

MARKERS OF THYROID AUTOIMMUNITY

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Summary: Autoimmune thyroid diseases (AITD), the most common endocrine disorders affecting humans, cause cellular damage and alter the thyroid gland function by humoral and cell-mediated mechanisms. Three principal thyroid autoantigens are involved in the development of AITD: thyroperoxidase (TPO), thyroglobulin (Tg) and thyroid stimulating hormone receptor (TSH-R). Other autoantigens have also been described, but as yet their diagnostic role in thyroid autoimmunity has not been established. Laboratory tests that determine the cell-mediated aspects of the autoimmune process are not currently available. However, tests of the humoral response, i.e. thyroid autoantibodies, can be assessed in most clinical laboratories. Thyroid autoantibodies are valuable for classification and reflect disease activity and progression. Lately, TPO antibodies assays have been used for the assessment of risk for developing AITD. Thyroid autoantibodies measurement is hampered by method specificity and sensitivity problems, as well as suboptimal standardization. Despite that, autoantibody tests have inherent clinical utility in a number of clinical situations.

Key words: autoimmune thyroid diseases, TPO, Tg, TSH-R, thyroid autoantibodies, clinical utility

Introduction

Activation of the immune response can be protective, as in infectious diseases, or destructive, as in autoimmune inflammatory diseases, or both. The immune response usually involves the activation of both T and B cells. Antibodies produced by the latter can be detected in sera and used to guide the clinical management of certain diseases. Here, we focus on autoimmune thyroid diseases (AITD), the most common organ-specific diseases affecting humans, i.e. laboratory support for the diagnosis and monitoring of thyroid diseases.

There are three intimately related syndromes associated with thyroid autoimmunity: 1) Graves' disease with goiter, hyperthyroidism and, in many patients, associated ophthalmopathy, 2) Hashimoto's thyroiditis with goiter and euthyroidism or hypothyroidism, and 3) primary thyroid failure or myxedema. Many variations of these syndromes are also recognized, including transient thyroid dysfunction occurring independently in pregnancy and 5–6% of postpartum

women, neonatal hypothyroidism and neonatal hyperthyroidism. The syndromes are bound together by their similar pathology, similar immune mechanisms, co-occurrence in the same family and transition from one form to another in the same individual over time. The immunological mechanisms involved in these three diseases must be closely related, while the phenotypes probably differ because of the specific type of immunological response that occurs (1).

Autoantigens in autoimmune thyroid disease

Three principal autoantigens are involved in AITD. These are: thyroglobulin (Tg), thyroperoxidase (TPO) and thyroid stimulating hormone receptor (TSH-R).

First to be recognized was *thyroglobulin*, the most abundant thyroid protein. Tg is the high molecular weight (670 kD) protein synthesized in thyroid cells in which triiodothyronine (T3) and thyroxine (T4) are produced. It is a soluble glycoprotein made up of two identical subunits, present with a high degree of heterogeneity due to differences in post-translational modifications (glycosylation, iodination, sulfation etc). During the process of thyroid hormone synthesis and release, Tg is polymerized and degraded. Consequently, the immunologic structure of Tg is extremely complex. The characteristics of Tg preparations may

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vary widely depending on the starting human thyroid tissue and the purification process used (2). The proteins prepared from different thyroid glands, especially those with Graves' disease and thyroid malignancy, react differently with polyvalent rabbit anti-Tg antiserum (3), suggesting that the fine structure of Tg differs from person to person. These are first clues explaining why Tg antibodies assays, as well as Tg assays, are so difficult to standardize. Four to six B cell epitopes of Tg are known to be involved in the human autoimmune response. Antibodies appear to recognize the conformation of large fragments of Tg, whereas T cells recognize peptide segments and their primary structure. The autoimmune response to Tg appears to play a lesser role in human thyroid autoimmunity than in animal models of thyroiditis (4).

