

Studies on Autoimmune Mechanisms of Thyroglobulin Autoantibody in Autoimmune Thyroid Disease

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ABSTRACT

In order to clarify the autoimmune mechanisms of anti-Tg antibody (anti-TgAb) in autoimmune thyroid disease (AITD), a series of examinations were conducted in patients with Graves' disease (n=59), Hashimoto's thyroiditis (n=63) and healthy controls (n=38). Our findings can be summarized as follows: 1) The distribution of anti-TgAb was measured by IgG subclass ELISA. IgG1 and/or IgG4 antibodies formed the major anti-Tg response, but in some patients, IgG2 anti-Tg tend to be the predominant response. We used ELISA to determine an IgG1 anti-Tg bound to Tg that had already interacted with IgG2 Tg-antibody (and vice versa). The results substantiated the view that TgAb of a different IgG subclass interacts with different epitopes on Tg. 2) Human antiidiotypic (anti-Id) antibodies to anti-Tg antibody occur spontaneously in the AITD. These anti-Id antibodies can be divided into two categories based on properties of their binding sites. One type acts like a 'internal image' of Tg antigen which shown Ab₂β activity. Another type has Ab₂α activity that recognizes Id determinants in the framework region common among anti-Tg antibody. 3) We also examined the competitive binding assay between TgAb and TgAb F(ab')₂ fragments, and demonstrated differences in the TgAb repertoires between patients. 4) Soluble IL-2 receptor (IL-2R) of patients significantly increased as compared with normal subjects and IL-2R values in GD were higher than those in HT (p<0.001). Therefore, IL-2R is regarded as a useful marker for disease activity.

Key words: *Autoimmune thyroid disease, Anti-thyroglobulin autoantibody, Anti-idiotypic antibody, IgG-subclass, Soluble IL-2 receptor*

Thyroglobulin (Tg), thyroid peroxidase (TPO) and thyrotrophin receptor (TSH-R) are the three main autoantigens in patients with autoimmune thyroid disease (AITD)⁴². Tg, a major glycoprotein constituent of the thyroid gland, is commonly involved in spontaneous and experimental thyroid diseases. Autoantibodies to Tg and TPO, predominantly of class IgG, not only are characteristic of patients with AITD^{29,35}, but occasionally also in low titers in thyroid carcinoma³⁴, subacute thyroiditis, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and in some normal individuals^{14,22}. Moreover, anti-TgAb only recognize a very limited number of epitopes, probably between four and six^{4,23,31} on the large Tg molecule (660,000 MW).

In the present study, we examined on the incidence of anti-Id antibodies in AITD, and on correlation between the levels of detected anti-Id antibodies and titers of anti-TgAb. Moreover, we wanted to establish if the distribution of Tg antibodies between the IgG subclasses tends to be restricted and to investigate the possibility that different IgG subclass interact with different epi-

topes on Tg. In addition, we studied whether the anti-TgAb repertoires of patients may differ also. This information of anti-TgAb production in AITD would increase our understanding of pathogenic mechanism of autoimmune thyroid disorders.

SUBJECTS

The studies concerned 63 patients with Hashimoto's thyroiditis (HT), 59 with Graves' disease (GD), and 38 normal subjects. GD (48 females, 11 males, mean ages 45±12 years, range 28~69 years) is diagnosed on the basis of clinical and laboratory data as well as signs of thyrotoxicosis with diffuse goiter, elevated serum thyroid hormone concentrations and the presence of microsome antibodies (MicAb) titres > 1:400. Sera were obtained from the patients before and after undergoing therapy with antithyroid drugs or iodine or thyroidectomy. The clinical diagnosis of HT (58 females, 5 males, mean ages 53±12 years, range 28~69 years) is usually based on clinical history and evidence of sera antibodies to microsome antigen and/or Tg, and all had MicAb

titers > 1:6400. Some patients were euthyroid on thyroxine.

Serum samples were obtained from 38 healthy persons who had undergone a physical examination: 25 females and 13 males, aged 28~69 years (mean \pm SD = 50 \pm 12). The patients H.Y and O.N were diagnosed with Hashimoto's disease. These two sera contained extremely high titers of TgAb, and H.Y serum was virtually restricted to IgG1, but O.N almost only to IgG2. Serum samples were stored at -20°C until used.

METHODS

Using an enzyme-linked immunosorbent assay (ELISA), anti-Tg antibody, anti-Tg IgG-subclass, anti-idiotypic antibody and soluble interleukin-2 receptor (IL-2R) were examined in the serum of patients with AITD, and by competitive binding assay, we measured TgAb repertoires difference. Statistical analysis of the results was performed using Student's *t*-test.

A) TgAb ELISA

Micro-plates (Nunc Co.) were coated with human thyroglobulin (hTg) of 2.5 $\mu\text{g}/\text{ml}$ (Paesel Co.) overnight at 4°C . Phosphate-buffered-saline (PBS) containing 1% bovine serum albumin (BSA) were used to block the nonspecific adsorption for 1 hr at room temperature, and the plates were incubated with either samples or diluted standard (high TgAb titre of HT). The wells were then incubated with HRP-anti-human IgG (Tago Co.) for 1 hr at room temperature. The colorimetric reaction was developed on the addition of 30% H_2O_2 into substrate containing 2.5 mg/ml O-phenylene-diamin (Sigma Co.) for 30 min. The absorbance was read at 492 nm (Sanko Junyaku Co.) by the ELISA reader.

