Possible reasons for different pattern disappearance of thyroglobulin and thyroid peroxidase autoantibodies in patients with differentiated thyroid carcinoma following total thyroidectomy and iodine-131 ablation

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ABSTRACT. The purpose of this study was to reveal some possible factors for the differences between the pattern of disappearance of thyroglobulin autoantibodies (anti-Tg) and thyroid peroxidase autoantibodies (anti-TPO) in patients with differentiated thyroid carcinoma following thyroidectomy and iodine-131 ablation. Patients with a history of follicular cell derived cancer (papillary, follicular, both papillary and follicular, Hürthle cell) and high pre-operative titers of anti-TPO and/or anti-Tg autoantibodies were retrospectively studied. Thyroglobulin (Tg) levels were measured using radio-immunometric assay (RIA). Anti-Tg and anti-TPO levels during the first 6 yr' follow-up were measured by passive agglutination, during the following 10 yr by ELISA method and during the last 2 yr by chemiluminescence assay. A statistically significant difference was observed between median time (72 months) of disappearance of anti-TPO and median time (39 months) of disappearance of anti-Tg in patients with complete ablation of thyroid tissue, following iodine-131 administration (p=0.0395, Logrank statistic=4.24, Kaplan-Meier method). A statistically significant difference was observed between median time (106 months) of disappearance of anti-TPO and median time (33 months) of disappearance of anti-Tg in patients >45 yr of age (p=0.034) and between median time (111 months) of disappearance of anti-TPO and median time (41 months) of disappearance of anti-Tg in patients with tumor size <2 cm (p=0.0175). We concluded that patients with differentiated thyroid carcinoma and pre-surgical elevated titers of both Tg and anti-TPO tend to become earlier anti-Tg seronegative. Although tumor size and age may influence the pattern of thyroid autoantibody reduction, the exact reasons for the different rhythm of autoantibodies decrease must further be evaluated.

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INTRODUCTION

Serum thyroglobulin (Tg) is the most reliable tumor marker in the follow-up of patients with differentiated thyroid carcinoma (DTC). However, its measurement can be seriously affected by the presence of thyroglobulin autoantibodies (anti-Tg). ELISA and IRMA methods used in Tg measurement can underestimate Tg in the presence of anti-Tg. RIA methods, which incorporate specific separation systems against heterologous antibodies (Abs), tend to overestimate Tg levels, while non-specific separation systems underestimate Tg levels. This interference affects the antibody levels and even the lowest detectable concentration may interfere with the accuracy of the assays (1). The underestimation of serum Tg values in these patients may have a deleterious effect, as it can mask a disease recurrence. There are several references indicating the role of anti-Tg measurement in DTC patients as a tumor marker, especially in those with negative whole-body scanning (WBS) and undetectable Tg, but there is no consensus for its clinical use yet (2).

Despite a few similar immunologic properties and serum fluctuations in response to therapy and pregnancy, thyroid peroxidase autoantibodies (anti-TPO) are a

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better measure of thyroid autoimmunity than anti-Tg. Moreover, antibodies to TPO correlate with thyroid damage and lymphocytic infiltration, can fix complement and may initiate antibody dependent cell mediated cytotoxicity against thyroid auto-antigenic targets (3) but their exact role as tumor markers in the follow-up of patients with DTC has to be further investigated. Approximately 20 to 40% of patients with thyroid cancer have thyroid autoantibodies, and their presence (indicating underlying immunoreactivity against thyroid cell autoantigens) may suggest a better prognosis (4). A falling titer or total loss of thyroid autoantibodies (especially of anti-Tg) in such patients, however, is an important and reliable prognostic sign indicating the absence of thyroid cell antigens (5). Although Tg serum half-life is approximately 3 days and levels decrease <5-10 ng/ml only 25 days after thyroidectomy (6), thyroid autoantibodies may persistently be detected for up to 18 yr following total thyroid antigens removal (7). Until now, no specific factors responsible for this long-lasting detection have been proved. Because total thyroid antigen removal is a target treatment option in patients with DTC, in this study we investigated the pattern of thyroid autoantibodies decrease following total thyroidectomy and ablation with iodine-131. Therefore, the aims of the study were: a) to reveal any differences between anti-Tg and anti-TPO pattern of disappearance, b) to examine the possible influence of some underlying clinical and epidemiological characteristics in the difference of prolonged autoantibody production and c) to propose a few factors which could explain the different immune thyroid autoantibody response.

