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Nonspecific Binding as a Source of Error in Thyrotropin Radioimmunoassay with Polyethylene Glycol as Separating Agent

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We investigated the effects of nonspecific binding on thyrotropin values obtained by radioimmunoassay in which polyethylene glycol is used as precipitant. Differences in nonspecific binding among individual samples were significant (*F*-test, $p < 0.001$, range 5.5 to 14.1%). Nonspecific binding and total serum protein were directly correlated ($r = 0.472$, $n = 59$, $p < 0.001$). Nonspecific binding increased with increasing concentrations of globulins but showed no relation to albumin concentration. If globulin concentration was <15 g/L, precipitation of the antigen–antibody complex by polyethylene glycol was incomplete. The mean value for thyrotropin in sera from 67 healthy subjects was 2.7 (SD 0.3) milli-international units per liter (milli-int. unit/L) without individual serum nonspecific binding correction, significantly ($p < 0.005$) higher than that with nonspecific binding correction (1.6, SD 0.1, milli-int. unit/L). Evidently, inter-sample variations in nonspecific binding may cause significant errors under these conditions, which can be minimized by taking into account the individual nonspecific binding of each serum sample.

Additional Keyphrases: *variation, source of · reference intervals*

In performing a thyrotropin radioimmunoassay, with use of polyethylene glycol (PEG) as the agent for separation of free and bound radiolabeled thyrotropin, we observed that the nonspecific binding values for some patients' serum samples were considerably lower than those of the equine serum used in the thyrotropin standards. Consequently, the values for thyrotropin we obtained using the nonspecific binding values of equine serum were considerably higher than those based on the nonspecific binding of individual patients' serum samples. This finding prompted us to further investigate the effects of nonspecific binding in radioimmunoassays for thyrotropin.

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Materials and Methods

Materials

Materials for the radioimmunoassay were from Abbott Laboratories, North Chicago, IL 60064. They included tris(hydroxymethyl)aminomethane buffer, pH 7.4, 100 mmol/L, containing, per liter, 3 g of bovine albumin and 2 g of sodium azide as preservative; rabbit thyrotropin antiserum in this buffer (300 000-fold dilution); a set of thyrotropin standards (0, 2.5, 10.0, 20.0, and 40 milli-int. units/L) in equine serum containing 2 g of sodium azide per liter as preservative; ^{125}I-labeled thyrotropin in the tris(hydroxymethyl)aminomethane buffer (about 0.1 mCi/L); and a solution of PEG 6000 (160 g/L in 90 mmol/L barbital buffer, pH 8.9).

Sera from normal persons and from patients were used in the present study. The normal sera were obtained from individuals who had a normal clinical history and a normal chemistry profile and who denied taking any medication within the last several weeks. The patients' sera were randomly selected from the samples sent to our laboratory for evaluation of thyroid function.

The control sera were Tri-Level Radioassay controls purchased from DADE Division, American Hospital Supply Corp., Miami, FL 33152.

Reagents required for the determination of total serum proteins concentration by the biuret colorimetric method (*1*), human serum albumin (Cohn Fraction V), and human serum globulin (Cohn Fraction II) were purchased from Sigma Chemical Company, St. Louis MO 63178.

Methods

The thyrotropin kit manufacturer's suggested assay procedure was modified by reducing the volume of all reagents by half. The modified assay involved incubating for 3 h at 37 °C a mixture of 50 μL of thyrotropin standard or sample and 150 μL of thyrotropin antiserum. This was followed by incubation with 50 μL of ^{125}I-labeled thyrotropin at 37 °C overnight, addition of 1.5 mL of PEG solution (final PEG concentration, 137 g/L), centrifugation, and counting the precipitate for radioactivity.

For determination of nonspecific binding, the exact buffer used to dilute thyrotropin antiserum was used in place of the thyrotropin antiserum solution. The final concentration of thyrotropin antiserum in the assay mixture was so low (1: 500 000) that the contribution to nonspecific binding of serum proteins in the thyrotropin antiserum was considered to be

negligible. Nonspecific binding was expressed as a percentage of the total radioactivity added to each incubation tube.