Thyroperoxidase, the primary protein involved in thyroid hormonogenesis, was initially identified as the »thyroid microsomal antigen«. TPO is a 107 kD, 933-amino acid residue cell-surface (membrane bound) glycoprotein. It has a single membrane-spanning segment and is present as a dimer on the apical surface of the thyroid follicular cell. Extracellular domain is large, while transmembrane and intracellular domains are short. TPO is involved in thyroid hormone synthesis at the apical pole of the follicular cell. Several isoforms related to differential splicing of TPO RNA have been described. TPO molecules may also differ with respect to their three-dimensional structure, extent of glycosylation and heme binding. Most of the TPO molecules do not reach the apical membrane and are degraded intracellularly (2). A stop codon introduced at the TPO ectodomain-plasma membrane junction converts the 933-amino acid membrane-associated molecule into an 845-residue secreted protein. Patients' autoantibodies recognize the TPO ectodomain to the same extent as the holoenzyme (5).

Thyroid stimulating hormone receptor, G protein-coupled receptor with seven membrane-spanning segments, is closely related to the receptors for the other glycoprotein hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (6). Activation of G proteins by the hormone receptor complex results in the stimulation of cyclic AMP production by adenylate cyclase and inositol phosphate turnover by phospholipases. TSH-R contains two subunits, an extracellular A subunit and a largely transmembrane B subunit, linked by disulfide bonds. TSH-R cleavage into A and B subunits has been found to be associated with the loss of an intervening C peptide segment corresponding approximately to a 50-amino acid »insertion« uniquely present in the TSH-R and not present in the noncleaving LH and FSH receptors (7). Of potential importance in the immune response to TSH-R is the fact that most A subunits are shed from the surface following cleavage (8). The 60kb TSH receptor gene located on the long arm of chromosome 14q31 has been cloned and sequenced. Exons 1–9 code for the extracellular domain of the receptor (397 amino acids) and exon 10 codes

for the transmembrane region (206 amino acids) (9). Site-directed mutagenesis has shown that the 3-dimensional receptor structure is important for the interaction with TSH and/or TSH-R antibodies (2).

Other autoantigens, such as the sodium iodide symporter (NIS), have also been described, but as yet their diagnostic role in thyroid autoimmunity has not been established (10). Antibodies to a variety of other thyroid cell components are also occasionally present in AITD, including antibodies that react with T4 or T3 (11), antibodies reacting with tubulin, megalin and calmodulin, and antibodies reacting to DNA or DNA-associated proteins (12–14). Antibodies that have T4 and T3 binding activity and represent another response to the Tg antigen do not alter the thyroid function, but can cause confusion in diagnosis due to artifacts in T4 and T3 assays.

Autoantibodies

Humoral response in AITD leads to the production of TPO, Tg and TSH-R antibodies. During the initiation of AITD, thyroid autoantibody formation presumably occurs in lymph nodes draining the thyroid. In fully developed AITD, the thyroid is clearly an important source of autoantibodies. Since there are relatively few circulating specific autoantibody-secreting B cells, it has been suggested that autoantibody formation occurs mainly or uniquely in the thyroid (15). However, lymph nodes, bone marrow, and possibly other organs, also contribute to the autoantibody production and this explains why patients with apparently destroyed thyroid tissue, or those with resected thyroids, continue to have circulating thyroid autoantibodies (16).

The nomenclature used for the principal thyroid autoantibodies has proliferated, particularly in the case of TSH-R antibodies (LATS, TSI, TBII, TSH-R and TRAb). Terms recommended internationally are: for TPO antibodies–TPOAb, Tg antibodies–TgAb and TRAb for TSH-R antibodies. These terms correspond to the molecular entities (immunoglobulins) which react with the specified autoantigens recognized by the laboratory test.

