Interactions of Standard Antibody with Australia Antigens in Au Ag-Ab Radioimmunoassay

HARRY DAUGHARTY, RUTH G. YOUNG, MILFORD H. HATCH, AND DONALD W. ZIEGLER
Center for Disease Control, Public Health Service, U.S. Department of Health, Education, and Welfare,
Atlanta, Georgia 30333

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Human antisera against Australia (Au) antigen have been characterized by liquid-phase radioimmunoassay (RIA) for their precipitation of $^{125}$I-labeled Au antigen. The end-point dilutions of sera (anti-Au) which precipitated 50% of $^{125}$I-Au antigen by RIA correlated well with complement fixation titers but had a much wider range, indicating a greater precision and perhaps a better sensitivity of assay. Anti-Au serum diluted to precipitate 50% of $^{125}$I-labeled Au antigen was used as standard antibody in RIA tests to detect either inhibition or enhancement of the reaction by preincubated mixtures of Au antigen and antibody specimens. Without free Au antigen or antibody in the resultant mixtures there was no inhibition or enhancement; the mixtures presumably contained immunoreactively equivalent proportions of Au antigen and antibody. RIA data for diagnostic specimens indicated an end-point sensitivity which was proportional to the dilution of the standard anti-Au sera used in the test. High concentrations of the standard antibody permitted detectable inhibition of $^{125}$I-Au antigen precipitation at lower antigen specimen concentrations. Similarly, low concentrations of the standard antibody permitted detectable enhancement of $^{125}$I-Au antigen precipitation at lower antibody specimen concentrations. Omitting the standard antibody altogether resulted in a more sensitive RIA for Au antibody in test sera.

Radioimmunoassay (RIA) has become a method of choice in detection of Australia (Au) antigen and antibody (anti-Au) because of its extreme sensitivity. The procedures generally involve either solid phase (12) or liquid phase (5, 12, 15) systems. In this report factors affecting the sensitivity of a liquid phase system are investigated.

Three liquid phase assay methods have recently been developed (1, 5, 12, 15). In each of these methods a known antiserum (standard anti-Au) and a known amount of $^{125}$I-labeled Au antigen are the basic components of the analytic system. When these components are mixed with test specimens, precipitation of the $^{125}$I-labeled Au antigen by the standard anti-Au is either enhanced (if antibody is present in the specimen) or inhibited (if antigen is present in the specimen).

RIA previously demonstrated in blind studies (14) had a high degree of sensitivity and requires the use of $^{125}$I-labeled Au antigen and standard anti-Au of high specificity to avoid false positive results. The important role of the standard anti-Au in the test was recognized by several investigators who have sought to produce anti-Au of high affinity and specificity. Purcell et al. (21) have noted variable responses in specificity and potency of antisera to Au relative to immunizing dose, time after inoculation, and animal species. Dreesman and colleagues (7) have carried out controlled studies to determine the most effective means for producing anti-Au of high specificity and sensitivity.

We have evaluated various Au antisera as standard anti-Au in our procedure, and have determined the sensitivity of RIA relative to the dilution of standard anti-Au. These determinations of sensitivity have permitted us to quantify antigen-antibody ratios in serum specimens containing both Au and anti-Au.

