can also be removed by clonal deletion. As mentioned earlier, transgenic mice have been generated that express the genes for the L chain and H chain variable region (linked to IgM) of a low-affinity human TSAb (104). These TSAb transgenic mice provide an opportunity to study B cell tolerance in a Graves' model. B cell numbers were reduced in the spleen and blood of TSAb-transgenic mice but accumulated in the peritoneal cavity. Moreover, after oral lipopolysaccharide administration, TSAb-producing B cells (probably in the peritoneum) were activated as reflected by increased T_4 and reduced TSH levels. Overall, these findings implicate a role for peritoneal B cells in the induction of TSAb, at least for the low-affinity antibody in this particular mouse strain.

VII. Th1 *vs.* Th2 Balance in the Immune Response to the TSHR

A. Cytokines involved in different Graves' models

The T helper 1/T helper 2 (Th1/Th2) cytokine paradigm provides a powerful framework for explaining the basis of immune responses (40). Generally, Th1 immune responses promote cell-mediated immunity and involve IFNγ, whereas Th2 responses enhance antibody-mediated immunity and involve IL-4, -5, and -13 (reviewed in Refs. 40 and 246). Because TSHR autoantibodies play a crucial role in the pathogenesis of disease, it is perhaps not surprising that Graves' disease in humans has long been thought to be a Th2-dominant autoimmune disease. Supporting evidence for this concept includes features of atopy (*e.g.*, Ref. 247) as well as induction of Graves's disease in humans by an anti-CD52 monoclonal antibody that induces Th2 immune deviation (248). However, many studies on cytokine profiles in thyroid tissues indicate a mixed Th1 and Th2 immune response (249–251). Moreover, and most important, human TSAbs are predominantly IgG1 (252), a Th1 type subclass in humans.

In mouse models, the cytokine profiles of induced immune responses to the TSHR are related to the different approaches used for induction. It should be appreciated that in mice, unlike in humans, IgG1 reflects a Th2-type response because IL-4 is required for its production. IgG2a generation, regulated by IFNγ, is a Th1-type response (reviewed in Ref. 41).

1. Shimojo model. Hyperthyroidism in this model can be enhanced by alum and pertussis toxin, and delayed by CFA (75). These two adjuvants induce contrasting cytokine production: Th1 cytokines (such as IFNγ) by CFA (253) *vs.* Th2

cytokines (IL-4 and IL-10) by alum and pertussis toxin, respectively (254) (Fig. 11, *middle panel*). The enhancing effect on disease of alum and pertussis toxin adjuvants has been independently confirmed (77). Therefore, *in vivo* data for this model suggest that a Th2 response plays an important role in disease development.

Other *ex vivo* studies have analyzed the Th1/Th2 balance of the TSHR-specific immune response in the Shimojo approach, namely determination of TSHR antibody IgG subclasses and splenocyte cytokine secretion in response to TSHR-antigen challenge. However, as mentioned previously, these approaches were hampered by nonspecific activation of the immune system, most likely arising from constitutive expression of the costimulatory molecule B7–1 on the MHC class II-positive fibroblasts (RT4.15HP cells) used to inject the mice (118). TSHR antibody subclass analysis by ELISA was not feasible because of nonspecific binding by sera from control-fibroblast-injected mice. However, in contrast to the *in vivo* reports, other *in vitro* evidence supports the possibility of a Th1 bias in the Shimojo model. Thus, splenocytes from these mice, whether injected with TSHR-expressing or control RT4.15HP cells, spontaneously secreted maximal amounts of IFNγ (118). In addition, when the Shimojo model for Graves' disease was modified to explore the immune response to TPO, IgG2a subclass antibodies to TPO also suggested a Th1 bias (118).

2. Naked TSHR-DNA vaccination. The Th1/Th2 balance in this Graves' disease model is also uncertain. In the original reports using this approach (84, 91), neither the IgG subclasses of serum TSHR antibodies nor splenocyte cytokine production was studied in BALB/c or outbred NMRI mice. However, mice developed thyroid lymphocytic infiltrates characterized by B cells and IL-4-producing T cells, reflecting Th2 responses (84, 91). In contrast, a monoclonal antibody (BA8), obtained from a mouse immunized in this manner, was of IgG2a subclass, reflecting a contribution from Th1 cytokines (84).

