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Negative Correlation Between The Conversion of Thyrotropin Receptor-bound Blocking Type Thyrotropin Receptor Antibody to The Stimulating Type by Anti-human IgG Antibodies and The Biological Activity of Blocking Type Thyrotropin Receptor Antibody

Bo Youn Cho, M.D., Min Ho Shong, M.D.,* Jae Hoon Chung, M.D.,
Hong Kyu Lee, M.D., Chang-Soon Koh, M.D. and Hun Ki Min, M.D.

*Department of Internal Medicine, Seoul National University College of Medicine, Seoul;
Department of Internal Medicine, College of Medicine, Chungnam University*, Taejon, Korea*

It has been reported that receptor-bound blocking type TSH receptor antibody (TRAb) can be converted to the stimulating type by anti-human IgG antibodies. To evaluate the relationship between the conversion of receptor-bound blocking type TRAb to the stimulating type and the biological activity of blocking type TRAb, we compared converting activities of blocking type TRAb from 10 patients with primary nongoitrous hypothyroidism with both the doses of blocking type TRAb which show 50% inhibition of ^{125}I-bTSH binding to the TSH receptor and those which show 50% inhibition of TSH-stimulated cAMP production in cultured rat thyroid cells (FRTL-5). The additions of anti-human IgG antibody to FRTL-5 cell-bound blocking IgGs resulted in the increase in cAMP production in a dose-dependent manner and the converting activities (percent increase of cAMP production) also depended on the doses of blocking IgGs. The converting activities were significantly correlated with the doses of blocking IgGs which showed 50% inhibition of ^{125}I-bTSH binding to the TSH receptor ($r=0.71$, $p=0.011$). And these converting activities were also significantly correlated with the doses of blocking IgGs which showed 50% inhibition of TSH-stimulated cAMP increase ($r=0.81$, $p=0.002$), and were negatively correlated with thyroid stimulation blocking antibody activities ($r=-0.58$, $p=0.02$). We have demonstrated that all cell-bound blocking type TRAb were converted to the stimulating type by anti-human IgG antibody and the degree of conversion was negatively correlated with the biological activity of blocking type TRAb. These findings support the possibility that binding sites for the blocking and stimulating antibodies are closely associated.

Key Words: Thyrotropin receptor antibody, blocking type thyrotropin receptor antibody, thyroid stimulating antibody

Address for correspondence: *Bo Youn Cho, Department of Internal Medicine, Seoul National University Hospital, Yongon-dong, Chongno-gu, Seoul 110-744, Korea*

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INTRODUCTION

Thyrotropin receptor antibody (TRAb) found in Graves' disease stimulates the thyroid gland and is widely thought to be responsible for hyperthyroidism (Burman & Baker, 1985; Rees Smith et al., 1988). On the other hand, TRAb found in patients

with primary nongoitrous hypothyroidism instead inhibits TSH-stimulated cAMP production (Konishi et al., 1983; Steel et al., 1984) and/or ^{3}H-thymidine incorporation (Iida et al., 1987; Cho et al., 1989) and is considered to play a pathogenic role in the development of hypothyroidism and thyroid atrophy in some patients with primary nongoitrous hypothyroidism (Cho et al., 1989). Both stimulating and blocking antibodies inhibit the binding of TSH to its receptor. However, it is still unclear whether these two types of TRAb bind to the same epitopes of TSH receptor. Recently, it has been reported that receptor-bound blocking type TRAb can be converted to the stimulating type by anti-human IgG antibodies and suggested that these two antibodies bind to the same epitope(s) of TSH receptor related antigens (Amino et al., 1987; Shong et al., 1991). Moreover, Taniguchi et al. (1990) reported that the addition of anti-human IgG antibody not only converts blocking type TRAb to the stimulating type but also recovers TSH activity via a postreceptor step. If the binding sites of these antibodies are the same, the conversion of blocking type TRAb to the stimulating type would be dependent on the binding affinity of the blocking type TRAb. To examine this assumption, we studied the conversion of blocking type TRAb to stimulating type by anti-human IgG antibodies in ten patients with primary nongoitrous hypothyroidism and compared it with both the doses of blocking type TRAb which show 50% inhibition of ^{125}I-bTSH binding to the TSH receptor and those which show 50% inhibition of TSH-stimulated cAMP production. In the present study, we observed that all blocking type TRAb were converted to the stimulating type by anti-human IgG antibodies and the degree of conversion was negatively correlated with the biological activity of blocking type TRAb.

