Note

Evaluation of a 1 step TRAb Assay for the Detection of High-affinity Components to hTSHR: Evidences Indicating Superiority of the Assay in the Lower TRAb Range

TAKASHI ISHIHARA, YASUHIKO SAIKI*, KATSUJI IKEKUBO*, MEGUMU HINO*, KAORI IKEDA, CYEOL SON, TOSHI OIWA, HIROMASA KOBAISHI AND TORU MORI**

Department of Internal Medicine, Kobe City General Hospital, Kobe 650-0046, Japan
*Department of Nuclear Medicine, Kobe City General Hospital, Kobe 650-0046, Japan
**Department of Internal Medicine, Kyoto Higashiyama Takeda Hospital, Kyoto 605-0932, Japan

Abstract. The aim of this study was to develop an assay to selectively detect high-affinity components among TRAb. Using an rhTSHR-coated tube system, a 1 step TRAb assay method was developed that included 1) co-incubation with $^{125}$I-bTSH, 2) a 50 μl serum sample, 3) an increased incubation volume (450 μl), and 4) a 1 hour incubation time. Sixty-one TRAb positive Graves’ sera were studied. When the regular TRAb assay (Reg) results were quantitatively compared to the 1 step assay (1 step) results, certain dispersions and overestimations using the latter were seen. Further, some 1 step positive results were observed in the low Reg range. Overestimations were considered mostly due to the differences between TRAb standard and patients’ serum TRAb in the binding competition against co-incubated $^{125}$I-bTSH, which was shown from a modified assay mimicking the 1 step conditions. Therefore, the 1 step results were decided to be expressed by % inhibition against $^{125}$I-bTSH. As for data dispersions, TRAb absorptions during the regular 1st incubation were studied. Individually, the absorption rates varied from 11 to 69%, and higher absorptions were observed in lower Reg range, especially in those negative by the 1 step. Observed 1 step positive results in the low Reg range were of interest, and 1 step/Reg ratios were calculated. The ratios with 1 step negative samples were significantly lower than those of 1 step positive samples. In conclusion, the 1 step assay was proved to detect a particular and biologically active TRAb, especially in those with low TRAb. The clinical significance of the 1 step results should be of future interest.

Key words: TRAb, TBII, TSHR, Graves’ disease, Competitive binding assay

TSH receptor antibody (TRAb) has been considered to be the strong causative factor for the development of Graves’ hyperthyroidism [1–4]. Autoantibodies including TRAb are essentially polyclonal, and therefore, heterogeneous TRAb components should exist in individual patient serum [4]. Recent progress in TRAb assay methods, such as the 2nd generation thyrotropin binding inhibitor immunoglobulin (TBII) assays and the high-sensitivity thyroid stimulating antibody (TSAb) assay, have made it possible to detect TRAb in nearly all Graves’ patient sera with very high specificity [5–9]. Clinical diagnosis of Graves’ disease became much easier and more relevant. Unfortunately, in addition to the clinical benefits, these highly sensitive assay results newly evoked another unfavorable problem. That is, these assays could detect TRAb even in some Graves’ patients during clinical remission after antithyroid drug (ATD) treatment, so that physicians are encountering difficulties when judging the timing of drug cessation based upon the TRAb results. Many investigators reported difficulty in the prediction of clinical remission or relapse using TRAb results [5, 10–14], and some have already tried to set up elevated cut-off points based on retrospective analyses of their Graves’ patients [15–17].
Through extensive analyses of the 2nd generation TBII assay system, we reported recently that 1) TSH receptor (TSHR) actions of Graves’ TRAb are very different from those of bTSH, 2) the 2 step incubation TRAb assay is not of competitive binding assay and 125I-bTSH in the 2nd incubation can not displace any of the previously bound TRAb to the hTSHR, 3) Graves’ IgG effects on the TSHR increase gradually depending on the TSHR reaction time, and 4) individual Graves’ IgGs should contain heterogeneous TRAb components with variable avidities to the TSHR [18]. We propose that highly sensitive TRAb assays may detect low-affinity TRAb, which may lack biological thyroid stimulatory activity. This may be one reason responsible for the clinically unmatched TRAb-positive results in some euthyroid or inactive patients with Graves’ disease, such as those under remission or with euthyroid Graves’ ophthalmopathy [18].

The purpose of our research was not simply to develop an assay method to elevate cut-off points, but to find a better assay method that could detect high-affinity TRAb components selectively.

