Clinical Significance of a Sensitive Assay for Thyroid-Stimulating Antibodies in Graves’ Disease Using Polyethylene Glycol at High Concentrations and Porcine Thyroid Cells

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Abstracts. The Inui and Ochi group recently reported that cAMP production by porcine thyroid cells (PTC) was augmented more by polyethylene glycol (PEG) 22.5% precipitated fractions from almost all Graves’ sera than those of PEG 12.5%. In the present study, thyroid stimulating immunoglobulin (TSI) activity was determined with PTC and prepared crude Ig fractions precipitated by two different concentrations of PEG (final concentrations 13.5% and 22.5%) from sera obtained from 117 Graves’ patients. The activity of TSI determined by the PEG 13.5% assay and activity determined by the PEG 22.5% assay were designated as thyroid-stimulating antibody (TSAb) and sTSAb, respectively.

At first we studied 55 TSAb-positive patients with untreated hyperthyroid Graves’ disease and classified them according to the TSAb activity—below 500% (group 1) and above 500% (group 2). The positive stimulatory effect, arbitrarily defined as the ratio of sTSAb to TSAb, being more than 1.2, was observed in 85% of patients, and group 1 had a significantly (P<0.025) greater stimulatory effect (34/35, 97.1%) than group 2 (13/20, 65%).

Subsequently, in 29 TSAb-negative patients, sTSAb was measured and detected in 26 (89.7%). Finally, sTSAb, TSAb and TBII were compared between patients presenting with recurrent Graves’ disease and those with silent thyroiditis after withdrawal of antithyroid drug treatment for Graves’ disease. sTSAb was detected in all 14 relapsed patients, but none of the 9 patients with silent thyroiditis had detectable sTSAb. In contrast, TSAb and TBII activities were found in only 7 (50.0%) of the 14 relapsed cases. The present paper demonstrated that the assay with a higher PEG concentration was found to be sensitive, specific and useful for the diagnosis and follow-up of Graves’ disease after drug withdrawal, although the underlying mechanism remains unclear.

Key words: TSAb assay, TSI, Porcine thyroid cells, Graves’ disease, Silent thyroiditis, Polyethylene glycol

THYROID hyperfunction in Graves’ disease is thought to arise from stimulation of the thyroid gland by thyroid stimulating immunoglobulin (TSI) [1, 2]. Since the detection of TSI is of fundamental importance both in the diagnosis of untreated active Graves’ disease and in following the course of the disease, a sensitive and practical assay for TSI is needed for its clinical application as a routine test. The TSI assay involves determination of cyclic adenosine monophosphate (cAMP) production by human thyroid cells, porcine thyroid cells (PTC) or FRTL-5 cells [3-11]. A crude γ-globulin fraction obtained from sera by the polyethylene glycol (PEG) precipitation method has been hitherto used in the routine assay (9). More recently the Inui and Ochi group (12) reported that cAMP production during a 5-hour incubation with PTC was significantly augmented by using PEG 22.5% precipitated fractions.
instead of PEG 12.5% from almost all Graves' sera. In the present study, we demonstrated that TSI activities measured by the assay with the PEG 22.5% precipitation method (the PEG 22.5% assay with the detected antibody designated as sTSAb) was detected in almost all patients with untreated or recurrent active Graves' disease, whereas in the routine assay system with a crude γ-globulin fraction obtained from sera precipitated with 13.5% PEG (the PEG 13.5% assay with the detected antibody designated as TSAb), thyroid stimulating antibody(TSAb) was detected in 85% of the patients with untreated Graves' disease and in 50% of the recurrent patients.

Materials and Methods

Patients

1. Untreated Graves' patients

Of 366 patients with active, untreated Graves' disease, TSI measured by the routine PEG 13.5% assay with the commercially available Yamasa kit was detected in 311 (85.0%). (Data not shown). Among these patients, 55 TSAb-positive untreated Graves' patients, 7 males and 48 females, aged 15-64 years and 29 TSAb-negative untreated patients, 4 males and 25 females, aged 21-61 years, diagnosed during the period between 1994 and 1998 were selected for the present study.

The diagnosis of hyperthyroidism due to Graves' disease was based on commonly accepted clinical criteria with the presence of diffuse goiter, high serum-free thyroxine (s-FT4) and serum free triiodothyronine (s-FT3) levels, undetectable s-TSH, a diffusely increased uptake of 99mTc by the thyroid, and hyperthyroid symptoms and signs.