Serum TSHR antibody levels in most other studies were undetectable or low (77, 85, 86). In occasional animals with higher titers, TSHR antibodies were Th1 and Th2, whereas animals with lower titers had Th1-type antibodies (112). Studies *in vitro* of splenocytes from TSHR-DNA-vaccinated mice supported a role for a Th1 immune response in this model. Thus, splenocytes from immunized BALB/c mice proliferated and produced Th1 cytokines (including IFNγ, TNFα, and IL-2) when challenged with TSHR antigen (85, 222). Attempts to enhance antibody production using IFNγ knockout mice (with a blunted Th1 response) were unsuccessful (112). Moreover, in another study, the combined intradermal injection of TSHR-DNA and IL-4-DNA attenuated induction of TSAbs whereas coinjection of IL-12 DNA had little effect (89). Taken together, the majority of data support the concept of a Th1 response in TSHR-DNA-vaccinated mice.

3. B cells and HEK293 cells. Mice immunized with M12 B cells or HEK293 cells stably expressing the full-length TSHR or the truncated TSHR (TSHR ectodomain), respectively, produce TSHR antibodies of both IgG1 and IgG2a subclasses. Moreover, because their splenocytes produced both IFNγ and IL-4 in response to *in vitro* stimulation with TSHR antigen, these data suggested mixed Th1 and Th2 responses (255). Further insight was provided using mice null for either IFNγ or IL-4 [or their associated signaling molecules, signal transducer and activator of transcription (Stat)-4 and Stat-6, respectively]. Hyperthyroidism was induced as readily in IFNγ knockout mice as in wild-type BALB/c. In contrast, IL-4 null mice were resistant to disease induction (255). Likewise, immunization with TSHR-expressing HEK293 cells induced TSAbs and hyperthyroidism in mice lacking Stat-4 (defective Th1 signaling) but not in mice lacking Stat-6 (defective Th2 signaling). These data indicate a role for Th2 signaling in this model (80).

Less informative evidence in the M12-TSHR model is the effect of transient immune deviation at the time of antigen priming. Thus, deviation to Th1 (using the cytokine Flt3L with or without IL-12) and deviation to Th2 (using granulocyte macrophage colony stimulating factor) had no effect on the induction of hyperthyroidism (255). However, because of the very long duration (many months) of the immunization protocols in the M12 or HEK293 cell models,

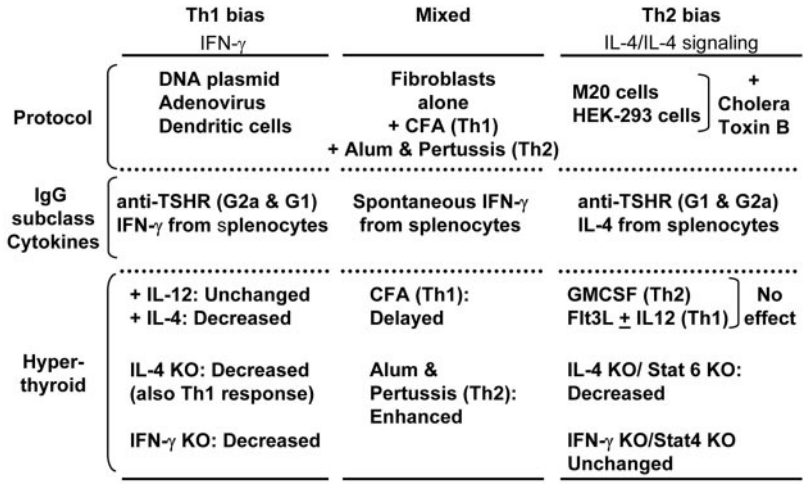

FIG. 11. Biasing cytokines toward either Th1 or Th2 regulates hyperthyroidism in different Graves' models. Models are classified as Th1 if splenocytes secrete IFNγ and if hyperthyroidism is unchanged by manipulating Th1 cytokines (IL-12/IFNγ) and decreased by IL-4. Conversely, models are classified as Th2 based on IL-4 secretion from splenocytes and decreased hyperthyroidism by altering IL-4 signaling. The Shimojo model is mixed because splenocyte secretion suggests a Th1 bias but cytokine coadministration indicates a Th2 bias. The IgG subclass distribution of TSHR antibodies (IgG1 and IgG2a) indicates that categorization as Th1 or Th2 is relative, not exclusive. KO, Knockout.

long-term immune deviation after antigen presentation might be necessary to suppress disease.

