Detection of Australia Antigen by Radioimmunoassay

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ABSTRACT A double-antibody radioimmunoassay has been developed for the detection of Australia antigen and antibodies to Australia antigen in human sera. The assay is simple to perform, and can be completed in 18 hr. Based on parallel studies of human sera, it is approximately 2000 times more sensitive than agar gel diffusion and 200 times more sensitive than quantitative complement fixation. Australia antigen or antibody to Australia antigen was demonstrated in 22 of 23 single serum samples obtained from patients acutely ill with the long-incubation form of hepatitis; eight of the sera were negative when evaluated by less sensitive techniques.

Since the Australia antigen (Au antigen) was first described in 1964 (1), considerable evidence has accumulated to link it with viral hepatitis (2, 3). This antigenic determinant (also called the hepatitis-associated antigen and the SH antigen) appears to reside on a virus-like particle about 200 Å in diameter (4). It has been observed most frequently in patients with the long-incubation form of viral hepatitis (2, 5).

Current methods most frequently used in the testing for Au antigen are agar gel diffusion (2, 3), complement fixation (5, 6), and various electrophoretic techniques (7, 8). We have described a radioimmunoassay for the demonstration of Au antigen that uses human serum with antibodies to the Au antigen (anti-Au antibody) (9). A more sensitive radioimmunoassay, using guinea pig serum that contains anti-Au antibody, has now been developed and is described in detail in this paper. The method is extremely sensitive, can be completed in less than 24 hr, and can be readily modified to test simultaneously for the presence of anti-Au antibodies.

MATERIAL AND METHODS

Au antigen and anti-Au antibodies were initially demonstrated in human serum both by Ouchterlony agar gel diffusion and quantitative complement fixation (5). Immunologic identity with the Au (1) antigen and anti-Au (1) antibody (2) was established by immunodiffusion with sera kindly supplied by Dr. Blumberg and his associates (Institute for Cancer Research, Fox Chase, Pa.).

Au antigen was purified by column chromatography and density gradient ultracentrifugation. Serum was first placed on a column (2.5 x 100 cm) containing Sepharose 2-B, equilibrated with the eluent, 0.15 M NaCl in 0.05% Na deoxycholate. Au antigen was eluted well ahead of the major protein peak. Fractions containing Au antigen were pooled, dialyzed to remove detergent, and centrifuged (Spinco SW25.1 rotor at 25,000 rpm for 28 hr) in a preformed linear gradient of CsCl (density 1.09-1.30). After rebanding in a second CsCl gradient, of density 1.15-1.25, Au antigen was recovered in a single symmetrical peak (fraction density of about 1.20) of absorbance at 280 nm. 5 ml of serum, containing about 300 mg of protein, yielded about 50 μg (1.2 absorbance units at 280 nm) of protein (range 30-100 μg, depending on the serum) by the Lowry technique (10); bovine serum albumin was used as the standard. The purified Au antigen preparation formed a single line of precipitation in agar gel diffusion with anti-Au antiserum. Human serum proteins were not detected by immunoelectrophoresis when tested against rabbit antiserum of high titer.

Anti-Au antibodies were prepared in guinea pigs by injections into the foot pad of about 25–100 μg of Au antigen, mixed in an equal volume of complete Freund's adjuvant. Animals were injected at about monthly intervals with 10–25 μg of Au antigen protein in Freund's adjuvant. Anti-guinea pig IgG and anti-human IgG antibodies, for use in the double radioimmunoassay procedure, were prepared in rabbits by immunization with the appropriate purified IgG (11).

