THYROID
Volume 10, Number 10, 2000
Mary Ann Liebert, Inc.

Differences Between Thyrotropin Receptor Antibody Bioactivity and Inhibition of ^{125}I-Bovine Thyrotropin Binding

Henri Wallaschofski,[1] Marina Kaczmarek,[1] Konstanze Miehle,[1] Betina Hentschel,[2] and Ralf Paschke[1]

Thyrotropin (TSHR) receptor antibodies that bind to the TSHR without stimulating the TSHR have been identified with a direct binding assay. Moreover, TSHR antibodies that exhibit thyroid epithelial cell stimulation without inhibition of ^{125}I-bovine thyrotropin (bTSH) binding and vice versa have been described. These data suggest that stimulation or blocking of the TSHR by stimulating (TSAB) or blocking (TSBAB) TSHR antibodies could be possible without detectable bTSH-displacement activity. However, to date, possible differences between TSAB or TSBAB activity and inhibition of ^{125}I-bTSH binding have not been systematically investigated. Therefore we compared inhibition of ^{125}I-bTSH binding and TSAB or TSBAB activity of sera from 113 patients with Graves' disease treated with antithyroid drugs. To exclude the different assay conditions of previous investigations as possible confounding factors, we determined TSAB or TSBAB and inhibition of ^{125}I-bTSH binding (TBIIW) with the same Chinese hamster ovary (CHO) cells expressing the human TSHR. Furthermore inhibition of ^{125}I-bTSH binding was also determined as thyrotropin-binding inhibitory immunoglobulin (TBII) with solubilized porcine thyroid membranes (TRAK, Brahms, Berlin Germany) and the highly sensitive recombinant human TSH receptor assay (hTRAK, Brahms, Berlin Germany). Only 78% (54/69) of TSAB-positive and 78% (21/27) of TSBAB-positive sera detected with JP26 cells exhibit inhibition of ^{125}I-bTSH binding measured as TBII or TBIIW. Furthermore, 59% (10/17) of sera without TSAB and TSBAB activity revealed inhibition of ^{125}I-bTSH binding measured as TBII or TBIIW. We found significant differences between TSHR bioactivities (TSAB or TSBAB) and inhibition of ^{125}I-bTSH binding. Moreover, there was no agreement between the detectable TSHR bioactivities (TSAB or TSBAB) and their detectable inhibition of ^{125}I-bTSH binding. Therefore, it is very likely that TSH displacement by TSHR antibodies and stimulation or blocking of the TSHR by TSHR antibodies are different functions that do not need to occur together.

Introduction

IT HAS BEEN SPECULATED for many years that thyrotropin receptor (TSHR) antibodies may exist that bind to the TSHR without stimulating (TSAB) or blocking (TSBAB) the TSHR. Recently antibodies which bind without stimulating the TSHR were detected in 1 of 38 sera of treated Graves' disease patients and 3 of 13 Hashimoto's patients using a direct binding assay respectively (1). Whereas, in another investigation of 50 Graves' disease sera it was demonstrated that TSAB activity is correlated with a direct binding assay for TSHR antibodies ($r = 0.58$, $p < 0.001$) and a thyrotropin binding inhibitory immunoglobulin (TBII) assay ($r = 0.76$, $p < 0.001$). Only 1 of these 50 sera that scored negative in the direct binding assay and was borderline positive in TBII assay showed TSAB activity and 5 of these 50 patients with Graves' disease showed direct binding or positive TBII values without TSAB activity (2). Previously, we found ^{125}I-bovine thyrotropin (bTSH) displacement in the porcine TBII assay for only 69 of 111 (62%) TSAB-positive and for only 18 of 34 (53%) TSBAB-positive sera of patients with Graves' disease treated with antithyroid drugs (3). Apart from demonstrating a higher sensitivity of TSAB (4) or TSAB and TSBAB assays (3), as opposed to TBII assays, for the detection of TSHR antibodies, these data could suggest the existence of TSAB or TSBAB that are not detectable by their inhibition of ^{125}I-bTSH binding in a TBII assay or in a highly sensitive direct binding assay. This would imply that stimulation or blocking of the TSHR by TSAB or TSBAB would be possible without ^{125}I-bTSH displacement, thus possibly opening new perspectives for the understanding of TSHR antibody or TSHR action. Different mechanisms of stimulation and ^{125}I-

[1]Department for Internal Medicine III, [2]Institute for Medical Informatics Statistics and Epidemiology, University of Leipzig, Leipzig, Germany.

bTSH displacement by TSHR antibodies are also suggested by the description of TSHR antibodies that exhibit thyroid epithelial cell stimulation without bTSH displacement and vice versa (5).

Inconsistent results for TSHR antibody determinations with TSAB and TBII assays have repeatedly been noted (3-17). It is therefore very likely that these different assays measure overlapping but not identical antibody activities (18). These discrepancies between TBII and TSAB assays have previously been explained that by the lack of comparability of assay conditions due to different TSHR numbers and the low levels of TSHR antibodies (19,20) or different TSHR species in TBII and TSAB bioassays (4,21) or a possible loss of TSH binding sites of solubilized porcine TSHR in the TBII assay (8,22). Therefore, it was suggested that the differences between TBII and TSAB or TSBAB could be related to the sensitivity of the assays. Possible differences between TSAB or TSBAB activity and inhibition of ^{125}I-bTSH binding have to date not been systematically investigated.

To exclude these possible confounding factors and to compare displacement and stimulating or TSH stimulation blocking activity of sera from patients with Graves' disease directly, we therefore determined inhibition of ^{125}I-bTSH binding of TSAB or TSBAB positive and TSAB and TSBAB negative sera of Graves' disease patients treated with antithyroid drugs with the same JP26 Chinese hamster ovary (CHO) cells expressing the hTSHR and with the same optimal (3,4) serum/buffer dilution. By determining the inhibition of TSH binding with the JP26 CHO cells expressing the human TSHR, we maintained the same assay conditions for the determination of stimulation, blocking, and displacement by TSHR antibodies. Ligand displacement studies with G-protein–coupled receptors expressed in cell lines are widely used for the characterisation of TSHR (23,28), luteinizing hormone (LH) receptor (29), follicle-stimulating hormone (FSH) receptor (30) and several other G-protein–coupled receptors (31). Because CHO cell lines expressing high numbers of TSHR have a lower sensitivity for the detection of TBII and TSBAB than those expressing low receptor numbers (3,20,21), we used the JP 26 CHO cell line (2,000 receptors per cell, equivalent to thyroid epithelial cells) for the detection of inhibition of TSH binding. The inhibition of ^{125}I-bTSH binding on the JP 26 CHO cells expressing the human TSHR was termed TBIIW to distinguish it from the inhibition of TSH binding determined with the TBII assay using solubilized porcine thyroid membranes (TRAK Brahms, Berlin, Germany). Using the recombinant immobilized human TSH receptor in a new TBII assay technique (hTRAK, Brahms) it was possible to improve the sensitivity and specificity for the detection of TSHR antibodies in Graves' disease patients to 98.8% or 99.6% respectively (32). Therefore, we also determined TBII with this hTRAK assay.

Subjects and Methods

Control group

Sera from 100 healthy individuals with no history of autoimmune thyroid disease and normal values for TSH, free triiodothyronine (FT_3), free thyroxine (FT_4), TBII (hTRAK and TRAK assay), and thyroid peroxidase (TPO) antibodies (Brahms) were used as control sera for TSAB and TSBAB determinations. Heat decomplementation of sera was performed at 56°C for 20 minutes. Sera were pooled, aliquoted and stored at −20°C.

Patients

Sera from 113 patients with Graves' disease were aliquoted and stored at −20°C after heat decomplementation. The diagnosis was based on clinical and laboratory criteria, including TSH, FT_3, FT_4, TBII (TRAK assay) and TPO-antibodies (Brahms), scintiscan, and ultrasound. In a previous study it has been demonstrated that changes in the TSHR antibodies occur more rapidly in the last 6 months of antithyroid drug treatment (ATDT) (13). Furthermore, differences between changes of TBII and TSAB were predominantly identified after ATDT (33). We therefore hypothesized that possible differences between binding and stimulating or blocking activity can be most successfully detected after several months of ATDT or after stopping ATDT. Furthermore, it has been demonstrated that some patients who do not relapse are positive for TSAB at the end of ATDT, while other patients relapse despite nondetectable TSAB (10,16). Therefore, only sera from patients with Graves' disease receiving ATDT longer than 6 months were selected. After determination of TSAB and TSBAB, the 113 sera of patients with Graves' disease were divided in to TSAB positive sera (n = 69), TSBAB positive sera (n = 27) and TSAB- and TSBAB-negative sera (17).