4. Dendritic cells infected with TSHR-adenovirus. Mice immunized by this method produced TSHR antibodies of both IgG1 and IgG2a subclasses, and their splenocytes secreted IFNγ in response to TSHR antigen challenge (Th2 cytokine production was not evaluated) (100). In this model, the Th2 adjuvants, alum and pertussis toxin, completely suppressed antibody production as well as hyperthyroidism, whereas Th1 adjuvant poly (I:C) augmented splenocyte production of IFNγ without affecting disease incidence.

5. TSHR-adenovirus. This immunization approach also induces a mixed Th1 and Th2 immune response against the TSHR, as reflected by IgG1 and IgG2a subclass TSHR antibodies, as well as by IFNγ and IL-10 (not IL-4) secretion by TSHR-challenged splenocytes from immunized mice (119). Concomitant injection of TSHR-adenovirus and IL-4-adenovirus (to transiently increase serum IL-4 levels) polarizes the TSHR-specific immune response toward Th2, as evidenced by increased IgG1/IgG2a TSHR antibody ratios. In addition, this protocol impaired IFNγ splenocyte secretion and also suppressed the induction of hyperthyroidism (256) (Fig. 11, *left panel*). Suppression of hyperthyroidism by transient immune Th2 polarization at the time of antigen presentation in the adenovirus approach is in a sharp contrast to the aforementioned results in the M12-TSHR model (255).

Other agents have been used to attain sustained physiological, or pathophysiological, Th2 deviation: injection of α-galactosylceramide or infection with the parasitic helminth *Schistosoma mansoni*. Coadministration of α-galactosylceramide with TSHR-adenovirus or *Schistosoma* infection before TSHR-adenovirus injections both deviated the TSHR-specific immune response away from Th1 and protected mice from hyperthyroidism (257). Immune deviation in these studies resulted from Th1 suppression rather than Th2 enhancement because TSHR IgG2a antibody (Th1) titers were reduced whereas IgG1 antibody (Th2) titers were not increased. Furthermore, the TSHR-induced IFNγ response was blunted (without increased Th2 cytokine secretion).

Overall, in the TSHR-adenovirus model, a Th1 immune response appears to be indispensable during the induction phase of TSAbs and hyperthyroidism. However, after anti-TSHR immune responses are established, Th2 immune deviation is ineffective in reverting hyperthyroidism. Therefore, at least in this Graves' disease model, Th2 immune deviation can prevent the initiation of disease but cannot cure established Graves'-like hyperthyroidism (257). Consequently, these data suggest that in human disease it will be difficult, if not impossible, to dampen the ongoing immune response to the TSHR by modulating the Th1/Th2 balance.

Consistent with the previous evidence for the importance of a Th1 response in the TSHR-adenovirus model, Th1 deviation by coinjection of IL-12 adenovirus (Th1 cytokine) induced Th1-type immune responses without changing disease incidence (256). More recent data, however, muddy the waters regarding the importance of a Th1 response in the TSHR-adenovirus model. Thus, prior infection with *Mycobacterium bovis Bacillus* Calmette-Guerin (*M. bovis* BCG) significantly suppressed TSHR-adenovirus induction of hyperthyroidism (258). In this study, the immune response against *M. bovis* BCG comprised mixed Th1 and Th2 elements. However, the anti-TSHR immune response was biased to a Th1 phenotype, as demonstrated by augmented IFNγ and loss of IL-10 production by TSHR-antigen-stimulated splenocytes. This protective effect of *M. bovis* BCG on induction of hyperthyoidism contrasts with that of IL-12 (see above), although both deviate from the immune response to Th1. Therefore, suppression of disease induction by *M. bovis* BCG infection may not be solely due to Th1 immune deviation. The possible interpretation of these unexpected results is discussed in *Section VII*.

Finally, BALB/c mice deficient in IFNγ or IL-4 by gene disruption are both resistant to TSHR-adenovirus-induced Graves' hyperthyroidism (259). At face value, suppression of induced disease in IL-4 null mice is inconsistent with the data, described above, that IL-4-adenovirus coinjection with TSHR-adenovirus suppressed the induction of hyperthyroidism (256). However, this apparently contradictory finding can be explained by impairment of both Th1 and Th2 immune responses in IL-4 null mice. For example, IFNγ production by splenocytes challenged with TSHR antigen is lost in IL-4 null mice.

