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Serum thyroglobulin: a model of immunoassay imperfection

Serum thyroglobulin is used to monitor and follow-up treatment of differentiated thyroid carcinomas. Available serum thyroglobulin assays are fraught with problems inherent to any immunometric detection system. This review summarises the clinical utility of serum thyroglobulin as a tumour marker, the problems associated with immunoassays for tumour marker detection and quantitation, and potential solutions to some of these problems.

by Dr A. Hoofnagle and Dr M. H. Wener

Thyroid nodules have a prevalence of approximately 4%. Fortunately, only 1 in 1,000 of these turns out to be malignant. A diagnosis of thyroid carcinoma is made using fine needle aspiration biopsy and radioactive iodine uptake scanning. Indeterminate cases require excisional biopsy for definitive diagnosis. Roughly half of patients diagnosed with thyroid cancer require aggressive surgery to remove the entire thyroid gland. These patients are older, have larger tumours, or have regionally or distantly disseminated disease. Following total or near-total thyroidectomy, radioactive iodine (^{131}I) is administered to ablate any remaining thyroid tissue. The loss of normally functioning thyroid tissue is treated with synthetic thyroid hormone at doses high enough to suppress pituitary release of TSH, the major growth factor for thyroid tissue. It is in this clinical setting that serum thyroglobulin can be used as a tumour marker to detect residual, recurrent, or metastatic disease in patients with differentiated thyroid cancer.

Thyroid cancers

Most thyroid cancer is well differentiated and carries a good prognosis. In patients under 45 years of age, for example, less than 1% will die of their disease in the first five years following diagnosis and less than 20% will die of their disease over the next 35 years. Older patients with more advanced disease have a poorer prognosis, however current therapeutic algorithms result in more than 50% 10-year survival with stage III disease. Differentiated tumours with these relatively good prognoses fall into three histological classes. Papillary carcinoma comprises the vast majority (>70%) of malignant thyroid neoplasms in countries with adequate dietary iodine. These tumours commonly metastasise to local lymph nodes, although this local dissemination does not alter prognosis to any great extent. Follicular carcinoma is the next most common tumour and comprises up to 45% of thyroid cancer in iodine deficient areas. It is a more aggressive tumour and can spread to distant sites, including bone and the lungs, via the blood stream. Hürthle cell neoplasms are rare and account for less than 5% of thyroid carcinomas. They are similar to follicular carcinomas in their histological diagnosis and are more frequently malignant than follicular or papillary neoplasms. Most patients (>90%) with these three histological classes of thyroid cancer can be followed with serum thyroglobulin measurements to assess for disease after treatment. Interestingly, up to 50% of undifferentiated anaplastic thyroid carcinomas produce thyroglobulin as well.

Following surgical removal of the thyroid body and radioablation of remaining thyroid tissue, patients are maintained on exogenous thyroid hormone. To detect residual disease, thyroid hormone is withheld for two weeks and the patient is maintained on a low-iodine diet to elevate serum concentrations of thyroid stimulating hormone (TSH) to more than 50 mIU/L. The presence of TSH in the plasma promotes uptake of radioactive iodine by any remaining thyroid tissue. This tissue, if present, can then be detected by full body scanning. In addition, serum thyroglobulin levels are determined, with values greater than 4 ng/mL suggestive of residual disease. In patients with detectable Tg and negative scans, other imaging modalities, including ultrasonography, computed tomography, positron emission tomography, and magnetic resonance imaging may be able to detect resectable disease. Regardless, patients with positive uptake scans or with significantly elevated thyroglobulin levels (>= 4ng/mL) receive high dose ^{131}I treatment and are followed up at 12 month intervals. At these times, thyroid hormone replacement is withdrawn or recombinant human TSH (rhTSH) is administered and the scan and serum thyroglobulin measurements are repeated. Subsequent surgery or radioablation are used to treat recurrent disease when appropriate. Unfortunately, patients often tolerate hormone withdrawal poorly and the rhTSH used to stimulate uptake and thyroglobulin production is expensive.