2. Patient with recurrent Graves' disease or silent thyroiditis after drug withdrawal in Graves' disease

The study was performed in 23 patients with Graves' patients who suffered from thyrotoxicosis within 1 year after cessation of the antithyroid drug. All patients had been given thiamazole for at least 2 years and alternative-day 5 mg thiamazole for one year after the establishment of negative thyrotropin binding inhibitor immunoglobulin (TBII) and had stopped taking medicine. The study group consisted of 9 female patients aged 19-48 years presenting with silent thyroiditis (group A) and 14 relapsed patients, 2 males and 12 females, aged 18 to 53 years (group B).

The diagnosis of relapse of Graves' disease or silent thyroiditis after cessation of thiamazole was based on greatly increased or decreased 99mTc thyroid uptake, high s-FT4 and s-FT3, suppressed s-TSH and a history of medication for Graves' disease.

Assays of TSAb and sTSAb activities

TSAb and sTSAb activities were determined according to the previously reported methods (9, 12, 13). Test sera (0.5 ml) were mixed with 1.5 ml of 18% or 30% PEG (6000) aqueous solution at 5°C, to make final PEG concentrations of 13.5% or 22.5%, respectively. The precipitated fraction (ppt fr) was obtained by aspiration of the supernatant fraction after centrifugation at 3000 rpm for 15 minutes. The ppt fr was recentrifuged and dissolved in 0.5 ml of Hank's buffer (salt-free), to bring them to the same volume of solution, because the ppt volume gradually increased in relation to the PEG concentrations. Of these dissolved fractions, 0.2 mL was used to incubate with cultured PTC for 4 hours at 37°C provided in the commercial kit (Yamasa -Shoyu Ltd., Tokyo, Japan). The assay was performed in a total volume of 0.25 mL according to the design of this kit. All experiments were performed in duplicate determinations.

The results of the TSAb assay were expressed as percentages of cAMP production: in the PEG 13.5% assay, [(cAMP produced in the presence of the precipitated fraction of patient sera treated with 13.5% PEG)/(cAMP produced in the presence of precipitated fraction of normal control sera treated with 13.5% PEG)] and in the PEG 22.5% assay, [(cAMP produced in the presence of the precipitated fraction of patient sera treated with 22.5% PEG)/(cAMP produced in the presence of the precipitated fraction of normal control sera treated with 22.5% PEG)].

The coefficients of intra-assay variation out of 8 single measurements of sTSAb were 7.4 and 6.0% at mean activities of 195 and 806%, respectively, and those of TSAb were 14.1 and 7.5% at mean activities.
SENSITIVE TSAb ASSAY USING HIGH PEG

of 216 and 720%, respectively. In normal controls the mean(±SD) TSAb and sTSAb activities were 140.4±22.0 (n=110) and 139.8±27.6% (n=55), respectively. The upper limits of the normal range determined according to the formula, mean+1.96 (statistical significance level of P<0.05)×SD, were 183.1% for TSAb and 193.9% for TSAb. The TSAb and sTSAb equal to or greater than 184% and 194% are considered positive.

Assay of thyrotropin binding inhibitor immunoglobulins (TBII)

TBII activity was measured in a radioreceptor assay with solubulized porcine thyroid membranes by a commercially available TSH receptor assay kit (Dade Behring Ltd., Stillwater, MN, USA.) as previously described in detail (13). The upper limit of the normal range was obtained from average and standard deviation at a statistical significance level of P<0.01. The upper limit of the normal range, which was determined from the average and the standard deviation of the values for 120 controls at a statistical significance level of P<0.01, was 8.9% and values over 10% were regarded as positive.

Other methods

Thyroid stimulating blocking antibody (TSBAb) was determined by a method previously described by Konishi et al. (14).

s-FT4, s-FT3 and s-TSH were measured by chemiluminescent immunoassay with ACS-180 (Bayer Co., Tarrytown, USA) and anti-thyroid peroxidase antibody and anti-thyroglobulin antibody by radioimmunoassay with commercially available kits (Cosmic Co., Tokyo, Japan).

Statistical analysis was done by χ2 statistic test.

Results

Comparison of TSAb and sTSAb in active, untreated Graves’ patients

As shown in Fig. 1, in most of the 55 TSAb-positive patients with untreated Graves’ disease sTSAb activities were higher than those of TSAb. And there was a significant correlation between sTSAb and TSAb (r=0.716; n=55; P<0.001).