B. Summary

TSHR immunization induces mixed Th1- and Th2-type responses in all animal models as reflected in the IgG subclass distribution of TSHR antibodies and TSHR-antigen-stimulated cytokine production by splenocytes. However, with one exception (the model involving dendritic cells), polarization to either Th1 or Th2 leads to two opposite results (Fig. 11). Th2 immune deviation is associated with decreased hyperthyroidism in genetic immunization approaches (plasmid or adenovirus). In contrast, a Th1 immune bias suppresses the induction of hyperthyroidism in intact cell immunization approaches involving TSHR expression on Shimojo fibroblasts, M12 B cells, and HEK293 cells. Consistent with these findings, studies with knockout mice revealed distinctly separate requirements, with the Th1 cytokine IFNγ being less important in the M12 cell model (255) but more important in the adenovirus model (259).

A strong Th1-dominant immune response against adenovirus itself (260) or CpG motifs in plasmid DNA (218) may explain the Th1 bias in TSHR genetic immunization models of Graves' disease. These data are consistent with the fact that mTSAbs isolated from mice subjected to genetic immunizations are IgG2a, a murine Th1 subclass (see *Section IV*). Unlike genetic immunization, there is no obvious explanation for the Th2 bias that occurs after injection of TSHR-expressing M12 or HEK293 cells alone. However, it should be appreciated that TSHR immunization using these cell types is usually combined with the cholera toxin B subunit, a potent Th2 adjuvant (79). On the other hand, induction of hyperthyroidism using dendritic cells infected with TSHR-adenovirus does not involve a separate adjuvant. Consequently, disease suppression by Th2 polarization in this cell model is likely attributable to the Th1-dominant immune response induced by adenovirus.

Despite the general segregation between genetic immunization (Th1) and intact cell immunization (Th2), this distinction is not clear cut. Thus, some contradictory data have been reported by different laboratories even when using the same mouse strains. For example, in the Shimojo model, splenocytes spontaneously produce the Th1 cytokine IFN-γ (85) whereas the TH1 adjuvant *CFA* reduced disease (75). Furthermore, after TSHR-DNA vaccination, thyroid infiltration with Th2 immune cells (84) does not conform to Th1 cytokine production by splenocytes challenged with TSHR antigen (222).

A consistent finding of potential importance among animal models of Graves' disease (with one exception) is that protection from hyperthyroidism by immune polarization, whether toward Th1 or Th2 or by cytokine gene disruption (IL-4, IFNγ, Stat-4, or Stat-6), is associated with a selective decrease in TSAb without changing TSHR antibody levels as measured by TBI or ELISA. TBAb titers were unaltered, at least in the case of coimmunization with IL-4 adenovirus (256). Consequently, these immune manipulations appear to inhibit the production of TSAb and the progression to clinically overt hyperthyroidism *without* altering the generation of nonstimulatory TSHR antibodies. The one exception is the TSHR-dendritic cell model, in which a Th2 adjuvant drastically suppressed not only titers of TSAb but also titers of nonstimulatory anti-TSHR antibodies (100).

Importantly, the inherent Th2 bias in BALB/c mice appears to be less important than the immunization protocol. For example, Graves' disease induced by injecting TSHR-expressing M12 B cells was reduced in the absence of IL-4 (255) whereas in the same mouse strain, deviation toward Th2 suppressed Graves' disease induced by TSHR-adenovirus (256). At present, it is unclear which model more closely mimics the pathogenesis of human Graves' disease. Nevertheless, in our view, it is important to be cautious in interpreting the data from a single animal model. As described by others, multiple animal models must be analyzed to gain an insight into the pathogenesis of Graves' disease in humans (261).

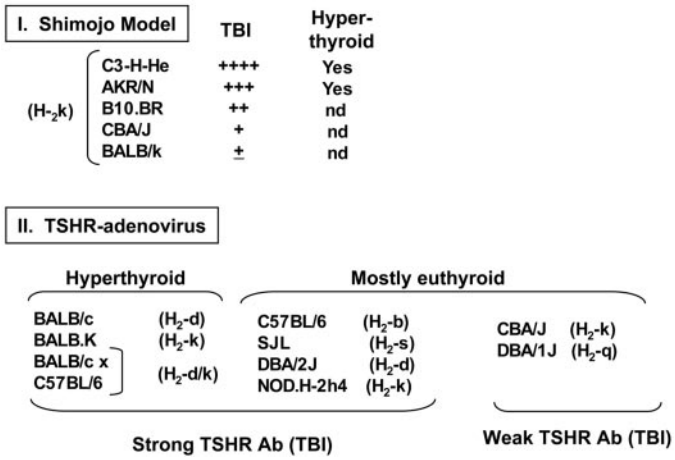

FIG. 12. Genetic differences in inbred mice to Graves' disease induced using the Shimojo approach or by TSHR-adenovirus. Injecting fibroblasts coexpressing the TSHR and H2-k MHC class II (Shimojo approach) provides information on the contribution of non-MHC genes to TSHR antibodies and, in some strains, to hyperthyroidism (74). Immunization with the TSHR-adenovirus approach can be performed in any mouse strain. This approach has been used to test the contribution of MHC and non-MHC genes to the generation of TSHR antibodies and disease (86, 96, 116). Ab, Antibody; nd, not determined.

