Thyroid-stimulating antibodies in sera from patients with Graves’ disease are heterogeneous in epitope recognition

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Two synthetic peptides, P354-14 (amino acid nos. 354 to 367) and P338-16 (nos. 338 to 353), corresponding to the partial amino acid sequences of the hTSH receptor structure were studied for their ability to bind specifically serum IgGs from patients with Graves’ disease and to inhibit thyroid stimulating TSH receptor antibody (TSH-R SAb) activity. IgG binding was measured by an ELISA using sera from 102 Graves’, 20 Hashimoto patients, and 9 normal subjects. Both peptides showed significantly increased IgG binding of Graves’ sera compared with those of Hashimoto and normal sera. There was a significant correlation (r = 0.529) between the amount of IgG bound by the two peptides, but neither of these values correlated well with their TSH-R SAb activity nor thyrotropin-binding inhibitor TSH receptor antibody (TSH-R IAb) activity. TSH-R SAb inhibiting effects of these peptides were then analysed by measuring TSH-R SAb activity after incubation with the peptides. Among eight Graves’ IgGs tested the TSH-R SAb activity of three was inhibited by both peptides, two were inhibited only by P354-14 and three were not affected by either. These TSH-R SAb inhibiting effects were dose-dependent and reproducible. To confirm these findings, a peptide-sepharose gel affinity absorption study was performed. Eleven Graves’ IgGs were applied to both peptide gels and the TSH-R SAb activity of the unabsorbed fraction was measured. The TSH-R SAb activity of five IgGs was strongly absorbed only by P354-14 and five others were absorbed by both peptides to an almost similar extent. One IgG was not affected by either of the peptides. None of the IgG activities tested was absorbed only or predominantly by P338-16. Most of Graves’ IgGs could bind to region no. 338 to no. 367 of the hTSH receptor. There was apparent heterogeneity among Graves’ TSH-R SAb IgGs as shown by their absorption by the peptides, and the existence of at least three heterogeneous species in epitopic recognition among Graves’ TSH-R SAb was demonstrated.

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Thyroid stimulating TSH receptor antibody (TSH-R SAb) has been postulated to play an important role in the development of Graves’ disease (1–5). Epitope analyses of the antibody have been performed extensively following the cloning and sequencing of the TSH receptor cDNA. Most of them indicated several epitopic regions on the extracellular domain, especially on the N-terminal region of the receptor (6–17). Nagayama & Rapaport recently reported different recognition sites on the hTSH receptor among TSH-R SAb using a chimeric cDNA expression technique (18).

We have also investigated the significance of a particular region of the hTSH receptor which is specific for this receptor and does not occur in the LH/CG or FSH receptor sequences (15, 19). Synthetic oligopeptides, such as P-195 (P353-11, hereafter) and P-218 (P354-14) are known to bind TSH-R SAb specifically and thus to inhibit TSH-R SAb activity. Further, P354-14 and P338-16 (P-231 in Ref. 19) were shown to exert quite different absorbing effects on TSH-R SAb activity with a Graves’ immunoglobulin, F-IgG (19). The TSH-R SAb activity was inhibited effectively by P354-14 but not affected by P338-16.

In the present article, we first compared IgG binding specificities of P354-14 and P338-16 using an ELISA method, and then analyzed the TSH-R SAb absorbing ability of both peptides using a preincubation method and an affinity absorption method with 18 Graves’ IgGs. We were able to demonstrate heterogeneous recognition of the hTSH receptor epitopes among these TSH-R SAb.
Materials and methods

Clinical subjects

Patients with Graves' disease and those with Hashimoto thyroiditis were diagnosed by typical clinical findings and routine and specific laboratory examinations, including TSH-R SAb measurements. All of 102 Graves' patients had TSH-R SAb and thyrotropin binding inhibitor TSH receptor antibody (TSH-R IAb) to a varying extent, while none of 20 Hashimoto patients had either of the TSH receptor autoantibodies. Nine normal healthy subjects served as controls.

Peptides

The synthetic peptides were designated as follows: P + (position of amino acid corresponding to the N-terminal amino acid of the peptide) - number of amino acids in the peptide. The numbering of the amino acid residues included the signal peptide region. For example, P354-14 indicates a peptide consisting of 14 amino acids from no. 354 valine. Both P354-14 (VFFEEQDEEIIFGF) and P338-16 (KEKSFOQDTHNNAHYY) were synthesized in vitro as described previously (11). Another peptide, P354-14 (reverse), having a completely reverse sequence of P354-14 was also prepared (P-262 in Ref. 19). All were soluble in water, did not change the pH of the solution, and were kept frozen until used.