MATERIALS AND METHODS

Patients

Patients with a history of follicular cell derived cancer (FCDC) (papillary, follicular, both papillary and follicular, Hürthle cell) and high pre-operative titers of TPO and/or Tg antibodies were retrospectively studied. All patients being studied were initially treated with near total or total thyroidectomy, with resection of metastatic lymph nodes when evident at surgery, followed by ablation of residual thyroid tissue with iodine-131. Only individuals who had the first follow-up examination at least 9 months after radio-iodine ablation therapy were included in the study. The 75 patients who fulfilled the above-mentioned criteria were categorized into two groups: patients with complete ablation of thyroid tissue following iodine-131 ablation (group A) and patients with residual thyroid tissue following iodine-131 ablation (group B). Most of the patients in the latter group were treated with additional iodine-131 therapy for complete thyroid tissue ablation. The clinical, epidemiological, and histological data of the 75 patients are shown in Table 1.

Table 1 - *The clinical, epidemiological, and histological data of the 75 patients participating in the study.*

Complete ablation group A				
Sex (no.)	Age on diagnosis median (range)	Histology (no.)	Maximum tumor diameter (mm) (mean±SD)	Stage* (no.)
Male (4)	50 (10-60)	PTC (3) FTC (1)	17.75±18.39	Stage I (2) Stage II (1) Stage III (1) Stage IV (0)
Female (37)	48 (18-73)	PTC (32) FTC (4) PTC and FTC (1)	11.72±10.20	Stage I (32) Stage II (3) Stage III (2) Stage IV (0)
Residual thyroid tissue group B				
Sex (no.)	Age on diagnosis median (range)	Histology (no.)	Maximum tumor diameter (mm) (mean±SD)	Stage* (no.)
Male (8)	51 (34-67)	PTC (6) FTC (1) HCC (1)	24.12±12.36	Stage I (4) Stage II (2) Stage III (2) Stage IV (0)
Female (26)	48 (22-71)	PTC (25) FTC (1)	12.46±11.99	Stage I (22) Stage II (3) Stage III (1) Stage IV (0)

*2002 size of primary tumor, regional nodal metastases, distant metastases (TNM) classification. PTC: papillary thyroid carcinoma; FTC: follicular thyroid carcinoma; HCC: Hürthle cell carcinoma.

Follow-up

Follow-up examination consisted of periodic chest radiography, iodine-131 WBS, serum Tg measurements, and serum TPO and Tg antibody measurements in patients not receiving L-T_4 (L-T_4 suppression therapy withdrawal). Complete ablation of thyroid tissue was defined by negative iodine-131 WBS, serum Tg levels <2 ng/ml in patients with normal Tg antibody levels having negative echographic and imaging findings. Patients with recurrent or metastatic disease during long-term follow-up were excluded from the study.

Thyroglobulin, thyroid peroxidase and thyroglobulin antibodies detection assays

Tg levels in all patients were measured using radio-immunometric assay (RIA). Since bi-directional (underestimation or overestimation of Tg levels) anti-Tg interference is possible with Tg RIA measurement, serum Tg was not used as a marker of persistent thyroid tissue in patients with high Tg antibody levels (no recovery tests could be carried out and no IRMA equipment was available). TPO and Tg antibodies during the first 6 yr' follow-up were measured by passive agglutination, during the following 10 yr by ELISA method and during the last 2 yr by chemiluminescence assay. Only patients with high titers of anti-Tg and/or anti-TPO, if confirmed in a second test were considered eligible. Patients with borderline thyroid autoantibody levels were excluded from the study in order to avoid both false positive and negative results.

