Immune Response of Mice Transgenic for Human Histocompatibility Leukocyte Antigen-DR to Human Thyrotropin Receptor-Extracellular Domain

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Background: Hyperthyroidism of Graves' disease is caused by auto-antibodies to human thyrotropin receptor (hTSH-R). To elucidate important T-cell epitopes in TSH-R, we studied three models of immunity to TSH-R in mice.

Methods: Mice transgenic for histocompatibility leukocyte antigen DR3 or DR2 were immunized with cDNA for hTSH-R-extracellular domain (hTSH-R-ECD), or hTSH-R-ECD protein, or hTSH-R peptide epitopes. Proliferative responses of immunized splenocytes to epitopes derived from the hTSH-ECD sequence, anti-TSH-R antibody responses, serum thyroxine and TSH, and thyroid histology were recorded.

Results: DR3 mice responded to genomic immunization with proliferative responses to several epitopes, which increased in intensity and spread to include more epitopes, during a 6-week immunization program. DR2 transgenic mice developed weak proliferative responses. Both types of mice developed anti-TSH-R antibodies measured by enzyme-linked immunosorbent assay or TSH-binding inhibition assay in 16–60% of animals. There was evidence of weak thyroid stimulation in one group of animals. Immunization of DR3 transgenic mice to hTSH-R-ECD protein induced a striking response to an epitope with sequence ISRIYVSIDVTLQQLES (aa78–94). Immunization to peptides derived from the TSH-R-ECD sequence (including aa78–94) caused strong responses to the epitopes, and development of immune responses to several other nonoverlapping epitopes within the hTSH sequence (epitope spreading) and antibodies reacting with hTSH-R. This implies that immunization with hTSH-R epitopes produced immunity to mouse TSH-R.

Conclusion: T-cell and B-cell responses to genetic immunization differ in DR3 and DR2 transgenic mice, and there is less genetic control of antibody than of T-cell responses. During both genomic and peptide epitope immunization there was evidence of epitope spreading during the immunization. Several functionally important epitopes are evident, especially aa78–94. However, if similar progressive epitope recruitment occurs in human disease, epitope-based therapy will be difficult to achieve.

Introduction

DEVELOPMENT OF THE AUTOIMMUNE REACTION in Graves' disease (GD) involves the uptake and presentation of immunogenic epitopes of human thyrotropin receptor (hTSH-R) by histocompatibility leukocyte antigen (HLA) class II molecules on the surface of antigen-presenting cells. This allows interaction of the DR-epitope complex with the T-cell receptor on CD4+ T cells, and leads to activation of the T cell,

which in turn produces signals that help B cells produce pathogenic antibodies (reviewed in Ref. 1). This autoimmune disease may involve dysfunction of central regulation (2) or peripheral regulation (reviewed in Ref. 3) of the immune system.

In some autoimmune disease, specific pathogenic epitopes and associated HLA-DR molecules inducing disease susceptibility have been identified. In type 1 diabetes, human glutamic acid decarboxylase 65 protein sequences and HLA-DR4

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(3) are believed to be pathogenic. In myasthenia gravis, human acetylcholine receptor sequences and HLA-DR3 (4) were defined as pathogenic.

In GD, an antibody against TSH-R is known to cause hyperthyroidism (reviewed in Ref. 1). Shed TSH-R-extracellular domain (TSH-R-ECD) may initiate the disease (5). Although important and reactive T-cell epitopes have been observed in both human patients and laboratory models, specific immunodominant epitopes have not been identified with certainty.

We found that memory T cells from GD patients can produce a model of GD in SCID mice (6). Mice immunized with TSH-R-ECD peptides developed a syndrome mimicking GD, and their T-cell lines produce anti-TSH-R antibodies in recipient mice (7). These data suggest the importance of T cells reactive to TSH-R-ECD in developing GD. Further, the importance of HLA-DR3 in susceptibility to GD has been clearly demonstrated (reviewed in Ref. 8).

Models of GD in mice have been developed in several laboratories through immunization using fibroblasts transformed with cDNA for TSH-R and major histocompatibility complex class II protein (9), plasmids expressing hTSH-R (10), adenovirus expressing hTSH-R (11) or hTSH-R-A subunit (12), or cDNA delivered by electroporation (13). Adenovirus immunization is reported to develop a strong T-cell response (reviewed in Ref. 14). Immunization of mice with TSH-R expressed using adenovirus (15) or in a plasmid (16) caused features of GD. Immunization with thyroglobulin induced thyroiditis in HLA-DR3 transgenic mice, but not in HLA-DR2 or HLA-DQ8 mice (reviewed in Ref. 17).

We have studied models of auto-immunity to TSH-R in mice using combined immunization with plasmid expressing hTSH-R-ECD and adenovirus expressing hTSH-R-ECD, or immunization with recombinant hTSH-R, or with peptides derived from the sequence of the TSH-R-ECD. To study epitope progression, the time course of the response to cDNA immunization was evaluated. To elucidate the relation of certain HLA-DR molecules to GD, we used mice transgenic for HLA-DR3 or HLA-DR2, as a GD susceptible or neutral model, respectively.

