Stimulating and Blocking Thyroid-Stimulating Hormone (TSH) Receptor Autoantibodies from Patients with Graves’ Disease and Autoimmune Hypothyroidism Have Very Similar Concentration, TSH Receptor Affinity, and Binding Sites

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Objective: The distinct biological properties of TSH receptor (TSH-R) autoantibodies (TRAbs) from patients with Graves’ disease (GD) are yet unexplained on the molecular level. Here we compare serum concentration, affinity to the TSH-R, and binding sites on the TSH-R of stimulating (TSAb) and blocking (TBAb) TRAbs.

Methods and Patients: Four-step affinity purification using human recombinant TSH-R was performed with 22 TRAb-positive sera from GD patients (11 with only TSAb and 11 with only TBAb) and five control sera. Antibody concentration, TSH binding inhibition (TBII), and TSAb/TBAb activity of the purified TRAb were assessed. Labeled purified TRAbs were used for displacement studies with TRAb and an additional 30 patients and 10 control sera.

Results: TRAbs could be purified to 80–93% purity with recovery of the TBII and TSAb and TBAb activity. No TRAbs could be purified from healthy individuals. The mean ± SD concentration of TRAb was 17.3 ± 5.4 μg/IU for the TSAb sera (range, 9.6–25.9) and 18.2 ± 8.5 μg/IU for the TBAb sera (range, 4.6–29.2), respectively (P = 0.79). Affinity was in the picomolar range for both TRAb subtypes with mean ± SD dissociation constant of 167 ± 109 pM (60–410 pM) for TSAb and 253 ± 132 pM (80–410 pM) for TBAb (P = 0.12). Purified and labeled TSAb and TBAb showed a very similar binding pattern to the TSH-R in displacement studies with unlabeled TSAb/TBAb or unpurified patients sera, indicating binding sites on the TSH-R in close proximity to each other.

Conclusion: TSAbs and TBAbs in the serum of patients with GD have similar characteristics. They are of low concentration with high affinity and have also similar binding epitopes on the TSH-R. (J Clin Endocrinol Metab 92: 1058–1065, 2007)

TSH RECEPTOR (TSH-R) autoantibodies (TRAbs) in patients with Graves’ disease (GD) are functionally heterogeneous; they include mainly thyroid-stimulating antibodies (TSAbs) and occasionally also thyroid-blocking antibodies (TBAbs). TSAbs activate the receptor and cause hyperthyroidism, whereas receptor occupancy by nonstimulatory TBAbs inhibits TSH action and may cause hypothyroidism. Together TSAbs and TBAbs comprise the TSH binding inhibition (TBII) activity detected by TRAb assays. Despite a plethora of information over the last decades, it is yet unclear what renders a TRAb a stimulator or a blocker.

In the present study, we apply this technology to purify TRAb from sera from patients with GD or autoimmune hypothyroidism (AH) with strong TSAb or TBAb activity. We then compared the purified subtypes with respect to autoantibody concentration, affinity to the TSH-R and competition with autoantibodies in serum.

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Abbreviations: AH, Autoimmune hypothyroidism; ATD, antithyroid drug; GD, Graves’ disease; InI, inhibition index; Kd, dissociation constant; RLU, relative light unit; SI, stimulation index; TBAb, thyroid-blocking autoantibody; TBII, TSH binding inhibition; TRAb, TSH-R autoantibodies; TSAb, thyroid-stimulating autoantibody; TSH-R, TSH receptor.

