Dynamics of Thyroid-Stimulating and -Blocking Antibodies to the Thyrotropin Receptor in a Murine Model of Graves’ Disease

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Graves’ disease is characterized by the presence of autoantibodies to the TSH receptor (TSHR). There are multiple antibodies to the TSHR, with thyroid-stimulating antibodies (TSAbs) and TSH-stimulating blocking antibodies (TSBAbs), which in patients can fluctuate over time, resulting in changes in disease activity. Recently, animal models of Graves’ disease have been developed, but it is not known whether the induced TSAbs and TSBAbs change spontaneously with time to influence disease. We used fibroblasts expressing major histocompatibility complex (MHC) class II and human TSHR murine model to study anti-TSHR antibody patterns in serial bleeds of 23 animals. Anti-TSHR antibody responses were first detectable after 7–8 wk of first immunization. Moreover, the pattern of the TSAbs or TSBAbs was selected early in the response. The majority of the animals showed anti-TSHR antibodies that were either TSAb or TS Bab responses and were maintained throughout the course of 17–24 wk of the experiment. Remarkably, a proportion of mice (13%) displayed presence of antibodies with both TSAbs and TSBAbs, which appeared to cycle over time and thus mimic the fluctuations described in some hyperthyroid patients. Analyses of the linear epitopes to TSHR by peptide scanning showed that there was no early restricted epitope response. Thus, despite using an inbred strain, the initial response appears to target different regions of the receptor in different animals. Our data show that anti-TSHR antibody epitopes in the model display heterogeneity in TSHR epitopes, which vary in individual animals as well as in their regulation. (Endocrinology 145: 1539–1545, 2004)

THE ROLE OF the TSH receptor (TSHR) as a target autoantigen in Graves’ disease is well recognized. The disease is mediated by autoantibodies to the TSHR, where thyroid-stimulating antibodies (TSAbs) mimic the action of TSH and act as agonists to stimulate thyroid hormone production with consequent hyperthyroidism and goiter (1, 2). Another type of anti-TSHR autoantibody acts as an antagonist by blocking the stimulatory action of TSH or TSAbs resulting in hypothyroidism (1, 2). It is well known that the levels of antibodies to TSHR in serum do not correlate with the clinical status of the patient, which has been attributed to the heterogeneity of the anti-TSHR response (3). Both stimulating and blocking antibodies can coexist in patients’ serum with the result that levels of the specific antibodies or their affinities change over time, leading to changes in the clinical presentation of disease (4, 5). Such dramatic shifts in patterns of epitopes of TSAbs and TSBAbs have been used to explain the remission of Graves’ disease patients during treatment with antithyroid drugs and radiiodine (6–8). Moreover, one of the explanations for the remission of thyrotoxicosis during pregnancy has been the demonstration of change from stimulatory to blocking type antibodies (9).

However, generally studies on spontaneous shifts in patterns of TSAbs and TSBAbs in Graves’ disease patients have been difficult to perform, due to the patients being placed on treatment immediately after diagnosis.

Over the past few years, a number of successful animal models of Graves’-like hyperthyroid disease have been developed in outbred and inbred strains of mice (10–15). One of these models is based upon multiple injections in H-2k mice of fibroblasts transfected to express major histocompatibility complex (MHC) class II and human TSHR, leading to 25–70% of the animals developing TSAbs, accompanied in some animals with thyroid enlargement and hyperthyroidism (10, 14, 16, 17). Although the regions on the TSHR recognized by the stimulating and blocking antibodies are not well characterized, it has been clear from a number of studies using in vitro mutagenesis and chimeric receptors that the antibodies were dependent for binding on conformational determinants, involving discontinuous regions of the receptor (18, 19). One of these important regions on the TSHR in the fibroblast injection model was localized to the amino terminal region of receptor (20). On the other hand, linear epitopes on the TSHR recognized by antibodies have also been studied (14, 15, 21), but their relevance has been debatable (22). Recently, a murine monoclonal antibody (mab) with strong thyroid-stimulating activity was shown by Western blotting to recognize a large TSHR recombinant fragment of residues 22–280 (23). In the fibroblast injection model, using synthetic peptides, the anti-TSHR antibodies recognized epitopes localized to the amino terminal region [amino acids (aa) 97–116], together with another cluster of overlap-