Cell culture

CHO cell line JP26 stably transfected with the recombinant hTSHR or with the vector pSVL-neo alone (clone JP02) to control for unspecific stimulation or displacement were used in this study (8,11,12,34). Cells were cultured in Petri dishes with Dulbecco's modified Eagle's medium (DMEM) (Gibco, Eggenstein, Germany) supplemented with 10% fetal calf serum, 100,000 IU/L penicillin, 100 mg/L streptomycin (Gibco) and 400 mg/L Genetecin (Sigma, Deisenhofen, Germany) at 37°C and 5% CO_2. Cells were harvested using a 1% trypsin ethylenediaminetetraacetic acid (EDTA) mixture (Gibco). Forty thousand cells per well were seeded in 96-well plates (Greiner, Solingen Germany). They were grown to confluence and used for the assay 24 hours after seeding.

TSAB and TSBAB bioassays

Serum samples were diluted in hypotonic Krebs Ringer Buffer containing 0.5 mmol/L isobutylmethylxanthine (Sigma). For the TSBAB assay, 10 mU/mL bovine TSH (Sigma) were added. After washing the cells twice with phosphate-buffered saline (PBS), the assays were performed with 200 μL of serum/Krebs Ringer Buffer mixtures. After 2 hours of incubation at 37°C, 5% CO_2, the supernatant was collected and stored at −20°C. Detection of the extracellular cyclic adenosine monophosphate (cAMP) in the supernatant was performed with a commercial cAMP radioimmunoassay (RIA) (Amersham, Braunschweig, Germany) according to the manufacturer's instructions. All assays were performed in duplicate in at least two separate experiments. The mean of the four separate values of each serum sample were calculated. The interassay and intra-assay variance of pooled sera and patients wera was 15% or less.

TSAB and TSBAB indices were calculated as follows. Stim-

ulation index: TSAB (%) = 100 × JP26 (cAMP patient/cAMP control)/JP02 (cAMP patient/cAMP control). Inhibition index: TSBAB (%) = 100 × {1 − JP26 (cAMP patient + TSH/cAMP control + TSH)/JP02 (cAMP patient + TSH/cAMP control + TSH)}.

The pooled 100 normal sera with and without 10 mU/mL bTSH were used as an internal standard in every assay.

Inhibition of ^{125}I-bTSH binding on intact CHO cells (TBIIW)

Assay conditions for the investigation of inhibition of ^{125}I-bTSH binding by serum samples were equivalent to those previously used for the characterization of ligand displacement on the TSHR (23,24,28), the LH receptor (29), the FSH receptor (30), and several other G-protein–coupled receptors expressed in COS-7 or CHO cell lines (31). After seeding 1,000,000 cells per well of a 12-well plate, CHO cells were grown to confluence for 24 hours.

Before displacement studies, the CHO cells were rinsed three times with 1 mL binding buffer (modified sodium-free Hank's buffer, supplemented with 278 mmol/L sucrose, 2.5% powdered milk, and 0.1% bovine serum albumin [BSA]). Afterwards, 85,000 counts per minute of ^{125}I-bTSH (Brahms) and serum diluted in 1 mL binding buffer were added to the CHO cells at the same time and were incubated at room temperature for 4 hours. After two washes with 1 mL of binding buffer, the cells were subsequently solubilized with 1 mL of 1N NaOH. TSH displacement was measured as bound radioactivity in counts per minute, determined in a γ-counter. The nonspecific ^{125}I-bTSH binding to the CHO cells was subtracted from the total counts bound. The nonspecific binding was determined using JP02 cells or by adding an excess (10–100 mU/mL) of unlabeled bTSH (Sigma) to JP26 cells. The specific inhibition of ^{125}I-bTSH binding (displacement of ^{125}I-bTSH) was determined as: total binding of ^{125}I-bTSH with JP26 cells (counts per minute [cpm]) − 2NSB (cpm). Inhibition of binding was calculated as: TBIIW index (%) = 1 − (cpm in the presence of test serum/cpm of normal pool sera) × 100. All assays were performed in duplicate in at least two separate experiments. The mean of the four separate values of each serum sample were calculated. The interassay and intra-assay variance of pooled sera and patients sera was 10% or less.

Inhibition of ^{125}I-bTSH binding on solubilized porcine thyroid membranes (TRAK) and recombinant human TSH receptor (hTRAK)

All samples were analyzed in the TRAK assay and the hTRAK assay (Brahms Diagnostica) performed according to the manufactor's instructions.

Detection of anti-bTSH antibodies in sera with TSBAB activity

To exclude anti-bTSH antibodies in TSBAB sera, 10,000–15,000 cpm of ^{125}I-bTSH in 0.9 mL of 0.1 M phosphate buffer (pH 7.4) were incubated with 0.1 mL of test serum or pooled control sera for 1 hour at 37°C, as previously reported (35,36). Subsequently 1 mL of 25% polyethylene glycol (Merck, Darmstadt, Germany) was added. After mixing well, the tubes were centrifuged at 3,000 r.p.m. for 30 minutes and counted after decantation of the supernatant. The mean binding of control sera was 13% ± 4% of total counts ($\bar{x}$ ± 2 SD of total binding). Precipitated radioactivity higher than 17% was considered as specific binding of ^{125}I-bTSH. All assays were performed in duplicate in at least two separate experiments. The mean of the four separate values of each serum sample were calculated.

Statistical analysis

For the evaluation of possible differences between the TSAB or the TSBAB assay and the TRAK, the hTRAK, and the TBIIW assay for detecting TSHR antibodies, McNemar's test was computed (37,38). Moreover, to quantify the agreement between the different assays for the detection of TSHR antibodies the κ coefficient was calculated. The κ coefficient has a maximum of 1, if the agreement is perfect, a value of zero indicates no agreement (39,40). The Pearson correlation coefficient was determined to assess the correlation between the values obtained in different assays. p Values of less than 0.05 were considered as statistically significant. Data were analyzed with SPSS for Windows (realized 8.0.0).

Results

Determination of the normal range for TSAB and TSBAB assays

One hundred sera from a control group of healthy individuals were investigated for cAMP production of 40,000 JP 26 cells per well of a 96-well plate with or without bTSH as previously described (3). The mean value for cAMP production stimulated by these normal sera was 74 ± 51 fmol per well ($\bar{x}$ ± 3 SD). Serum samples inducing a cAMP stimulation 3SD above the mean value of the 100 control sera (125 fmol per well) were considered positive for TSAB.

The mean value for cAMP production induced by 100 serum samples from the control group incubated together with 10 mU/mL bTSH was 1350 ± 525 fmol per well ($\bar{x}$ ± 3 SD). Serum samples blocking bTSH stimulated cAMP production 3 SD below the mean value of the 100 control sera (850 fmol per well) were considered positive for TSBAB. For further assays, the 100 sera of the control group were pooled and used as control sample. The cAMP production of the 100 pooled sera of the control group was in the range of 3 SD of the separate determined cAMP values of the control group. The interassay and intra-assay variance for TSAB and TSBAB determinations was 15% or less.

Determination of the normal range for inhibition of ^{125}I-bTSH-binding on CHO cells expressing the human TSH receptor (TBIIW)

In previous studies, total binding was measured using a pool of normal sera (8,11,12,20) or one negative reference serum (21). In our TSAB or TSBAB assay we investigated the TSAB or TSBAB activity of the 100 control sera separately, and performed all further investigations with a pool of these 100 sera as control. To define the unspecific ^{125}I-bTSH binding of the pooled sera, we determined the binding of 30 control sera separately. The total binding of the pool of the 100 control sera and of the 30 individual control sera did not differ significantly in the JP26 assay (3.6 ± 0.1% vs. 3.8% ± 0.08% of total cpm, data not shown). Therefore, the pooled

100 control sera were used as the control in the inhibition of ^{125}I-bTSH binding assays.