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Patients and Methods

Patient sera

GD sera and sera from patients with AH were obtained from selected patients on the basis of their high TRAb levels and their TSAb or TBAb antibody subtypes. Patients with a history of GD were selected from blood donors recruited for the development of in vitro diagnostics (Invent GmbH, Hennigsdorf/GmbH, Hennigsdorf, Germany). This blood...
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TABLE 1. Characteristics of patients selected for purification

| Patient no. | Age (yr) | Sex | Diagnosis | Duration of disease (months) | Free T4 (pmol/liter) | TSH (mIU/liter) | TgAb (IU/liter) | TSAb SI (percent) | TBAb InI (Ul/L) | TPOAb (Ul/L) |
|-------------|----------|-----|-----------|-------------------------------|---------------------|----------------|----------------|------------------|----------------|--------------|
| TSAb 1      | 49       | W   | GD        | 20                            | 0.038               | 431            | 150            | 0.1              | 0              | ND           |
| TSAb 2      | 47       | M   | GD        | 34                            | 0.523               | 750            | 153            | 0.1              | 0              | ND           |
| TSAb 3      | 48       | W   | GD        | 24                            | 1.88                | 280            | 156            | 0.1              | 0              | ND           |
| TSAb 4      | 49       | W   | GD        | 30                            | 0.41                | 384            | 134            | 0.1              | 0              | ND           |
| TSAb 5      | 54       | W   | GD        | 5                              | 0.001               | 426            | 136            | 0.1              | 0              | ND           |
| TSAb 6      | 45       | W   | GD        | 1                               | 0.001               | 246            | 136            | 0.1              | 0              | ND           |
| TSAb 7      | 45       | W   | GD        | 1                              | 0.001               | 341            | 136            | 0.1              | 0              | ND           |
| TSAb 8      | 48       | W   | GD        | 1                              | 0.001               | 426            | 136            | 0.1              | 0              | ND           |
| TSAb 9      | 54       | W   | GD        | 1                              | 0.001               | 341            | 136            | 0.1              | 0              | ND           |
| TSAb 10     | 54       | W   | GD        | 1                              | 0.001               | 426            | 136            | 0.1              | 0              | ND           |
| TSAb 11     | 54       | W   | GD        | 1                              | 0.001               | 341            | 136            | 0.1              | 0              | ND           |

Normal range of values: TSH, 0.3–4 mIU/liter; free T4, 10–25 pmol/liter; TBII, less than 1.0 IU/liter; OP, Operation; RIT, radioiodine treatment; TgAb, thyroglobulin antibodies.

TRAb purification from serum and acridiniumester labeling

TRAb was affinity purified from patients and control serum using a four-step protocol as described in detail elsewhere (7). The first step involved total IgG preparation on solid phase bound streptococcal Protein G (Upstate Biotechnology, Lake Placid, NY). Briefly, the detection was carried out in low salt conditions using JPO9 Chinese hamster ovary cells transfected with the human TSH-R (12). The second step was removal of IgG directed to the expression cell line K562 using wild-type K562 cells, which were produced in a 30-liter fermenter as described (13). Cells were washed with PBS and resuspended with the IgG preparation at a concentration of 10% (vol/vol) in buffer. The total amount of cells was 5 × 10^8 cells for 1 IU of TRAb. After incubation at 4°C overnight under slow agitation, the suspension was centrifuged and the IgG containing supernatant was removed by aspiration to reduce contamination by cell debris.

The third step was to repeat this procedure with K562 cells expressing the human TSH-R (9) under identical conditions. The optimal purification was obtained at a ratio of 5 × 10^5 cells for 1 IU of Graves’ IgG. After incubation at 4°C overnight under slow agitation, cells were washed twice with PBS, three times with HEPES buffer and once with water.

Donation was approved by a national ethical committee. GD was defined on clinical terms by a physician. Sera of 11 patients with high TBII and TSAb but no TBAb activity were selected for antibody purification and are termed TSAb 1–11 in this study. These patients, who presented initially with hyperthyroidism, were at the time of blood withdrawal either under antithyroid drug (ATD) treatment (n = 5, two for < than 1 month) or had had an operation (n = 3) or radioiodine treatment (n = 4) during the course of their disease. A further 21 serum samples from patients with similar characteristics (high TBI; high TSAb, no TBAb) were used for displacement studies.