Statistical analysis

Kaplan-Meier statistical method of estimating "survival" was used to calculate the time needed for 50% of patients to become anti-TPO and/or anti-Tg seronegative. The probability (p_i) of a patient of remaining seropositive (defined by the last follow-up data entry time that a patient was seropositive) at a month i was estimated by the formula: $p_i=1-q_i$, whereas the probability of seroconversion (defined by the first data entry time that a patient had normal antibody levels) each time a patient became seronegative was estimated by the formula: q_i=dini (d_i is the number of seroconvertions and n_i is the number of seroconverted patients). The standard error of cumulative seropositivity estimate S_i was worked out using the formula: $SE(S_i)^2=\Sigma\, d_i/n_i(n_i-d_i)$. The Logrank test was used to compare "seroconversion" curves in both group A and B, male and female subjects, patients < or >45 yr of age, patients' tumor size equal or less than 2 cm, between 2,1-4 cm and >4 cm, patients with PTC, FTC, HCC and PTC+FTC and in patients with tumor stage I, II, III. An approximate chi-square test, with 1 or 2 degrees of freedom was used to test the null hypothesis that the "seroconversion" distributions were the same in comparing groups. Chi-square test with >2 degrees of freedom was used for the comparison of more than two categorical stratification variables (strata analysis). Calculations were performed using the Number Cruncher Statistical System and Power Analysis System (NCSS/PASS 2004, Dawson Edition) and Statistical Package for Social Sciences (SPSS V.10). $p<0.05$ was considered statistically significant.

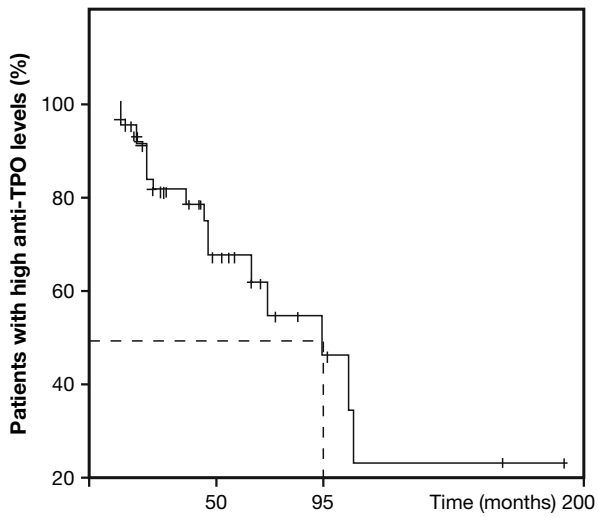


Fig. 1 - *Survival plot showing the percentage of thyroid peroxidase autoantibodies (anti-TPO) seropositive patients over time.*

RESULTS

Thyroid peroxidase autoantibodies level reduction

Fifty-nine patients had high anti-TPO levels preoperatively. The median time to disappearance of TPO antibodies was 95 months (standard error 18 months, 95% confidence interval: 61-129 months, range 12-192 months), as shown in Figure 1.

No statistically significant differences in median time to disappearance of anti-TPO were observed between patients' group, age, sex, histological type of follicular cell derived cancer, tumor size and stage, as shown in Table 2.

Table 2 - Post-hoc *comparisons in thyroid peroxidase autoantibodies (anti-TPO) seroconversion curves.*

	Patient' group		Age (yr)		Sex		Histology			Tumor size** (cm)			Stage		
	A	B	<45	≥45	Male	Female	PTC	FTC	HCC	0-2	2.1-4	>4	I	II	III
Median time to disappearance (months)	72	107	115	95	120	95	95	156*	–	95	107	48	105	48	72
Statistical significance (Logrank test) (p)	0.1793		0.6813		0.4852		0.6513			0.3257			0.9494		

*The mean time to disappearance of anti-TPO was estimated because 4 out of 5 patients were censored. **According to 2002 size of primary tumor, regional nodal metastases, distant metastases (TNM) classification.