Enzyme-linked immunosolvent assay (ELISA) of bound IgG

A 96 well flat-bottom plate was coated with 5 µg/well of peptide dissolved in PBS for 3 h at 30°C. The plate was washed with 0.1% Tween 20 with four times concentrated PBS (4 x PBS) and then with PBS. After blocking by 1% BSA overnight at 4°C, 50 µl/well of 1 to 100 diluted solution samples were incubated for 2 h at 28°C. The plate was washed with 0.1% Tween 20, with 4 x PBS and RIA solution (PBS containing 0.5% BSA), then with 50 µl/well of 1 to 1000 diluted anti-hlgG F(ab)2, rabbit antiserum (Immunotech. S.A., France) conjugated with alkaline phosphatase was added. After 1 h at room temperature, the plate was washed again and 100 µl/well of substrate solution (2 mg/ml p-nitrophenylphosphate in 100 mM sodium bicarbonate, 10 mM MgCl2 pH 9.5) was added and the optical density at 405 nm in each well was read by a microplate reader (BIO-RAD). The data were expressed as optical density (OD) values at 405 nm.

TSH-R SAb and TSH-R IAb measurements

TSH-R SAb assays were carried out using 2 x 10^4/well of FRTL-5 cells under low salt incubation conditions as described previously (19). Generated cAMP after incubation with bTSH or 400 µg/well of purified IgG for 2 h at 37°C was measured by an RIA (Eiken Chemicals, Tokyo). Values exceeding 150% of those of normal IgG were taken as significant. TSH-R IAb was measured using a kit (Baxter-Travenol, Cardiff, UK), and the normal range was less than 10% after correction of individual non-specific binding (20).

Peptide application

Four hundred µg/well aliquots (40 µl) of IgG dissolved in low salt Hank's solution were mixed with 10 µl each of peptide solutions (0.5 mg/ml) in a microtube. After incubation for 20 h at 4°C with gentle shaking, 5 µl of 5 mM IBMX was added, mixed well, and then 50 µl aliquots were applied in triplicate for TSH-R SAb assay. Except for the dose-dependent study (Fig. 3), all the tested samples including normal IgG and/or bTSH contained the same amount of peptides. These peptides were known to exert no effects on the basal cAMP or those induced by bTSH, forskolin or blocking type TSH receptor antibody of FRTL-5 cells (19).

Affinity absorption study

Affinity gels were prepared in microtubes by conjugation of 2 mg of peptides with 1 ml of CNBr-sepharose gel. A 1 ml precolum containing glycine-saturated sepharose gel was prepared. After equilibration of the gel with low-salt Hank's solution 200 µl serum equivalent (approximately 2 mg) of IgG in 0.5 ml was applied. Two ml each of the eluted fractions was collected and then transferred onto the microtubes, which were also equilibrated with low-salt Hank's solution. The microtubes were rotated gently overnight and centrifuged for 10 min at 3000 rpm. The unabsorbed fractions (supernatants) were recombined to the initial volume and then subjected to TSH-R SAb measurements. As shown previously, most of TSH-R SAb activity of one Graves' IgG, no. 1 IgG in Table 1, was known to be absorbed effectively by a P354-14 affinity gel and to be recovered in the acid-eluate (19).

Results

We first attempted to establish an ELISA system which could detect TSH-R SAb-IgG specifically. For this purpose, P354-14 was chosen because this peptide is known to inhibit most of the TSH-R SAb activity by preincubation (15, 19). As a control, P338-16 was used, which is adjacent but non-overlapping with P354-14 and also known to have no inhibiting effect on one of the TSH-R SAb (no. 1 in Table 1).

Fig. 1 shows the results obtained measuring specifically bound IgG to either P354-14, P338-16 or P354-14 (reverse) coated plastic plate. For P354-14, sera from 9 normal subjects showed OD values (mean and SD: 0.282 ± 0.098) which did not differ significantly
Table 1. Changes in TSH-R SAb activity of eight Graves' IgGs after incubation with two oligopeptides corresponding to the partial sequences of the hTSH receptor and amounts of IgG bound to the peptides.