We also compared responses of T cell immunized *in vivo* to recent published studies describing *in vitro* TSH-R-ECD peptide binding to HLA-DR molecules (18).

Materials and Methods

Mice transgenic for DR3 and DR2

Breeding stock mice transgenic for HLA-DR3 and HLA-DR2 were very kindly provided by Dr. Chella David (Mayo Clinic). These mice have a mixed genetic background, being approximately 75% black/6, but having minor contributions of about 10% CBA and black 10 genes. Peripheral blood mononuclear cells were stained with fluorescein isothiocyanate-labeled anti-DR antibody and evaluated by fluorescent activated cell sorting (FACS). Animals with DR expression on 17% or more of cells were selected for studies. All studies were performed under a protocol approved by the Institutional Animal Care Committee.

Peptide synthesis

Thirty-one 16–20mer peptides spanning the sequence of TSH-R-ECD with overlaps of five to six amino acids were

synthesized as previously reported (19). In addition, we synthesized 10 new peptides (Chi Scientific, Maynard, MA) (Table 1). These were predicted by Epimatrix (Epivax, Inc., Providence, RI) to have high-binding affinity for HLA-DR3, or to multiple DRs, and thus to be possibly important epitopes.

TABLE 1. SEQUENCE, POSITION, AND ARBITRARY NUMERICAL DESIGNATION OF PEPTIDES

No.	Position	Amino acid sequence
1	20–35	GGMGCSSPPCECHQEE ^a
2	30–49	ECHQEEDFRVTCKDIQRIPS
3	44–62	IQRIPSLPPSTQTLKLIET
4	57–75	LKLIETHLRTIPSHAFSNL
5	70–88	HAFSNLPNISRIYVSIDVT
6	83–102	VSIDVTLQQLESHSFYNLSK
7	97–112	FYNLSKVTHIEIRNTR
8	109–124	IRNTRNLTYIDPDALKE
9	119–137	PDALKELPLLKFLGIFNTG
10	132–150	GIFNTGLKMFDPDLTKVYST
11	145–163	TKVYSTDIFFILEITDNPY
12	158–176	ITDNPYMTSIPVNAFQGLC
13	172–186	FQGLCNETLTLKLYN
14	183–198	KLYNNGFTSVQGYAFN
15	195–210	YAFNGTKLDAVYLNKNKY
16	207–222	AYYLNKNKYLTVIDKDAFG
17	217–232	DKDAFGGVYSGPSLLD
18	227–242	GPSLLDVSQTSVTALP
19	237–252	SVTALPSKGLEHLKEL
20	248–263	HLKELIARNTWTLKKL
21	258–277	WTLKKLPLSLSFHLTRADL
22	272–291	LTRADLSYPHCCAFKNQKK
23	286–305	FKNQKKIRGILESMLCNESS
24	301–320	CNESSMQSLRQRKSVNALNS
25	315–334	VNALNSPLHQEYEENLGDSI
26	329–348	NLGDSIVGYKEKSKFQDTHN
27	343–362	FQDTHNNAHYVFFEEQEDE
28	357–376	EEQEDEIIGFQELKNPQEE
29	371–390	KNPQEEETLQAFDSHYDYTIC
30	385–404	YDYTICGDSSEDMVCTPKSDE
31	399–418	TPKSDEFNCPEDIMGYKFLR
32 (like 8)	105–118	HIEIRNTRNLTYID
33 (like 10)	137–150	GLKMFDPDLTKVYST
34 (overlap of 21–22 ^a)	267–282	LSFLHLTRADLSYPHCC
35 (overlap of 26–27 ^a)	339–352	EKSKFDQTHNNAHY
36 (like 29)	376–389	ETLQAFDSHYDYTI
37 (overlap of 5–6 ^a)	78–94	ISRIYVSIDVTLQQLES
38 (overlap of 7–8 ^a)	103–119	VTHIEIRNTRNLTYIDP
39 (overlap of 15–16 ^a)	201–217	KLDAVYLNKNKYLTVID
40 (overlap of 21–22 ^a)	266–284	SLSFLHLTRADLSYPHCC
41 (overlap of 22–23 ^a)	283–297	CCAFKNQKKIRGILE

Overlapping TSH-R-ECD epitopes used in this study, numbered (1–31) from the start of the coding sequence. Mature TSH-R protein starts at position 20. Peptides 32–36 are modifications of the original sequences, designed by Epivax to have higher DR3 binding affinity. Numbers 37–41 were determined by Epivax to have strong affinity across multiple DR alleles. The numbering 1–31, or 1–41, is used in the abscissa of Figures 1–5.

^aAn epitope overlapping two original epitopes.

TSH-R-ECD, thyrotropin receptor-extracellular domain.

These sequences partially overlapped some of the original peptides, or included a binding motif that was severed in the original peptide sequences. The sequences of all peptides and purity were confirmed by reverse phase high-performance liquid chromatography, and purity was 90–95% (Table 1).