Thyroglobulin assays

All thyroglobulin assays currently available are immunoassays. Radioimmunoassays and enzyme linked immunosorbent assays (ELISA) permit detection of this important tumour marker with a sensitivity of 0.01 ng/mL in some systems. More commonly, because radioactivity is being removed from clinical laboratories whenever possible and because they are less expensive to operate, automated "sandwich" immunoassays are used to detect thyroglobulin antigen in serum samples. These automated platforms enable rapid detection, usually with a sensitivity of approximately 0.1 ng/mL, however they suffer from all of the common problems associated with immunoassays. What follows is a discussion of these problems in the context of thyroglobulin detection. However, all of the problems discussed are potential complications in any immunoassay for tumour antigen detection.

Characteristics of thyroglobulin

Before discussing these complications, it is important to point out the unusual characteristics of the thyroglobulin polypeptide. It is a very large macromolecule with an apparent molecular weight of approximately 660 kDa. The predicted mass of one monomer of the homodimeric antigen is only 302 kDa. Post-translational modifications account for the remainder. These post-translational modifications include oxidation and iodination of tyrosine residues, which occurs as thyroglobulin is modified into the thyroid hormones triiodothyronine and thyroxine. Thyroglobulin exists predominantly in the colloid of the thyroid gland where it serves as a reservoir for the thyroid hormones released upon TSH stimulation of the gland. It leaks into the lymphatics surrounding functioning thyroid tissue, which subsequently circulates in the plasma. In addition, thyroglobulin is modified by phosphorylation at serine and tyrosine residues, by sulphation, and by O- and N-linked glycolsylation, which may also be sulphated and phosphorylated. Importantly, around 10% of plasma thyroglobulin mass is composed of variable carbohydrate moieties.

Problems with assays

Development of antibodies for clinical assays starts with antigen selection. Immunisation of animals with purified antigen in the form of whole antigen, domains of the antigen, or peptides from the antigen have all been shown to yield useful antibodies. These antibodies are directed toward antigenic sites, or epitopes, on the surface of the tumour antigen and will bind with high affinity in many cases. The differences in immunogens provide the underpinnings of the first major problem of immunoassays. Thyroglobulin protein present in human plasma represents a heterogeneous mix of protein

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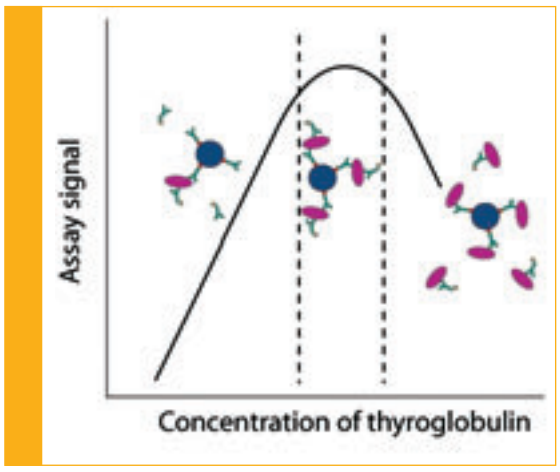

Figure 1. One-step thyroglobulin immunoassays can be characterised by three ratios of antigen to reagent antibody. As the concentration of thyroglobulin increases (left), the successful formation of antigen-antibody sandwich complexes results in increased signal and antibody is in excess. At some point, the concentration of thyroglobulin saturates available antibody binding sites (middle), generating the maximum signal for the system. Additional thyroglobulin prevents successful formation of sandwich complexes (right), reducing the signal detected. This latter situation typifies the hook effect seen in one-step immunoassays.