MATERIALS AND METHODS

Antigen source and iodination. The Au antigen used in these studies was a purified, concentrated form of hepatitis B associated antigen. It was obtained from Electro-Nucleonics Laboratories, Inc. Bethesda, Md., lot no. 86-24-1. The immunodiffusion titer prior to iodination was 1:8, and the protein concentration was 633 µg/ml.
For iodination the antigen was diluted in phosphate buffer (pH 7.4) containing EDTA and 1.0 mM MgCl₂ at pH 7.4. Iodination followed the procedure of Greenwood et al. (10) with modifications by Hollinger et al. (12). While 50 µg of purified Au antigen was being mixed in an ice bath, 1.0 mcI of ¹²⁵I in 0.1 M NaOH and an equal volume of 0.1 M HCl were added. After the suspension was thoroughly mixed, 2.5 mg of chloramine T were added dropwise. The oxidation reaction was allowed to proceed for 4 to 5 min while stirring was continued. The reaction was stopped by adding 5.0 mg of potassium metabisulfite. After the sample was mixed for 4 to 5 min, it was exhaustively dialyzed against borate saline buffer (BSB; 0.15 M NaCl, 0.002 M H₂BO₃, pH 7.4). Iodinated antigen was further purified by filtration on a Sephadex G200 column (2.2 by 32 cm) with BSB. The eluted fractions associated with the initial radioactive peak were pooled and used as the ¹²⁵I-Au antigen for the RIA procedure. The radioactivity of this pool was 90 to 85% precipitable with 10% trichloracetic acid and approximately 80% precipitable with our standard antiserum against Au antigen. Assuming about 80% recovery of protein from the Sephadex column, the specific activity of our ¹²⁵I-Au preparations was about 2.25 x 10⁶ counts per min per µg.

Specimens and reagents. The standard anti-Au reagent used in these studies was purchased from Medical Sciences International, Inc., Medical Services Division, Stoneham, Mass. The antiserum had been obtained from a hemophilic individual who had received multiple therapeutic transfusions. Compared to several other anti-Au sera, the selected serum exhibited maximal precipitation of ¹²⁵I-Au antigen in our RIA so as to preclude any possible false positive reactions. Additional human sera tested for Au antibody were also from hemophiliacs or they were selected from diagnostic specimens supplied by the Epidemiology Program, Viral Diseases Branch, Center for Disease Control, Atlanta, Ga.

The specimen of Au antigen positive plasma was obtained from the Atlanta Chapter of the American Red Cross. It was found to be positive for Au antigen by agar-gel diffusion, by crossover electrophoresis (titer 1:2), and by complement fixation (titer 1:128).

RIA procedure. Evaluation of standard anti-Au sera and quantitation of both Au antigen and Au antibody in specimens were performed by RIA. The RIA procedure was essentially the double antibody method of Hollinger et al. (12) and Purcell et al. (15). The double antibody procedure was modified by using Coller and his colleagues’ (5) method of ammonium sulfate precipitation for separating bound ¹²⁵I-Au antigen from unbound ¹²⁵I-Au antigen.

The titration of standard anti-Au and the assays of specimens for Au antigen or antibody are essentially similar procedures. In these procedures 0.5 ml of twofold diluted standard anti-Au is used for the titration. For antigen or antibody assay, preincubated (2 h at 37 C and 18 h at 4 C) mixtures of 0.5 ml of standard anti-Au and 0.05 ml of 1:10-diluted test specimens are reacted in glass tubes (10 by 75 mm) with 0.5 ml of a dilution of ¹²⁵I-Au antigen having approximately 3,000 counts/min. The final volume is adjusted to 1.1 ml by the addition of BSBSA diluent (0.1 M borate, 0.85%; NaCl buffer containing 0.04% bovine serum albumin, pH 8.4). After incubation of this final mixture for 2 h at 37 C and 18 h at 4 C, the specimens either synergistically enhances the precipitation of ¹²⁵I-Au antigen if it contains Au antibody or competitively inhibits precipitation if it contains Au antigen.

In both the standard anti-Au titration and the specimen assay, double antibody complexes are induced by incubating the final mixtures for 2 h at 37 C and 18 h at 4 C with 0.05 ml of a 1:2 dilution of goat antiserum to human IgG. Double antibody complexes which form are precipitated by adding 0.82 ml of 60.9% ammonium sulfate (final concentration of 25.0%) and incubating for 1 h at room temperature. The precipitate is then sedimented for 1 h at 20 C in a Sorvall RC 3 centrifuge at 7,000 x g. Supernates are aspirated into separate test tubes. The distribution of ¹²⁵I-Au antigen between the precipitate (containing bound antigen) and its corresponding supernate (containing unbound antigen) is determined by counting the respective fractions in a gamma-scintillation spectrometer. The end point for a standard anti-Au titration is defined as the dilution of the antiserum which precipitates 50% of the ¹²⁵I-Au antigen (the 50% ¹²⁵I-Au precipitating level). With each group of specimens assayed, a standard set of 10 randomly selected negative control sera is tested to establish a mean baseline value and standard deviation of ¹²⁵I-Au antigen precipitated by negative sera. Minimal precipitation of Au-antigen by these control sera gave a Gaussian (bell-shaped) distribution (14). A positive specimen is inferred when the percentage of ¹²⁵I-Au antigen precipitated by the specimen deviates more than 2 standard deviations from the mean value of ¹²⁵I-Au antigen precipitated by the negative controls.

