Attenuation of Induced Hyperthyroidism in Mice by Pretreatment with Thyrotropin Receptor Protein: Deviation of Thyroid-Stimulating to Nonfunctional Antibodies

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Graves’-like hyperthyroidism is induced by immunizing BALB/c mice with adenovirus expressing the thyrotropin receptor (TSHR) or its A-subunit. Nonantigen-specific immune strategies can block disease development and some reduce established hyperthyroidism, but these approaches may have unforeseen side effects. Without immune stimulation, antigens targeted to the mannose receptor induce tolerance. TSHR A-subunit protein generated in eukaryotic cells binds to the mannose receptor. We tested the hypothesis that eukaryotic A-subunit injected into BALB/c mice without immune stimulation would generate tolerance and protect against hyperthyroidism induced by subsequent immunization with A-subunit adenovirus. Indeed, one sc injection of eukaryotic, glycosylated A-subunit protein 1 wk before im A-subunit-adenovirus immunization reduced serum T4 levels and the proportion of thyrotoxic mice decreased from 77 to 22%. Prokaryotic A-subunit and other thyroid proteins (thyroglobulin and thyroid peroxidase) were ineffective. A-subunit pretreatment reduced thyroid-stimulating and TSH-binding inhibiting antibodies, but, surprisingly, TSHR-ELISA antibodies were increased. Rather than inducing tolerance, A-subunit pretreatment likely expanded B cells that secrete nonfunctional antibodies. Follow-up studies supported this possibility and also showed that eukaryotic A-subunit administration could not reverse hyperthyroidism in mice with established disease. In conclusion, glycosylated TSHR A-subunit is a valuable immune modulator when used before immunization. It acts by deviating responses away from pathogenic toward nonfunctional antibodies, thereby attenuating induction of hyperthyroidism. However, this protein treatment does not reverse established hyperthyroidism. Our findings suggest that prophylactic TSHR A-subunit protein administration in genetically susceptible individuals may deviate the autoantibody response away from pathogenic epitopes and provide protection against future development of Graves’ disease. (Endocrinology 150: 3944–3952, 2009)
erythematous in New Zealand Black (NZB) mice (2, 3) as well as from investigating experimentally induced disease, notably from collagen-induced arthritis and experimental autoimmune encephalitis, models for rheumatoid arthritis and multiple sclerosis, respectively (4, 5).

Graves’ disease can be induced in susceptible mouse strains such as BALB/c by immunization with adenovirus expressing the full-length human TSHR (6) or its A-subunit (7). Immune deviation away from T helper 1 toward T helper 2 type responses using cytokines (8, 9) or Schistosoma infection (10) reduces the proportion of mice that become hyperthyroid, but neither of these protocols can treat animals with established hyperthyroidism. Using decoy molecules of the TNF family ligand inhibitors B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) to target B cell proliferation or survival factors, hyperthyroidism was reduced in mice with ongoing Graves’ disease (11). Moreover, a monoclonal antibody to B cells (rituximab) is being used to treat patients with Graves’ hyperthyroidism or ophthalmopathy and likely acts by interrupting antigen presentation to T cells (e.g. Refs. 12–14). However, these non-antigen-specific immune manipulations carry the risk of unforeseen and potentially serious side effects (reviewed in Ref. 15).

Dendritic cells (DCs) play critical roles in antigen presentation. Immune responses are initiated by mature DCs that express major histocompatibility complex class II antigens and costimulatory molecules. For example, Graves’ disease is induced by transferring DCs infected with TSHR-expressing adenovirus (16) or the TSHR A-subunit (17) to recipient mice. However, in the absence of maturation signals, immature DCs induce antigen-specific peripheral T cell tolerance (e.g. Ref. 18).

Receptors present on macrophages and DCs, such as the mannose receptor, enhance endocytosis of glycosylated antibodies and increase the efficiency of antigen presentation to T cells (19). The mannose receptor has eight carbohydrate recognition domains and an amino-terminal cysteine-rich domain that binds sulfated carbohydrates (20). All three thyroid autoantigens, the TSHR A-subunit, thyroglobulin (Tg), and thyroid peroxidase (TPO), are glycosylated and the glycan moieties of Tg are sulfated (21, 22). The mannose receptor interacts with Tg via its cysteine-rich domain (23, 24). More importantly, despite no interaction with TPO, the carbohydrate recognition domains of the mannose receptor bind to Tg and very strongly to the TSHR A-subunit (24).