The Au antigen preparation described above (or, more recently, material isolated by zonal centrifugation *) was radio-labeled by the Chloramine-T method of Hunter and Greenwood (12). The reaction mixture contained Au antigen (about 0.004 absorbance unit at 280 nm), 1 mCi of K131I, and 50 μg of Chloramine-T in 0.2 M phosphate, pH 7.4 (final volume 50 μl). After 15 min at 4°C, 250 μg of sodium metabisulphite and 2 mg of KI were added, and free I– was removed on a Sephadex G-25 column equilibrated in phosphate-saline (0.15 M NaCl-0.01 M phosphate, pH 7.4) or 0.15 M veronal buffer, pH 7.4. In selected experiments, reaction conditions were systematically altered to determine maximum radiolabeling efficiency. This included varying the amount of Chloramine-T from 25–200 μg with proportionate changes of sodium metabisulphite, the use of different buffers and pH (ethylene-diamine, 0.2 M, pH 7.4; veronal, 0.2 M, pH 7.5; borate, 0.2 M, pH 8.5), and variations in the reaction time and temperature. Radiolabeled Au antigen was stored in standard diluents (phosphate-saline or veronal buffer, 0.5% bovine serum albumin) at 4°C and 20°C. Under these conditions, precipitability with anti-Au antibody remained constant for at least a month.

* Electronucleonics, Bethesda, Md. 1 ml of this preparation contains 2 x 1014 Au antigen particles; A524 = 0.740. Human serum proteins were not detected by immunoelectrophoresis or immunodiffusion with rabbit anti-human serum.

Abbreviation: Au (antigen), Australia antigen (hepatitis-associated antigen).
The radioimmunoassay was performed by the double-antibody technique using guinea pig anti-Au (0.05 ml of a 1:10,000 dilution) as the first antibody and rabbit serum containing antibodies to guinea pig IgG (in excess) as the second antibody. The human serum under evaluation (0.05 ml at various dilutions) was incubated with the first antibody and [125I]Au antigen (8000 cpm/tube) for 1 hr at 37°C, and then the second antibody was added (final reaction volume 0.35 ml). After another 15 hr at 4°C, the precipitate was isolated by centrifugation, washed twice with ice-cold standard diluent, and counted in an automatic gamma scintillation counter. In the presence of Au-antigen-containing serum, up to 98% inhibition of precipitation of radiolabeled Au antigen was obtained (by comparison with the cpm precipitated when normal guinea pig serum was substituted for first antibody). The average variation between triplicate samples was 5% or less. In serial experiments with the same serum, the variation in the mean value for counts precipitated was 10% or less.

For the detection of anti-Au antibodies in human sera, the samples were incubated with [125I]Au antigen for 1 hr at 37°C, then an excess of rabbit anti-human IgG was added and the incubation was continued for another 15 hr at 4°C.

86 serum samples, each from a different individual, were evaluated initially by agar gel diffusion and complement fixation. Study groups included: (a) 15 sera from patients with post-transfusion hepatitis, "positive" for Au antigen; (b) 8 sera from patients with post-transfusion hepatitis (drawn during the acute phase of their disease), "negative" for Au antigen; (c) 5 sera, which contained anti-Au antibodies, from patients with hemophilia; (d) 8 samples from patients with other forms of liver disease (alcoholic liver disease, 5; drug-induced hepatitis, 3); and (e) 50 control sera obtained from healthy adults and hospitalized patients with no evidence of liver disease as judged by history, physical examination, and laboratory screening procedures (serum bilirubin, transaminase, and alkaline phosphatase).

RESULTS

The technique for iodination of Au antigen described above results in incorporation of only about 1% of the 125I used. Systematic variation of the reaction conditions failed to increase the labeling efficiency. The limited incorporation of 125I on the Au antigen particles could be the result of a low tyrosine content or of low tyrosine reactivity (steric hindrance or hydrogen bonding). The precipitability of radiiodinated Au antigen prepared by sonal ultracentrifugation was consistently in the range of 65-75% at high concentrations of guinea pig anti-Au antibody. Incubation of [125I]Au antigen with rabbit anti-human serum failed to reveal human serum protein contaminants after precipitation with goat serum that contained antibodies to rabbit IgG, which suggests that the protein that remained soluble may be Au antigen rendered nonimmuno-reactive during purification or iodination. Although Au antigen prepared by column chromatography and CsCl ultracentrifugation was free of human serum protein components by immunoelutriphoresis and immunodiffusion, the maximal precipitability of radiiodinated preparations of this material with anti-Au antibody was 58%. The relatively low precipitability of this preparation seemed to be due to trace amounts of contaminating proteins that radiolabel more readily than Au antigen, since rabbit anti-human serum, in the presence of goat anti-rabbit IgG, precipitated up to 20% of the total radioactivity. The presence of radiolabel on protein other than Au antigen did not alter the specificity of the immunoassay (either with guinea pig or human anti-Au antibody), as indicated by the fact that precipitation of [125I]Au was completely inhibited by Au-antigen-containing sera, whereas various dilutions of normal human serum did not inhibit. However, subsequent studies were conducted with radiolabeled Au antigen purified by zonal ultracentrifugation.

