Immunoadsorption of IgG onto Second Antibody Covalently Attached to Amino-Dylark Beads for Radioimmunoassays

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We present a solid-phase immobilization method for radio-ligand assays, using an immunoadsorption coating procedure of anti-triiodothyronine rabbit IgG (anti-T3 IgG) on second antibody (sheep anti-rabbit IgG) covalently bound to Amino-Dylark beads. The second antibody was in excess, compared with the first antibody, thus eliminating reproducibility problems between immunoadsorptions. Beads coated with second antibody can be used to immobilize a variety of antigen-specific first antibodies. The amount of anti-T3 antibody required for solid-phase T3 radioimmunoassay (RIA) was only 10% more, per assay tube, than that utilized in liquid-phase T3 RIA, in which polyethylene glycol solution was the separation reagent; characteristics of assay performance were comparable. The immobilization procedure requires high-titer antisera or antigen-specific IgG and seems advantageous because of the decrease in antibody requirements without significant modification of antibody functionality.

Additional Keyphrases: triiodothyronine · solid-phase vs liquid-phase assays · double-antibody immunoassays

The use of solid-phase immobilized antibodies in radioimmunoassays, given their technological advantages, has been established as a method of choice over liquid-phase methods in the routine clinical laboratory (1). Most coating procedures utilize simple physical adsorption of antibody or antigen to plastic surfaces (2). However, in principle, this is reversible and in some cases lacks reproducibility (3). Chemical immobilization, involving the formation of covalent bonds between residues of antibody and functionalized carriers, diminishes desorption and provides precise immobilization conditions (4). Nevertheless, both physical adsorption and chemical immobilization have limitations (1, 2) related to the requirement of high-titer antisera, the decrease in the apparent affinity constant of the coated antibody or even its failure to retain immunoreactivity, the slower kinetics, the chemical inactivation of antibody during storage, the breaking or making of new covalent bonds, and others.

In this study, we present part of our effort to eliminate some of the problems of solid-phase immobilization for radio-ligand assays. To do this, we used an immunoadsorption coating procedure of anti-triiodothyronine rabbit IgG (anti-T3 IgG) on second antibody (sheep anti-rabbit IgG) (5) covalently bound to Amino-Dylark or Dylark beads.4 Amino-Dylark beads retain amino-functions; they are prepared from ethylenediamine and a partial hydrolysate of Dylark, which is a copolymer of styrene and maleate.

Materials and Methods

Chemicals and reagents. All reagents were analytical grade. Carrier-free Na125I (specific radioactivity 17 kCi/g, radiochemical purity 99.9%, iodate <2%), obtained from Radiochemical Co., Atomic Energy of Canada, Ottawa, Canada, was used to prepare a 250 Ci/L radiiodine working solution for radiolabeling in 0.25 mol/L phosphate buffer, pH 7.5. Complete Freund's adjuvant was from Difco Labs., Detroit, MI. Sephadex G-25 was a product of Pharmacia, Uppsala, Sweden. The 6.35-mm-diameter Dylark and Amino-Dylark beads were products of C-BE21 and C-BE23, respectively) of Sekiaku Chemical Co., Kita-Ku, Osaka, Japan. Free acid 1-T3, normal rabbit IgG, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, bovine thyroglobulin, Chloramine-T, thimerosal sodium salt, sodium metabisulfite, 1,5-pentanediol (dilutaric acid), and T3-free bovine serum albumin (BSA; Cohn Fraction V) were obtained from Sigma Chemical Co., St. Louis, MO. That the BSA did not contain T3 was confirmed by radioimmunoassay. 8-Anilinonaphthalene-1-sulfonic acid, dimethylformamide, Polystyrene Glycol 6000 (PEG), and all other reagents were products of Merck, Darmstadt, F.R.G., except as otherwise indicated.

Anti-T3 antiserum. The anti-T3 antiserum was produced by immunizing rabbits as described in the accompanying paper (6).