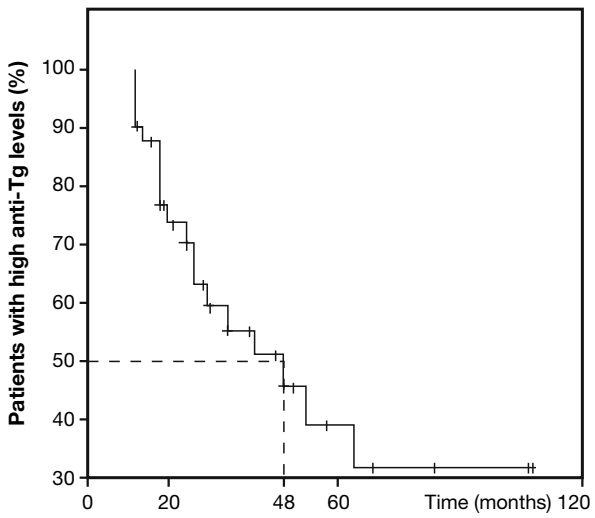


Fig. 2 - *Survival plot showing the percentage of thyroglogulin autoantibodies (anti-Tg) seropositive patients over time.*

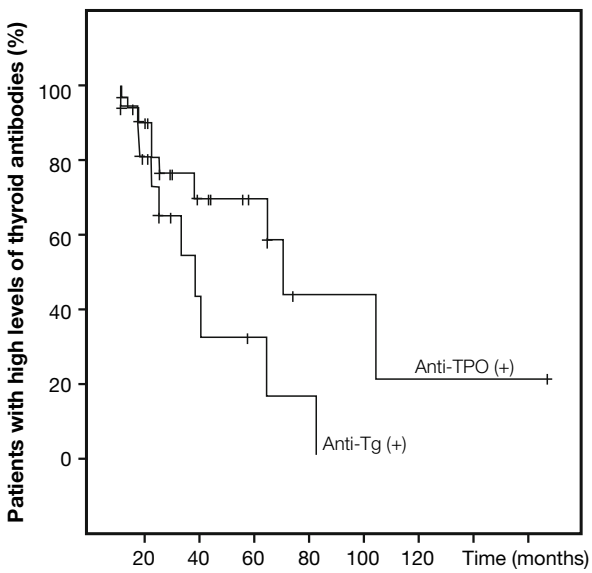


Fig. 3 - *Difference in survival plots between thyroid peroxidase autoantibodies (anti-TPO) and thyroglobulin autoantibodies (anti-Tg) seropositive patients over time.*

Thyroglobulin autoantibodies level reduction

Forty-one patients had high anti-Tg levels preoperatively. The median time of disappearance of Tg antibodies was 48 months (standard error 11 months, 95% confidence interval 26-70 months, range 12-108 months), as shown in Figure 2.
No statistically significant differences in median time of disappearance in anti-Tg were observed between patients' group, age, sex, histological type of follicular cell derived cancer, tumor size and stage, as shown in Table 3.

Thyroid antibody reduction in patients with complete ablation of thyroid tissue following iodine-131 administration

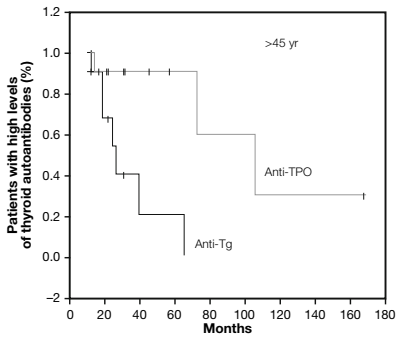
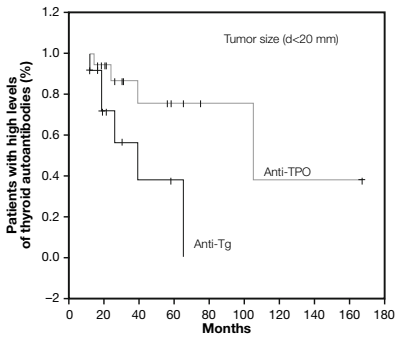
A statistically significant difference was observed between median time (72 months) of disappearance of TPO antibodies and median time (39 months) of disappearance of Tg antibodies in patients with complete ablation of thyroid tissue (group A) following iodine-131 administration (p=0.0395, Logrank statistic 4.24), as shown in Figure 3.
Furthermore, in the same group of patients (group A) with complete ablation of thyroid tissue following iodine-131 ablation, a statistically significant difference was observed between median time (106 months) of disappearance of TPO antibodies and median time (33 months) of disappearance of Tg antibodies in patients >45 yr of age (p=0.034, Logrank statistic 5.09), but not in patients <45 yr of age. In addition, a statistically significant difference was observed between median time (111 months) of disappearance of TPO antibodies and median time (41 months) of disappearance of TPO antibodies in patients with tumor size <2 cm (p=0.0175, Logrank statistic 5.64), as shown in the Table 4.
No further statistical differences in the pattern of anti-Tg and anti-TPO reduction were observed

