

Characterization of Apparently Paradoxical Thyrotropin Binding Inhibitory Immunoglobulins With Neutral Bioactivity

Tetsuya Tagami^{1,2}  and Kenji Moriyama³

¹Department of Endocrinology and Metabolism, National Hospital Organization Kyoto Medical Center, Kyoto 612-8555, Japan

²Clinical Research Institute for Endocrine and Metabolic Diseases, National Hospital Organization Kyoto Medical Center, Kyoto 612-8555, Japan

³Department of Medicine and Clinical Science, Faculty of Pharmaceutical Sciences, Mukogawa Women's University, Hyogo 663-8179, Japan

Correspondence: Tetsuya Tagami, MD, PhD, Division of Endocrinology and Metabolism, Clinical Research Institute, National Hospital Organization Kyoto Medical Center, Mukaihata-cho 1-1, Fukakusa, Fushimi-ku, Kyoto 612-8555, Japan. Email: ttagami@kuhp.kyoto-u.ac.jp.

Abstract

Context: The thyrotropin (TSH) receptor (TSH-R) autoantibody activity is clinically measured by inhibition of labeled ligand (TSH or M22) binding to the TSH-R (TSH-binding inhibitory immunoglobulin [TBII]) or by stimulation (TSH-R stimulating antibody [TSAb]) or inhibition (TSH-R blocking antibody [TSBA]) of 3',5'-cyclic adenosine 5'-monophosphate (cAMP) production in isolated cells.

Objective: We experienced a patient with hypothyroid Graves disease (GD) having strong positive TBII but with almost neutral bioactivities on the TSH-R. The aim of this study is the characterization of this apparently paradoxical TBII (serum sample S).

Methods: We first compared the TBII, TSAb, and TSBA activities of serum sample S with mixtures of stimulating (S-mAb) and blocking monoclonal Ab (B-mAb). Next, we serially measured cAMPs stimulated by various serum samples in the presence or absence of TSH.

Results: Mixtures of S-mAb and B-mAb did not reproduce the characteristics of serum sample S. Instead, serum sample S had a unique feature that blocked the TSH-stimulated cAMP initially but disappeared the blocking activity thereafter to reach the control level.

Conclusion: We present here the TBII with neutral bioactivities found in the patient with autoimmune thyroid disease, which strongly inhibit TSH binding to the TSH-R but exerts neither TSAb nor TSBA activity. Differences in the methods of detecting TRAb between TBII in vitro and bioassay may cause the discrepancy. Although serum sample S may be an extreme example, a variety of TRAb that not only stimulates or blocks but also interferes with TSH-R binding for only a short time may exist in the serum samples of GD patients.

Key Words: TSH receptor-stimulating antibodies, TSH receptor-blocking antibodies, TSH-binding inhibitory immunoglobulins

Abbreviations: B-mAb, blocking monoclonal antibody; bTSH, bovine thyrotropin; cAMP, 3',5'-cyclic adenosine 5'-monophosphate; EIA, enzyme immunoassay; GD, Graves disease; mAb, monoclonal antibody; N-mAb, neutral monoclonal antibody; rhTSH, recombinant human thyrotropin; rhTSH-R, recombinant human TSH-R; S-mAb, stimulating monoclonal antibody; TSBA, TSH receptor blocking antibody; TBII, thyrotropin binding inhibiting immunoglobulin; TSAb, thyrotropin receptor-stimulating antibody; TSH, thyrotropin; TSH-R, thyrotropin receptor.

Thyrotropin receptor (TSH-R) autoantibodies (TRAbs) are found in the serum samples of hyperthyroid patients with Graves disease (GD) and rarely in those of hypothyroid patients with primary myxedema. TRAbs are clinically detected using the methods based on inhibition of labeled ligand (TSH or M22) binding to the TSH-R thyroid-binding inhibitory immunoglobulins (TBIs). The stimulating biological activity that usually causes hyperthyroidism is measured by stimulation of 3',5'-cyclic adenosine 5'-monophosphate (cAMP) production (TSH-R stimulating antibodies [TSAb]) in isolated cells. The blocking biological activity that rarely causes hypothyroidism is measured by inhibition of cAMP production induced by TSH (TSH-stimulation blocking antibody [TSBA]) in the same isolated cells. Alternatively, both stimulating TRAb and blocking TRAb can be detected by TBII as well. Previously, we have isolated both monoclonal autoantibodies (mAbs) to the TSH-R, one with stimulating activity

(S-mAb) and one with blocking activity (B-mAb), obtained from the same blood sample [1]. Using these mAbs, we demonstrated that dynamic fluctuation of TRAb between stimulation and inhibition in the serum samples of GD patients can be partially reproduced by their mixture in various ratios [2]. However, we also found the serum samples in a patient with Hashimoto thyroiditis, which are strongly positive for TBII, but had disproportionately weak TSAb activity and no TSBA activity [2]. Here, we characterized this apparently paradoxical TRAb, which appears strongly positive for TBII but shows almost neutral bioactivity.

Materials and Methods

Patient

Patient S, who had TBIs with neutral bioactivities on the TSH-R, and other GD patients have been described

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elsewhere [2]. Briefly, patient S, a 52-year-old woman, was diagnosed with hypothyroidism and treated with 100 µg of levothyroxine. Her TBILs varied between 82 and 233 IU/L by ECLusys and between 141 and 191 IU/L by DYNOfest. Interestingly, her TSABs varied between 131% and 204% but her TSBABs were completely negative (-26.3% to 0.9%) (Fig. 1), suggesting that her TRABs are almost neutral, with a slight TSAB activity. Her hypothyroidism was considered due to Hashimoto thyroiditis (thyroglobulin antibody and thyroid peroxidase antibody tests were both positive). Patient M, an 80-year-old man, presented with subclinical hyperthyroidism, thyroid-associated orbitopathy, and had high serum TSAB activity (817%-5760%) but traces by TBII (< 0.3 to

3.1 IU/L). Patient K, a 52-year-old woman, was hypothyroid without thyroid-associated orbitopathy, with high TBILs (152-184 IU/L) and highly positive TSBAB activity (98.6%-99.3%) but low TSAB activity (180%-309%), and was the donor of K1-18 and K1-70. She became hyperthyroid recently and her TSAB activity rose (384%-1480%). The serum samples of patient B were kindly provided by Dr Ochi [3]. Institutional ethics committee approval for the study was obtained and, after obtaining written informed consent, serum samples at 752 time points from 2015 to 2021 were obtained from 442 GD patients in total, who were either untreated, treated with antithyroid drugs, or in remission, in addition to the previous report [2].