Construction of pcDNA3-TSH-R-ECD and pCA13-TSH-R-ECD

A pair of primers was used to amplify the TSH-R19-417 sequence by polymerase chain reaction (PCR) from pRSETC-TSH-R-19-417. The forward primer (5'-CAAGATATCACC ATGCTGGGCGGAATGGGGTGTTCG-3') contains the start codon, Kozak sequence, and EcoRV sequence. The reverse primer (5'-TSH-R-ECD end, 5'-TACCTCGAGCTACAGGAA CTTGTAGCCCATTAT-3') contains the stop codon and Xho-I sequence.

PCR conditions were as follows: 94°C for 5' (94°C for 30", 55°C for 30", and 72°C for 30") × 5 cycles, (94°C for 30", 62°C for 30", and 72°C for 30") × 25 cycles, 72°C for 5', and hold at 10°C. The PCR product TSH-R19-417 was a single 1200 bp band on 1.2% agarose gel. Eluted DNA was digested with *EcoRV* and *XhoI*, to integrate into pcDNA3 (Invitrogen, Carlsbad, CA) or pCA13 (Microbix, Toronto, Canada), which were also digested with *EcoRV* and *XhoI*.

Propagation of AdCMVTSH-R-ECD

pCA13-TSH-R-ECD and pJM17 (Microbix) were co-transfected in 293 cells (Microbix) to make AdCMVTSH-R-ECD. Adenoviruses were propagated, and purified by CsCl gradients (20).

Preparation of human recombinant TSH-R-ECD protein

TSH-R-ECD was prepared in *Escherichia coli* BL21 cells transformed with pRSET-TSH-R 19-417 (TSH-R-ECD) as previously described (21). To increase purity of the TSH-R-ECD, in some studies the eluted material was subjected to a second preparative electrophoresis and the same elution and concentration procedure. Gel purified recombinant hTSH-R-ECD was subjected to further purification using a nickel affinity column, or antibody affinity column, for use in some assays. A Ni-NTA (Invitrogen) column was equilibrated with phosphate-buffered saline (PBS, pH 7.4), and the sample was loaded. The column was washed with 50 mM NaPO₄ and 0.5 M NaCl (pH 8.0), including 20 mM of imidazole. TSH-R-ECD protein was eluted with 50 mM NaPO₄ and 0.5 M NaCl (pH 8.0), including 250 mM of imidazole. The fraction containing TSH-R-ECD was collected, dialyzed against PBS at 4°C, and stored at -60°C until use. Protein purity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentration was determined by BCA protein assay kit (Thermo Scientific-Pierce, Rockford, IL).

We also prepared an affinity column using the monoclonal anti-TSH-R antibody T5-317 obtained from American Type Culture Collection (Manassas, VA). Electrophoretically purified material was past through the affinity column in 0.02 M sodium phosphate (pH 7.0) buffer, and the column was then washed with 0.02 M sodium phosphate (pH 7.0). Bound protein was eluted by changing buffer to 0.1 M glycine-HCl (pH 2.7). One milliliter aliquots were collected and immedi-

ately neutralized with 1 M Tris-HCl (pH 9.0). The protein peak occurred at about 6 mL eluate volume, and this peak was concentrated. This material, showing one band on electrophoresis, was used for enzyme-linked immunosorbent assay (ELISA) of anti-TSH-R antibodies.

We have also used an hTSH-ECD protein prepared by Chesapeake Protein Expression and Recovery Labs (Savage, MD) using recombinant baculovirus technology to express proteins in *Trichoplusia ni* (cabbage looper caterpillar). cDNAs encoding histidine-tagged TSH-R ECD 19-417 were cloned into baculovirus for expression under control of the polyhedron promoter. *T. ni* larvae are then infected with recombinant virus by oral inoculation for whole insect protein expression under temperature- and humidity-controlled conditions for 96 hours. Larvae are harvested and mechanically disrupted to recover expressed protein. Histidine-tagged protein was purified by immobilized nickel affinity chromatography and dialyzed against PBS.

Immunization of HLA transgenic mice with TSH-R-ECD peptides, or TSH-R-ECD plasmid and adenovirus

Plasmid-adenovirus vaccination was done as follows. On day 1, about 7 µg of cardiotoxin was injected into the muscle of one leg of each mouse. On days 6 and 20, 100 µg pcDNA3-TSH-R-ECD plasmid was injected in the same leg. pcDNA3 plasmid was used as a control plasmid. On day 34, about 1 × 10¹¹ particles of AdCMVTSH-R-ECD were given into the leg muscle of the experimental mice, and the same amount of AdCMVbeta-galactosidase was used as a control. On day 48, all mice were sacrificed. In a study of the time course of the response, the immunized mice were sacrificed on days 19, 33, and 48. Groups consisted of 4–11 mice for each comparison. For immunization with hTSH-R-ECD protein, or bovine serum albumin (BSA), 100 µg protein was emulsified in complete freund adjuvant (CFA) and given intracutaneously, followed by two sub-cutaneous injections of protein in incomplete freund adjuvant (IFA) at 2-week intervals.