B) Anti-Id antibodies ELISA

The plates were coated with mouse anti-hTg monoclonal antibody (MoAb) (Dako Co.) at 2.5 $\mu\text{g}/\text{ml}$ as above, and then incubated with either patient or normal sera for 1 hr at room temperature. All sera were inactivated for 30 min at 56°C . The plates were processed further as described above, and results were expressed as an ELISA Index calculated from optical density (OD) at 492nm as follows:

$$\text{Index} = \frac{\text{OD}_{492} \text{ of sample}}{\text{OD}_{492} \text{ of a reference serum}}$$

C) Competitive inhibition assay

The microELISA plates were coated with mouse anti-hTg MoAb at 2.5 $\mu\text{g}/\text{ml}$ as above, and then incubated at the same time with equal volume of hTg at rising concentrations, and patient sera from type 1 and type 2 for 2 hr at room temperature. The plates were processed as described

above, and the results were expressed as a percentage of inhibition according to the formula:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{OD in the presence of inhibitor}}{\text{OD in the absence of inhibitor}} \right) \times 100$$

D) IgG subclass ELISA

Polystyrene plates were coated with Tg as described above. Appropriate dilutions of sera were made. Monoclonal antibodies (MoAb) to IgG1, IgG2, IgG3 and IgG4 respectively (California Co.) were used at dilutions (1:4000). The second antibody was an peroxidase-conjugated anti-serum to mouse IgG (Tago Co.) at 1:8000. The distribution of anti-TgAb activity among the IgG subclass in sera was estimated by expressing OD_{492} for each subclass as a percentage of the OD_{492} subclass 1-4. Determinations for all four subclass were made simultaneously.

Anti-Tg IgG1 and IgG2 antibodies bind to Tg simultaneously. Duplicate human Tg-coated microELISA plates were incubated with serial dilutions of H.Y serum, which was virtually restricted to IgG1, and subsequently exposed to various concentrations of O.N serum, which was almost only to IgG2, or buffer. One plate was then developed with a monoclonal anti-IgG1 antibody to identify H.Y serum, and the other with a monoclonal anti-IgG2 to identify O.N serum followed by horseradish peroxidase-conjugated goat anti-mouse IgG. In the reverse experiment, duplicate plates were first incubated with serial dilution of O.N serum, and then with H.Y serum or buffer. Individual plates were developed with anti-IgG1 or anti-IgG2.

E) Preparation of F(ab')₂ fragments of TgAb and Conjugation of horseradish peroxidase (HRP) with F(ab')₂

Serum from AITD (3 ml) was equilibrated with 0.1 M Tris buffer pH 8.0, and an IgG fraction was isolated by chromatography on a column of DEAE cellulose. The IgG solution was digested with pepsin (1:50, pepsin:IgG) for 16 hr at 37°C in 0.07M acetate buffer pH 4.5, neutralized with 2M Tris pH 8.0 and the products were separated by Sephacryl S-200 in PBS pH 7.2. Following, the method reported by Nakane et al³⁰ was modified. 1% 1-fluoro-2, 4-dinitrobenzene ethanol solution was added to 4 mg of HRP (Boehringer Minim, Germany) in 1 ml of 0.3M NaOH buffer pH 8.1, and incubated for 20 min at room temperature. The solution was then dialyzed overnight at 4°C with 0.01M NaOH buffer PH 9.5. After adding 1.0 ml of 0.06M NaCl, the solution was incubated at room temperature for 30 min. 1 ml of 0.16M ethylene glycol was added, and then dialysis was performed overnight at 4°C using 0.01M NaOH buffer pH 9.5. The solution was mixed with 1.0 ml of 5 mg/ml F(ab')₂ equilibrated with 0.01M

NaOH buffer pH 9.5 and incubated at room temperature for 3 hr. Following this, the solution was dialyzed overnight at 4°C with PBS, and gel filtered by Sephacryl S-200 equilibrated with PBS to collect fractions with HRP-conjugated antibodies.

F) Competitive binding assay

The plates were coated with Tg at 2.5 µg/ml as above, and then an equal volume of serum and F(ab')₂ fragments conjugated with HRP were simultaneously applied for 1 hr at room temperature. Finally, the process was performed as described above. Sera were diluted to yield an ELISA appropriate OD. The dilution of F(ab')₂ producing OD=0.8 was determined for each of the six preparations. This dilution was used against the 102 diluted sera from the patients with AITD.

G) Assay for Soluble IL-2R

Ta60a and Ta60b MoAb which recognize different IL-2R α-chains were used for the detection of soluble IL-2R by sandwich ELISA³⁹. 96-well was coated overnight at 4°C with purified Ta60b MoAb of 10 µg/ml (Aichi Cancer Center). 1% PBS-BSA, samples and diluted a reference standard that was cultured supernatant of the HUT₁₀₂ for human T cell leukemia virus (HTLV)-1-positive T cell line were added in turn to be incubated. Following this, dilutions of HRP-conjugated Ta60a MoAb were added.

