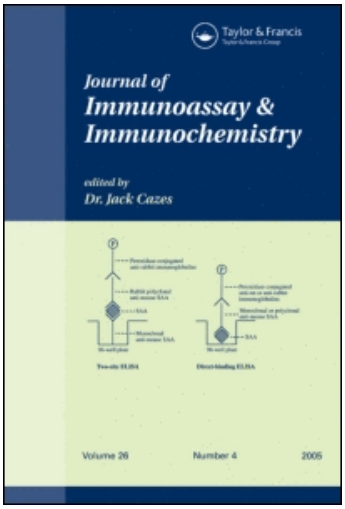

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ANTIBODY COATING APPROACH INVOLVING GAMMA GLOBULINS FROM NON-IMMUNIZED ANIMAL AND SECOND ANTIBODY ANTISERUM

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ABSTRACT

An alternative protocol for immobilization of antibodies onto plastic solid supports is presented. According to the proposed protocol, tubes are first coated with γ-globulins from non-immunized animal of the same species as that from which the antigen-specific antibody has been developed. Then, an excess of second antibody is added to the tubes and the anti-species specific antibodies present in the antiserum are immunoadsorbed on the immobilized γ-globulins. Finally, the antigen specific antibody is immunoadsorbed on the immobilized second antibody. We found that the coating protocol developed allows the use of antigen-specific and second antibody antisera dilutions, thus avoiding the need for affinity purification of antibodies. Additionally, it provides solid-phase

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second antibody with increased binding capacity compared to the directly adsorbed onto the solid second antibody. The advantages of the proposed coating protocol were demonstrated through the development of a solid-phase radioimmunoassay for the determination of total triiodothyronine in human serum samples.
(**KEY WORDS**: solid-phase antibody; second antibody immobilization protocol; triiodothyronine; solid-phase radioimmunoassay)

INTRODUCTION

The use of immobilized second antibody as a universal solid-phase reagent for the development of immunoassays is an attractive alternative to direct coupling of antigen-specific antibodies onto solid supports (1). The advantages offered by this immobilization approach over the direct coupling of the antibodies are the saving of the relatively expensive affinity purified antigen-specific antibody, the preservation of immobilized antibody binding capacity and finally, the ability to use solid supports coated with the same second antibody to immobilize antibodies against different antigens (2-4).

Following the second antibody coating approach, a certain number of applications can be carried out using the γ-globulins fraction of the second antibody antiserum. In most of the applications, however, an affinity purified second antibody has to be used in order to obtain satisfactory loading of the antigen-specific antibody onto the solid surface. In these cases, relatively high amounts of second antibody are required due to the loss of the antibody binding capacity after its adsorption onto the solid. Apart from the affected binding capacity, the use of affinity purified second antibody increases the cost of the coating procedure by affecting negatively the time, labor and reagents required.

In this work we present an alternative approach for the immobilization of antibodies onto plastic solid supports which provides increased binding capacity of solid-phase antibodies using antigen-specific and second antibody antisera dilutions. The proposed

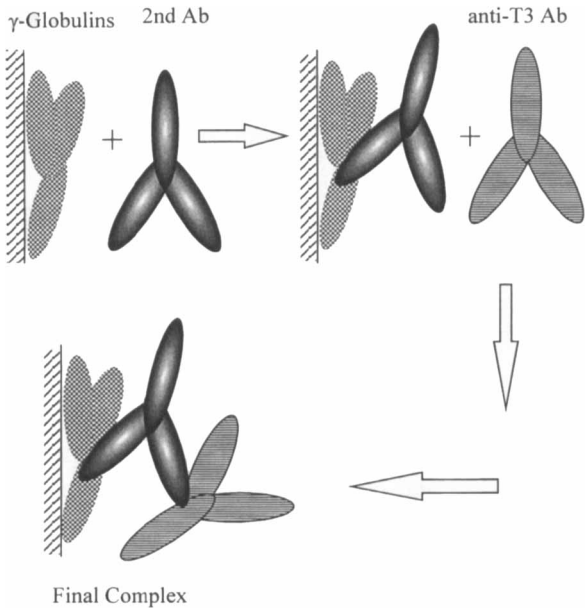

FIGURE 1. Schematic representation of the proposed coating approach.

coating approach, which is schematically presented in Figure 1, is completed in three steps. At first, γ-globulins from non-immunized animal of the same species as that from which the antigen-specific antibody has been developed are adsorbed onto the solid (5). Then, the immobilized γ-globulins are incubated with an excess of second antibody (antiserum dilution). During this step, the anti-species specific antibodies present in the antiserum are immunoadsorbed on the immobilized γ-globulins, while the non- specific γ-globulins do not bind and are discarded at the end of this step. Finally, an antigen-specific antiserum dilution is added to the tube. During incubation of this solution with the tube, part of the existing antigen specific and non-specific γ-globulins is immobilized onto the tube through binding with the solid-phase second antibody.