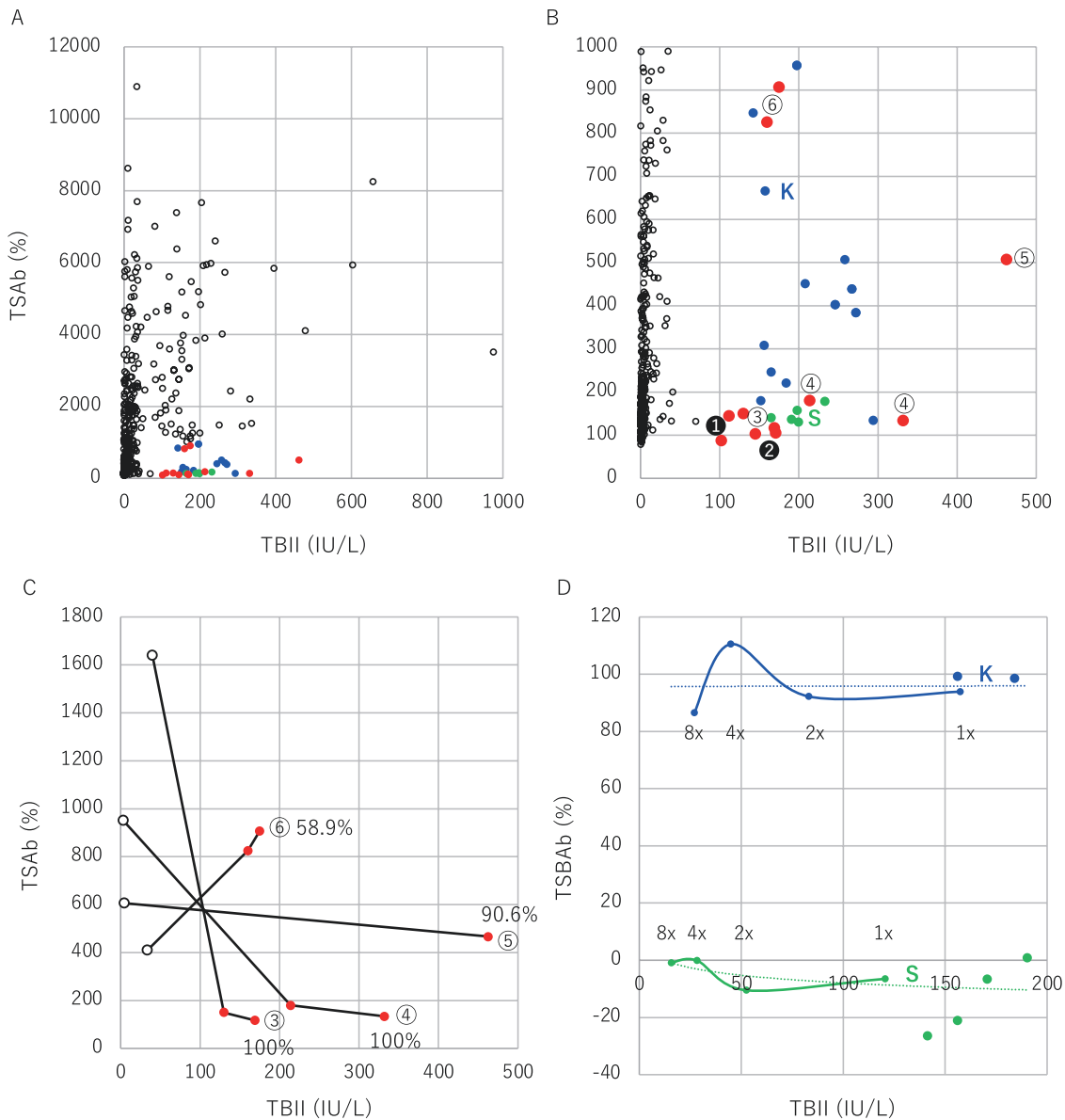


Figure 1. Relationship between TSH-binding inhibitory immunoglobulin (TBII) and TSH-R stimulating antibody (TSAb) in patients with Graves disease (GD). A, TSAb values were plotted against TBII values in the sera at the different time points of patients S, Nos. 1 to 6, and an additional 434 patients with GD (open circles), who are untreated or treated with antithyroid drugs. B, The area less than 1000% in TSAb of Fig. 1A is shown. C, The changes in the relationship between TBII and TSAb before (open circles) and after (closed circles) were shown in the serum samples of Nos. 3 to 6 in Fig. 1B. The numbers in the figure indicate the TSAB values. D, TSAB values were plotted against TBII values in the serum samples at the different time points of patients K and patients S. The results of dilution assay up to 8-fold using these serum samples are also shown. Exponential approximate curves were drawn using Microsoft Excel.

Laboratory Evaluation

TRAb was measured with an electrochemiluminescence immunoassay based on inhibition of M22 binding (ECLusys, TRAb M22, Roche Diagnostics) [4, 5] and with an assay based on inhibition of ^{125}I -bTSH binding (DYNtest TRAb [human], BRAHMS) [6]. Results of both TBII assays are reported in international units per liter based on a World Health Organization standard, and the normal range is 2.0 IU/L for ECLusys and 1.0 IU/L for the DYNtest. The upper limits of detection of ECLusys and DYNtest were both 40 IU/L, and levels greater than 40 IU/L were remeasured by diluting by pooling serum samples obtained from healthy blood donors. TSAb was assessed using a bioassay based on stimulation of cAMP production by porcine thyroid cells (TSAb enzyme immunoassay [EIA] kit, Yamasa Corporation) [7, 8]. TSBABs were assessed using a modified TSAb EIA kit in the presence or absence of 100 mU/L of bovine thyrotropin (bTSH) [2]. The bTSH was provided by the kit. The values of TBII measured with ^{125}I -TSH (DYNtest TRAb human) and with M22 (ECLusys, TRAb M22) were almost equivalent among the GD patients' serum samples (Fig. 2A).

TSAb values were calculated as follows: $\text{TSAb} (\%) = 100 + (\text{cAMP produced by patient serum} - \text{cAMP produced by negative control serum}) / (\text{cAMP produced by positive control serum} - \text{cAMP produced by negative control serum}) \times 650$. The negative control serum (TSAb = 100%) and positive control serum (TSAb = 750%) were provided in the kit. The normal range of TSAb is less than 120% according to the package insert.

TSBAB values were calculated as follows: $\text{TSBAB} (\%) = [1 - (\text{cAMP produced by 100 mU/L bTSH and patient serum} - \text{cAMP produced by patient serum}) / (\text{cAMP produced by 100 mU/L bTSH and negative control serum} - \text{cAMP produced by negative control serum})] \times 100$. The normal range of TSBAB is less than 34% [9].

The outline of the new bioassay for TSAb and TSBAB is as follows. The assay set consists of human embryonic kidney 293 (HEK293) cells expressing a human TSH-R and GloSensor (cAMP-binding domain-inserted luciferase: Promega Corp) [10]. TRAb in a patient serum binds to the TSH-R expressed in HEK293 cells, and cAMP is produced via activation of adenylate cyclase. When cAMP binds in the cAMP-binding domain of GloSensor, the GloSensor changes in structure and reacts with luciferin, increasing the amount of luciferase luminescence. The luminescence is measured every 10 minutes for the initial 1 hour and then every 30 minutes until 240 minutes with a luminometer. TSAb activity is quantified by measuring the amount of luminescence. The bTSH or recombinant human TSH (rhTSH) was used for this assay. The rhTSH was purchased from Sanofi K.K.

Characterization of Monoclonal Antibodies

Two stimulating, M22 [11], K1-18 [12] mAbs, and one blocking, K1-70 [13] mAb, were provided by Dr B. R. Smith at FIRS Laboratories, RSR Ltd. Different concentrations of M22, K1-18, and K1-70 were diluted in healthy blood donor serum and assayed for TBII, TSAb, and TSBAB.