In previous investigations, the nonspecific binding (NSB) was determined either in the presence of unlabelled bovine TSH or with untransfected CHO cells (8,11,12,20). To exclude changes of NSB due to different methods that might have influenced our displacement data, we therefore determined NSB in both ways. The NSB was determined by adding an excess (10, 20, 20, 30, 50, and 100 mU/mL) of unlabeled bTSH (Sigma). The mean value of the NSB was 0.5% ± 0.02% of total cpm of ^{125}I-bTSH ($\bar{x}$ ± 3 SD) for the JP26 assay. The mean value for the NSB of the CHO cell line JP02 transfected with pSVL-neo alone was 0.5% ± 0.01% of the total cpm of ^{125}I-bTSH ($\bar{x}$ ± 3 SD). Because no significant differences between the different determinations were observed, the pooled normal sera with and without 10 mU/mL of bTSH were used as the control sample in further assays. The inter and intra assay variance was 15% or less.

There were no differences for inhibition of ^{125}I-bTSH binding on CHO cells by adding sera before ^{125}I-bTSH and vice versa or both at the same time in the TBIIW assay (data not shown). Therefore we started sera/^{125}I-bTSH incubation in TBIIW assay together for all further experiments.

Determination of the normal range for the hTRAK assay

It is recommended by the manufacturers that each laboratory establishes its own reference ranges for normal control sera. Therefore, 100 sera from a control group of healthy individuals with normal values in the TRAK assay (<9 U/L) were analyzed for TSHR antibodies with the hTRAK assay. The new hTRAK assay was calibrated with TSAB World Health Organization (WHO) standard 90/672, therefore, 1 IU in the hTRAK is equivalent to 1 IU of TSAB standard WHO 90/672. The assays were performed according to the manufacturer's instructions. The mean value of these normal sera was 0.6 ± 0.9 IU/L ($\bar{x}$ ± 3 SD). Serum samples with hTRAK values 1.6 IU/L or less were considered as negative, values greater than 1.6 IU/L as positive for TSHR antibodies.

TBIIW and TBII activity of 69 TSAB-positive sera from 113 patients with Graves' disease

Sixty-nine of 113 sera from patients with Graves' disease treated with antithyroid drugs in which only TSAB could be detected (Fig. 1a) with the JP26 assay (TSAB activity range: 283%–1400%) were investigated for TBIIW and TBII (hTRAK and TRAK). Only 54 of the 69 (78%) TSAB-positive sera detected with JP26 cells exhibit inhibition of ^{125}I-bTSH binding measured as TBII or TBIIW (Fig. 1 and Fig. 2). In 35 of the 69 (50%), TSAB-positive sera TBIIW were detectable, whereas 45 of the 69 (65%) revealed positive TBII values. Forty-two (93%) of these 45 TBII-positive sera were positive in the hTRAK whereas 24 (53%) were positive in the TRAK assay respectively (Tables 1A and 1B, Fig. 1). 21 of the 45 (47%) TBII-positive sera were overlapping positive in the hTRAK as well as in the TRAK assay (Fig. 1).

Nine of the 69 TSAB positive sera were only TBII positive (displacement activity range: 19%–26%) shown in Figure 2, whereas 26 of the 69 (38%) TSAB-positive sera showed TBIIW as well as TBII activity (17/24 [70%] TRAK-positive and 26/42 [62%]) hTRAK-positive sera (Fig. 1). Furthermore, 19 of the 69 (27%) TSAB-positive sera were positive for TBII only (Fig. 2); 12 of these 19 TBII-positive sera showed inhibition of ^{125}I-bTSH binding in the hTRAK assay only (Fig. 1), whereas 4 were positive in the TRAK and the hTRAK and 3 in the TRAK assay only (Fig. 1).

There was no significant correlation between TSAB and TRAK ($r = 0.0659$; $r = 0.59$) or hTRAK ($r = 0.21$; $p = 0.0833$) values, whereas TSAB and TBIIW values showed a moderate correlation ($r = 0.38$; $p < 0.001$). Moreover, the comparison of the 113 sera of treated patients with Graves' disease using the McNemar's test revealed statistical differences between the determination of TSHR antibodies by detecting TSAB activity in the JP26 assay and inhibition of ^{125}I-bTSH

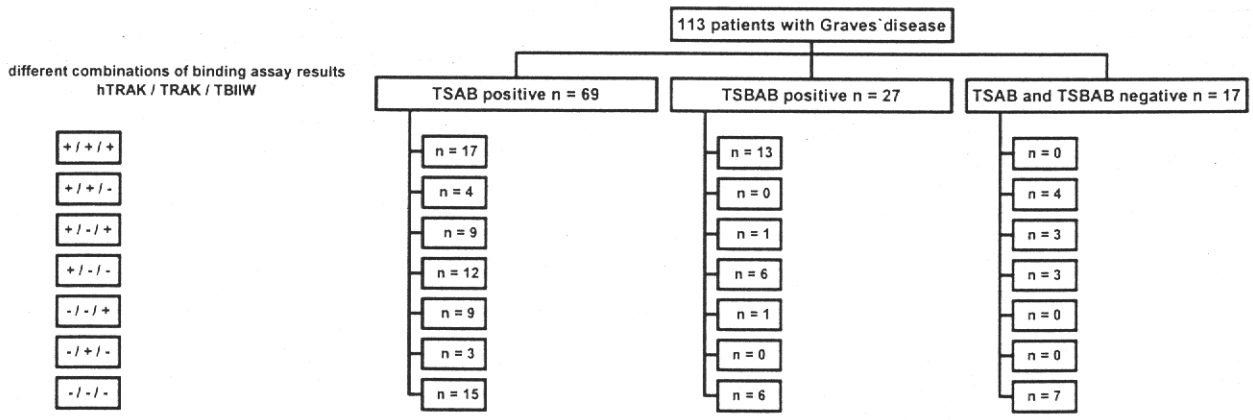

FIG. 1. Binding assay results (hTRAK/TRAK/TBIIW) for thyrotropin receptor stimulating antibody (TSAB)- or blocking (TSBAB)-positive and TSAB- and TSBAB-negative sera of 113 patients with Graves' disease treated with antithyroid drugs. Sera of 113 patients with Graves' disease receiving antithyroid drug treatment longer than 6 months were selected and divided into TSAB-positive ($n = 69$), TSBAB-positive ($n = 27$), and TSAB- and TSBAB-negative sera ($n = 17$). Thyrotropin-binding inhibitory immunoglobulin (TBII) (hTRAK and TRAK) and TBIIW activity were detected as described in materials and methods. The different combination of hTRAK/TRAK/TBIIW assay results are shown.