Abbreviations: aa, Amino acid; bTSH, bovine TSH; HBSS, Hanks’ buffered salt solution; mab, monoclonal antibody; MHC, major histocompatibility complex; TPO, thyroid peroxidase; TSAbs, thyroid-stimulating antibodies; TSBAbs, TSH-stimulating blocking antibodies; TSHR, TSH receptor; TT₄, total thyroid hormone.

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ping peptides in the carboxyl terminus (aa 322–401) (14). More recently, in other models such as the plasmid DNA vaccination and the adenovirus models in BALB/c (H-2b) mice, the dominant epitopes have been localized to the extreme amino terminus, cysteine-rich region of the receptor (aa 22–41) (aa 1–21 comprise the signal peptide that is excised in the mature, cell surface expressed TSHR) (21). Thus, the mouse models appear to show differences in their recognition of the dominant, linear epitopes on the receptor that may be related to different genetic backgrounds (14, 20, 21). Nevertheless, despite the differences in linear epitope recognition, the models lead to successful induction of Graves’-like hyperthyroid disease in these animals.

Although the epitopes of the anti-TSHR antibodies have been extensively studied in these models, there is little information on whether both TSAbs and TSBAbs can coexist in the serum, which may influence clinical presentation of the disease. Using the fibroblast injection model, we show that some animals display cycling levels of TSAbs or TSBAbs, whereas others show different patterns of expression of these antibody specificities. Moreover, analysis of linear epitope patterns on TSHR suggest that they are heterogeneous and do not show any evidence of epitope spreading.

Materials and Methods

Establishment of experimental murine model of Graves’ disease

The fibroblast injection model in AKR/N (H-2k) mice using syngeneic fibroblasts transfected to express MHC class II and human TSHR, was used (10, 17). Briefly, 6-wk-old female mice (Harlan UK Ltd., Bicester, UK) were injected with MHC class II and TSHR transfected RT12 fibroblasts (20 × 10⁶ cells) by ip injection in alun/pertussis toxin adjuvant (14, 17). Immunizations were repeated every 2 wk for a total of eight injections. Mice were bled from the saphenous vein every 2 wk. Two separate groups of animals, composed of 10 (group 1) and 13 (group 2) animals, were studied. All tests on immune sera were carried out on individual sera. Total thyroid hormone (TT₄) was determined by RIA using 20 µl undiluted serum (DYNOTest TT₄, BRAHMS AG, Berlin, Germany). Animals were housed under non-barrier-free conditions, approved by UK Home Office regulations and the institution.