Observation of a factor in the serum of patients with Graves' disease causing long-acting stimulation of thyroid hormone release from the thyroid, in contrast to the short-acting stimulation produced by TSH, led directly to the knowledge about TRAb. These antibodies are currently separated into three broad types. Some antibodies bind in TSH-R and activate the receptor, producing the same effects as TSH, by inducing post-receptor signal transduction and cell stimulation, in particular causing the generation of cyclic AMP. These antibodies may be referred to as TSAb – thyroid stimulating immunoglobulins or thyroid stimulating antibodies. TSAb appear to bind the N-terminal portion of the extracellular domain. Other antibodies bind to different, or the same, epitopes and interfere with radiolabeled TSH binding in

certain assays – thus they are known as TSH binding inhibitory immunoglobulins (TBII). The C-terminal region is more important for the TSH receptor blocking antibodies (abbreviated TBAb or TSBAb) which block stimulation by either TSAb or TSH, causing hypothyroidism. These may have other less well characterized inhibitory effects (17). Numerous other names are used.

Clinical utility of thyroid autoantibodies

Laboratory tests that determine the cell-mediated aspects of the autoimmune process are not currently available. However, tests of the humoral response, i.e. thyroid autoantibodies, can be assessed in most clinical laboratories.

Since in autoimmune diseases the immune response is itself part of the disease process, it is possible to use autoantibodies as markers of disease activity and severity. In patients with established disease, autoantibodies can help define the nature of the disease and provide markers to classify the disease. Under some circumstances, autoantibodies should be able to predict disease. This approach is especially promising for diseases with a long preclinical period, a feature of many organ-specific autoimmune diseases like AITD. Thyroid autoantibodies can reflect disease activity and progression and be valuable in disease prediction and classification (18).

According to the recommendations made by the National Academy of Clinical Biochemistry (NACB) of the United States, *TPOAb* should be measured:

- for diagnosis of AITD;
- as a factor for AITD;
- as a risk factor for hypothyroidism during Interferon alpha, Interleukin-2 or Lithium therapy;
- as a risk factor for thyroid dysfunction during amiodarone therapy;
- as a risk factor for hypothyroidism in Down's syndrome patients;
- as a risk factor for thyroid dysfunction during pregnancy and for postpartum thyroiditis;
- as a risk factor for miscarriage and *in vitro* fertilization failure.

TPOAb is the most sensitive test for detecting autoimmune thyroid disease. *TPOAb* is typically the first abnormality to appear in the course of developing hypothyroidism secondary to Hashimoto's thyroiditis. When *TPOAb* is measured by a sensitive immunoassay, >95% of subjects with Hashimoto's thyroiditis have detectable levels of *TPOAb*, as do most (~85%) patients with Graves' disease (19).

Autoantibodies rarely develop before 20 years of age, but they may presage subsequent clinical disease (primary hypothyroidism) (20). The appearance of *TPOAb* usually precedes the development of thyroid dysfunction. Longitudinal studies suggest that

TPOAb may be a risk factor for future thyroid dysfunction. According to the 20-year follow-up study of the Whickham cohort, the probability of developing overt hypothyroidism within the next 20 years in women who are *TPOAb* negative with the TSH levels lower than 2 mU/L is less than 5%. That probability increases logarithmically to 55% when the TSH is greater than 6 mU/L in *TPOAb* positive subjects, with annual rate of progression of 4.3%, as compared with just 2.6% for those with only elevated TSH and 2.1% for those with only *TPOAb*. Higher rates of progression have been found in men – who are five times more likely than women to progress to overt disease; in women aged 45 years or older; in patients with TSH levels greater than 20 mU/L (21).

TPOAb may be a risk factor for future thyroid dysfunction including the development of autoimmune complications from treatment by a number of therapeutic agents, amiodarone therapy for heart disease, Interferon-alpha therapy for chronic hepatitis C and Lithium therapy for psychiatric disorders (22–24). Patients with Down's syndrome have an increased risk of autoimmune thyroid dysfunction, and annual screening for TSH and *TPOAb* is therefore recommended (25). Patients with *TPOAb* detected in early pregnancy are at risk of developing postpartum thyroiditis (26). Recent reports have suggested that the IQ of children born to mothers with increased TSH and/or detectable *TPOAb* during pregnancy may be compromised (27). This has prompted recommendations that all pregnant women should have TSH and *TPOAb* levels measured in the *first trimester* of their pregnancy. Since high *TPOAb* levels are associated with a high risk of miscarriage and failure to conceive with *in vitro* fertilization, *TPOAb* measurements may have a role in infertility (28).

