Interference of Iodine-125 Ligands in Radioimmunoassay: Evidence Implicating Thyroxine-Binding Globulin

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Although there is abundant published evidence that radiiodinated antigens interfere in digoxin radioimmunoassays, other radioimmunoassays are similarly affected. We investigated the relationship of radiiodinated antigen structure to its function in the immunoassay. Carrier-free 125I-labeled iodotyrosine and iodohistamine derivatives were incubated with human serum, and the bound and free fractions were separated. We demonstrated that diiodotyrosyl analogs bind avidly to serum proteins. Because protein binding could be reduced with competitors that inhibit thyroxine-binding globulin, such as 1,8-anilinonaphthalene sulfonate and thyroxine, thyroxine-binding globulin was clearly implicated. Diiodotyrosyl compounds also bound to solutions of purified thyroxine-binding globulin, and this binding was inhibited by the same two competitors. We postulate that thyroxine-binding globulin is the major source of the hereetofore unexplained interference of radiiodinated hapten. We present recommendations for eliminating or minimizing such interference.

Radioimmunoassay (RIA) is now widely used in medical diagnosis to determine hormones and drugs in blood. Initially, in procedures for measuring hapten, tritiated labels were used, but Oliver et al. (1) developed an alternative method by synthesizing tyrosine-hapten analogs, which could be easily radiiodinated by the method of Hunter and Greenwood (2). Although this provided many advantages, subsequent reports indicate that some ligands labeled with iodine-125 behave anomalously in radioimmunoassays, behavior not shared by the tritiated analogs (3–21). The cause of this interference has not been explained.

Reviewing the evidence and the structures of common tracers used in RIA, we noted that iodine-labeled tyrosine derivatives give rise to compounds in which the terminal structure is identical to the terminal structures of the thyroid hormones thyroxine (T₄) and triiodothyronine (T₃) (Figure 1). We postulated that thyroxine-binding globulin (TBG), which binds T₄ and T₃ with very high affinities, could interfere in direct RIA of serum by binding the structurally similar radiiodinated tyrosine analogs. The binding interference in a particular clinical specimen would vary, depending upon the thyroid status of the patient.

The effect of TBG interference would be to remove a portion of the labeled ligand, thus disturbing the competitive equilibrium between antibody and label, and increasing the apparent concentration when the bound fraction is determined by immunoprecipitation methods. The problem of aberrant sample values with radiiodinated ligands has shown up critically in RIAs for digoxin, a widely prescribed cardiac glycoside (3–18).

Having seen this effect in the development of several RIAs, we decided to study structure–function relationships of several different radiiodinated hapten. We measured the binding of 125I-labeled hapten by incubating them with hypothyroid sera and by separating protein-bound and unbound radioactivity by gel-filtration chromatography.

As a model system, we diluted carrier-free monoiiodine-125 and diiodine-125 digoxigenin-3-O-succinyltyrosine (MID and DID, respectively) to about 100 000 cpm per 50 μL.

We used human serum samples that we had assayed for T₄ and for T₃ uptake and classified as being from hypothyroid individuals.

A 1 × 12 cm G-25-150 Sephadex column was prepared and washed with 0.1 mol/L phosphate-buffered isotonic saline, pH 7.0. The column was calibrated by using 125I-labeled human placental lactogen as an index of the fraction at which the protein-bound 125I would elute. The analytical recovery of total radioactivity from the column was 96%.

Reaction mixtures consisted of 100 μL of serum diluted with an equal volume of (a) water, (b) aqueous 1,8-anilinonaphthalene sulfonate (5 g/L), or (c) aqueous T₄ (the free acid, 0.5 g/L). Reaction mixtures were incubated for at least 2 h at room temperature. We then added MID or DID, incubated the mixture at room temperature for 1 h, applied 100 μL of the mixture to the column, and collected 0.5 mL.

Figure 2 shows that when DID was incubated with hypothyroid serum, about 26% of the MID was avidly bound to protein. Addition of 1,8-anilinonaphthalene sulfonate or T₄ to the serum sample before DID was added decreased binding of DID to protein to 3 and 7%, respectively, which is essentially the degree of nonspecific binding in this system. Added barbital had no effect in the presence or absence of 1,8-anilinonaphthalene sulfonate or T₄. When MID was added to hypothyroid serum, less than 5% of the labeled MID was found in the protein fraction.

