Original Article

Incidence and Specificities of Labeled Thyrotropic Hormone (TSH) Binding Immunoglobulins (LTB-Igs) in Patients with Graves' Disease and Other Thyroid Disorders

Takashi AKAMIZU, Toru MORI*, Hitoshi ISHII, Toshihiko YOKOTA, Hirotoshi NAKAMURA and Hiroo IMURA

Using [125I]bTSH, labeled TSH binding (LTB) in sera from 203 patients with various thyroid disorders was studied. Four of them with known potent anti-TSH antibody showed extremely high LTB, as has been reported previously. Excluding these 4 sera, the mean ± s.d. of serum LTB from 199 patients was calculated to be 8.6 ± 2.1%. LTB exceeding 10.7% (mean + 1 s.d.) was observed in 16 sera; these were taken as increased. LTB measured by polyethyleneglycol (PEG) precipitation correlated significantly with the serum IgG concentration; however, sera with increased LTB had high values irrespective of the serum IgG concentration. Specificities of increased LTB in 13 sera were further analyzed by means of binding to Protein A-sepharose and displacement studies using bTSH and nonradioactively iodinated bTSH. A significant correlation was observed between the LTB obtained by PEG and those by Protein A-sephrose. BTSH specificity was confirmed in 5 of the 13 sera; 7 of the remaining 8 sera showed displacement only by iodinated bTSH. None of the control Graves' sera showed any significant displacement. Comparisons of the results of measurement of LTB by Protein A-sephrose and those by the displacement studies disclosed that most of displaced sera had increased LTB to the IgG fraction. Disease distributions of 203 overall cases and 20 increased LTB cases revealed that apparently higher incidence (22.9%) of increased LTB in untreated Graves' patients than the others, though some increased LTB cases were also observed in patients with inactive Graves' or other thyroid disorders. In conclusion, increased LTB was observed in sera from approximately 10% of the patients with various thyroid disorders; most of them were found to be specific to either bTSH or iodinated bTSH. A frequent association of increased LTB with active Graves' disease suggests a significance of these antibody like products to the disease process.

Key Words: Anti-TSH antibody, [125I]TSH binding, Graves' disease, TSH receptor antibody, antibody to iodinated TSH

We have previously reported the existence of abnormal TSH binding immunoglobulin, anti-TSH autoantibody, in 2 patients with active Graves' disease[1]. These cases were recognized from unusually high PEG precipitable [125I]bTSH in the thyrotropin binding inhibitor immunoglobulin (TBII) assay. Less potent anti-TSH antibody may be produced more commonly in Graves' patients. Under experimental conditions, Biro[2, 3] and Beall and Kruger[4] have already reported the existence of TSH binding globulins extensively in sera from patients with Graves' disease and

From The Second Division, Department of Internal Medicine, Faculty of Medicine Kyoto University, Kyoto.
*The Second Division, Department of Internal Medicine, and Division of Diagnosis and Therapy, Department of Molecular Medicine, Faculty of Medicine Kyoto University, Kyoto.
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Reprint request to: Takashi Akamizu, MD, The Second Division, Department of Internal Medicine, Kyoto University Faculty of Medicine, 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606, Japan.

also from normal subjects. These considerations led us to study labeled TSH binding (LTB) of sera from patients with Graves' disease, using the conventional PEG precipitation method.

This paper describes the incidence and specificities of increased LTB.

MATERIALS AND METHODS

Clinical materials

LTB was measured in sera from 203 patients with various thyroid disorders, 92% of which had Graves' disease (Table 1).

[125I] bTSH

Purified bovine TSH was kindly gifted from Dr. J. G. Pierce (UCLA, USA) and was labeled by the lactoperoxidase method[5]. The specific activity was 150 μCi/μg.

BTSH for displacement study and for cold iodination

BTSH was purchased from Sigma, USA, and a 50 mU/ml solution was used for the displacement study. Further, 1 mg of this bTSH was iodinated with nonradioactive NaI under a molar to molar base by the chloramine T method[6]. The labeling efficiency was monitored by a tracer dose of Na[125I] and was calculated as 70%. A solution of 100 mU/ml iodinated TSH was also used for the displacement study.

Procedures of LTB determination

One hundred μl aliquots of test serum were incubated with [125I]bTSH for 1 hr at 37°C, and then overnight at 4°C.

In the polyethyleneglycol (PEG) precipitation, PEG with NaCl was added to the incubate to make the final concentration of 15 w/v% and 0.5M, respectively. After mixing and centrifuging, the pellet was counted by an autogramma counter.