Table 3 - Post-hoc *comparisons in thyroglobulin autoantibodies (anti-Tg) seroconversion curves.*

	Patient' group		Age (yr)		Sex		Histology			Tumor size* (cm)			Stage		
	A	B	<45	≥45	Male	Female	PTC	FTC	PTC+FTC	0-2	2.1-4	>4	I	II	III
Median time to disappearance (months)	41	48	41	53	80*	41	48	12	–	53	41	48	41	39	24
Statistical significance (Logrank test) (p)	0.8932		0.8722		0.2405		0.7022			0.9050			0.8286		

* The mean time of disappearance of anti-Tg was estimated because 5 out of 7 patients were censored. **According to 2002 size of primary tumor, regional metastases, distant metastases (TNM) classification system.

Table 4 - *Differences in thyroglobulin autoantibodies (anti-Tg) and thyroid peroxidase autoantibodies (anti-TPO) pattern of disappearance according to tumor size and age of patients with complete ablation of thyroid tissue following iodine-131 administration.*

	Age				Tumor size					
	<45 yr		>45 yr		<20 mm		21-40 mm		>40 mm	
	anti-Tg	anti-TPO	anti-Tg	anti-TPO	anti-Tg	anti-TPO	anti-Tg	anti-TPO	anti-Tg	anti-TPO
Mean time to disappearance (months)	50	54	33	106	41	111	24	47	34	41
Diagrams										
Logrank test	*p*=0.024				*p*=0.0175					

concerning patients' group, sex, histological type of DTC and tumor stage (size of primary tumor, regional nodal metastases, distant metastases (TNM)).

DISCUSSION

In patients with a history of DTC, pre-operative thyroid autoantibody production could be stimulated by pre-existent autoimmune thyroid disease (AITD) (8), antigen exposure to immune cells following tissue infiltration and destruction by the tumor itself and neoantigen expression in the form of chimeric proteins, which present an unusual altered self target for T cell recognition and may promote thyroid autoimmunity (9). Antibody production by differentiated CD19+ (plasma cells) requires sufficient exposure of thyroid autoantigens in antigen presenting cells (APC), mainly B cells, dendritic cells and macrophages. With the exception of human leucocyte antigen (HLA) class I restricted exogenous "non-self" antigen presentation (10), it has been suggested that the first step for the entire process is inappropriate autoantigen presentation in the context of HLA class II expression, in the form of HLA-DR molecules newly expressed on thyroid epithelial cells, in patients with thyroid autoimmune responses (11). Helper and/or cytotoxic T cells with Th1-like and/or Th2-like responses are involved in anti-Tg and anti-TPO production, but their specific pathogenic roles remain to be clarified. What is more likely to follow is thyroid cell killing by T cell-mediated and antibody-dependent cell-mediated cytotoxicity induced by anti-TPO (3), although apoptosis may play a significant role in the final pathway of thyroid cell destruction (12). Lymphoid infiltrates in the thyroid gland often organize themselves as follicle-like structures containing germinal centers similar to those in secondary lymphoid follicles of the corresponding regional lymph nodes or bone marrow. Both intra- and extra-thyroidal lymphoid follicles with germinal centers are crucial sites, not only for the production of thyroid autoantibodies but also for the development and maintenance of autoimmune response, because they are the sites where lymphocytes undergo somatic hypermutation and affinity maturation (13). Within lymphoid tissue, thyroid antibody-secreting cells are found mainly in extra-follicular sites, such as the red pulp of the spleen and the medulla of the lymph nodes. These cells also migrate to the bone marrow, which may be a major site of antibody production 2 to 3 weeks following exposure to thyroid antigens. Thyroid autoantibody-specific producing plasma cells in extra-thyroid tissues are probably the main cell population responsible for the gradual disappearance of anti-Tg and anti-TPO in patients with complete thyroid tissue removal, and this will be further discussed below.