Because binding of DID to protein could be inhibited with TBG-sensitive competitors such as 1,8-anilinonaphthalene sulfonate and T₄ but was not inhibited by prealbumin-sensitive blocking agents such as barbital, TBG was clearly implicated.

Purified TBG was diluted to a concentration of 20 mg/L with isotonic saline and incubated with water, 1,8-anilinonaphthalene sulfonate (5 g/L), or T₄ (0.5 g/L) for at least 2 h at room temperature before adding MID or DID.
Figure 3 shows the results of incubating DID with a normal physiological concentration of TBG (20 mg/L). About 23% of the DID was bound avidly. Addition of either 1,8-anilinonaphthalene sulfonate or T₄ to TBG before the addition of DID decreased TBG-bound DID to zero. When MID was added to TBG, 15% was bound. Although MID also bound to charcoal-stripped sera, we could demonstrate no MID binding to unadulterated normal or hypothyroid serum. Under the same conditions, TBG bound 43% of an equimolar concentration of [¹²³I]T₄.

To determine conclusively the interfering moiety, we prepared diiodine-125 tyrosine by exchange labeling and found that the binding characteristics of DID and diiodotyrosine were nearly identical.

We then synthesized digoxigenin-3-O-succinyl histamine and labeled the histamine moiety with iodine-125. When this compound was incubated with serum in the absence of exogenous T₄ or 1,8-anilinonaphthalene sulfonate, the protein-bound radioactivity was less than 3%.

These data indicate that TBG is the major cause of the discrepancies previously reported for radiiodinated haptons. The addition of 1,8-anilinonaphthalene sulfonate, which specifically inhibits the binding of the tyrosine-like hormones T₃ and T₄ to TBG, also inhibited the binding of diiodotyrosyl haptons to TBG and hypothyroid serum. Adding T₄ to serum or to TBG, before adding DID, filled unsaturated TBG binding sites, thus preventing the binding of DID to TBG.

In reviewing the literature, we found many reports describing similar interferences. The first investigators to use [¹²³I]labeled tyrosine derivatives in radioimmunoassay reported that extraction of digitoxin from the protein-containing aqueous phase was necessary for the assay to be valid (1). Another approach, enzymic digestion of all serum proteins before assaying, was reported by one of us previously (20). Several investigators suggested that an individual protein-binding blank should be run for each sample (3-5).

Confusion in the literature regarding the usefulness of radiiodinated derivatives is compounded by the fact that commercial suppliers of radioimmunoassay kits do not remove the 10-20% of diido-labeled tyrosyl derivative when they purify the radiiodinated product. In one case this may have led to the erroneous conclusion that a radiiodinated tyrosine-free acid derivative was preferable to a tyrosine methyl ester derivative (6). The original paper on tyrosine derivatives, which required an extraction step, separated only free iodine from the labeled compound mixture of MID and DID (1).

Interference with radiiodinated tyrosine analogs is not limited to digoxin, but has also been reported for angiotensin (21), gonadoliberin (19), and cortisol (20), and there are many reports of the preferred use of histamine rather than tyrosine derivatives (22-24).

The interference of tyrosine analogs can be eliminated by several methods. Pure MID might be used, but MID will bind to the proteins in the charcoal-stripped serum that is frequently used as the matrix for preparing standards. However, we did not test a large number of different sera, and MID might bind to some human or animal sera. Other preventive measures include addition of 1,8-anilinonaphthalene sulfonate, T₄, or any 2,6-diiodinated phenol; extraction; heat denaturation; or enzymic digestion. However, from our studies we conclude that the problem can be essentially eliminated by use of iodine-labeled histamine derivatives.

In summary, this report accounts for the heretofore unex-
plained interference of 125I-labeled tyrosyl ligands in RIA and provides evidence implicating TBG.

We thank Dr. Elliott Block, Becton Dickinson Immunodiagnostics, for supplying purified MID and purified DID; and Dr. Carlos Bonilla, Department of Physiology and Biophysics, Colorado State University, for supplying the purified thyroxine-binding globulin.

References