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Abstract. Recently a new procedure for measuring serum TSH receptor (TSHR) autoantibody (TRAb) was reported by Smith et al. in which the autoantibodies inhibit binding of a human monoclonal thyroid stimulating antibody M22 (labeled with biotin) to TSHR-coated ELISA plate wells (pTRAb\textsubscript{3rd} assay). The aim of this study was to compare the performance of pTRAb\textsubscript{3rd} assay with pTRAb\textsubscript{2nd} assay based on inhibition of TSH-biotin binding to TSHR-coated ELISA plate wells. In addition, we evaluated the applicability of TRAb3rd assay to discriminate between untreated Graves’ disease (GD) and painless thyroiditis (PT). Analysis of sera from 230 healthy controls indicated that only 1 (0.43%) gave inhibition of M22-binding values of greater than 15% (32.8% inhibition). To define the clinical cut-off point for a positive serum with autoantibodies to the TSHR, we performed receiver operating characteristic curve of the data from 244 untreated GD and three different control groups for pTRAb\textsubscript{3rd} assay. With a sensitivity of 99.6% at a cut-off of 14.5%, 22.0% and 22.0% inhibition of M22 binding, the specificity of healthy controls without PT, with PT and with PT excluding postpartum PT and PT during remission of GD was 99.6%, 96.6% and 97.5%, respectively. The pTRAb\textsubscript{3rd} assay was closely correlated to pTRAb\textsubscript{2nd} assay in the 244 untreated Graves’ sera (r = 0.911). The pTRAb\textsubscript{3rd} assay detected 243 of 244 (99.6%) untreated GD, whereas 9.2% of PT and 6.7% of the subacute thyroiditis (SAT) were detectable. In contrast, pTRAb\textsubscript{2nd} assay detected 242 of 244 (99.2%) Graves’ sera, while 16.8% from PT’s sera and 13.3% from SAT were detectable. In conclusion, pTRAb\textsubscript{3rd} assay has significantly (p = 0.0026) superior diagnostic accuracy for GD and PT, compared to that of pTRAb\textsubscript{2nd} assay.

Key words: M22, 3rd generation TRAb assay, 2nd generation TRAb assay

THE pathologic role of autoantibodies (TRAb) to the TSH receptor (TSHR) in sera of patients with Graves’ disease has been clearly established. At present, there are two established principles to detect autoantibodies to the TSHR. One is TRAb assay based on the porcine TSHR or human recombinant TSHR, where autoantibodies and bovine TSH (labeled with I-125 or biotin) compete for the binding sites of the receptor. The other method is based on the ability of some autoantibodies similar to TSH to induce the second messenger cAMP in cell lines. These bioassays, as they are called, are able to distinguish between stimulating antibodies (TSAb) and blocking antibodies (TSBAb), based on their biological activity to either enhance or inhibit the cAMP production.

More recently a new procedure for measuring serum TSHR autoantibodies was first described by Smith et al. [1] in which the autoantibodies inhibit binding of a human monoclonal thyroid stimulating antibody M22 (labeled with biotin)[1] to TSHR-coated enzyme-linked immunoabsorbent assay (ELISA) plate wells [2]. The aim of this study was to compare the performance of this new M22-based assay (3rd generation TRAb assay) with a similar ELISA based on inhibition of TSH-biotin binding to TSHR-coated well (2nd genera-
tion TRAb assay) [3]. Furthermore, we evaluated the applicability of the new assay to discriminate between patients with thyrotoxicosis due to Graves’ disease (GD) and painless thyroiditis (PT).

**Patients and Methods**

**Patients**

Included in the study were 244 untreated patients with GD (mean age, 40 years; range, 10–78 years; 210 females). GD was diagnosed initially according to standard clinical criteria (suppressed TSH, elevated FT$_3$ and/or FT$_4$, diffusely increased thyroidal uptake of Technetium-99m, goiter, ultrasonography, and signs of Graves’ ophthalmopathy when present). Furthermore we included 155 patients with PT (diagnosed on the bases of suppressed TSH levels, elevated serum FT$_3$ and/or FT$_4$, diffusely decreased thyroidal uptake of Technetium-99m, goiter, ultrasonography, and signs of thyrotoxicosis) including 124 unrelated to delivery (mean age, 41 years; range, 15–76 years; 117 females), 8 postpartum painless thyroiditis (mean age, 29 years; range, 24–37 years) and 23 developed during remission of Graves’ disease (mean age, 47 years; range, 13–70 years; 22 females), subacute thyroiditis (diagnosed on the bases of painful and tender struma, suppressed TSH levels, elevated serum FT$_3$ and/or FT$_4$, elevated C-reactive protein, hypoechoic lesion at painful portion) (n = 15; mean age, 44 years; range, 24–67 years; 13 females), and 232 healthy individuals (mean age, 34 years; range, 7–76 years, 183 females) without a history of thyroid disease who were euthyroid, negative for TgAb and TPOAb and revealed normal thyroid volume estimated by sonography. All patients were informed of the purpose of this study.