Sera from patients with autoimmune thyroid disease, who were clinically hypothyroid but contained high levels of TBI, were recruited from Singapore General Hospital, Singapore. Again, sera of 11 patients with high TBI were selected for antibody purification. In contrast to the first lot, these patients had strong TBI but no TSAb activity and are termed TBAb 1–11 in this study. Four of these patients had hyperthyroid GD at the onset and turned hypothyroid during the course of their disease, whereas seven patients presented at the beginning with AH without a prior episode of hyperthyroidism. A further 10 serum samples from patients with similar characteristics (high TBI; high TBA, no TSAb) were used for displacement studies. These patients are described in detail elsewhere (8).

As reference control, the purification protocol was also used with five serum samples from healthy individuals, who had neither TBI nor TSAb or TBAb. These are termed controls 1–5 in this study. Another 10 serum samples from healthy individuals were used for displacement studies. Written consent was given by all blood donors. Table 1 shows the clinical and biochemical characterization of the patients at the time of blood withdrawal.

Assays for TRAb detection

TBI activity was measured with the TRAK human LIA (B.R.A.H.M.S. AG, Henningsdorf/Berlin, Germany) according to the manufacturer’s instructions. The technical and clinical evaluation of this product is described in detail elsewhere (9, 10). This assay is calibrated 1:1 to the World Health Organization (WHO) standard 90/672, and data are expressed as international units (international units per liter), where 1 IU/liter represents about 10% inhibition of TSH binding, 8 IU/liter about 50% inhibition, and 40 IU/liter (the highest standard) greater than 85% inhibition (10). All samples with TRAb greater than 40 IU/liter were diluted to obtain quantitative data.

TSAbs and TBAbs were measured in a bioassay as described (11). Briefly, the detection was carried out in low salt conditions using JPO9 Chinese hamster ovary cells transfected with the human TSH-R (12). cAMP was measured in a commercial RIA (Amersham, Aylesbury, UK). Thyroid stimulating index (SI) was calculated as: SI (percent) = 100 × (cAMP patient/cAMP euthyroid control). For TRAb detection, bovine TSH (1 mU/ml; Sigma, St. Louis, MO) was added with either euthyroid serum samples from healthy individuals were used for displacement studies. These sera were designated as controls. For TBAb detection, bovine TSH (1 mU/ml; Sigma, St. Louis, MO) was added with either euthyroid or test serum. The inhibition index (InI) was calculated as: InI = TSH (100%) - TSH (patient) / TSH (100% - TSH (patient)) × 100%.

Normal range of values: TSH, 0.3–4 mIU/liter; free T4, 10–25 pmol/liter; TBII, less than 1.0 IU/liter; OP, Operation; RIT, radioiodine treatment; TgAb, thyroglobulin antibodies.
Supernatant was removed by aspiration. TRAbs were eluted three times by centrifugation with elution buffer [25 mM citric acid, 100 mM NaCl (pH 2.1)]. Fractions were combined, neutralized with 200 mM Tris-HCl (pH 7.5) and centrifuged at 100,000 x g to remove any cellular debris. To prevent subsequent adsorption of TRAb to surfaces, 1% (wt/vol) BSA (Sigma) was added, and the preparation was stored at −80 °C.

The fourth and final purification round was performed using recombinant TSH-R bound to a specific monoclonal antibody [BA8 (9)] on Carbolink gel (Pierce Biotechnology, Rockford, IL). Coupling was performed overnight according to the manual with a yield of 70–90%. TSH-R extract from KS62 cells was obtained as described (9), and 200 ml membrane extract were incubated with 2.5 ml gel in batch for 5 h at 4 °C under mild agitation. Two hundred milliliters membrane extract represent the detergent preparation of around 5 x 10^7 cells. Gel material was three times resuspended with washing buffer and centrifuged. After the last washing step, the gel material was resuspended in the TRAb-enriched buffer from the third purification step at a ratio of 1 ml g to 15 IU TRAbs and incubated overnight at 4 °C under mild agitation, allowing the TRAb to bind specifically to the solid-phase bound human TSH-R. The gel material was washed three times with 50 ml washing buffer, and acidic elution was done with 15 ml freshly prepared elution buffer [50 mM citric acid, 1% BSA, 150 mM NaCl (pH 2.1)]. Immediately after elution, the fraction was neutralized by adding 15 ml PBS containing 1% BSA and as additional neutralization buffer 200 mM phosphate buffer (pH 10) to a final pH of 6.8. Before freezing, the buffer was centrifuged at 100,000 x g to remove precipitated TSH-R and other cellular debris.