The use of thyroid autoantibody measurements for monitoring the treatment of AITD is generally not recommended, since treatment of AITD addresses the consequence (thyroid dysfunction) and not the cause (autoimmunity) of the disease. However, changes in the autoantibody concentrations often reflect a change in disease activity (29).

NACB recommendations for *TgAb* measurement are divided, depending on patient diagnosis.

In non-neoplastic conditions:

- in iodide sufficient areas, it is not usually necessary or cost-effective to order both *TPOAb* and *TgAb* measurement, because *TPOAb*-negative patients with detectable *TgAb* rarely display thyroid dysfunction;
- in iodide deficient areas, serum *TgAb* measurements may be useful for detecting autoimmune thyroid disease when patients have a nodular goiter;
- monitoring iodide therapy for endemic goiter.

In Differentiated Thyroid Carcinomas (DTC):

- *TgAb* should be measured in every serum specimen sent to the laboratory for *Tg* testing;

- serial TgAb measurements should be made on all TgAb-positive DTC patients using the same manufacturer's method because serial TgAb values have prognostic significance for monitoring response to DTC treatment;

- TgAb method should be an immunoassay, not agglutination, and serial measurements must be quantitative, not qualitative;
- serum Tg recovery tests do not reliably detect the presence of TgAb and should be discouraged as a method for detecting TgAb;
- before changing the TgAb method, the laboratory should inform the physicians using it and evaluate the relationship between the old and proposed new method values. Patients should be re-baselined if the difference between the methods is >10% CV.

There is some debate over the clinical utility of serum TgAb measurement for assessing the presence of thyroid autoimmunity. The United States NHANES III study reported that 3 % of subjects with no risk factors for thyroid disease had detectable TgAb without the associated presence of TPOAb (30). Since this cohort had no associated TSH elevation, TgAb measurements do not appear to be a useful diagnostic test for AITD in areas of iodide sufficiency (31). In iodide deficient areas, however, TgAb is believed to be useful for detecting AITD, especially for patients with nodular goiter. TgAb measurements are also useful for monitoring iodide therapy for endemic goiter, since iodinated Tg molecules are more immunogenic.

Serum TgAb testing is primarily used as an adjunct test when serum Tg measurements are requested. The clinical utility of TgAb measurements in sera from DTC patients is two-fold. First, sensitive and specific TgAb screening of sera in these cancer patients is necessary, because even low antibody concentrations can interfere with the Tg measurements made by most Tg methods. Second, serial TgAb measurements themselves may serve as a surrogate tumor marker test for TgAb-positive patients in whom Tg testing may be unreliable. Specifically, TgAb-positive patients who are rendered disease-free typically become TgAb-negative within 1–4 years. In contrast, patients who have persistent disease after treatment retain detectable TgAb concentrations. In fact, a rise in the TgAb level is often the first indication of recurrence in such patients (32).

As with TPOAb methods, because both older and newer methods are still being used concurrently in clinical laboratories, the sensitivity and specificity of available methods can vary widely depending on the laboratory.

The NACB recommendations for clinical uses of TRAb measurement are:

- to investigate the etiology of hyperthyroidism when the diagnosis is not clinically obvious;

- a declining TRAb concentration during long-term antithyroid drug therapy is suggestive of remission;

- TRAb measurements are useful to diagnose Graves' disease patients and for relating TRAb values to a treatment algorithm;
- to evaluate patients suspected of »euthyroid Graves' ophthalmopathy;
- for pregnant women with a past or present history of Graves' disease;
- euthyroid pregnant women (\pm L-T4 treatment) who have had prior radioiodide treatment for Graves' disease;
- pregnant women who take antithyroid drugs (ATD) for Graves' disease to maintain a euthyroid state during pregnancy should have TRAb measured in the third trimester;
- the assessment of the risk of fetal and neonatal thyroid dysfunction necessitates the detection of either blocking or stimulating TRAb when mothers have no intact thyroid following past therapy for Graves' hyperthyroidism;
- to identify neonates with transient hypothyroidism due to the presence of TSH receptor blocking antibodies.