The advantages offered by the proposed coating approach are demonstrated through the development of a radioimmunoassay (RIA) for the determination of total triiodothyronine (T_3) in human serum samples.

MATERIALS AND METHODS

Chemicals, Reagents, and Instrumentation

All reagents were of analytical grade. Carrier-free $Na^{125}I$ (specific radioactivity 17 KCi/g, radiochemical purity 99.9%, iodate < 2%) obtained from Nordion Europe S.A. (Belgium), was used for radiolabeling. Rabbit γ-globulins (Cohn Fraction I, II), bovine serum albumin and L-thyroxine (free acid) were obtained from Sigma Chemical Co. (St Louis, MO, USA). 8-Anilino-naphthalene-1-sulfonic acid ammonium salt, sodium azide, sodium salicylate, maleic acid, Chloramine-T, mercaptoethanol, and all other reagents were products of Merck (Darmstadt, Germany), except as otherwise indicated. T_3-free human serum for the preparation of T_3 standard solutions was purchased from Scantibodies Laboratory Inc. (San Diego, CA, USA). Polystyrene RIA tubes (75x12 mm) were from VIVE Co. (Athens, Greece). Anti-T_3 antiserum was purchased from O.E.M. Concepts Inc. (Toms River, NJ, USA). Sheep anti-rabbit γ-globulins antiserum was a product of the RIA Lab., I/R-RP, NCSR "Demokritos" (Athens, Greece). The γ-globulins fraction of this antiserum was obtained following the ammonium sulfate precipitation method. The precipitate corresponding to the fraction containing the γ-globulins (40% saturated ammonium sulfate) was filtered and exhaustively dialyzed against isotonic saline (6). Affinity purified sheep anti-rabbit γ-globulins antibodies were obtained from the antiserum γ-globulins fraction by affinity chromatography on a 2-mL AminoLink column (Pierce Chemical Co., Rockford, IL, USA) loaded

with rabbit γ-globulins. The coupling buffer used was 0.15 M Tris-HCl, pH 8.25, whereas the elution buffer was 0.1 M glycine-HCl, pH 2.8. The concentration of the resultant protein solutions was estimated in all cases by determining their absorbance at 280 nm considering a A_{280} value of 1.4 for a sheep γ-globulins solution of 1 g/L. Following the above described procedure, 4.1 mg of specific anti-rabbit IgG antibodies were isolated per mL of antiserum. Radioactivity measurements were performed in a 12-well γ-counter (NE-1612, Nuclear Enterprises Ltd., UK), facilitated with software appropriate for interpretation of RIA results.

Buffers

The following buffers were used in the immobilization procedure: a) a 0.05 M carbonate buffer, pH 9.2, was used for the adsorption of rabbit γ-globulins or antibodies (coating buffer), b) a 0.15 M Tris-HCl buffer, pH 8.25, containing 1 g of BSA and 0.2 g of ethyl-mercurithiosalicylic acid (sodium salt) per liter was used for the immunoadsorption steps (coupling buffer), c) the blocking solution was 0.1 M $NaHCO_3$ containing 10 g of BSA and 0.5 g of sodium azide per liter, d) a 0.01 M Tris-HCl buffer, pH 8.25, was the washing solution used throughout the coating process. The ^{125}I-T_3 radiotracer diluent was 0.1 M Tris-malate buffer, pH 8.25, containing 0.15 g of 8-anilino-naphthalene-1-sulfonic acid (ammonium salt), 0.10 g of salicylic acid (sodium salt) and 1.0 g of NaN_3 per liter.

T_3 standards and control sera

T_3 standards were prepared by spiking T_3-free serum with known amounts of T_3 and verifying their concentrations using a commercially available RIA kit (T3 Amerlex-M, Amersam, UK). Human sera with low, medium and high concentration of T_3 were pooled apart in order to prepare three control sera.