Immunized guinea pigs produced a high titer of anti-Au IgG antibodies. A standard titration curve of guinea pig anti-Au antisera is compared to our highest titer human (hemophilia) anti-Au antisera in Fig. 1. The two antibody dilution curves are parallel, indicating that the same antigenic determinants are being measured. The linear portion of the titration curve for guinea pig antisera fell between dilutions of 1:10,000 and 1:120,000; precipitation of radiolabeled Au antigen was still evident at an antibody dilution of 1:200,000. Addition of various amounts of normal human serum did not change the amount of 125I recovered in the precipitate.

Standard inhibition curves of several hepatitis sera containing Au antigen are shown in Fig. 2. The curves are essentially parallel on a semilog plot. The limits of detection correspond to 0.010 to 0.002 microliter of the undilutedAu-positive sera (dilutions of 1:10,000 to 1:40,000).

The effect of varying the length of the incubation period and the sequence in which the various reactants are added was evaluated. The greatest precipitation of the labeled Au antigen (70%) occurred with the longest incubation period (6 days at 4°C). However, 52% precipitation of [125I]Au antigen was obtained after 16 hr at 4°C. Incubation of a mixture of first and second antibody before the addition of 125I essentially eliminated the precipitation of labeled antigen.

The relative sensitivity of the radioimmunoassay (completed in an 18-hr period) is compared to agar gel diffusion

![Graph](image-url)
Fig. 2. Inhibition curves of three Au-antigen-positive human sera by the double-antibody technique (guinea pig anti-Au antibody 1:10,000). 0.05 ml of the indicated dilutions of the three human sera were used. In the absence of Au-antigen-containing serum, 52% of the added [125I]Au antigen was specifically precipitated. Control precipitates (normal guinea pig serum, rather than guinea pig anti-Au antisera) contained only 4% of the added radiolabeled Au antigen.

and quantitative complement fixation in Table 1. Based on parallel studies with Au-antigen-"positive" sera, serially diluted either in normal human serum or standard diluents, the radioimmunoassay was 1200–3500 times more sensitive than agar gel diffusion and 160–240 times more sensitive than quantitative complement fixation in the detection of Au antigen.

![Graph showing inhibition curves of Au-antigen-positive human sera](image)

**Fig. 2.** Dilution curves of three Au-antigen-positive human sera by the double-antibody technique (guinea pig anti-Au antibody 1:10,000). 0.05 ml of the indicated dilutions of the three human sera were used. In the absence of Au-antigen-containing serum, 52% of the added [125I]Au antigen was specifically precipitated. Control precipitates (normal guinea pig serum, rather than guinea pig anti-Au antisera) contained only 4% of the added radiolabeled Au antigen.

**TABLE 1. Dilution of Au-antigen-positive sera**

<table>
<thead>
<tr>
<th>Patient</th>
<th>AGD</th>
<th>CF</th>
<th>RAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:16</td>
<td>1:256</td>
<td>1:40,000</td>
</tr>
<tr>
<td>2</td>
<td>1:8</td>
<td>1:128</td>
<td>1:30,000</td>
</tr>
<tr>
<td>3</td>
<td>1:8</td>
<td>1:64</td>
<td>1:10,000</td>
</tr>
</tbody>
</table>

Relative sensitivities of agar gel diffusion (AGD), quantitative complement fixation (CF), and radioimmunoassay (RAI) in the detection of Au antigen. The values represent the maximal dilution at which Au antigen was still detectable. Each sample was tested at a volume of 0.05 ml. The radioimmunoassay curves for these sera are shown on Fig. 2.

