Effects of genetic immunization of Swiss outbred mice with human thyroid stimulating hormone receptor cDNA plasmids harboring gain-of-function mutations

Su-Chin Ho, Sui-Sin Goh¹, Irene H C Kee², Pierce K H Chow², Chin-Pin Yeo³ and Daphne H C Khoo¹

SingHealth Research Facilities, Singapore Health Service, 5, Hospital Drive, Block A, #03-04, Singapore 169609, Republic of Singapore

Departments of ¹Endocrinology, ²Experimental Surgery and ³Pathology, Singapore General Hospital, Outram Road, Singapore 169608, Republic of Singapore

(Requests for offprints should be addressed to S-C Ho; Email: ho.su.chin@sgh.com.sg)

Abstract

Animal models of Graves' disease have been generated in recent years with various vaccination protocols using wild-type TSH receptor. In this study, we report the findings of genetic immunization of Swiss outbred mice with three different mutated human TSH receptor plasmids, each containing one constitutive activating mutation located at the ectodomain (S281N), exoloop (I486F), and transmembrane segment (D633H) respectively. Although the overall rate of thyrotoxicosis in the mice was <10%, anti-TSH receptor antibodies could be detected in many animals by flow cytometry, radioreceptor assay, and functional bioassays using recombinant human TSH receptor. Mice injected with plasmids harboring activated mutants (S281N and D633H) showed production of predominantly stimulating antibodies, whilst those treated with wild-type receptor plasmids generated mainly blocking sera. Most of these antibodies displaced radiolabeled bovine TSH, and their epitopes, independent of functional characteristics, were mapped to the first 271 amino acids of the TSH receptor. This supports recent findings that binding of stimulatory or blocking antibodies lie in close proximity within the leucine-rich repeat region.

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Introduction

Graves' disease (GD) is an autoimmune thyroid disorder resulting from agonistic action of anti-thyroid stimulating hormone (TSH) receptor antibodies (TRAB) on the TSH receptors (TSHR), leading to thyroid hyperplasia and thyrotoxicosis (Weetman 2000, Davies *et al.* 2005). Only humans develop GD spontaneously and over the years, much effort has been directed towards the development of animal GD models that will serve as vital tools for understanding the disease pathogenesis of thyroid autoimmunity. Using novel immunization approaches, several groups have succeeded in inducing hyperthyroidism and thyroid stimulating antibodies (TSAB) in various mouse strains. These include *in vivo* expression of TSHR by injecting syngeneic cells coexpressing the TSHR, major histocompatibility complex (MHC) class II antigen, and a costimulatory molecule, or alternatively genetic immunizations using naked DNA plasmids or adenovirus vector encoding the TSHR (Shimojo *et al.* 1996, Costagliola *et al.* 1998a, 2000, Kaithamana *et al.* 1999, Nagayama *et al.* 2002, Nagayama 2005). These methods vary in the incidence of hyperthyroidism, prevalence of TSAB, and intrathyroidal inflammatory infiltrates.

Various attempts have also been made to improve the rate of hyperthyroidism. One such study succeeded in inducing a high rate of hyperthyroidism by using adenovirus that expresses only the free A subunit of the TSHR instead of the full-length holoreceptor (Chen *et al.* 2003). Thus far, most studies have employed wild-type TSHR in their animal experimentation. Investigation into the effect of Y601H polymorphism, a mutation that results in low constitutive cyclic AMP (cAMP) production, found no influence on the outcome of DNA vaccination in BALB/c mice (Pichurin *et al.* 2002). However, the reverse strategy, i.e. investigating the effects of activating TSHR mutations on induction of hyperthyroidism, has not been explored. We hypothesize that if constitutively active TSHR mutants are used in the immunization process, the mutants that are synthesized and expressed by the antigen-presenting cells (i.e. myocytes, Langerhan's cells, or dendritic cells) may potentially affect endosomal processing into peptides, thereby exposing novel epitopes that may be important in receptor activation and otherwise absent in wild-type receptor. This process can potentially result in the formation of a repertoire of antibodies that is different from immunization with wild-type receptor and hence affect the rate of GD

induction. In this study, we immunized 45 Swiss outbred mice with plasmids encoding the cDNA of three constitutively active TSHR mutants, each harboring a mutation located at codon 281 (S281N) in the ectodomain, codon 486 (I486F) in the first exoloop, and codon 633 (D633H) in the sixth transmembrane segment of the TSHR. They are spontaneous mutations which have been identified in toxic multinodular goiter, autonomous functioning thyroid adenomas/cancer, and familial non-autoimmune hyperthyroidism (Parma *et al.* 1995, 1997, Russo *et al.* 1996, 1997, Duprez *et al.* 1997, Kopp *et al.* 1997, Gruters *et al.* 1998, Tonacchera *et al.* 1998, Camacho *et al.* 2000). These single amino acid alterations have been known to cause structural and conformational changes in the protein that are important in constitutive receptor activation in the absence of its ligand TSH. We tested our hypothesis and found that, indeed, the prevalence of TSAB was higher in mice immunized with activating TSHR mutants compared with those injected with wild-type receptor.