To study the effects of proteins on the nonspecific binding in this assay, we used 50 μL of a physiological saline solution containing either a fixed amount of human serum albumin (50 g/L) and various amounts of human serum globulins, or a fixed amount of serum globulins (30 g/L) and various amounts of human serum albumin, in place of the serum sample.

The significance of differences between the nonspecific binding values of individual serum samples was tested by analysis of variance (the *F*-test) (*2*). In this study, we determined the nonspecific binding of each serum sample in duplicate. The interindividual variance (variation between the mean values of duplicates of serum samples between individuals) and the intra-individual variance (variation between duplicates of serum samples within individuals) were estimated and the variance ratio (*F*-ratio) was calculated for testing the significance of differences (*2*).

Because thyrotropin was undetectable in about 14% of the healthy subjects by the radioimmunoassay described above (minimum detectable value, 1 milli-int. unit/L), the maximum likelihood method (*3*) was used to estimate the mean and the normal range of thyrotropin values.

The difference between the thyrotropin values with and without correction for nonspecific binding was expressed as a coefficient of variation (CV) of difference, defined by the formula

$$\text{CV of difference} = \sqrt{\Sigma d^2/2n}/\bar{\bar{x}}$$

where d is the difference between the paired data, n is the number of data pairs, and $\bar{\bar{x}}$ is the mean of means of each data pair.

Results

In our modified thyrotropin radioimmunoassay procedure, the amounts of reagents used were only one-half of those suggested by the kit manufacturer. All other assay conditions were kept the same. This modification did not alter the precision of the assay. Nonspecific binding, percent binding, and the standard curve obtained compared well with those obtained by the kit manufacturer's suggested procedure. Within- and between-assay coefficients of variation were less than 10% for thyrotropin concentration of 5 to 30 milli-int. units/L. The modified method required only 50 μL of serum and thus was especially advantageous for assaying pediatric samples. Furthermore, by decreasing the assay volume, we could use the standard 12 × 75 mm test tubes commonly used in our other radioimmunoassays.

The thyrotropin standards prepared in equine serum were proposed by the manufacturer to maintain assay conditions in the standard tubes as close as possible to those in the sample tubes. The mean nonspecific binding of equine serum determined in 31 assays performed over a period of about five months was 9.2 (SEM 0.2)%, compared with a mean nonspecific binding of 7.44 (SEM 0.12)% in 96 patients' serum samples measured during the same time (unpaired *t*-test, $p < 0.001$). Nonspecific binding in the patients' samples varied from 5.5 to 14.1%, and their serum thyrotropin ranged from undetectable to 37.0 milli-int. units/L, after correction for nonspecific binding for each individual. If the nonspecific binding correction were not made or if the equine nonspecific binding were used for the correction, then the thyrotropin concentrations would have been significantly different (paired *t*-test, $p < 0.001$).

These results indicated that the mean nonspecific binding of patients' sera was significantly different from the nonspecific binding of equine serum, but they did not tell us whether the nonspecific binding of the patients' sera differed from one another. To answer this question, the nonspecific binding of each patients' serum was measured in duplicate and subjected to the *F*-test. The ratio of inter- to intra-individual variance was 10.51 (*df* 65 and 66), which indicates that nonspecific binding for each serum sample was significantly ($p < 0.001$) different.