We also investigated the ratios of sTSAb to TSAb in the TSAb-positive patients and classified them according to their TSAb activities-below 500% (group 1) and above 500% (group 2), as shown in Table 1. If the positive stimulatory effect was defined arbitrarily as the ratio of sTSAb to TSAb being equal to or greater than 1.2, it was observed in 85.5% of all cases (47/55) and 97.1% (34/35) of group 1 patients and 65.0% (13/20) of group 2 patients. The difference between group 1 and group 2 in the stimulatory effect was statistically significant (P<0.025).

sTSAb was then tested for the 29 TSAb-negative patients with untreated Graves’ disease and detected in 26 (89.7%), as shown in Fig. 2.

TBII activity was detected in only 16 (61.5%) of 26 TSAb-negative and sTSAb-positive patients and in 52 (94.5%) of 55 TSAb- and sTSAb-positive patients with untreated Graves’ disease.

In untreated Graves’ patients, both TSAb and sTSAb were significantly correlated with 99mTc thyroid uptake (r=0.468; n=42; P<0.001 and
Comparison of sTSAb activity in patients with relapse and those with silent thyroiditis after cessation of antithyroid drug in Graves' disease

The age, gender and results for 99mTc thyroid uptake, s-FT4 and s-FT3 levels, TSAb, sTSAb and TBII activity recorded immediately after the discovery of the development of thyrotoxicosis subsequent to the withdrawal of thiamazole in Graves' disease are summarized in Table 2.

Fig. 3 shows that sTSAb activity in group B was detected in all 14 patients, but none of the 9 patients in group A had detectable sTSAb. In contrast, the results for TSAb and TBII in group B were positive in only 7 (50.0%) of the 14 recurrent patients. One patient with a elevated TBII level (66.4%) in group A probably had blocking-type TSH-receptor antibody, as TSBAb activity increased at 97.5% (normal, <40%).

Discussion

The salient features of these studies include the finding that, in untreated hyperthyroid Graves' disease, the PEG 22.5% assay is definitely more sensitive in determining TSI than the PEG 13.5% assay. The positive stimulatory effect of TSI occurred in 85% of TSAb-positive patients, and a detectable sTSAb was found in approximately 90% of TSAb-negative patients (26/29, 89.7%), although TBII activity was found in only 62.0% (18/29). We also found a significantly higher frequency of the positive stimulatory effect of TSI in group 1 patients with TSAb activities of <500% than in group 2 (>500%). Since there was a significant correlation between sTSAb and TSAb in TSAb-positive patients, it seemed likely that sTSAb reflected TSAb to some extent.

The prevalence of detectable TSI in untreated hyperthyroid Graves' patients reported in the litera-
Table 2. Summary of data for patients with (A) silent thyroiditis or (B) recurrent Graves' disease after withdrawal of antithyroid drug in Graves' disease

<table>
<thead>
<tr>
<th>age/sex</th>
<th>TSAb activities %</th>
<th>sTSAb activities %</th>
<th>TBII %</th>
<th>s-FT&lt;sub&gt;4&lt;/sub&gt; ng/dl</th>
<th>s-FT&lt;sub&gt;3&lt;/sub&gt; pg/ml</th>
<th>99mTc thyroid uptake %</th>
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<td>(A)</td>
<td>48/F</td>
<td>120</td>
<td>110</td>
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<td>41/F</td>
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<td>133</td>
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<td>22/F</td>
<td>158</td>
<td>164</td>
<td>0.1</td>
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<tr>
<td></td>
<td>38/F</td>
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<td>155</td>
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<td>19/F</td>
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<tr>
<td>(B)</td>
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<td>441</td>
<td>3.5</td>
<td>2.02</td>
<td>3.9</td>
</tr>
<tr>
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<td>615</td>
<td>12.4</td>
<td>3.05</td>
<td>8.4</td>
</tr>
<tr>
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<td>45/M</td>
<td>296</td>
<td>701</td>
<td>0.5</td>
<td>2.47</td>
<td>4.4</td>
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</table>

Normal Range: <184, <195, <10, 0.75, 2.1, 0.5

Fig. 3. Comparisons of sTSAb, TSAb and TBII activity in (A) the state of silent thyroiditis after cessation of antithyroid drug in Graves' disease and (B) relapse of Graves' disease. Parenthesis denotes the ratio of positive cases to total examined cases. (−: upper limit of normal range; ○: positive and ●: negative).
ture was as follows: 59 (92.2%) of 64 (3), 13 (92.8%) of 14 (4), 38 (92.7%) of 41 (5), 57 (93.4%) of 61 (6) and 28 (96.6%) of 29 (7) in human thyroid cell assay; 41 (97.6%) of 42 (9) in PTC assay; 25 (89.3%) of 28 (8), all of 50 (10) and 76 (90.5%) of 84 (11) in FRTL-5 thyroid cell assay.