Other methods of Au antibody titrations. The passive hemagglutination (PHA) (23) tests on the serum specimens were performed at the American National Red Cross, Washington, D.C., to determine the Au antibody PHA titers.

Complement fixation (CF) titers were determined by the Hepatitis and Enteric Virology Section, Center for Disease Control. The micro method for CF (4) was used for the block titration of antigen and antibody.

RESULTS

Standard anti-Au titration. The potency of an antiserum intended for use as a standard reagent in the RIA procedure was evaluated. In the titration of the standard Au antiserum twofold dilutions were reacted with constant amounts of ¹²⁵I-Au antigen. An RIA titration curve for a standard anti-Au preparation with ¹²⁵I-Au antigen is presented in Fig. 1. At dilutions between 1:1,280 and 1:81,920, a decreasing proportion of the ¹²⁵I-Au antigen was precipitated. A maximum of 80% of the ¹²⁵I-Au antigen was precipitated at standard anti-Au dilutions of 1:1,280 or less. At dilutions greater than 1:81,920 a negligible (less than 10%)
precipitation of $^{125}$I-Au antigen occurred. By interpolation, 50% of the $^{125}$I-Au antigen was estimated to be precipitated at a dilution between 1:5,120 and 1:10,240.

Titration of several sera by both CF and RIA showed a good correlation between CF titers and RIA titers (the dilution factor associated with precipitation of 50% of the $^{125}$I-Au antigen). Sera with the highest CF titers also exhibited highest RIA titers. These results, presented in Table 1, indicate that whereas CF results exhibited a fourfold range (8 to 32) in titers, the RIA titers varied over a 32-fold range (160 to 5,120).

**Equivalence proportions (1:1) of Au antigen and antibody determined by standard anti-Au.** The potential of the RIA method for detecting either reactive Au antigen or antibody in specimens was investigated by preincubating fixed concentrations of Au antigen positive plasma (0.025 ml) with variable dilutions of Au antiserum (0.5 ml). The reaction mixtures as specimens were incubated for 2 h at 37 °C and then for 18 h at 4 °C. These reaction mixtures were added to the test system consisting of $^{125}$I-Au antigen and standard anti-Au at a 50% precipitating concentration. Excess Au antigen in the reaction mixtures was indicated by inhibition of precipitation of $^{125}$I-Au antigen. Conversely, excess anti-Au was indicated by enhancement of $^{125}$I-Au antigen precipitation. In Fig. 2 each precipitation curve represents a constant amount of Au antigen plasma reacted with increasing dilutions of anti-Au in the reaction mixture.

Greatest inhibition of $^{125}$I-Au antigen precipitation was observed when anti-Au was preincubated with the highest concentration of Au antigen positive plasma; also, inhibition was greatest when Au antigen positive plasma was preincubated with the lower concentrations of anti-Au. Thus, precipitation below the 50% level was not attained when the reaction mixture consisted of anti-Au and the higher dilutions (1:1,000, 1:10,000) of Au antigen positive plasma. However, precipitation below the 50% level was readily attained with mixtures of higher dilutions of anti-Au and antigen positive plasma dilutions at 1:100 and 1:10. The broken line parallel to the abscissa indicates the 50% precipitation of $^{125}$I-Au antigen observed when the negative control instead of the reaction mixtures was added to the standard test system. When there is a 50% precipitation of the $^{125}$I-Au antigen in the final mixture with the standard test system, one may assume that there is neither free Au antigen nor antibody in