The results of the evaluation of 86 human sera are shown in Fig. 3. In no instance was there overlap between Au-antigen-"positive" or anti-Au-antibody-"positive" serum (as delineated by agar gel diffusion and complement fixation) with the control group. Differences in the mean values were statistically significant ($P < 0.001$) by Student's $t$ test. Six of the 8 samples obtained from patients with a clinical diagnosis of post-transfusion hepatitis that were Au-antigen-"negative" exhibited an intermediate degree of inhibition between the 15 known Au-antigen-"positive" samples and control sera. One post-transfusion hepatitis sample was positive for anti-Au antibodies. Thus, of 23 serum samples obtained from patients in the acute stage of their disease, only one fell within the control range by radioimmunoassay. It should be emphasized that single serum samples (one from each patient) were used in this evaluation and that the sera were collected at various times during the acute phase of the disease. One of the 50 control sera appeared to fall clearly outside the rather tight grouping of the other 49 samples, in the range of the serum from the "Au-negative" hepatitis patients, raising the possibility that it contained a small amount of Au antigen not otherwise detectable. Review of this patient's chart revealed that he had received one unit of plasma in another hospital for a third-degree burn 4 days before admission to Barnes Hospital. None
of the sera from patients with other forms of liver disease was positive for Au antigen or anti-Au antibodies.

**DISCUSSION**

This report describes the development of a sensitive and specific radioimmunoassay for Australia antigen. The assay utilizes 125I-labeled Au antigen, guinea pig anti-Au antibody (the first antibody), and rabbit serum with antibodies to guinea pig gamma globulin (second antibody). The radioimmunoassay for Au antigen differs from most other double-antibody immunoassay systems in that it is necessary to incubate the Au antigen with the anti-Au antibody for at least 30 min before the second antibody is added. If the first and second antibodies are mixed before, or at the time of, addition of [125I]Au antigen, coprecipitation of labeled antigen is markedly inhibited. Evidently anti-Au antibody that has combined with second antibody is unable to bind Au antigen effectively. This observation is in contrast to what is observed with the insulin-anti-insulin immunoassay system (13), where preformed first antibody-second antibody complexes can be used without reducing the sensitivity of the assay. In certain phage-anti-phage immune systems, the presence of second antibody greatly enhances the stability of the phage-anti-phage complexes (14). The physical basis for the impaired binding of [125I]Au is uncertain. Most or all of the Au antigen appears to be present on a virus-like particle about 200 Å in diameter (4). Presumably, antigenic determinants on the exterior surface of this particle would be readily accessible to anti-Au antibody, regardless of whether it was combined with rabbit anti-guinea pig antibody or not. Quite possibly, the antigen is partially buried within the matrix of the particle where sizable immune complexes have difficulty penetrating. Treatment of Au antigen particles with organic solvent gives results that support this explanation. After exposure to ether or genetron, particle size decreases to a diameter of about 160 Å, but antigenic reactivity, as defined by quantitative complement fixation, increases (15). However, difficulty in achieving equilibrium between the two particulate systems (Au antigen particles and preformed, insoluble immune complexes) can not be excluded.