MATERIALS AND METHODS

Test samples

The IgG fractions were isolated from serum samples, by means of affinity chromatography on columns of protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden), from 10 patients with primary nongoitrous hypothyroidism (all women, age range 25-68 yr). All had nonpalpable thyroid glands, low serum T3 and T4 levels, markedly decreased ^{131}I thyroid uptake (24 h) and elevated serum TSH levels at the time of diagnosis. All patients were euthyroid due to thyroxine replacement therapy at the time of experiment. Primary hypothyroidism due to ectopic goiter was ruled out by thyroid scintigraphy. All ten patients had potent thyrotropin binding inhibitor immunoglobulin (TBII) activities (more than 70% inhibition of ^{125}I-bTSH binding) and four of them (patients 1 to 4 in Table 1.) had been reported in 1989 (Cho et al., 1989). In addition, IgG was prepared from a pool of serum samples from 10 normal subjects. The protein concentration of the IgG fraction was determined according to the method of Lowry et al. (1951).

Table 1. Summary of clinical data on ten patients with primary nongoitrous hypothyroidism with blocking type TSH receptor antibody

Case No.	Age (yr)	Sex	MCHA	TGHA	TBII* (%)	TSBAb** (%)
1	46	F	160^{2}	10^{2}	100	100
2	25	F	160^{2}	10^{2}	95.2	99.8
3	30	F	320^{2}	80^{2}	80.2	75.1
4	68	F	40^{2}	(-)	78.0	96.2
5	39	F	(-)	(-)	80.0	70.1
6	40	F	80^{2}	10^{2}	82.0	82.0
7	32	F	(-)	(-)	73.8	96.2
8	34	F	(-)	40^{2}	96.6	96.2
9	28	F	40^{2}	10^{2}	71.4	88.7
10	27	F	320^{2}	160^{2}	99.1	96.2
Normal range			$<10^{2}$	$<10^{2}$	<15.0	<37.0

MCHA: Antimicrosomal hemagglutination antibody, values are reciprocals of titers. TGHA: Antithyroglobulin hemagglutination antibody, values are reciprocals of titers.
* Measured with 50 ul of 10 g/l IgG. ** Measured with 300 ul of 2 g/l IgG

Measurement of thyrotropin binding inhibitor immunoglobulin(TBII)

TBII activity was measured using a TSH receptor antibody kit prepared by R. S. R. Ltd. (Cardiff, Wales, UK) as previously described (Southgate et al., 1984). TBII was expressed as percent inhibition of ^{125}I-bTSH binding to the TSH receptor. All samples were run in duplicate. The intra-assay variance was 1.7 - 24.5% and the inter-assay variance 3.7 - 10.5%. The normal range of TBII in our laboratory was from -10 to 14.9%, and a TBII value exceeding 15% was considered abnormal or positive. To calculate the doses of IgGs which show 50% inhibition of ^{125}I-bTSH binding to the TSH receptor, all IgGs were serially diluted with normal

pooled IgG and TBII activities were measured in each dilution (50 ul).

Measurement of thyroid stimulation blocking antibody (TSBAb) activities

For the assay of TSBAb activity, FRTL-5 cells, kindly supplied by Dr. L. D. Kohn (NIH, Bethesda, MD, USA) were maintained as previously described (Ambesi-Impiombato et al, 1980) and maintained for 7 days in medium without TSH before assay. The medium was exchanged with 300 ul of test IgG (2 g/l) with or without bTSH (0.1 U/l). IgGs were dissolved in Hank's balanced salt solution (HBSS) without NaCl containing 0.5 mmol/l 3-isobutyl-l-methyl-xanthine (IBMX), 20 mmol/l HEPES, and 1% BSA, pH 7.4. After 2 h incubation at 37°C, cAMP released into the medium was measured by RIA (Immunonuclear, Stillwater, MN, USA). The assay system was sensitive to 5 mU/l bTSH with a response of 1.71±0.7 times the basal cAMP level. All samples were run in triplicate. The intra-assay variance was 8.2 - 12.1% and the inter-assay variance 17.1 - 30.5%. TSBAb activity was expressed as percent inhibition of TSH-stimulated cAMP increase, and was calculated as follows:

$$\text{TSBAb (\%)} = 100 \times \left(1 - \frac{\text{cAMP(TSH + test IgG) - cAMP(normal IgG)}}{\text{cAMP(TSH + normal IgG) - cAMP(normal IgG)}}\right)$$

TSBAb was defined as positive when the value was greater than 2 SD above the mean value produced by the IgG fraction from 24 normal subjects (>37%). To calculate the doses of IgGs which showed 50% inhibition of TSH-stimulated cAMP increase, all IgGs were serially diluted with normal pooled IgG and TSBAb activities were measured in each dilution.