Materials and methods

Animal experimentation

DNA vaccination was performed as previously described by Costagliola *et al.* (2000) with minor modifications. A total of 45 female Swiss outbred mice, aged 6- to 8-weeks-old, were used. All animals were pretreated with 100 µl cardiotoxin (10 mM, purified from the venom of *Naja nigricollis*; Calbiochem, La Jolla, CA, USA) by i.m. injection at the anterior tibialis muscles. Control mice ($n=5$) received 100 µg of the empty vector pCDNA3 at the same site, 5 days later, while study mice were each vaccinated with 100 µg DNA containing either (1) wild-type TSHR ($n=10$), (2) S281N TSHR ($n=10$), (3) I486F TSHR ($n=10$), or (4) D633H TSHR ($n=10$). DNA injections were repeated at week 4 and 8 and the animals were killed at week 16 by chloroform inhalation. Blood was collected by intra-cardiac puncture and sera used for subsequent study. The mice were housed under pathogen-free conditions and fed standard chow at the Department of Experimental Surgery, Singapore General Hospital. Weights of the animals were monitored at each injection and at death. The study adhered to IACUC guidelines and was approved by the local IACUC committee.

Serological parameters

Total T4

Total T4 levels were measured by Vitros ECI Immuno-diagnostic System (Ortho-Clinical Diagnostics, Rochester NY, USA).

Flow cytometry using TSHR expressing cells

Flow cytometry was performed as described previously using 5 µl mouse sera in a 100 µl reaction and results were expressed in arbitrary fluorescence units (FU) (Ho *et al.* 2001, 2005). The cell lines used in this study were: (i) JP19, which expressed wild-type full-length TSHR, (ii) GT14, which expressed only the ectodomain of the TSHR anchored on a glycosylphosphatidylinositol insert (ECD-GPI) (Ludgate *et al.* 1992, Costagliola *et al.* 1998b), and (iii) TLT, which expressed a chimera TSHR-LH receptor (LHR) ectodomain on a GPI anchor. Construction of this chimera is described below.

TSH-binding inhibitory immunoglobulin (TBII)

JP19 cells plated at a density of 30 000 cells per well were incubated for 4 h with 100 µl binding assay buffer (NaCl-free HBSS containing 277 mM sucrose, 5% BSA, and 25 mM HEPES, pH 7.4) containing 30 000 c.p.m. 125 I-labeled bovine TSH (bTSH) and 5 µl serum. Cells were washed twice with cold buffer and incubated with 100 µl 1M NaOH for another 15 min before counting, as described previously (Ho *et al.* 2001, 2005). Experiments were performed in triplicate for each serum sample and the intra-assay coefficient of variation for c.p.m. obtained was 5.6%. The TBII value of each sample was obtained using the formula: $(1 - (\text{TSH binding in the presence of test serum} / \text{mean TSH binding in presence of control sera})) \times 100$.

Thyroid-stimulating antibody (TSAB) and thyroid-stimulating blocking antibody (TSBAB)

For TSAB, 30 000 JP19 cells per well were incubated at 37 °C for 4 h in the presence of 5 µl mouse serum and 95 µl buffer consisting of 124 mM NaCl, 5 mM KCl, 1.25 mM KH_2PO_4 , 1.25 mM MgSO_4 , 1.45 mM CaCl_2 , 8 mM glucose, 25 mM Rolipram (Sigma Aldrich Corp.), 25 mM HEPES, and 0.05% BSA at pH 7.4. Cyclic AMP released into the supernatant was collected and measured by RIA kits (NEN TM, Boston, MA, USA). Experiments were done in triplicate for each mouse serum and the results were expressed as pmol/ml. For TSBAB assay, the same procedure was performed with the addition of 200 µIU/ml bTSH and the sample result was also expressed in pmol/ml. The intra-assay coefficients of variation of cAMP obtained for TSAB and TSBAB were 10.8 and 12.8% respectively.