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Original</th>
<th>TSH-R SAb activity (%)</th>
<th>ELISA binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>P354-14</td>
<td>P338-16</td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>5912 ± 37</td>
<td>1129 ± 32 (−81)</td>
<td>5746 ± 45 (−3)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8293 ± 40</td>
<td>3782 ± 41 (−54)</td>
<td>8331 ± 40 (−2)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9873 ± 47</td>
<td>5952 ± 30 (−40)</td>
<td>6681 ± 27 (−32)</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>3639 ± 35</td>
<td>2246 ± 25 (−38)</td>
<td>2210 ± 33 (−39)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>479 ± 21</td>
<td>292 ± 10 (−39)</td>
<td>373 ± 15 (−22)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3062 ± 40</td>
<td>2619 ± 13 (−14)</td>
<td>2990 ± 23 (−2)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2829 ± 60</td>
<td>2646 ± 116 (−6)</td>
<td>2771 ± 64 (−2)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>583 ± 10</td>
<td>542 ± 12 (−7)</td>
<td>516 ± 10 (−11)</td>
</tr>
</tbody>
</table>

TSH-R TSAb activity was expressed as the percent increase of generated cAMP compared to that of normal IgG.

( ) Percent change of TSH-R TSAb activity from original. Figures shown mean ± sd.

Bound IgG is measured by the percent change of OD at 405 nm compared to that of normal IgG.

from those of 20 Hashimoto patients (0.315 ± 0.124). In 102 sera from Graves’ patients, however, significantly increased IgG binding was observed (0.483 ± 0.189) (Fig. 1a). Similar results were observed in the case of P338-16 and significantly higher IgG bindings of Graves’ sera (0.490 ± 0.227) than those of Hashimoto (0.336 ± 0.190) or normal sera (0.262 ± 0.082) were observed (Fig. 1b). Specificity of the binding was shown by the finding of no significant difference in IgG amounts bound to P354-14 (reverse) coated plates among sera from the three groups (Fig. 1c; 0.245 ± 0.058 for 33 Graves’, 0.217 ± 0.085 for 20 Hashimoto and 0.214 ± 0.100 for 9 normal subjects, respectively). When the amounts of IgG in 102 Graves’ sera bound to both peptides were compared (Fig. 2), a significant relationship between them was found (r = 0.529, p < 0.001) and preferential IgG binding to P354-14 over P338-16 was not apparent. Further, none of these values was found to correlate linearly with TSH-R SAb or TSH-R IAb activities (data not shown). Therefore, Graves’ IgGs demonstrated some binding affinity with both of these peptides, but the binding did not exhibit any biological significance.

When 8 Graves’ IgGs with various TSH-R SAb activities were incubated with 5 µg each of either P354-14 or P338-16, the degree of inhibition of TSH-R SAb activity varied considerably depending upon the IgG and peptide used, as shown in Table 1. No. 1 and no. 2 IgGs showed considerable inhibition in TSH-R SAb activity with P354-14 but no inhibition with P338-16 (Group A). Both peptides inhibited TSH-R SAb activity

Fig. 1. Amounts of IgG bound to two oligopeptides corresponding to the partial amino acid sequence of the hTSH receptor in sera from 102 Graves’, 20 Hashimoto patients, and 9 normal subjects. (a) Binding to P354-14, (b) Binding to P338-16, (c) Binding to P354-14 (reverse). Bars show the mean of each group.
in sera 3, 4 and 5 (Group B). There were three IgGs (nos. 6, 7 and 8) which showed no diminution of their TSH-R SAb activity with these two peptides (Group C). There was no significant relationship in these samples between the original TSH-R SAb activity or the degree of TSH-R SAb inhibition by the peptides and the amount of bound IgG to the peptides. Fig. 3 shows the dose-dependent TSH-R SAb inhibiting effects of both P354-14 and P338-16 on IgG no. 3 of Table 1, which were dose-dependent. The observed differences in TSH-R SAb inhibiting effects of these peptides were therefore considered to relate with constituents of the individual IgG.

In order to minimize possible non-specific effects of the peptides on the assay system, affinity absorption studies were performed. Figure 4 summarizes the results of TSH-R SAb absorption of 11 Graves’ IgGs by two affinity gels with each of P354-14 or P338-16. There were five IgGs including no. 1 IgG (A-3 in the Figure) categorized as Group A in which P354-15 showed predominant absorptions of TSH-R SAb activity. The Group B pattern was seen in five IgGs whose TSH-R SAb activities were absorbed with both P354-14 and P338-16. After affinity absorption studies, 1 IgG still exhibited no change in TSH-R SAb activities with either of the peptides (Group C). Thus, together with the results of Table 1, 6 out of 18 IgGs (33%) were found to be in Group A, 8 (44%) in Group B and 4 (22%) in Group C, respectively. Of note was that none of 18 IgGs studied was found to be affected predominantly by P338-16.