H) Measurement of free T3, free T4, TSH and Thyroglobulin and Microsomal antibodies by Particle Agglutination (PA) Method

The free T4 or free T3 concentrations in sera were assayed using the free T4 RIA kit or free T3 RIA kit, (respectively Dainabot Co.). Serum TSH was measured in each sample, using TSH RIA kit (Dainabot Co.). Thyroglobulin and thyroid micro-

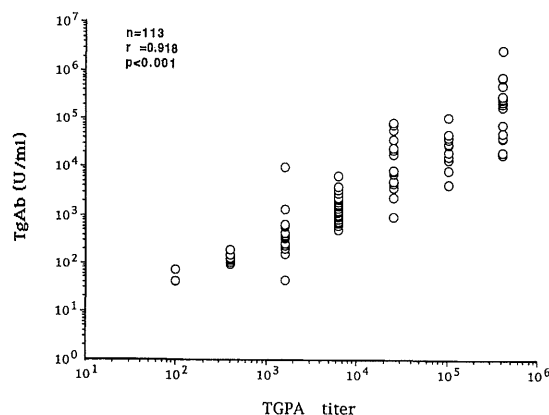


Fig. 1. Correlations of the sera TgAb values between ELISA and Particle Agglutination (PA) method.

somal antibody titres were measured by a commercial kit (Serodia-ATG, Serodia-AMC, Fujirebio Co.). Sera were considered positive when particle agglutination (PA) was observed at a dilution of 1:100 for both ATG and AMC.

I) Human Thyroglobulin

The concentration of thyroglobulin in sera was assayed using RIA kit (Eiken Co.).

RESULTS

A) Comparison of the TGPA and TgAb tests

As shown in Fig. 1, a significant correlation ($r=0.92$, $p<0.001$) was found between the levels of Tg antibodies measured by particle agglutination (PA) and the those determined by ELISA in the 113 sera from patients with AITD. Although a good correlation between ELISA and PA method was present, TgAb positive activity of ELISA was higher than those of PA (data not shown).

Table 1. Comparison of the IgG subclass distribution of Tg antibody in Graves' and Hashimoto's patients. The predominant contribution by each IgG subclass to TgAb are shown.

	Contribution made to thyroglobulin antibody									
	Graves' patients					Hashimoto's patients				
	n	%IgG1	%IgG2	%IgG3	%IgG4	n	%IgG1	%IgG2	%IgG3	%IgG4
Predominantly IgG1	2	70 ± 2 ^a	8 ± 1	5 ± 4	16 ± 8	6	73 ± 8	13 ± 5	3 ± 2	12 ± 8
Predominantly IgG2	2	24 ± 7	67 ± 6	2 ± 3	7 ± 2	7	19 ± 6	71 ± 6	2 ± 2	7 ± 7
Predominantly IgG4	7	17 ± 6	3 ± 4	4 ± 2	76 ± 5	16	12 ± 7	9 ± 4	1 ± 1	78 ± 7
IgG1 and IgG2	2	42 ± 5	52 ± 5	2 ± 1	4 ± 1	4	47 ± 5	42 ± 7	2 ± 1	9 ± 3
IgG1 and IgG4	6	41 ± 4	8 ± 8	6 ± 4	45 ± 5	7	42 ± 6	15 ± 4	4 ± 2	39 ± 4
IgG2 and IgG4	3	14 ± 4	39 ± 2	2 ± 1	45 ± 2	2	15 ± 1	40 ± 4	2 ± 1	43 ± 3
IgG1, IgG2 and IgG4	3	33 ± 3	29 ± 3	4 ± 1	34 ± 3	3	32 ± 8	34 ± 6	2 ± 1	32 ± 3
Total	25					45				

^a The distribution of TgAb activity among the IgG subclass in sera of patient were estimated by expressing the OD₄₉₂ for each subclass as a percentage (Mean ± SD) of the sum of the OD₄₉₂ for their own subclasses 1-4.

B) IgG subclass

The mean percentage obtained from the IgG subclass distribution of patients in the above study is shown in Table 1. IgG subclass of TgAb in 25 Graves' disease and 45 Hashimoto's thyroiditis, each of which was assessed by mean percentage, was either predominantly IgG4 (7 GD and 16 HT), predominantly IgG1 (2 GD and 6 HT), or both IgG1 and IgG4 (6 GD and 7 HT). Percentages of IgG3 were usually low in all patients, but some patients (2 GD and 7 HT) which contained extremely high titers of Tg antibodies tended to have higher levels of IgG2 subclass. The other patients (8 GD and 9 HT) were either both IgG1 and IgG4, or both IgG2 and IgG4, or meanly distributed in IgG1, IgG2 and IgG4.

As shown in Fig. 2a, the optical density (OD) values for anti-Tg IgG1 antibody were similar for serially diluted H.Y serum dominated by IgG1 subsequently exposed to either buffer or various dilutions of O.N serum dominated by IgG2. In the parallel plate developed with anti-IgG2 (Fig. 2c), OD values were unchanged to the concentration of O.N serum in IgG1 Tg antibody, the correspondence in IgG2 Tg antibody OD was observed. In the reverse experiment, the dilution curves for IgG1 Tg antibody in H.Y serum were virtually identical in the presence or absence of O.N serum (Fig. 2b). Furthermore, OD values for IgG2 antibody corresponded to the concentration of O.N serum added, and were unaffected by the addition of H.Y serum (Fig. 2d). These data suggest that the anti-Tg IgG1 and IgG2 antibodies can bind to Tg simultaneously, and indicate that these anti-Tg antibodies recognize different epitopes on Tg.

We investigated the interaction of the two Tg antibodies, by a parallel series of experiments in which dilutions of H.Y and O.N sera were allowed to bind to Tg-coated ELISA plates. Tg was bound to the test in the presence of H.Y serum Tg antibody and anti-human IgG1 to yield an ELISA OD of ≈ 0.8 , and this level of binding was unaffected by the addition of O.N serum in amounts which bound $OD \approx 0.8$ Tg in the presence of anti-IgG2 antiserum (Fig. 3). Since the anti-Tg antibodies of H.Y and O.N were not MoAb, each one of them had contained a few IgG subclass of the opposite side. When these two sera bind to Tg together, the obtained absorbance values were higher than those from unitary serum. However, the difference was not significant.