### Table 1. CF titers of standard anti-Au compared with RIA end points for precipitating 50% $^{125}$I-Au antigen

<table>
<thead>
<tr>
<th>Serum specimen</th>
<th>CF titer*</th>
<th>RIA titers* (50% $^{125}$I-Au antigen precipitating end point)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F.Z.</td>
<td>&lt;8</td>
<td>160</td>
</tr>
<tr>
<td>D.G.</td>
<td>8</td>
<td>1,000</td>
</tr>
<tr>
<td>No. 1407</td>
<td>16</td>
<td>1,300</td>
</tr>
<tr>
<td>No. 1402</td>
<td>16</td>
<td>1,800</td>
</tr>
<tr>
<td>No. 1397</td>
<td>16</td>
<td>3,500</td>
</tr>
<tr>
<td>No. 1369</td>
<td>32</td>
<td>3,500</td>
</tr>
<tr>
<td>No. 1393</td>
<td>32</td>
<td>4,000</td>
</tr>
<tr>
<td>G.K.</td>
<td>32</td>
<td>5,120</td>
</tr>
</tbody>
</table>

* End-point dilution factors.
the reaction mixture (ratios of Au antigen and antibody are equivalent, i.e., 1:1). Thus addition of disproportionate amounts of either Au antigen or antibody in the reaction mixture will cause precipitation of $^{125}$I-Au antigen in amounts greater or less than 50%. Precipitation greater than 50% indicates enhancement by excess Au antibody (Au antigen:antibody ratio less than one), and precipitation less than 50% indicates inhibition by excess Au antigen (Au antigen:antibody ratio greater than one).

**RIA sensitivity relative to standard anti-Au concentration.** It was observed that the sensitivity of the RIA method for assaying Au antigen and antibody was related to the concentration of standard anti-Au in the test system. Variable concentrations of standard anti-Au were used in the test system to determine the sensitivity for the assay of Au antigen (Fig. 3) and anti-Au (Fig. 4) in specimens. These dilutions of standard anti-Au antibody represented a full range for minimum (10%) to maximum (80%) precipitation of $^{125}$I-Au antigen. The end point for a test specimen may be considered as the highest dilution of the specimen at which there is detectable inhibition (antigen) or enhancement (antibody) of $^{125}$I-Au antigen precipitated. These specimen end points increase or decrease relative to the dilution of standard anti-Au antibody used in the test (Fig. 3). Although less obvious in Au antigen determinations, higher concentrations of standard anti-Au generally resulted in higher end points for the antigen positive plasma specimen tested. Similarly (Fig. 4), lower concentrations of standard anti-Au usually resulted in higher end points for the antibody positive specimen tested.

The increased sensitivity attained in these experiments suggested that even greater sensitivity in detecting Au antibody could be attained by the complete omission of standard anti-Au in the test system. Therefore, RIA was carried out for a group of serum specimens, with and without standard anti-Au. In Table 2 these results are compared with titers determined by the conventional PHA method. When a dilution of standard anti-Au antibody precipitating 50% of the $^{125}$I-Au antigen was included in the test,

![Fig. 3. RIA of Au antigen in serial dilutions of an antigen positive plasma. Higher concentrations of standard anti-Au antibody resulted in higher end points for the Au antigen positive specimen as indicated by later occurring plateau regions. Standard anti-Au dilutions: Δ, 1:320; ▲, 1:3,200; ○, 1:5,120; ●, 1:20,000; x, 1:80,000.](http://aem.asm.org/)

![Fig. 4. RIA of Au antibody in serial dilutions of an antibody positive plasma. Lower concentrations of standard Au antibody resulted in greater end points for the Au antibody positive specimen as indicated by later occurring plateau regions. Standard anti-Au dilutions: Δ, 1:320; ▲, 1:3,200; ○, 1:5,120; ●, 1:20,000; x, 1:80,000.](http://aem.asm.org/)