VIII. Genetic *vs.* Environmental Factors

A. Genetic factors

1. Non-MHC genes. In humans, the pathogenesis of Graves' hyperthyroidism is multifactorial, involving both environmental and genetic factors. Among the latter are MHC and non-MHC genes (reviewed in Ref. 93). Several lines of evidence obtained with three different mouse models indicate the importance of non-MHC genes. First, in five different mouse strains, all with the same H-2k MHC haplotype but with different non-MHC genetic backgrounds, injecting fibroblasts coexpressing MHC class II and the TSHR (Shimojo model) induces variable titers of TSHR antibodies (74) (Fig. 12). These data provided strong evidence of a crucial role for non-MHC genes in generating TSHR antibodies in the Shimojo model. Second, induction of Graves' disease by TSHR-DNA vaccination of outbred NMRI mice, but not BALB/c mice (H-2d), also supports the importance of non-MHC genes because most hyperthyroid outbred mice had the same MHC (H-2 q/q) (91).

A third line of evidence against an important role for MHC in this phenomenon is that TSHR-adenovirus injection induces Graves'-like hyperthyroidism in both BALB/c (H-2d) and BALB.K (H-2k) mice. These two susceptible strains are H-2 congenic, *i.e.*, they bear different H-2 haplotypes on the same non-MHC genetic background, thereby excluding a role for MHC genes in developing hyperthyroidism. Further evidence is that other mice with the same MHC haplotypes as the foregoing susceptible strains, namely DBA/2J (H-2d) and CBA/J (H-2k), are resistant to the induction of Graves' hyperthyroidism (Fig. 12). Although not directly compared, NOD.H.2h4 mice, also with an H2-k haplotype, are only weakly susceptible to disease (116).

Other mouse strains with different MHC haplotypes [C57BL/6 (H-2b), DBA/1J (H-2q), and SJL/J (H-2 s)] are resistant to induction of hyperthyroidism (86, 96) (Fig. 12). However, a role for MHC genes in these resistant phenotypes cannot be deduced without comparison to mice with the same MHC haplotypes. Of potential future importance in defining the non-MHC genes underlying susceptibility to TSHR-adenovirus-induced hyperthyroidism is that the F1 cross between susceptible BALB/c and resistant C57BL/6 mice remains susceptible to disease. These data suggest the presence of dominant, non-MHC susceptibility genes in the BALB/c background (262).

Interestingly, some mice that are resistant to TSHR-adenovirus-induced hyperthyroidism (C57BL/6, SJL/J, DBA/2J, and NOD.H-2h4) are nevertheless good responders in terms of TSHR antibody production as measured by TBI and ELISA (86, 96, 116, 262). In contrast, CBA/J and DBA/1J mice are poor antibody responders, suggesting the existence

of additional non-MHC genes that regulate the amplitude of the TSHR antibody response (86, 96).

Finally, it is important to emphasize that genetic susceptibility to disease in different mouse strains can only be interpreted if the same immunization protocol is used. Discrepancies have been reported in the same strain studied by different approaches. For example, BALB/c and C57BL/6 mice are susceptible and resistant, respectively, to Graves'-like hyperthyroidism in the TSHR-adenovirus model (86) although C57BL/6 mice produce antibodies more readily than BALB/c in response to TSHR-DNA vaccination (92). In addition, BALB/k mice are highly susceptible to developing hyperthyroidism in the TSHR-adenovirus model (86) but are poor responders in the Shimojo model (74).