C) Anti-idiotypic antibody

Anti-Id antibody activities against anti-Tg MoAb in the sera of patients with AITD were measured by ELISA (Fig. 4). 14 of 61 (23%) sera from patients with HT and 10 of 58 (17%) from patients GD exhibited elevated (above upper-limit of normal range) anti-Id antibody activity against anti-Tg and in none of 38 normal subjects. More-

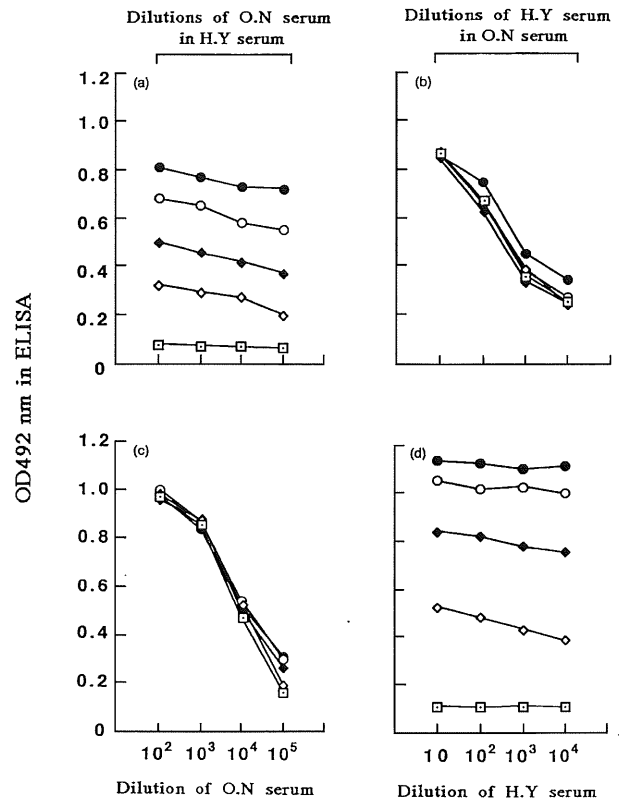


Fig. 2. Anti-Tg IgG1 antibody (a and b) and IgG2 antibody (c and d) measured by ELISA for dilutions of O.N serum in buffer or decreasing concentrations of H.Y serum (a and c) and dilutions of H.Y serum in buffer or decreasing concentrations of O.N serum (b and d). O.N serum dilutions 1:100 (●); 1:1000 (○); 1:10000 (◆); 1:100000 (◇); H.Y serum dilutions 1:10 (●); 1:100 (○); 1:1000 (◆); 1:10000 (◇). (□) Buffer.

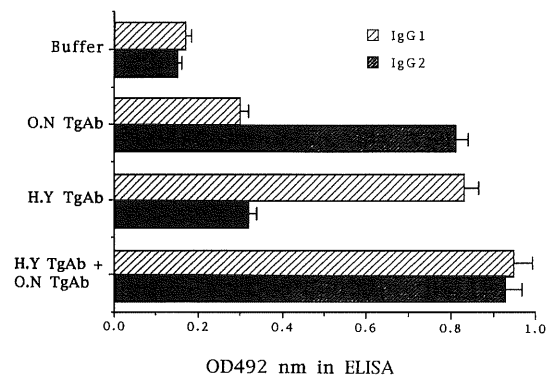


Fig. 3. Binding of Tg by anti-Tg IgG1 antibody (in serum from patient H.Y diluted 1:100) incubated in absence or presence of IgG2 Tg antibody (O.N patient serum diluted 1:2000). Results are expressed in terms of OD bound of closely agreeing duplicates.

over, the levels of anti-Id antibodies from HT were significantly different from those GD ($p < 0.05$). The positive activities of anti-Id antibodies in HT were higher than those in GD ($p < 0.001$).

The typical titration curves of Hashimoto's thyroiditis, Graves' disease anti-Id-positive and -negative HT patient sera against Id MoAb are shown in Fig. 5. Serum HT-1 exhibited the highest anti-Id activity. The binding was dose dependent, and titers of anti-Id antibody varied from 1:160 to 1:2560. There was no correlation between anti-TgAb titers and anti-Id antibody titers. Also, no correlation between anti-Id activity in patient sera and anti-microsomal antibody titers, FT4,

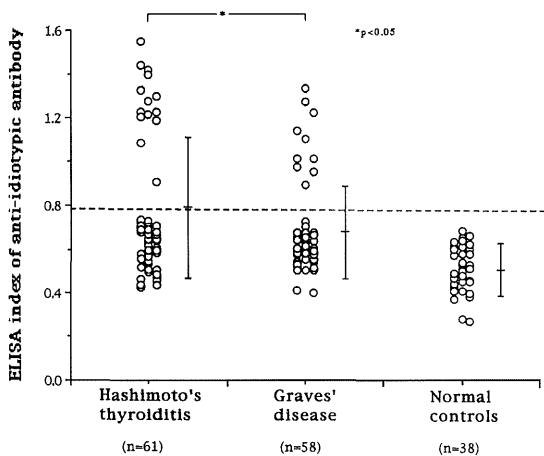


Fig. 4. Serum antibody against mouse anti-hTg MoAb (2.5 μ g/ml) in patients with HT, GD and in normal subjects assessed in an ELISA with HRP-anti-human IgG antiserum. Results are expressed as ELISA index. Sera were diluted 1/ 100. A broken line showed the upper limit of normal range (Mean \pm 2SD).