Purified TRAbs were diluted with buffer [20 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1% BSA, 10% glycerol] to a concentration of 20 IU/liter TRAbs and incubated over night at 4 °C with an equal amount of 7.5), 150 mM NaCl, 1% BSA, 10% glycerol] to block unbound tracer. IU/liter TRAbs and incubated over night at 4 °C with an equal amount of 7.5), 150 mM NaCl, 1% BSA, 10% glycerol] to a final pH of 6.8. Before freezing, the buffer was centrifuged at 100,000 x g to remove precipitated TSH-R and other cellular debris.

Calculation of dissociation constant (K_d) of TRAb

Different concentrations (5–3000 pm) of indirectly labeled TRAbs were added in 0.3 ml buffer [20 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1% BSA, 10% glycerol] to a concentration of 20 IU/liter TRAbs and incubated over night at 4 °C with an equal amount of acidinium ester (MACN; Invitro GmbH, Henningsdorf, Germany) labeled goat antihuman antibodies. At the end of the incubation, 1 mg/ml total human IgG was added to block unbound tracer.

Displacement studies

For antibody displacement studies, 0.125 mIU of purified and labeled TRAbs were added in duplicate to 0.3 ml of buffer [20 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1% BSA, 10% glycerol] together with increasing amounts of unlabeled purified TRAbs to TSH-R-coated tubes (TRAK human RIA). Tubes were incubated for 24 h at room temperature, washed four times with 2 ml washing buffer, and bound relative light units (RLUs) were measured in a luminometer. Nonspecific binding, determined in excess of unlabeled purified TRAbs, was subtracted. The K_d was calculated at 50% saturation by nonlinear regression analysis (Prism 4; GraphPad Software, San Diego, CA). Data are shown as saturation curve and in the conventional Scatchard plot (14).

Results

TRAb were purified from 22 selected patients with GD or AH. All sera were tested for TBI, TSAb, and TAb. All patients had very high levels of TBI, ranging from 135 to 6550 IU/liter based on the WHO standard 90/672 calibration. In terms of inhibition of TSH binding, all patients had near total inhibition of binding, even at high dilutions. All 11 patients with a history of hyperthyroidism had high levels of TSBs (mean ± SD stimulation index 121 ± 41.7) but no TAb. Inversely, all 11 patients with hypothyroidism had high levels of TSBs (mean ± SD inhibition: 98.9 ± 9.0%) but were negative for TSBs. Thus, the patients selected for this study represent both extreme ends of the autoimmune response to the TSH-R. Table 2 lists all sera grouped according to their bioactivity. The mean ± SD TBI activity in the TSB patients was 1736 ± 1778 IU/liter, which is significantly higher (P = 0.03) than the mean TBI activity in the TSB-positive patients (432 ± 242 IU/liter). Serum obtained from the five healthy blood donors had no TBI or TSB/TAb activity.

Four-step affinity purification was performed with all 22 patients and five control sera (Table 2). Purification was successful with 21 of 22 patients sera. The purified TRAbs of patient TSB 7 were lost due to technical difficulties with the elution buffer. This sample was excluded from further calculations. After affinity purification, TSB and TSB/TAb activity of the TRAbs was still present. Figure 1 shows dose-response curves with increasing TRAb concentration in a cAMP bioassay for two purified TSBs (Fig. 1A) and one purified TSB sample (Fig. 1B). The complete cAMP bioassay data of all purified TRAbs is shown in Table 2 at a TRAb concentration of 10 IU/liter.