Results

The distribution of TSAb to TBII was scattered (Fig. 1A) and some serum samples showed extremely high TBII but relatively low TSAb (colored points in the graph). In detail

(TSAb < 1000%), several points from 6 different patients distributed in the same area ($100 < \text{TBII} < 500 \text{ IU/L}$) as patient S (green) or K (blue) (Fig. 1B). Of these 6 patients, 2 (Nos. 1 and 2) were GD patients with primary hypothyroidism and their serum samples were inhibitory (strongly positive TSBAB). The other 4 (Nos. 3-6) were GD patients who became hypothyroid with increased TBII and positive TSBAB after radioiodine therapy (Fig. 1C). As we reported previously [2], the TSBAB activity of the serum samples of patient K (serum sample K) was strongly positive, whereas that of the serum samples of patient S (serum sample S) was completely negative (Fig. 1D). In addition, dilution of these serum samples reduced TBII without altering TSBAB activity until at least an 8-fold dilution. The addition of an increasing amount of B-mAb reduced the bTSH-induced cAMP with increased TBII activity in a dose-dependent manner (Fig. 2A). In contrast, the addition of serum sample S drastically increased TBII activity (from left to right of green dotted arrows) but had little effect on cAMP induced by bTSH (Fig. 2B), S-mAb, or B-mAb (Fig. 2C). For TSBAB assays, the cAMPs were compared between the absence and presence of 100 mU/L bTSH (Fig. 2D). In the presence of 100 mU/L bTSH, cAMPs produced by serum samples S (green solid lines) increased equally to that in normal serum samples (a black line), indicating that those TSBABs are negative. In contrast, in the presence of 100 mU/L bTSH, cAMPs produced by K1-70 B-mAb (blue lines) increased a half (100 mg/L K1-70) or very little (500 mg/L K1-70) of those in normal serum samples or serum samples S, indicating that K1-70 B-mAb has strong TSBAB activity. Again, the addition of serum samples S increased TBII activities with little effect on the cAMPs of B-mAb in the absence and presence of 100 mU/L bTSH (from left to right of green dotted arrows). Therefore, serum samples S have little activities both for TSAb and TSBAB, indicating they are neutral TRAb with strong TBII.

First, we tried to reproduce the characteristics of TRAb in serum samples S using the S-mAb and B-mAb. As shown in Fig. 3A, 100 mg/L of S-mAb (M22 or K1-18) showed 10 IU/L TBII and 11 000% TSAb. In contrast, 100 mg/L of B-mAb (K1-70) showed 10 IU/L TBII and approximately 100% TSAb (Fig. 3A), whereas 200 mg/L of K1-70 showed 20 IU/L TBII and approximately 90% TSBAB (Fig. 3B). We then mixed the S-mAb and B-mAb in different combinations and assayed TSAb and TSBAB activities because autoantibodies of TSH-R in the serum samples of GD patients are polyclonal. However, an increase of B-mAb to decrease strong TSAb activity of S-mAb increased TSBAB activity, and the mixture of S-mAb and B-mAb is unlikely to be achieved to reach the much higher values of TBII found in serum samples S in keeping with both negative for TSAb and TSBAB (Fig. 4).

Next, we attempted to reproduce the characteristics of TRAb in serum samples S by assuming they consist of a kind of very weak agonist for TSH-R, a virtual neutral mAb (N-mAb) that shows very weak TSAb and no TSBAB. Alternatively, a high amount of virtual N-mAb may be able to reproduce strong TBII activities with weak TSAb and no TSBAB activities. Because the relationship between mAb concentration and TBII value was linear ($\text{TBII (IU/L)} = \sim [\text{mAb}] / 11 \text{ (mg/L)}$), and no difference was observed among mAb species [2], the TBII values ($\sim 200 \text{ IU/L}$) of the serum samples S roughly correspond to approximately 2200 mg/L of virtual N-mAb. On the other hand, 500 mg/L of K1-70 showed 50 IU/L TBII and approximately 100%