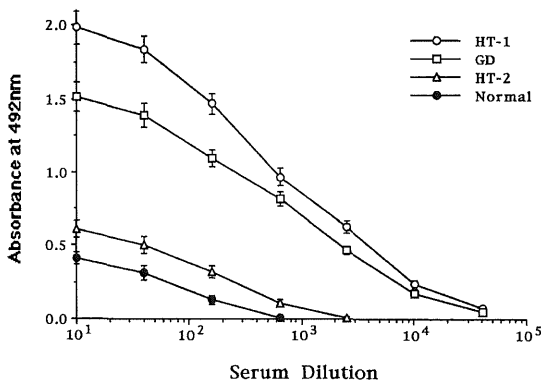


Fig. 5. Titration curves of patient sera binding to anti-hTg MoAb. Sera at increasing dilutions from patients with HT and GD, who exhibited anti-Id activity and from normal subject were incubated with anti-hTg MoAb. Results are expressed as absorbance at 492 nm.

FT3 and TSH levels, or soluble IL-2 receptor levels was observed (Data not shown).

On the other hand, positive anti-Id antibodies can be divided into two types. The patients (7 HT and 4 GD) were parallel with positivity both anti-Id antibodies and human Tg, and defined as type 1. The others (5 HT and 1 GD) showed the positive anti-Id antibodies but negated hTg, and then defined as type 2 (Fig. 6).

We used a competitive inhibition ELISA to study further the anti-Id antibody specificity. Inhibition produced by sera of anti-Id activity that had bound directly, was measured by diluting each to a concentration that produced half-maximal binding before being mixed with inhibitors. The results of the competitive inhibition studies were shown in Fig. 7 and the tested samples were from type 1 and type 2. The binding of type 1 to anti-Tg MoAb were apparently inhibited by the addition of Tg antigen, but that of type 2 were not.

D) TgAb Repertoires

TgAb from 94 of 102 patients were partially (30~60%) or strongly (>60%) competitively inhibited from binding to Tg by one or more of the six F(ab')₂ preparations. TgAb from 21 patients (group 1) were very strongly inhibited (>70%) by F(ab')₂ fragments from patient M.H and U.K, while there was little or no inhibition of these same TgAb by F(ab')₂ fragments from patient M.S and M.K. Conversely, TgAb from 13 other patients (group 2) were very strongly inhibited by F(ab')₂ fragments from M.S and M.K, with little inhibition by fragments M.H or U.K (Fig. 8). But it is not different that TgAb from group 1 and 2 were inhibited by F(ab')₂ fragments from W.T and

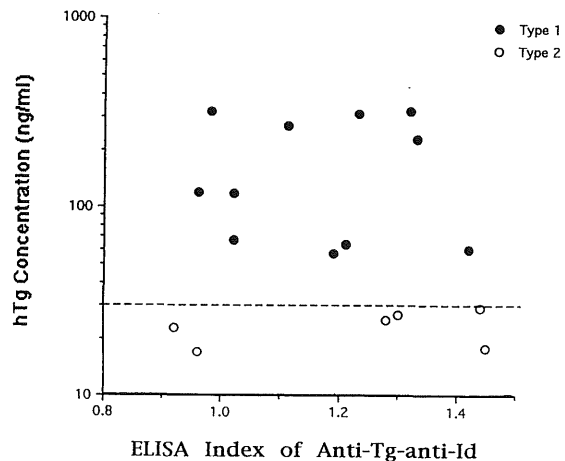


Fig. 6. Correlation between positive anti-Id antibodies against anti-Tg and hTg concentration in patients with AITD. Based on hTg levels, anti-Id antibodies can be divided into two types. (a broken line, the upper limit represents the positive.)

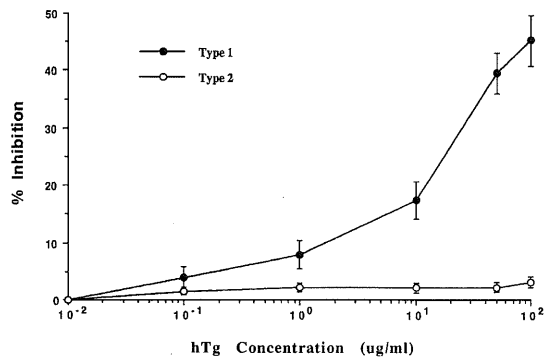


Fig. 7. Competitive inhibition of anti-Id binding to mouse anti-hTg MoAb by hTg measured in ELISA. The plates coated with anti-hTg MoAb at $2.5 \mu\text{g/ml}$ were incubated with type 1 and 2 sera exhibiting anti-Id activity in the presence of inhibitor at rising concentration of hTg, the sera were diluted 1/200. The binding was detected with HRP-anti-human IgG. Results are expressed as percentage inhibition.