Rabbit IgG antiserum. This antiserum was obtained by immunizing sheep with five injections (one endopterneal, two maxillary, two inguinal) monthly, over a period of two years, with 5 mL of Freund's complete adjuvant emulsion in NaCl, 0.85 g/L, containing 0.1 g of rabbit IgG per liter. Blood sampling (from the jugular vein) was started 10 days after the fifth administration and continued at the same rate after every immunization. The antiserum was separated by centrifugation (2500 × g) for 30 min at 4°C and remained usable for more than two years when stored at −25°C.

For utilization in the T3-second antibody liquid-phase assay, we prepared an immunoprecipitating reagent as follows: We diluted the rabbit IgG antiserum 33-fold with 20 mmol/L Tris buffer, pH 8.5, containing 80 mg of rabbit IgG, 20 g of PEG, and 2 g of NaN3 per liter, after preincubation with a suitable amount of anti-T3 antiserum, so that 500 μL of the final solution should contain the same amount of anti-T3 IgG as that originally present in 300 μL of anti-T3 antibody solution. The immunoprecipitating reagent was stored at 4°C.

Anti-T3 IgG and anti-rabbit IgG fraction. To obtain the γ-globulin fraction of the anti-T3 antisem (native anti-T3 IgG) and the γ-globulin fraction of the anti-rabbit antisem, we precipitated 10 mL of each antiserum with caprylic acid, following the method of Steinbuch and Uran (7). The solutions obtained were diluted with isotonic saline (8.5 g/L), separated into aliquots containing 150 μg

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4 Nonstandard abbreviations: BSA, bovine serum albumin; PEG, polystyrene glycol; and T3, triiodothyronine.
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of protein, lyophilized, and stored at 4 °C until use. The total protein yield was 12–14% of the original antiserum protein content, determined by the method of Bradford (8).

Radioiodination of T₃. The radioiodination was performed by a modification of the method of Greenwood et al. (9), as described in detail in the accompanying paper (6). The specific radioactivity (10) was 3400 Ci/g.

Immunoadsorption protocol. We washed 100 Amino-Dylark beads with 2 g/L Tween 20 solution, then exhaustively washed them with water and degassed the resulting beads in water. The beads were immersed in diglutaraldehyde solution, 10 mL/L (11, 12), incubated for 1 h on an orbital shaker at 20–22 °C, and washed four times with 100 mL of phosphate-buffered saline (PBS), pH 7.0. We then placed the beads into 50 mL of 6 mg/L anti-rabbit IgG solution in 50 mmol/L phosphate buffer, pH 7.4, containing 1 g of NaN₃ per liter, and incubated them for 24 h at 4 °C. After incubation we washed the beads four times with 100 mL of 50 mmol/L phosphate buffer, pH 7.0, containing 5.85 g of NaCl, 1 g of BSA, and 1 g of NaN₃ per liter (wash buffer), then transferred them into 30 mL of anti-T₃ antiserum solution (1:290 000 titer) in wash buffer. We incubated the beads for 24 h at 4 °C, then washed them with PBS, and stored them in the wash buffer at 4 °C for at least one month. We used the same procedure to covalently bind anti-T₃ IgG onto Amino-Dylark beads.

For the adsorption of anti-T₃ IgG onto the surface of the Dylark beads, we incubated the beads at 22 °C for 24 h, using 0.5 mL of coating solution per bead. The coating solution was prepared by diluting the IgG with 1.0 mol/L phosphate buffer, pH 7.0, to give a final concentration of 1 mg of anti-T₃ IgG per liter.

T₃ assay protocol. The solid-phase T₃ radioimmunoadsorption and the liquid-phase T₃ assay were described in detail in the accompanying paper (6). For the solid-phase assay we used anti-T₃ immunoadsorbed onto Amino-Dylark or Dylark beads, instead of polystyrene beads.

We used the same protocol for the second-antibody liquid-phase method, replacing the 300 µL of the anti-T₃ antiserum with 500 µL of immunoprecipitating reagent (see above), and incubating for 2 h. After centrifuging the samples at 3000 × g for 10 min, we counted the radioactivity of the precipitate.