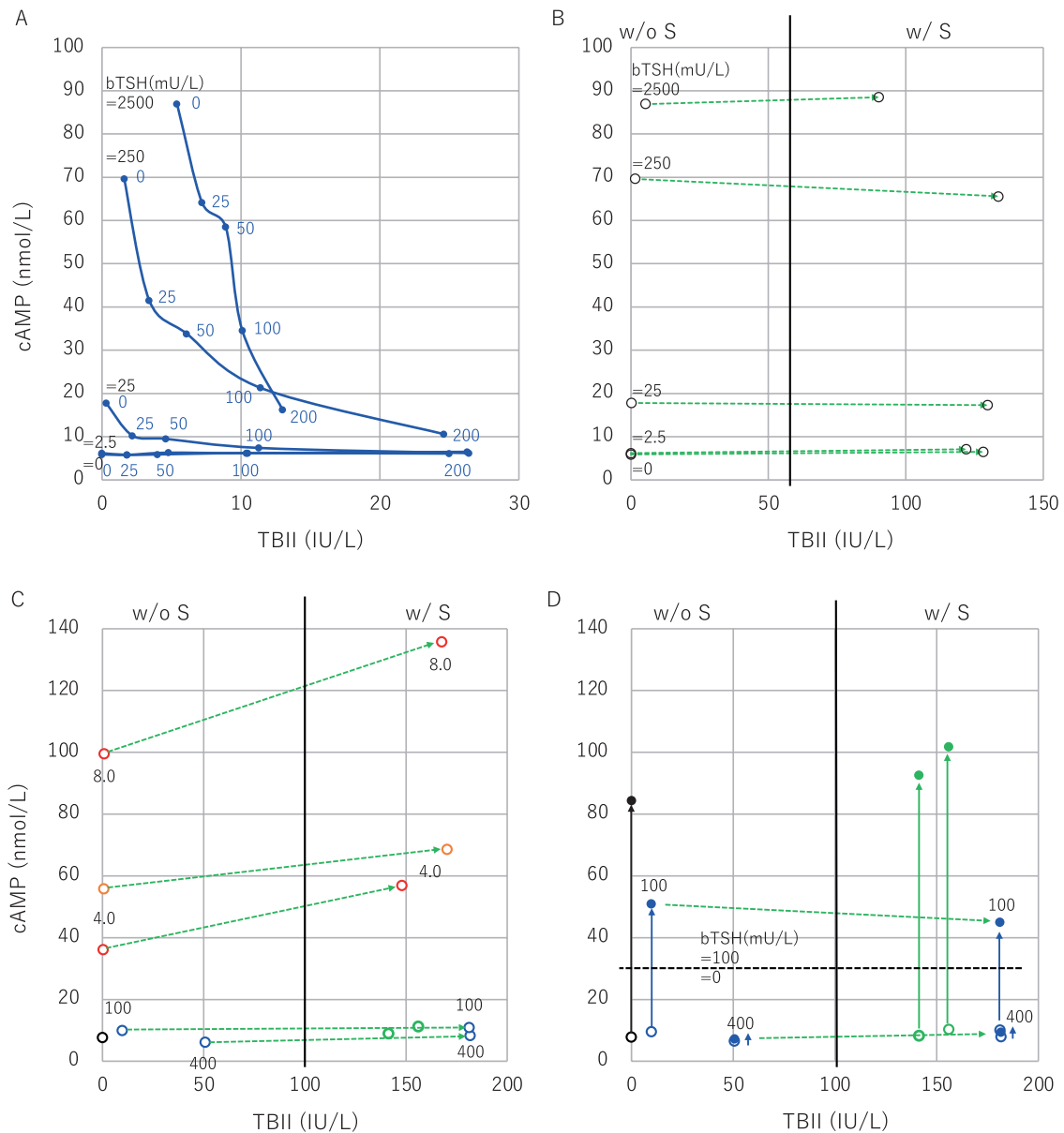


Figure 2. Effect of additions of the patient S's serum samples on bovine thyrotropin (bTSH), M22 and K1-18 stimulating monoclonal antibodies (S-mAbs) or K1-70 blocking monoclonal antibodies (B-mAbs). A to C, Assays for TSH-R stimulating antibody (TSAb). A, TSH-binding inhibitory immunoglobulins (TBIs) and 3',5'-cyclic adenosine 5'-monophosphates (cAMPs) induced by various concentrations (0-2500 mU/L) of bTSH and effects of the addition of various amounts (0-200 mg/L) of K1-70 B-mAb were measured. B, TBIs and cAMPs induced by various concentrations (0 to 2500 mU/L) of bTSH and effects of the addition of the serum sample S were measured. C, TBIs and cAMPs of normal serum samples (an open circle on the lowest at 0 of TBII), the serum samples of patient S (two open circles on the lowest around 150 of TBII) obtained at 2 different time points, 4.0 and 8.0 mg/L M22 (open circles labeled as 8.0 on the top and the lower ones labeled as 4.0 in the middle), 4.0 mg/L K1-18 (the upper open circles labeled as 4.0 in the middle), 100 and 400 mg/L K1-70 (open circles labeled as 100 and 400 on the bottom), and effects of the addition of the serum sample S were measured (w/ S). The arrowheads of dotted lines, B and C, indicate that additions of the serum samples of patient S (w/S) increased TBIs but had little effect on the original cAMPs (w/o S). D, Assays for TSH-R blocking antibody (TSBAbs). TBIs and cAMPs of normal serum samples (circles at the far left), the serum samples of patient S (circles around 150 of TBII) obtained at 2 different time points, and 100 and 400 mg/L K1-70 (labeled as 100 and 400) were measured, with (closed circles) or without (open circles) 100 mU/L bTSH. The arrowheads of dotted lines indicate that additions of the serum samples of patient S (w/S) increased TBIs but had little effect on the original cAMPs (w/o S) also in the presence of 100 mU/L bTSH.

TSBAbs (Fig. 3B), which indicates no increase in cAMP by the addition of 100 IU/L TSH (Fig. 2D). Alternatively, 100 mU/L bTSH cannot activate TSH-Rs anymore, which are completely occupied with 500 mg/L of K1-70. Therefore, TSH should not be able to activate TSH-R in the presence of virtual N-mAb, the amount of which is 3 times that of 500 mg/L of K1-70.

Accordingly, the discrepancy seems to come from other factors, such as the chronological difference between an in vitro

assay (TBII) and bioassay in cells, for example. In contrast, S-mAb or B-mAb can compete with bTSH-binding to the TSH-R both in vitro and in cells and stay on the receptor to keep off bTSH; TBIs with little bioactivity may depart from the receptor as time goes by and bTSH can access the receptor thereafter to exert its activity. To observe the time course of bioactivity, we used a new bioassay system, in which the GloSensor measures the real-time cAMP levels in the cells induced by TSH-R activation. The cAMP activities were serially

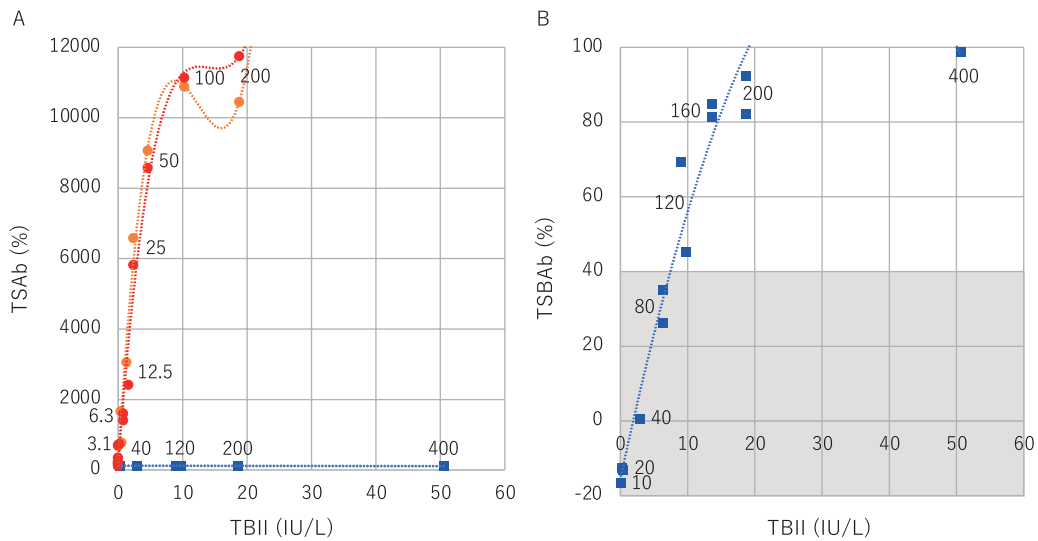


Figure 3. Dose response curve of M22 and K1-18 for TSH-R stimulating antibody (TSAb) and K1-70 for TSAb and TSH-R blocking antibody (TSBAb). A, TSAb values in various concentrations of M22, K1-18 (circles), and K1-70 (squares) are plotted against the corresponding TSH-binding inhibitory immunoglobulin (TBII) values measured by a DYNOfest TRAb assay based on inhibition of ¹²⁵I-TSH binding. Numbers by the points indicate the concentrations of mAbs (mg/L) used for this assay. B, TSBAb values in various concentrations of K1-70 are plotted against the corresponding TBII values measured by a DYNOfest TRAb assay. A shadow indicates the reference (negative) range of TSBAb. Numbers by the points indicate the concentrations of K1-70 (mg/L) used for this assay.