Radioiodination of T_3

T_3 was radioiodinated by a modification of the method of Greenwood et al. (7). Briefly, in a polystyrene tube containing 10 μL of 0.95 M T_2 solution, pH 7.4, 10 μL of $Na^{125}I$ solution (250 Ci/L) in 0.25 M phosphate buffer, pH 7.4, (radioiodination buffer), and 10 μL of 1.775 M Chloramine-T solution in the same buffer were added. After 60 s of incubation while vortexing, the reaction was terminated by adding 10 μL of a 0.14 % (v/v) mercaptoethanol solution in radioiodination buffer. The radiolabeling yield, determined by paper electrophoresis (8), was higher than 90 %. The ^{125}I-T_3 was purified on a Sephadex G-25 column by elution with 50 mM phosphate buffer, pH 7.4, containing 1 g of BSA and 0.5 g of NaN_3 per liter. Fractions having binding capacity >45 % for a fixed amount of ^{125}I-T_3 and high radioactivity were pooled and diluted with radiotracer diluent to a storage activity of 0.10 μCi/mL (^{125}I-T_3 radiotracer solution). The specific radioactivity of the ^{125}I-T_3 was 3400 Ci/g as it was determined by the method of Chiang (9).

Preparation of Radioiodinated Rabbit γ-Globulins (^{125}I-RγG)

A protocol similar to that used for radioiodination of T_3 was followed. In this case, 20 μL of a 1 mg/mL protein solution in radioiodination buffer and 5 μL of a 100 Ci/L solution of $Na^{125}I$ were used. Labeled protein was separated from free iodine on a PD-10 column (Pharmacia, Sweden) eluted with 50 mM phosphate buffer, pH 7.4, containing 3 g of BSA, and 1 g of NaN_3 per liter. Fractions with high radioactivity were collected and diluted to a storage activity of 0.114 μCi/mL with coupling buffer (^{125}I-RγG radiotracer solution). The radiolabeling yield was approximately 85%, and the specific radioactivity 21 Ci/g.

Solid-Phase Immobilization Protocols

Protocol Developed using Affinity Purified Second Antibody. One mL of rabbit γ-globulins solution in coating buffer was added to each tube and the tubes were incubated for 22 h at room temperature (RT). Then, the tubes were washed twice with 2 mL of washing solution. One mL of affinity purified second antibody solution (0.2-20 mg/L) in coupling buffer was added per tube and the tubes were incubated for 22 h at RT. After that, the tubes were decanted and washed as previously. One mL of anti-T_3 antiserum dilution (1/80,000) in coupling buffer was then added into each tube and the tubes were incubated for 22 h at RT. The tubes were decanted, washed as in the previous step, and blocked with 2 mL/tube of blocking solution for 18 h at RT. Finally, the blocking solution was decanted and the tubes were washed twice prior to use. Alternatively, the tubes can be kept, filled with the washing solution, for 1 week at 4 °C without losing their activity.

Protocol Developed using Second Antibody Antiserum Dilution. This protocol was same as the previous one but the solutions of the affinity purified antibody were replaced by second antibody antiserum dilutions (1/20,000 to 1/500).

Comparison Protocol. A modified literature protocol was applied for the direct coating of tubes with second antibody (3). Briefly, 1 mL of a 4 mg/L affinity purified second antibody solution in coating buffer was added in each tube and the tubes were incubated for 22 h at RT. Then, after washing the tubes twice with 2 mL of washing solution, 1mL of anti-T_3 antiserum dilution (1/80,000) in coupling buffer was added per tube. Incubation, washing, blocking and subsequent handling of these tubes was as for those prepared according to the proposed protocol.

T_3-assay Protocol

In tubes coated with anti-T_3 antibodies, add 100 μL of T_3 standards (0-5.9 μg/L in T_3-free human serum) or serum sample, 1 mL of ^{125}I-T_3 radiotracer solution and mix by vortexing. Incubate the tubes without shaking for 1.5 h at 37 °C. Decant the liquid phase, leave the tubes inverted for a few minutes (or aspirate the remaining droplets) and measure the radioactivity bound onto the solid in the γ-counter. Calculate the binding capacity of the solid-phase antibody as the fraction of radiotracer bound in presence of T_3-free serum. The standard curve of the assay can obtained by plotting the $(B_x/B_o)x100$ values vs. the logarithm of the T_3 concentration in the standards and drawing the curve between the points. B_o and B_x represent the radioactivity bound onto the solid in the presense of zero standard and standards containing known amounts of unlabeled T_3, respectively.

Determination of the Apparent Affinity Constant of Second Antibody

The apparent K_{aff} of the second antibody immobilized following the protocol developed was determined using tubes coated with 100 μg/L of rabbit γ-globulins combined with 100 μg/L of affinity purified sheep anti-rabbit γ-globulins antibody. When the comparison coating protocol was used, the tubes were coated with 400 μg/L of sheep anti-rabbit γ-globulins antibody. Into tubes coated with sheep anti-rabbit γ-globulins antibody following either protocols, 1 mL of ^{125}I-RγG solution containing increasing amounts of unlabelled rabbit γ-globulins (2.5-160 μg/L) was added. The tubes were incubated for 18 h at RT and then, washed twice with 2 mL of washing solution prior to measurement of the radioactivity. In each case, the data were analyzed by the Steward-Petty method to determine the apparent K_{aff} (10).