Peptide immunization was done using 25 µg of individual peptides, or a pool of 100 µg of peptides, on days 0, 7, 14, and 21. The peptides were mixed with complete Freund's adjuvant on day 0 and incomplete Freund's adjuvant (Sigma, St. Louis, MO) on other days. The mice were killed on day 28 or as noted below.

Cell proliferation assay

About 10⁵ splenocytes were incubated in each well of round bottom microplates with or without 10 µg/mL antigens. About 1 µCi of [3H] thymidine was added to each well 48 hours later. After 72 hours incubation, [3H] thymidine incorporation was measured by liquid scintillation counting. Results were expressed as a stimulation index (SI): the ratio of [3H] thymidine uptake in samples with antigen, to samples incubated without antigen. The peptide epitopes used in the studies are listed in Table 1 as numbers 1–41. In some studies we used the peptides listed in Table 1 as numbers 1–36, and in most studies the complete list of epitopes, 1–41. Recombinant hTSH-ECD protein prepared in *E. coli* caused stimulation of T cells in the unimmunized mice, which is presumed to represent contamination with endotoxin. hTSH-R-ECD protein derived from insect larvae was free of this problem.

All assays were done in triplicate for each epitope studied. Data presented in figures are the average SI of all mice in the group for each epitope. Experiments were repeated 2–4 times, and representative studies are presented.

TSH-R-ECD protein ELISA of mouse serum

TSH-R-ECD proteins were diluted in PBS–0.1% TritonX100 and used to coat 96-well plates (250 ng/50 μ L/well) overnight at 4°C. After washing with washing buffer (PBS–0.05% Tween20), the plate was blocked with 5% BSA in PBS at room temperature. Sera were diluted to 1:500–1:4000 in PBS, 0.05% Tween20, 3% BSA, and 1 mM ethylenediaminetetraacetic acid, and 50 μ L of aliquots were added to each well. After incubation for 3 hours at room temperature, the plate was washed with washing buffer, and 50 μ L of a 1:1000 dilution of peroxidase-labeled goat anti-mouse Fc were added to each well. After 1 hour at room temperature, the wells were washed, and 75 μ L o-phenylenediamine dihydrochloride- H_2O_2 solution was added as substrate. The reactions were stopped with 50 μ L of 1 M H_2SO_4 , and absorbance at 450 nm was determined.

TSH binding inhibition assay

TSH binding inhibition (TBII) assay was also done using the TRAK assay kit (ALPCO, Salem, NH). Data are reported as percent inhibition of binding of 125 I-labeled TSH.

Thyroid histology

Preparation of microscope slides for examination of thyroid histology was done at the Brown University Molecular Pathology Lab, Providence, RI.

Results

Genomic immunization and comparison of immunization of mice transgenic for DR3 versus DR2

Groups of DR3 and DR2 transgenic mice were immunized by the standard protocol including pretreatment with cardiotoxin, two injections of plasmid expressing hTSH-R-ECD, and one injection of adenovirus expressing hTSH-R-ECD, and sacrificed 2 weeks after the last injection. Response of splenocytes to hTSH-R peptides, serum TSH, thyroxine (T4), TRAb, hTSH-R ELISA, and thyroid histology were determined.

Mouse splenocytes from immunized DR3 transgenic animals responded to hTSH-R-ECD, and to multiple peptides, including peptide numbers 8, 10, 17, 18, 23, 24, 29, and 30 (SI >2, p vs. control <0.05), and also to 2 and 21 (SI >2) (Table 1; Fig. 1, upper panel). An SI >2 is considered positive, and usually is also statistically significant (p < 0.05) on comparison to control cells reacting to the same peptide. In these studies there did not appear to be one or a few dominant epitope(s), and individual mice or groups of mice (presumably largely congenic) responded strongly to different peptides (see Figs. 1 and 2). There was an increased average response to all TSH-R-ECD epitopes in the DR3+ mice compared to controls (Fig. 1, p < 0.001 by analysis of variance), although the response did not reach an SI of 2 for many peptides. This implies development of a modest response to all TSH-R-ECD peptides induced by cDNA immunization. Since controls were immunized with a similar control plasmid, and with a similar adenovirus, the observed difference is caused by the TSH-R cDNA immunogen, not the plasmid or virus backbone used in the immunization process.

FIG. 1. (A) response of DR3+ mice immunized to thyrotropin receptor–extracellular domain (TSH-R-ECD) cDNA, and control mice, to individual TSH-R-ECD peptides 1–41. Series 1 (diamonds) represents controls, and series 2 (squares) represents experimental animals. The average SI values for the experimental group exceed those for the control group (p < 0.001) and individual peptides induced responses in splenocytes of SI >2. (B) responses of splenocytes from DR2 transgenic experimental and control mice (11 mice in each group) immunized to human TSH-R-ECD (hTSH-R-ECD) cDNA as described. No responses were equal to or greater than SI of 2.

FIG. 2. Response over time of DR3+ mice to immunization. Data are shown for average responses at 2, 4, and 6 weeks after start of immunization to hTSH-R-ECD cDNA.

