Characterization of Thyroglobulin Epitopes in Patients with Autoimmune and Non-Autoimmune Thyroid Diseases Using Recombinant Human Monoclonal Thyroglobulin Autoantibodies

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Context: Thyroglobulin (Tg) epitopes of serum Tg autoantibodies (TgAb) have been characterized using inhibition of Tg binding by human monoclonal TgAb in autoimmune thyroid diseases (AITD) [Hashimoto's thyroiditis (HT) and Graves' disease (GD)] but not in non-AITD [nontoxic multinodular goiter (NTMG) and papillary thyroid carcinoma (PTC)].

Objective: Our objective was to compare Tg epitopes of serum TgAb from patients with AITD, non-AITD, and PTC associated with histological thyroiditis (PTC-T) using inhibition of Tg binding by four recombinant human TgAb-Fab (epitopic regions A–D).

Design: Inhibition of Tg binding of 24 HT, 25 GD, 19 NTMG, 15 PTC, and 25 PTC-T TgAb-positive sera by each TgAb-Fab was evaluated in ELISA. Inhibition by the pool of the four TgAb-Fab was evaluated using labeled Tg.

Results: Levels of inhibition were different for TgAb-Fab regions A ($P = 0.001$), B (0.007), and D (0.011). Inhibition by region A TgAb-Fab was significantly higher in HT, GD, and PTC-T than in NTMG and PTC patients. Inhibition levels by region B TgAb-Fab were significantly higher in HT compared with NTMG and PTC patients and in GD compared with NTMG patients. Inhibition by D region TgAb-Fab was significantly lower in NTMG than in the other groups. Inhibition by the pool ranged from 44% (NTMG) to 72% (GD).

Conclusions: The pattern of Tg recognition is similar when HT patients are compared to GD and NTMG to PTC patients and differs when AITD are compared with non-AITD patients. In PTC-T patients, it is similar to that of AITD patients. **(*J Clin Endocrinol Metab* 93: 591–596, 2008)**

Thyroglobulin (Tg), thyroperoxidase (TPO), and the TSH receptor (TSH-R) are the major thyroid autoantigens. Circulating autoantibodies (Ab) to Tg (TgAb) and TPO (TPOAb) are hallmarks of the two most common types of autoimmune thyroid diseases (AITD), *i.e.* chronic autoimmune thyroiditis [Hashimoto's thyroiditis (HT), with its classical goitrous form and the atrophic variant] (1) and Graves' disease (GD) (2). TgAb, usually at low concentrations, may also be detected in patients with non-AITD, including nontoxic multinodular goiter (NTMG) (3) and papillary thyroid carcinoma (PTC) (4, 5), and in individuals with no apparent thyroid disease (normal individuals) (3, 6). TgAb can interfere with Tg measurement, which represents an essential tool for the follow-up of patients with differentiated thyroid cancer (DTC) (PTC and follicular thyroid carcinoma) (7).

Whether different pathogenetic mechanisms are involved in the production of TgAb in AITD and non-AITD is not known.

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Abbreviations: Ab, Autoantibodies; AITD, autoimmune thyroid disease; DTC, differentiated thyroid cancer; GD, Graves' disease; HT, Hashimoto's thyroiditis; NTMG, nontoxic multinodular goiter; PTC, papillary thyroid carcinoma; PTC-T, PTC associated with histological thyroiditis; Tg, thyroglobulin; TPO, thyroperoxidase; TSH-R, TSH receptor.

Characterization of TgAb, and in particular of their epitopes, may help to elucidate this question. In addition, recognition of different TgAb epitopes in AITD compared with non-AITD may contribute to the discrepant results obtained using different methods to measure TgAb. If correct, such an observation would be particularly relevant in DTC patients (8).