Prior to interpretation, the data were corrected for the amount of radioactivity non-specifically bound to tubes as well as for the amount

of the non-bindable ^{125}I-RγG. In both protocols, the non-specific binding was determined in tubes coated with bovine γ-globulins in the first step. The amount of the non-bindable ^{125}I-RγG was determined from the amount of ^{125}I-RγG bound to tubes coated with high amounts of sheep anti rabbit γ-globulins antibody capable of binding the whole amount of the label added, and it was less than 5%.

RESULTS

The proposed coating approach, which is presented schematically in Figure 1, consists of three steps, namely, adsorption of rabbit γ-globulins, immunoadsorption of second antibody, and immunoadsorption of anti-T_3 antibody. The reaction conditions and the concentrations of the reagents involved in each one of the steps of the coating procedure were optimized on the grounds of the capacity of the immobilized anti-T_3 antibody to bind a fixed amount of ^{125}I-T_3 as well as of the coating repeatability.

Optimization of Coating Conditions - General Physicochemical Parameters

The general physicochemical parameters of the coating protocol as well as the T_3 assay conditions were optimized using certain rabbit γ-globulins and second antibody concentrations. Optimum conditions concerning the time and temperature of the different incubation steps as well as the pH and the ionic strength of the buffers used were those described under Materials and Methods. The dilution of the anti-T_3 antiserum required to obtain satisfactory binding capacity was also determined. We found that using the anti-T_3 antiserum at dilutions ranging between 1/150,000 and 1/80,000 satisfactory binding capacity (45-50%) and coating repeatability (CV less than 5%) was obtained. However, throughout this study, we used a 1/80,000 dilution of the antiserum, since it provided similar binding capacity values with

either the protocol developed or that using directly adsorbed second antibody, in order to facilitate comparisons.

Optimization of Coating Conditions - Reagents Concentration

Significant parameters of the coating protocol developed are the concentration of the rabbit γ-globulins used for the initial coating, as well as the concentration of the second antibody used in the following step. The rabbit γ-globulins concentration in the coating solution was determined with respect to the concentration of the second antibody used in the subsequent step. At first, we used affinity purified second antibody, instead of antiserum dilution, in order to compare on a quantitative basis the results obtained by the protocol developed and the comparison protocol.

Several concentrations of affinity purified second antibody were tested using tubes coated with rabbit γ-globulins solutions in the range of 0.2 to 10 mg/L. As it is shown in Figure 2, for a given second antibody concentration between 0.5 and 2 mg/L, maximum binding capacity values were reached when the rabbit γ-globulins concentration in the coating solution was increased to become equal to the concentration of the second antibody used. Exceeding this concentration, however, a more or less sharp decline in the binding capacity was observed, depending on the concentration of the second antibody. When the concentration of the second antibody was higher than 2 mg/L, the binding capacity values were increased and reached maximum plateau values by increasing the rabbit γ-globulins concentration up to 1 mg/L. Further increase in rabbit γ-globulins concentrations up to 10 mg/L did not affect the binding capacity value obtained. Taking it overall, we found that using rabbit γ-globulins concentration equal to or higher than 1 mg/L, the binding capacity was increased and reached maximum plateau values by increasing the second antibody concentration up to 5 mg/L. Further increase of the