Splenocytes from DR2 transgenic mice immunized by the same protocol typically did not respond with $SI > 2$ to any of the peptides (Fig. 1, lower panel), although as shown below, they produce anti-TSH-R-ECD antibodies as determined by ELISA and TBII assay. Please note the difference in ordinate scale. None of the peptides induced an $SI > 2$ in these animals.

Epitope recruitment

In a further effort to recognize dominant epitopes, we studied the time course of the response to immunization in DR3+ mice. At 2 weeks after the first immunization, splenocytes responded to peptides 2 and 15. Mice studied at 4 weeks responded to peptides 7, 15, 35, and 36. At 6 weeks, in this experimental group, splenocytes responded to TSH-R-ECD peptides with an SI of > 2 to 17 of the studied peptides, and were significantly positive ($SI \geq 2$) to 20 peptides of the 36 tested (Fig. 2). Again there were many differences in the response of individual mice to the peptides, differences in responses at different times after start of immunization, and no single dominant peptide. However, there was a striking increase in responsivity to particular epitopes (10, 11, 12, 15, 35, and 37) as immunization progressed, as well as recruitment of additional epitopes (5, 14, and 18).

There was significant variability in results from different groups of DR3+ mice studied under an identical protocol, and the exact reason remains unclear. However, the data reported always represent the average of 4–11 similarly treated animals, each tested in triplicate with each epitope. Mice were 6–8 weeks of age at the start of each experiment, and were selected because they expressed human DR3 on $> 17\%$ of peripheral blood mononuclear cell. The pups were the

offspring of six different breeding pairs; thus, possibly there were genomic differences related to the gene insertion site or other unrecognized genomic differences. We do not believe that the variability was an assay problem. The reproducibility of responses to individual peptides within an assay is well shown in Figure 2, and reproducibility when studying two groups of unimmunized animals is shown in Figure 3. Series 1 DR2 mice were immunized to BSA protein using the standard protocol. Series 2 mice were untreated. Spleen cells from both were tested with TSH-R-ECD peptides. The responses are almost identical, and hover around an SI of 1 indicating no response.

Response to immunization with recombinant hTSH-R

The T-cell response to immunization with recombinant hTSH-R produced in *E. coli* was weak. Mice immunized four times with intracutaneous and subcutaneous hTSH-R-ECD in adjuvant developed reactivity to several epitopes with responses significantly greater than in controls, and individual peptide response reached an SI of 2. The TSH-R preparation used in this study was a denatured and nonglycosylated protein, which would probably not develop B-cell responses to native TSH-R. However, presumably it would be taken up by antigen-presenting cells and processed to produce T-cell epitopes similar to those derived from processing native protein.

Results of immunization with hTSH-R-ECD produced in insect larvae were quite different. Mice developed a striking early response to one epitope (#37, 5–6, aa78–94) (Fig. 4) and to the immunizing protein with SI of 7.8 versus 1 in controls (data not shown in graph). These mice also developed

FIG. 3. Reproducibility of peptide assay results. Series 1 were immunized to bovine serum albumin, and series 2 mice were not treated. Spleen cells of both groups of animals were tested with TSH-R-ECD peptides.

FIG. 4. Response of DR3 mice to immunization with hTSH-R-ECD protein. Mice were immunized with 100 μ g rhTSH-R given once sc in the foot pad, and once sc in IFA in the groin. Spleen cells collected and tested 14 days after the second immunization against the panel of epitopes. Peptide 5–6 (shown on the abscissa as #37) is the unique strongly response epitope, while epitope 22–23 (abscissa #41) is also positive, and the cells reacted strongly to the immunizing antigen. The mice displayed positive enzyme-linked immunosorbent assay responses in dilution of sera $>1/4000$ at this time.

anti-TSH-ECD antibodies with titer $>1/4000$ as determined by dilution in the ELISA. The antibodies reacted with both *E. coli* and insect larvae–derived protein. The mice also reacted to other epitopes, with less intensity, as immunization was repeated.

Response to immunization with TSH-R-ECD peptides

Mice were immunized as described in the Materials and Methods section with peptides 12 + 13 + 32 + 33 in complete Freund's adjuvant once, and boosted with the same peptides

in incomplete adjuvant on two occasions. Peptides were selected based on experience in a previously reported study (7). Immunized mouse splenocytes responded to peptides 5, 10, 15, and 33, and borderline to 37 (data not shown). Peptides 10 and 33 share sequence. Responses to some of the peptides used for immunization (peptides 12 and 13, and peptide 8, which shares sequence with #32) were not significant, although responses to other peptides tested were strongly positive (SIs of 3–8).

DR3-transgenic mice were immunized to peptides 5–6 or 32 (Fig. 5). Peptide 5–6 induced a positive response only to the

FIG. 5. (A) responses to TSH-R-ECD epitopes by splenocytes of DR3+ mice immunized to peptide 5–6, or peptide 32. Series 1 represents controls, series 2 shows responses to epitope 5–6, and series 3 shows responses to epitope 32. (B) responses to TSH-R-ECD epitopes by splenocytes of DR2+ mice immunized to peptide 5–6, or peptide 32.