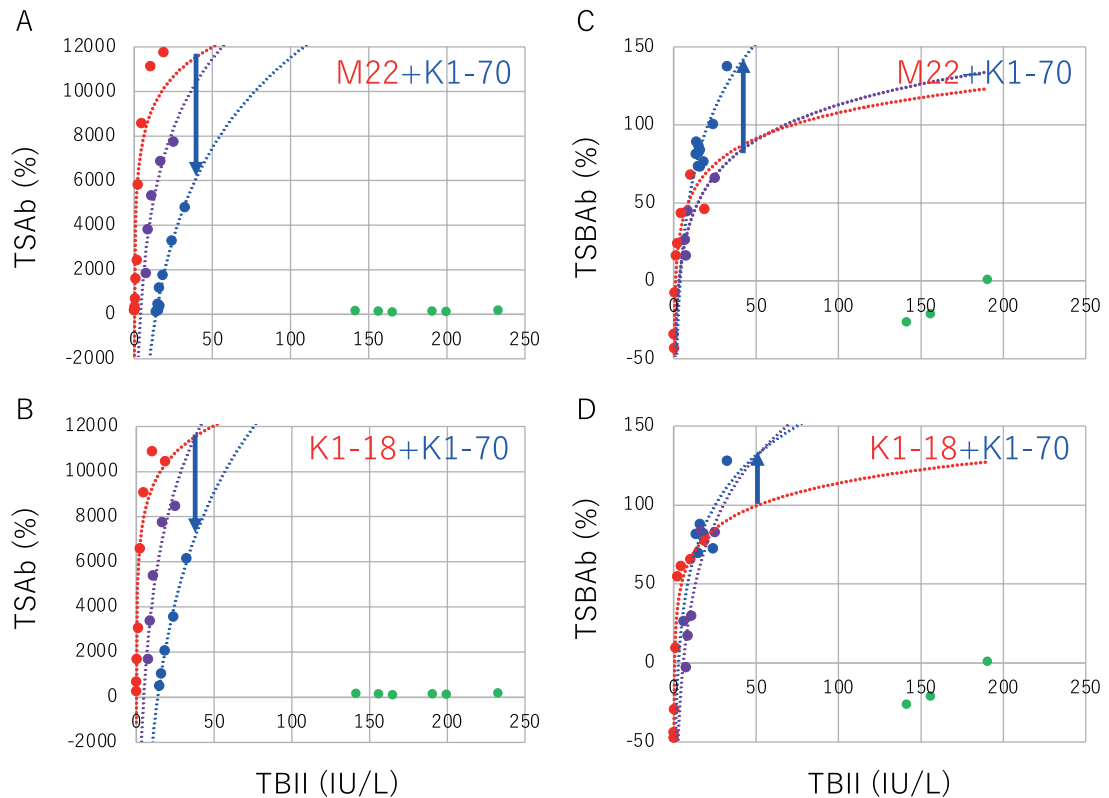


Figure 4. Relationship between TSH-binding inhibitory immunoglobulin (TBII) and TSH-R stimulating antibody (TSAb) in the mixture of M22 or K1-18 and K1-70, and a comparison with patient S. TBII, TSAb, and TSH-R blocking antibody (TSBAb) were measured using increasing concentrations of A and C, M22, or B and D, K1-18 (0-200 mg/L) together with increasing concentrations of K1-70 (0, the upper series in A and B and the lower series in C and D; 80, the middle series in A to D, and 160 mg/L, the lower series in A and B and the upper series in C and D: concentrations increased along the arrow) and the A and B, TSAb, or C and D, TSBAb were expressed against the TBII measured by DYNOfest or ECLusys TRAb assay. TBII values in the serum samples at the different time points of patient S (distributed from 150 to 230 of TBII in A to D) measured by DYNOfest or ECLusys TRAb assay are superimposed onto each figure. Exponential approximate curves were drawn using Microsoft Excel.

measured with a luminometer. First, we measured cAMP activities produced by various concentrations of bTSH (Fig. 5A). Increasing concentrations of bTSH produced cAMP in

a dose-dependent manner. Similarly, both serum samples M [2], and serum samples No. 6 in Fig. 1 stimulated cAMP even in the absence of bTSH (Fig. 5B). The serum samples No. 6

contain some TBAb components (TSBAb = 58.9% in Fig. 1C) and the cAMP returned to normal level at the end of assay. Next, we examined cAMP activities produced by serum samples S, serum samples K, and serum samples No. 5 in Fig. 1 or normal serum samples in the absence or presence of various concentrations of bTSH (Fig. 6). In the absence of bTSH (Fig. 6A), both serum samples K and serum samples No. 5 stimulated cAMP, but serum samples S as well as normal serum samples did not. Note that the TSAb and TSBAb of sera K used in this assay were 666% and 94%, respectively, and those of serum samples No. 5 were 507% and 91%, respectively,

indicating that serum samples K and serum samples No. 5 contain both stimulating and blocking components. The addition of 25 to 2500 mU/L bTSH to serum samples K or serum samples No. 5 had little effect on the pattern of cAMP stimulation (A-D in Fig. 6). In contrast, the addition of 25 to 2500 mU/L bTSH to serum samples S or normal serum samples stimulated their cAMP productions in a dose-dependent manner. As a result, the difference in the peak stimulation in cAMP among the 4 serum samples almost disappeared in the presence of 250 mU/L bTSH (Fig. 6C). However, closer observation revealed a difference between serum samples S and

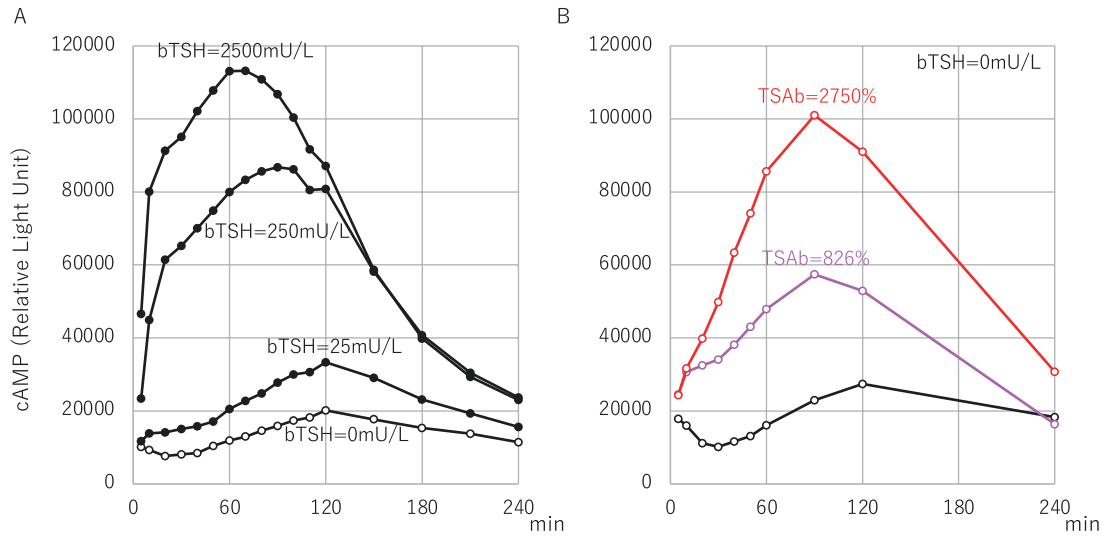


Figure 5. Time course of 3',5'-cyclic adenosine 5'-monophosphates (cAMP) activities measured as luciferase activities of bovine thyrotropin (bTSH) or the serum samples of patients. A, The cAMP activities of various concentrations (0-2500 mU/L) of bTSH were measured every 10 minutes for the initial 120 minutes and then every 30 minutes until 240 minutes with a luminometer. B, The cAMP activities of the serum samples of patient M (TSAb=2750%) or No. 6 in Fig. 1 (TSAb=826%) in the absence of bTSH were measured every 10 minutes for the initial 1 hour and then at 90, 120, and 240 minutes with a luminometer.

