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Immunization with Rat Thyrotropin Receptor Peptides Corresponding to Two Specific Extracellular Domains Alters Thyroid Function *in vivo*

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Abstract: The relationship between the specificities of rat thyrotropin receptor antibodies and their biological effects on the rat thyroid *in vivo* was studied. Sixteen male Wistar rats were immunized with one of two synthetic peptides; one corresponding to the N-terminal region, amino acid residues 29-57, termed RAT-N, and the other corresponding to the C-terminal region of the extracellular domain, amino acid residues 341-370, termed RAT-P. Rats immunized with RAT-N possessed thyroid-stimulating antibody (TSAb) activity and significantly higher serum concentrations of 3, 5, 3'-triiodothyronine (T_3) than those of the control groups. In contrast, antibodies generated in rats immunized with RAT-P possessed TSH-binding inhibitor immunoglobulin (TBII) activity and lower T_3 levels than those in the control group. Histologically, there were no inflammatory or destructive reactions in the thyroid glands of any of the rats. These findings suggest that thyrotropin receptor antibodies against the N-terminus have stimulatory effects on thyrocytes, and that antibodies generated against the C-terminal region of the extracellular domain play an inhibitory role in thyroid function *in vivo*.

Key words: thyrotropin receptor, autoantibody, hyperthyroidism, hypothyroidism

INTRODUCTION

It has previously been shown that thyrotropin receptor (TSH-R) autoantibodies play an important role in the pathogenesis of autoimmune thyroid disease[1]. One such autoantibody has a stimulatory effect on thyrocytes, resulting in the thyrotoxicosis seen in Graves' disease. In contrast, patients with idiopathic myxedema produce an antibody which is thought to block the action of thyrotropin (TSH), causing hypothyroidism[1]. Thus, determining the recognition sites and biological activities of these TSH-R antibodies is important in the study of the pathogenesis of Graves' disease and idiopathic myxedema. Human and rat TSH-R cDNA has recently been cloned, enabling researchers to study the importance of the various domains in the actions of TSH or TSH-R autoantibodies [2-5]. Many groups have found that the N-terminal region of the TSH-R is required for the binding of the thyroid-stimulating antibody (TSAb)[6-8]. Conversely, the thyroid stimulation-blocking antibody (TSBAb) is directed toward the C-terminal region of the extracellular domain[9, 10]. Recently, Kosugi et al. identified a threonine located at residue 40 of the rat TSH receptor that was required for binding of the TSAb[11] and reported that tyrosine 385 and cysteine 390 formed a critical epitope for the TSBAb[12]. However, all these studies were performed *in vitro*, limiting their use in specifying recognition sites

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of TSH-R antibodies which exhibit biological effects on the thyroid *in vivo*.

We showed previously that experimentally produced antibodies to the N-terminal segment (amino acid residues 29–57) had TSAb activities[13, 14], and those to the C-terminal segments of the extracellular domain (residues 341–358 and 372–397) possessed TSBAb activities[15]. In the present study, we examined the biological effects of these rat TSH-R antibodies on rat thyroid functions to determine the relationship between the recognition sites of TSH-R antibodies and their role in thyroid functions *in vivo*.

Materials and Methods

Peptide synthesis and antibody production

Two different peptides corresponding to the N-terminal region (amino acid residues 29–57), termed RAT-N, (Fig. 1A), and upstream sequences of the transmembrane region (amino acid residues 341-370), termed RAT-P, (Fig. 1B) of the rat TSH-R were synthesized by Automatic Peptide Synthesizer (Pharmacia, Uppsala, Sweden), as previously reported[15]. Ten mg of purified peptide was conjugated to 2 mg of bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.).

Eight male Wistar rats were inoculated with each conjugated peptide prepared in an emulsification of Freund's complete adjuvant every 2 weeks. The presence of anti-peptide antibodies in the serum was detected as follows: 300 ng of peptide was applied to nitrocellulose sheets and incubated with rat serum diluted 1:200 in Tris-buffered saline containing 2% gelatin for 2 hr at room temperature. Blots were washed in Tris-buffered saline containing 0.3% Tween-20 for 30 min and the sheets were further exposed to peroxidase-labeled anti-rat IgG (1:400) for 1 hr (DAKO Co., Ltd. Glostrub, Denmark). The sheets were then washed again, and peroxidase activity was detected with diaminobenzidine / H_2O_2.