The epitopes recognized by human Ab on the large Tg molecule are usually conformational and cannot be determined using synthetic peptides. Instead, human TgAb epitopes are investigated by determining the ability of other, well-characterized polyclonal or monoclonal antibodies generated in animals to inhibit serum TgAb binding to Tg. This approach has been used to generate epitopic data for TgAb in patients with AITD and non-AITD (9–11) (reviewed in Refs. 12–14). More recently, human monoclonal TgAb have been isolated as Fab molecules (TgAb-Fab) from Ig combinatorial libraries and used for epitopic analysis (12, 15–17). Such human TgAb-Fab have been used to study TgAb in patients with AITD but not in individuals with non-AITD (15).

In the present investigation, we used human recombinant TgAb-Fab that map four epitopic regions on Tg to study TgAb recognition by sera from a large number of individuals including patients with AITD, HT, and GD. In addition, we studied patients with non-AITD including NTMG and PTC and a group comprising PTC patients with histological evidence of thyroid lymphocytic infiltration [PTC associated with histological thyroiditis (PTC-T)].

Patients and Methods

Study group

A total of 108 patients with serum TgAb (AIA-Pack; Tosoh Bioscience, Shiba, Japan), evaluated at the Thyroid Clinic of the Department of Endocrinology, University Hospital of Pisa, were selected for the present study. Routinely, patients were examined for free T_4 and T_3 (free T_4 and free T_3 reagent packs; Ortho-Clinical Diagnostics, Amersham, Little Chalfont, UK) and TSH (Immulite 2000; Euro/DPC, Gwynedd, UK; normal values, 0.4–3.6 μU/ml) as well as for TPOAb (AIA-Pack; Tosoh Bioscience). Thyroid ultrasound (Technos, Esaote Biomedica, Genova, Italy; with a 7.5-MHz linear transducer) was performed in all patients.

Twenty-four patients had HT, as diagnosed by the presence of high levels of TPOAb and/or TgAb, associated with a hypoechogenic pattern at thyroid ultrasound with or without hypothyroidism (defined by TSH $>$ 3.7 μU/ml). In 25 patients, GD was diagnosed, on the basis of hyperthyroidism, serum Ab to the TSH-R associated with TPO and/or TgAb and a diffuse, hypoechogenic goiter at thyroid ultrasound. In addition we evaluated 19 NTMG, 15 PTC, and 25 PTC-T patients that underwent total thyroidectomy. Lymphocytic infiltration was classified by pathologists as follows: absent ($<$10 lymphocytes per field), mild (10–50 lymphocytes per field), moderate (50–100 lymphocytes per field), and massive ($>$100 lymphocytes per field) (18). NTMG patients were selected because of absent or mild lymphocytic infiltration. In PTC, thyroid lymphocytic infiltration was absent in three and mild in 12 patients. In PTC-T, the lymphocytic infiltration was moderate in five and massive in 20 patients; secondary follicles and oxyphilic transformation of thyroid follicular cells were present in 13 patients. Patients were all euthyroid (with or without treatment) when sera were collected. None of the PTC and PTC-T patients had been previously treated with ^{131}I.

Serum TgAb characterization

To determine the most appropriate serum dilution for epitopic analysis, wells coated with human Tg (obtained from thyroid glands of postmortem individuals and from surgical specimens of NTMG or GD patients; Calbiochem, San Diego, CA) (4 μg/ml at 4 C overnight) were incubated with serial dilutions of sera (1:100 to 1:33,000). Antibody binding was detected with antihuman IgG-Fc conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, MO). After addition of substrate (*o*-phenylenediamine plus H_2O_2) and H_2SO_4 to stop the reaction, the OD was read at 490 nm. Serum dilutions yielding OD of approximately 1 were used in subsequent inhibition experiments.

Expression of monoclonal human TgAb-Fab

Using the phage display combinatorial library technique, we recently cloned seven human monoclonal TgAb expressed as Fab molecules (16, 17). To avoid confusion with serum TgAb, we will refer to these monoclonals as TgAb-Fab. Four TgAb-Fab corresponding to different epitopic regions, nos. 37 (region A), 26 (region B), 6 (region C), and 32 (region D) (Fig. 1), were used in the present study. As previously reported (16, 17), TgAb-Fab were expressed in bacteria, and their binding to Tg was assessed by ELISA. All four expressed TgAb-Fab at similar levels (OD ~2.0 in Tg ELISA).