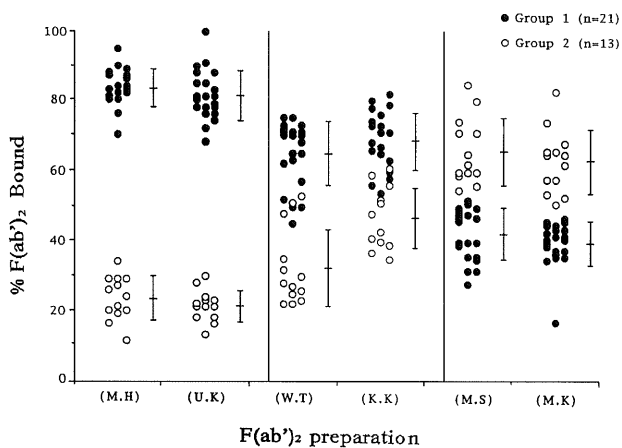


Fig. 8. Competitive inhibition of TgAb from patient sera binding to human Tg by $F(ab')_2$ fragments-HRP measured in ELISA. Micro-plates coated with Tg were incubated simultaneously with $F(ab')_2$ fragments-HRP and TgAb from patients of groups 1 and 2. The dilution of $F(ab')_2$ fragments-HRP producing $OD \approx 0.8$ was determined for each of six preparations. Results are expressed as percentage binding.

K.K.

Significant correlations were high ($r=0.9$, $p<0.001$) in the degree of TgAb inhibition caused by the $F(ab')_2$ preparation from patients M.H and U.K in all 102 samples and slightly low ($r=0.7$, $p<0.001$) from patients M.S and M.K. The remaining 60 patients (group 3) were partially inhibited ($>30\%$) by all six $F(ab')_2$ preparations. For the other 8 patients, there was less than 30% inhibition by one or more of the six $F(ab')_2$ preparations.

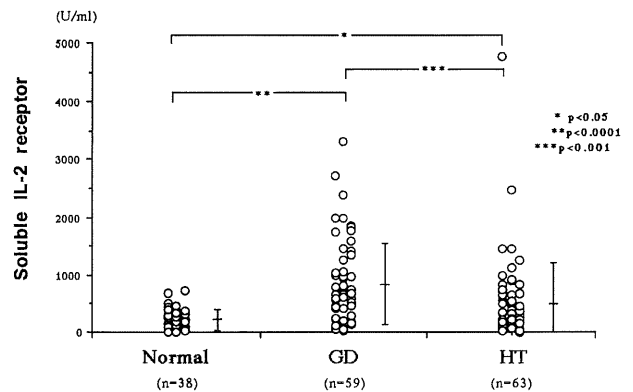


Fig. 9. Soluble IL-2 receptor levels in sera of patients with GD, HT and normal subjects.

E) Soluble IL-2 Receptor

Soluble IL-2 receptor (IL-2R) in sera was measured in order to study the immunological condition of the T cell in AITD. In 59 patients with GD and 63 patients with HT (Fig. 9), the means of soluble IL-2R were 837 ± 709 and 505 ± 704 respectively, and significantly higher than 215 ± 187 U/ml in 38 normal controls ($p<0.001$). Thirty-two of 59 GD patients (54%) and seventeen of 63 HT patients (25%) had soluble IL-2R values over the upper normal limit of 600 U/ml. Moreover, soluble IL-2R values in GD were significantly higher than in HT ($p<0.001$). In addition, there was no correlation among soluble IL-2R levels with FT3, FT4 and TSH levels, or ATG, AMC titers (Data not shown).

DISCUSSION

The causal mechanisms for AITD are multiple and complicated, and any single factor does not explain the process⁶. Recently, the immunological mechanisms involved in GD and HT two diseases must be closely related, while the phenotypes probably differ because of the specific type of immunological response that occurs. For example, if immunity leads to production of thyroid stimulating antibodies, GD is produced, whereas if a cell-destructive process occurs, the result is HT. TgAb have been documented as one of the important autoantibodies concerned with the aberrant immunity of AITD, and the analysis of its auto-immune mechanisms has been refined^{11,13,20,31,32,38}. A discussion between our results and findings in the previous studies is done in the following.

A) IgG subclass

Autoantibodies to Tg and TPO are characteristic of patients with AITD, and they are predominantly of class IgG. Many findings have confirmed that these antibodies are principally

restricted to subclass IgG1 or IgG4^{5,32}). The clinical significance of this subclass distribution is described by Jansson et al¹⁹). We investigated the IgG subclass distribution of anti-TgAb in 25 GD and 45 HT sera using an ELISA technique based on monoclonal anti-subclass antibodies. Considerable subclass restrictions were observed to be predominantly associated with anti-TgAb, but not exclusively with IgG1 and/or IgG4 (GD mean 60%, HT mean 64%). Titres of IgG3 antibody were generally low. However, IgG2 anti-Tg was high in some patients, even forming the major anti-Tg response. Antibodies in nine of 70 patients (12.9%) were mainly restricted to the subclass IgG2, and contained an extremely high concentration of anti-Tg antibody (ATG titres > 1:25600~409600). These different subclass distribution probably reflect the heterogeneity of the immune status concerning TgAb production among patients with AITD.

Much researches have been done on mechanisms of IgG subclass restriction of autoantibodies. For example, studies of antibodies to exogenous antigens in mice and man indicate that subclass restriction depends on a number of factors including the type of 'help' provided by T cells or T cell derived lymphokines^{17,26}). It has also been reported that the IgG subclass distribution of an antibody may change from IgG1 to IgG4, as the result of repeated exposure to the same antigen¹).

The high circulating thyroid autoantibody levels in AITD patients indicated that these individuals are chronically stimulated by thyroid autoantigens. Recently, it has been demonstrated that the IgG subclass distribution of thyroid autoantibody is usually unaffected by changes in total serum autoantibody levels, expressed as the fingerprint of an individual's autoimmune response to thyroid autoantigens²⁰). However, the IgG2 antibody subclass showed distinct changes which parallel changes in total serum anti-Tg antibody values²⁴), and showed a highly restricted affinity heterogeneity, suggesting that anti-Tg of this subclass are clonally restricted⁸). Recent evidences have shown that IgG2 and IgG4 anti-Tg antibodies recognize different epitopes on Tg¹¹) and studies on responses to bacteria suggested that IgG2 antibody may recognize a carbohydrate epitope¹⁵). These findings have documented the difference between IgG2 and IgG4 antibodies.