Conversion Experiment

For study of the conversion of blocking type TRAb to the stimulating type, FRTL-5 cells grown in 24 well plates (Corning, Medfield, MA, USA) for 5-7 days were switched to 5H medium (without TSH), and after 7 days the experiments were performed. FRTL-5 cells were first incubated with 300 ul of IgG solution dissolved in hypotonic HBSS containing 0.5 mmol/l IBMX, 20 mmol/l HEPES, and 1% BSA for 30 min at 37°C. Then, the supernatant was aspirated, and the cells were washed twice with Ca^{++}, Mg^{++} free HBSS. Next, the cells were incubated with 300 ul of various types of goat anti-human IgG antibodies or normal goat serum first for 1.5 h at 4°C (second incubation) and then for 3 h at 37°C. Finally cAMP in the supernatant was measured in the same way as in the TSBAb assay. All samples were run in triplicate. The converting activity was expressed as percent increase of cAMP production by the addition of anti-human IgG antibodies and was calculated as follows : converting activity (%) =

$$100 \times \left(\frac{\text{cAMP (anti-human IgG antibodies)}}{\text{cAMP (normal goat serum)}}\right)$$

Statistical analysis

All data are presented as the mean ± SD. Statistical analysis was done with Student's t test and correlation coefficient was calculated by linear re-

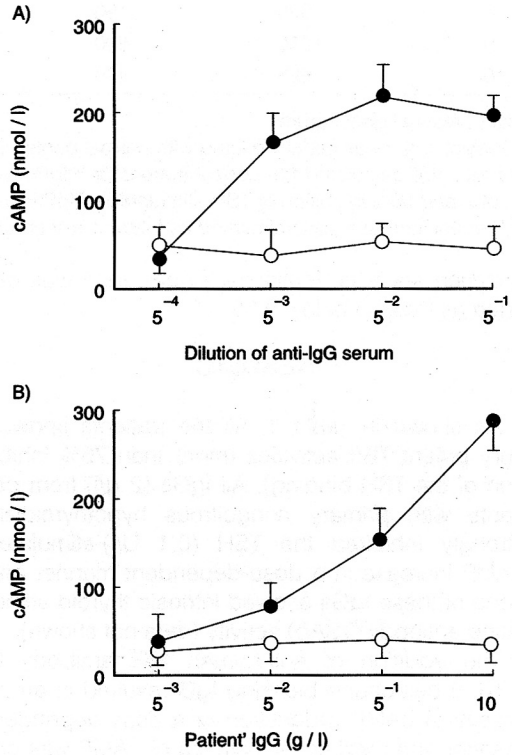

Fig. 1. A) cAMP production by subsequent addition of goat anti-human IgG antibody (whole molecule). FRTL-5 cells were incubated with patient's (patient 8 in Table 1) IgG (2 g/l) for 30 min at 37°C. After washing the cells with HBSS, the cells were incubated with various concentrations of anti-human IgG antibody for 4.5 h. The cAMP concentrations in the supernatant were then measured. B) Dose-dependent effect of blocking type TRAb on subsequent cAMP production by anti-human IgG antibody (whole molecule, 1:25 dilution). Each result represents the mean SD of triplicates in one of three experiments which yielded closely comparable results. (●)blocking type IgG; (○)normal pooled IgG.

Table 2. Doses of blocking IgGs which show 50% TBII and TSBAb activities and conversion of blocking type TRAb to the stimulating type by anti-human igG antibody in 10 patients with primary nongoitrous hypothyroidism

No.	50% inhibition doses of blocking type IgG (ug/ml)*		cAMPproduction (nmol/l)		% Increase#
	TBII	TSBAb	NGS	Anti-hIgG	
1	235	62	13 ± 2	60 ± 5	460
2	410	170	13 ± 1	128 ± 8	981
3	830	340	23 ± 1	725 ± 39	3166
4	820	320	12 ± 1	293 ± 29	2306
5	570	410	13 ± 0	701 ± 16	5434
6	350	76	13 ± 1	328 ± 33	2600
7	530	110	22 ± 4	297 ± 5	1359
8	635	190	17 ± 3	217 ± 19	1299
9	1250	520	14 ± 1	950 ± 95	6834
10	625	470	14 ± 1	1598 ± 53	4184