Recently it was shown that an adaptive immune response to antigens captured by the mannose receptor on antigen-presenting cells also requires innate immune system activation, such as by coadministering endotoxin (25). Antigen presentation in the absence of the latter signal induces tolerance. Because highly glycosylated TSHR protein is avidly captured by mannose receptors on antigen-presenting cells (24), we hypothesized that preadministering such protein without activating the innate immune system would attenuate the induction of hyperthyroidism by subsequent immunization with A-subunit adenosivus. The present study confirms this hypothesis but not by the anticipated mechanism. Unexpectedly, rather than inducing tolerance, TSHR protein pretreatment diverted the antibody response away from functional thyroid-stimulating antibodies (TSAbs) toward production of nonstimulatory TSHR antibodies.

Materials and Methods

Eukaryotic TSHR A-subunit, TPO, and Tg

The recombinant A-subunit (TSHR-289) is expressed in Chinese hamster ovary (CHO) cells, purified by affinity chromatography, and dialyzed against 10 mM Tris (pH 7.4) and 50 mM NaCl (26). Two forms of this recombinant protein can be isolated. Inactive TSHR-289 is recognized by a mouse monoclonal antibody (3BD10) but not human TSHR antibodies; conversely, active TSHR-289 is recognized by and neutralizes TSHR antibody activity in Graves’ sera (27, 28). To preclude the possibility of passive neutralization of TSHR antibody neutralization, we used inactive TSHR-289, hereafter referred to as A-sub-CHO. Recombinant human TPO was purified by affinity chromatography from the supernatants of transfected CHO cells (29). Human Tg (>96% pure) was purchased from Calbiochem (San Diego, CA).

Prokaryotic TSHR-A-subunit

TSHR-289 was expressed in bacteria as a fusion protein coupled to the C terminus of thioredoxin (Trx). Briefly, the cDNA for TSHR-289 with 6-terminal histidine residues and BamHI and XhoI restriction sites (5’ and 3’, respectively) was amplified by PCR from pcDNA-TSHR289 (30). After digestion with BamHI and XhoI, the DNA fragment was ligated into the plasmid pET-32a(+) (Novogen, La Jolla, CA) to generate pET-TSHR289. Escherichia coli strain Origami cells (Novogen) were transformed with this plasmid and grown at 37 °C in Luria-Bertani broth (LB) medium containing 50 μg/ml ampicillin. After the OD 600 nm reached 1.0, protein expression was induced by adding 0.3 mM isopropyl-β-D-thiogalactoside (Sigma-Aldrich, St. Louis, MO) and incubation continued at 30 °C for 3 h. The cell suspension was centrifuged (7,000 rpm, 10 min), the pellet dissolved in 20 ml phosphate buffer, sonicated by ultrasound, and centrifuged (4 °C, 12,000 rpm, 10 min) to obtain inclusion bodies. The pellet was solubilized (6 M guanidine in phosphate buffer), the lysate centrifuged (12,000 rpm, 4 °C, 10 min), and the supernatant applied to a His-Trap HP column (Amersham, GE Healthcare Bio-Sciences Corp., Piscataway, NJ) pre-equilibrated with 8 M urea in phosphate buffer. After extensive washing (8 M urea, 100 mM imidazole in phosphate buffer), TSHR289-Trx (subsequently referred to as A-sub-Trx) was eluted (8 M urea and 1000 mM imidazole in phosphate buffer) and dialyzed against PBS containing 1 M urea (1 h) and then against PBS (overnight). Trx protein was expressed as a soluble protein using the empty pET-32a(+) plasmid and purified without guanidine or urea. The specificity of A-sub-Trx was confirmed by immunoprecipitation (not shown) using an in-house mouse monoclonal antibody (3H9) that recognizes the N terminus of the TSHR.

Mice, pretreatment, and induction of Graves’ disease

Female BALB/c mice (6 wk old) were obtained from Jackson Laboratory (Bar Harbor, ME). Adenosivus expressing the human TSHR A-subunit (A-subunit-Ad, amino acids I–289) and Null-adenovirus (Con-Ad) have been described (7, 31). Adenosivues were propagated in HEK293 cells (American Type Culture Collection, Manassas, VA), purified on CsCl density gradients, and viral particle concentration determined from the absorbance at 260 nm (32).