The question was further approached in terms of ELISA to determine whether an anti-Tg IgG2 antibody could bind to Tg that had already interacted with IgG1 Tg antibody (and vice versa). Our data (Fig. 2) suggested that the IgG2 and IgG1 antibodies can bind to Tg simultaneously. However, it is possible that, if the Tg molecules on the ELISA plates had not all been bound by the first antibody, they could have subsequently

interacted with the second antibody. Therefore, we investigated the interaction of the two anti-Tg antibodies using the same MoAb to IgG1 and IgG2 followed by anti-mouse IgG-HRP (Fig. 3). Neither H.Y antibody nor O.N antibody appeared to significantly interfere with each other in binding to Tg.

We have shown by ELISA that binding of an anti-Tg IgG1 antibody occurred at the same time as binding of an anti-Tg IgG2 antibody indicating that these anti-Tg antibodies recognize different epitopes on Tg. As exogenous carbohydrate antigens tend to induce IgG2 antibodies, it is possible that the anti-Tg IgG2 antibody recognizes the carbohydrate residues on Tg. Furthermore, as exogenous proteins generally induce IgG1 and/or IgG4 antibodies¹⁵), the anti-Tg IgG1 antibody may interact with a protein epitope.

Despite extensive screening or our finding, HT patients have Tg antibodies that are predominantly of IgG1 and/or IgG4 subclasses²⁴). The antibodies described here (O.N and H.Y) are the only ones presently available to us that are restricted to one subclass. However, the epitopes on Tg recognized by O.N and H.Y do not appear to be unique. For example, binding of O.N or H.Y antibodies to Tg in an ELISA in the presence or absence of other patients sera has shown that Tg antibodies in two HT patients recognize the same epitopes as IgG1 and in two GD patients recognize the same epitopes as IgG2.

Investigation between idiotypes and isotypes of mice suggest that preferential VDJ combinations occur with certain heavy chain C regions²⁷) and at least in part, the mechanism could also explain the IgG subclass associations of specific antibodies with different types of antigens. Moreover, the study of Delves & Roitt⁷) established that the autoimmune disease appears to be stable in terms of autoantibody spectrotyping and idio-type in patients with HT.

On the basis of these studies and our observations, it is possible to substantiate the view that the IgG subclass 'fingerprints' reflect the ability of individuals to recognize different autoantigenic epitopes on human Tg. IgG subclass are linked to inherited determinants on the immunoglobulin allotype (Gm), and some Gm allotype have been associated with increased susceptibility to GD and HT²⁰). If it can be established that a direct relationship exists between IgG subclass and the capacity of the antibody to mediate destruction of the thyroid, the IgG subclass distribution of anti-Tg antibody could be used (in conjunction with TSH-R antibody levels) to help predict the development of disease in genetically predisposed individuals in AITD.

B) Anti-Id antibody

It is now well known that antiidiotypic antibo-

dies play an essential regulatory role in immune network systems²⁰). Idiotype immunity is thought to be an important mechanism for the maintenance of immunological tolerance to self-antigens, and autoimmunity may be attributed to the disturbance of down-regulation of autoreactive clones in an Id network⁴⁴). Clinical investigations have revealed the spontaneous presence of anti-Id antibodies to self-reacting antibodies in human as SLE^{2,41}), hepatitis⁴²), and AITD^{18,38,46}). It is interesting that the Id of anti-DNA antibodies was found in the active stage of SLE, whereas the anti-Id antibodies occur spontaneously in the inactive stage²). Anti-Id antibodies act in the immune system by recognizing the complementary Id on the surface of immune competent cells²¹). Thus, elucidating of the properties of anti-Id antibodies would be important not only for understanding the pathophysiological role of Id-anti-Id interaction in autoimmunity, but also for manipulating antibody production in autoimmune disease^{2,10,12,25}). Those reports supported that anti-Id antibodies are spontaneously produced as an intrinsic concomitant of the immune response, and concord with network controls may regulate the formation of pathologic autoantibodies. Therefore, it is possible that the autoimmune response results from abnormalities in the Id-anti-Id system.

This paper describes the binding of anti-Id antibodies to mouse anti-Tg monoclonal antibody. The results found naturally occurring anti-Id antibodies to Tg idiotype in sera of patients with AITD. Although the frequency of occurrence of anti-Id antibodies was low in this study, the specific positive reaction was observed overall in 10 of 58 (17%) patients with GD, in 14 of 61 (23%) patients with HT, and in none of 38 normal subjects. Such a low incidence of anti-Id appearance could be explained by low sensitivity of the assay, by fluctuation of anti-Id titer, depending on the course of disease and treatment. Moreover, one should consider the formation of Id-anti-Id complexes in circulating sera²⁸). Therefore, it is possible that some of the naturally occurring anti-Id antibodies are already in the form of Id-anti-Id complexes in patients sera. These complexes might have been missed in my assay, and thus the real anti-Id activity values were underestimated. The detected binding was due to the presence of anti-Id antibodies to anti-human Tg and not to Tg-anti-Tg circulation immune complexes, as anti-Tg antibodies were not found in the some positive sera by ELISA. The binding was dose dependent, and titers of anti-Id antibodies varied from 1/160 to 1/2560. The observed sera of normal individuals with high absorbance values in ELISA were due to crossreactivity between the anti-human IgG second antibody and mouse Ig. There is a possibility that non-specific interaction

between the human and mouse antibodies occur via Fc receptors³⁸).