The total TRAb international units recovered based on TBI activity was 26.9 ± 17.2% for TSB samples and 21.9 ± 15.2% for TSBs, respectively. Measurement of total IgG in purified samples of patients and controls showed a clear difference. Patients had mean ± SD total IgG concentrations of 375 ± 151 µg/liter for TSBs and 458 ± 88 µg/liter for TSBs, respectively, whereas controls had only 47.6 ± 15.6 µg/liter. However, the purified material from both TSB and TSB patients still had strong TBI activity, whereas the control material did not show any interaction with the TSH-R. The IgG still present in control samples after the same purification protocol indicates nonspecific background carried through the process and allows the estimation of the purity of IgG from patients samples to be 80–93% pure TRAbs.

A comparison of total IgG and TRAb international units allowed calculation of the amount of IgG per international unit of TRAb. This was very similar for each individual serum purified and showed no difference between TSBs and TSBs. The mean ± SD was 17.3 ± 5.4 µg/IU for the TSB sera (range 9.6–25.9), and 18.2 ± 8.5 µg/IU for the TSB sera (range 4.6–29.2), respectively (P = 0.78). Therefore, the concentration of TRAbs in the serum of patients with GD is between 5 and 300 µg/liter for 1 international WHO unit (IU), irrespective of the TSB or TSB subtype. The patients in this study with very high levels of TRAbs had antibodies in the milligram per liter range, whereas the average patient with GD (usually with TSB/TBI < 40 IU/liter) is likely to have antibodies in the medium to high microgram per liter range.

Affinity of the purified TRAbs to the TSH-R was measured...
by saturation experiments, in which increasing amounts of indirectly labeled purified TRAbs were incubated on TSH-R-coated tubes. Figure 2 shows the Scatchard plot for two representative TSAb and two TBAb patients. The Kd for all TRAb samples is shown in Table 3, again grouped according to TSAb or TBAb activity. Affinity was in the picomolar range for both TRAb subtypes, and there was no difference between TSAb and TBAb samples. Mean $K_d$ for TSAb was 167 ± 109 pm (60–410 pm) and 253 ± 132 pm (80–410 pm) for TBAb, respectively ($P = 0.12$).

### Table 2. Affinity purification of TSH-R autoantibodies from patients' serum

| Serum no. | TBII (IU/liter) | TSAb % | TRAb added (mIU) | Recovery (%) | TBII (IU/liter) | TSAb % | TRAb InI (%) |
|-----------|-----------------|--------|------------------|-------------|----------------|--------|--------------|
| TSAb      |                 |        |                  |             |                |        |              |
| 1         | 431             | 150    | 0                | 431         | 66             | 15.3   | 18.8         |
| 2         | 750             | 153    | 0                | 750         | 178            | 23.7   | 19.8         |
| 3         | 295             | 41     | 0                | 295         | 80             | 27.1   | 21.8         |
| 4         | 250             | 156    | 0                | 250         | 46             | 18.4   | 11.5         |
| 5         | 889             | 149    | 0                | 889         | 196            | 22.0   | 21.8         |
| 6         | 274             | 71     | 0                | 274         | 37             | 13.5   | 10.5         |
| 7         | 318             | 148    | 0                | 318         | 48             | 15.1   | 13.8         |
| 8         | 281             | 134    | 0                | 281         | 176            | 62.6   | 19.5         |
| 9         | 426             | 136    | 0                | 426         | 78             | 18.3   | 22.3         |
| 10        | 707             | 128    | 0                | 707         | 147            | 20.8   | 21.0         |
| 11        | 135             | 62     | 0                | 1193        | 700            | 58.7   | 28.0         |
| Mean      | 432             | 121    | 0                | 529         | 159            | 26.9   | 19.0         |
| SD        | 242             | 41.7   | 0                | 312         | 188.8          | 17.2   | 5.2          |