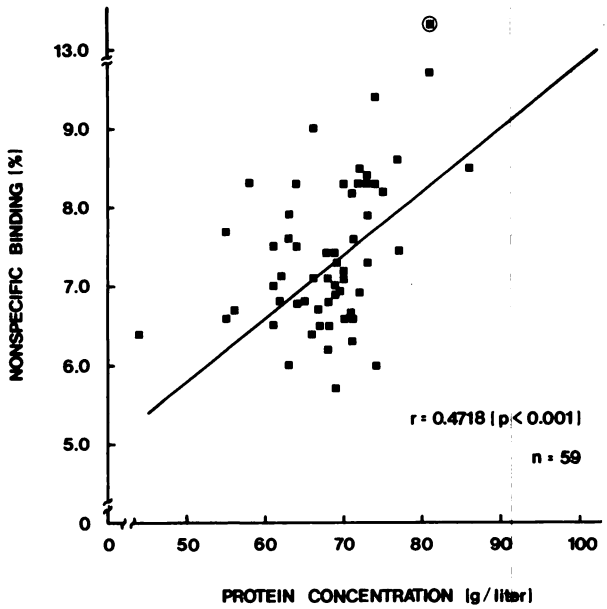

Fig. 1. Correlation of serum protein concentrations with nonspecific binding in a thyrotropin radioimmunoassay with PEG as precipitant.
(■) indicates a value above the break in the ordinate

The importance of this variation is evident from the normal ranges of serum thyrotropin values we determined from 67 healthy subjects, using either equine nonspecific binding or individual sample nonspecific binding for calculation. The unpaired *t*-test revealed that the mean thyrotropin value of 2.7 milli-int. units/L, based on equine nonspecific binding, was significantly higher ($p < 0.005$) than the mean value of 1.6 milli-int. units/L, based on the nonspecific binding of each individual sample. Consequently, the upper limit of the normal range was also higher (7.49 vs 5.52 milli-int. units/L).

We also found a significant direct correlation between individual serum nonspecific binding and the individual total serum protein concentration ($r = 0.472$, n = 59, $p < 0.001$) (Figure 1). The effects of proteins on the percent of nonspecific binding and on the percent of binding (percent of total radioactivity bound to thyrotropin antiserum in the absence of added thyrotropin after nonspecific binding correction) were investigated further by measuring the nonspecific binding in the presence of a fixed amount of albumin and various amounts of globulin and vice versa. The results, summarized in Figure 2, show a greater than six-fold increase in nonspecific binding when the globulin concentration was increased from 3 to 60 g/L and the albumin concentration was kept constant. The percent binding stayed fairly constant at about 33% when the globulin concentration was in the range of 15 to 60 g/L, but decreased sharply when the globulin concentration was reduced further. When the globulin concentration was kept constant at 30 g/L and the albumin concentration was varied from 5 to 100 g/L, nonspecific binding and trace binding both decreased slightly, from 9.2 to 6.8% and from 36 to 33%, respectively. To rule out the possibility that the increase in nonspecific binding with increasing globulin concentrations was due to contamination of the human globulin we used by thyrotropin antibodies, we determined nonspecific binding of a 60 g/L globulin solution in the presence of 750 milli-int. units of thyrotropin per liter, a quantity sufficient to com-