Preparation of plasmids for injection

Activating mutations, S281N, I486F, and D633H were introduced into the wild-type full-length human TSHR in pCDNA3 by PCR using the following primers:

281-Forward(F) CTCACACGGGCTGACCTTTCTT-
ACCCAAATCACTGCTGTGC
281-Reverse(R) GGGTAAGAAAGGTCAGCCCGTG-
TGAGGTGAAGGAACTCAAGG
486-F ACTCACTCTGAGTACTACAACCATGCCTT-
TGACTGGCAG
486-R GGTTGTAGTACTCAGAGTGAGTGTAGAG-
GTCTACAGAGGC
633-F CCAAGAGGATGGCTGTGTTGATCTTCAC-
CCACTTCATATGC
633-R CAACACAGCCATCCTCTTGGCAATTTTGG-
TATCTTTGTC

Each pair of primers, complementary to the opposite strands, was extended with Pfu DNA Polymerase (Promega, Madison, WI, USA) according to the manufacturer's protocol using the following conditions: initial denaturation at 95 °C for 30 s for one cycle, followed by denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 14 min for 15 cycles, and final extension at 72 °C for 4 min. The PCR product was digested overnight with DpnI (New England Biolabs, MA, USA) and followed by transformation in competent *Escherichia coli*. Colonies were picked and DNA was extracted using QIAprep Miniprep kit (QIAGEN GmbH, Germany) followed by Big Dye sequencing (Applied Biosystems, CA, USA) for detection of mutations. Constructs harboring the three desired mutations were then digested with XhoI and XbaI and the fragments ligated into the vector pCDNA3. Larger-scale plasmid preparations for injections were done with Maxiprep kit (QIAGEN GmbH, Germany) and the DNA pellet dissolved in PBS.

Construction of stable cell lines expressing TSHR–LHR chimera

Two chimera constructs, TLT and LTT in pSVL, containing segments of LHR in the ectodomain of full-length TSHR were reported previously and kindly given by Dr S Costagliola (Costagliola *et al.* 2002a,b). In brief, TLT contains TSHR sequences from amino acids 1–271 followed by homologous LHR sequences from codon 272 to the CEDIM motif located at the insertion into the cell membrane. LTT, on the other hand, contains LHR sequences from amino acids 1–271 followed by TSHR sequences from codon 272 onwards right up to the CEDIM motif. The ectodomains of TLT and LTT were excised by restriction enzymes XhoI and EcoRV and transferred into similarly cut TSHR ECD–GPI in pCDNA3 vector. TLT and LTT ectodomains anchored on GPI thus created were subsequently lifted out by XbaI and KpnI digestion and cloned into pEFIN as followed: TSHR ECD–GPI in pEFIN vector was first thoroughly cut with XbaI, followed by brief digestion with KpnI to allow the harvest of a 5·9 kb

fragment bearing the correct ends to allow the insertion of TLT and LTT ectodomains. The final products were verified by direct sequencing. For construction of stable cell lines expressing the LHR–TSHR chimeras, 2 µg DNA were used for transfection of 200 000 CHO cells with Geneporter (Gene Therapy Systems Inc., CA, USA) followed by the addition of Geneticin G418 (Gibco Invitrogen Corp., CA, USA) at 48 h for selection. Cells were then detached, divided, and plated in smaller quantities to make a few cell lines. The surface expression of chimera proteins in these lines were assessed by flow cytometry using two mouse monoclonal antibodies IRI-SAb1 and 15·2 (both kind gifts from Dr S Costagliola). IRI-SAb1, whose linear epitope is located in the first 280 residues of the ECD, was used for assessing TLT lines, while 15·2, whose linear epitope is located close to the C-terminal portion of the ECD from amino acid residue 371–400 immediately upstream of the first transmembrane helix, was used for LTT lines (Costagliola *et al.* 2002a,b). The LTT chimera was poorly expressed at cell surface and was sequestered intracellularly (stable transfection repeated twice; data not shown). The TLT construct, on the other hand, showed good cell-surface expression and one cell line with the highest level of receptor protein was selected for subsequent experiments.

Statistical analyses

Data were analyzed and graphs plotted using SPSS 9.0 software. Non-parametric data were expressed as medians and inter-quartile ranges (IR 25–75%). In the determination of serum performance on various assays, mean values of controls were first calculated and cut-off set arbitrarily at 2 s.d. from the mean. Fisher's exact test was used to determine the significance of differences between the number of mice positive or negative for a parameter in a particular group. Differences in the magnitude of responses between groups of mice were evaluated for significance using Mann–Whitney *U*-test. Correlations between parameters were calculated by Spearman coefficient. A *P* value of <0·05 was taken as statistically significant.