Inhibition of serum TgAb binding to Tg by TgAb-Fab in ELISA

The ability of single TgAb-Fab to inhibit the binding of serum TgAb to Tg was evaluated in ELISA. Briefly, 50 μl/well of TgAb-Fab (or culture medium) was incubated with an equal volume of serum (diluted to provide a final OD of ~1.0) in Tg-coated ELISA wells (60 min at room temperature) (16). Binding of serum TgAb was detected with horseradish peroxidase-conjugated antihuman IgG-Fc (Sigma), which binds to IgG but not to Fab molecules. Color was developed as described above. The inhibition by TgAb-Fab was calculated from the OD values for serum TgAb alone − serum TgAb + TgAb-Fab and expressed as a percentage of serum TgAb alone. Nonspecific binding of a TgAb-negative serum (~12%) was subtracted in calculating percent inhibition. Inhibition experiments were performed three times, each time in triplicate.

Inhibition of serum TgAb binding to radiolabeled Tg by the four TgAb-Fab pool

The ability of the four TgAb-Fab pool (containing equal binding activities of the four TgAb-Fab) to inhibit serum TgAb binding to Tg was determined using [^{125}I]Tg (specific activity 2 μCi/μg; a kind gift of Dr. A. Bergmann, Brahms, Berlin, Germany). Tg had been iodinated by the chloramine T method, and the radiolabeled Tg had been purified by gel chromatography. Fifty microliters of serum (diluted up to 1:33,000) were incubated with 50 μl [^{125}I]Tg (15,000 cpm) and 100 μl culture

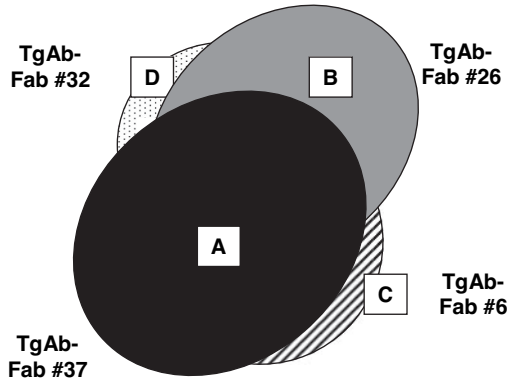

FIG. 1. Schematic representation of overlapping epitopes on Tg recognized by human TgAb-Fab (16). One representative TgAb-Fab from each group was used for inhibition experiments: nos. 37 (group A), 26 (group B), 6 (group C), and 32 (group D).

medium (60 min at 25 C). After addition of 50 μl protein A (Pansorbin; Calbiochem) (60 min at 25 C), tubes were centrifuged (2500 rpm for 15 min), the supernatant was discarded and [^{125}I]Tg binding to protein A was determined. Protein A binds serum TgAb but not TgAb-Fab. For inhibition studies, 50 μl serum, diluted to yield a final binding equal to 10% of maximum, was incubated with 50 μl [^{125}I]Tg and 100 μl of the four TgAb-Fab pool. The percentage of inhibition by the TgAb-Fab pool was calculated as for ELISA. Nonspecific Tg binding of a TgAb-negative serum to [^{125}I]Tg (~7%) was subtracted in calculating inhibition.

Inhibition of serum TgAb binding to Tg by the four TgAb-Fab pool using surface plasmon resonance

A carboxy-methylated dextran sensor chip (CM5, Biacore) was coupled with human Tg (Calbiochem; 5 μg/ml), and inhibition was performed by surface plasmon resonance (Biacore X; Biosense SRL, Cinisello Balsamo, Italy) (16), After injection of the four TgAb-Fab pool to saturate Tg binding sites, the binding of IgG purified with a protein G column (HiTrap Protein G HP; Amersham Biosciences Europe, Cologno Monzese, Italy) (19) was measured. Informative results were obtained only from the serum with the highest TgAb concentration (data not shown).