**Table 2. Au antibody detected at lower levels of concentration when standard antibody was absent**

<table>
<thead>
<tr>
<th>Specimen no.</th>
<th>PHA titer</th>
<th>RIA results with standard Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Present*</td>
</tr>
<tr>
<td>1576, 1843</td>
<td>1:8</td>
<td>-</td>
</tr>
<tr>
<td>1664, 1864</td>
<td>1:16</td>
<td>-</td>
</tr>
<tr>
<td>1574, 1566</td>
<td>1:32</td>
<td>-</td>
</tr>
<tr>
<td>1086, 1494</td>
<td>1:64</td>
<td>-</td>
</tr>
<tr>
<td>1987, 2157</td>
<td>1:128</td>
<td>-</td>
</tr>
<tr>
<td>1568, 2877</td>
<td>1:256</td>
<td>-</td>
</tr>
<tr>
<td>1026, 1830</td>
<td>1:512</td>
<td>-</td>
</tr>
<tr>
<td>1619, 1844</td>
<td>1:1,024</td>
<td>-</td>
</tr>
<tr>
<td>1495, 1643</td>
<td>1:2,048</td>
<td>-</td>
</tr>
<tr>
<td>1100</td>
<td>1:5,120</td>
<td>+</td>
</tr>
<tr>
<td>1800, 2036</td>
<td>1:10,240</td>
<td>+</td>
</tr>
</tbody>
</table>

*Standard anti-Au present at a dilution which precipitated approximately 50% of the $^{125}$I-Au.

*BSBSA diluent (0.5 ml) substituted in place of standard antibody.
only the anti-Au specimens with PHA titers 1:5,120 or greater were classified as positive. However, when only BSBSA diluent was substituted for standard anti-Au in the test, almost as many positive specimens were detected by RIA as by the PHA method.

**DISCUSSION**

Several characteristic reactions of Au antibody have been noted in the RIA procedures. For example, complete precipitation of $^{125}$I-Au antigen by anti-Au has not been attained. When we tested standard anti-Au dilutions for the percent of $^{125}$I-Au antigen which they were able to precipitate, values greater than 80% were rarely obtained. This may be due to soluble, nonprecipitable Au antigen-antibody complexes or aggregates, or perhaps free 125-I-iodine. Our value of 80% for maximum precipitation is consistent with results of others (5, 12, 15). Although all of our Au antisera have been from humans, antisera from other species have also been compared. It was shown that specific and potent immunoreactivity of Au antibody was the greatest for human, guinea pig, and rabbit antisera, in that order (21).

There was good correlation between CF titers and RIA end-point dilutions of Au antibody precipitating 50% of the $^{125}$I-Au antigen. Although sera with high CF titers also had high RIA end-point dilutions, there was a much wider range of titers by the RIA method than by the CF method. It is evident that Au antisera that react quite similarly in the CF test may vary widely in their capacity to precipitate $^{125}$I-Au antigen (Table 1). The greater variability of the RIA method reflects a definite qualitative difference in Au antisera having equal CF activity. This qualitative difference among Au antisera of the same CF activity is obviously a function of antibody dilution since certain sera contain more reactive (higher degree of affinity) antibodies at lower dilutions. This greater reactivity of certain sera is evident (Table 1) where higher dilutions are just as capable of precipitating 50% of the $^{125}$I-Au antigen as lower dilutions of other sera which may have exactly the same CF titers.

Those sera which precipitate larger amounts of $^{125}$I-Au antigen, presumably from their higher affinity constants, would serve more effectively as standard anti-Au in RIA than sera of lower precipitating capacity. At a fixed dilution the sera with high precipitating titers are more sensitive to inhibition by Au antigen in specimens because $^{125}$I-Au antigen precipitation is proportional to the concentration of standard anti-Au (Fig. 1). Antiserum of extremely high affinity for Au antigen as determined by RIA would also be very useful in simplifying Au antigen purification procedures. Such elaborate procedures involving gradient and rate-zonal centrifugation (7, 8, 17) may be eliminated by using affinity chromatography and anti-Au serum of high potency. The feasibility of this method of affinity chromatography has previously been demonstrated for the isolation of Aleutian disease virus (13).