For pretreatment, mice received one sc injection on the back with A-sub-CHO (0.1–10 μg) or A-sub-Trx (8 μg) in 50 μl saline. Control injections were saline alone (0 μg) or the following proteins: Tg (50 μg), TPO (10 μg), or Trx (3 μg) (also in 50 μl saline). [Non-A-sub-CHO proteins (A-sub-Trx, Trx, Tg, TPO) were injected in approximately equivalent molar concentrations as 10 μg A-sub-CHO]. One week later, mice were immunized im in the thigh with A-sub-Ad (pAdHIM4CM vector; 109 particles/injection). Three weeks later, the second A-sub-Ad immunization was performed. As controls for A-sub-Ad, mice were pretreated with saline and immunized twice with control adenosivus (Con-Ad; 109 particles/injection (31). One week after the second adenosivus injection, blood was drawn for analysis or mice were euthanized.

In the second part of the study (detailed in the text), two different protocols were used: 1) mice pretreated with A-subunit-CHO or A-sub-
with human TSHR-expressing CHO cells (not shown). TSAb values are presented as the mean ± SEM for cAMP release expressed as a percentage of the response from Con-Ad immunized mice.

**TBI**

TBI was measured using a commercial kit (Kronus, Boise, ID). Serum aliquots (25 μl) were incubated with detergent solubilized TSHR; 125I-TSH was added and the TSHR antibody complexes were precipitated with polyethylene glycol. TBI values were calculated from the formula: $[1 - (\text{TSH binding in test serum-nonspecific binding})/(\text{TSH binding in normal serum-nonspecific binding})] \times 100$.

**ELISA for TSHR antibody**

The ELISA was performed using wells coated with A-subunit-CHO protein (see above; 5 μg/ml) as previously described (7). After incubation with test sera (duplicate aliquots, 1:100 dilution), antibody binding was detected with horseradish peroxidase-conjugated goat antinuine IgG (Sigma Chemical Co., St. Louis, MO), and the signal was developed with o-phenylenediamine and H$_2$O$_2$. TSHR ELISA antibodies are reported as the OD at 490 nm.

**Statistical analyses**

The statistical significance of differences between responses were determined by Student’s t test or ANOVA for normally distributed values or by nonparametric tests (rank sum and ANOVA on ranks). A χ² analysis was used to determine the significance of proportional differences between groups of mice.

**Results**

**A-subunit protein and protocol for pretreatment**

We used two types of TSHR protein: TSHR A-subunit generated in eukaryotic (CHO) cells, referred to as A-sub-CHO, and the A-subunit generated in bacteria fused to Trx, abbreviated A-sub-Trx. On SDS-PAGE (Fig. 1A), A-sub-Trx and Trx (the bacterial fusion partner) run as sharply focused bands (~47 and 20 kDa, respectively). In contrast, A-sub-CHO runs as a broad, diffuse band (~50–70 kDa), reflecting its extensive glycosylation (e.g. Ref. 24).

In a preliminary study, we first tested a wide concentration range of TSHR A-sub-CHO to examine whether pretreatment with this protein would ameliorate the induction of hyperthyroidism by A-sub-Ad immunization. Mice were injected once with TSHR A-sub-CHO (0, 0.1, 0.3, 1, and 3 μg) 1 wk before the first of two A-sub-Ad immunizations; sera were tested 1 wk after the second immunization (Fig. 1B). Baseline serum T₄ values were obtained from mice immunized with Con-Ad. As expected, the majority of mice pretreated with saline (0 μg) before A-sub-Ad immunization developed elevated T₄ levels. Pretreatment with A-sub-CHO did reduce the induction of hyperthyroidism but only with the highest concentration (3 μg) (Fig. 1C). Consistent with lower T₄ levels, TSAb activity was decreased in mice pretreated with 3 μg of A-sub-CHO (Fig. 1D).
Specificity of A-sub-CHO and relevance of glycosylation

To expand on these observations, in two subsequent experiments, we used higher doses of A-sub-CHO (up to 10 µg) and larger numbers of mice (10 vs. five per group). In the first of these two studies, we included the thyroid antigens Tg and TPO to provide specificity controls. As mentioned, the TSHR A-subunit and Tg (but not TPO) bind to the mannose receptor (23, 24). Moreover, carbohydrate is required for TSHR A-subunit binding to the mannose receptor (24). Therefore, in the second of these two studies, we compared the outcome of pretreatment with highly glycosylated A-subunit-CHO vs. nonglycosylated A-subunit expressed in bacteria as a fusion protein (A-sub-Trx). For both studies, we used the protocol outlined in Fig. 1B: a single sc injection of A-sub-CHO, A-sub-Trx, or control proteins (Tg, TPO, or Trx) a week before the first of two A-sub-Ad immunizations followed by tail bleeding 1 wk later (wk 5).