Statistical analysis

Statistical analysis was performed using the SPSS 13.0 for Windows. Inhibition among the five groups of patients for each TgAb-Fab was calculated by the Kruskal-Wallis test, and the groups were compared with each other by the Mann-Whitney *U* test. P values < 0.05 were considered statistically significant.

Results

Titers of TgAb and TPOAb

Concentrations of TgAb and TPOAb (measured with immunoenzymatic assays) in 24 HT, 25 GD, 19 NTMG, 15 PTC, and 25 PTC-T patients are shown in Fig. 2. Because patients were included only if they had positive TgAb, the present study overestimates the prevalence of TgAb in comparison with that of TPOAb in both AITD and non-AITD.

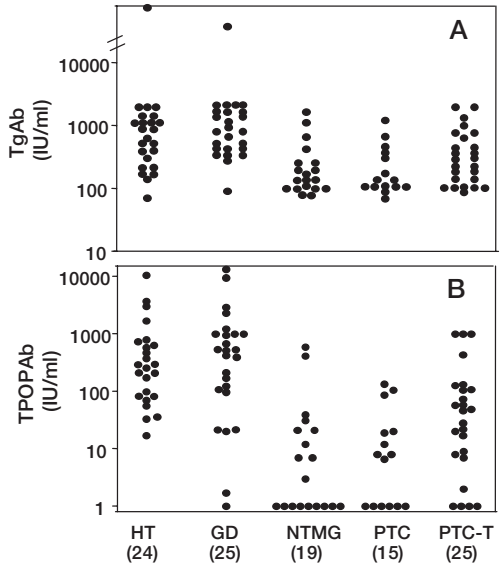

FIG. 2. Concentrations of TgAb (A) and TPOAb (B) in patients with HT, GD, NTMG, PTC, and PTC-T. TgAb and TPOAb were measured by immunoenzymatic methods. The number of patients in each group is indicated in *parentheses.* The detection limit was 1 IU/ml for both TgAb and TPOAb.

Inhibition of individual TgAb-Fab on serum TgAb binding to Tg

Inhibition by the TgAb-Fab (epitopic regions A–D) on Tg binding by serum Ab is illustrated for representatives of the five patient groups (Fig. 3A) and as the mean (± SD) for all individuals within each group (Fig. 3B). The highest levels of inhibition for all patient groups were induced by TgAb-Fab of region A. Indeed, for all 108 patients, A region TgAb-Fab inhibited serum binding to Tg by 44.9 ± 20.9% (mean ± SD) (Table 1). Lesser inhibition was observed for region B TgAb-Fab (24.3 ± 19.9%) with lower and more variable inhibition levels for TgAb-Fab regions C (19.4 ± 18.4) and D (13.4 ± 15.9%) (Table 1).

Turning to different patient groups, statistically significant differences were observed for inhibition by TgAb-Fab corresponding to regions A ($P = 0.001$), B (0.007), and D (0.011) but not region C (0.147) (Table 1). The specific differences between data for patient groups were as follows.

Group A TgAb-Fab

Inhibition levels were comparable for patients with HT, GD, and PTC-T. However, values were significantly higher in patients with AITD (HT and GD) than in patients with non-AITD (NTMG and PTC).

Group B TgAb-Fab

As for group A TgAb-Fab, there was no difference between the data for AITD (HT and GD) and non-AITD (NTMG and PTC) patients. However, inhibition levels were significantly higher in HT than in NTMG, PTC, and PTC-T patients. Also, the values for GD patients were greater than those with NTMG.

Group C TgAb-Fab

None of the differences between groups were significantly different.

Group D TgAb-Fab

Values were significantly lower in NTMG than in the other four groups.

Inhibition of serum TgAb binding to [^{125}I]Tg by the pool of the four TgAb-Fab

We estimated recognition by serum TgAb of all epitopic regions A–D by determining the inhibition of serum binding to [^{125}I]Tg by the four TgAb-Fab pool (Fig. 4). Sera from the fol lowing 42 patients were available for these experiments: nine HT, nine GD, eight NTMG, eight PTC, and eight PTC-T. Inhibition in the total of 42 patients was 58.0 ± 19.9 (mean ± SD). The highest levels were obtained in GD (71.7 ± 11.6%), the lowest in NTMG (44.3 ± 20.0%) patients. Differences were not statistically significant ($P = 0.127$).