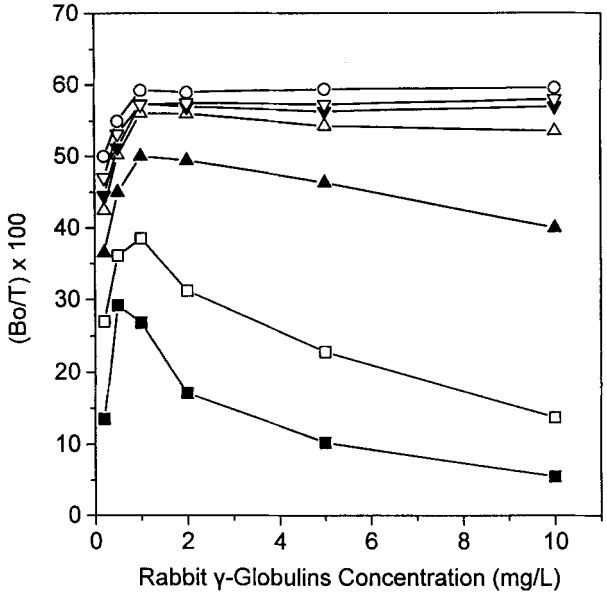

FIGURE 2. Effect of the concentration of rabbit γ-globulins and affinity purified second antibody on the binding capacity of the immobilized anti-T_3 antibody The concentrations of affinity purified sheep anti-rabbit IgG antibody used were : 0.5 mg/L, (■); 1 mg/L, (□); 2 mg/L, (▲); 5 mg/L, (Δ); 7.5 mg/L, (▼); 10 mg/L, (∇); and 20 mg/L, (○). (Bo/T)x100 represents the ^{125}I-T_3 bound onto the solid as a percentage of the total ^{125}I-T_3 added per tube in the presence of zero standard.

second antibody concentration up to 20 mg/L improved the results marginally (less than 5% increase).

When second antibody antiserum dilution was to be used, optimum concentrations of rabbit γ-globulins solution required for coating were determined following the same experimental set-up. Binding capacity patterns similar with those obtained using affinity purified antibody were observed. We found that maximum plateau binding capacity values were obtained using a rabbit γ-globulins concentration of 1 mg/L in the coating solution along with a second

antibody antiserum dilution equal to or lower than 1/2,000. However, we found that a 1/7,000 dilution of second antibody antiserum was adequate for our application and was selected for the development of the T_3 RIA.

Comparison of the Coating Protocol Developed with Other Protocols

The abilities of the proposed coating protocol were investigated by comparing tubes coated directly with second antibody, with tubes prepared according to the proposed coating approach in terms of the binding capacity values provided. In both cases, optimum immobilization conditions and affinity purified second antibody were used. As is indicated in Figure 3, the binding capacity values provided by the proposed coating protocol were superior to those obtained by the comparison protocol. In fact, for second antibody concentrations between 0.5 and 2 mg/L, the protocol developed provided 2.6 to 1.3 times higher binding capacity values than those obtained using the comparison protocol. Higher differences in the binding capacity values between the protocol developed and the comparison one were observed for lower second antibody concentrations (e.g. 0.2 mg/L), although these values were not adequate for our application. As the second antibody concentration was increased above 2 mg/L, the difference in the binding capacity values obtained with the two coating protocols was gradually decreased. Using concentrations of second antibody equal to or higher than 5 mg/L, the proposed coating protocol provided 4-7% higher binding capacity values than those provided by the comparison protocol. Concerning the amount of second antibody required, when it was directly adsorbed onto the solid, a 2-fold higher concentration (10 mg/L) was required to achieve maximum binding capacity values compared with the proposed protocol. Additionally, following the protocol developed a 40-50% binding capacity, which was selected to perform the T_3 RIA, can be

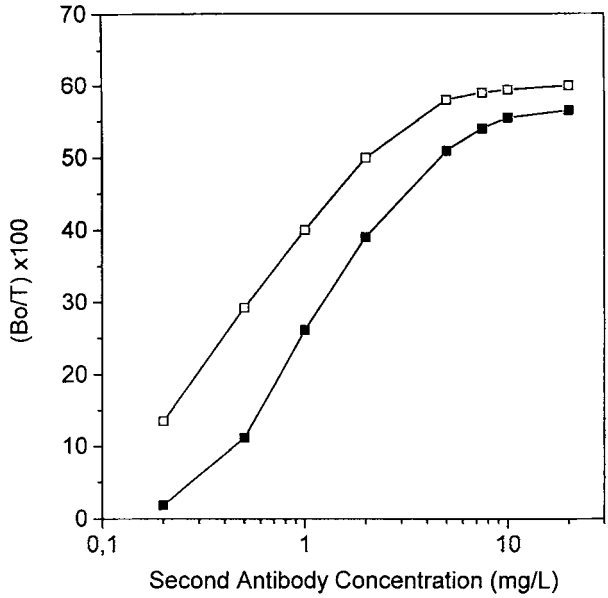

FIGURE 3. Comparison of the binding capacity of the anti-T_3 antibody immobilized into plastic tubes at a 1/80,000 antiserum dilution through affinity purified sheep anti-rabbit IgG antibody which was: a) directly adsorbed onto the solid (■), or b) immuno-adsorbed onto immobilized rabbit γ-globulins (□). The concentration of rabbit γ-globulins was 0.5 mg/L for second antibody concentrations of 0.2 and 0.5 mg/L, whereas it was 1 mg/L for all the other second antibody concentrations.

obtained using 1-2 mg/L of second antibody, whereas 2-5 mg/L were required when the comparison protocol was used.