**Methods**

Before any therapy, a blood sample was taken and serum stored at −20°C until assay. Fig. 1 shows the assay principles of M22-based assay (labeled 3rd generation TRAb assay, the values are presented as pTRAb$_{3rd}$) and ELISA based on inhibition of TSH-biotin binding to TSHR coated well (labeled 2nd generation TRAb assay, their values are presented as pTRAb$_{2nd}$). Second generation TRAb assay was carried out as described previously [3] and 3rd generation TRAb assay was performed by a newly developed assay which was first described by Smith et al. [2]. The assay procedure was taken from the report by Smith et al. [2] and is briefly described as follows. Assay buffer was added to each TSHR-coated plate well (75 µL per well) followed by test sera (75 µL) and incubated with shaking at room temperature (20–25°C) for 2 hours. The sera were then discarded and the plate wells washed once with 300 µL of wash buffer and M22-biotin added to each well. After 25 minutes at room temperature without shaking, the plate wells were washed once with wash buffer, 100 µL of streptavidin peroxidase added and incubation continued for 20 minutes (without shaking). Then the plate wells were washed twice with wash buffer, once with water, 100 µL tetramethyl benzidine added and after 30 minutes (in the dark without shaking) the reaction was stopped with 50 µL of 0.5 M H$_2$SO$_4$. Absorbance of each well was then read at 450 nm on ELISA plate reader.

**Definition of cut-off point and statistical analysis**

To obtain the optimal decision threshold level for positivity, receiver operating characteristic (ROC) analysis was performed. The sensitivity (true positive result) was calculated from 244 patients with untreated GD. On the other side, the specificity (true negative results) was calculated from three different controls including (a) 230 healthy persons, (b) 385 controls containing 230 healthy persons and 155 patients with PT and (c) 354 controls containing 124 patients with PT excluding postpartum painless thyroiditis (PPT) and...
PT occurring during the remission of GD. The cut-off of 2nd generation TRAb assay with 100% specificity and 99.2% sensitivity was 22%.

Correlation analysis was performed with Pearson’s Correlation. For method comparison of the sensitivities of 3rd and 2nd generation TRAb assays, McNemar’s chi-square test was used. P<0.05 was considered statistically significant.

Results

To define the clinical cut-off point for a positive serum with autoantibodies to the TSHR, an ROC plot analysis was performed to determine diagnostic sensitivity and specificity. The sensitivity and specificity were calculated based on the normal controls and/or painless thyroiditis. Fig. 2 shows the calculated cut-off for positivity from ROC plot analysis in 3rd generation TRAb assay and with a sensitivity of 99.6% at a cut-off of 14.5% (a), 22.0% (b) and 22.0% (c) inhibition of M22 binding, the specificity of healthy controls without PT (a), with PT (b) and with PT excluding PPT and PT during remission of GD (c) was 99.6% (a), 96.6% (b) and 97.5% (c), respectively. The result confirms the recommended cutoff of 15%. Using this cut-off for same sera from patients with newly diagnosed GD, PT and subacute thyroiditis (SAT), the sensitivity and calculated diagnostic accuracy for differentiation between GD and PT of 3rd generation TRAb assay compared to those of 2nd generation TRAb assay is shown in Table 1 and their distribution is shown in Fig. 3. Third generation TRAb assay detected 243 out of 244 (99.6%) patients with untreated GD, although 9.2% of patients with PT and 6.7% of patients with subacute thyroiditis (SAT) were detectable. On the other hand, 2nd generation TRAb assay detected 242 (99.2%) of 244 Graves’ same sera, whereas it detected 16.8 % of PT and 13.3% of SAT in the same sera. In conclusion, the 3rd generation TRAb assay has significantly (p = 0.0026) superior diagnostic accuracy for GD and PT, compared to that of 2nd generation TRAb assay. There was a close correlation (r = 0.911) between TRAb\textsubscript{3rd} and TRAb\textsubscript{2nd} in 244 patients with untreated GD, as shown in Fig. 4.
The epidemiology of nosological types of hyperthyroidism varies considerably. In Japan, an area with high iodine intake, the majority of the cases of thyrotoxicosis are due to GD and PT, whereas in areas with low iodine intake both GD and multinodular toxic goiter are common causes of hyperthyroidism [4].

In the present clinical setting, TSHR antibodies are assayed in patients with various forms of thyrotoxicosis to identify those with GD. In the present study, we have confirmed the high sensitivity of ELISA based on inhibition of M22-biotin binding to TSHR-coated plate wells in diagnosing GD and furthermore, in discriminating between patients with thyrotoxicosis due to GD and PT. Comparison between TSH-based ELISA and M22-based ELISA has been made by Smith et al. [1]. They concluded that inhibition of M22 binding was almost always greater, resulting in improved sensitivity and precision. In the present study, we demonstrated that inhibition of M22 binding to the TSHR was closely correlated to inhibition of TSH binding in the untreated 244 Graves’ sera (r = 0.911). In addition, the sensitivity of pTRAb\textsubscript{3rd} in diagnosing GD versus pTRAb\textsubscript{2nd} in the same sera from the 244 untreated GD was 99.6% versus 99.2%. In our group of patients...
with PT, the 3rd generation TRAb assay indicated a positive percent inhibition in 9.2%, while we were able to detect positive pTRAb\textsubscript{2\text{nd}} in 16.8%. While its reason remains unclear, one possible mechanism seemed to involve its different bonding site on TSHR. Interestingly, pTRAb\textsubscript{3\text{rd}} above 50% was observed in 8 out of 119 patients with PT, although further studies including analysis of TSBAb are needed. Finally, our results demonstrate 96.7% sensitivity of 3rd generation TRAb assay based on inhibition of M22-biotin binding to TSHR-coated plate wells in hyperthyroid patients with GD and PT. Thus 3rd generation TRAb assay is significantly ($p = 0.0026$) superior to 93.9% of sensitivity of the second generation TRAb assay based on inhibition of TSH-biotin binding to TSHR-coated plate wells. Overall, the new assay for TSHR autoantibodies based on M22-biotin appears to have considerable advantage over earlier methods particularly in terms of differentiation between GD and PT, although it must be necessary to clarify the clinical value of a new assay for estimating prognosis of GD after stopping medication and its relation to Graves’ ophthalmopathy.

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**References**