molecules in which different levels and sites of post-translational modification are present. Even monomers within the homodimer are only identical at the level of the primary sequence of amino acids, and even this can differ after partial cleavage with plasma proteases. For this reason, concentrations of thyroglobulin determined with one assay are not identical to those determined with another assay. It is therefore imperative that patients be followed with the same assay and that the method used in determining serum thyroglobulin concentration be included in the clinical laboratory report in the patient's medical record. While a standard preparation for thyroglobulin was developed in cooperation with the Community Bureau of Reference of the Commission of the European Communities (the standard is named CRM-457), available thyroglobulin assays yield different results for patient specimens. Each of the assays gives nearly identical results for the standard, but may still differ for patient specimens by as much as 4-fold. This is due to the heterogeneity of thyroglobulin in patient specimens.

Automated "sandwich" immunoassays can also suffer from saturation of the analytical antibody binding sites. In single-step assays in which antibody-antigen complexes form in the presence of both the reporter enzyme-conjugated antibody and the selective antibody that is used to separate the antigen-antibody complexes from the unbound reporter antibody, it is possible for very high concentrations of thyroglobulin to bind to all of the antibodies and not successfully form the complex needed for antigen detection [Figure 1]. This is not a problem for radioimmunoassays in which thyroglobulin from the patient sample competes with radiolabelled thyroglobulin for antibody binding sites prior to precipitation of the anti-thyroglobulin antibody. It is also generally not of concern in sandwich assays with a wash step, unless the wash step does not completely remove excess antigen. It should be stressed that while this is a concern for thyroglobulin assays, this so-called "hook effect" would only result in a false negative result in patients with extremely high plasma concentrations of thyroglobulin, e.g. >30,000 ng/mL, which fortunately happens very rarely. However, the prudent laboratory will assess for potential hook effects using dilution of negative patient specimens, recovery of exogenously spiked antigen, or measurement of pooled patient specimens, and compare with expected results.

Besides a lack of standardisation and the hook effect, thyroglobulin assays are also plagued by heterophilic antibodies, which are directed non-specifically toward the reagent antibodies used in the assay. It has been demonstrated that these heterophilic antibodies are present in approximately 3% of patients and can cause low false positive results in most cases and significantly false positive serum thyroglobulin results in others [1]. There are methods available to remove heterophilic antibody interference from patient samples. These usually contain mammalian immunoglobulins at concentrations high enough to bind the antibodies that would interfere with testing. Unfortunately, they are not universally effective and false positives still occur in patients with heterophilic antibodies.

In addition to heterophilic antibodies, patients may also have interfering autoantibodies. Even in the absence of differentiated thyroid carcinoma, up to 10% of subjects have autoantibodies directed toward thyroglobulin, which have been implicated in some forms of autoimmune thyroid disease. Interestingly, a higher percentage of patients, typically about 25%, with malignant thyroid tumours have autoantibodies. The higher prevalence in patients with cancer may be due to altered thyroglobulin

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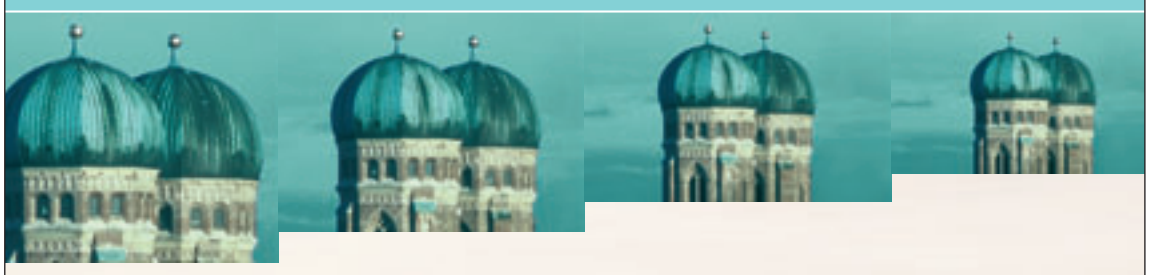