Preparation of IgG fractions

Serum samples were diluted with equal vol-

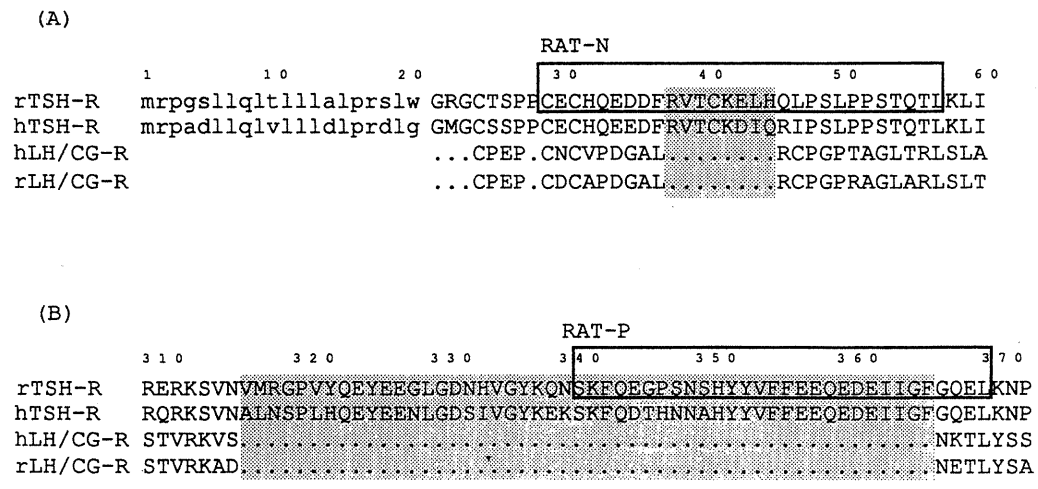

Fig. 1. Comparison of the amino acid sequences of a part of the rat TSH receptor (rTSH-R), human TSH receptor (TSH-R), human LH/CG receptor (hLH/CG-R) and rat LH/CG receptor (rLH/CG-R). Numbering of amino acid residues is based on Ref.2. Dots indicate gaps. The sequences corresponding to the synthetic peptide, RAT-N and RAT-P, are boxed. The unique tract in the TSH-R is shaded.

ume of PBS and mixed with saturated ammonium sulfate to give a final concentration of 50 %. They were centrifuged at 6000 rpm for 30 min. The pellets were dissolved in 50 mM Tris-HCl (pH 7.2) buffer and dialyzed over night against the same buffer. The concentration of IgG in solution was calculated by absorption at 280 nm.

T_3/T_4 concentrations

Concentration of 3, 5, 3'-triiodothyronine (T_3) and thyroxine (T_4) were measured using commercial kits (Amersham International plc, Amersham, Buches, UK.) according to the manufacturer's specifications.

Assessment of TSAb/TSBAb/TBII activities

TSAb and TSBAb activities were measured in FRTL-5 cells as previously described[16]. cAMP was measured by radioimmunoassay (RIA) using RIA kits (Yamasa Shoyu Co., Choshi, Japan) in duplicate determinations. TSBAb activities were defined as the percent inhibition of cAMP increase normally observed in the presence of 100 mU/m*l* bovine TSH. TBII activity was assayed using a commercial kit (Baxter, Cardiff, UK.).

TSAb activity (%) was calculated as follows:

$$\left(\frac{\text{cAMP increase in the presence of test IgG}}{\text{cAMP increase in the presence of normal control IgG}}\right) \times 100$$

TSBAb activity (%) was calculated as follows:

$$\left(1 - \frac{\text{cAMP increase in the presence of test IgG and 100 mU/l bTSH}}{\text{cAMP increase in the presence of normal control IgG and 100 mU/l bTSH}}\right) \times 100$$

TBII activity (%) was calculated as follows:

$$\left(1 - \frac{\text{labeled TSH specifically bound in the presence of test serum}}{\text{labeled TSH specifically bound in the presence of normal control serum}}\right) \times 100$$

The twenty-four hour thyroidal uptake of [^{125}I] iodine

We injected [^{125}I] NaI into the peritoneal space of the rats. After 24 hr they were anesthetized with ether, and left lobar thyroidectomies were performed. The radioactivity of the lobe was measured and the value was expressed as a percentage of the injected radioactivity.

Statistical analysis

All results are presented as the mean ± SD. Statistical differences from control groups (age-matched eight rats) were calculated by Student's t-test. Significance was set at $p < 0.05$.