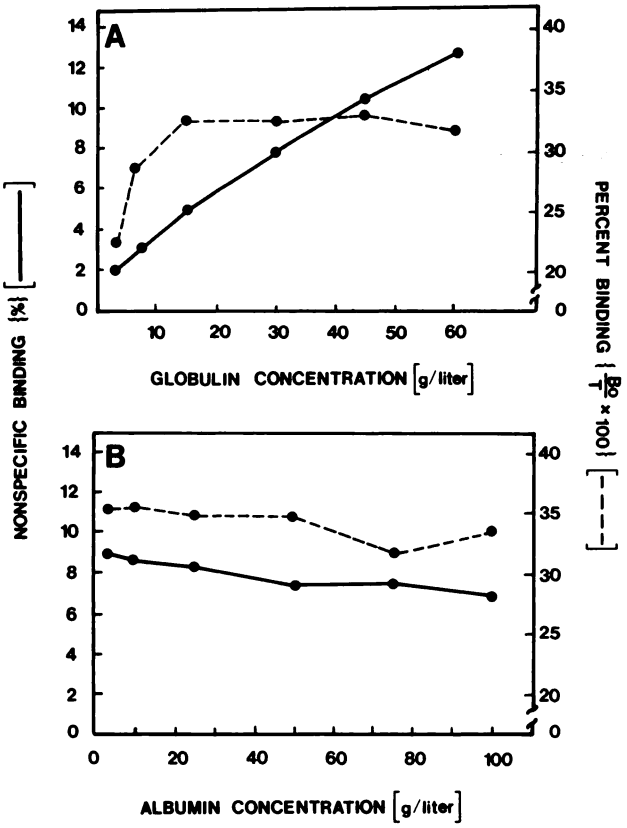

Fig. 2. Effects of human serum albumin and globulins on the nonspecific binding (●—●) and the percent binding (●- -●) in a thyrotropin

A, radioimmunoassay with PEG at constant albumin concentration, 50 g/L; *B*, at constant globulin concentration, 30 g/L. B_0 is the antibody-bound radioactivity in the absence of added thyrotropin and T is the total radioactivity added. All values of the percent binding have been corrected for their respective nonspecific binding

pletely suppress the binding of ^{125}I-labeled thyrotropin to thyrotropin antibodies under the routine assay condition. We observed no decrease in the nonspecific binding, indicating that the observed increase in nonspecific binding was not due to contaminating thyrotropin antibodies.

Tables 1 and 2 show the effect of added globulins on the nonspecific binding and serum thyrotropin values. In this study, 0.8 mg of human serum globulins in 2 μL of physiological saline was added to three control sera just before addition of PEG. This addition increased the globulin concentration of the control sera to about 4 g/L and caused a significant increase in nonspecific binding. The CV's of difference between nonspecific binding without and with added globulin exceeded 19.3% (Table 1). When equine nonspecific binding was used in the calculation, the thyrotropin values in the presence of added globulin were higher in all control sera; the CV's of difference exceeded 15.1% (Table 2). This discrepancy could be minimized when the nonspecific binding of each individual control serum was used in calculation.

Discussion

Since the introduction by Desbuquois and Aurbach (*4*) of PEG as a separating agent for radioimmunoassay, it has been used in many commercial radioimmunoassay kits because of the rapidity and simplicity of the technique. Precipitation of globulins by PEG depends on protein and salt concentrations (*4, 5*).

In our routine thyrotropin radioimmunoassay with PEG as precipitant, the mean nonspecific binding for the equine serum used in the standards is significantly higher than that

Table 1. Effects of Added Globulins on Values for Nonspecific Binding by Three Control Sera

Nonspecific binding, mean (SD), % [a]		
No globulin added	Globulin added [b]	CV of difference, %
5.3 (0.5)	8.1 (1.2)	32.4
5.7 (0.3)	7.4 (0.7)	19.3
5.7 (0.3)	7.5 (1.0)	23.2

[a] n = 7 in each case.
[b] See text.

Table 2. Effects of Added Globulins on Thyrotropin Values of Three Control Sera

Thyrotropin, mean (SD), milli-int. units/L [a]		
No globulin added	Globulin added [b]	CV of difference, %
With equine nonspecific binding		
5.6 (0.2)	7.6 (0.2)	22.9
10.9 (0.2)	13.3 (0.4)	15.1
31.9 (1.2)	47.6 (1.0)	39.8
With individual serum sample nonspecific binding		
5.3 (0.4)	4.8 (0.4)	9.9
10.0 (0.5)	9.2 (0.6)	7.8
25.9 (3.1)	24.2 (0.3)	6.2

[a] n = 7 in each case.
[b] 0.8 mg of human serum globulin in 2 μL of isotonic saline added to 300 μL of incubation mixture just before adding polyethylene glycol.

in patients' sera. Moreover, there is a significant variation of nonspecific binding between individual serum samples, which affects the mean and the normal range of thyrotropin values. The upper limit of normal varied from 5.5 to 7.5 milli-int. units/L, depending on whether the individual correction for nonspecific binding was made. Without the individual correction, individual thyrotropin values could be overestimated by as much as 2 milli-int. units/L at this clinically important concentration.