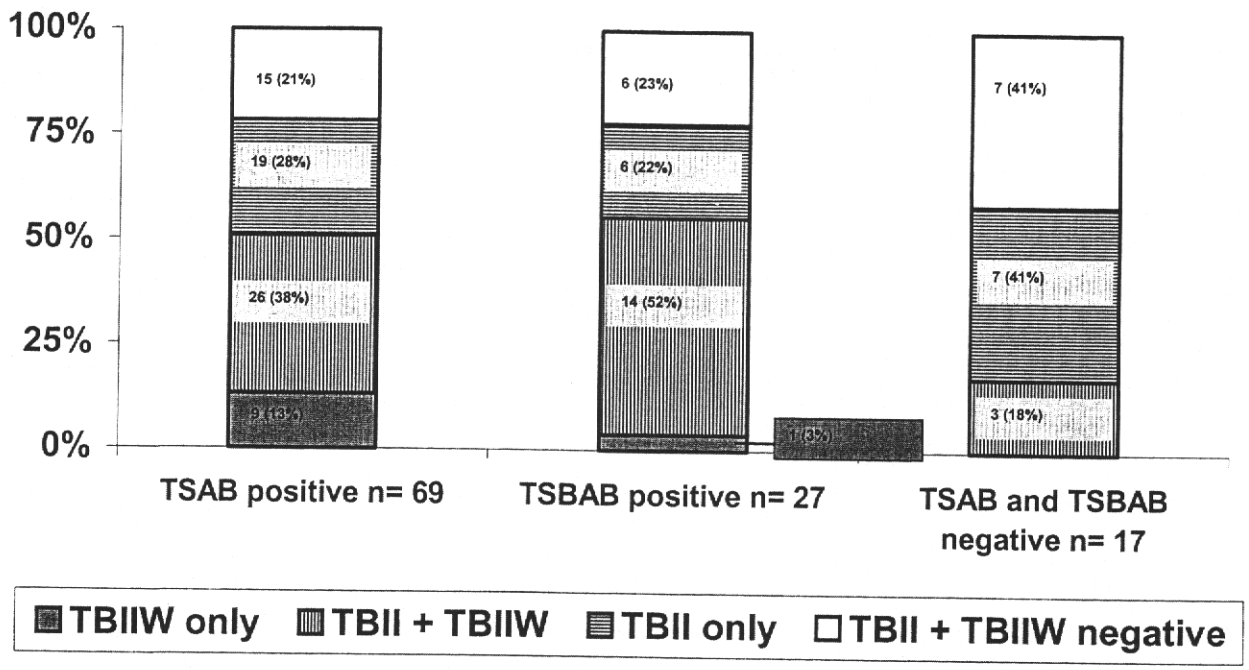

FIG. 2. Prevalence of thyrotropin-binding inhibitory immunoglobulin (TBII) (hTRAK and TRAK) and TBIIW in thyrotropin receptor stimulating antibody (TSAB)- or thyrotropin receptor blocking antibody (TSBAB)-positive and TSAB- and TSBAB-negative sera of 113 patients with Graves' disease treated with antithyroid drugs. TSAB (243%–1400% stimulation of pooled normal sera) or TSAB (38%–86% blocking of maximal bovine thyrotropin [bTSH] stimulation) activity and TBIIW were determined using the Chinese hamster ovary (CHO) cell line JP26 with 2,000 hTSH receptors per cell as described in Materials and Methods. TBIIW index was calculated as described in materials and methods. TBII (TRAK and hTRAK) were detected with the TRAK and hTRAK assay (Brahms, Berlin, Germany).

binding in the TBIIW ($p < 0.0001$), the TRAK ($p < 0.0001$), or the hTRAK assay ($p = 0.008$) respectively (Table 1). To qualify the agreement between the different assays, the κ coefficient was calculated. There was no agreement between the TSAB activity in the JP26 assay and the inhibition of ^{125}I-bTSH binding in the TRAK ($\kappa = 0.059$; $p = 0.375$) or the hTRAK assay ($\kappa = 0.015$; $p = 0.877$), whereas the JP26 assay and the TBIIW assay results showed a low agreement ($\kappa =$

TABLE 1A. DIFFERENCES BETWEEN TSHR BIOACTIVITY (TSAB OR TSBAB) AND INHIBITION OF ^{125}I-bTSH BINDING (hTRAK, TRAK AND TBIIW) IN 113 SERA OF PATIENTS WITH GRAVES' DISEASE TREATED WITH ANTITHYROID DRUGS

113 patients with Graves' disease	*hTRAK-positive*		*TRAK-positive*		*TBIIW-positive*	
	n	p	n	p	n	p
69 TSAB-positive	42	0.008	24	<0.0001	35	<0.0001
27 TSAB-positive	20	0.629	13	0.031	15	0.035

TABLE 1B. LACK OF AGREEMENT OF THE TSAB OR TSBAB ASSAY AND INHIBITION OF ^{125}I-bTSH BINDING (hTRAK, TRAK AND TBIIW) IN 113 SERA OF PATIENTS WITH GRAVES' DISEASE TREATED WITH ANTITHYROID DRUGS

113 patients with Graves' disease	*hTRAK-positive*			*TRAK-positive*			*TBIIW-positive*		
	n	κ	p	n	κ	p	n	κ	p
69 TSAB-positive	42	0.0155	0.877	24	0.059	0.375	35	0.196	0.014
27 TSAB-positive	20	0.0158	0.029	13	0.222	0.102	15	0.345	0.013

Comparison of 113 sera of treated patients with Graves' disease revealed differences between the determination of thyrotropin receptor (TSH) antibodies by detecting thyrotropin-stimulating antibody (TSAB) or thyrotropin-blocking antibody (TSBAB) activity in the JP26 assay and ^{125}I-bovine thyrotropin (bTSH) displacement determined as TBIIW, TRAK, and hTRAK respectively. Analysis of possible differences between TSAB or TSBAB activity and ^{125}I-bTSH displacement for detecting TSHR antibodies was computed using McNemar's test (Table 1A). To quantify the agreement between the different assays the kappa coefficient was calculated (Table 1B). p values of less than 0.05 were considered as statistically significant. Data were analyzed with SPSS for Windows. TSAB and TSBAB values were detected with standard in vitro assay conditions (40,000 cells per well of a 96-well plate, serum buffer dilution 1/10) as described in Materials and Methods. To obtain comparable assay conditions, TBIIW were also detected with JP26 cells at serum/buffer dilution 1/10 as described in Materials and Methods. Thyrotropin-binding inhibitory immunoglobulin (TBII) were detected with the TRAK or hTRAK assay, respectively (Brahms, Berlin, Germany).

0.196; $p = 0.014$) only (Table 1B). However, inhibition of ^{125}I-bTSH binding detected with the hTRAK assay was significantly correlated with the TBIIW values ($r = 0.66$; $p < 0.0001$) or TRAK assay ($r = 0.78$; $p < 0.0001$).

TBIIW and TBII activity of 27 TSBAB-positive sera from 113 patients with Graves' disease

Twenty-seven of 113 sera from patients with Graves' disease treated with antithyroid drugs in which only TSBAB could be detected (Fig. 1) with the JP26 assay (range: 38%–86% blocking of maximal bTSH stimulation) were investigated for TBIIW and TBII (hTRAK and TRAK). Only 21 of the 27 (78%) TSBAB-positive sera detected with JP26 cells exhibit inhibition of ^{125}I-bTSH binding measured as TBII or TBIIW (Figs. 1 and 2). Fifteen of the 27 (55%) TSBAB-positive sera showed TBIIW, whereas 20 of these 27 (65%) sera were TBII positive. All 20 TBII positive sera were positive in the hTRAK assay and 13 were positive in the TRAK assay respectively (Tables 1A and 1B, Fig. 1). Thirteen of the 20 (65%) TBII positive sera were overlapping positive in the hTRAK as well in the TRAK assay (Fig. 1).

One of the 27 (3%) of the TSBAB-positive sera showed TBIIW only (Fig. 2), whereas 14 of the 27 (52%) TSBAB-positive sera showed TBIIW as well as TBII activity (Fig. 2). All of these 14 TBII positive-sera were positive in the hTRAK and 13 were positive in the TRAK assay, respectively (Fig. 1). Furthermore, 6 of the 27 (23%) of the TSBAB positive sera were positive for TBII only (Fig. 2). These 6 sera showed inhibition of ^{125}I-bTSH binding in the hTRAK assay only (Fig. 1).

There was no significant correlation between TSBAB and TRAK ($r = 0.1193$; $p = 0.5616$) or hTRAK ($r = 0.3243$; $p = 0.1061$) values, whereas TSAB and TBIIW values showed a moderate correlation ($r = 0.4928$; $p = 0.009$). Moreover, the comparison of the 113 sera of treated patients with Graves' disease using the McNemar's test revealed statistical differences between the TSBAB activity in the JP26 assay and the inhibition of ^{125}I-bTSH binding in the TBIIW ($p = 0.035$) or the TRAK assay ($p = 0.031$), whereas the difference of the TSBAB assay and the hTRAK assay were not statistically significant ($p = 0.629$) (Table 1A). Furthermore, there was no significant agreement between the TSBAB activity in the JP26 assay and ^{125}I-bTSH displacement in the TRAK ($\kappa = 0.222$; $p = 0.102$) or hTRAK assay ($\kappa = 0.158$; $p = 0.29$). However, TSBAB activity and TBIIW values showed a moderate agreement ($\kappa = 0.345$; $p = 0.013$) (Table 1B). Moreover, inhibition of ^{125}I-bTSH binding detected with the hTRAK assay was significantly correlated with the TRAK assay ($r = 0.55$; $p < 0.0035$), whereas no correlation between hTRAK and TBIIW values were detectable ($r = 0.11$; $p < 0.58$).