Results

Production of anti TSH-R peptide antibodies

As shown in Fig. 2, twelve weeks after the first immunization, each rat immunized with RAT-N or RAT-P produced antibodies against the corresponding synthesized TSH-R peptide.

TSAb, TSBAb, and TBII activities

TSAb, TSBAb and TBII activities were measured in the presence of control or test IgGs in FRTL-5 cells. Normal ranges for each parameter were defined as the mean ± 2SD of the values obtained in the presence of IgG from eight age-matched non-immune control rats. The data are summarized in Table 1. Anti-RAT-N antibodies from eight rats possessed TSAb activity of 108 to 216 % of control values with a mean of 144 %, significantly higher than that of the control groups (100 ± 19 %, $p<0.01$). Four of the eight rats immunized with RAT-N peptide had

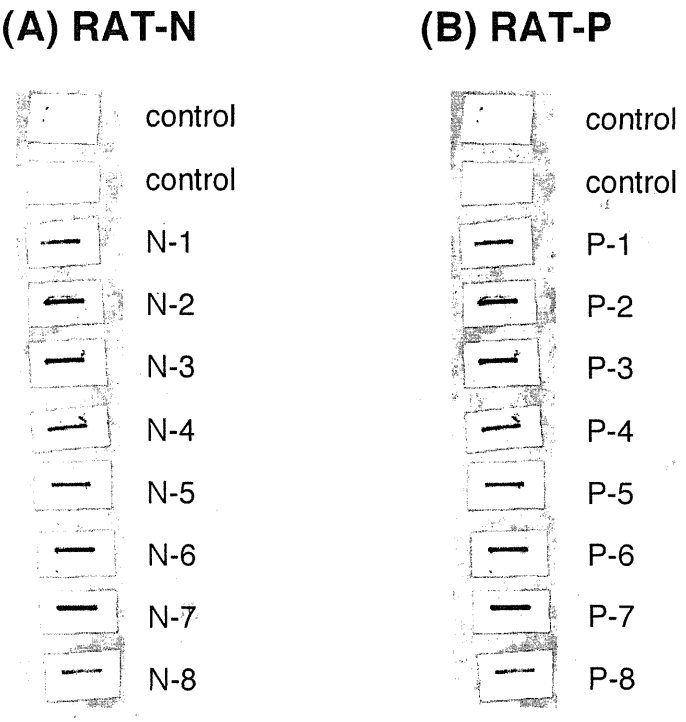

Fig. 2. Immunodetection of synthesized peptides by rat antibody. Synthesized RAT-N (A) or RAT-P (B) peptide (300 ng) was slot blotted onto nitrocellulose sheets. The sheets were incubated with antiserum from control rats (control), immunized rats with RAT-N peptide (N-1~N-8) and immunized rats with RAT-P peptide (P-1~P-8).

Table 1. Effects of immunization with rat TSH receptor peptides on rat thyroid functions

	TSAb %	TSBAb %	TBII %	^{125}I uptake%	T_3 ng/ml	T_4 μg/ml
Control (n = 8)	100 ± 19	0.0 ± 32.9	0.0 ± 7.4	14.2 ± 2.7	0.47 ± 0.06	4.39 ± 0.49
RAT-N (n = 8)	144 ± 35*	−16.1 ± 8.8	11.5 ± 16.2	12.0 ± 12.1	0.66 ± 0.12*	4.34 ± 0.53
RAT-P (n = 8)	102±31	14.4±15.4	7.7 ± 5.3*	14.1±1.7	0.29 ± 0.19*	3.91±0.60

Eight rats were used for each group. ^{125}I uptake, T_3 and T_4 were also measured in each rat. The data were expressed as the mean ± SD.
*is significant vs. control ($P < 0.05$).

TSAb activities higher than the normal range. However, TSBAb (-16.1 ± 8.8 %) and TBII activities (11.5 ± 16.2 %) were not significantly different from those of the control group. Antibodies generated in rats immunized with RAT-P possessed TBII activity (7.7 ± 5.3 %), at levels significantly ($p < 0.05$) higher than that of the control group (0.0 ± 7.4 %). However, TSAb and TSBAb activities were not significantly different from those of the control group, although they