In order to gain more insight concerning the coating protocol developed, we determined the apparent affinity constants (K_{aff}) of the second antibody immobilized onto the solid surface either directly or through immunoadsorption. This was performed by developing a RIA for rabbit γ-globulin and analyzing the data obtained by Steward-Petty and Scatchard plots. The concentration of the antibody used in each protocol was selected so that a binding capacity of about 50%

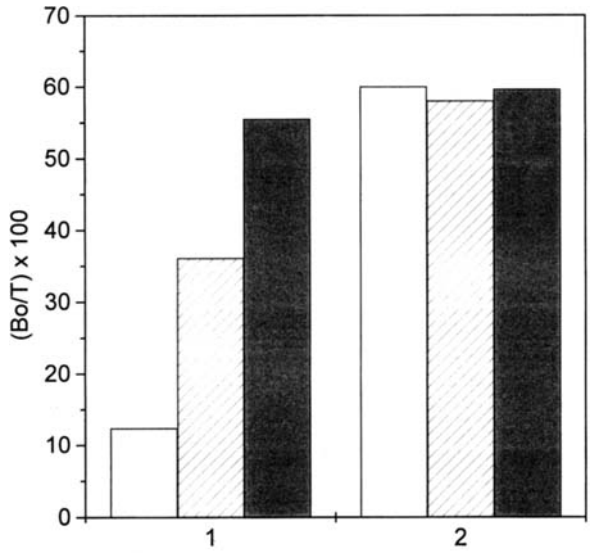

FIGURE 4. Comparison of the binding capacity values obtained using anti-T_3 antibody immobilized following either the comparison coating protocol (bar group 1) or the protocol developed (bar group 2). Three different second antibody preparations were used : neat antiserum dilution (plain bars); antiserum γ-globulins fraction (hatched bars); and, affinity purified antibody (gray bars). In all cases, the concentration of the reagents used for coating were selected so as to provide maximum plateau binding capacity values.

would be achieved. For the protocol developed this was realized using 100 μg/L of sheep anti-rabbit γ-globulins antibody combined with equal concentrations of rabbit γ-globulins in the coating solution. In the comparison protocol 400 μg/L of sheep anti-rabbit γ-globulins antibody were used. The apparent K_{aff} value of the second antibody immobilized following the protocol developed was 9.55×10^{10} M^{-1}, whereas that determined for the directly adsorbed second antibody was 2.05×10^{10} M^{-1}.

The two coating approaches were also compared in terms of the

binding capacity obtained using different preparations of second antibody, namely, affinity purified antibody, γ-globulin fraction of the antiserum, neat antiserum dilution. Appropriate reagents concentration were used in either protocol so as to provide maximum plateau binding capacity values. As is shown in Figure 4, when the second antibody was directly adsorbed onto the solid, binding capacity values adequate to perform T_3 radioimmunoassay could be achieved only by using affinity purified second antibody, whereas the neat antiserum dilution and the γ-globulins fraction of the antiserum provided poor results. On the other hand, using the coating protocol developed, high binding capacity (approximately 60%) was obtained using each of the three different second antibody preparations.

Analytical Characteristics of the T_3 Radioimmunoassay Developed

A typical standard curve of the T_3 RIA-assay obtained using tubes prepared according to the protocol developed is shown in Figure 5. For comparison a curve obtained using tubes coated directly with affinity purified second antibody is also provided. In both cases, the anti-T_3 antibody binding capacity was about 46%.

The detection limit of the assay developed, defined as the analyte concentration corresponding to the mean radioactivity value of 20 replicates of zero standard -2SDs, was 0.03 μg/L. The corresponding detection limit of the assay performed with tubes coated with affinity purified second antibody directly adsorbed onto the solid was 0.1 μg/L.

The within-run precision of the assay was determined by 12 replicate determinations of three control serum samples in a single assay and the between-run precision by duplicate determinations of control samples in 20 different runs. At mean T_3 concentrations of 1.30-3.75 μg/L, the within-run CVs were between 0.51% and 5.6% whereas the between-run CVs were ranged between 3.0% and 8.7%.