In patients being studied, high thyroid autoantibody levels were persistently detected for >16 yr, following complete thyroid tissue ablation. The pattern of

Table 5 - *Possible reasons for extrathyroidal autoantibody production in patients with total thyroid tissue ablation.*

Suggested reason	Mechanism	Limitations	Ref.
Undetectable amounts of thyroid autoantigens, which may be displayed on follicular dendritic cells for months or years, may continually stimulate the production of thyroid autoantibodies by thyroid-specific memory B cells	Memory B cells typically bear high-affinity (mutated) autoantigen receptors and Ig molecules of switched isotypes more commonly than do naïve B lymphocytes. The production of large quantities of isotype-switched, high affinity thyroid autoantibodies by memory B cells is greatly accelerated after secondary exposures to thyroid autoantigens	The production of variant forms of Tg by some tumors may not always be detected by immunoassays. Low levels of thyroid antigens not detected by techniques with high sensitivity may be sufficient for memory B cells immune responses	15, 16
Tg production by blood cells	Alternative or illegitimate Tg gene transcription by circulating blood cells	Unknown if such Tg transcripts can maintain memory B cells immune responses	17
B cell infection from EBV	A large proportion of circulating EBV infected B cell precursors from healthy subjects are able to produce antibodies against Tg and thyroid microsomal antigen (TPO). High prevalence of EBV infection in normal subjects	Antibodies are polyreactive; bound to a variety of self and exogenous antigens and have lower affinity than monoreactive monoclonal antibodies	18, 19
Molecular mimicry or cross-reaction with some *Escherichia coli* proteins	TPO peptide 114-126 and Tg peptide 2746-2765 share a common epitope, which is also present in some *E. coli* proteins	It has not been proven that these homologies have a pathogenetic role in thyroid autoantibody production	20
Molecular mimicry with *Candida albicans*	Treatment of thyroid antibody positive sera with *Candida albicans* causes reduction in thyroid antibody levels	More *in vivo* and *in vitro* studies are necessary	21
Cross reaction with chronic *Helicobacter pylori* infection	The chronic immune response induced by *H. pylori* activates cross-reactive B and T lymphocytes against thyroid antigens	Sufficient amount of thyroid antigens are requisite for cross-reactive immune response	22

Tg: thyroglobulin; EBV: Epstein-Barr virus; TPO: thyroid peroxidase.

gradual thyroid antibody disappearance over time could not only be attributed to the removal of intrathyroid lymphocytes. If such a suggestion was the main reason for the decrease in autoantibody concentration, a sharper curve would be expected. On the other hand, minimal remnant detection of thyroid tissue requires more sensitive imaging and laboratory tests, not yet available in the clinical routine (14), as has already been proposed by Chiovato et al. (7). Furthermore, it is not known whether limited (<2 ng/ml) but detectable levels of circulating Tg can maintain immune responses concerning anti-Tg production. Some of the factors, which potentially could be involved in extra-thyroidal anti-Tg and anti-TPO production, are summarized in Table 5 (15-22).

The quality of the thyroid autoimmune response is characterized by different parameters including the concentration and persistence of thyroid autoantibodies, as well as their affinity. Until now, no data are available about the immunologic factors, which could influence the long-lasting thyroid autoantibody production. Furthermore, the factors responsible for the prolonged biological half-life of TPO and anti-Tg have not yet been established. In the present study, subjects with recurrent or metastatic disease were excluded from group A and complete removal of thyroid antigens could be certified by negative imaging studies during long-term follow-up, low Tg levels in anti-Tg negative individuals and gradual disappearance of thyroid autoantibodies. Patients with similar imaging and biochemical characteristics could be ideal candidates to investigate some of the prominent factors involved in extrathyroidal specific B cell autoantibody production, such as the presence of complement C3b-antigen complexes (considering that anti-TPO can fix complement) (23), the chemical composition of the corresponding epitope (24), the density of CD19 expression on B lymphocytes (25) as well as the impact of CD40 stimulation on memory B cells (26).