Discussion

We recently isolated a large group of recombinant TgAb-Fab from the thyroid of a patient with GD and associated HT (16). Seven representatives of these TgAb-Fab recognized a cluster of overlap-

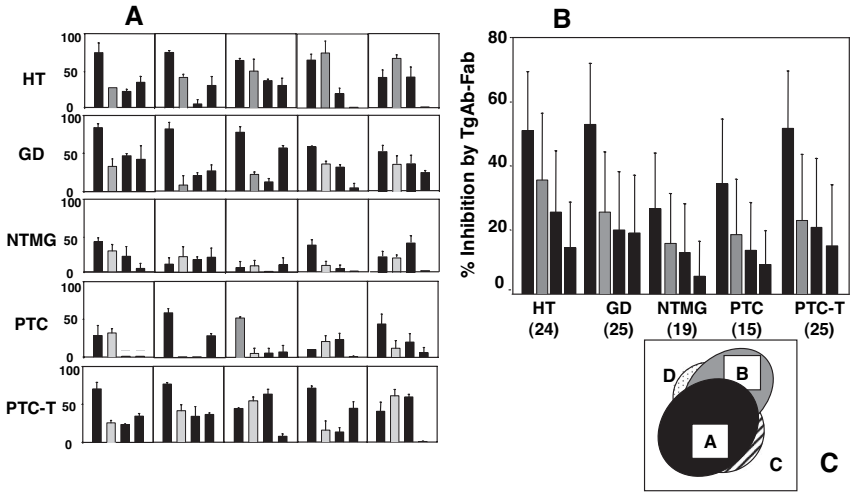

FIG. 3. Inhibition of serum Ab binding to Tg by TgAb-Fab for regions A, B, C, and D. A, TgAb in the five groups of patients: HT, GD, NTMG, PT, and PTC-T. Representative data for five individuals from each patient group are shown. Percent inhibition of serum TgAb binding to Tg-coated ELISA wells in presence of each TgAb-Fab is shown. Data shown are the mean of OD readings in ELISA (three experiments in triplicate); *error bars* represent SD. *Shading* corresponds to epitopic regions in C. B, Mean + SD for all individuals in each patient group. *Shading* corresponds to epitopic regions in C. C, Scheme to indicate epitopic regions recognized by TgAb-Fab.

ping epitopes on Tg (16). We have now used four of these TgAb-Fab, which identify regions A–D, to characterize the epitopes recognized by serum TgAb from patients with AITD, HT, and GD as well as non-AITD patients, namely patients with NTMG and PTC. In addition, we have evaluated patients with a mixed form of PTC who had lymphocytic infiltration of the thyroid (PTC-T), a feature absent from the NTMG and PTC groups.

Previous observations on TgAb epitopes are summarized to provide the context for our findings. Using labeled Tg to precipitate TgAb, a molar ratio of TgAb to Tg between 2:1 and 4:1 was reported in HT patients (20), indicating restricted recognition of Tg epitopes. Consistent with this finding, when recombinant human TgAb-Fab were used as probes to inhibit serum Ab binding to Tg, Prentice *et al.* (15) demonstrated that two major epitopes were recognized by TgAb from patients with HT and GD. Other studies, employing TgAb from immunized animals to inhibit serum Ab binding to Tg, confirmed the epitopic restriction of TgAb in AITD (21–25). It should be emphasized that the epitopes recognized by animals immunized with Tg differ markedly from those of spontaneously arising Ab in humans. Unlike the numerous studies indicating restricted epitopic recognition by human TgAb in AITD patients (10, 15, 16, 20, 26), TgAb induced in animals recognize a large number of epitopes (22, 24, 25, 27, 28). For example, sera from rabbits hyperimmunized against hog Tg recognized 40 Tg epitopes (29). Cross-inhibition studies of Tg binding showed incomplete and variable overlap between human (obtained from AITD patients) and murine TgAb (10, 11, 22). Hence, TgAb from immunized animals include epitopes outside the human immunodominant regions. These non-immunodominant epitopes may be recognized to a greater extent by TgAb from non-AITD patients. With a large panel of mouse monoclonal TgAb, Bresler *et al.* (28) observed a broader pattern of recognition by TgAb from HT patients. Moreover, some evidence was obtained for broader recognition of Tg epitopes by Ab from non-AITD *vs.* AITD patients (23). Like-