NGS : Normal goat serum.
* Patient's IgG was serially diluted with normal pooled IgG and TBII and TSBAb activities were measured in each diluted IgG. We calculated the concentrations of ^{125}IgG which showed 50% inhibition of ^{125}I-bTSH binding to the TSH receptor and 50% inhibition of TSH-stimulated cAMP increase from each dilution curve.
# Percent increase was calculated as follows: % increase = 100 x cAMP (anti-human IgG)/cAMP (normal goat serum).

gression analysis. Statistical significance was defined as P-value below 0.05.

RESULTS

As shown in Table 1, all the patients showed very potent TBII activities (more than 70% inhibition of the TSH binding). All IgGs (2 g/l) from patients with primary nongoitrous hypothyroidism strongly inhibited the TSH (0.1 U/l)-stimulated cAMP increase in a dose-dependent manner and none of these IgGs showed intrinsic thyroid stimulating antibody (TSAb) activity (data not shown).

The addition of anti-human IgG antibody to FRTL-5 cell-bound blocking IgG resulted in an increase in cAMP production in a dose-dependent manner and maximal stimulation of cAMP was observed with a goat anti-human IgG dilution of 1:25 (Fig. 1A). The converting activity (percent increase of cAMP production) also depended on the doses of blocking IgG (Fig. 1B). When normal IgG was preincubated with FRTL-5 cells, no cAMP increase was observed. When the IgGs with high antimicrosomal antibody and/or antithyroglobulin antibody titer, which had neither TBII nor TSAb activity, was added at preincubation, no significant cAMP production was observed after anti-human IgG antibody treatment(data not shown).

The IgGs obtained from the ten patients all stimulated the thyroid cells after addition of the anti-human IgG antibody. The converting activity of individual IgG was variable in the range of 460% to 6834% (Table 2). To determine whether these converting activities are correlated with the biological activity of blocking IgG, we measured TBII and TSBAb activities in serially diluted IgG from each patient and calculated the doses of blocking IgG which showed 50% inhibition of the ^{125}I-bTSH binding to the TSH receptor and 50% inhibition of TSH-stimulated cAMP increase from the dilution curves. The converting activities were significantly correlated with the doses of blocking IgGs which showed 50% inhibition of ^{125}I-bTSH binding to the TSH receptor ($r=0.71$, $p=0.01$, Fig. 2). And these converting activities were also significantly correlated with the doses of blocking IgGs which showed 50% inhibition of TSH-stimulated cAMP increase ($r=0.81$, $p=0.002$, Fig. 3), and were negatively correlated with TSBAb activities ($r=-0.58$, $p=0.02$, Table 2).

DISCUSSION

In this study, we also confirmed that cell-bound blocking type TRAb can be converted to the stimulating type by anti-human IgG antibody in a dose-dependent manner. Although the converting activities were varied in individual IgGs, all blocking type IgGs from 10 patients with primary nongoitrous hypothyroidism showed the converting

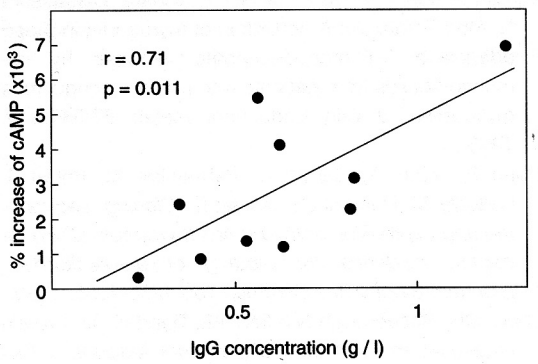

Fig. 2. Correlation between the percent increase of cAM prod uction by subsequent addition of goat anti-human IgG antibody and the doses of blocking type IgGs which showed 50% TBII activities. FRTL-5 cells were first incubated with patient's IgG (2 g/l) for 30 min. Then, the cells were washed and incubated with anti-human IgG antibody (whole molecule, 1:25 dilution) for 4.5 h. Patients' IgGs were serially diluted with normal pooled IgG and TBII activities were measured in each dilution(50 ul). And then doses of IgGs which showed 50% inhibition of ^{125}I-bTSH binding to the TSH receptor were determined from dilution curves.