The selectivity of RIA for anti-Au of high affinity would also have application for therapeutic procedures. In the past, randomly pooled human globulin has been used in treatment of persons accidentally exposed or under high risk of infectivity with hepatitis virus. RIA testing and PHA assays (20) now offer a better basis for selection of antisera with higher titers for use in globulin pools. These pools should be more effective in the elimination of hepatitis virus. This application is consistent with the demonstration by Alpers et al. (2) that high affinity antibody is more effective than low affinity antibody in eliminating circulating protein antigen.

We have described a procedure whereby equivalence proportions of Au antigen and antibody can be determined. Mixtures of immunoreactively equal amounts of the Au antigen and antibody result in nonreactivity of either unbound antigen or unbound antibody. This is a result of complex formation between the added antigen and antibody so that neither is able to react by inhibiting (excess antigen) or enhancing (excess antibody) the precipitation of the $^{125}$I-Au antigen used in the test. Thus, one can quantitate combining ratios of Au antigen and antibody for varying ratios of antigen-antibody complexes. Similar complexes of amylase and specific antibody have been demonstrated to have greater immunogenicity than free antigen (19). The complexes were also shown to be phagocytosed and degraded more rapidly in eliciting a primary response than was the free antigen. At the other extreme, antigen in fourfold excess was shown to have only approximately 0.5% the immunogenicity of unbound antigen (11).

With our proposed method of RIA one can obtain Au antigen-antibody complexes in mixtures of the antigen and antibody in immunoreactively equivalent proportions. Disproportionate amounts of either Au antigen or antibody result in antigen-antibody complexes which may resemble human globulin as antigen in combination with excess globulin-specific
antibody whereby immune precipitates are metabolically processed differently by the host (11). These complexes can be used for determining the role of Au antigen in the pathogenesis of virus-induced immune complex disease and for studying their interactions with rheumatoid factor and complement. Similar investigations with herpes (18) and influenza (6) viruses have indicated increased virus sedimentation characteristics and enhanced neutralization of the virus-antibody complexes upon interaction with rheumatoid factor and complement. A more precise understanding of the roles of Au antigen in immune complex diseases will require further study of the factors which influence the association and dissociation of these complexes.

There was an inverse relationship between the concentration of standard anti-Au and sensitivity in Au antibody determinations (Fig. 4). However, the direct relationship between standard anti-Au concentration and Au antigen determinations was not as obvious (Fig. 3). Plateau regions indicating end points for Au antigen assays did occur later with higher concentrations of standard anti-Au. However, the trend is less noticeable and has less reproducibility than with Au antibody determinations. For this reason one should perhaps use as low a dilution of standard anti-Au as possible in Au antigen assays for achieving maximum 125I-Au precipitation (Fig. 1). On the other hand, higher dilutions of standard anti-Au or omitting it entirely would afford a more sensitive Au antibody assay.

The advantages of RIA for detecting Au antigen and antibody have been demonstrated in recent epidemiological investigations. Numerous surveys for determining the prevalence of Au antigen and antibody (3, 9, 15, 22) have depended heavily upon the sensitivity of the RIA methodology. Without the sensitivity of RIA for carrying out these surveys our present state of knowledge concerning the incidence, transmission, and persistence of an agent associated with hepatitis B would be far more incomplete. Development of more refined RIA techniques will be essential for future epidemiological endeavor in subtyping of Au antigen and antibody. Presently, characteristic dilution curves for the major Au antigen subtypes serve as a basis for RIA differentiation of "ad" and "ay" specimens (9). However, labeling a mono-specific typing serum of single serological specificity has proven useful in determining type-specificities of hepatitis B viral antigen (16). These methods of RIA subtyping will require more selective and more specific standard Au antisera for subtyping purposes.

LITERATURE CITED
nology 23:545-557.