Only pretreatment with A-sub-CHO (10 µg), not Tg or TPO, significantly reduced serum T4 levels compared with saline (0 µg), Tg, or TPO (Fig. 2A). Moreover, unlike glycosylated A-sub-CHO, pretreatment with TSHR bacterial fusion protein did not lower T4 levels compared with controls (saline or Trx) (Fig. 2B). These data confirmed the specificity of A-subunit protein pretreatment, as well the requirement for glycosylated TSHR, to block hyperthyroidism induced by A-sub-Ad immunization.

Combining the data from the foregoing studies, A-sub-Ad immunization induced hyperthyroidism in 77% (50 of 76) of mice pretreated with control substances (saline, Tg, TPO, or Trx) in contrast to only 22% (five of 23) of animals injected with 10 µg glycosylated A-subunit protein (P = 0.001, χ² test). Thus, after pretreatment with a single 10 µg dose of A-subunit-CHO protein, thyrotoxicosis developed only in one third of the expected number of animals.

Characterization of TSHR antibodies after pretreatment with TSHR protein

To understand the basis for reduced hyperthyroidism described above, TSHR antibodies were measured using three assays. Consistent with findings from the preliminary experiment (Fig. 1D), TSAb activity was reduced after pretreatment with A-sub-CHO (3 and 10 µg) but not Tg, TPO, or bacterial A-subunit protein (A-sub-Trx) (Fig. 2, C and D). These TSAb data were obtained using mouse TSHR-expressing CHO cells; comparable observations were made with human TSHR-expressing CHO cells (supplemental Fig. 1, published as supplemental data on endo.endojournals.org).
The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). TSHR antibodies measured by TBI were also decreased in mice pretreated with A-sub-CHO (10 and 3 μg) but not Tg, TPO, or bacterial A-subunit protein (Fig. 2, E and F).

Unexpectedly, measuring TSHR antibodies by ELISA revealed a different pattern. A-subunit-CHO pretreatment moderately but significantly enhanced TSHR antibody levels; no enhancement was observed in control mice pretreated with saline, Tg, TPO, A-sub-Trx, or Trx (Fig. 2, G and H). These surprising observations indicated that, contrary to our hypothesis, tolerance had not been induced. Instead, A-subunit protein injected before A-sub-Ad immunization deviated antibody generation away from those detected in Tg, TPO, or bacterial A-subunit protein (Fig. 2, E and F).

Persistence of the suppressive effect of A-subunit protein pretreatment

We investigated the long-term outcome of pretreatment with A-subunit protein before A-sub-Ad immunization. Mice that had received two cycles of A-subunit protein pretreatment (0, 1, 3, and 10 μg) followed by A-sub-Ad and immunization were maintained without manipulation for a further 7 wk until euthanasia (total of 12 wk). As observed at the 5-wk interval (Fig. 2), T₄ levels were significantly reduced at 12 wk in mice pretreated with 10 μg A-sub-CHO vs. saline (supplemental Fig. 2A). Likewise, TBI and TSAb levels were lower after pretreatment with 10 μg or 3 μg A-sub-CHO vs. saline or A-sub-Trx (supplemental Fig. 2, B and C). Overall, serum T₄, TBI, and TSAb values remained stable. Thus, these data provide no evidence for escape from the efficacy of pretreatment with A-subunit CHO and instead demonstrate its long-term effects.

Effect of TSHR A-subunit protein injection on an established TSHR immune response

A-sub-CHO protein pretreatment attenuated the hyperthyroid response to A-sub-Ad immunization, as described above. The question then arose whether A-sub-CHO protein administered for the first time after the development of hyperthyroidism would influence the course of the latter. Two experiments were performed.