TBIIW and TBII activity of 17 TSAB- and TSBAB-negative sera from 113 patients with Graves' disease

In 17 of the 113 sera from patients with Graves' disease treated with antithyroid drugs, neither TSAB nor TSBAB could be detected (Tables 1A and 1B, Figs. 1 and 2) with the JP26 assay. These 17 sera were investigated for TBIIW and TBII (hTRAK and TRAK). Ten of these 17 (58%) sera without TSAB and TSBAB activity revealed inhibition of ^{125}I-bTSH binding measured as TBII or TBIIW (Fig. 1). Three of these 17 (18%) sera showed TBIIW activity, whereas 10 of these 17 (58%) sera were TBII positive. All 10 TBII positive sera were positive in the hTRAK assay and 4 were positive in the TRAK assay respectively (Tables 1A and 1B, Fig. 1). Four of the 10 (40%) TBII-positive sera were overlapping positive in the hTRAK as well in the TRAK assay (Fig. 1).

Three of the 17 (18%) TSAB- and TSBAB-negative sera showed positive values in the TBIIW and the hTRAK assay (Figs. 1 and 2), whereas 7 of these 17 (41%) sera were only TBII positive. Six of these 10 TBII-positive sera were positive in the hTRAK assay and scored negative in the TRAK assay (Fig. 1).

Determination of TSAB, TSBAB, and TBIIW with different serum dilutions

To investigate further the previously identified differences between the inconstant TSAB or TSBAB with lower blocking activity (<60% of maximal bTSH stimulation) and the constantly high TSBAB activity (>60% of maximal bTSH stimulation) over a wide range of serum dilutions for its possible differences in ^{125}I-bTSH displacement, we determined TSBAB and TBIIW activities with JP26 cells at serum/buffer dilutions 1/10, 1/50, and 1/100. Previously investigated sera

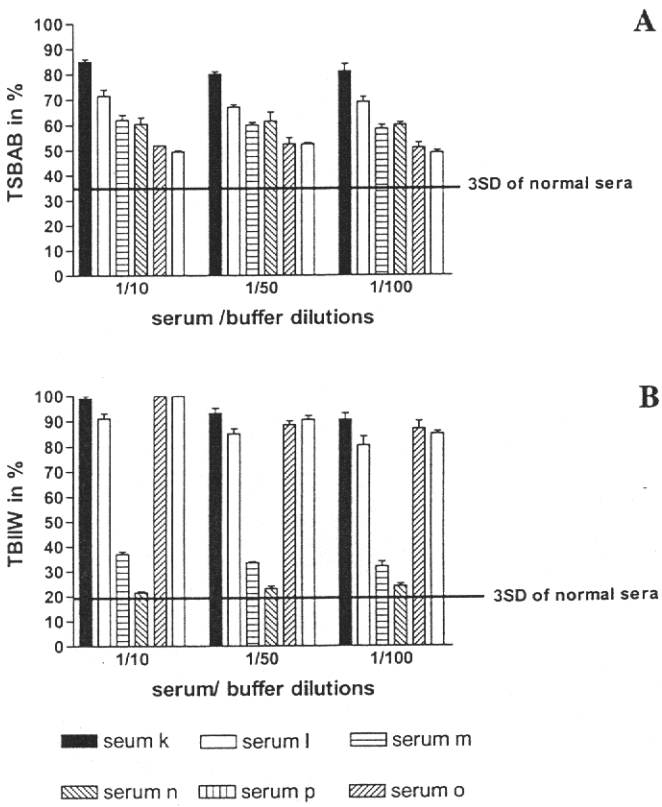

FIG. 3. A and B: Thyrotropin receptor blocking antibody (TSBAB)-positive sera with constant inhibition of ^{125}I-bovine thyrotropin (bTSH) binding (TBIIW) and constant blocking activity. TSBAB and TBIIW values of 6 of 17 TSBAB-positive sera with constant blocking activity (>50% blocking of maximal bTSH stimulation) assayed at different serum/buffer dilutions and with standard conditions (TBIIW: 1,000,000 JP26 cells per well of a 12-well plate; TSBAB: 40,000 JP26 cells per well of a 96-well plate) as described in Materials and Methods. The interassay and intra-assay variance was ≤15%.

revealed the highest differences for TSAB and TSBAB activity at serum dilutions 1/10 to 1/100 (3).

For all 12 sera with TSBAB activity less than 60% of maximal bTSH stimulation, blocking activity was only detectable at a serum buffer dilution of 1/10. Only for 1 of these 12 sera TBIIW activity was detectable at a serum buffer dilution of 1/10 to 1/100. None of these 12 sera showed TSBAB activity at serum/buffer dilutions 1/50 or 1/100 (data not shown). In 13 of 15 sera with high TSBAB activity (60%–86% blocking of maximal TSH stimulation) at a serum/buffer dilution of 1/10, constant TSBAB activity was detectable at the dilutions 1/10, 1/50, and 1/100 (data not shown). Only 6 of these 13 sera with constant TSBAB activity showed constant TBIIW activity at serum dilutions of 1/50 and 1/100 (Figs. 3a and 3b), whereas the other 7 sera with constant TSBAB activity revealed inconstant TBIIW activity (data not shown). In contrast, only 6 of the 69 TSAB-positive sera showed constant TBIIW activity for dilutions 1/10, 1/50 and 1/100 (data not shown). However, none of these 6 sera had constant TSAB activity when diluted 1/50 or 1/100 (Figs. 4a and 4b).

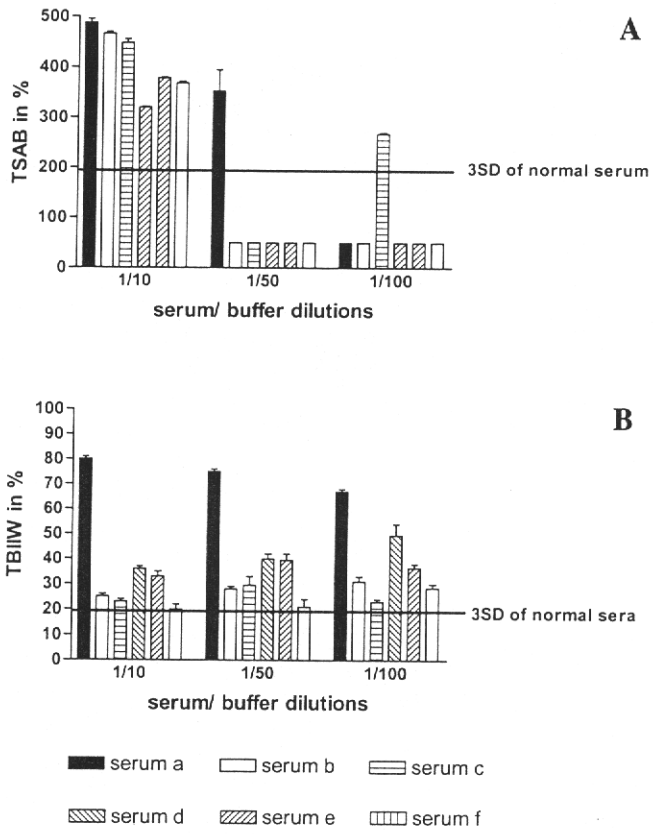

FIG. 4. **A** and **B:** Thyrotropin receptor stimulating antibody (TSBAB)-positive sera with constant inhibition (TSAB)-positive sera with constant inhibition of ^{125}I-bovine thyrotropin (bTSH) binding (TBIIW) and variable TSAB activity. TSAB activity of the only six serum samples ± SEM with constant TBIIW activity determined with different serum/buffer dilutions and standard conditions (TBIIW: 1,000,000 JP26 cells per well of a 12-well plate; TSBAB: 40,000 JP26 cells per well of a 96-well plate). TSAB activity was determined using the Chinese hamster ovary (CHO) cell line JP26 with 2,000 hTSH receptors per cell as described in Materials and Methods. Serum samples inducing a cyclic adenosine monophosphate (cAMP) stimulation 3 SD above the mean value of 100 control sera (125 fmol per well) were considered positive for TSAB. The TBIIW index was calculated as described in materials and methods. The interassay and intra-assay variance was ≤15%.

Detection of anti-bTSH antibodies in sera with TSBAB activity

None of the 27 TSBAB-positive sera detected with the CHO cell line JP 26 showed positive binding to ^{125}I-bTSH indicating the absence of anti-bTSH-antibodies (data not shown).