2. MHC genes. Despite the foregoing evidence against a role for MHC genes in mouse models of hyperthyroidism, evidence of a role for MHC class II genes in response to the TSHR comes from studies using TSHR-DNA to vaccinate mice that express *human* HLA molecules. These mice are null for endogenous mouse MHC class II. One study has been performed using mice transgenic for HLA-DR3 or for HLA-DQ6b, which are or are not (respectively) associated with Graves' disease in Caucasians. In these strains, both on mixed C57BL10 backgrounds, some DR3 mice (but not DQ6b animals) developed TSHR antibodies as well as thyroid lymphocytic infiltration (94). In another study, approximately 30% of DR3-transgenic on a NOD background (and also lacking endogenous class II) developed TSAbs and elevated T_4 as well as thyroid lymphocytic infiltration (95). Moreover, these responses were associated with thyroid damage and the appearance of Tg antibodies, consistent with data for wild-type NOD that lack the human MHC transgene (263).

Overall, the evidence from mouse models of Graves' hyperthyroidism is that non-MHC genes play a greater role than MHC genes in determining susceptibility to hyperthyroidism, a finding compatible with that for autoimmune Graves' disease in humans (reviewed in Ref. 93).

B. Environmental factors

Environmental factors associated with Graves' disease in humans include iodine, smoking, infection, and stressful life events (reviewed in Ref. 264). The possible involvement of specific pathogen(s), such as *Yersinia enterocolitica*, have long been of interest because of suggested cross-reactivity with the TSHR (reviewed in Ref. 265). To date there are no definitive data linking *Y. enterocolitica* (or any other organism) to Graves' disease in humans. However, mouse models have provided the opportunity, intentional or otherwise, to explore the role of environmental factors in responses to the TSHR as well as for the development of Graves' disease.

1. TSHR-DNA vaccination. The outcome of naked DNA vaccination is variable in BALB/c mice. TSHR antibodies were readily induced in one laboratory (84) but not in others (77, 85, 86, 112). As discussed earlier (*Section III*), the reasons for these differences are unclear and include different vaccination protocols (cardiotoxin pretreatment *vs.* DNA in sucrose; single *vs.* multiple injections) and possible genetic drift between BALB/c strains separated early in the 20th century. However, one factor that seems increasingly likely to play a role is the use of conventional *vs.* pathogen-free housing facilities for the mice. It is well recognized that diabetes incidence varies widely in NOD mouse colonies around the world, despite similar breeding protocols, and likely reflects environmental factors including housing conditions (266). In this context, it is of interest that TSHR antibodies are reported to be readily induced in mice housed conventionally (84) but only rarely in animals immunized using the same protocol in pathogen-free facilities (85, 112).

Until recently, this hypothesis received little support. However, a new study clearly indicates the importance of environmental factors in a Graves' model. In this model, BALB/c mice are primed by TSHR-DNA vaccination and their splenocytes are removed and boosted *in vitro* with TSHR antigen before transfer into naive recipient mice. When performed in Brussels, recipient mice developed thyroiditis and ocular pathology resembling GO without TSHR antibodies (87). However, attempts to reproduce the model in Cardiff were unsuccessful. Using the same mouse substrains and identical protocols (including transfer of bedding, food, and water from Brussels to Cardiff), thyroiditis and orbital changes were absent although some mice developed TSAbs and became hyperthyroid (88). Both animal facilities (Brussels and Cardiff) are conventional, and common pathogens in each facility may be different from each other as well as at different times when the studies were performed. Consequently, although unlike conditions in human societies, pathogen-free animal housing is likely to be preferable to ensure experimental consistency.

2. TSHR-adenovirus. In the adenovirus model, disease incidence is essentially the same in mice maintained in a specific pathogen-free condition and those in a conventional housing condition (96). Moreover, simultaneous administration of microbial components, *Escherichia coli* lipopolysaccharide or *Saccaromyces cerevisae* zymosan A [the ligands for Toll-like receptors 4 and 2, respectively, and stimulators of innate immunity (reviewed in Ref. 267)] also had little effect (96). Therefore, unlike for naked DNA vaccination (see above), in the TSHR-adenovirus model environmental microbial agents do not have a substantial impact on the induction of Graves'-like hyperthyroidism in BALB/c mice. It is possible that the powerful adjuvant effect of adenovirus obscures any effect of microorganisms.