In experiment A (Fig. 3, left side), we studied three groups of mice that received control pretreatment (saline, Tg, or TPO) before two A-sub-Ad immunizations. On testing at 5 wk, these groups had similar levels of T₄ and TSHR antibodies (shown below). At 7 and 8 wk, these mice were challenged with A-sub-CHO protein alone, A-sub-Ad alone, or the combination of pretreatment with A-sub-CHO protein followed by A-sub-Ad. Not shown in Fig. 3, mice previously immunized twice with Con-Ad received a third injection of control adenovirus.

In experiment B (Fig. 3, right side), mice pretreated with saline before each of two A-sub-Ad immunizations were challenged at wk 7 with glycosylated or nonglycosylated A-subunit protein (A-sub-CHO protein or A-sub-Trx). Control mice for this experiment (not shown in Fig. 3) were maintained without further treatment.

For both experiments A and B, mice were euthanized at wk 12 and their sera tested for T₄ and TSHR antibodies. The influence of A-sub-CHO protein on the established immune response was assessed by comparing serum T₄ and TSHR antibody levels at the 5- and 12-wk time points.

There were no major differences in the levels of T₄ or TSHR antibodies measured by TBI at wk 5 and 12 in experiment A (Fig. 4, A and B). Consequently, unlike the efficacy of pretreatment, A-sub-CHO protein was unable to reverse hyperthyroidism or reduce TBI activity in mice with established immune responses to the TSHR. Remarkably, completely different observations were made for TSHR antibodies measured by ELISA (Fig. 4C). Compared with the 5-wk parameters, injecting A-subunit-CHO protein markedly enhanced TSHR ELISA antibody levels and even more so when this injection was followed 1 wk later by an immunization boost with A-sub-Ad. Without the injection of A-subunit-CHO protein, the A-subunit-Ad immunization boost only modestly increased TSHR antibodies measured by ELISA.

Recombinant TSHR A-subunits generated in CHO cells are very heavily glycosylated (~40% of their mass). As demonstrated above (Fig. 2), prokaryotic, nonglycosylated TSHR A-subunits administered before A-subunit-Ad immunization did not attenuate induction of TSAb, TBI, or hyperthyroidism. Therefore, in experiment B, we compared the ability of glycosylated A-subunit-CHO vs. nonglycosylated A-subunit-Trx proteins to influence the course of an established immune response after A-sub-Ad immunization. Mice were pretreated with saline, immunized with A-sub-Ad, and then at the 7-wk time point were injected with A-sub-CHO or A-sub-Trx (Fig. 3, experiment B).

As in experiment A, there were no significant differences in serum T₄ or TBI levels when assayed 5 wk later (12 wk time point) (Fig. 5, A and B). These data confirmed the inability of A-sub-CHO to reverse hyperthyroidism or reduce TBI in mice with established immunity to the TSHR. In contrast, TSHR antibodies detected...
by ELISA were boosted by A-subunit-CHO protein and, although to a lesser extent, by A-sub-Trx protein (Fig. 5C).

Together, experiments A and B indicate that A-subunit protein, even if glycosylated, administered after A-subunit adenovirus immunization is unable to reduce pathogenic TSHR antibody levels and ameliorate hyperthyroidism. Unexpectedly, however, both glycosylated and prokaryotic A-subunit protein markedly boosted the levels of nonfunctional TSHR antibodies: antibodies that are detectable by ELISA but do not stimulate the TSHR or block the action of TSH.

**Discussion**

Antigens targeted to the mannose receptor in the absence of stimuli (like endotoxin) to the innate immune system induce tolerance (e.g. Ref. 25). Previously we observed that highly glycosylated TSHR A-subunits bind to the mannose receptor (24). We therefore tested the hypothesis that pretreatment of mice with this TSHR A-subunit protein would establish tolerance to the TSHR A-subunit and prevent or attenuate Graves’-like hyperthyroidism induced by immunization with adenovirus expressing the A-subunit. We injected TSHR A-subunit protein sc, a route known to favor peripherally located mannose receptor-expressing DCs (25).

Incidentally, we had previously attempted to block the induction of hyperthyroidism in mice using adenoviruses encoding costimulation decoys to interfere in the signaling between antigen-presenting cells and T cells. Nonspecific inhibition was an unforeseen consequence of administering a decoy adenovirus (or control adenovirus) together with low dose A-subunit adenovirus, and, therefore, we could draw only limited conclusions from these experiments (31).