Discussion

In clinical practice, TSHR antibodies are mostly determined as TBII by their ability to inhibit the binding of radiolabelled bTSH to its receptor in solubilized porcine thyroid membrane preparations (18,41) or to the immobilized human TSHR in a new assay technique. The latter showed superior sensitivity and specificity for detecting TSHR antibodies in untreated patients with Graves' disease when compared to the TRAK assay (32). Serum samples with detectable TSAB but no TBII have been described for small numbers of sera from patients with Graves' disease treated with antithyroid drugs (1,3,17). These unexpected findings were mainly explained by the lack of comparability of assay conditions and by the different TSAB, TSBAB, or TBII assay sensitivities. Therefore, we excluded these possible methodologic interferences in the comparison of different TSHR antibody activities by parallel determinations of TSAB, TSBAB, and TBIIW, using the same experimental conditions for the determination of all TSHR antibody qualities.

Our results show that 96 of the 113 (85%) sera were positive for TSAB or TSBAB (Tables 1a and 1b and Fig. 1 and Fig. 2). Anti-bTSH antibodies as a possible reason for blocking activity without inhibition of ^{125}I-bTSH binding were excluded in all 27 TSBAB-positive sera. Only 78% (54/69) of the TSAB-positive and 78% (21/27) of the TSBAB-positive sera of patients with Graves' disease treated with antithyroid drugs exhibit TBII or TBIIW activity (Fig. 1 and Fig. 2). To the best of our knowledge, our study is the first to compare TSAB and TSBAB assays with the results for inhibition of ^{125}I-bTSH binding using the same experimental conditions or the highly sensitive hTRAK assay.

In a recent study, only 1 of 50 investigated Graves' disease sera with TSAB activity scored negative in a direct binding assay and only borderline positive in a TBII assay (2). We detected inhibition of ^{125}I-bTSH binding (hTRAK, TRAK, and TBIIW) in only 78% of TSAB (54/69) or TSBAB (21/27) positive sera. Therefore our results suggest that TSH displacement by TSHR antibodies and stimulation and blocking the TSHR are different functions, which do not always need to occur together. Furthermore, our results imply that the stimulating and blocking mechanisms of TSAB or TSBAB are not always related to the bTSH-binding epitopes. This interpretation is further supported by the computed differences between TSAB and the TBIIW activities ($p < 0.001$), the TRAK ($p < 0.0001$) or the hTRAK values ($p = 0.008$) respectively (Table 1a). The computed κ coefficient for the sera that score positive in the TSAB and the TBIIW ($\kappa = 0.196$; $p = 0.014$), the TRAK assay ($\kappa = 0.059$; $p = 0.375$) and the hTRAK assay ($\kappa = 0.015$; $p = 0.877$) revealed no agreement between

stimulation of the TSHR and inhibition of ^{125}I-bTSH binding (Table 1b). Furthermore, we detected differences between TSBAB activity and the TRAK ($p = 0.031$) or the TBIIW ($p = 0.035$) values (Table 1a). There was no significant difference between TSBAB activity and the values of the hTRAK assay ($p = 0.629$). Both assays showed no agreement ($\kappa = 0.0155$; $p = 0.877$) for the detection of TSHR antibodies. The low agreement between the TSBAB assay and the TBIIW assay ($\kappa = 0.34$; $p = 0.013$) was not supported by the computed differences between the TSBAB and the TBIIW assay ($p = 0.035$) in the McNemars' test (Table 1A). The analyzed κ coefficient for the sera that score positive in the TSBAB and the TRAK ($\kappa = 0.222$; $p = 0.102$) or the hTRAK ($\kappa = 0.158$; $p = 0.29$) revealed no agreement between TSBAB activity and ^{125}I-bTSH displacement (Table 1B).

These sera that are negative for inhibition of ^{125}I-bTSH binding, but TSAB- or TSBAB-positive could interact with other non-bTSH-biding regions of the TSHR. It was previously suggested that high-affinity binding sites of TSH and the binding sites of TSHR antibodies are not identical but partly overlapping (18,43). In contrast to the hypotheses that the stimulating and blocking mechanisms of TSAB or TSBAB are not always related to the bTSH-binding epitopes, recently published studies suggest that TSHR antibodies detectable in these assays bind principally to the same region of the TSH receptor as TSH itself. This seemed to be independent of their TSAB or TSBAB activity (1,2,42). These studies demonstrated close correlations for direct TSHR antibody binding assays with TBII values in 38 ($r = 0.881$, $p < 0.001$), 63 ($r = 0.92$, $p < 0.001$) or 50 ($r = 0.69$, $p < 0.001$) Graves' disease patients (1,2,42). However, the values of different direct binding assays correlated less well with the detected TSAB activity in 36 ($r = 0.582$, $p < 0.001$) or 50 patients with Graves' disease ($r = 0.58$, $p < 0.001$) respectively (1,2). Furthermore, differences in the changes of TBII and TSAB values in 29 patients with Graves' disease during a 12-month course of ATDT imply that TSAB and TBII are not identical. This could be a further explanation for the different assay sensitivities in the previously reported as well as in our present study (33). Moreover, these different changes of TBII and TSAB values support our conclusion that TSH displacement by TSHR antibodies and stimulation and blocking the TSHR are different functions, which do not always need to occur together.

TSH and TSHR antibody binding studies using either TSH-LH-receptor chimeras (18,44) or synthetic peptides corresponding to regions of the extracellular domain (ECD) (18,45) suggest that the amino terminal region of the ECD is responsible for the TSAB binding, whereas epitopes of the carboxyl region bind TSBAB. Whether the three extracellular loops of the transmembrane domain or interaction of TSHR antibodies with the intracellular domain contribute to stimulation or blocking activity of the TSHR without detectable inhibition of ^{125}I-bTSH binding is unknown (2,18). However, TSHR antibodies binding to these three loops could lead to conformational changes of these regions resulting in activation or inactivation of the receptor without detectable TBII activity. TSBAB without inhibition of ^{125}I-bTSH binding has not previously been demonstrated. However, TSHR stimulation without binding and vice versa have previously been reported (2,3,5,33). It has been speculated for many years that antibodies may exist that bind to the TSHR but neither stimulate nor block the TSHR. Using a direct binding assay for TSHR antibodies only 1 of 38 sera of Graves' disease patients treated with antithyroid drugs and 3 of 13 Hashimoto's patients and 5 of 50 Graves' disease patients revealed TSHR binding and/or positive TBII values without stimulation of the TSHR (2). We identified 10 serum samples with ^{125}I-bTSH displacement but no stimulating or blocking activity (Tables 1A and 1B). It is therefore likely that TSH displacement by TSHR antibodies and stimulating or blocking activity of TSHR antibodies are different functions that do not always need to occur together. Other reasons for these discrepancies like a mixture of TSAB and TSBAB (excluded in our study), limitations of the different assays and isolated receptor binding or TSAB activities (5) have also been suggested (2).

Studies of the constitutive activity of the wild-type TSHR and of TSHR mutants suggest a model for TSHR activation in which the binding of TSH to the receptor would activate the receptor by stabilizing the active conformation of the receptor. Binding of TSAB or TSBAB would lead to stabilization of the active or inactive conformation only with detectable ^{125}I-bTSH displacement. However, activation of the receptor by partial proteolysis that could include conformational changes which activate the receptor is a further possibility (27,46). The activation of the TSHR by trypsin, which removes epitopes of the extracellular domain, supports the latter concept (27,47).

A very tight interaction of TSBAB with high blocking activity (>60% of maximal bTSH stimulation) with the TSHR has previously been demonstrated in three sera (3). This concept is further supported by the finding that in 13 of 15 sera with high TSBAB activity (60%–86% blocking of maximal TSH stimulation) at a serum/buffer dilution of 1/10, constant TSBAB activity was detectable at the dilutions of 1/10, 1/50, and 1/100 (data not shown). Furthermore, in all 12 sera with lower TSBAB activity (<60% of maximal bTSH stimulation) blocking activity was only detectable at a serum buffer dilution of 1/10. The most likely reason for these findings might be a higher affinity of TSBAB with high blocking activity than TSAB. Due to the high constitutive activity of the TSHR it seems to be easier for TSHR antibodies without high-affinity binding to activate the receptor than to inhibit TSH stimulation. This could be consistent with the hypothesis that TSAB without ^{125}I-bTSH displacement could activate the TSHR by proteolysis (18,22,27,46,47) or interact with the intracellular domain (2).