The differential diagnosis of hyperthyroidism can be resolved in most patients without resorting to TRAb testing. Nevertheless, the presence of TRAb may distinguish Graves' disease from factitious thyrotoxicosis and other manifestations of hyperthyroidism, such as subacute or postpartum thyroiditis and toxic nodular goiter.

TRAb measurements have also been proposed as a means for predicting the course of Graves' disease. A meta-analysis of the relationship between TRAb levels and the risk of relapse has shown that 25% of patients are misclassified by TRAb assays. This suggests that after ATD therapy, a follow-up of the patients is necessary whatever the TRAb level at the time of AITD withdrawal, and that TRAb measurement is not cost-effective for this purpose (29). There is general agreement that TRAb measurements can be used to predict fetal and/or neonatal thyroid dysfunction in pregnant women with a previous history of AITD. High levels of TRAb in the mother during the *third trimester* of pregnancy suggest a risk of thyroid dysfunction in the offspring. Two to 10% of pregnant women with very elevated TRAb deliver newborns with hyperthyroidism. The risk of neonatal hyperthyroidism is negligible following successful treatment of hyperthyroidism with antithyroid drugs, but can develop after radioiodide treatment if TRAb levels remain elevated. Euthyroid pregnant women (+/- L-T4 treatment) who have had prior radioiodide therapy for Graves' disease should have TRAb levels measured both in early pregnancy, when an elevated value is a significant risk factor for fetal hyperthyroidism, and during the third trimester, to evaluate the risk

of neonatal hyperthyroidism (33). Pregnant women who take ATD for Graves' disease should have TRAb measured in the third trimester. High TRAb levels in such patients should prompt a thorough clinical and biochemical evaluation of the neonate for hyperthyroidism, both at birth (cord blood) and after 4–7 days, when the effects of the transplacental passage of ATD have disappeared (34). It is worth noting that the TBII receptor assays are often used for this purpose since they detect both TSAb and, in rare cases, TBAb/TSBAb which cause transient hypothyroidism in 1:180,000 of newborns (35). It is also advisable to test for both stimulating and blocking antibodies because the expression of thyroid dysfunction may be different in the mother and the infant (36).

Thyroid antibody tests methodology

Thyroid autoantibodies measurement is hampered by method specificity and sensitivity problems, as well as suboptimal standardization. Current methods differ widely in epitope recognition, which results in sensitivity differences. This produces in vastly different reference intervals, even when methods are standardized to the same international reference preparation. Thyroid autoantibodies are clearly not unique molecular entities but, rather, mixtures of immunoglobulins that only have in common their ability to interact with Tg, TPO or the TSH receptor. Differences in the sensitivity of autoantibody tests may arise from the design of the assay (e.g. competitive RIA versus two-site IMA) as well as the physical method used for the signal (e.g. radioisotope versus chemiluminescence). Differences in specificity may occur as a result of contamination of the autoantigen preparation by other autoantigens (Tg and/or variations in the three-dimensional structure of TPO) (2). Beside analytical sensitivity, it is necessary to determine the functional sensitivity and referent ranges of thyroid antibody tests.

Most clinical laboratories use assays for TBII (receptors assays), that merely measure the ability of a serum or IgG preparation, to block the binding of a TSH preparation and do not measure the biological response. Bioassays that use cell preparations to measure the biologic effects of TRAb (stimulation, inhibition of TSH activity or growth) can detect functional changes in TRAb heterogeneity. This fundamental difference in assay design explains why bioassays and receptor assays usually display a weak correlation ($r = 0.31-0.65$) (37–39).