| TBAb      |                 |        |                  |             |                |        |              |
| 1         | 2050            | 0      | 99               | 2000        | 329            | 16.5   | 36.5         |
| 2         | 225             | 0.6    | 98               | 250         | 44             | 17.6   | 12.5         |
| 3         | 6550            | 0      | 100              | 3000        | 749            | 25.0   | 83.3         |
| 4         | 335             | 1      | 97               | 335         | 51             | 15.2   | 14.5         |
| 5         | 2100            | 0      | 99               | 2000        | 421            | 21.1   | 46.8         |
| 6         | 1030            | 1.7    | 99               | 1100        | 83             | 7.5    | 16.5         |
| 7         | 2000            | 0.4    | 99               | 1000        | 1.5            | 0.1    | 96           |
| 8         | 1700            | 0.4    | 100              | 850         | 128            | 15.1   | 23.3         |
| 9         | 260             | 0      | 99               | 260         | 58             | 23.3   | 14.5         |
| 10        | 2140            | 0.7    | 100              | 735         | 118            | 15.7   | 21.5         |
| 11        | 710             | 0      | 98               | 1428        | 900            | 63.0   | 36.0         |
| Mean      | 1736            | 0.4    | 98.9             | 1179        | 262            | 21.9   | 30.5         |
| SD        | 1778            | 0.5    | 0.9              | 865         | 308            | 15.2   | 21.8         |

| Controls  |                 |        |                  |             |                |        |              |
| 1         | 0.7             | 0      | 0                | 0.1         | 22.0           | 48.0   |              |
| 2         | 0.2             | 0      | 0                | 0.2         | 64.0           | 60.0   |              |
| 3         | 0.4             | 0      | 0                | 0.2         | 54.0           | 54.0   |              |
| 4         | 0.1             | 0      | 0                | 0.1         | 22.0           | 22.0   |              |
| 5         | 0.4             | 0      | 0                | 0.1         | 50.0           | 50.0   |              |
| Mean      | 0.4             | 0.0    | 0.0              | 0.1         | 47.6           | 47.6   |              |
| SD        | 0.2             | 0.0    | 0.0              | 0.1         | 15.6           | 15.6   |              |

**Fig. 1.** cAMP bioassay with purified TSAbs and TBAbs. Increasing concentrations of purified TRAb were incubated in a Chinese hamster ovary cell bioassay with cAMP readout. Shown is the dose-response curve for two purified TSAbs and one TBAb.
Finally, we tested purified TRAb material in competition studies to see whether a different displacement pattern indicates differences in the binding sites of TSAbs and TBAbs. First, competition was done among the purified samples in a cross-over design. Different TSAb samples as tracer were competed with increasing concentration of other TSAbs (Fig. 3A) or TBAbs (Fig. 3B). The same was done with labeled TBAb samples, which were brought into competition with TSAb samples (Fig. 3C) or other TBAb samples (Fig. 3D). The dose-response curves shown are examples of several experiments and demonstrate similar displacement among all purified TRAb and no difference between TSAbs and TBAbs.

Then competition studies were done with labeled TSAb or TBAb samples and unpurified sera from additional patients (20 patients with GD, 10 patients with autoimmune thyroid disease containing only TBAbs, and 10 controls). Figure 4 shows six representative experiments (three with labeled TSAbs and three with labeled TBAbs), which demonstrate a very similar binding pattern of both TSAbs and TBAbs. In all experiments, purified TSAbs competed with patients sera containing only TSAbs but also with sera containing only TBAbs. The identical pattern was seen for purified TBAbs, which competed with not only TBAb-containing sera but also TSAb-positive GD sera. In all experiments, the interaction with TBAb-containing sera was higher than with TSAb sera, irrespective of the tracer used. No interaction was seen with control sera. Using the standard bovine TSH tracer from the TRAb kit gave very similar results (Fig. 5).