In the protein A-sepharose binding, 20 mg of protein A-sepharose (Pharmacia, Sweden) suspension was added to the incubate, as described above. After another 1 hr incubation at 37°C under constant but gentle mixing, 2 ml of Tris-HCl with 1% BSA, pH 7.4, was poured. The supernatant was aspirated after 5 min of still-

Table 1. Disease distribution of 203 cases overall and 20 cases with increased LTB, and summary of displacement studies.

Diseases	No. of cases tested	No. of cases with increased LTB	Results of displacement study[a] by bTSH	by iodinated bTSH	Total
Active Graves'					
Untreated	35	8* (22.9%)	4	3	7
Relapsed	12	0 (0.0%)	0	0	0
Treated	61	5+ (8.2%)	3	1	4
Subtotal	108	13 (12.0%)	7	4	11
Inactive Graves'					
Treated	61	5° (8.2%)	1	3	4
In remission or euthyroid Graves'	18	0 (0.0%)	0	0	0
Subtotal	79	5 (6.3%)	1	3	4
Hypothyroidism due to blocking type TBII	1	0 (0.0%)	0	0	0
Hashimoto's thyroiditis	11	1 (9.1%)	1	0	1
Others	4	1# (25.0%)	0	0	0
Total	203	20 (9.9%)	9	7	16

*: Seven of 8 sera were subjected to the displacement study.
+: Four of 5 sera were subjected to the displacement study.
°: One of them was not displaced by either bTSH or iodinated bTSH.
#: A patient with simple goiter, who was not subjected to the displacement study.
a: show the number of patients significantly displaced by bTSH and/or iodinated bTSH.

stand. This washing procedure was repeated 5 times and the final sediment was counted.

LTB was expressed by net CPM or was calculated by the following formula:

$$\text{LTB}(\%) = \frac{\text{bound } [135\text{I}]\text{bTSH}}{\text{total } [125\text{I}]\text{bTSH}} \times 100$$

Displacement study by bTSH or iodinated bTSH

One hundred μl of bTSH (50 mU/ml) or iodinated bTSH (100 mU/ml) was added to the serum incubate and bound [125I]bTSH was separated by PEG as described above. The bound percent was compared with that obtained by the addition of 100 μl Tris-HCl BSA instead.

Measurement of serum IgG concentrastion

The serum IgG was measured by using the Nor-Partigen R-IgG kit (Hoechst, West Germany).

Statistical analysis

For statistical analysis, the Student's t test and chi-square test were used, and p values smaller than 0.05 were taken as significant.

RESULTS

Relation of serum [125I]bTSH binding with IgG concentrations and selection of increased LTB sera

Fig. 1 shows the relation between LTB by PEG precipitation and serum IgG concentration. A significant correlation (N=203, r=0.738, $p < 0.001$) was observed. However, there were some sera with high LTB irrespective of the IgG concentration.

Through 203 determinations, LTB averaged 9.4 ± 8.1 (s.d.)%. In this series there were 4 sera with known potent anti-TSH antibody[1]. When these 4 sera were excluded, LTB of 199 sera averaged 8.6 ± 2.1%. Sixteen sera with LTB of 10.7% or higher, that is, exceeding mean + 1 s.d., were selected as increased.

Results of Protein A binding study

As has been shown in Fig. 1, PEG precipitation did not directly indicate the specific binding of a serum component to [125I]bTSH. To see the specific [125I]bTSH binding to the serum IgG, a Protein A-sepharose study was performed. Fig. 2 shows the relation between LTB by PEG and LTB by Protein A. A significant correlation between them was observed in 13 cases with

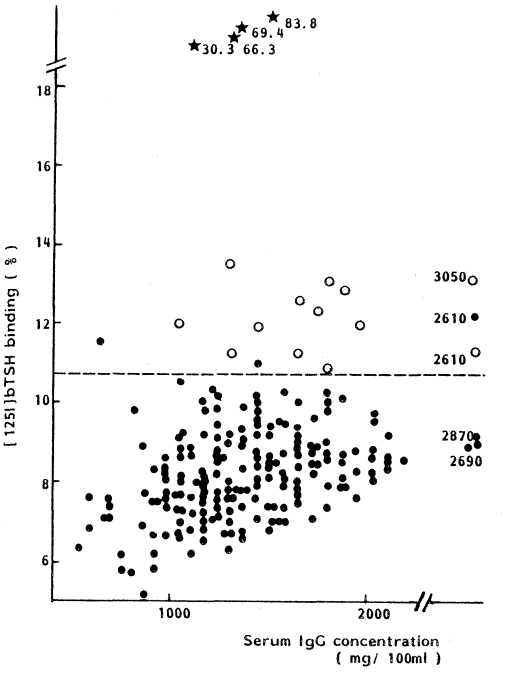


Fig. 1. Relation between serum [125I]bTSH binding (LTB) and serum IgG concentration in 203 patients with various thyroid disorders.