The effects of varying nonspecific binding became more pronounced at higher concentrations of thyrotropin. An increase in the mean nonspecific binding from 5.7 (SD 0.3)% to 7.5 (SD 1.0)% milli-int. units/L, due to the addition of human serum globulins (Table 1), resulted in an increase in the mean thyrotropin value from 31.9 (SD 1.2) to 47 (SD 1.0) milli-int. units/L when the equine nonspecific binding value was used; this discrepancy could be minimized, however, by using the individual nonspecific binding correction (Table 2). These results indicate that, to obtain more nearly accurate results, every individual serum sample should be corrected for its proportion of nonspecific binding in thyrotropin radioimmunoassays that involve a PEG separation technique.

A direct correlation between the total serum protein concentrations and the nonspecific binding of individual serum samples (Figure 1) suggests that proteins in serum play an important part in the distribution of radiolabeled thyrotropin between the bound and free fractions in the PEG separation system. In our thyrotropin radioimmunoassay, precipitation was carried out at a PEG concentration of 137 g/L at pH 7.4. Under this condition, fibrinogen and most globulins generally precipitate, while albumins stay in the solution (*6*). When we added globulins to three control sera in an amount about double the original amount of globulin in the sera, there were significant increases in the nonspecific binding of all three control serum samples (Table 1), suggesting that globulins were responsible. This increase did not seem to correlate with the amount of thyrotropin in the control serum samples,

however, being 32.4, 19.3, and 23.2%, respectively, for the serum samples containing 5.3, 10.0, and 25.9 milli-int. units of thyrotropin per liter (Tables 1 and 2).

As our studies clearly indicate, labeled thyrotropin can be adsorbed to or coprecipitate with the PEG-precipitated globulins, probably because of increased entrapment of free labeled thyrotropin. This process results in an increased value for nonspecific binding. Shaw et al. (7) did not observe such a phenomenon when they used PEG in the radioimmunoassay of digoxin, a much smaller antigen than thyrotropin. They were able to recover all added tritiated digoxin in the supernate, in the absence of added digoxin antiserum with various amounts of PEG (8.8–247 g/L) and of added albumin or globulins (10–100 g/L). Our results suggest that the globulin-induced increase in nonspecific binding in the PEG separation system may be dependent on the molecular size of the antigen involved.

More recently, it has been reported that about 5% of the total ^{125}I-labeled digoxin in a radioimmunoassay system with PEG as a separating agent (final PEG concentration, 138 g/L) coprecipitates with globulin, and this nonspecific coprecipitation increases with increasing amount of added immunoglobulin G (Ortho Radioassay Reports Bulletin, Ortho Digoxin RIA PEG kit, Ortho Diagnostics Inc.). The discrepancy between this observation and that of Shaw et al. could be due to the difference in the labeled digoxin used (^{3}H vs ^{125}I).

Our studies (Figure 2) also indicate that the globulins, not the albumin, are responsible for the increased nonspecific binding. In fact, there was a slight decrease in nonspecific binding when albumins were increased in the presence of constant amounts of globulins (Figure 2*B*), probably because of a decrease in the globulin precipitation in the presence of increasing amounts of albumin. The sharp decrease in the trace binding at low globulin concentrations was probably due to incomplete precipitation of the antigen–antibody complex by PEG because of insufficient amounts of globulins in the system.

Variations in the nonspecific binding of each patient's sample may cause significant errors in the thyrotropin values reported. These errors can be minimized by correcting the thyrotropin values for the nonspecific binding of each serum sample. A low globulin concentration in a serum sample (less than 15 g/L) may result in incomplete precipitation of the antibody-bound ^{125}I-labeled thyrotropin and thus may give a falsely high thyrotropin value.

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