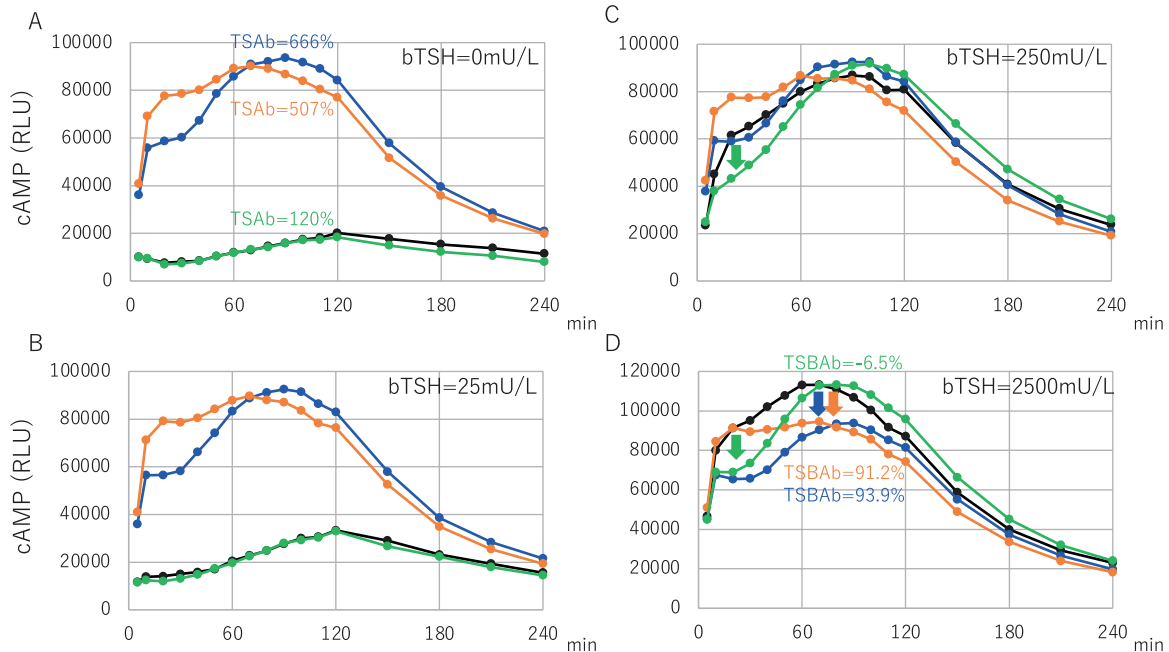


Figure 6. Time course of 3',5'-cyclic adenosine 5'-monophosphates (cAMP) activities measured as luciferase activities of the serum samples of patients. The cAMP activities of the serum samples of patients S (TSAb=120% and TSBAb=-6.5%), K (TSAb=666% and TSBAb=93.9%), No. 5 in Fig. 1 (TSAb=507% and TSBAb=91.2%) or normal control (the highest where 30-60 min in D), with various concentrations (0-2500 mU/L) of bovine thyrotropin (bTSH), were measured every 10 minutes for the initial 120 minutes and then every 30 minutes until 240 minutes with a luminometer. Arrows indicate the difference of cAMP activities between the serum samples of patients and normal control.

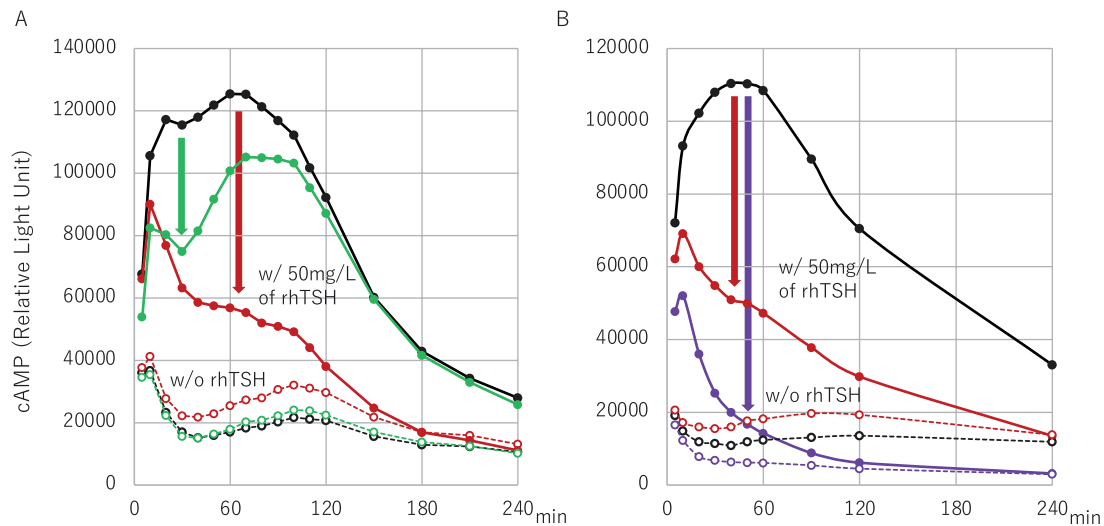


Figure 7. Time course of 3',5'-cyclic adenosine 5'-monophosphates (cAMP) activities measured as luciferase activities of the serum samples of patients. A, The cAMP activities of the serum samples of patient B (the lowest w/ rhTSH and the highest w/o rhTSH), S (in the middle both w/ and w/o rhTSH among three), or normal control (the highest w/ rhTSH and the lowest w/o rhTSH), with or without 50 mg/L recombinant human thyrotropin (rhTSH), were measured every 10 minutes for the initial 1 hour and then every 30 minutes until 240 minutes with a luminometer. B, The cAMP activities of the serum samples of patient B (in the middle w/ rhTSH and the highest w/o rhTSH), No. 1 in Fig. 1 (the lowest both w/ and w/o rhTSH among three) or normal control (the highest w/ rhTSH and in the middle w/o rhTSH), with or without 50 mg/L rhTSH, were measured every 10 minutes for the initial 1 hour and then at 90, 120, and 240 minutes with a luminometer. Arrows indicate the difference of cAMP activities between the serum samples of patients and normal control.

normal serum samples at approximately the first 60 minutes in the presence of 250 to 2500 mU/L bTSH (green arrows in Fig. 6C and 6D). Alternatively, the initial elevation in cAMP in serum samples S was blunted compared to normal serum samples. On the other hand, the peak cAMP activities at 60 to 90 minutes produced by serum samples K and serum samples No. 5 in the presence of 2500 mU/L bTSH were lower than those by serum samples S or normal serum samples (blue and orange arrows in Fig. 6D), indicating that both serum samples K and serum samples No. 5 have blocking activities. The similar or more prominent phenomenon was observed in the coexistence of 50 mg/L of rhTSH as shown in Fig. 7. The differences among serum samples S, serum samples B [3], serum samples No. 1 in Fig. 1B, and normal serum samples were remarkable. Both potent blocking serum samples B (red) and serum samples No. 1 (purple) inhibited rhTSH-induced cAMP production from 10 to 240 minutes (also shown by red and purple arrows, respectively). The serum samples B produced a little cAMP in the absence of rhTSH (red dotted lines in Fig. 7A and 7B) but strongly inhibited (red solid lines) rhTSH-induced cAMP production compared to normal serum samples (black solid lines). The inhibitory effects of serum samples No. 1 were more potent. The rhTSH-induced cAMP activities were completely blocked at the end of assay by serum samples No. 1 (see Fig. 7B). On the other hand, the time course of serum samples S in the presence of rhTSH was also unique in this assay. The rhTSH-induced cAMP production in coexistence of serum samples S were lower than those in normal serum samples during the initial 120 minutes (see Fig. 7A). Thereafter, the inhibitory effect of serum samples S decreased and rhTSH-induced cAMP production in coexistence of serum samples S caught up with those in normal serum samples.