Discussion

An attempt to measure TSH-R SAb activity indirectly by an ELISA system using P354-14 did not succeed. P354-14 or a smaller peptide P353-11 (P-195) is known to have specific and significant inhibiting effects on TSH-R SAb activities by pre-incubation (15, 19). Indeed, significantly increased binding of Graves’ IgGs to P354-14 was observed compared to Hashimoto, or normal IgGs. However, IgG binding to P354-14 did not correlate significantly with TSH-R SAb activity, and furthermore, similar and Graves’ specific IgG binding was also seen with P338-16. Graves’ specific IgG binding to the peptides corresponding to the partial sequences of hTSH-R has been reported previously (11, 12, 15, 23, 24), but our observations indicate that bound IgG amounts do not exhibit any biological activity directly. The significance of such binding cannot be explained at present, but it appears that TSH-R autoantibody activity cannot be measured simply by an IgG-peptide binding system.

The observation of Graves’ specific IgG binding to P338-16 prompted us to analyze the TSH-R SAb inhibiting effect of the peptide on Graves’ IgGs other than F-IgG (19). The effects were compared with those obtained by P354-14. Addition of these synthetic peptides to various Graves’ IgGs resulted in three types of inhibiting effect on their TSH-R SAb activity. Our group A IgGs were characterized by TSH-R SAb inhibition only with P354-14, and no inhibition with P338-16. Group B IgGs were inhibited almost to the same extent with both peptides, while some IgGs, Group C, did not show any significant inhibition with either of the peptides.

The possibility that the different amino acid compositions of both peptides may have had some effect on the TSH-R SAb assay, or the addition of 5 μg peptide with IgG may have induced some non-specific effects were
considered unlikely. A reverse sequence peptide of P354-14, P354-14 (reverse), is known to have no inhibitory effects on the biological activity of Graves' IgG (19), and in this series this peptide was found not to show any increased IgG binding using sera from patients with Graves' disease either. As for the amounts of peptide used, we have shown that such amounts of P354-14 did not exert any significant effects on the basal cAMP levels of FRTL-5 cells as well as those induced by hTSH, forskolin or TSH-R BAb (19). Further, such large amounts are also known to be necessary for the specific inhibitory effects of the peptides on TSH-R SAb activity (15, 19). In a TSH-peptide binding study from another group, similar or even larger amounts of peptide were needed (27). In the present study dose-dependent TSH-R SAb inhibiting effects of both peptides were confirmed and further, three types of TSH-R SAb absorbing effects with both peptides were observed.

As for the significance of the TSH receptor specific region of the No. 317 to 366 amino acid sequence, studies using cDNA deletion and chimeric cDNA indicated no apparent necessity of this region for TSH binding or cAMP production by TSH or TSH-R SAb (8, 9, 10, 14, 16, 17). However, applications of synthesized peptides revealed specifically increased binding of Graves' IgG, TSH-R SAb inhibiting effects, and bioactive (thyroid stimulatory or stimulation blocking) antibody production by immunization of animals with these peptides (15, 16, 19, 21–23). Further, this particular region is now considered to be the immunodominant domain of the TSH-R (23–25).

Kosugi et al. (23) observed that approximately 80% of Graves' IgG could bind specifically to a peptide (amino acids 352 and 367), and in the present study both P354-14 and P338-16 showed inhibition of TSH-R SAb activity in 78% of Graves' IgG. P354-14 showed TSH-R SAb inhibiting effects with all Graves' IgGs in Group A and B, while P338-16 showed effects in Group B, and none of the TSH-R SAb IgGs tested showed specific inhibition of activity only by the latter. This indicates that TSH-R SAb inhibiting effects may reside at least within P354-14 and several consecutive N-terminal amino acids may also be recognized fre-
quently. Shifting the frame of the peptide in this region by a few residues on either side is known to result in very different activities and so adjacent peptides should have different activities (26). We do not mean to imply that this region is the only epitope for TSH-R SAb but we consider this to be one of the epitopic regions. Ikeda et al. reported that four peptides from the hTSH receptor, including one close to the present study, are recognized specifically but variably by Graves’ IgGs (28). Nagayama and Rapoport showed different recognition of no. 25 to 30 regions of the hTSH receptor by 10 Graves’ IgGs tested (18). However, they were unable to demonstrate the dose-response effect on cAMP production because their cDNA transfected CHO cells were not sensitive enough to detect TSH-R SAb activity in the diluents. Our present observations though, indicate clearly the heterogeneity of sites recognized by TSH-R SAb on the TSH receptor.

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