Results

All the 45 mice completed the entire immunization protocol without mortality.

Weight

The weights of animals in the various treatment groups did not differ significantly from control either before or after the immunization (before: control 24·4 g (23·5–26·7), wild type 24·5 g (23·6–26·3), S281N 24·9 g (22·0–26·2),

I486F 23.9 g (22.2–26.2), D633H 23.9 g (22.2–26.5) and after: control 33.9 g (32.8–50.1), wild type 40.0 g (34.3–47.8), S281N 37.7 g (33.1–43.0), I486F 34.1 g (31.9–38.2), D633H 39.4 g (33.0–43.2). All animals gained a significant amount of weight during the immunization period ($P < 0.001$).

Total T4

The median total T4 levels at death for treatment groups did not differ significantly from those of controls (control 60.2 nmol/l (55.6–87.3), wild type 63.8 nmol/l (55.9–79.4), S281N 72.0 nmol/l (63.0–94.7), I486F 65.3 nmol/l (49.2–76.0), and D633H 67.4 nmol/l (52.6–80.9)). For the mice injected with wild type, I486F, and D633H TSHRs, only 1 out of 10 mice in each group showed elevated total T4. No thyrotoxicosis was found in mice immunized with S281N TSHR. There was no statistical significant difference in the rates of thyrotoxicosis amongst groups.

TBII

Wild-type and D633H TSHR-vaccinated mice were 90% positive for TBII activity, while S281N and I486F TSHR injected groups were both positive for TBII in 50% of cases. This difference was not significant. However, TBII-positive cases in S281N and I486F TSHR groups generally had much lower TSH displacing ability when compared with the wild-type TSHR group. Their median TBII values were also significantly lower when compared with the wild-type TSHR group (wild type: 25.6% (14.5–52.1); S281N: 6.7% (2.8–9.1), $P = 0.007$; I486F: 5.7% (3.2–8.0), $P = 0.002$; Fig. 1).

TSAB

Immunization with wild-type TSHR generated only two sera positive for TSAB (20%), while S281N, I486F, and D633H TSHR groups had 80, 50, and 90% positive cases respectively (Fig. 2). Differences in prevalence were significant for S281N and D633H TSHR injected groups when compared with wild-type receptor injection ($P = 0.023$ and 0.005 respectively). All the three mutant TSHR groups achieved significantly higher TSAB levels compared with those in wild-type receptor group (wild type: 1.25 pmol/l (0.88–1.92) vs S281N: 2.50 pmol/l (2.06–5.00), $P = 0.005$; I486F: 2.15 pmol/l (1.72–2.90), $P = 0.013$; D633H: 3.28 pmol/l (2.67–3.76), $P = 0.001$).

TSBAB

Wild-type TSHR injected mice developed the highest incidence of blocking antibody TSBAB (50%), while the incidences in S281N and I486F TSHR injected groups were lower at 10 and 30% respectively (Fig. 3).

The difference was not significant. Of interest, none of the mice immunized with D633H TSHR tested positive for blocking bioactivity and this was significantly different compared with the wild-type TSHR group ($P = 0.033$). The median TSBAB levels achieved with mutant receptor immunization also did not differ significantly from that of wild-type receptor injection. However, among the TSBAB-positive sera, those obtained from wild-type receptor vaccination showed clear suppression of TSH-mediated cAMP production, while those obtained from mutant injections tended to be only marginally positive.

Flow cytometry

Figure 4 shows the flow cytometry readings obtained using GT14 and JP19 cell lines from each individual serum with cut-off levels set at 13.1 and 10.7 FU respectively. There was concordance in TRAB detection among the 10 mice injected with wild-type TSHR: eight (80%) were positive for TRAB on both cell lines, while the remaining two mice were negative on both cell lines. In contrast, TRAB from mutant receptor groups recognized holoreceptors on JP19 cells poorly. Detection rates were only 30, 10, and 20% positive in S281N, I486F, and D633H TSHR groups respectively compared with 80% in the wild-type TSHR group and this decline was significant in the latter two ($P = 0.005$ and 0.023 respectively). Flow cytometry performed with GT14 cells was a more efficient and sensitive method. Using these cells, the prevalence of positive sera rose for all the three mutant groups, especially for D633H TSHR-injected animals (S281N 50%, I486F 40%, and D633H 90%) and their prevalence was comparable with that of wild-type group.