Discussion

The narrow sense of neutral TRAb is against the hinge region, an inert scaffold connecting the leucine-rich ectodomain that binds

to TSH, to the transmembrane region of the receptor, and it does not basically affect TSH binding to the TSH-R [14-16]. It seems that some patients with GD have these type of autoantibodies that bind to the hinge region of TSH-R [17-20]. The neutral antibodies, in a narrow sense, do not interfere with TSH-binding to the TSH-R and, therefore, they should be negative for TBII. In contrast, the TBIIs with neutral bioactivities on the TSH-R, as we describe here, are strongly positive for TBII but have neutral (neither stimulating nor blocking) biological activities to the TSH-R. This is very curious because positive TBII indicates replacement of TSH on the TSH-R, which should stimulate, otherwise inhibit. We would like to call this kind of TRAb an apparently neutral TRAb in contrast to the neutral TRAb against the hinge region of TSH-R, whose TBII values are negative. In clinical practice, we sometimes experience TBIIs with neutral bioactivities (negative both for TSAb and TSBAb) on the TSH-R with mildly positive TBII in the serum samples of GD patients in remission [2], while their TBII values are usually small. Because high concentrations of B-mAb (at least ~100 mg/L, which corresponds to ~8 IU/L TBII) are required to show TSBAb activity in contrast to S-mAbs (see Fig. 3), it is conceivable that negative TSBAb in TRAb detected in the serum samples of GD patients in remission is simply because they are insufficient in quantity to show TSBAb activity. Alternatively, the TRAb can be blocking if their TBII activities are under 8 IU/L.

First of all, because both TBII values in serum samples S measured by ^{125}I -TSH (DYNtest) and M22 (ECLusys) were equally high, it is conceivable that the positivity for TBII in serum samples S is not an artifact. There are several fundamental conditions to discuss the issues on the TSH- and mAb-binding to the TSH-R. First, one molecule of either TSH or M22 binds to one molecule of TSH-R [21, 22]. Second, the values of TBII measured by ^{125}I -TSH (DYNtest) and M22 (ECLusys) were almost equivalent (Fig. 8A). Third, up to about 25 mg/L of M22 produced cAMP almost linearly (Fig. 8B) and so did up to more than 300 mU/L of bTSH (Fig. 8C). Fourth, the relationship between mAb concentration and

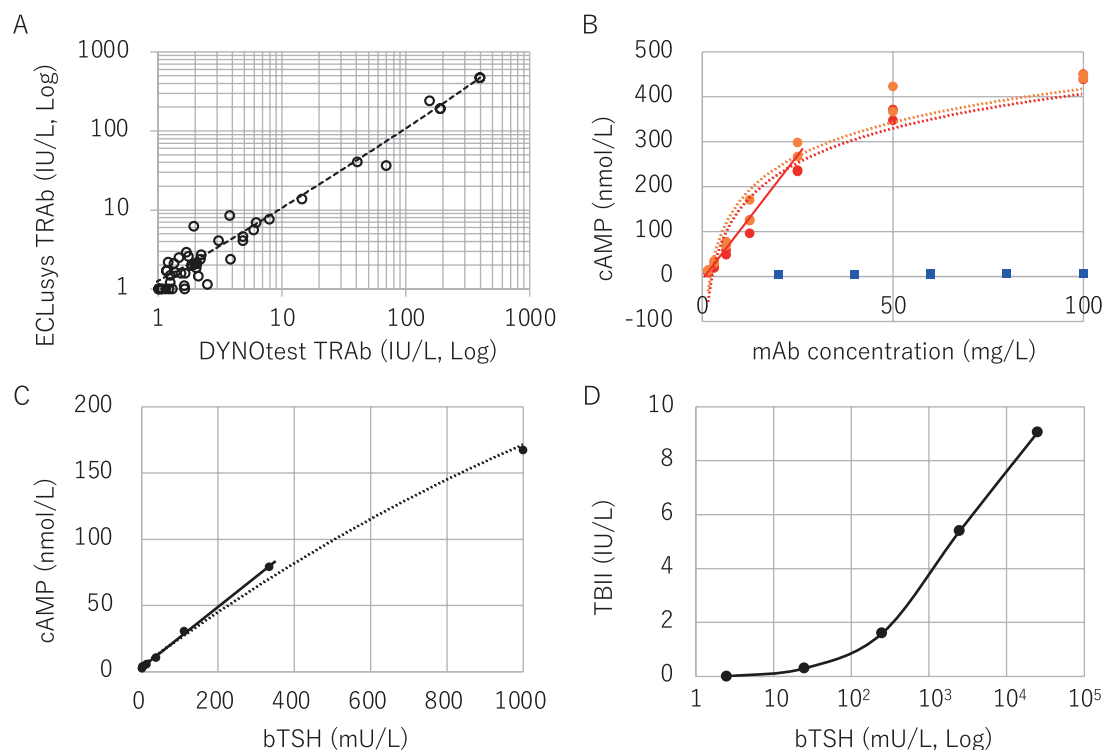


Figure 8. Relationship between TSH-binding inhibitory immunoglobulin (TBII) measured by DYNtest TRAb and TBII measured by ECLusys TSH-R autoantibodies (TRAb) in the patients with Graves disease (GD) and the 3',5'-cyclic adenosine 5'-monophosphates (cAMPs) produced by bovine thyrotropin (bTSH) or stimulating monoclonal antibody (S-mAb). A, TBII values measured by DYNtest based on inhibition of ¹²⁵I-TSH binding on the x-axis were plotted against the corresponding TBII values measured by ECLusys based on inhibition of M22 binding on the y-axis in the serum samples from 162 patients with GD, who are untreated or treated with antithyroid drugs. A dotted line indicates the linearity between the 2 assays. B, Various concentrations of bTSH are plotted on the x-axis against the corresponding cAMP values on the y-axis. Polynomial approximate curve was drawn using Microsoft Excel and a solid line indicates the linearity at least within the drawn areas. C and D, Various concentrations of M22, K1-18 (circles), and K1-70 (squares) are plotted against C, the corresponding cAMP values, or D, TBII measured by DYNtest TRAb. Polynomial approximate curves were drawn using Microsoft Excel. Solid lines indicate the linearity at least within the drawn areas.

TBII activity measured with ¹²⁵I-TSH was linear without a difference between S-mAb and B-mAb [2]. Fifth, TSBAb are evaluated, briefly, by the comparison between cAMP produced by 100 mU/L bTSH with and without patient serum [9, 23]. Under these conditions, because 200 mU/L bTSH produced about 50 nmol/L cAMP (see Fig. 8C) and 20 mg/L M22 produced about 200 nmol/L cAMP (see Fig. 8B), 100 mU/L bTSH is calculated to be almost equivalent to 2.5 mg/L M22. In fact, the TBII of 100 mU/L bTSH was less than 1.0 IU/L (Fig. 8D).

Accordingly, the titer of TBII is assumed to reflect the number of TRAbs that occupy the TSH-R, by competing ¹²⁵I-bTSH or M22 S-mAb binding. The concentration of 200 mg/L K1-70 B-mAb, which is equivalent to 20 IU/L TBII, almost completely inhibited cAMP induced by 100 IU/L bTSH (see Fig. 3B). Alternatively, 20 IU/L TBII of K1-70 B-mAb can almost completely prevent TSH binding to the TSH-R by 100 IU/L bTSH in cultured cells. In contrast, although TBII of serum samples S were as high as 200 IU/L, which is 10 times of B-mAb that can almost completely occupy the TSH-binding sites of TSH-R, the patient's TSBAb were around 0%. Alternatively, serum samples S allowed the full stimulation by 100 IU/L bTSH in cultured cells, while completely competing with bTSH binding to the TSH-R.