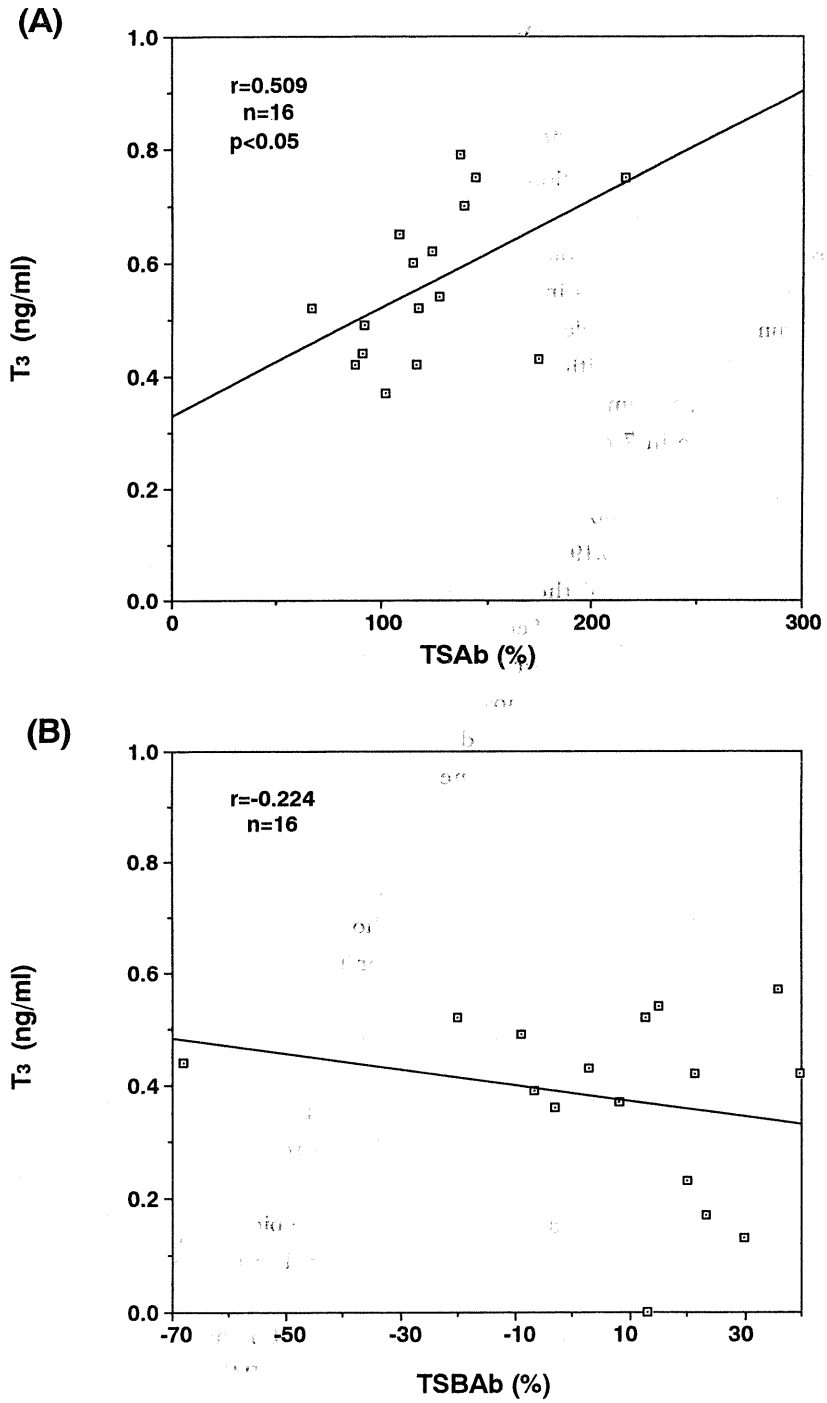

Fig. 3. (A) Correlation between serum concentrations of T_3 and TSAb activity in the sera obtained from eight control rats and eight rats immunized with RAT-N. (B) Correlation between serum concentrations of T_3 and TSBAb activity in the sera obtained from eight control rats and eight rats immunized with RAT-P. The line shows the linear regression line of best fit.

had a tendency for an increase in TSBAb activity.

Serum T_3, T_4, and thyroidal uptake of [^{125}I] iodine

We next examined thyroid functions of the immunized rats by measuring serum concentrations of thyroid hormone and thyroidal uptake of [^{125}I] iodine (Table 1). In rats immunized with RAT-N, serum T_3 levels were elevated, ranging from 0.43 to 0.79 ng/ml, with a mean of 0.66 ng/ml, $p < 0.01$). Serum T_3 levels were above the normal range in 7 of 8 rats immunized with RAT-N.