Ratios of FU obtained from GT14 to JP19 cells were calculated and used as a means of comparing antibody affinity between treatment groups. Data obtained from control mice were expectedly almost uniform 0.95 (0.80–1.18), while mice injected with D633H TSHR had the highest ratio (3.50, 2.79–6.93) followed by wild-type TSHR (2.59, 1.44–2.91), I486F TSHR (1.74, 0.94–2.41), and S281N TSHR (1.67, 0.85–3.34). Only the difference between wild-type TSHR and D633H TSHR groups reached statistical significance ($P = 0.035$), indicating a preferential binding of antibodies for the ECD–GPI over the full-length receptor in the latter group of animals. Comparisons of FU ratios were also made between groups according to TSAB, TSBAB, and TBII status. GT14 to JP19 FU ratio were significantly lower for TSBAB- and TBII-positive cases than negative cases (TSBAB: positive 1.44 (1.34–2.11) vs negative 2.81 (1.67–5.08), $P = 0.031$ and TBII: positive 1.39 (0.93–2.26) vs negative 2.80 (1.87–4.34), $P = 0.017$), indicating that antibodies with TSH displacement properties or blocking activities bound

Figure 1 TBII activities of Swiss outbred mice immunized with wild-type, S281N, I486F, and D633H TSHRs. Each serum was assayed in triplicates. Bars represent mean of triplicate results and error bars represent s.e. of mean. Solid grid line represents cut-off for TBII assay set at 6.1%. Mean values above this cut-off are considered positive for the assay. Wild-type and D633H TSHR-injected groups achieved 90% prevalence, while S281N and I486F groups achieved 50% prevalence (results not statistically significant). Note that wild-type TSHR group achieved significantly higher TBII activity levels when compared with S281N and I486F TSHR injected mice (wild-type: 25.6 (14.5–52.1) vs S281N: 6.7 (2.8–9.1), $P=0.007$ and I486F: 5.7 (3.2–8.0), $P=0.002$). Tables below bar charts show individual serum's flow cytometry status performed with GT14 and JP19 cell lines: + and - denote positive and negative results respectively. Sera with strong TBII activities tended to be positive for both GT14 and JP19 flow cytometric assays. TBII values correlated significantly with GT14 ($r=0.74$, $P<0.0001$) and JP19 ($r=0.57$, $P<0.001$) FU readings.

JP19 holoreceptors to a larger extent. The ratios for TSAB positive and negative sera were both similar (positive 2.47 (1.52–5.51) vs 2.46 (1.36–3.07)).

To delineate antibody-binding regions of these TRAB on the ectodomain of TSHR, flow cytometry experiments were repeated for all sera with the TLT

lines generated. The prevalence of sera positive on TLT cells was 90% with wild-type TSHR, 60% with S281N TSHR, 40% with I486F TSHR, and 70% with D633H TSHR groups. Overall, stratification of sera based on established cut-off levels showed concordance between the GT14 and TLT data ($P<0.0001$).

Figure 2 TSAB activities of Swiss outbred mice immunized with wild-type, S281N, I486F, and D633H TSHRs. Each serum was assayed in triplicate. Bars represent mean of triplicate results and error bars represent s.e. of mean. Solid grid line represents cut-off for TSAB assay set at 2.14 pmol/l. Mean values above this cut-off are considered positive for the assay. Immunization with wild-type TSHR generated only 20% positive sera, while S281N, I486F, and D633H TSHR groups had 80, 50, and 90% positive cases respectively. Increased prevalence in S281N and D633H TSHR groups was found to be significant ($P=0.023$ and 0.005 respectively). Injection with three activating mutants generated higher TSAB levels compared with wild-type receptor (wild type: 1.25 pmol/l ($0.88\text{--}1.92$) vs S281N: 2.50 pmol/l ($2.06\text{--}5.00$), $P=0.005$; I486F: 2.15 pmol/l ($1.72\text{--}2.90$), $P=0.013$; D633H: 3.28 pmol/l ($2.67\text{--}3.76$), $P=0.001$). Note that two mice achieved extremely high TSAB levels: mouse 6 in S281N TSHR group and mouse 5 in D633H TSHR group. Tables below bar charts show individual serum's flow cytometry status performed with GT14 and JP19 cell lines: + and - denote positive and negative results respectively. TSAB values did not correlate with flow cytometric readings on GT14 or JP19 cells.