TSHR antibody assays

Antibodies to TSHR were assessed by different assays, including TSH-binding inhibition activity using the radioreceptor (human) TRAK II kits (BRAHMS). The assay was performed using 100 µl undiluted serum (single determination) according to kit instructions, and the results expressed as percentage of inhibition of radiolabeled TSH binding (17). Inhibition of more than 10% was considered positive. The TSAb and TSBAb activity of sera was assayed in TSHR expressing JOP9 cells, essentially as described (17), except the assay was performed in salt-free, isotonic Hanks’ buffered solutions (HBSS) containing sucrose and HEPES. JPI9 cells were grown overnight from 30,000 seeded cells per well in a flat-bottomed 96-well plate. Before the assay, the medium was replaced with the isotonic sucrose HBSS buffer (pH 7.2) (20 mM HEPES, 1.26 mM CaCl₂, 5.33 mM KCl, 0.44 mM KH₂PO₄, 0.5 µM MgCl₂, 0.4 mM MgSO₄, 0.37 mM NaHCO₃, 5.6 mM glucose, and 222 mM sucrose) supplemented with 1.5% BSA and 0.5% isotybul-1-methylxanthine (Sigma-Aldrich, Poole, UK). For measurement of TSAb, test serum (3 µl) was added to each well containing 87 µl isotonic HBSS buffer and incubated at 37 C for 4 h. The cAMP released into the medium was measured by RIA (R&D Systems, Oxford, UK) after 1.4 h dilution in the ED2 dilution buffer in the kit. The cAMP concentrations are expressed as picomoles/milliliter. As controls, prebleed serum from animals before immunization was used to determine the normal + 3 sn range. In all assays, serum from two or three nonimmunized AKR/N female animals was always used as normal controls. For TSBAbs, the assay was carried out as above, except after 2 h incubation, a suboptimal concentration of bovine TSH (bTSH) (40 µU/ml) was added, and the incubation continued for an additional 2 h. All serum samples were initially tested in single determination, followed by subsequent assay in duplicate samples. TSAb index was calculated as cAMP (test serum) ÷ cAMP (control serum) and values above 3 were considered positive. TSBAbs were calculated as described (24) using the following formula: (1 – [cAMP test serum in presence of 40 µU/ml bTSH + cAMP control serum in presence of 40 µU/ml bTSH]) × 100. Values of at least 30% were considered positive. For determination of inter- and intraassay coefficients of variation, serum from three immune mice (bleeds obtained by cardiac puncture at the time mice were killed) known to contain high, moderate, and low levels of cAMP stimulatory activity with JP09 cells were used for calculations, with the samples run in duplicates on three separate runs and calculated using INOVA. The inter- and intraassay coefficients of variation for TSAb in the laboratory were 15.7% and 13.6%, respectively. For TSBAbs, these were calculated to be 23.8% and 19.0%, respectively.

Epitope mapping with synthetic peptides of TSHR by ELISA

A complete set of 26 synthetic peptides encompassing the entire TSHR ectodomain, where each peptide was 20 aa (except peptide no. 26 of 14 aa) (aa 397–415) with a 5-a spacer, were provided by Dr. John Morris (Rochester, MN) (25). Three control peptides of similar length [antisense human thyroid peroxidase (TPO) sequence peptides which do not react with anti-TPO antibodies], were provided by Professor Andrzej Gardas (Warsaw, Poland). All peptides were solubilized at 1 mg/ml in dimethyl sulfoxide and stored at −20 C. Before the assay, the peptides were freshly diluted to a final concentration of 2 µg/ml in carbonate bicarbonate buffer [15 mM Na₂CO₃, 35 mM NaHCO₃ (pH 9.6) containing 0.02% sodium azide], and 100 µl added to each well of MaxiSorp (Nunc, Roskilde, Denmark) flat bottom ELISA plates, overnight in the cold room (26). After washing, the wells were blocked 2 h with 1% BSA in PBS-Tween. Mouse serum (diluted 1:500 in PBS-Tween/2% BSA), was incubated in antigen coated microtiter wells at room temperature for 1 h. After washing, a 1:5000 dilution of alkaline phosphatase conjugated goat antimouse IgG Fc antibody (Sigma-Aldrich) was added (100 µl/well) for 1 h. After washing, freshly prepared substrate solution containing p-nitrophenylphosphate in citrate buffer pH 4.0 was added to each well for 40 min. Substrate conversion was measured at 405 nm using a Titertek Plus reader (EFLAB, Helsinki, Finland).

Results and Discussion

At the end of the immunization scheme, analysis of serum for TSAbs and/or TSBAbs by bioassay in two separate groups of mice showed eight animals (80%) and 11 animals (85%) developing anti-TSHR antibodies (Table 1). In TSH-binding inhibition assays, seven animals (70%) were positive for TSHR antibodies (Table 1). Assessment of thyroid function showed two animals with significantly (greater than mean + 3 sn) elevated levels of serum TT₄ (animal nos. 6 and 13, Table 1). As described (10), the thyroid glands from all animals were free of lymphocytic infiltration. These results are in complete agreement with our earlier study (17).