In our present studies, we focused on the A-subunit protein itself as a potential tolerogen. Indeed, injecting A-subunit protein before A-sub-Ad immunization reduced serum T4 and significantly decreased the proportion of thyrotoxic mice. This reduction occurred using glycosylated eukaryotic, but not nonglycosylated prokaryotic, A-subunits. Consistent with the attenuation of hyperthyroidism, TSAbs and TBI declined in mice pretreated with A-subunit-CHO. The effect of the A-subunit was specific because neither Tg (which binds) nor TPO (which does not bind) to the mannose receptor (24) was effective. Indeed, injecting A-subunit protein before A-sub-Ad immunization reduced serum T4 and significantly decreased the proportion of thyrotoxic mice. This reduction occurred using glycosylated eukaryotic, but not nonglycosylated prokaryotic, A-subunits. Consistent with the attenuation of hyperthyroidism, TSAbs and TBI declined in mice pretreated with A-subunit-CHO. The effect of the A-subunit was specific because neither Tg (which binds) nor TPO (which does not bind) to the mannose receptor (24) was effective. Overall, these data support the concept of tolerance induction by glycosylated A-
subunits interacting with the mannose receptor (or other lectin-like receptors on antigen presenting cells) in the absence of signals to activate the innate immune system. It is of interest that Kong and colleagues induced tolerance and prevented experimentally induced thyroiditis by pretreating mice with deglycosylated mouse Tg or by TSH infusion to raise circulatory mouse Tg levels (36). This tolerance induced by mouse Tg was mediated by regulatory T cells of the CD4+ CD25+ subset (37).

Surprisingly, however, our deduction that we had induced tolerance by pretreatment with A-subunit-CHO protein was overturned in subsequent analysis of the type of TSHR antibodies generated. There are a number of assays for TSHR antibodies. In the context of hyperthyroidism, the most relevant are a bioassay for thyroid stimulation (TSAb) and a competitive inhibition assay for TSH binding to the native receptor on the cell surface (TBI). In Graves’ disease and hyperthyroidism induced in mice, serum TBI and TSAb are neutralized by a highly conformational form of recombinant, glycosylated TSHR A-subunits generated in eukaryotic cells (27, 28, 38). Coating of ELISA wells with this same antigen abolishes recognition by Graves’ sera. However, nonfunctional TSHR antibodies readily bind to this material on ELISA wells (e.g. Ref. 39). Consequently, in our numerous studies on induced hyperthyroidism in mice, we do not always measure TSHR antibodies by ELISA. Fortuitously, in the present study, we also assayed TSHR antibodies by ELISA. We observed that although pretreatment with A-subunit-CHO protein reduced the subsequent serum Tg, TSAb, and TBI responses to A-subunit adenovirus immunization, TSHR antibody levels measured by ELISA were moderately increased. Therefore, contrary to our expectations, rather than inducing tolerance (such as via the mannose receptor), the injected A-subunit protein diverted the immune response toward the generation of nonfunctional TSHR antibodies.

We propose a mechanism to explain our unexpected findings. Recombinant A-subunits generated in CHO cells are purified in two conformational forms: active, recognized by functional TSAb and TBI, and inactive, recognized only by antibodies without functional activity (27). Active A-subunits are conformationally unstable and spontaneously convert to the inactive form. Injecting microgram amounts of A-subunit protein (largely inactive) targets B cells bearing receptors for nonfunctional (ELISA type) antibodies (Fig. 6). Unlike injection of TSHR protein generated in vitro, immunization with adenovirus generates very small (nanogram) amounts of TSHR A-subunits in vivo, much of which is in the active conformation and therefore captured and processed by B cell precursors that ultimately secrete pathogenic antibodies (measured by TBI and TSAb). The adenovirus immunization provides the immune signals required to activate both B cell populations. However, because A-subunit protein pretreatment administers a much larger dose of A-subunit protein than A-subunit adenovirus, B cells secreting ELISA-type TSHR antibodies dominate and attenuate the induction of functional (or pathogenic) antibodies.