The two flow cytometry readings also showed a high degree of correlation ($r=0.81$, $P<0.0001$). TLT readings showed correlation with TBII levels ($r=0.73$, $P<0.0001$) but not with TSAB or TSBAB activities.

These findings suggest that regardless of functional profile, most of the TRAB found in both wild-type and mutant TSHR immunized mice bind to regions in the first 271 amino acid segment of the TSHR.

Figure 3 TSBAB activities of Swiss outbred mice immunized with wild-type, S281N, I486F, and D633H TSHRs. Each serum was assayed in triplicate. Bars represent mean of triplicate results and error bars represent s.e. of mean. Solid grid line represents cut-off for TSBAB assay set at 98.8 pmol/ml. Mean values below this cut-off are considered positive for the assay. Immunization with wild-type TSHR generated 50% positive sera, while S281N and I486F TSHR groups had 10 and 30% positive cases respectively (results not statistically significant compared with wild-type TSHR group). D633H TSHR group sera were all negative for TSBAB and this absence was significant compared with wild-type group ($P=0.033$). Median TSBAB levels in mutant receptor groups did not differ from wild-type group. However, among the TSBAB-positive sera, the majority obtained from wild-type receptor vaccination showed clear suppression of TSH-mediated cAMP production, while those obtained from mutant injections tended to be only marginally positive. Tables below bar charts show individual serum's flow cytometry status performed with GT14 and JP19 cell lines: + and - denote positive and negative results respectively. TSBAB values did not correlate with flow cytometric readings on GT14 or JP19 cells.

Correlation of various TRAB measurements

TBII was significantly correlated with TSBAB activity ($r=-0.314$, $P=0.026$), a finding that was not surprising because measurement of TSBAB activity entails the

displacement of ligand TSH. TBII, in addition, was also significantly correlated with FU readings obtained with JP19 cells ($r=0.57$, $P<0.001$) and GT14 cells ($r=0.74$, $P<0.001$). Although there was increased recognition by sera from mice immunized with mutant TSHRs for

Figure 4 Flow cytometric readings obtained with each individual serum from GT14 (■) and JP19 (□) cells. Solid and dotted horizontal lines indicate cut-off values for GT14 and JP19 cell lines respectively, set at 2 s.d. from the mean of control group (13.1 FU for GT14 and 10.7 FU for JP19). In wild-type TSHR injected mice, results of GT14 and JP19 cells were concordant and showed high rate of prevalence (80%). Sera from mice immunized with mutant receptors recognized JP cells poorly: 30% in S281N (result not significantly different from wild-type group), 10% in I486F ($P=0.005$ compared with wild-type group), and 20% in D633H ($P=0.023$ compared with wild-type group) TSHR groups. Usage of GT14 cells reversed this situation and increased detection rate to 50, 40, and 90% respectively. *Fluorescence unit ratios of GT14 to JP19 cells were examined and found to be significantly higher in D633H TSHR group compared with wild-type receptor.

GT14 compared with JP19 cells, and of these mice often possessed stimulating antibodies, the correlation of TSAB with GT14 flow cytometric readings was not significant.

Description of the three mice with elevated total T4 levels

Only three mice showed elevated total T4 and they came from the groups immunized with wild-type (mouse number 10), I486F (mouse number 9), and D633H (mouse number 8) TSHRs. Their total T4 levels were 123.4, 113.3, and 144.9 nmol/l respectively (cut-off: 107.2 nmol/l). These mice did not exhibit the highest level of TSAB in each of their respective

cohorts. In fact, TSAB was negative in the thyrotoxic mouse (number 10) immunized with wild-type receptor (TSAB 1.55 pmol/ml, cut-off: 2.14 pmol/ml). It was also negative for TSBAB but showed 60% displacement of radiolabeled bTSH on TBII assay. The thyrotoxic mouse immunized with I486F TSHR showed the highest FU with GT14 cells but its sera was blind to JP19 cells. It yielded a cAMP of 2.80 pmol/ml on TSAB assay, fourth in rank amongst its cohort (maximum TSAB in group: 3.53 pmol/ml). It was negative for TSBAB and showed 9% displacement of radiolabeled ligand. The most thyrotoxic mouse in the entire batch came from D633H receptor vaccination. Its TRAB showed the highest FU for both GT14 and JP19 cells

in its own cohort. It also had the greatest TBII activity with 75% displacement of radiolabeled ligand. It was negative for TSBAB and generated 4.33 pmol/ml cAMP on TSAB assay, second in rank in its cohort where the maximum cAMP produced was 29.4 pmol/ml.