Moreover, only 6 of these sera with constant TSBAB activity showed constant TBIIW activity at serum dilutions of 1/50 and 1/100 (Figs. 3a and 3b), whereas the other 7 sera with constant TSBAB activity revealed inconstant TBIIW activity (data not shown). In contrast, only 6 of the 69 TSAB positive sera showed constant TBIIW activity for dilutions 1/10, 1/50 and 1/100. However, none of these 6 sera had constant TSAB activity at dilutions of 1/50 and 1/100 (Figs. 4a and 4b). These differences between TSAB or TSBAB activities and TBIIW activity at different serum dilutions further demonstrate that TSAB and most TSBAB activities are not correlated with TBIIW or TBII (Tables 1A and 1B).

In the hTRAK assay 72 (63%) of all 113 sera of patients with Graves' disease were positive (Fig. 1 and Fig. 2). However, only 41 (36%) or 53 (46%) of all 113 investigated sera were detectable in the TRAK or the TBIIW assay, respectively

(Fig. 1). Therefore, the hTRAK assay showed a higher sensitivity than the TBIIW or the TRAK assay for detecting TSHR antibodies in patients with Graves' disease treated with antithyroid drugs with or without TSAB or TSBAB activity (Table 1A and 1B and Fig. 1). However, 96 of the 113 (85%) sera were positive for TSAB or TSBAB (Tables 1a and 1b, Fig. 1 and Fig. 2). Therefore, our results could imply that the JP26 bioassay has an even higher sensitivity for the detection of TSHR antibodies than the hTRAK assay. However, it has also been shown that the hTRAK assay (32) has a higher sensitivity for detecting TSHR antibodies than the JP09 TSAB bioassay (4) in sera of untreated Graves' disease patients. These diverse results can be best reconciled by the hypothesis that TSH displacement by TSHR antibodies and stimulation or blocking of the TSHR are different functions, which do not always need to occur together. Consequently sensitivities of binding assays and bioassays should not be compared since these assays determine different qualities of TSHR antibodies.

Sera from Graves' disease patients treated with antithyroid drugs with detectable TSAB or TSBAB activity without detectable TBII and vice versa could also explain the low sensitivity of TBII detection alone or in combination with TSAB (10,13,16) as a prognostic marker for remission or relapse after 1 year of ATDT of Graves' disease. Due to the reported transitions from TSAB to TSBAB and vice versa (48,50) and the different changes of TSAB and TBII in the course of ATDT (33), most likely only a combined highly sensitive determination of TBII, TSAB, and TSBAB during the course of Graves' disease can possibly predict the individual course of Graves' disease. Further follow-up studies of Graves' disease patients with simultaneous TBII, TSAB, and TSBAB determinations should evaluate the prognostic significance of changes in the individual TSHR antibody profile in Graves' disease.

Acknowledgments

We would like to thank G. Vassart for the CHO cell clones and Brahms, Berlin, Germany for providing labeled bTSH. This study was supported by the Wilhelm Sander Stiftung.

References

1. Chazenbalk GD, Pichurin P, McLachlan SM, Rapoport B 1999 A direct binding assay for thyrotropin receptor autoantibodies. Thyroid **9**:1057–1061.
2. Sanders J, Oda Y, Roberts S, Kiddie A, Richards T, Bolton J, McGrath V, Walters S, Jaskolski D, Furmaniak J, Smith BR 1999 The interaction of TSH receptor autoantibodies with 125I-labelled TSH receptor. J Clin Endocrinol Metab **84**:3797–3802.
3. Wallaschofski H, Paschke R 1999 Detection of thyroid stimulating (TSAB)- and thyrotropin stimulation blocking (TSBAB) antibodies with CHO cell lines expressing different TSH-receptor numbers. Clin Endocrinol (Oxf) **50**:365–372.
4. Vitti P, Elisei R, Tonacchera M, Chiovato L, Mancusi F, Rago T, Mammoli C, Ludgate M, Vassart C, Pinchera A 1993 Detection of thyroid-stimulating antibody using Chinese hamster ovary cells transfected with cloned human thyrotropin receptor. J Clin Endocrinol Metab **76**:499–503.
5. Worthington J, Byfield PG, Himsworth RL 1991 Heterogeneity of circulating TSH-receptor antibodies in thyroid disease demonstrated directly by chromatography. Clin Endocrinol (Oxf) **34**:147–154.
6. Chiovato L, Vitti P, Santini F, Lopez G, Mammoli C, Bassi P, Giusti L, Tonacchera M, Fenzi G, Pinchera A 1990 Incidence of antibodies blocking thyrotropin effect in vitro in patients with euthyroid or hypothyroid autoimmune thyroiditis. J Clin Endocrinol Metab **71**:40–45.
7. Chiovato L, Vitti P, Bendinelli G, Santini F, Fiore E, Capaccioli A, Tonacchera M, Mammoli C, Ludgate M, Pinchera A 1994 Detection of antibodies blocking thyrotropin effect using Chinese hamster ovary cells transfected with the cloned human TSH receptor. J Endocrinol Invest **17**:809–816.
8. Costagliola S, Swillens S, Niccoli P, Dumont JE, Vassart G, Ludgate M 1992 Binding assay for thyrotropin receptor autoantibodies using the recombinant receptor protein. J Clin Endocrinol Metab **75**:1540–1544.
9. Derwahl M, Schatz H, Bolle B, Pohl A, Meyer K 1992 Measurement of stimulating TSH receptor antibodies in sera of patients with Graves' disease by a recombinant TSH receptor bioassay. Exp Clin Endocrinol **100**:75–79.
10. Feldt-Rasmussen U, Schleusener H, Carayon P 1994 Meta-analysis evaluation of the impact of thyrotropin receptor antibodies on long term remission after medical therapy of Graves' disease. J Clin Endocrinol Metab **78**:98–102.
11. Ludgate M, Perret J, Parmentier M, Gerard C, Libert F, Dumont JE, Vassart G 1990 Use of the recombinant human thyrotropin receptor (TSH-R) expressed in mammalian cell lines to assay TSH-R autoantibodies. Mol Cell Endocrinol **73**:R13–R18.
12. Ludgate M, Costagliola S, Danguy D, Perret J, Vassart G 1992 Recombinant TSH-receptor for determination of TSH-receptor-antibodies. Exp Clin Endocrinol **100**:73–74.
13. Michelangeli V, Poon C, Taft J, Newnham H, Topliss D, Colman P 1998 The prognostic value of thyrotropin receptor antibody measurement in the early stages of treatment of Graves' disease with antithyroid drugs. Thyroid **8**:119–124.
14. Michelangeli VP, Munro DS, Poon CW, Frauman AG, Colman PG 1994 Measurement of thyroid stimulating immunoglobulins in a new cell line transfected with a functional human TSH receptor (JPO9 cells), compared with an assay using FRTL-5 cells. Clin Endocrinol (Oxf) **40**:645–652.
15. Michelangeli VP, Poon CW, Arnus EE, Frauman AG, Connelly J, Colman PG 1995 Measurement of TSH receptor blocking immunoglobulins using 3H-adenine incorporation into FRTL-5 and JPO9 cells: Use in a child with neonatal hypothyroidism. Clin Endocrinol (Oxf) **42**:39–44.
16. Schleusener H, Schwander J, Fischer C, Holle R, Holl G, Badenhoop K, Hensen J, Finke R, Bogner U, Mayr WR 1989 Prospective multicentre study on the prediction of relapse after antithyroid drug treatment in patients with Graves' disease [published erratum appears in Acta Endocrinol (Copenh) 1989 Aug;121(2):304]. Acta Endocrinol (Copenh) **120**:689–701.
17. Zhu Y, Portmann L, Denereaz N, Lemarchand-Beraud T 1994 Simultaneous assay for three types of thyrotropin receptor antibody activities using FRTL-5 cells in patients with autoimmune thyroid diseases. Eur J Endocrinol **131**:359–368.
18. Rapoport B, Chazenbalk GD, Jaume JC, McLachlan SM 1998 The thyrotropin (TSH) receptor: Interaction with TSH and autoantibodies [published erratum appears in Endocr Rev 1999 Feb;20(1):100]. Endocr Rev **19**:673–716.
19. Jaume JC, Kakinuma A, Chazenbalk GD, Rapoport B, McLachlan SM 1997 Thyrotropin receptor autoantibodies in serum are present at much lower levels than thyroid peroxidase autoantibodies: Analysis by flow cytometry. J Clin Endocrinol Metab **82**:500–507.
20. Kakinuma A, Chazenbalk GD, Jaume JC, Rapoport B,