Support for the foregoing concept is provided by follow-up studies. Animals not pretreated with A-subunit-CHO protein before A-subunit adenovirus immunization develop mainly pathogenic and some nonfunctional antibodies. In these mice, once the immune response to A-subunit immunization has been established, subsequent injection of A-subunit-CHO protein markedly enhances generation of antibodies detected by TSHR ELISA but not TBI or TSAb. Moreover, although ineffective in the pretreatment phase, prokaryotic A-subunits also enhance ELISA TSHR antibody generation in mice with an established immune response (Fig. 6). Taking all our data together, only glycosylated A-subunits are capable of initially redirecting pathogenic antibodies toward nonfunctional antibodies and attenuating hyperthyroidism. However, once the immune response to the TSHR is established, both glycosylated and nonglycosylated A-subunit proteins can expand the population of TSHR ELISA antibody-secreting B cells. At this time point and despite high levels, these nonfunctional antibodies cannot reduce the production or ongoing effects of TSAb.

Observations in mice transgenic for a hapten-specific antibody provide parallels for our study. Transgenic monoclonal B cells are fixed and unable to undergo antibody affinity maturation that occurs after immunization with the hapten conjugate in nontransgenic normal B cells. Adoptive transfer of transgenic B cells to nontransgenic animals dramatically impairs the affinity maturation process (40). By analogy, B cells that recognize the A-subunit protein that we used for pretreatment correspond to the low-affinity monoclonal hapten-specific population because their antibodies have a low affinity for (that is, do not recognize) the functional TSHR. In contrast, B cells activated by the A-subunit expressed in vivo (by adenovirus) secrete antibodies that recognize (and activate) the TSHR. This B cell population corresponds to the normal B cells that are capable of undergoing affinity maturation after hapten immunization.

Our observations have implications for the murine model of Graves’ disease. First, it is possible to redirect (deviate) antibodies by pretreatment with microgram amounts of TSHR protein. Second, A-sub-CHO treatment cannot reverse hyperthyroidism in mice with ongoing immune responses to the TSHR. Third, we provide insight into the time frame during which TSHR antibodies and/or their effects persist. After two TSHR A-sub-Ad
immunizations, regardless of the A-sub-CHO concentration used for pretreatment, TBI and TSAb levels were essentially unchanged for the following 7 wk. Because the mice were maintained in pathogen-free conditions, environmental antigen exposure could not provide bystander stimulation to maintain antibody responses. The half-life of antibodies is of the order of weeks (e.g. Ref. 41). Therefore, the persistent TSHR antibody levels are a testament to the extended time period (>1 yr) for which antibody-secreting plasma cells can survive (42).

Unlike many autoimmune conditions with multiple and/or unknown autoantigens, the TSHR (particularly the shed A-subunit) is the unequivocal immune target in Graves’ disease. Even for antibody-mediated diseases with recognized autoantigens, stimulatory autoantibodies are rare. An important question arising from our studies is whether A-subunit deviation toward nonfunctional antibodies could be applied therapeutically in humans. Because we were unable to block hyperthyroidism in mice with an established immune response, this treatment is unlikely to succeed in Graves’ patients. Instead, it may be possible to vaccinate against pathogenic antibodies in individuals at risk of developing Graves’ hyperthyroidism, namely the relatives of Graves’ patients. However, apart from the practical hurdles of generating sufficient A-subunit protein, redirecting antibody epitopes may not preclude activating TSHR-specific T cells. Clearly, before the A-subunit protein vaccination approach could be used in humans, it would be essential to ensure that the process did not activate TSHR-specific T cells with the potential to home to the orbit and precipitate or enhance Graves’ ophthalmopathy.

A recent study in humans provides a potential parallel with our findings. As mentioned in the introductory text, anti-CD20 antibody (rituximab) is currently being explored to treat Graves’ disease (12–14). When added to methimazole therapy, rituximab reduced TSAb but not TBI activity when compared with methimazole treatment (44). Differences between the two studies include disease severity as well as time frame. However, if the TSAb (but not TBI) reduction by rituximab is confirmed, these findings will demonstrate the feasibility of deviating away from pathogenic TSHR antibodies in humans.

In conclusion, a single injection of glycosylated TSHR A-subunit before TSHR immunization deviates immune responses away from pathogenic, and toward nonfunctional, antibodies, thereby protecting mice from developing Graves’ hyperthyroidism. The effect is antigen specific and is sustained, at least in the short term, but this approach does not reverse established hyperthyroidism in mice. Consequently, although TSHR A-subunit protein is a potential immune modulator, it cannot be used to treat immunized animals and is unlikely to be useful in patients diagnosed with Graves’ disease. However, in genetically susceptible individuals, vaccination with TSHR A-subunit protein has the potential for deviating away from pathogenic antibodies and providing long-term protection against the development of Graves’ disease.

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