**Materials and Methods**

1. **TRAb assay methods**

   TRAb was measured using the DYNO test TRAb human kit (Yamasa Corporation, Tokyo, Japan) [6]. All activities measured, using the kit, are referred to as TRAb, regardless of the different assay conditions.

1) **Regular assay procedures:**

   Two step incubations were performed as described previously [6, 18]. The 1st incubation was performed by adding 100 μl of serum sample or standard substance to 200 μl of assay buffer containing anti-hTSH antibody to an rhTSHR-coated tube. After 2 hour-incubation, the tube was washed and 200 μl of 125I-bTSH solution was added. The 2nd incubation (1 hour) was terminated by washing out the solution, and radioactivity bound to the tube was counted using a gamma-counter.

   To perform quantitative analyses, TRAb activities were calculated as IU/L. For each assay, a standard curve was constructed with data obtained using varying amounts of the international TRAb standard substance. By extrapolation of individual data, TRAb was calculated in IU/L.

   As will be detailed subsequently, we performed TRAb assays under various conditions. In these assays we applied the standard substance at the 1st incubation simultaneously with serum samples, and TRAb activities were calculated from the data.

   As described previously [18], TRAb activities were also expressed as % inhibition of 125I-bTSH binding, which was calculated as; \( \{1-(\text{bound count by sample/ maximal bound count})\} \times 100 \% \). Normal ranges were defined as less than 1.5 IU/L for quantitative expression and 15% for % inhibition, respectively.

2) **1 step incubation assay procedures:**

   As described previously [18], 50 μl of serum sample, 200 μl of assay buffer and 200 μl of 125I-bTSH solution were mixed together and then poured into an rhTSHR-coated tube. After 1 hour incubation, the tube was washed and directly counted for bound radioactivity. The assay results were mainly expressed as % inhibition of 125I-bTSH binding as above, and also expressed by IU/L read on the standard curve constructed with the standard substances handled data. Normal ranges were defined as less than 15% for % inhibition [18] and less than 1.5 IU/L for quantitative expression.

3) **Re-assay procedures of the unabsorbed fraction after the 1st incubation:**

   After the 1st incubation regularly for 2 hours, the unabsorbed fraction was aspirated, collected and then subjected newly to the regular assay (2nd-regular) and also to the 1 step assay (2nd-1step). At least four tubes were used for each sample, and the unabsorbed fractions were collected after the 1st incubation. These aliquots (300 μl for the regular assay and 150 μl for the 1 step assay, respectively) were subjected to re-assay.

4) **Modified assay procedures:**

   To study time and volume effects of the 1 step assay condition, a modified 1-hour incubation using a 50 μl sample with 200 μl of assay buffer and 200 μl of PBS instead of 125I-bTSH (to mimic the 1 step assay condition), was used for the 1st incubation. The 2nd incubation was performed as usual.

   Fig. 1 shows flow diagrams of 4 different assay procedures.

   At least 2 tubes were applied to each assay, and their means were used for all calculations.
2. Clinical materials

TRAb-positive (regular assay) sera from 61 Graves’ patients were used after receiving written informed consent individually. Clinical diagnoses were established using thyroid function test results such as elevated fT4, fT3 and radio-pertechnetate uptake, suppressed serum TSH concentrations, and positive for TRAb.

3. Statistical analyses

Student’s t-tests were performed, and p<0.05 was taken as significant.

Results

1. Correlation between regularly assayed TRAb activities and those by the 1 step assay

The 1 step assay was considered useful to detect high-affinity TRAb, and quantitative assay results for both the regular and 1 step assays were compared. As shown in Fig. 2, a roughly proportional correlation is seen ($r=0.892$), but certain dispersions are also observed. Further, most of the 1 step assay results distributed above the line, $y=x$, indicating that these values were overestimated compared to the regular assay results.
To see the effects of the 1 step assay conditions, a modified assay mimicking the 1 step assay was investigated (see Materials and Methods). Quantitative results of the regular assay and the modified assay revealed quite a good linear correlation \((r = 0.981)\) (Fig. 3). Therefore, we considered that the different TRAb results observed by comparing the regular and 1 step assay were not simply due to the effects of incubation condition and/or experimental errors. Overestimations seen by the 1 step assay results suggest that the standard TRAb substance had a smaller competitive effect than TRAb in most patients’ sera on the rhTSHR binding inhibition of co-incubated \(^{125}\text{I}\)-bTSH. So, the 1 step assay results were determined to be unsuitable for quantitative expression, and relative expression using % inhibition of \(^{125}\text{I}\)-bTSH binding is used, hereafter.