Chiovato et al. (7) observed a different pattern of decrease between anti-Tg and anti-TPO longstanding production following total thyroid tissue ablation in patients with DTC and our observations confirm these data. No data are available to support the sharper decrease in anti-Tg production in comparison to anti-TPO. The lack of detectable serum Tg even in the presence of demonstrable disease may be accounted to dedifferentiation of thyroid cancer cells with loss of Tg production and to the production of an

Table 6 - *Possible reasons for the difference between thyroglobulin autoantibodies (anti-Tg) and thyroid peroxidase autoantibodies (anti-TPO) pattern of disappearance in patients with total thyroid tissue ablation.*

Possible reasons	Mechanism	Limitations	Ref.
Degree of sequence similarity between TPO and MPO	Alignment studies and structural homologies have shown that three distinct discontinuous domains form TPO: a MPO-like, a CCP-like, and an EGF-like domain. Three restricted regions (353-363, 377-386, 713-720) in the MPO-like domain and region 766-775 in the CCP-like domain delineate the immuno-dominant binding surface on the three dimensional structure of TPO. Memory B cells could be stimulated by release of small amounts of MPO	The autoimmune response to TPO is focused to two dominant conformation determinants. TPO Abs from patients' sera preferentially recognize discontinuous epitopes on TPO. It is not known if cross reactivity between TPO conformational epitopes and MPO linear homology epitopes is sufficient to maintain memory B cells immune responses	29, 30
Magnitude of CD4+ activation and duration of immune responses concerning thyroid autoantibody production	Whether strong CD4+ activation will occur depends on several crucial factors: HLA-II-peptide complex dissociation constant, affinity of the TCR for the HLA-II-peptide complex, the density of TCR and HLA-II-peptide, expression of co-receptors, adhesion molecules and co-stimulatory molecules on the interacting cells	Although anti-TPO and anti-Tg have similarly high affinities for their respective autoantigens, no data are available for HLA-II-peptide interaction (provided that the peptide contains the allele specific anchor motifs) or HLA-II-peptide and TCR interaction in patients with DTC and pre-existent AITD. Studies are necessary for further evaluation	31, 32
Tumor Tg reduced antigenicity	Weak immune responses to tumor Tg epitopes are consistent with previous reports that tumor Tg has reduced antigenicity as a result of lower iodine content	Unknown if this lower iodine content is different in pre- and post-thyroidectomized patients	33

MPO: myeloperoxidase; CCP: complement control protein; TCR: T-cell receptor; HLA: human leucocyte antigen; Abs: antibodies.

abnormal form of Tg, which is not measured by available immunoassays (27). It could be supposed that undetected minimal thyroid remnants or metastatic micro-foci which maintain the ability to transport and oxidize iodine but have lost the ability of Tg production (28) could be a possible reason for the aforementioned different pattern of thyroid autoantibody level decrease, but the prevalence of this phenomenon is rare and in patients being studied seems unlikely. Although exclusion of statistical error type II or I would be certified in a larger cohort of subjects, we observed a sharper decrease of anti-Tg levels in older patients (>45 yr) with total thyroid antigen removal compared to younger patients (<45 yr old) or to anti-TPO levels in the same age group (>45 yr). Similarly, the mean time of disappearance of anti-Tg was inversely related to d_{max} of ablated tumor. No data are available to give reasons for these observations. Since increased prevalence of thyroid autoantibodies has been observed in patients with DTC (5), an enhanced presentation of thyroid tumor autoantigens to the immune system of studied groups could be a reasonable explanation. Some of the underlying immunological factors, which could be involved in the sharper disappearance of anti-Tg are shown in Table 6 (29-33).

In conclusion, patients with DTC and pre-surgical elevated titers of both Tg and anti-TPO tend to become earlier anti-Tg seronegative. Although tumor size and age may influence the pattern of thyroid autoantibody reduction, the exact reasons for the different rhythm of autoantibodies decrease must further be evaluated.

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