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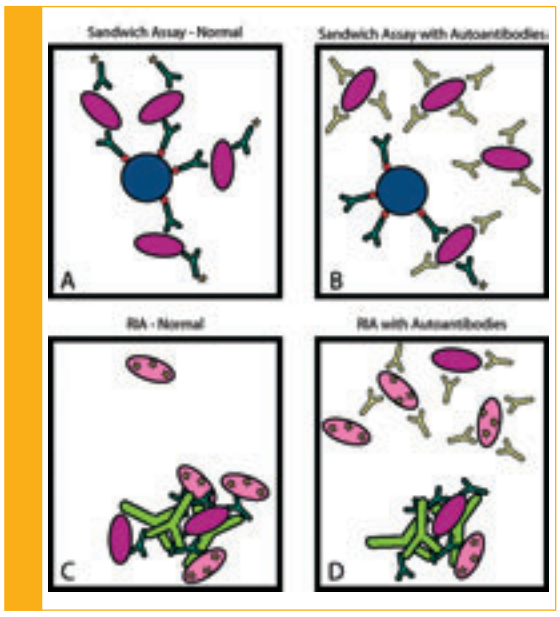

Figure 2. (A) One representative automated thyroglobulin immunoassay uses streptavidin-coated paramagnetic beads (blue circles) to isolate complexes formed by biotin-labelled primary monoclonal antibody (cyan Y with red circles) endogenous thyroglobulin (purple ovals) and enzyme-conjugated secondary monoclonal antibody (cyan Y with yellow stars). (B) Autoantibodies (yellow Y) interfere with complex formation leading to a reduction in signal. (C) Radioimmunoassays quantitate endogenous thyroglobulin as the reduction in radiolabelled thyroglobulin (pink ovals with yellow stars) precipitated with precipitating antibodies (large green Y) competed off with endogenous thyroglobulin. (D) Autoantibodies reduce the amount of precipitated radiolabelled thyroglobulin leading to false elevations in serum thyroglobulin quantitation.

epitopes generated in malignant tissue, thence recognised as foreign by the patient's adaptive immune system. Because thyroglobulin is detected with antibodies that bind to surface epitopes, the presence of autoantibodies may interfere with detection. In fact, depending on the type of assay, the autoantibodies can result in false-positive as well as false-negative results [Figure 2]. For this reason, many laboratories determine the recovery of spiked thyroglobulin from patient samples with autoantibodies. By comparing the assay signal gained from the addition of exogenous purified thyroglobulin to that expected, one can assess the extent to which detection of endogenous thyroglobulin is blocked by interfering autoantibodies. A cut-off of 80% has been suggested as a recovery at which negative results can be interpreted as truly negative. However, there are many examples of autoantibody-positive patients with recoveries greater than 80% and undetectable serum thyroglobulin concentrations in one assay with detectable levels in another assay. For this reason, all negative serum thyroglobulin results in patients with autoantibodies in standard immunoassays should be interpreted cautiously. In addition, low positive results from radioimmunoassays should also be considered carefully.

By focusing on the basic biochemistry of the antigen-autoantibody complexes, one night find a way around the autoantibody problem. There are myriad ways to disrupt protein-protein interactions used regularly in laboratories such as detergents, low or high pH, and high-salt concentrations, to name a few. Indeed, the first assays to detect the p24 antigen as a marker of human immunodeficiency virus (HIV) replication were complicated by antibodies to the p24 antigen itself, antibodies now used to detect HIV seropositivity when screening different patient populations. Two insightful approaches were described to help correct the problem. The first involved a pretreatment step at pH 2.5 with subsequent neutralisation and analysis [2]. The second used a more aggressive strategy [3]. Patient samples were incubated with sodium dodecyl sulphate and diethylenetriaminepentaacetic acid and heated to near boiling for four minutes. These treatments were effective in denaturing the antibodies, but left the p24 epitopes mostly intact, improving the recovery. A number of other approaches have been tried using differences in ionic strength and pH to dissociate interfering antigen-antibody interactions. An interesting study demonstrated that the most efficient method for dissociating chicken IgY antibodies from antigen used high concentrations of magnesium chloride [4]. Our attempts at using many different dissociation methods for thyroglobulin revealed the antigen to be unstable and easily denatured. However, it appears to be relatively stable in the presence of chaotropic ions, which change the hydrogen-bonding network of water and weaken hydrophobic interactions and can often disrupt antigen-antibody complexes. Pretreatment with magnesium chloride or sodium bromide improves the recovery of thyroglobulin in antibody-positive patients.