Fig. 4a shows the correlation between the regular assay TRAb results and the 1 step assay results expressed as % inhibition. Data dispersions shown in Fig. 2 did not improve much, \((r = 0.875)\) and were attributed to indicate the presence of individual heterogeneity. When % expressed results of the regular and 1 step assay were compared, overestimation seen by the 1 step assay was improved but data dispersions remained unimproved (Fig. 4b). Of note was that, as shown in Fig. 4a insert, some of lower TRAb samples by the regular assay revealed positive results by the 1 step assay for unmatched assay conditions (3-fold dilution and a shorter incubation for 1 hour). There were...
17 samples with the regular TRAb levels < 3 IU/L, and 7 of these were positive from the 1 step assay.

2. TSHR absorption of TRAb during the 1st incubation

If heterogeneity among TRAb is of considerable extent, then it should be observed as the differences in TRAb absorption to the rhTSHR during the 1st incubation. Then, the unabsorbed fractions after 2 hour-1st incubations were newly subjected to the regular TRAb assay. The details are not shown, but original TRAb activities and those in the unabsorbed fraction revealed an excellent linear correlation over the entire assay range (r = 0.993). The TRAb recovery in the unabsorbed fraction was calculated to be 69.6 ± 14.5%. TRAb absorptions by hTSHR were also analyzed in relation to the 1 step assay results. As shown in Fig. 5, higher absorption rates were seen mostly in samples with negative 1 step assay results, and positive results from the 1 step assay showed relatively low TSHR absorptions. Four negative samples and only 1 of 21 positive samples, using the 1 step assay, had absorption rates ≥ 48%. Thus, the higher absorption rates by the TSHR were not associated with positive results from the 1 step assay. On the contrary, 1 step positive samples were found to have lower absorption rates even if the original TRAb levels were low.

Further, to see the rhTSHR-absorbed TRAb qualitatively, unabsorbed fractions after a regular 2 hour-1st incubation were re-assayed using the 1 step assay. Original TRAb levels ≥ 5.4 IU/L were known to remain positive using this 2nd-1 step assay and these showed a fairly good correlation with the 2nd-1 step assay results (data not shown). During the 2 hour-1st incubation, most of samples with lower TRAb levels were found to lose competitive activity against ¹²⁵I-bTSH binding, but samples with higher TRAb levels remained positive. When these 2nd-1 step assay results were compared with the 1 step assay results, samples with higher TRAb levels showed an excellent correlation to each other (Fig. 6, r = 0.976). Among 17 cases with negative results from the 2nd-1 step assay, there were 10 positive cases from the 1 step assay.

The samples with a positive 1 step assay and negative 2nd-1 step assay result were of interest, and in another series using 37 Graves’ patients, the ratios of {1 step assay results/regular assay results (expressed as % inhibition) × 100 %} were calculated and compared. As shown in Fig. 7a, there was some overlap,
but a significant difference was seen between samples with negative 1 step assay results (Group A) and samples with 1 step positive and 2nd-1 step negative results (Group B) \( p = 0.002 \). Further, no significant difference was observed between samples with 1 step positive and 2nd-1 step negative and samples that were positive using both assays (Group C). When the ratios of the 2nd-1 step negative samples were analyzed in relation to TRAb using the regular assay, as shown in Fig. 7b, those with the 1 step positive (Group B) were clearly separated from and distributed much higher than those with the 1 step negative (Group A). The 1 step assay positive samples were found to inhibit \( ^{125}\text{I}-\text{bTSH} \) binding efficiently even if the regular TRAb levels were not high enough, but the 1 step assay negative samples were known to be absorbed by hTSHR well but have poor activity for competitively inhibiting \( ^{125}\text{I}-\text{bTSH} \) binding.

The 1 step assay was concluded to detect particular and biologically active TRAb, which was included in but could not be accessed fully by the regular 2 step assay.