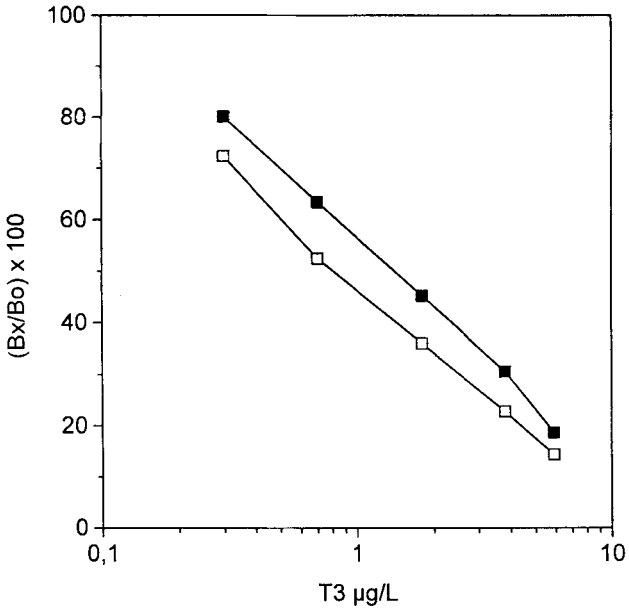

FIGURE 5. Typical T_3 standard curves obtained with tubes prepared following the proposed coating protocol using a 1/7,000 second antibody antiserum dilution (□), and with tubes prepared according to the comparison coating protocol using a 4 mg/L of affinity purified second antibody solution (■). The binding capacity values were 45.1 and 46.5%, respectively. Each point represents the mean of triplicate determination (standard error was <1% throughout standards range).

The recovery was assessed by analyzing patient samples before and after the addition of known concentrations of exogenous T_3 and then subtracting the estimated concentration of endogenous T_3. The recovery of added exogenous T_3 was found to be between 91% and 111% (Table 1). The dilution linearity of the assay was evaluated by assaying serum samples serially diluted with T_3-free serum. We found that there was a good agreement between the measured and the expected values which were derived from the initial concentrations of T_3 in undiluted samples ({Determined value} = 1.032x{Expected value}-0.08; r=0,998; P<0.001; N=22).

TABLE 1

Recovery of Exogenous T_3 Added to Human Serum Samples

Serum Sample	T_3 (μg/L) Amount Added	T_3 (μg/L) Amount Determined	Recovery %
1	0	0.61	
	0.58	1.25	110
	1.18	1.89	108
	1.98	2.81	111
	4.55	5.04	97
2	0	1.26	
	0.58	1.83	98
	1.18	2.50	105
	1.98	3.26	101
	4.55	5.64	96
3	0	2.55	
	0.58	3.15	103
	1.18	3.62	91
	1.98	4.36	92

The RIA developed RIA presented high specificity for T_3. Cross-reactivity of <0.1% was found for 3,5-diiodo-L-tyrosine, 3-iodo-L-tyrosine, 5,5'-diphenylhydantoin, phenylbutazone and reverse T_3. 3,5-Diiodothyronine and T_4 cross-reactivity values were 0.12% and 0.45%, respectively, while 3,3',5'-triiodothyroacetic acid exhibited 100% cross-reaction.

The mean T_3 concentration in sera from 65 apparently euthyroid subjects found to be 1.4±0.7 (±2SD) μg/L and the determined normal range was 0.7-2.1 μg of T_3/L.

The results obtained by assaying serum samples with the developed assay were in good agreement with the results obtained by two commercially available RIA kits for the determination of total T_3, namely the T3-SOLID-RIA (NCSR "Demokritos"), and the T3 Amerlex-M RIA (Johnson & Johnson Clinical Diagnostics) kit. The linear regression equations were $Y = 1.126xX + 0.03$ ($r=0.984$; $P< 0.001$; $N=37$) and $Y = 1.046xX + 0.20$, ($r=0.983$; $P<0.001$; $N=30$) for the T3-SOLID-RIA and the T3 Amerlex-M RIA kit, respectively, where X=comparative assay and Y = RIA developed.

DISCUSSION

The aim of the present work was to develop a method for coating of plastic solid supports with antigen-specific antibodies, using second antibody and antigen-specific antibody antisera dilution. The developed coating approach, which is illustrated in Figure 1, is completed in three steps and allows the use of antisera dilution instead of affinity purified antibodies.

The protocol developed requires excess of second antibody with respect to the immobilized rabbit γ-globulins in order to provide optimum results (Figure 2). When excess of second antibody is used, it seems that most of the antibody molecules bind through one of their binding sites with the immobilized γ-globulins, whereas the other

binding site remains free. This is in agreement with the affected binding capacity values observed when low concentrations of second antibody (e.g. 0.5 mg/L) were combined with relatively high concentrations of rabbit γ-globulins (e.g. higher than 1 mg/L). In this case, the affected binding capacity values can be ascribed to the consumption of both second antibody binding sites for coupling with the immobilized rabbit γ-globulins. From Figure 2, it is also concluded that using rabbit γ-globulins at a concentration of 1 mg/L and second antibody concentration equal to or higher than 1 mg/L, binding capacity values appropriate for use in a T_3 RIA can be achieved.