LTB obtained by PEG precipitation was expressed as % of total [125I]bTSH added. The dotted line (-----) indicates 10.7% binding; samples above the line were taken as increased. Those shown by stars (★) and open circles (○) were 4 cases with known anti-bTSH antibody and 13 selected cases used in the displacement study, respectively.

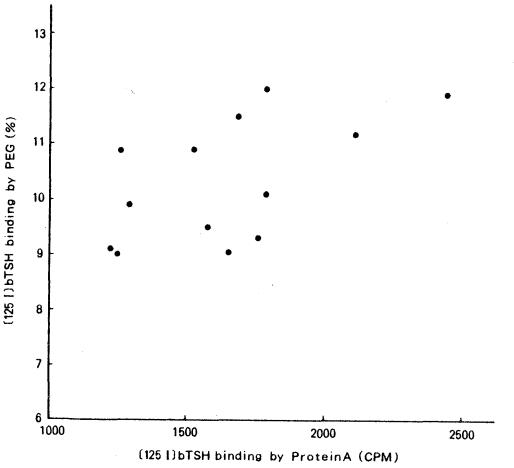


Fig. 2. Comparisons of serum LTB determined by PEG and by Protein A-sepharose. Used sera were 13 selected cases with increased LTB by PEG (Fig. 1). Bound [125I]bTSH by Protein A-sepharose was expressed by CPM.

increased LTB ($r=0.58$, $p < 0.05$).

Displacement study by bTSH and iodinated bTSH

Excluding the 4 sera with known anti-TSH antibody and 3 in insufficient amount, 13 of the 20 sera which showed increased LTB and 4 control Graves' sera with normal LTB were subjected to the displacement studies.

Fig. 3a shows the relation between bound [125I]bTSH with and without bTSH. An almost linear correlation was observed, but 5 sera were found to be shifted upwards and left which implies a displacement of bound [125I]bTSH by the addition of bTSH.

The number of sera showing specific displacement by bTSH was limited, and then another displacement study using iodinated bTSH was performed. Fig. 3b shows the relation between bound [125I]bTSH with and without iodinated bTSH. Control sera and 2 sera with increased LTB were found to be distributed close to the y=x line. However, most of the increased LTB sera were observed to be shifted upwards and to the left. The degree of displacement by iodinated bTSH appeared greater than those obtained by bTSH.

Fig. 4 shows the relation between bound [125I]bTSH by Protein A and the degree of displacement by bTSH (a) and that by iodinated bTSH (b). Five sera which were displaced by bTSH were found to have increased specific LTB by Protein A also. Four sera with increased LTB by PEG but not displaced by bTSH were found to have low LTB by Protein A. However, there were 4 sera showing increased LTB by Protein A but not displaced by bTSH. As shown in Fig. 4b, all but one of these sera which were not displaced by bTSH were found to be newly displaced by iodinated bTSH. Control sera and one of the 5 sera displaced by bTSH were not displaced by iodinated bTSH.

Table 1 shows the disease distribution of 203 cases overall, and that of 20 increased LTB cases. In the overall cases, the incidences of active Graves' disease (untreated, relapsed, or treated but still active) and inactive Graves' (treated and nontoxic, in remission, or euthyroid Graves') were 53.2 and 38.9%, respectively. On the other