Description of two mice with the highest TSAB levels

Two mice stood out amongst the entire study in having extremely high TSAB activities (Fig. 2). They were mouse number 6 (S281N TSHR group) and mouse number 5 (D633H TSHR group). They generated cAMP of 23.2 and 29.4 pmol/ml respectively, which represented 17- and 22-fold increases above the median of control group. Although positive for potent TSAB, neither were thyrotoxic. Number 6 had a T4 level of 90.8 nmol/l. Number 5 had the lowest T4 level of the 45 animals, 33.7 nmol/l, which fell just short of the cut-off for hypothyroidism set at 32.8 nmol/l. Number 6 displaced 36% and number 5 displaced 6% of TSH in TBII assays. Both the mice were negative for TSBAB and their sera showed preferential recognition of GT14 cells over JP19 cells (Fig. 4).

Discussion

Genetic immunization with human TSHR cDNA is an effective means of generating TRAB although the rate of hyperthyroidism is generally low in mice. Vaccination of female BALB/c mice produced predominantly TSBAB-positive antibodies with only 7.1% (1 out of 14) mice showing TSAB activity. All animals had normal thyroid hormones (Costagliola *et al.* 1998a). Results in female NMRI outbred mice were better with 17.2% (5 out of 29) mice positive for TSAB. The mice also showed T4 elevation and TSH suppression (Costagliola *et al.* 2000). Our study in Swiss outbred mice resulted in a lower induction rate with this protocol (7.5% (3 out of 40)), contributed by additional factors such as genetic makeup of mouse strains and environmental differences between animal laboratories (Ludgate 2000). However, TRAB were commonly detected using various assays, demonstrating the dissociation between thyroid status and immunological responses against the antigen. Indeed, thyrotoxic mice were not the strongest producers of TSAB and neither did mice with high TSAB activities develop thyrotoxicosis. The discrepancies between T4 and TSAB, and in some studies thyroid histology, had been observed in both inbred and outbred mice (Yamada *et al.* 2002, Rao *et al.* 2003, Chen *et al.* 2004). Although dominance of TSBAB over TSAB and different serum dilution requirements for bioassays have been proposed as possible explanations (Chen *et al.* 2004), our TSAB-positive euthyroid animals lacked blocking antibodies and thus could not account for the findings. Another possibility is the production

of TRAB with epitopes unique to human antigen, especially with the usage of mutated TSHRs, which interacts only with assays using human recombinant TSHR without generating any biological action on the mouse thyroid. Performing the TSAB assay using murine TSHR could potentially circumvent this shortcoming.

Murine GD models have been optimized by varying immunization methods and/or murine strains, with most studies using wild-type human TSHR or TSHR variants (Shimojo *et al.* 1996, Costagliola *et al.* 1998a, 2000, Kaithamana *et al.* 1999, Nagayama *et al.* 2002, Nagayama 2005). Vaccination with free A subunit instead of the full-length holoreceptor by adenovirus induced high rates of hyperthyroidism (Chen *et al.* 2003). Loss-of-function silencing mutation at codon 601 (Y601H) did not influence DNA vaccination outcome in BALB/c mice (Pichurin *et al.* 2002). The reverse strategy, i.e. immunization with activating TSHR mutants, was explored in this study, which compared their serological profiles with wild-type receptor vaccination. The three mutations, located in the ectodomain, exoloop, and transmembrane segments, each results in structure alteration and the adoption of active conformation important in receptor activation. The authors hypothesized that immunization with such mutants containing key amino acid changes may affect endosomal processing into peptides thereby exposing novel epitopes that are otherwise absent in wild-type receptor. This could be potentially important in generating an antibody repertoire critical for receptor activation. Indeed, findings from this study support this hypothesis. Vaccination with gain-of-function mutant TSHRs increased the prevalence of TSAB-positive sera and their median cAMP production when compared with injection with wild-type receptor. It also consistently generated a greater number of stimulating sera compared with blocking sera, while immunization with wild-type TSHR tended to produce more blockers than stimulators. Induction of TSAB was unrelated to the degree of gain-of-function (in descending order: I486F, S281N, and D633H (Ho *et al.* 2005)) since the weakest mutant D633H TSHR generated the highest prevalence of stimulating TRAB. Good cell-surface expression of D633H mutant (surface expression in descending order: D633H, S281, and I486F; Ho *et al.* 2005) or properties intrinsic to its conformational change might have contributed. Nevertheless, it is interesting that a mutation located in a domain not exposed on the cell surface could significantly influence immunization results. This is in contrast to the negative findings of Y601H loss-of-function mutant (Pichurin *et al.* 2002).