The standard curve of the assay was obtained by plotting B/B₎ vs concentration of unlabeled T₃ standards on linear paper and drawing the optimum curve between the points. The concentration of T₃ in serum samples was determined from this curve. We could also plot B/B₅ vs standards on logit-log scale and determine the unknown serum values by computer, using a linear equation.

Results

The immobilization of antibodies, e.g., anti-T₃ rabbit IgG (first antibody), onto Amino-Dylark beads was simple to perform. We pre-activated solid-carrier surface aminogroups with diglutaraldehyde, followed by exhaustive washing with water, and incubated with appropriately diluted sheep anti-rabbit IgG (second antibody).

Chemical immobilization of the first antibody onto Amino-Dylark beads failed to retain the functional antibody characteristics for acceptable T₃ radioimmunoassay performance, independent of the amount of the protein immobilized. This was tested by quantifying the capacity of the immobilized anti-T₃ IgG to bind a fixed amount of [1²⁵I]T₃ in the presence of various concentrations of unlabelled T₃. On the other hand, physical adsorption of anti-T₃ IgG onto Dylark beads yielded appropriate immobilized immunoreactivity for utilization in T₃ RIA, although 10-fold more antibody was required than in the common liquid-phase RIA of T₃ involving PEG.

The immunoabsorption process involved fixation of the first antibody onto a second one, covalently attached through activated aminogroups, onto Amino-Dylark beads. The amount of immobilized second antibody was in excess, compared with the anti-T₃ IgG required for the assay, and did not demand special quantification after the initial calibration studies. For the solid-phase T₃ RIA, the amount of the first antibody required for the immunoabsorption process was determined by incubating the second-antibody-coated beads with serial dilutions of the anti-T₃ antiserum (Figure 1). We selected immunoabsorption conditions that provided a 40–50% binding capacity, to be comparable with that used in liquid-phase PEG radioimmunoassay. Under these conditions, the estimated amount of anti-T₃ IgG immunoabsorbed per bead was approximately 28 ng; 25 ng of IgG per assay tube was required for the liquid-phase T₃ PEG method.

The interbatch coating precision (CV), determined for 12 batches of 50 beads per batch over a period of 12 months, was 5.3%. The intrabatch coating CV was 2.3%, evaluated by dividing 200 coated beads into five groups and assaying over a period of one month.

As indicated in Figure 2, the calibration curve obtained by using the above coated beads in the T₃ solid-phase assay was comparable with that obtained by the liquid-phase PEG method and was superimposable on that obtained by the second-antibody T₃ liquid-phase method. The estimated apparent affinity constant of immunoabsorbed antibody for complexing with T₃ (13) was 5.67 × 10⁰ L · mol⁻¹ vs 7.03 × 10⁹ L · mol⁻¹ when in soluble state, and 6.91 × 10⁹ L · mol⁻¹ when in suspension form with the second antibody. The characteristics (14) of the solid-phase T₃ assay were comparable with those of liquid-phase methods: the detection limit for each was 0.11 µg of T₃ per liter. The interassay CV of the standard curves determined from 15 measurements (three sets of reagents) run in duplicate during 15 weeks averaged about 5.8–6.1% over the range of

![Fig. 1. Anti-T₃ antiserum titer curve obtained with T₃ PEG RIA method (Δ) compared with the capacity of anti-T₃ IgG immunoabsorbed onto Amino-Dylark beads (Ⅺ), at the corresponding antiserum dilutions, to bind a fixed amount of [1²⁵I]T₃](image-url)
the standards, and 6.9–10.4% for the control sera. The intra-assay CV of the standard curves, run in duplicate during five days, averaged about 3.9–4.8% over the range of the standards and 4.3–4.8% over the range of the control sera.