To explain this phenomenon, we considered 2 possibilities. Because the polyclonal autoantibodies in the serum samples of GD patients are assumed to consist of different kinds of mAbs, the first possibility is that the combination

of S-mAb and B-mAb may be able to reproduce the situation both of negative TSAb and TSBAb. This is because 2 different S-mAbs, M22 and K1-18, cloned from distinct patients, had similar stimulatory properties (see Fig. 3A), and because K1-18 S-mAb and K1-70 B-mAb have been cloned from a single patient. Accordingly, we mixed S-mAb and B-mAb in various proportions to reproduce the activities to serum samples S (see Fig. 4). However, the increase of B-mAb in amount to reduce the TSAb of S-mAb resulted in the increase the TSBAb without reaching the TBII values (150-200 IU/L) of serum samples S. The second possibility is that there may be a kind of N-mAb (virtual N-mAb) that binds to the TSH-binding site of TSH-R and exhibits very weak activities of both TSAb and TSBAb. For example, the fact that a large amount of human chorionic gonadotropin can induce gestational transient hyperthyroidism suggests that some kind of autoantibodies may weakly stimulate the TSH-R. From the point of view of TSH-R, the fact that some constitutively activating TSH-R mutations found in some toxic thyroid adenomas weakly produce cAMP in the absence of TSH indicates that the incomplete conformational change of the receptor by some kind of autoantibodies can weakly produce cAMP. In this case, it is necessary that virtual N-mAbs occupy a certain proportion of TSH-Rs, leaving some place for 100 mU/L bTSH. However, it is theoretically impossible to tolerate that 20 IU/L TBII equivalent of B-mAb can almost completely prevent 100 mU/L bTSH-binding while 200 IU/L TBII equivalent of virtual N-mAb freely allows 100 mU/L

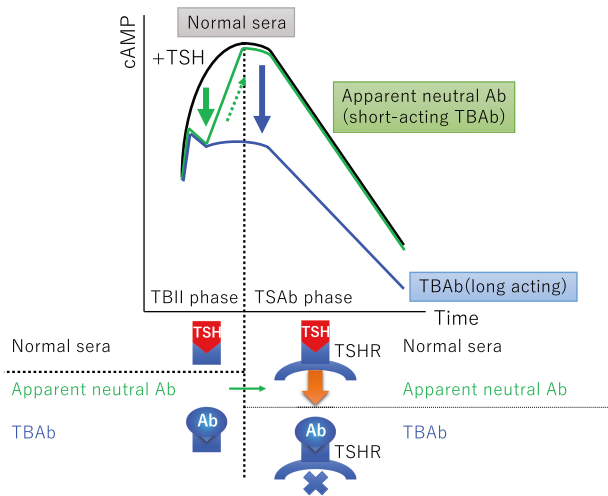


Figure 9. Schematic presentation of 3',5'-cyclic adenosine 5'-monophosphate (cAMP) induction in the cells by thyrotropin (TSH) and its repression by thyroid-blocking antibody (TBAb) or apparently neutral Ab (aN-Ab). Initially, the rise of cAMP by TSH diminished in the presence of TBAb or aN-Ab. In the case of TBAb such as serum sample B, the increase of cAMP by TSH was diminished the entire time and reached the basal level at the end of the assay. In contrast, in the case of aN-Ab such as serum sample S, cAMP rapidly increased after that and caught up with the level of normal serum samples (with TSH). Alternatively, compared with the normal serum sample, the TSH-induced cAMP increase is blocked for 1 hour (solid arrow in the TBII phase) by aN-Ab but the suppressive activity disappears after that (dotted arrow). In contrast, TBAb suppresses the cAMP induced by TSH the entire time (arrow in the TBAb phase). Accordingly, the initial delay in the rise of cAMP by aN-Ab suggests temporary binding to the thyrotropin receptor (TSH-R), which corresponds to the positive TSH-binding inhibitory immunoglobulin (TBII). However, aN-Ab may dissociate TSH-R afterward and TSH can increase cAMP; in contrast to TBAb, which continues to occupy TSH-R.

bTSH to bind. Therefore, it is also difficult to explain the phenomenon in serum samples S even using a virtual N-mAb. In fact, although some weak mAbs created in mice showed weak stimulatory activities to the TSH-R, the TBII activities were also weak [24]. The TBII activities of human chorionic gonadotropin were weak, as well [3].

From the aforementioned results, we had to change our view point. That is, serum samples S may inhibit the bTSH-binding to the TSH-R *in vitro* but do not inhibit the bTSH-induced activity in cells. In other words, a chronological difference between TBII assay *in vitro* and bioassay *in vivo* may cause the discrepancy. To clarify this question, we conducted a real-time analysis of cAMP activities in the presence of TSH and found interesting differences among TRAbs. The serum samples S inhibited the cAMP production induced by bTSH (see Fig. 6) or by rhTSH (see Fig. 7) for 1 or 2 hours after the reaction with rhTSH-R. After that, cAMP activities in serum samples S caught up to the level of the control. In contrast, potent blocking serum samples B [3], or serum samples No. 1 in Fig. 1B inhibited TSH-stimulated cAMP activities compared with normal serum samples and serum samples S the entire time from 10 minutes after the reaction (see Fig. 7). Similarly, serum samples K, or serum samples No. 5 in Fig. 1B and 1D, which exerted both TSAb and TSBAb activities, stimulated cAMP activities even in the absence of TSH but inhibited TSH-stimulated cAMP activities compared with normal serum samples (see Fig. 6D). In terms of serum samples S, the initial delays in the elevation of cAMP suggest binding to the TSH-R, which corresponds to the positive TBII, and the

following catching up with the control suggests the dissociation from the TSH-R, which corresponds to negative TSBAb activity (Fig. 9). Alternatively, serum samples S only transiently inhibit TSH binding to the TSH-R. In contrast, TBAb constantly suppresses the TSH-induced cAMP. Interestingly, a phenomenon similar to the delay in the initial elevation of TSH-induced cAMP in serum samples S compared to normal serum samples (see Fig. 6D) is observed between serum samples K and serum samples No. 5 in the absence of TSH (also in the presence of TSH). This suggests that serum samples K may contain “serum samples S-like components” more than serum samples No. 5. Alternatively, there may exist a short-acting type and long-acting type in TBAb, and it may be that serum samples S consist of solely short-acting TBAb but serum samples K consist of both types of TBAb in addition to TSAb. In contrast, serum samples No. 5 may contain long-acting TBAb and TSAb but not short-acting TBAbs.

There are some limitations in this study. Because serum samples S are polyclonal, it may be necessary to isolate mAbs from the patient blood and to characterize more precisely their properties, including the binding site in the TSH-R.

In conclusion, we present here the TBII with neutral bioactivities on the TSH-R found in the patient with autoimmune thyroid disease, which strongly inhibit TSH-binding to the TSH-R but exerts neither TSAb nor TSBAb activity. Neither the mixture of S-mAb and B-mAb nor a virtual N-mAb, which theoretically exhibits both weak TSAb and weak TSBAb, can explain this phenomenon. Instead, the discrepancy may result from the chronological difference in TSH-R binding between patient TRAbs and TSH *in vivo*. A variety of TRAbs, not only stimulating and blocking but also interfering with TSH-R binding for only a short time, may exist in the serum samples of GD patients.

Future Directions and Recommendations

TRAbs in the serum samples of Graves hyperthyroidism are polyclonal and may contain apparent neutral Abs (or short-acting TBAb) in addition to TSAb and TBAb (long acting). Therefore, the measurement of TSAb is useful to assess the remission of the patient after treatment with antithyroid drugs [25]. For a complete understanding of TRAb, the isolation of apparent N-mAb is necessary.

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Disclosures

The authors have nothing to disclose.

Data Availability

All data generated or analyzed during this study are included in this published article or in the data repositories listed in “References.”

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