Besides functional differences, TRAB obtained from wild-type and mutant immunization also differed in their binding to ECD–GPI moiety and full-length

holoreceptor on flow cytometry. In human, detection of TRAB binding to TSHR by flow cytometry was rarely achieved with full-length holoreceptors until the generation of ECD–GPI as a membrane protein (Costagliola *et al.* 1998b, Da Costa & Johnstone 1998). TRAB present in GD sera also preferentially bound ECD–GPI over holoreceptor and ECD–GPI cytometry readings significantly correlated with TSAB activity. In contrast, TSBAB sera from hypothyroid patients recognized both ECD–GPI and full-length receptors equally well (Chazenbalk *et al.* 2002). Our mouse data shared many similarities. TRAB obtained from wild-type group often contained TSH-blocking activity and showed full concordance of JP19 and GT14 cytometric readout. Calculation of GT14 to JP19 FU ratios also showed that TBII- and TSBAB-positive sera significantly bound holoreceptor to a greater extent. In contrast, antibodies obtained from mice immunized with activating mutants often had TSAB bioactivities and preferentially recognized GT14 over JP19 cells, although the correlation between TSAB and GT14 flow cytometry data was negative. Discrepant binding to these two TSHR species has been attributed to steric hindrance found in holoreceptor to TSAB binding, which comes from the plasma membrane, the extracellular loops, or receptor multimerization (Chazenbalk *et al.* 2002). The ECD–GPI may thus be more accessible to antibody binding due to the greater flexibility offered by its anchor and the absence of receptor multimerization (Chen *et al.* 2001, Chazenbalk *et al.* 2002). Conversely, activating mutations at key residues could potentially convert the receptor into open conformations by the release of negative constraint exerted by the ectodomain acting as an inverse agonist on the transmembrane domain, thereby exposing crucial epitopes otherwise hidden in holoreceptor (Zhang *et al.* 2000, Vlaeminck-Guillem *et al.* 2002). This situation is akin to vaccination with purified A subunit (Chazenbalk *et al.* 1999).

Findings obtained with TLT chimera indicated that most of the TRAB, be it TSAB or TSBAB, had their binding regions confined to the first 271 amino acids of the TSHR. This region contains nine leucine-rich repeats that form a succession of β -strands and α -helices organized into a horseshoe-shaped structure with which TSH and autoantibodies interact (Szkudlinski *et al.* 2002, Smits *et al.* 2003). Our data corroborate with findings that stimulating antibodies from GD patients and TSH-blocking antibodies from autoimmune hypothyroidism patients can both be neutralized by purified TSHR A subunits (Chazenbalk *et al.* 2002). TSH-blocking antibodies have, in addition, epitopes located on the holoreceptor. Thus, the previous concept that the epitopes for blocking antibodies are largely confined to the C-terminus of the ectodomain is oversimplified. Our findings are more in keeping with

recent TRAB epitope studies, which indicate that binding sites for stimulating and blocking autoantibodies lie close together, not on distinct or distant parts of the TSHR molecule (Morgenthaler *et al.* 2003, Ando *et al.* 2004, Costagliola *et al.* 2004, Sanders *et al.* 2004, 2005, Schott *et al.* 2005).

Conclusions

Genetic immunization of Swiss outbred mice in our laboratory using either wild-type or constitutive activating mutant TSHRs was not as efficient in producing mouse thyrotoxicosis as previously reported (Costagliola *et al.* 2000). However, it was effective in generating TRAB, which was detected by methods utilizing human recombinant TSHR. Moreover, immunization with active receptor conformations, especially with D633H mutant, generated higher prevalence of stimulating sera with higher median TSAB levels. In contrast, vaccination with receptor under basal state of activity in a constrained conformation would appear to preferentially generate blocking sera. It will be of interest to investigate the outcome of this strategy when applied to the other immunization methods. Regardless of functional activity, these autoantibodies could displace TSH, and their epitopes were mapped to the first 271 amino acids of the TSHR, a region where the leucine-rich repeat region resides and interacts with TSH and TRAB. Therefore, this study corroborates findings that both stimulating and blocking antibodies overlap in their binding sites and are not exclusively located at the N- and C-termini of the receptor as previously suggested (Tahara *et al.* 1997, Kohn & Harii 2003). Potentially, this study opens a novel approach in generating a spectrum of stimulating antibodies for the better understanding of receptor activation, a process that entails generation of monoclonal antibodies and definition of their epitopes.

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