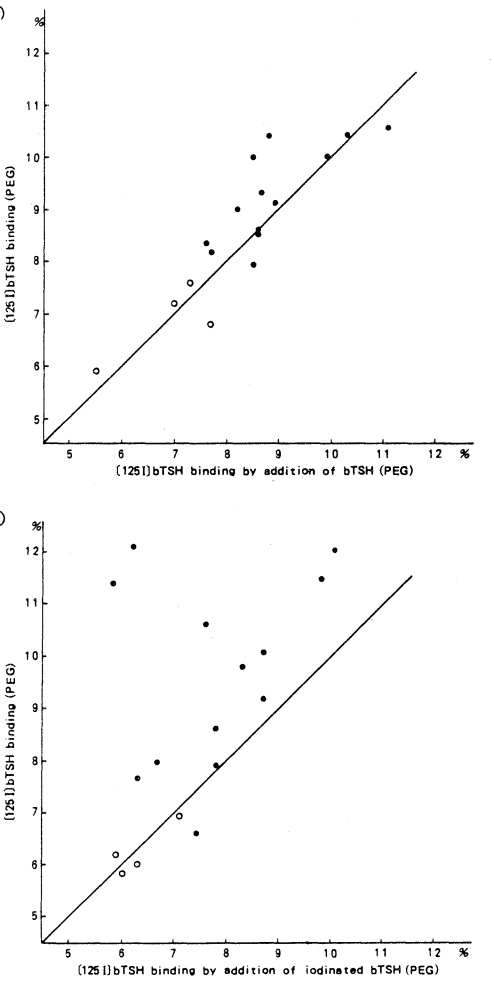
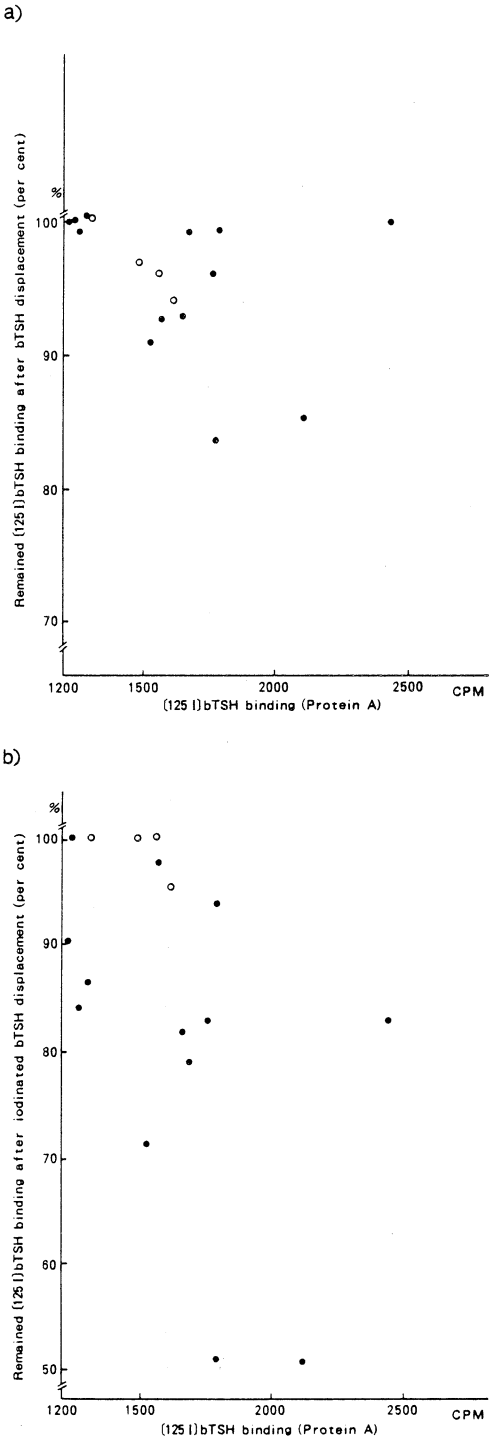


Fig. 3. Displacement study results of serum LTB of 13 selected cases by bTSH (a) or iodinated bTSH (b).
Bound [125I]bTSH % with (absissa) and without (ordinate) the addition of bTSH or iodinated bTSH were compared. Closed circles (●) show 13 sera with increased LTB, and open circles (○) show control sera. In (a), 5 samples located upwards and to the left side of the y=x line were taken as significant while in (b), 11 samples were taken as significant.

hand, in increased LTB cases these incidences were 65.0 and 25.0%, respectively, and the ratio of active vs. inactive Graves' disease was significantly higher ($p < 0.005$) than that of overall cases. Further, all but one of 17 sera with increased LTB were displaced by bTSH and/or iodinated bTSH. Higher associations of bTSH specific displacement were observed in patients



with active Graves' disease than in inactive Graves' disease. In a patient with Hashimoto's thyroiditis, LTB was displaced only by bTSH; while in a patient with inactive Graves' disease having LTB of 11.9%, neither bTSH nor iodinated bTSH showed displacement.