We next examined the appearance of anti-TSHR antibodies in serial bleeds from these groups of animals. Due to the limited blood volume that could be collected in the serial bleeds, anti-TSHR antibody measurements were restricted to examining the TSAb and TSBAb responses. Analysis of anti-TSHR antibodies in both groups 1 and 2 showed that the earliest appearance of anti-TSHR antibodies occurred within 7–8 wk after the first injection of fibroblasts (Fig. 1, A–C). Further analysis of serial samples showed a variety of pat-
TABLE 1. Induced anti-TSHR antibody production and thyroid function (TT4) in female AKR/N mice after injection of transfected fibroblasts expressing MHC class II and human TSHR (RT12) cells

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Mouse no.</th>
<th>TBII (%125I-TSH inhibition)</th>
<th>TT4 (nmol/liter)</th>
<th>TSAbs (pmol/ml, SI)</th>
<th>TSBAbsb (pmol/ml, &gt;30% +ve)</th>
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<td>Group 1</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>RT12 cells + alum/pertussis toxin as adjuvant ip</td>
<td>1</td>
<td>&lt;10</td>
<td>46</td>
<td>67.1 (SI, 22.6)</td>
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<tr>
<td></td>
<td>2</td>
<td>&lt;10</td>
<td>57</td>
<td>3.0</td>
<td>&lt;30</td>
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<tr>
<td></td>
<td>3</td>
<td>58.8</td>
<td>70</td>
<td>51.0 (SI, 17.2)</td>
<td>41.4</td>
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<td>1.3</td>
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<tr>
<td></td>
<td>23</td>
<td>ND</td>
<td>28</td>
<td>0.8</td>
<td>85.6</td>
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Two separate groups (groups 1 and 2) of AKR/N mice were immunized with 20 million indicated cells in saline, ip in adjuvant every 2 wk for a total of eight injections. Data with serum from animals killed on different dates after wk 18 are shown. Values greater than mean ± 3 sd were considered positive and are represented in bold face type. TSH binding inhibitory Igs using a TRAK II kit with 100 μl undiluted serum. Values above 10% are considered positive and are represented in boldface type.

a TSAbs and TSBAbs were measured in bioassay using JP09 cells by stimulation of cAMP in salt-free, hypotonic HBSS buffer. All serum samples for group 1 or 2 were analyzed together in assay on the same day; normal mouse serum backgrounds were 2.96 and 0.75 pmol/ml, respectively. The stimulation index (SI) was calculated with reference to the respective background of the experiment.
b TSH-stimulating blocking antibodies (TSBAbs) values greater than 30% were considered positive (24).
c ND, TBII activity not determined. This group of animals was not killed at the end of experiment, but instead continued to be used as immune animals for a different experiment. Data from serum bleed at wk 17 are shown.

terms of TSAbs and/or TSBAbs during the course of the induced disease. For example, a number of anti-TSHR antibody positive animals showed exclusive TSAb responses that appeared to be maintained beyond 13–15 wk of the course of the experiment (Fig.1A, animal nos. 1, 5, and 6). During this time, there were no detectable TSBAbs in any of the serial samples from these animals (Fig. 1A). In contrast, other TSHR antibody-positive animals showed high levels of TSBAb activity (Fig. 1B, animal nos. 11, 13, and 14). Moreover, a proportion of TSHR antibody positive animals were positive for both TSAbs and TSBAbs (Fig. 1C, animal nos. 3, 8, and 10). Remarkably, qualitative differences in the anti-TSHR response were observed in animal nos. 8 and 10, where different serial bleeds were dominated by either TSAbs or TSBAbs (Fig. 1C). In animal no. 8, for example TSAbs appear first in wk 8 and continue to fluctuate until wk 13, after which at wk 15, TSBAbs show a strong response with no measurable TSAbs (Fig. 1C, animal no. 8). Similarly, animal no. 10 (Fig. 1C) shows a strong TSHR response from wk 8, but as the blocking response declines in wk 13, it is followed by the appearance of measurable TSAbs (Fig. 1C, animal no. 10). Thus, these animals showed anti-TSHR responses that appear to cycle over time with the functional TSAbs or TSBAbs.