TABLE 2. SEQUENCE AND POSITION OF IMMUNIZING AND RESPONDING EPITOPES AS SHOWN IN FIGURE 5

Position and number	Amino acids	Sequence
#37 in graph (5-6)	78-94	ISRIYVSIDVTLQQLES
6	83-102	ISRIYLSIDATLQRLEP VSIDVTLQQLESHSFYNSLK LSIDATLQRLEPHSFYNSLK
#38 in graph (7-8)	103-119	VTHIEIRNTRNLTYIDP
7	97-112	MTHIEIRNTRSLTYIDP FYNSLKVTHIEIRNTR FYNSLKMTHIEIRNTR
32	105-118	HIEIRNTRNLTYID HIEIRNTRSLTYID

Human TSH-R-ECD sequence is shown above the mouse TSH-R-ECD sequence, with differences in the mouse sequence in bold.

cognate peptide *in vitro* (Table 3). Immunization to peptide 32 induced reactions to the cognate peptide, and also to peptides 6, 7, 32, 37 (5-6), and 38 (7-8). All of these peptides differ by 1-3 amino acids from the mouse sequence. Some of these epitopes do not share any sequence. The peptide sequences involved are shown in Table 2. Immunizing peptide 32 shares no sequence with peptide 6 or 37, but is next to them in the TSH-R sequence. Thus, it appears that the response to sequence aa105-118 induced a response in mouse TSH-R to a neighboring sequence, as an example of epitope recruitment, or spreading. This must indicate that a response developed to epitopes present in the mouse TSH-R, which are similar but not identical to the hTSH-R epitopes used in the immunization and analysis.

A similar phenomenon was seen on immunization of DR2 transgenics with the same peptides. Strikingly, immunization to peptides 5-6 induced responses to peptides 1 through 6, an area spanning 100 bases in the amino-terminal region of the receptor, as well as to the cognate peptide (Fig. 5, lower panel). All of these peptides differ from the mouse sequence in 1-3 amino acids.

Thyroid function

In addition to identification of potential T-cell epitopes involved in the response to TSH-R-ECD, we investigated the effect of immunization on mouse thyroid function. Data are shown in Table 3. We measured antibody response to the TSH-R by ELISA and TBII assay. The recombinant hTSH-R-ECD used in the ELISA was presumed to have possible contaminants. This would not present a problem when assaying responses to genomic or peptide immunization, but could cause confusion when measuring responses to TSH-R-ECD protein immunization since the response could be to a contaminant. The antigen used in the ELISA is a denatured protein, and probably is recognized by antibodies differing from those recognized in the TBII assay. There was, however, a strong concordance in assay responses, suggesting that mice developing an immune response to genomic immunization formed a variety of antibodies to the receptor. A cut-off value of ≥ 20 U in the TBII assay was found to provide the most clear

separation between groups. The TSH assay is a sensitive, heterologous, disequilibrium, double-antibody precipitation radioimmunoassay developed in the laboratory of S. Refetoff. The normal range for murine TSH in this assay is 10-180 mIU/L. Female mice often have low TSH levels.

In these studies, using DR3+ or DR2+ animals, both ELISA and TBII assay indicated the development of antibodies reacting with TSH-R. In the protocol studying the time course of immunization, the average ELISA and percent positive TBII assays increased during 2-6 weeks, as expected. T4 levels in the experimental groups were significantly above controls only in the DR2 animals. TSH levels were lower in experimental groups than in control animals, but the wide range of results made the data statistically insignificant. Many of the samples were hemolyzed, which may cause some error in the determinations.

We can conclude that antibodies to TSH-R were appropriately formed, and had an apparent mild stimulatory effect on thyroid function, but clear-cut thyrotoxicosis was not detected in any of the animals. DR2+ mice were able to mount an antibody response to TSH-R-ECD roughly equal to DR3+ mice, although their response to T-cell epitopes was clearly lower.

Immunization to hTSH-R-ECD protein produced in insect larvae caused production of high-titer antibodies that reacted in equal titer to protein produced in *E. coli*, or insect larvae. Presumably, the *E. coli* protein was not glycosylated, while that from insect larvae was. However, we presume that the glycosylation was not identical to that produced in thyroid cells.

Immunization to four TSH-R-ECD peptides also induced positive ELISA and TBII responses, but no significant difference in average T4 or TSH levels. This experiment reproduced our previous study of immunization to some of the same peptides (7).

Histology

Mice with elevated T4 levels showed histology with colloid ballooning and thyrocyte hyperplasia, unaccompanied by lymphocyte infiltration, which is compatible with mild thyroid stimulation.