**Discussion**

Autoantibodies including TRAb are considered to be polyclonal, and individual sera contain antibodies with variable avidities to autoantigens, such as TSHR [4]. As for TRAb indeed, the presence of heterogeneity has been known already by the epitope recognition and the IgG sequence of monoclonal human TRAb antibodies [19–24]. We have studied TRAb actions to TSHR in detail, and reported that there are rapid-acting and slow-acting components among TRAb [18]. Recently, high-affinity mouse monoclonal TRAb antibodies (mAb) were produced by 3 research groups [25–27]. These mAb are quite useful for understanding Graves’ hyperthyroidism, and developing new TRAb assays [28]. Minich *et al.* labeled 3 mAb types...
recognizing different epitopes on the hTSHR, and performed TRAb assays using various serum samples [29]. None of the mAb or even a mixture of them could improve the assay results obtained by $^{125}$I-bTSH application, which suggests the heterogeneity and complexity of epitope recognition among TRAb. On the other hand, Morgenthaler et al. have succeeded in the affinity purification of human TRAb to 3420-fold [30]. The purified TRAb was useful for the TRAb assay, and virtually identical results were obtained whether the labeled TRAb were TSAb or thyroid-stimulation blocking antibody (TSBAb). Assuming TRAb is heterogeneous, when measured under certain conditions, the assay results should represent only part of the heterogeneous components, or if the results could detect most of the heterogeneous activities, then these may not be able to reveal details of the ingredients.

In our previous paper, we applied a 1 step assay system that differed from the regular 2 step assay by 4 points, co-incubation with $^{125}$I-bTSH, a shorter incubation time (1 hour), less applied serum sample (50 μl), and larger incubation volume (450 μl) [18]. This 1 step assay was considered useful to detect high-affinity components among TRAb. In the current study, we analyzed the 1 step assay results quantitatively. When the 1 step assay results were compared with the regular assay results, a fairly good correlation but not a linear one was obtained, and further certain dispersions and apparent overestimations by the 1 step assay were also seen. The observed overestimations were considered to be due to the differences in binding inhibition of co-incubated $^{125}$I-bTSH between serum TRAb in most of Graves’ patients and TRAb in the provided standard substance. Further, observed dispersions appeared to indicate the presence of heterogeneity among individual TRAb.

We considered that if individual TRAb contains heterogeneous components with variable affinities against hTSHR, then the TSHR absorption rate of TRAb during the 1st incubation should be different between individual components. However, TRAb in the unabsorbed fractions showed an excellent linear correlation with the original TRAb activities. In general, heterogeneity among TRAb appeared to be rather limited as suggested by Morgenthaler et al. [30]. The quality of TSHR-absorbed TRAb was then studied measuring the unabsorbed fractions by the 1 step assay subsequently. Those with an original TRAb level ≥5.4 IU/L were found to remain positive even by the 2nd-1 step assay, indicating that TRAb activities measurable by the 1 step assay remained unabsorbed when TRAb were present in excess.

There were some 1 step positive samples in the lower TRAb range with unmatched conditions (more dilution and shorter assay time). To further investigate the significance of these findings, the relative potencies of inhibiting $^{125}$I-bTSH binding were analyzed by calculating (TRAb by 1 step assay/TRAb by regular assay) ratios. These ratios were found to be significantly different whether the 1 step assay results were positive or not, irrespective of the 2nd-1 step assay results. Further, even in the lower TRAb range apparently higher 1 step/regular ratios were seen in samples with the 1 step positive than those with negative. The 1 step assay proved to indicate most of the competitive activities related to inhibiting $^{125}$I-bTSH binding. As reported previously [18], the regular 2 step TRAb assay was not known to be a competitive binding assay but to be measuring the hTSHR occupancy by TRAb within the 1st incubation, which could not be displaced by $^{125}$I-bTSH during the 2nd incubation.

The 1 step assay is similar to the 1st generation TBII assay [31], which is thought to be more insensitive but a better assay for determining clinical hyperthyroidism in Graves’ patients compared to the 2nd generation TRAb assay and/or sensitive TSAb assay [2, 5, 10, 32]. However, recently, in order to improve the assay sensitivity, the 1st generation TBII assay has been using a 2 step incubation system with serum first and $^{125}$I-bTSH afterwards [33, 34]. The present 1 step TRAb assay was known to have a good sensitivity close to the 2nd generation assay, superior accuracy, and simplicity due to its use of a solid phase-system [18].

Finally, it was concluded that the 1 step TRAb assay detected specific TRAb activity that could not be accessed fully by the regular 2 step TRAb assay. This activity was proved to be of biological significance. Evaluation of the 1 step assay in relation to clinical findings from Graves’ patients, especially those with lower TRAb levels, should be of great future interest. We have already observed some interesting findings in this regard, and these will be presented in a separate paper.
Acknowledgment

The authors would like to express their sincere thanks to the Yamasa Corporation, Tokyo, Japan for providing the TRAb kits used in this study.

References

21. Ueda Y, Sugawara H, Akamizu T, Okuda J, Ueda M,