Use of pathogen-free facilities provides the opportunity to study the outcome of planned infections. As described in *Section VII*, prior infection with *S. mansoni* or *M. bovis* BCG suppressed Graves' disease significantly, indicating that certain infectious microorganisms may possibly negatively influence disease occurrence. Because 1) *S. mansoni* and *M. bovis* BCG infections induce Th2 and Th1 immune deviations, respectively, as mentioned above (257, 258), and 2) Th1 polarization by adenovirus expressing IL-12 had little effect on disease development (256), the protective effect of *M. bovis* BCG cannot solely be explained by altered Th1/Th2 balance. Instead, these results may fit the hygiene hypothesis or counterregulatory model (268, 269). This concept proposes that reduced exposure to either Th1- or Th2-promoting microorganisms during childhood in developed countries impairs

the development of an appropriately educated immune system. Under these conditions, there is increased likelihood that the individual will develop not only Th1-type autoimmune diseases but also Th2-type allergic diseases in adults. Similar results have been reported for other models of Th1 autoimmune disease including type 1 diabetes, experimental autoimmune encephalomyelitis, and arthritis (reviewed in Ref. 270). More importantly, the incidence of these diseases in humans is increasing in developed countries (reviewed in Refs. 268 and 269).

Overall, the development of Graves' disease in particular, and autoimmune diseases in general, may be affected by certain infectious pathogens regardless of their ability to modify Th1/Th2 balance. Animal models provide an opportunity to test experimentally these epidemiologically derived hypotheses.

3. Iodine. Excess iodide intake in humans is associated with thyroid dysfunction, particularly in individuals predisposed to thyroid autoimmunity (reviewed in Ref. 271). NOD.H-2h4 mice develop Tg autoantibodies and thyroiditis spontaneously, and the process is enhanced by iodide administration (272–274). Potential interactions between induced TSHR antibodies, spontaneous thyroid autoimmunity, and iodide intake were investigated in NOD.H-2h4 mice immunized with TSHR-adenovirus (116). Tg autoantibody levels and thyroiditis severity were enhanced by dietary iodide but were unchanged by TSHR immunization. Conversely, iodide intake had no effect on induction of TSHR antibodies (116). A high-iodide diet reduced hyperthyroidism in TSHR-adenovirus-immunized mice, probably by a mild suppression of thyroid hormone secretion with thyroid hormone levels generally remaining in the normal range, an effect demonstrated in humans (275). Overall, iodide intake influenced spontaneous thyroid autoimmunity, but not induced immunity, to the TSHR.

IX. Summary

Graves' disease can be induced in mice or hamsters by novel approaches that involve *in vivo* expression of the TSHR. TSAbs and hyperthyroidism develop in animals injected with TSHR-expressing cells (fibroblasts, B cells, and dendritic cells) (73, 79, 100) or by vaccination with TSHR-DNA (91) or TSHR-adenovirus (86). Moreover, virtually alone among models of experimental autoimmunity, coadministration of a traditional adjuvant (Freund's, lipopolysaccharide, or pertussis) is not required. As would be expected, mTSAbs also induce hyperthyroidism, either acutely (within days) after injection of purified IgG (161, 177) or gradually (from 3 months of age) in transgenic mice expressing the Ig genes for a low-affinity human TSAb (104). Thyroiditis is associated with TSHR antibodies and/or hyperthyroidism in some models (78, 79, 84, 91, 94, 95) but not in others (73, 75, 86). However, lymphocytic infiltration of the thyroid gland is variable, and its development may be related to environmental factors (88).

The first Graves' model was reported in 1996. Since that time, the field has been expanding rapidly, extending information reviewed earlier (220, 276) as well as more recent overviews (7, 277). Much has been learned about the models themselves, and important insights have been gained into Graves' disease in humans:

1. Depending on the model, induction of hyperthyroidism can be modified by manipulating immune responses toward Th1 or Th2, using adjuvants, cytokines, or appropriate knockout mice. Biasing immune responses toward Th1 and away from Th2 reduced hyperthyroidism in some (75, 80, 255), but not all (100), cell-based models. Conversely, immune manipulation toward Th2 prevented Graves' disease induced by injecting TSHR-adenovirus (256, 257). The IgG subclass of human TSAbs suggests a role for Th1 cytokines (252). However, Graves' disease in humans may be heterogeneous and may be subject to differential regulation in individual patients.

2. Shedding of the TSHR A subunit, consequent to receptor cleavage, appears to be important for the development of TSAbs (97, 98) and for the balance between TSAbs and TBAbs (97). The underlying hypothesis arose from observations for human TSHR antibodies (120) and could only be tested in an experimental animal model. Although not intuitive, high levels of TSHR antibodies do not equate with high TSAbs and hyperthyroidism. Instead, high titers are associated with spreading of antibody epitopes and increased TBAb activity (99). These findings are consistent with elevated TSHR antibody levels in rare patients with hypothyroidism due to TBAbs (*e.g.*, Refs. 120 and 131).