**Discussion**

This study took advantage of a recently established purification method for TRAbs (7) and examined the differences between TSAbs and TBAbs purified from patients with GD and hypothyroid autoimmune disease. The results can be summarized as follows. First, the concentration of TRAbs in patients with GD is about 18 μg/liter IgG (5–30 μg/liter) per international WHO unit, with no difference between TSAb and TBAb. Second, the affinity of both TSAbs and TBAbs to the TSH-R is in the picomolar range (60–410 pm), again without a significant difference between TSAbs and TBAbs. Third, both TSAbs and TBAbs show a very similar binding pattern to the TSH-R in displacement studies with purified TRAbs and patients' sera, indicating binding sites on the TSH-R and no difference between TSAbs and TBAbs. Because the binding of TSH shows the same pattern, this binding site of TSAbs and TBAbs is most likely in the TSH binding part of the TSH-R, which according to present models of the receptor lies within the horseshoe of the ectodomain (15–17).

This study confirms our preliminary report, that TRAbs are of low concentration with high affinity (7). The 14–28 μg/liter per international unit reported initially are well in concordance with the figures in this study. Although there were earlier estimates of TRAb concentration based on neutralizing studies with soluble TSH-R (18, 19), the concentration (and also affinity) of TRAb is a constant matter of debate. It should be pointed out that the TRAb concentration per 1
IU is relatively low, and most GD patients have less than 40 IU/liter TRAbs. However, a few patients may have several thousand international units per liter TRAbs, which is equivalent to a TRAb concentration in the milligram per liter range. The affinity of TRAbs to the TSH-R in this study confirms our preliminary report. It is noteworthy that the linear Scatchard plot indicates the presence of TRAbs with very similar $K_d$ in each patient preparation. This is also supported by the comparable results in all experiments. TRAbs may be heterogeneous within and between patients, but all TRAbs have a very similar affinity to the TSH-R. This affinity is comparable with that found for bovine TSH (7, 19) and with a human TSAb monoclonal antibody (5).

The identification of the binding sites of TSAbs and TBAbs to the TSH-R is still controversial. Over decades data were presented that supported a rather simple concept: TSAbs bind to the N-terminal part of the TSH-R ectodomain and TBAbs to the C-terminal part close to the membrane (20–22). Following this concept, we tried to develop different in vitro assays for the discrimination of TSAbs and TBAbs by changing either the receptor (23) or tracer (24), albeit with moderate results. After the establishment of monoclonal antibodies with TSAb or TBAb activity (2, 5, 6, 25), the model of distinct and distant TSAb and TBAb epitopes could no longer be supported. Three groups examined independently the interaction of labeled monoclonal antibodies with autoantibodies from patients to the TSH-R and reported similar results: monoclonal TSAbs seem to interact with binding sites for TSH, TSAbs, and TBAbs (2, 5, 6, 25), whereas monoclonal TBAbs likewise do exactly the same (6). Our study offers independent proof for this with purified TRAbs from pa-