TABLE 1. Epitopic region TgAb-Fab (percentage of inhibition)

	Mean ± SD			
	A	B	C	D
HT	51.3±18.1	35.5 ± 20.8[b]	25.6 ± 18.9	14.4 ± 14.3
GD	52.7±19.3	25.6 ± 18.8[c]	20.0 ± 18.1	19.1 ± 17.9
NTMG	25.9 ± 16.4[a]	14.5 ± 15.4	13.1 ± 15.1	5.5 ± 11.0[d]
PTC	34.5 ± 20.1[a]	18.5 ± 17.2	13.7 ± 14.9	9.2 ± 10.6
PTC-T	51.7 ± 17.9	22.9 ± 20.4	20.8 ± 21.4	15.0 ± 19.0
All groups	44.9 ± 20.9	24.3 ± 19.9	19.4 ± 18.4	13.4 ± 15.9
Differences among the five groups of patients (Kruskal-Wallis test) (*P*)	0.001	0.007	0.147 (NS)	0.011

Comparison of serum TgAb binding to Tg-coated ELISA wells by TgAb-Fab (epitopic regions A–D) in different groups of patients: HT, GD, NTMG, PTC, and PTC-T. Data are reported as mean ± SD. Significant differences of inhibition for each TgAb-Fab among the five groups of patients were tested by the Kruskal-Wallis test, and the groups were compared each other by the Mann-Whitney *U* test ([a–d]). NS, Not significant.

[a] $P < 0.05$ *vs.* HT,GD, and PTC-T.

[b] $P < 0.05$ *vs.* NTMG, PTC, and PTC-T.

[c] $P < 0.05$ *vs.* NTMG.

[d] $P < 0.05$ *vs.* HT, GD, PTC, and PTC-T.

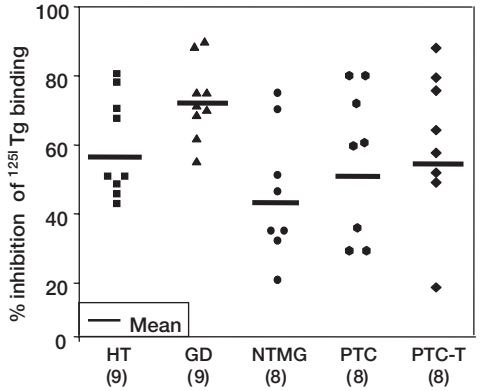

FIG. 4. Inhibition of serum TgAb binding to [^{125}I]Tg by the pool of four TgAb-Fab (A, B, C, and D) in 42 patients. Data represent the mean of one experiment in triplicate. The *bars* indicate the mean for each patient group. There was no statistically significant difference ($P = 0.127$ by Kruskal-Wallis test).

wise, another study (involving members of our group) observed less restricted recognition in NTMG patients *vs.* restricted TgAb recognition patterns in DTC and HT (9).

Our studies clearly identify region A as the major component of the immunodominant region on Tg recognized by Ab from AITD and non-AITD patients. Indeed, binding to Tg by sera from patients with HT, GD, and PTC-T patients is inhibited to more than 50% by region A TgAb-Fab. Epitopic region B is recognized to the greatest extent by HT patients (35% inhibition) and to a lesser extent in all other patient groups. Regions C and D are on the fringe of the immunodominant region in all five patient groups. The D region is particularly marginal in NTMG patients. When HT patients were compared with GD patients, levels of inhibition were similar, in particular for group A monoclonal TgAb. B region appeared to be more represented in TgAb from HT than GD patients, although this difference was not statistically significant. TgAb from NTMG and PTC patients recognized A and B regions at similar levels and at a lower degree than TgAb of AITD patients.

