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DEVELOPMENT OF CHICKEN ANTIBODIES TOWARD THE HUMAN THYROTROPIN RECEPTOR PEPTIDES AND THEIR BIOACTIVITIES

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SUMMARY We have synthesized four peptides (P2, P4, E3 and P1) corresponding to different segments of human thyrotropin (TSH) receptor. We have obtained antibodies by immunizing them to chickens, and antibodies are evaluated for their thyroid stimulating antibody (TSAb), thyroid stimulation blocking antibody (TSBAb) and TSH-binding inhibitor immunoglobulin (TBII) activities. None of the antibodies had TSAb activity. Antibodies against human TSH receptor specific region such as P2 and P4 (P2: No.372-397, P4: No.341-358) had TSBAb and TBII activities. Anti-E3 antibody (E3: the third putative extracellular loop, No. 649-661) had only TSBAb activity. Anti-P1 antibody (P1: high homology with pig LH/CG receptor, No. 398-417), however, had none. These results suggest that anti-TSH receptor antibodies to different antigenic epitopes show heterogeneity in their biological activities. © 1991 Academic Press, Inc.

It is widely believed that autoantibodies to TSH receptor play a central role in the pathogenesis of Graves' disease and idiopathic myxedema (1). However, TBII is not detected in about 10% of patients with Graves' disease and does not always reflect their hyperthyroid state (1). Furthermore, there is a case report of Graves' disease with both TSBAb and TSAb (2). These phenomena suggest the existence of heterogeneity in anti-TSH receptor antibodies, but the detail remains unknown.

Recently, human TSH receptor-encording cDNA was cloned and its deduced amino acid sequence has been reported by several groups (3,4). TSH receptor is a member of the G protein-coupled receptors which have seven putative transmembrane domains. It has a much larger extracellular domain than that of β-adrenergic receptor (3).

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In this study, we have obtained four anti-TSH receptor antibodies by immunizing synthesized peptides corresponding to different segments of the TSH receptor. To investigate functional activities of anti-TSH receptor antibodies, we examined their TSAb, TSBAb and TBII activities.

MATERIALS AND METHODS

Peptide synthesis: Four different peptides in human TSH receptor were synthesized. Segment of P2 (No. 372-397 amino acid of human TSH receptor) and P4 (No. 341-358) are specific for human TSH receptor compared to pig LH/CG receptor (5). Segment of E3 (No. 649-661) is the third putative extracellular loop. Segment of P1 (No. 398-417) has high homology with the corresponding portion of pig LH/CG receptor. These peptides (each consisting of 13-20 residues long) were synthesized by Automatic Peptide Synthesizer (Pharmacia, BioLynx).

Antibody production: Four chickens were inoculated with 4 mg of conjugated peptide prepared in an emulsification of LES + STM adjuvant (RIBI ImmunoChem Reseach, Inc., MT, USA). After 12 days, booster was done, and after 22 days, antigen-antibody reaction was carried out in essentially the same way as previously described by Moeremans, et al. (6). Briefly, 200 ng of each antigen peptide was applied on nitrocellulose sheets, incubated with the corresponding chicken serum (1:50) in Tris buffered saline containing 2% of gelatin for 2 h at room temperature. They were then washed in Tris buffered saline containing 0.3% of Tween 20, further incubated with gold-labelled secondary serum (1:50) for 1 h. The sheets, washed as above, were additionally dipped in 1% of glutaraldehyde solution and washed in distilled water. Finally, the sheets, incubated in silver intensification solution for 20 min, were washed and air-dried.

TSAb/TSBAb: TSAb and TSBAb activities were measured using FRTL-5 rat thyroid cells as previously described by Kasagi, et al. (7). Crude Ig fractions were prepared and used at a final concentration of 5 mg/ml. The cAMP measurement for radioimmunoassay was performed in triplicate determinations. TSBAb activity was defined as the ability to inhibit 100 μU/ml bTSH-induced cAMP increase.

TBII: TBII activity was measured using a commercial kit (TRAb kit, Baxter), but rat thyroid membranes from FRTL-5 cells (8) were used instead of porcine membranes in the kit, because FRTL-5 rat thyroid cells were used in measurement of TSAb/TSBAb activity.