DISCUSSION

Increased serum LTB was observed in 20 (9.9%) of the 203 studied. There have been reports indicating the existence of serum factors which interfere with the binding of labeled hormone, including [125I]bTSH, to the specific antibody or receptor[7, 8]. Shewring et al.[9] have further reported that approximately 9% of [125I]bTSH could be precipitated by 15 w/v% of PEG and that the amount of precipitable radioactivities was related to the serum IgG concentration. We have also observed a significant correlation between LTB measured by PEG and the serum IgG concentration. Therefore, the LTB level seems not to be free from such nonspecific serum factors. Increased LTB was then selected as exceeding the mean + 1 s.d. of 199 LTB excluding 4 extremely high LTB of known anti-TSH antibody cases, and this selection was considered reasonable in regard to the IgG concentrations.

Further, these sera with increased LTB by PEG were found to be correlated significantly with LTB determined by Protein A-sepharose, which indicates that the binding is specific to the serum IgG fraction. Displacement studies further proved that most of these sera with increased

Fig. 4. Relation between Protein A determined LTB and the grade of displacement of the bound [125I]bTSH by bTSH (a) or by iodinated bTSH (b).
The abscissa shows bound [125I]bTSH determined by Protein A and the ordinate shows the retained percent of LTB after addition of bTSH (a) and iodinated bTSH (b). Remarks were the same as Fig. 3. Taking displacement of more than 5% as significant, all displaced cases were found to have high LTB. However, there were observed 4 cases showing high LTB by protein A which was not displaced by bTSH (a). In the case of iodinated bTSH (b), most of the cases showing increased LTB were displaced, and increases in number and grade of displacement were also observed.

LTB were specific to TSH. However, only 9 of 17 sera studied were displaced by 50 mU/ml of bTSH. Taking the remainder as nonspecific seemed unlikely, and a subsequent displacement study using iodinated bTSH of 100 mU/ml was performed which resulted in finding 7 additional sera with significant displacement. The grade and frequency of displacement by iodinated bTSH were much higher than those by bTSH. This may be ascribed to the difference in TSH concentration used, and those, whose LTB was displaced by both cold materials, are considered to have their antigenic site(s) apart from the iodinated portion of the TSH molecule. There might be pertinent arguments related to the TSH specificity of the sera displaced only by iodinated bTSH. However, a similar study using [125I]hTSH did not show any proportional increase in LTB except in one serum which was displaced by cold hTSH (data not shown). To our knowledge, this is the first report indicating the existence of an antibody-like serum component against iodinated TSH. Similar binding materials to the iodinated hormone in sera have been reported in cases of insulin[10] and gastrin[11]. One of the increased LTB sera was displaced only by bTSH but not by iodinated bTSH. Another serum with increased LTB by PEG did not show any significant displacement by either bTSH or iodinated bTSH, and LTB by Protein-A sepharose was not increased, either. The reasons of these 2 are not clear.

In the present study we used bTSH. One of the reasons was mentioned above, and our previous experience[1] also revealed that very potent TSH antibodies in 2 patients with Graves' disease had much higher binding affinity to bTSH than to hTSH. The reasons for favorable binding to bTSH are not clear yet, but Biro also has reported [131I]bTSH binding globulins in sera from Graves' patients and even from healthy subjects. Using [125I]hTSH, Beall and Kruger[4] failed to find any significant binding with raw Graves' IgG, and they reported significant binding only after receptor absorption of IgG. They proposed a hypothesis that anti-TSH receptor antibody should be an antiidiotype antibody against idiotypic anti-TSH antibody. They considered that under the existence of the antiidiotype antibody in excess, the idiotypic antibody could not be detected, and receptor absorption of the former induced the detectability of the latter[4]. We have also confirmed their observation, but the increased LTB after TSH receptor absorption was found to be heat labile (unpublished observation). We previously reported that the TSH receptor was greatly damaged after treatment at 56°C for 30 min[1], and there have been reports showing the existence of LATS absorbing substance in the soluble fraction of thyroid homogenates[12, 13]. Therefore observations of Beall and Kruger might be well explained by the possible contamination of the TSH receptor or its fragments into the supernatant after the absorption procedure.

Disease distribution of the increased LTB cases showed higher incidence in active Graves' patients than those in inactive Graves' patients and in other thyroid disorders. Even though there observed one each patient with Hashimoto's thyroiditis and simple goiter who had an increased LTB, the frequent association of increased LTB in active Graves', especially untreated Graves' patients appears to suggest certain role(s) of the increased LTB on the disease process of Graves' disease. Clinical significance of the increased LTB and whether this could be a real idiotypic antibody against TSH receptor antibody are of great interest. These should be elucidated further.

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