Inhibition of sera binding to [^{125}I]Tg by the four TgAb-Fab pool confirmed that epitopic regions A–D are well represented in both AITD and non-AITD patients. Indeed, inhibition exceeded 50% in HT, GD, PTC, and PTC-T groups and was a little lower in the NTMG group. Because Tg epitopic regions A–D partially overlap, inhibitions by the four TgAb-Fab pool were lower than the sum of inhibitions induced by each TgAb-Fab. These data highlight that in addition to Tg epitopic regions A–D, other epitopes are recognized by serum TgAb from AITD and, to a higher degree, non-AITD patients.

Thus, the pattern of Tg recognition is similar comparing HT with GD patients or NTMG with PTC patients. However, the pattern of AITD (HT and GD) patients differs from that of non-AITD (NTMG and PTC) patients. The data obtained from non-AITD patients were more variable than those of AITD patients, indicating greater heterogeneity of TgAb in the former group. To our knowledge, no previous studies have evaluated Tg epitopes of patients with PTC and concomitant thyroiditis (PTC-T group), which are distinguished from PTC alone by the presence of extensive thyroid lymphocytic infiltrates. Intriguingly, the pattern of recognition of TgAb from PTC-T patients is more similar to HT than to PTC patients. Our results clearly establish that in PTC patients, the pattern of TgAb epitopes is quite variable, being of non-AITD type in the absence of lymphocytic infiltration and AITD-like in its presence.

The different pattern of TgAb epitopes recognition in AITD compared with non-AITD could be due to the presence of molecular differences of the large (660-kDA) Tg glycoprotein in the two groups of diseases (30, 31). The glycoprotein portion of Tg is variable, in particular in DTC (32–35). These discrepancies of Tg molecules could cause the different TgAb pattern of AITD and non-AITD diseases. However, carbohydrate is not involved in serum TgAb recognition (36). Another hypothesis for this discrepancy is the existence of different mechanisms of antigen presentation or processing of Tg in AITD and in non-AITD diseases. In particular, it is possible that the immune mechanisms involved in TgAb appearance in AITD and in PTC-T differ from those induced by the stimulation of the immune surveillance that is elicited by the development of non-AITD. Our finding that the association of lymphocytic infiltration with PTC (PTC-T group) switches the TgAb epitopes response from PTC- to HT-like is in keeping with this hypothesis. More studies are needed to corroborate this supposition.

The characteristics of the Tg used for TgAb binding are crucial. Tg derived from the thyroids of patients with AITD differ from that obtained from patients with non-AITD (37). In the present study, we have used two different preparations of Tg, one for inhibition of binding by single TgAb-Fab in ELISA and another, radiolabeled, for inhibition by the four TgAb-Fab pool. The results were comparable. However, it would be interesting to evaluate the interaction of our TgAb-Fab with the Tg selectively obtained from the thyroids of AITD and non-AITD patients. This issue is currently under investigation.

Measurement of TgAb is mandatory in patients with DTC, because they interfere with assays for Tg (38). Hence, their presence renders measurement of Tg, which is an essential tool for the follow-up of DTC patients (7), unreliable. Based on our results, one can hypothesize that the pattern of Tg epitopes recognition of TgAb is relevant for their effect on Tg measurement.

Our recent observation that in patients with DTC and thyroid autoimmunity, total thyroid ablation is required for the disappearance of circulating TgAb, TPOAb, and Ab to the TSH-R (39) suggests that measurement of TgAb may be used in adjunct to that of Tg in the management of DTC (4, 14). However, an important caveat of TgAb measurement is the discrepancy of the results obtained with different kits, particularly in DTC patients, as confirmed by a recent report (8). Thus, it is commonly advised to use the same manufacturer's method for serial TgAb measurements in DTC patients (40). Based on our observations, these discrepancies might be related to the different pattern of TgAb in PTC compared with PTC-T patients and to the heterogeneity of TgAb in PTC patients.

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