The present study examining a series of a large number of untreated Graves’ sera noted that the incidence of detectable TSAb, 85% (311/366), was insufficiently sensitive, and the sensitive PEG 22.5% assay can demonstrate the existence of TSI in almost all untreated Graves’ patients.

Also of interest is the observation that sTSAb was a diagnostically determinant marker of Graves’ disease in patients who developed recurrent thyrotoxicosis after cessation of the antithyroid drug. In the patients with silent thyroiditis after withdrawal of the antithyroid drug for Graves’ disease, sTSAb was negative in all patients studied thus far. Conversely, all of the recurrent patients had positive sTSAb when tested, although TSAb and TBII were detected in only 50% of the recurrent cases. On this basis, it seems clear that sTSAb may be able to accurately differentiate the relapse from silent thyroiditis after antithyroid drug therapy in Graves’ disease. Kasagi et al. (9) also demonstrated that TSAb measured by the sensitive assay with FRTL-5 thyroid cell was present in all patients with recurrent hyperthyroid Graves’ disease. Similar studies performed by Momotani et al. in postpartum thyrotoxicosis revealed that TBII activity was present not only in all 33 patients with recurrent hyperthyroid Graves’ disease but also in 12 of the 23 patients with low radioiodine uptake who had a history of Graves’ disease. In the present study, TBII was found in only one patient with silent thyroiditis who had TSBAAb, whereas 7 of 14 relapsed Graves’ patients were positive for TBII. This difference could be dependent on the clinical situation at the time of discontinuation of the antithyroid drug. In our study, the antithyroid drug was stopped after TBII became negative. In contrast, in most of the cases reported by Momotani et al., the antithyroid drug was discontinued during pregnancy. Regarding the clinical usefulness of TSI for predicting the prognosis in patients with Graves’ disease, Kasagi et al. (16) demonstrated that TSAb, measured by a sensitive assay (9), at the time of discontinuation of treatment was present in Graves’ patients even in remission (12/23, 56.5%). Our study also revealed sTSAb at withdrawal of the antithyroid drug was found in 29.0% (20/69) of cases who remained in remission. (Data not shown). Therefore, it seems likely that TSI measured by sensitive assay may not be useful in predicting the prognosis of Graves’ patients at the end of therapy.

The use of PEG (final concentration 15%) instead of ammonium sulfate, for the preparation of crude Ig fractions was first introduced by Shewring and Smith in an assay for TSH. (17).

The method of adding PEG-precipitated crude Ig fractions directly into the PTC culture medium in the TSAb assay, as done in the present study, was originally developed by Kasagi et al. (8). According to their data, the PEG sediment proteins consisted of 27% albumin, 3.8% α1-globulin, 7.2% α2-globulin, 12% β-globulin, and 49.5% γ-globulin. Since Inui et al. (12) demonstrated that the recovery of IgG in the PEG 12.5% ppt fr and PEG 22.5% ppt fr is more than 85%, the positive stimulatory effect is not dependent on the difference in the recovered IgG concentration.

Although the mechanism by which a high PEG concentration increases the thyroid stimulation activity by TSI remains unclear, Inui et al. (12) proposed two hypotheses. One is that cAMP production is increased by the increased TSH receptor binding by TSI resulting from the conformational change due to PEG conjugation in PTC. Another possibility is the existence of some other factors that influence the ability of TSI to stimulate thyroid cells. Consistent with this hypothesis, Kasagi et al. (9) demonstrated by using porcine thyroid cells that cAMP response to the crude Ig fraction obtained from Graves’ sera was stronger than that to the purified IgG. It was also demonstrated that coinubation of purified TSI with the PEG 22.5% ppt fr from normal human serum induced a significant increase in cAMP production (12). These observations have led to the conclusion that the assay with a high concentration of PEG is sensitive, specific and useful for the diagnosis and follow-up of Graves’ disease after drug withdrawal. Further studies on the mechanism of the increase in TSAb activity stimulated by high concentrations of PEG are necessary.
References