Conversely, serum concentration of T_3 in rats immunized with RAT-P (0.29 ± 0.19 ng/ml) was significantly lower than that of the control group (0.47 ± 0.06 ng/ml, $p < 0.05$). Serum T_4 levels in rats immunized with RAT-N or RAT-P were not significantly different from those of the control group. There was no significant difference in thyroidal uptake of [^{125}I] iodine between the groups.

Correlation between serum concentration of T_3 or T_4 and TSAb or TSBAb activity

There was a significant positive correlation between TSAb values and serum T_3 concentrations in the sera obtained from eight control rats and eight rats immunized with RAT-N ($r = 0.509$) (Fig. 3A). However, there was no significant correlation between TSBAb values and serum T_3 concentrations in the sera obtained from control rats and rats immunized with RAT-P ($r = -0.224$) (Fig. 3B). Additionally, there was no correlation between TSAb or TSBAb and T_4 concentrations in any group (data not shown).

Histological findings

There were no changes which suggested inflammatory or destructive reactions in the thyroid gland of any of the rats.

This experiment was performed in accordance with the Guidelines for Animal Experiments, Yamanashi Medical University.

Discussion

Identification of the recognition sites of TSH-R autoantibodies is extremely important in the study of the pathogenesis of Graves' disease. Cloning of TSH-R encording cDNA has revealed that TSH-R has two unique insertions not present in the luteotropin/chorionic gonadotropin receptor (LH/CG-R), amino acid residues 38-45 and amino acid residues 317-366 [2,17] (Fig. 1). These regions were therefore considered as potential sites for the binding of TSH or autoantibodies to the receptor.

We previously demonstrated that experimentally produced rabbit antibodies to the unique N-terminal region (amino acid residues 29-57) of the human TSH-R have TSAb activity[13,14] and that chicken antibodies to the second unique region (amino acid residues 341-370) possess TSBAb activity[15]. Other investigators have also shown the importance of the N-terminal region for TSAb binding by using site directed mutagenesis[6], synthetic peptides[7], and point-mutations[11]. The C-terminal region of the extracellular component is thought to be one of the epitopes required for TSBAb binding[9,10,12], but this remains controversial[18].

We immunized rats with rat TSH-R peptide since the biological activities of their IgGs were measured in rat FRTL-5 cells. The significantly positive correlation between the serum concentrations of T_3 and TSAb values in rats immunized with RAT-N and control rats indicated that antibodies to the TSH-R peptide, which have a stimulatory effect on FRTL-5 cells *in vitro*, also stimulate thyroid function *in vivo*.

We recently demonstrated that TSAb values

of IgGs from patients with Graves' disease in porcine thyrocytes and Chinese hamster ovary (CHO) cells transfected with human TSH-R were heterogeneous in some cases[19]. Therefore, it seems that the thyroid stimulating activity of TSH-R autoantibodies needs to be evaluated within the same species.

We are uncertain as to why serum T_4 levels and thyroidal uptake of [^{125}I] iodine did not change in the presence of autoantibodies. This may partially be explained by our short observation period. Another possibility is that the RAT-N and RAT-P regions are only two of multiple immunogenic sites involved in the regulation of thyroid function. Alternatively, the relative weakness of antibodies for biological activities may result only in an elevation of serum T_3, as has been observed in some patients with Graves' disease.

In the present study, we found elevated concentrations of T_3 associated with high TSAb activities in sera obtained from rats immunized with the RAT-N peptide. T_3 levels paralleled TSAb activity. In contrast, rats immunized with RAT-P had decreased serum T_3 concentrations and higher TBII activities, although the elevation of TSBAb was not significant. Immunization with either peptide did not result in any inflammatory or destructive reactions in the rat thyroid. These findings demonstrate that antibodies to amino acid residues 29-57, have a stimulatory effect on the thyroid, leading to hyperthyroidism, and that antibodies directed against residues 341-370 might play an inhibitory role in thyroid function resulting in hypothyroidism. This is the first report demonstrating that experimentally generated anti-TSH-R antibodies of the same species can induce an increase or decrease in thyroid hormone.

In summary, we have demonstrated that antibodies which recognize separate sites of the TSH-R have differential effects on the thyroid. Accordingly, the biological activities of TSH-R antibodies were specific for their recognition sites in the receptor. We are now in the process of investigating the biological activity of antibodies directed against point-mutated TSH-R peptides.

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