### Table 3. Affinity of purified TRAb to the TSH-R

| Purified TRAb | $K_d$ (10^{-12} M) | 95% CI of $K_d$ | Bmax (10^{-12} M) |
|---------------|-------------------|----------------|------------------|
| TSAb          |                   |                |                  |
| 1             | 80.8              | 50.7–111       | 20.0             |
| 2             | 78.5              | 59.5–97.4      | 18.7             |
| 3             | 260               | 174–346        | 31.8             |
| 4             | 228               | 152–303        | 55.0             |
| 5             | 119               | 84.2–154       | 20.6             |
| 6             | 410               | 194–627        | 4.3              |
| 7             | 92.1              | 40.6–144       | 35.4             |
| 8             | 81.0              | 38.0–124       | 27.6             |
| 9             | 59.7              | 14.8–105       | 22.9             |
| 10            | 192               | 105–278        | 34.0             |
| 11            | 240               | 200–265        | 30.0             |
| Mean ± SD    | 167 ± 109         |                | 30.9 ± 11.1      |
| TBAb          |                   |                |                  |
| 1             | 195               | 109–281        | 55.2             |
| 2             | 370               | 318–422        | 35.4             |
| 3             | 128               | 87.12–169.0    | 36.9             |
| 4             | 155               | 113–198        | 24.5             |
| 5             | 303               | 182–424        | 72.6             |
| 6             | 109               | 57.8–161       | 13.2             |
| 7             | 381               | 221–542        | 85.4             |
| 8             | 397               | 149–645        | 41.5             |
| 9             | 109               | 60.3–92.8      | 12.0             |
| 10            | 80                | 40.6–144       | 35.4             |
| 11            | 240               | 200–265        | 30.0             |
| Mean ± SD    | 253 ± 132         |                | 43.5 ± 24.3      |

CI, Confidence interval; Bmax, maximal binding capacity.

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**Fig. 3.** TSAb tracer and TBAb tracer displacement by purified autoantibodies. Shown are representative examples of a purified TSAb sample as tracer in competition with increasing concentration of other purified TSAbs (A) or purified TBAbs (B). Inversely, a labeled TBAb sample competed with purified TSAb samples (C) or other purified TBAb samples (D).
patients. We also found no differences between TSAb and TBAb interaction with the TSH-R in competition studies with purified TRAb or patient sera.

If concentration, affinity for, and binding site on the TSH-R are similar for TSAbs and TBAbs, what makes the biological difference? A possible explanation was proposed by a recent study on the interaction of TSAb and TBAb monoclonal antibodies and TSH-R mutants with single amino acid substitutions in the crucial leucine-rich horseshoe part of the receptor (26). This study showed that TSAb and TBAb epitopes are in very close steric proximity, but they are nevertheless distinct on the molecular level (6). Single amino acid differences in the binding site could be the reason for the dramatic difference in bioactivity. However, alternative explanations may exist. The recent crystal structure of the FSH-R together with its ligand FSH (27) suggests that similar approaches for the TSH-R and TSH or TSAb/TBAb could be helpful in solving this riddle.

Two limitations of our study should be addressed. First, the patients selected for this study had high levels of TRAbs, some even after treatment, which may not be representative for the average patient with GD. Second, the stringent purification conditions might have been selective for high-affinity TRAbs. Whereas the average recovery of TRAbs after purification was reasonable (27%), low-affinity TRAbs could have been lost during the process. Therefore, our study does not exclude the existence of TRAb with lower affinity than reported here.

Finally, it should be noted that we were unable to purify TRAbs from healthy individuals. This is in contrast to a study on affinity enrichment of TRAbs from GD patients and healthy individuals (28). In this study the evidence for TRAbs in healthy individuals was (besides fluorescence-activated cell sorter analysis) based on a weak interaction of the enriched material in a competitive TBII system. It is important to keep in mind that the detection of TRAbs by any competitive radioreceptor assay is an indirect method and therefore subject to artifacts. Any substance (i.e. residual chemicals from the purification protocol or a high salt concentration) that negatively influences the TSH-R TSH interaction will reduce the binding of the tracer, which results in a positive readout. However, it is also possible that this discrepancy between the two studies is due to differences in the affinity purification protocol, particularly the loss of low-affinity TRAbs in our system.

As we have pointed out before (7), the strongest evidence for a successful purification of TRAbs by our protocol is not...
necessarily the recovery of TBII activity but a clear stimulation in the bioassay and the fact that we are able to label the purified TRAbs for saturation and displacement studies. Here the purified autoantibodies from patients behave similar to studies we did with monoclonal TSH-R antibodies (6, 24), indicating a high level of purity and functionality.

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