Examination of thyroid function in the immunized mice reveal some discrepancies between the TT4 measurements and the presence or absence of TSAbs (Table 1). It is difficult to speculate precisely the reasons for these discrepancies in thyroid function in some animals in this and other studies (11, 17), although thyroid hormone levels in mice are known to be much more variable, which may influence the results (27).

The present results show that the induction of anti-TSHR response in the experimental model leads to the selection of antibodies with TSAb and/or TSBAb specificities. The data also indicate that, in some animals, there may be cycling patterns of TSAbs and TSBAbs, with perhaps an inverse correlation in these specificities over time. We next determined the epitope specificities of the anti-TSHR antibody positive serial sample bleeds. For this purpose, we relied on ELISA with overlapping synthetic peptides of TSHR ectodomain to measure antibodies to linear epitopes on the receptor. The specificity of the peptides was shown using our panel of mabs to TSHR, with known epitopes localized to the amino, middle, and carboxyl regions of the TSHR ectodomain. As shown in Fig. 2A, the mabs bind to their respective peptides (26), confirming the integrity of the peptides in ELISA. We examined whether epitope spreading of linear peptides on TSHR was present in the model by comparing the first serial bleed positive for anti-TSHR antibodies with subsequent serial sample from the same animal. Addition-
ally, we also examined whether there were qualitative differences in epitope recognition in serum samples with TSAb or TSBAbs activities. Representative examples for animals highly positive for TSBAbs and their recognition of peptides for linear epitope antibody specificities are shown in Fig. 2, B and C. On wk 7, when the anti-TSHR response was first detectable, animal no. 11 reacts with P1 (aa 22–41) (Fig. 2B), whereas animal no. 16 recognized P21 (aa 322–341) and P23 (aa 352–371) (Fig. 2C). Examination of the sera 5 wk later showed that the peptide responses had declined. Moreover, no new antibody response to other peptide specificities were detectable (Fig. 2, B and C). A similar pattern of reactivity was

**Fig. 1.** Anti-TSHR antibody profiles in serial bleeds of mice, assessed by bioassay in JP09 cells for TSAbs and TSBAbs. The assay was performed in salt-free, hypotonic HBSS containing sucrose and HEPES, the stimulated cAMP measured and TSAb and TSBAb activity calculated as described in Materials and Methods. The TSAb activity is shown in terms of the induced cAMP in picomoles/milliliter and the TSBAb activity in terms of percentage of TSH-stimulating blocking activity. The *dashed line* represents values of TSBAbs of more than 30%, which were considered positive. A, Serial bleeds from representative animals, mice nos. 1, 5, and 6, which showed the presence of TSAbs only during the entire course of disease. Serial blood samples shown are [1], wk 0; [2], wk 8; [3], wk 11; [4], wk 13; [5], wk 15; [6], sample at the time of killing, which was variable, depending on the time the mice were killed between wk 17 and 20. B, Serial bleeds from representative animals, mice nos. 11, 13, and 14, which showed the dominant presence of TSBAbs during the course of disease. Serial bleed samples shown are [1], wk 0; [2], wk 7; [3], wk 10; [4], wk 11; [5], wk 13. C, Serial bleeds from representative animals, mice nos. 3, 8, and 10, which showed the presence of TSAbs and TSBAbs during the course of disease. Serial bleed samples shown are [1], wk 0; [2], wk 8; [3], wk 11; [4], wk 13; [5], wk 15; [6], sample at the time mice were killed, which was variable between wk 17 and 20.
FIG. 2. Epitope mapping of the anti-TSHR antibodies by ELISA in two different time points in different animals to study epitope spreading and differences in epitope recognition between transition from TSBAβ to TSAb activity. Overlapping synthetic peptides to the TSHR ectodomain were used. A, Determination of the integrity of the peptide ELISA using mouse mabs A7, A9, and A10, whose epitope specificities are known (26). As expected, A7 shows binding to P26, A9 reacts with P13, whereas A10 reacts strongly with P1 peptide, with weaker binding to P14 (26). B, Mouse no. 11, with blocking antibodies only during the entire course of the experiment reacts solely with P1 peptide. C, Mouse no. 16, with blocking antibodies only during the course of the disease, reacts on this occasion with different peptides, P21 and P23. D, Mouse no. 17, with TSAbs.
seen in other animals with TSBAb activity (not shown). In addition, antibodies to linear epitopes were not detectable in serum with only TSAb activity, as shown for a representative example for no. 17 (Fig. 2D). Moreover, there was no evidence of linear epitope spreading in this model, because the early restricted response to linear epitopes was not seen to diversify in subsequent serum samples. The fibroblast model of Graves’-like hyperthyroid disease appears to be characterized by an initial response to different linear epitopes that is restricted, but which fails to spread rapidly.