3. The first mTSAbs were isolated from animals that developed hyperthyroidism after TSHR-DNA vaccination (158, 160) or TSHR-adenovirus injection (159). The demonstration that high-affinity mTSAbs have TBI activity definitively answered a long-standing controversy, namely that TSAbs and TBI represented different antibodies (as others postulated) rather than as activities of the same antibody in different assays. As previously observed for human TSAbs, the epitopes of murine mTSABs are conformational (158, 160, 161, 166) and cannot be defined with synthetic peptides. Importantly, their binding sites overlap with those of human TSAbs as well as human TBAbs (158, 161, 167).

4. The role of APCs and T cells in the immune response to the TSHR has been explored in the mouse models. These studies (85, 112) arose from the difficulties encountered with some strategies for inducing TSHR antibodies and subsequently addressed issues of antigen presentation (90) and the immune cells (89) involved in TSHR immunization. Moreover, analysis of antigen-specific lymphoid cell responses *in vitro* provided information concerning the Th1/Th2 cytokine bias in different models (85, 112, 118, 255, 256) and provided an approach for establishing the T cell epitopes on the TSHR recognized by different mouse strains (119, 222).

5. Immune tolerance toward the TSHR is being investigated using Graves' models. With one exception (79), the human TSHR is used to induce antibodies that cross-react with mouse (or hamster) TSHR to cause hyperthyroidism. Whether these protocols break self-tolerance to the host TSHR is not clear. Because central T cell tolerance involves deletion of T cells with high affinity for self-proteins expressed ectopically in the thymus, TSHR null mice cannot develop self-tolerance for this protein. Unexpectedly, TSHR knockouts were no more responsive to vaccination with hu-

man TSHR-DNA (a non-self-protein) than wild-type mice (223), suggesting limited or absent central TSHR tolerance for the murine TSHR in normal mice. However, transgenic mice with the human A subunit targeted to the thyroid exhibited profound tolerance because neither T cell responses nor antibodies were induced by immunization with adenovirus (244). In transgenics expressing the Ig genes of a low-affinity TSAb (104), self-reactive B cells were deleted from all immune compartments except the peritoneum.

6. Inbred mouse strains provide invaluable information on the contribution of genetic (74, 86, 96, 262) and environmental factors (including iodine) (88, 116) to induced Graves' disease. The contribution of nonmajor MHC, rather than MHC, genes in mice parallels the findings for human thyroid autoimmunity (reviewed in Ref. 93) and confirms the relevance of these models to human disease. It is of interest that some mice transgenic for the Graves' MHC susceptibility allele HLA-DR3 develop thyroiditis (94, 95) in response to TSHR-DNA vaccination under conditions that do not induce thyroiditis in other strains.

7. The models suggest that the development of Graves' disease is affected by infectious pathogens regardless of their ability to modify Th1/Th2 balance (257, 258). These data provide evidence for the hygiene hypothesis in murine autoimmune hyperthyroidism and suggest that this hypothesis is also applicable in human Graves' disease.

X. Conclusions

It must be recognized that all animal models, particularly those that are experimentally induced, have their limitations. Nevertheless, as suggested by Taneja and David (261), studies of the same disease in multiple models provide greater insight than investigation of a single model. Thus, individual models provide information on different aspects of Graves' disease, such as the outcome of different induction protocols, the role of particular genes, and the contribution of background genes and environmental factors to disease. Integrating these data will ultimately enhance the overall understanding of this particular autoimmune disease in humans.

The database on Graves' models is rapidly expanding to provide exciting, unexpected, and sometimes controversial information. Future studies, some ongoing, will address the role of T-regulatory cells (Treg), a "hot topic" in immune responses to infectious organisms and autoimmunity (278). Manipulating Treg alone or together with costimulatory signals (279) will be explored to elucidate tolerance and control over immune responses to the TSHR. Moreover, the contribution of receptors involved in innate immunity including Toll receptors (267) and C-type lectin receptors [such as the mannose receptor (179)] is likely to be a fruitful topic. The models are revealing the potential obstacles of preventing and, even more difficult, treating Graves' disease induced in animals, indicating that a "quick fix" immunological treatment is unlikely in the near future. Instead, developing immunospecific forms of therapy for Graves' disease will require painstaking dissection of immune recognition and responses to an intriguing and unusual member of the glycoprotein hormone G protein-coupled receptor family, the TSHR.

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