Id determinants can potentially occur in any area of the V region of antibodies: antigen-combining site, framework, or D or J regions. Thus, anti-Id antibodies can be separated into at least two categories based on the properties of their binding sites. One type was directed towards Id determinants in the antigen-combining site of antibody molecules. This anti-Id antibodies acted like a 'internal image' of antigen ($Ab_2\beta$), other type recognizes Id determinants in the framework region (non-binding site, $Ab_2\alpha$) of antibodies²¹). The results of competitive inhibition studies (Fig. 7) suggest that there are two different specificities of anti-Id antibodies. The binding of type 1 to anti-Tg MoAb could be blocked by Tg antigen. But type 1 showed positive not only in anti-Id antibodies but also in Tg levels. These anti-Id antibodies may belong to the categories of $Ab_2\beta$. Type 2 also reacted with the anti-Tg antibody. However, the sera of type 2 binding to anti-Tg antibody were not blocked by Tg, and the Tg levels showed negative. Therefore, type 2 had $Ab_2\alpha$ activity that seemed to be directed towards the framework region common among anti-Tg antibody.

The results here show that the positive of anti-Id antibodies was found in some patients with very low or undetectable anti-TgAb titers, whereas other patients with high anti-TgAb titers had very little or no anti-Id antibodies. The present study evidenced no correlation between anti-Id activity and anti-Tg antibodies in patient sera. However, the negative correlation has also been observed in SLE^{2,41}), hepatitis⁴²) and AITD³⁸) by other investigators. The negative correlation of high levels of anti-Id with low levels of Id suggested that there may be a suppressive effect of anti-Id on Id expression.

Our findings confirm the network theory that anti-Id antibodies do occur naturally during the course of thyroid autoimmune disorders. In animal models of autoimmune thyroiditis⁴⁴), administration of anti-Id against the pathogenic antibodies suppressed the level of autoantibodies and led to improvement of the autoimmune lesions. Their findings thus raise the interesting possibility of using anti-Id as a new treatment for human immunemediated diseases.

C) TgAb repertoires

Although anti-TgAb are characteristic of patients with AITD, the autoepitopes of human Tg were not fully identified⁹). It has been suggested, however, that the restricted anti-TgAb response in AITD is directed towards the same few autoepitopes in different patients³¹). In a recent report using monoclonal antibodies, Schulz et al have observed that MoAb Tg 13 is specific for an

autoimmunodominant B cell epitope of hTg³⁷). One of the Tg epitope detected by Mireille et al¹⁶) confirmed the presence of an immunodominant domain in the central part of the hTg molecule. The data exhibited above demonstrated the differences in the anti-TgAb repertoires between patients with AITD.

We have identified two major group of patients with restricted repertoires of TgAb, each accounting for about 16.5% (34/102) of the samples studied. The patterns of competition between anti-TgAb and F(ab)₂ fragments indicate that the anti-TgAb of these two groups are directed against restricted and exclusive subsets of anti-TgAb. The results of other researchers¹³) have also demonstrated that the phenomenon is not due to differences in anti-TgAb affinities. The majority of the patients studied had anti-TgAb which were partially inhibited by F(ab)₂ preparations from both group 1 and 2, indicating that most patients possess a wider repertoire of anti-TgAb comprising features of both subgroups. Eight patients were observed to have anti-TgAb which were poorly inhibited by one or more of the six F(ab)₂ preparations (but not all F(ab)₂ preparations) used in own study.

The present study has demonstrated a spectrum of anti-TgAb repertoires in patients with AITD by competitive binding assay using TgAb F(ab)₂ fragments. Groups of patients having the same anti-TgAb repertoire have been defined: two of these repertoires, defined by F(ab)₂ groups 1 (M.H and U.K) and 2 (M.S and M.K), are restricted and show no overlap. The majority of patients, however, possess elements present in each of the two restricted repertoires. Characterization of the specific Tg autoepitopes involved in AITD will be assisted by selection of informative samples with restricted repertoires of anti-TgAb. Although the pathogenic role of the autoantibodies in autoimmune thyroiditis is not yet clear³³), their restricted range of characteristics suggests that examination might reveal general aspects of the autoimmune response to a specific antigen.

D) Soluble IL-2 receptors

The increase in the serum levels of the soluble IL-2 receptors might be due to its release from activated cells³⁶). Therefore, soluble IL-2R levels in serum may serve as an index of lymphocyte activation. Since in various autoimmune diseases, circulating levels of soluble IL-2R levels are related to progressive disease activity³). We measured soluble IL-2R levels in 122 patients with AITD. The mean levels of soluble IL-2R of GD and HT were both significantly higher than the normal controls, and IL-2R values in GD were higher than those in HT ($p < 0.001$). Therefore, it is regarded as parameters of disease activity in AITD. We conclude that soluble IL-2R levels are

elevated in AITD patients and that might be a useful marker for disease activity.

From the findings mentioned above, we conclude that the different anti-Tg antibodies with a variety of immunologies were found in the patients with AITD, and infer that the characteristics common to IgG subclass and anti-TgAb repertoire might document that generally GD is more or less similar to HT, but that some differences in anti-Id antibody and IL-2R levels might exhibit that GD is more relatively active than HT.

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