The utility of pretreatment in tumour marker assays is likely not limited to thyroglobulin. Other tumour markers are known to be immunogenic and trigger production of autoantibodies. One example is the MUC1 gene product, which comprises the CA27.29 and CA 15-3 tumour antigens. It is possible that pretreatment of patient sera in this assay would effectively limit the interference from autoantibodies. Interestingly, at least one manufacturer employs a pretreatment step of 0.08 N sodium hydroxide [5], which may provide a more uniform recovery across clinical specimens.

It is our prediction that the ultimate reference method that completely removes autoantibody interference from tumour marker assays, such as thyroglobulin, will utilise methods not depending solely on antibodies. One approach to this problem would employ tandem mass spectrometry (MS/MS) with stable isotopically labelled internal standards. Digestion of intact or partially purified human serum with trypsin or another protease would liberate peptides from thyroglobulin and degrade interfering immunoglobulins. When peptide digests are introduced via nanoelectrospray ionisation, excellent sensitivity and precision are possible using triple quadrupole MS/MS instruments. Experiments have demonstrated sensitivity in complex plasma digests down to the femtomolar level needed to detect thyroglobulin postoperatively in patients with DTC [6]. Techniques using anti-peptide antibodies to enrich for tumour antigen specific peptides also show great promise [7]. Experiments aimed at establishing this thyroglobulin reference assay will likely yield exciting results.

In summary, thyroglobulin is very useful as a tumour marker in the follow-up of patients diagnosed with differentiated thyroid carcinoma. In conjunction with standard imaging, recurrent, residual, and metastatic disease can be detected and treated accordingly. As with all immunoassays, serum thyroglobulin detection is complicated by several limitations, including interference with heterophilic and autoantibodies, and heterogeneity in antigens and antibodies. Efforts aimed at solving these limitations will benefit patients with thyroid disease and may improve other clinical assays, as well.

References

1. Preissner CM, O'Kane DJ, Singh RJ, Morris JC and SK Grebe. Phantoms in the assay tube: heterophile antibody interferences in serum thyroglobulin assays. J Clin Endocrinol Metab 2003; 88: 3069-74.
2. Nishanian P, Huskins KR, Stehn S, Detels R and Fahey JL. A Simple Method for Improved Assay Demonstrates That HIV P24 Antigen Is Present as Immune-Complexes in Most Sera from HIV-Infected Individuals. Journal of Infectious Diseases 1990; 162: 21-28.
3. Steindl F, Armbruster C, Pierer K, Purtscher M and Katinger HWD. A simple and robust method for the complete dissociation of HIV-1 p24 and other antigens from immune complexes in serum and plasma samples. Journal of Immunological Methods 1998; 217: 143-151.
4. Kummer A and Li-Chan ECY. Application of an ELISA-elution assay as a screening tool for dissociation of yolk antibody-antigen complexes. Journal of Immunological Methods 1998; 211: 125-137.
5. ADVIA Centaur Assay Manual for CA 27.29; Document 11675, Rev. D, 2002-02.
6. Anderson L and Hunter CL. Quantitative Mass Spectrometric Multiple Reaction Monitoring Assays for Major Plasma Proteins. Mol Cell Proteomics 2006; 5: 573-88.
7. Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW and Pearson TW. Mass Spectrometric Quantitation of Peptides and Proteins Using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). J Proteome Res 2004; 3: 235-44.

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