**Discussion**

Here we describe a solid-phase assay utilizing first antibody (anti-T<sub>3</sub> rabbit IgG, 1:290 000) immunoabsorbed onto second antibody (sheep anti-rabbit IgG), covalently attached to a water-insoluble matrix. The Amino-Dylark beads used as carriers were produced under thorough quality-control inspection, to maintain consistency of their surface characteristics.

Preliminary experiments indicated that, by using physical adsorption of anti-T<sub>3</sub> IgG, at pH 7.0, onto Dylark beads, 10-fold more antibody was required for the solid-phase T<sub>3</sub> radioimmunoassay than for the liquid-phase PEG assay. Use of acidic-pH-pretreated anti-T<sub>3</sub> IgG (15) immobilized onto the same beads required only eightfold more antibody than in the liquid-phase—still a high reagent consumption.

When the first antibody was chemically immobilized onto Amino-Dylark beads, it did not retain its functional characteristics, as was indicated in the T<sub>3</sub> solid-phase method. The quantities of anti-T<sub>3</sub> IgG selected for immobilization in these studies were less than, equal to, or as much as fivefold greater than those used in the physical adsorption process of this antibody onto Dylark beads.

To eliminate some of the problems related to the immobilization procedures, we decided to utilize a different fixation process by inserting a second antibody between the solid-phase and the first antibody (5). Thus, we could avoid direct interactions between the binding sites of the first antibody and the active functional groups of the insoluble carrier.

The second antibody attached to Amino-Dylark beads was resistant to successive washing steps, and retained sufficient binding capacity for the first antibody to satisfy the T<sub>3</sub> solid-phase assay requirements. The estimated amount of second antibody needed for immobilization was ~3 μg per bead. We considered this quite a large quantity, related to the low content of the specific anti-rabbit IgG in the sheep serum; however, it could easily be obtained in adequate quantities from big animals, without significant problems. The immunoabsorbed anti-T<sub>3</sub> IgG retained its binding capacity and specificity as well as the assay characteristics (sensitivity, precision, reproducibility) and provided satisfactory solid-phase assay results comparable with those of the liquid-phase method. The calculated amount of antibody utilized in the proposed method during immobilization was only 10% more than that required in the liquid-phase assay; i.e., the liquid-phase assay required about 25 ng of antibody per tube, the immunoabsorption procedure about 28 ng per bead. This comparable antibody consumption in the liquid- and the solid-phase assays was accompanied by similar functional characteristics of antibodies, leading to practically superimposed standard curves with equally good inter- and intra-assay precision.

The second antibody was always used in excess compared with the amount of the first antibody; thus, even when there were small differences between the amount of second antibody attached to the beads, equal amounts of first antibody could be immunoabsorbed onto them, eliminating reproducibility problems between the different production batches. High-titer antisera could be used diluted directly in the immunoabsorption step. On the other hand, using the IgG fraction of low-titer antisera provided no significant advantage over the use of whole antiserum, owing to the immunoabsorption of nonspecific IgG onto second antibody. In that case, we recommend utilizing antigen-specific IgG (first antibody), isolated through affinity chromatography. In this way, the proposed method could have the advantage of a decrease in antibody requirements without significant modification of the antibody functionality.

In conclusion, we present the potential utilization of Amino-Dylark beads as immobilizing matrices in radioimmunoassays, using an immobilization approach by immunoabsorption of first antibody onto second antibody covalently bound to Amino-Dylark beads. The significant saving of first antibody, compared with the quantities used in conventional immobilization processes, was an advantage of this method. Although this work has been exemplified with use of anti-T<sub>3</sub> rabbit antiserum, brief experiments with rabbit antisera against thyrotropin, follitropin, and lutropin indicate that these beads, so coated, can be used successfully for other assay systems as well.

**References**


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Ed. note: This journal recently published a somewhat similar approach (Clin Chem 1989;35:110-4), in which a mouse monoclonal antibody was coupled indirectly to particles of polyvinylidene chloride via polyclonal goat anti-mouse immunoglobulin. The present study provides much new information about "indirect" coupling and, moreover, utilizes a different method to immobilize antibody to the solid support.