With regard to the epitopes recognized by the TSBAbs, of particular interest was mouse no. 11 with high blocking activity that recognized peptides in the amino terminal region of the receptor, an immunodominant region that is believed to be targeted predominantly by stimulating antibodies (2, 18, 19, 28). It is interesting that in other studies with human autoantibodies, serum containing pure blocking antibodies like those present in primary myxoedema hypothyroid patients, show heterogeneity in their epitopes, whose location may be spread broadly in the receptor ectodomain (28, 29). Thus, the ability of mouse no. 11 with high blocking activity to react with regions disparate from the carboxyl terminal region parallel the findings using human blocking antibodies and highlight their heterogeneity, with the epitopes spread throughout the entire ectodomain of the receptor. Moving to the determinants recognized by serum with strong TSAb activity, it was interesting that no linear epitope specificities were readily recognized in this study. Considerable evidence suggests that the cysteine-rich, amino terminal region of the TSHR is important for the binding of these disease inducing antibody specificities (30), with the cysteine residues contributing to the formation of highly conformational epitopes. This may explain our failure to identify peptide reactivity with murine sera containing strong stimulating activity, as this population of anti-TSHR antibodies may be critically dependent on the tertiary structure of the determinant.

In the fibroblast injection model in H-2k mice (10), earlier studies on epitope mapping of the induced TSHR antibodies had localized the epitopes using chimeras of TSHR-LHR to a large segment in the amino terminal region of the receptor (20). Another study by peptide ELISA with the same panel of peptides used in this study, led to a more precise definition of the epitopes, where in individual animals, the anti TSHR antibody response was directed specifically to P6 (aa 97–116) and another cluster of peptides in the C-terminal region, comprising of P21 to P25 (aa 322–401) (14). Interestingly, analysis of the anti-TSHR response in individual animals in this study also showed antibody responses to the C-terminal peptide cluster in a number of the animals, but there were also dramatic differences. In particular, none of the animals in this study, which were positive for TSHR antibodies, irrespective of the timing of the serum sample, showed the presence of antibodies specific for P6 (aa 97–116). Instead, a few animals showed responses for another region in the amino terminus of the receptor (P1, aa 22–41), and thus appear to be similar to those described in TSHR DNA-induced models using plasmid or adenovirus vehicles for injection (21).

In summary, this study highlights the diversity of the anti-TSHR response in animals undergoing Graves’-like hyperthyroid disease. This heterogeneity is present despite using inbred strains of mice. Indeed, different animals within the same group of immunizations also continued to show a heterogeneous response. The initial anti-TSHR antibody response appears to be limited to a small number of epitopes, which does not show epitope spreading. An understanding of the non-genetic and molecular factors responsible for the heterogeneity of the anti-TSHR response may give an insight into the mechanisms of epitope selection on the receptor, which may well ultimately determine the development of hyper or hypothyroidism in patients with Graves’ disease.

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T.M. and J.A.G. contributed equally to this study.

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