Discussion and Conclusions

In this study DR3 transgenic mice responded to immunization using TSH-R-ECD DNA with a much more intense T cells response to TSH-R epitopes than did DR2+ transgenics, but DR2+ mice produced a similar antibody response. The implication of this difference is not certain, but DR3 is associated with GD, and this may imply that the T-cell response is as important in determining GD as is the ability to produce antibodies. The antibodies produced clearly reacted with TSH-R protein and mildly stimulated the thyroid production of T4, but did not cause clear hyperthyroidism, indicating that they are primarily TSH-R-ECD binding, but not stimulating, antibodies. DR3+ transgenic mice were studied on the assumption that they would be more prone to develop anti-TSH-R immunity than other mice, in parallel with the known importance of DR3 in propensity to develop GD in humans. DR2+ transgenics were presumed to represent a relatively neutral DR genotype. While we would have preferred to study DR7+ transgenics, since DR7 is a protective

TABLE 3. SERUM ASSAYS ON IMMUNIZED MICE

			ELISA average OD	ELISA OD ₄₅₀ > 0.2	TBII ≥ 20	T4 $\mu\text{g/dL}$	TSH mIU/L
DR3 mice (Fig. 1)	Experimental	$n = 6$	0.518	4/6 (67%)	4/6 (67%)	3.88 ± 0.74	70.5 ± 49.5
	Control	$n = 6$	0.060	0/6 (0%)	1/6 (16%)	3.85 ± 0.49	105 ± 38.0
DR3 mice (Fig. 3)	2 weeks	$n = 4$	0.138	1/4 (25%)	1/4 (25%)	3.45 ± 0.21	57 ± 46.6
	4 weeks	$n = 4$	0.208	2/4 (50%)	2/4 (50%)	3.3 ± 0.14	72.5 ± 34.6
	6 weeks	$n = 4$	0.517	2/4 (50%)	2/4 (50%)	3.75 ± 1.76	96 ± 93.3
DR2 mice (Fig. 2)	Experimental	$n = 5$	0.525	3/5 (60%)	3/5 (60%)	3.19 ± 0.89^a	55.5 ± 35.6
	Control	$n = 4$	0.052	0/4 (0%)	1/4 (25%)	2.18 ± 0.45	95.1 ± 62.1
DR3 mice (peptide immunization)	Experimental	$n = 8$	0.401	6/8 (75%)	6/8 (75%)	3.1 ± 0.55	75.3 ± 63.0
	Control	$n = 8$	0.067	0/8 (0%)	1/8 (12%)	2.9 ± 0.74	82.8 ± 31.7

^a $p < 0.01$ to control.

ELISA, enzyme-linked immunosorbent assay; TSH, thyrotropin; TBII, TSH-binding inhibition; T4, thyroxine; OD, optical density.

genotype for GD, such animals were not available. Nevertheless, the difference in the T-cell response between DR3+ and DR2+ mice is quite striking, and supports the relative importance of DR3 in development of anti-TSH-R immunity. Epitope responses after cDNA immunization were variable from animal to animal, and group to group, despite the apparent similarity of the procedure and genetic background. This may relate to different levels of DR expression, or other genetic differences, including the insertion site of the transgene, since we used mice derived from six different breeding pairs.

During the course of immunization to TSH-R-ECD cDNA-expressing plasmid and virus, there was a progressive and marked recruitment of epitopes, as determined by incubation of splenocytes with synthetic peptide epitopes spanning the ECD. It is tempting to consider this as a model for the progression of immunity in humans exposed to TSH-R. However, the circumstances are radically different. Nevertheless, the study does suggest that, as expected, progressive exposure to immunogen leads to development of reactivity to more and more pieces of the antigen. This has important implications for epitope-based immunotherapy, since it might require therapy directed at multiple differing epitopes in different patients, and as well at differing DR proteins.

Immunization with *E. coli*-derived protein produced weak T-cell responses, but protein derived from insect larvae was powerfully immunogenic and produced high-titer antibodies recognizing two different preparations of recombinant TSH-R-ECD. Possible differences may relate to purity of the preparations, the amount of specific antigen employed, or differences in glycosylation. However, the immunization with the second preparation of TSH-R-ECD did provide evidence for the importance of one strongly reactive T-cell epitope (aa78–94).

Immunization with peptides 12, 13, 32, and 33, derived from the TSH-R-ECD molecule, also produced immunity to the TSH-R-ECD as demonstrated by positive ELISA and TBII assays, as well as reactivity to several peptide epitopes. This must mean that the peptides were taken up by and presented to T cells, by endogenous antigen-presenting cells. These peptides all had moderate or high affinity for binding to DR3 molecules, with IC₅₀s of, respectively, 50, 18, 10, and 0.3 μM .

Although strong reactivity developed to peptides 5, 10 (sharing sequence with peptide 33), 15, and 33, and 37, we could not demonstrate reactivity to the immunizing peptides 12, 13, and 32. The reason for this observation is uncertain, but may suggest that at some point during induction of immunity, T cells specific for these peptides were either deleted or rendered anergic by exposure to the specific peptide. Immunization to epitopes 5–6, and 32 (chosen because of evidence of strong immunogenicity), in both DR3 and DR2 mice gave strong reactivity, and caused development of responses to epitopes entirely distinct from the immunizing epitope. We take this as further evidence for epitope spreading during the immune response. It also must mean that the host immune system recognized these peptides as part of TSH-R-ECD, and developed T-cell responses to other sections of the mouse TSH-R protein. It is of interest that all of the immunizing and responding peptides had some sequence differences between the mouse and human sequence, but in each case apparently shared T-cell specificity.