McLachlan SM 1997 The human thyrotropin (TSH) receptor in a TSH binding inhibition assay for TSH receptor autoantibodies. J Clin Endocrinol Metab **82**:2129–2134.
21. Kakinuma A, Morimoto I, Kuroda T, Fujihira T, Eto S, McLachlan SM, Rapoport B 1999 Comparison of recombinant human thyrotropin receptors versus porcine thyrotropin receptors in the thyrotropin binding inhibition assay for thyrotropin receptor autoantibodies. Thyroid **9**:849–855.
22. Foti D, Russo D, Costante G, Filetti S 1991 The biological activity of bovine and human thyrotropin is differently affected by trypsin treatment of human thyroid cells: Thyroid-stimulating antibody is related to human thyrotropin. J Clin Endocrinol Metab **73**:710–716.
23. Parma J, Duprez L, Van Sande J, Cochaux P, Gervy C, Mockel J, Dumont J, Vassart G 1993 Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas [see comments]. Nature **365**:649–651.
24. Parma J, Van Sande J, Swillens S, Tonacchera M, Dumont J, Vassart G 1995 Somatic mutations causing constitutive activity of the thyrotropin receptor are the major cause of hyperfunctioning thyroid adenomas: identification of additional mutations activating both the cyclic adenosine 3′,5′-monophosphate and inositol phosphate-Ca2+ cascades. Mol Endocrinol **9**:725–733.
25. Paschke R, Tonacchera M, Van Sande J, Parma J, Vassart G 1994 Identification and functional characterization of two new somatic mutations causing constitutive activation of the thyrotropin receptor in hyperfunctioning autonomous adenomas of the thyroid. J Clin Endocrinol Metab **79**:1785–1789.
26. Tonacchera M, Van Sande J, Cetani F, Swillens S, Schvartz C, Winiszewski P, Portmann L, Dumont JE, Vassart G, Parma J 1996 Functional characteristics of three new germline mutations of the thyrotropin receptor gene causing autosomal dominant toxic thyroid hyperplasia. J Clin Endocrinol Metab **81**:547–554.
27. Van Sande J, Massart C, Costagliola S, Allgeier A, Cetani F, Vassart G, Dumont JE 1996 Specific activation of the thyrotropin receptor by trypsin. Mol Cell Endocrinol **119**: 161–168.
28. Wonerow P, Schoneberg T, Schultz G, Gudermann T, Paschke R 1998 Deletions in the third intracellular loop of the thyrotropin receptor. A new mechanism for constitutive activation. J Biol Chem **273**:7900–7905.
29. Hakola K, Haavisto AM, Pierroz DD, Aebi A, Rannikko A, Kirjavainen T, Aubert ML, Huhtaniemi I 1998 Recombinant forms of rat and human luteinizing hormone and follicle-stimulating hormone; comparison of functions in vitro and in vivo. J Endocrinol **158**:441–448.
30. Spetzler JC, Meldal M, Meinjohanns E, Steinaa L, Mouritsen S, Bock K 1997 Synthetic hFSH peptide constructs in the evaluation of previous studies on the hFSH receptor interaction. J Pept Sci **3**:397–414.
31. Dorje F, Wess J, Lambrecht G, Tacke R, Mutschler E, Brann MR 1991 Antagonist binding profiles of five cloned human muscarinic receptor subtypes. J Pharmacol Exp Ther **256**: 727–733.
32. Costagliola S, Morgenthaler NG, Hoermann R, Badenhoop K, Struck J, Freitag D, Poertl S, Weglohner W, Hollidt JM, Quadbeck B, Dumont JE, Schumm-Draeger PM, Bergmann A, Mann K, Vassart G, Usadel KH 1999 Second generation assay for thyrotropin receptor antibodies has superior diagnostic sensitivity for Graves' disease. J Clin Endocrinol Metab **84**:90–97.
33. Yamano Y, Takamatsu J, Sakane S, Hirai K, Kuma K, Ohsawa N 1999 Differences between changes in serum thyrotropin-binding inhibitory antibodies and thyroid-stimulating antibodies in the course of antithyroid drug therapy for Graves' disease. Thyroid **9**:769–773.
34. Perret J, Ludgate M, Libert F, Gerard C, Dumont JE, Vassart G, Parmentier M 1990 Stable expression of the human TSH receptor in CHO cells and characterization of differentially expressing clones. Biochem Biophys Res Commun **171**:1044–1050.
35. Ochi Y, Inui T, Hachiya T, Nakajima Y, Ishida M, Kajita Y, Fujikawa H, Ogura H 1990 Coexistence of autoantibody to human thyrotropin (TSH) and autoantiidiotypic antibody to antihuman TSH antibody in a case with simple goiter. J Clin Endocrinol Metab **71**:1163–1167.
36. Raines KB, Bakerr JRJ, Lukes YG, Wartofsky L, Burman KD 1985 Antithyrotropin antibodies in the sera of Graves' disease patients. J Clin Endocrinol Metab **61**:217–222.
37. Eliasziw M, Donner A 1991 Application of the McNemar test to non-independent matched pair data. Stat Med **10**:1981–1991.
38. Krummenauer F, Kalden P, Kreitner KF 1999 [Cohen's kappa or McNemar's test? A comparison of binary repeated measurements]. Rofo Fortschr Geb Rontgenstr Neuen Bildgeb Verfahr **171**:226–231.
39. Cyr L, Francis K 1992 Measures of clinical agreement for nominal and categorical data: The kappa coefficient. Comput Biol Med **22**:239–246.
40. Liehr P, Dedo YL, Torres S, Meininger JC 1995 Assessing agreement between clinical measurement methods. Heart Lung **24**:240–245.
41. Rees SB, McLachlan SM, Furmaniak J 1988 Autoantibodies to the thyrotropin receptor. Endocr Rev **9**:106–121.
42. Minich WB, Weymayer JD, Loos U 1999 Immunoprecipitation analysis of pathological autoantibodies in Graves' patients sera using biotinylated human thyrotropin receptor labeled with 125I-neutravidiny. Exp Clin Endocrinol Diabetes **107**:555–560.
43. Banga JP, Harris PE 1998 Potential pathogenicity of autoantibodies to thyrotropin receptor in treated, euthyroid patients with Graves' disease. Eur J Endocrinol **139**:139–142.
44. Watanabe Y, Tahara K, Hirai A, Tada H, Kohn LD, Amino N 1997 Subtypes of anti-TSH receptor antibodies classified by various assays using CHO cells expressing wild-type or chimeric human TSH receptor. Thyroid **7**:13–19.
45. Mori T, Sugawa H, Piraphatdist T, Inoue D, Enomoto T, Imura H 1991 A synthetic oligopeptide derived from human thyrotropin receptor sequence binds to Graves' immunoglobulin and inhibits thyroid stimulating antibody activity but lacks interactions with TSH. Biochem Biophys Res Commun **178**:165–172.
46. Duprez L, Parma J, Costagliola S, Hermans J, Van Sande J, Dumont JE, Vassart G 1997 Constitutive activation of the TSH receptor by spontaneous mutations affecting the N-terminal extracellular domain. FEBS Lett **409**:469–474.
47. Vu TK, Wheaton VI, Hung DT, Charo I, Coughlin SR 1991 Domains specifying thrombin-receptor interaction. Nature **353**:674–677.
48. Cho BY, Shong YK, Lee HK, Koh CS, Min HK 1989 Graves' hyperthyroidism following primary hypothyroidism: sequential changes in various activities of thyrotropin receptor antibodies. Acta Endocrinol (Cophenh) **120**:447–450.
49. Kung AW, Jones BM 1998 A change from stimulatory to

blocking antibody activity in Graves' disease during pregnancy. J Clin Endocrinol Metab **83**:514–518.
50. Tamai H, Kasagi K, Takaichi Y, Takamatsu J, Komaki G, Matsubayashi S, Konishi J, Kuma K, Kumagai LF, Nagataki S 1989 Development of spontaneous hypothyroidism in patients with Graves' disease treated with antithyroidal drugs: Clinical, immunological, and histological findings in 26 patients. J Clin Endocrinol Metab **69**:49–53.

Address reprint requests to:
Prof. Dr. med. R. Paschke
Universität Leipzig
Zentrum für Innerre Medizin
Medizinische Klinik und Poliklinik III
Phillipp-Rosenthal-Str. 27
D-04103 Leipzig, Germany

E-mail: pasr@medizin.uni-leipzig.de