RESULTS AND DISCUSSION

Each chicken has produced an antibody against the corresponding synthesized TSH receptor peptide (Figure 1),

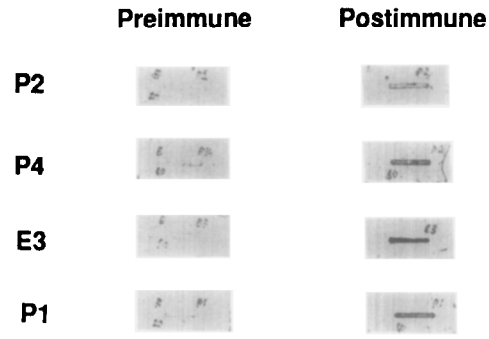

Figure 1. Immunoblotting of antigen peptide with chicken sera. Each peptide was incubated with preimmune and postimmune sera, and specific reaction was obtained in the latter.

which showed no cross-reactivity among them (data not shown).

Table 1 shows TSAb, TSBAb and TBII activities of each anti-receptor antibody. None of antibodies produced in this study had TSAb activity. Based on TSAb, TSBAb and TBII activities, we classified the antibodies into three types.

Anti-P2 and P4 antibodies belong to the first type. Since Nagayama, et al. proposed that TSH receptor specific region such as P2 and P4 is a good candidate for TSH binding site (3), anti-P2 and P4 antibodies are supposed to inhibit TSH binding and suppress cAMP augmentation. The fact that the first type antibodies showed strong TSBAb and TBII activities is compatible with their expectation. However, it should be noted that neither of the antibodies had TSAb activity. Since P2 and P4 cover most of the TSH receptor specific region, our data suggest the existence of different mechanism of receptor-adenylate cyclase activation between a hormone: TSH and a receptor antibody: TSAb.

Second type (anti-E3 antibody) shows TSBAb but not TBII activity. Though we don't know the mechanism of anti-E3 antibody action allowing TSH to bind to the receptor while inhibiting TSH to enhance cAMP production, a conformational change of the receptor induced by anti-E3 antibody binding is certainly a possibility.

Third type shows none of these activities. It seems that anti-P1 antibody is related to neither TSH binding nor inhibition of cAMP increase.

TSAb activity has not been demonstrated in our experiment, suggesting that this phonomenon might be due to

Table 1. Activities of TSAb, TSBAb and TBII (Mean ±SD)

	(a) TSAb activity (%)	(b) TSBAb activity (%)	(c) TBII activity (%)
anti-P2 antibody	70.5 ± 5.0	38.5 ± 13.8	25.6 ± 13.0
anti-P4 antibody	83.9 ± 4.9	47.4 ± 3.6	20.9 ± 1.4
anti-E3 antibody	89.5 ± 8.3	49.8 ± 14.5	4.5 ± 2.5
anti-P1 antibody	92.2 ± 1.5	3.1 ± 4.0	10.9 ± 0.4

(a) TSAb activity (%) was calculated as follows:

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

(b) TSBAb activity (%) was calculated as follows:

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

Specific blocking was obtained in the presence of anti P2, P4 and E3 IgG.

(c) TBII activity was calculated as follows

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

Activity higher than 15% was judged positive.

the differnt antigenic target between TSAb and TSBAb. We guess that there may exist other factors such as anti-idiotype antibody, which play pivotal roles in interaction with anti-receptor antibodies (9).

Recently, several studies on heterogeneity of anti-TSH receptor antibodies has been carried out. TBII activity is reported to be not always parallel with TSAb activity (1). Zakarija, et al. reported the presence of a case with Graves' disease which had TSBAb as well as TSAb (2). Valente, et al. reported different type of antibodies toward the TSH receptor derived from the lymphocytes of patients with Graves' disease (10). However, these studies have limitation for analyzing the heterogeneity of autoantibodies from patients with autoimmune thyroid diseases, since the

autoantibodies may recognize different epitope of TSH receptor or TSH receptor-related antigens in the thyroid. To clarify these, we have produced antibodies against synthesized TSH receptor peptides based upon the published amino acid sequence, and found that there exist at least three types of anti-TSH receptor antibody as judged by TSAb, TSBAb and TBII activities.

Very recently, Wadsworth et al. reported that eight-amino acid tract near the amino terminus of the TSH receptor (No. 38-45) is an important site of interaction for both TSH and TSAb (11). Further studies are in progress in our laboratory to evaluate the interaction between TSAb and TSBAb.

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