International Reference Preparations, MRC 65/93 for TgAb, MRC 66/387 for TPOAb, are available from the National Council for Biological Standards and Control in London, UK. These preparations

were made from a pool of serum from patients with autoimmune thyroid disease and were prepared and lyophilized 35 years ago. Lyophilized antibodies are prone to degradation over time. Commercial kits contain secondary standards that differ for each method, and vary with the experimental conditions as well as the antigen preparation used by the manufacturer. In the case of TRAb situation is somewhat better – the reference preparation MRC 90/672 is more recent (1990), but currently used by few manufacturers.

Reference values (RV) for TPOAb and TgAb are highly dependent on the sensitivity and specificity of methods used. There appear to be two classes of TPOAb and TgAb immunoassays. One class is characterized by low detection limits (<10 kIU/L) and an undetectable normal reference limit. Such methods suggest that the presence of these antibodies is a pathologic finding. The other class of assays reports higher detection limits (>10 kIU/L) and cites normal reference range. The likelihood is that these detectable »normal range« values merely represent non-specific assay »noise« and may not be pathologically meaningful.

RV for TPOAb assays are highly variable and often arbitrarily established, so that a large majority of patients with AITD test positive, and most subjects without clinical evidence of AITD test negative. Referent ranges should be determined from 120 male subjects, free from any history of thyroid disease (personal or family), no goiter, less than 30 years of age, and biochemical euthyroids (serum TSH levels between 0.5 and 2.0 mIU/L) (2).

In cooperation with the Medical Military Center in Podgorica, we determined the RV for TPOAb. When choosing the reference population, we followed the mentioned recommendations. TPOAb were measured on Elecsys System 2020, Roche Diagnostics GmbH (electrochemiluminescence). The used assay cites RV 0.0–34.0 kIU/L. Subjects were 123 healthy males (age 24.2 ± 3.5 , TSH 1.03 ± 0.41). All subjects had TPOAb values below the detection limit of the test, <5 kIU/L. So, we obtained an undetectable normal reference limit. These data suggest that TPOAb are not normally present in a healthy population and that the used method showed satisfying sensitivity and specificity. The clinical significance of low levels of thyroid autoantibodies in euthyroid subjects is still unknown and remains to be established. Whether individuals with low levels of TPOAb should be considered normal remains in question until long-term follow-up studies on such individuals show that they do not have an increased risk of developing thyroid dysfunction. Results will very much depend on the sensitivity and specificity of the methods employed.

MARKERI TIREOIDNE AUTOIMUNOSTI

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Kratak sadržaj: Autoimune tireoidne bolesti (AITB), najčešći endokrinološki poremećaj kod ljudi, dovode do oštećenja ćelija i promjene funkcije žlijezde humoralnim i ćelijski posredovanim mehanizmima. Tri glavna antigena uključena u razvoj ovih bolesti su: tireoperoksidaza (TPO), tireoglobulin (TG) i receptor tireostimulirajućeg hormona (TSH-R). Opisani su i drugi antigeni, ali za sada nije utvrđen njihov klinički značaj. Laboratorijski testovi koji bi se koristili za procjenu ćelijski posredovanih mehanizama kod AITB još uvijek nisu dostupni. S druge strane, procjena humoralnog odgovora, tj. određivanje tireoidnih antitijela, izvodi se u većini kliničkih laboratorija. Vrijednosti ovih parametara od značaja su za klasifikaciju, a pored toga reflektuju aktivnost i progresiju bolesti štitne žlijezde. U novije vrijeme, određivanje TPO antitijela koristi se za procjenu rizika za razvoj AITB. Mjerenje tireoidnih antitijela još uvijek prati niz problema vezanih za razlike u osjetljivosti i specifičnosti metoda, kao i odsustvo adekvatne standardizacije. Uprkos tome, njihovo određivanje od velikog je značaja za niz kliničkih situacija.

Ključne riječi: autoimune tireoidne bolesti, TPO, TG, TSH-R, tireoidna autoantitijela, klinička upotreba

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