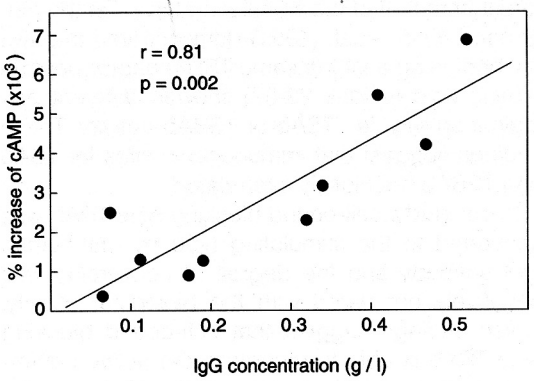

Fig. 3. Correlation between the percent increase of cAMP production by subsequent addition of goat anti-human IgG antibody and the doses of blocking type IgGs which showed 50% TSBAb activities. FRTL-5 cells were first incubated with patient's IgG (2 g/l) for 30 min. Then, the cells were washed and incubated with anti-human IgG antibody (whole molecule, 1:25 dilution) for 4.5 h. Patients' IgGs were serially diluted with normal pooled IgG and TSBAb activities were measured in each dilution. And then doses of IgGs which showed 50% inhibition of TSH-stimulated cAMP increase were determined from dilution curves.

activities. Since most IgGs contained antimicrosomal and/or antithyroglobulin antibody and antimicrosomal antibody may attach to cell surface at preincubation, we should consider the effect of antimicrosomal antibody on the conversion phenomenon. However, after preincubation with IgGs containing a high titer of antimicrosomal and/or antithyroglobulin antibody, no significant cAMP increase was observed by subsequent anti-human IgG antibody treatment. Therefore, it is less likely that the antimicrosomal antibody in the serum of patients affected the cAMP production.

Interestingly, in our study, the degree of conversion of blocking type TRAb to the stimulating type was significantly correlated with both the doses of blocking IgGs which showed 50% inhibition of the ^{125}I-bTSH binding to the TSH receptor and those which showed 50% inhibition of TSH-stimulated cAMP increase. And it was negatively correlated with TSBAb activities. These results suggest that the converting activity of blocking IgG is negatively correlated with the biological activity of blocking type IgG. In this study, we did not measure directly the binding affinity of individual blocking type IgG. However, if we assume that the biological activity of blocking IgG depends on the binding affinity of antibody, we can speculate that the conversion of blocking type TRab to the stimulating type may be dependent on the binding affinity of blocking antibody. This new finding further supports the possibility that the blocking and stimulating antibodies bind to the same or closely related regions of TSH receptor and the very small differences in binding energy or ionic charge influence the biologic response (Amino et al., 1987).

There is still controversy whether the binding site of the blocking type TRab is the same as that of the stimulating type. Since human TSH receptor-encoding cDNA was cloned and its deduced amino acid sequence has been reported (Libert et al., 1989; Nagayama et al., 1989), several investigators have tried to determine the epitope(s) for TRAb. Murakami & Mori (1990) recently reported that there are at least two divergent immunogenic regions in the extracellular domains of human TSH receptor as important features of antibody-binding epitopes. These two synthetic peptides were significantly precipitated by sera of patients with Graves' disease. Although they did not evaluate whether antibodies raised against each peptide are functionally different, it is suggested that the epitope of the blocking type TRAb is dif-

ferent from that of the stimulating type. On the other hand, Endo et al. (1991) demonstrated that two antibodies against the same TSH receptor peptide (amino acid residue 29-57) showed different biological activity, ie., TSAb or TSBAb activity. These findings suggest that immunogenic sites for TSAb and TSBAb are closely interrelated.

In our study, cell-bound blocking type TRAb was converted to the stimulating type by anti-human IgG antibody and the degree of conversion was negatively correlated with the biological activity. These findings suggest that cell-bound blocking type TRAb is able to recognize the active conformation of a critical domain in the TSH receptor molecule by the addition of anti-human IgG antibody (Rebios & Fishman, 1984; Taniguchi et al., 1990). Since major epitopes on TSH receptor in vivo are conformational rather than sequential (Ludgate & Vassart, 1990), it seems likely that binding sites for the blocking and stimulating antibodies are closely associated, even though there may be several epitopes on the TSH receptor and epitope(s) for the blocking type TRab may be different from that for the stimulating type.

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