Our laboratory has sought dominant epitopes of the TSH-R-ECD in a variety of studies, and we have previously reported peptides that seem important in humans and animals (22). The present studies confirm the multiplicity of epitopes to which immunized animals respond, and fails to identify one, or a very few epitopes that are uniquely important. Some epitopes (32, 5–6) do produce very strong responses in both DR3+ and DR2+ animals, suggesting that they may be important in the pathology of GD. Epitope 5–6 is especially reactive after immunization with TSH-R protein. Important TSH-R T-cell epitopes have been recognized by other groups. Arima *et al.* found an epitope comprised of aa121–140 (similar to our peptide 9) to be a dominant peptide in studies using cDNA immunization in mice expressing IA-k (23). Pichurin *et al.*, also using cDNA immunization in similar DR3 transgenic animals, noted a peptide with sequence 142–161 (like our peptide 11) to be most responsive (24). In our studies neither of these sequences seemed to represent important epitopes. We also noted that individual animals that are largely congenic develop responses to different epitopes. However, considering that intense reactivity is developed to several epitopes, it seems probable that one dominant epitope may not be present in TSH-R. This would differ from observations in experimental

models of MS, and in human subjects with type 1 DM. In general, epitope-based therapies designed to remove or inhibit reactive T cells would be most useful if there are truly dominant epitopes against which the therapy can be directed.

In these studies, using DR3+ or DR2+ animals, both ELISA and TBII assay indicated the development of antibodies reacting with TSH-R. In the protocol studying the time course of immunization, the average ELISA and percent positive TBII assays increased during 2–6 weeks, as expected. T4 levels in the experimental groups were significantly above controls only in the DR2 animals. TSH levels were lower in experimental groups than that in control animals, but the wide range of results made the data statistically insignificant. Many of the samples were hemolyzed, which may cause some error in the determinations.

We can conclude that antibodies to TSH-R were appropriately formed and had an apparent mild stimulatory effect on thyroid function, but clear-cut thyrotoxicosis was not produced in any of the animals. DR2+ mice were able to mount an antibody response to TSH-R-ECD roughly equal that of DR3+ mice, although their response to T-cell epitopes was clearly lower.

Immunization to hTSH-R-ECD protein produced in insect larvae caused production of high-titer antibodies that reacted in equal titer to protein produced in *E. coli*, or insect larvae. Presumably, the *E. coli* protein was not glycosylated, while that from insect larvae was. However, whether the glycosylation is identical to that produced in thyroid cells is unknown.

Immunization to four TSH-R-ECD peptides also induced positive ELISA and TBII responses, but no significant difference in average T4 or TSH levels. This experiment reproduced our previous study of immunization to some of the same peptides (7).

Mice with elevated T4 levels showed histology with colloid ballooning and thyrocyte hyperplasia, unaccompanied by lymphocyte infiltration, which is compatible with histology seen in hyperthyroidism. Others, including Nagayama *et al.* (11), have reported that immunization with whole TSH-R coding adenovirus induced a model of GD efficiently with elevated T4 and TSH-R-binding activity, and sometimes increased TBII and TSH-R-ELISA values.

In a recent study we reported the IC50 values for TSH-R-ECD synthetic peptide epitopes binding to several purified HLA molecules, including DR3 (18). We found that peptides 8, 10, and 13, bearing sequences IRNTRNLTYIDPDALKE (109–124), GIFNTGLKMFDPDLTKVYST (132–150), and GLKMFDPDLTKVYST (137–150) of TSH-R-ECD, bound with very high affinity to HLA-DR-3. It is of interest that these peptides were recognized (among others) by T cells from immunized animals, and that peptide 10 induced immunity to the ECD and peptide 13 when used for immunization. This must mean that, at least in the case of this experimental immunization, binding of these peptides to DR3 correlates with T-cell reactivity, and that such T cells are present in the animals, thus not deleted by virtue of high affinity binding to DR.

In our work, immunization with the cDNA for TSH-R-ECD administered via plasmid or viral vectors produced T-cell and B-cell responses, but did not produce clear hyperthyroidism, as reported by other laboratories (11), and appeared to be less effective than immunization using cells expressing both DR protein and TSH-R (9). The T-cell responses of DR2 transgenic mice were clearly lower than that of DR3 transgenics, but this

difference did not appear in antibody responses. A striking feature of both genomic immunization and immunization with peptide epitopes was the spreading of the epitope responses during immunization. This was especially clear during peptide immunization, which induced responses, indicating that unrelated portions of the mouse (host's) TSH-R-ECD had become immunogenic. The responses to immunization also supported, again, the view that single specific dominant epitopes in the TSH-R-ECD are either not present, or somehow well hidden.

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Disclosure Statement

The authors declare that no competing financial interests exist.

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