An important finding of our study was that higher binding capacity values were obtained with tubes prepared according to the proposed protocol, compared with those obtained by the comparison one, for the whole range of the second antibody concentrations tested (Figure 3). This finding is even more impressive taking into consideration that only one binding site per second antibody molecule is available when it has been immobilized through the protocol developed, whereas two free binding sites exist per molecule when it has been adsorbed onto the solid. However, as it has been reported in the literature, extensive loss of the antibody binding activity can be observed when it is adsorbed directly onto plastic solid supports (5, 11). The antibody inactivation is more obvious when low antibody concentrations are used for coating and has been ascribed to the interaction of the antibody molecules with the solid surface. Our findings are in agreement with these literature data, since the greater differences in the binding capacity values between the two protocols were obtained for low second antibody concentrations (<1 mg/L). In addition, the apparent affinity constant determined for the second antibody when it was directly adsorbed onto the solid was significantly lower (approximately 5 times) compared with that determined for the immunoadsorbed second antibody. These results

indicated that the direct adsorption of second antibody affected considerably its binding activity. On the other hand, it seems that when the proposed protocol is used, the γ-globulin layer, which is interposed between the solid surface and the second antibody, prevents its inactivation.

The gain in the binding capacity of the second antibody, when the developed coating approach was applied, was translated to significant saving of the second antibody reagent. Thus, in general, half the amount of affinity purified second antibody was adequate when the proposed protocol was used in order to achieve similar binding capacity values with those obtained using directly adsorbed second antibody.

A main advantage arising from the immobilization principle of the protocol developed was that it allowed for the successful replacement of affinity purified second antibody by the neat antiserum (Figure 4). This was not possible when the direct adsorption of the second antibody was employed. In the latter case, the amount of the finally immobilized anti-species specific antibodies was very low due to the high concentration of the non-specific γ-globulins in the antiserum. On the contrary, only the anti-species specific antibodies existing in the second antibody antiserum were immobilized onto the solid surface by coupling to the immobilized γ-globulins when the proposed coating protocol was applied. Thus, we were able to develop a solid-phase RIA with appropriate analytical characteristics for the determination of T_3 using 1 mL per tube of a 1/7,000 second antibody antiserum dilution instead of at least 2 mg/L of affinity purified second antibody. Concerning the specific second antibody preparation and our affinity purification data, we found that 7,000 tubes could be prepared per mL of second antibody antiserum following the proposed protocol, whereas 2,000 tubes could be prepared by direct adsorption of affinity purified second antibody isolated from 1 mL of antiserum. Thus, besides the elimination of the

affinity purification step, a considerable saving of second antibody was achieved.

Since the second antibody used in our protocol was in excess against both the immobilized rabbit γ-globulins and the anti-T_3 antibody, small fluctuations of the rabbit γ-globulins and second antibody concentrations did not affect the binding capacity values obtained. This feature adds to the coating repeatability of the proposed protocol. In fact, the variation of the binding capacity values obtained for several batches of tubes (500-1000 tubes per batch) prepared over a period of 1 year was less than 5% (data not shown).

The assay based on the proposed coating protocol presented significantly higher sensitivity compared with that developed using tubes coated with anti-T_3 antibody through directly adsorbed second antibody (Figure 5). This finding was significant for our application and might be a result of the immobilization approach followed; general conclusions, however, cannot be drawn from it, since a similar effect was not observed for all of the antibodies used in this study.

The assay developed was accurate as was indicated by the recovery (91-111%) and the dilution linearity values (86-102%) obtained. The low between- and within-run CVs indicated that the assay was reliable and reproducible. Additionally, the T_3 values obtained by assaying human serum samples with the assay developed were in good agreement with those provided by two commercially available RIA kits, as it was indicated by the correlation study.

Finally, taking into account that the immobilized second antibody can serve as a universal separation reagent, the protocol developed can be used for the development of solid-phase immunoassays for the determination of other substances of clinical interest. Our preliminary results concerning assays for thyroxine, free-thyroxine, and thyrotropin indicated that the application of the proposed protocol for these hormones is feasible.

In conclusion, we presented a method for the preparation of antibody coated polystyrene tubes which uses second antibody and anti-T_3 antibody antiserum. Following this approach significantly increased binding capacity of the immobilized anti-T_3 antibody was achieved, compared with that obtained using affinity purified second antibody directly adsorbed onto the solid. As a result, the preparation cost of the solid-phase reagent was significantly reduced. Based on this coating protocol we developed an accurate, sensitive, and reliable solid-phase RIA for the determination of T_3 in human serum samples. The proposed protocol should be helpful for the development of immunoassays for the determination of other antigens.

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