In a radioimmunoassay for Au antigen, there is a choice between the use of human anti-Au antibody obtained from the serum of multiply-transfused patients and anti-Au antibody prepared in animals. With the reagents available to us, the sensitivity of the immunoassay with guinea pig anti-Au as first antibody is about 20-fold greater than the sensitivity with the best human anti-Au antibody. This finding is not surprising, since the sensitivity of a radioimmunoassay depends in part on the affinity of the antibody for antigen. Immunoassay with moderate amounts of antigen in complete Freund’s adjuvant favors the development of high-affinity antibody, especially if late bleedings are obtained (16). It seems probable that with still later bleedings to prepare guinea pig antisera, the sensitivity of the assay may be further increased. Thus, the use of selected animal antisera may be a significant advantage of our assay procedure over the recently described immunoassay system developed independently by Walsh et al. (17), in which human anti-Au antibody is used. Their system also differs in that chromatoelectrophoresis is used to separate free from antibody-bound antigen, and antigen and antibody are incubated together for 48-72 hr before separation.

In the past several years, a number of methods have been described for the detection of Au antigen in serum. The first, agar gel diffusion, has been the least sensitive; it requires 24 hr or longer to become positive. Complement fixation by quantitative semi-micro (5) and micro titer methods (6) is considerably more sensitive, but is difficult to quantitate and poses problems in interpretation because of the occurrence of anticomplementary activity in certain human sera. More recently, various electrophoretic techniques have been reported (7, 8, 18) that reduce the time required for completion of the assay to 1-2 hr, but the sensitivity for antigen as compared with agar gel diffusion is increased no more than 10- to 20-fold. To date, none of these techniques has been positive in all patients with serum hepatitis during both preicteric and icteric phases. Possible explanations for the negative results are: (a) clinically similar infections by agents which do not contain Au antigen; (b) variable concentrations of Au antigen in serum, depending on the disease pattern and the point in the illness at which serum is obtained; and (c) complexes of Au antigen to anti-Au antibody so that antigenic sites are no longer detectable. In extensive agar gel diffusion studies of sera collected from widely scattered areas, the rare occurrence of additional specificities that cross-react with Au antigen and anti-Au antibody has been described (2). In the samples of serum we have evaluated, individual sera have parallel inhibition curves in the radioimmunoassay, indicating that identical, or very closely related, antigens are being detected. Further, using the radioimmunoassay described in this paper, Au antigen or anti-Au antibody was demonstrated in the sera of 22 of 23 patients with parenterally transmitted hepatitis, including seven who were Au-antigen “negative” by less sensitive techniques. Therefore, despite the possibility of variations of Au antigen specificity, it is logical to assume that previous negative results in other studies of long-incubation hepatitis were largely due to the use of less sensitive methods of detection.

The question of immunoassay sensitivity may be equally important in the screening of blood and blood products for Au antigen prior to their administration to patients. In studies with volunteers conducted by the NIH from 1951-1954, involving subcutaneous inoculations of known infectious material, it was demonstrated that 1 ml of an infected plasma pool produced hepatitis in 22 of 37 recipients (19). The same volume of a 1:10⁴ and 1:10⁴ dilution of the plasma caused hepatitis in 2 of 5 recipients and 1 of 5 recipients, respectively. In 15 subjects given plasma diluted to 1:10⁵, 1:10⁶, or 1:10⁷, no cases of hepatitis occurred. In recent Au antigen studies on this material (stored frozen at -20°C or as a lyophilized powder at 4°C), there was a complement fixation titer of 1:10, whereas immunodiffusion results were negative even with the undiluted material (20). Thus, concentrations of Au-antigen-containing material that were at one time infectious gave negative results both by complement fixation and immunodiffusion. Loss of antigen reactivity during storage is unlikely, since our own results and those of others indicate that the antigen is remarkably stable to extremes of pH and temperature. Moreover, Gocke et al. (21) have recorded the occurrence of serum hepatitis in 4 of 69 patients that received blood that was negative for Au antigen by immunodiffusion. The radioimmunoassay reported here appears to be about 2000 times more sensitive than immunodiffusion, and about 200 times


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more sensitive than quantitative complement fixation. It seems likely that the disparity between infectivity of serum and detection of Au antigen can be reduced, or even eliminated, by the application of sensitive test systems such as that described in this paper.

In its present form, the radioimmunoassay can be completed within 18 hr, which makes it an effective method for routine screening of donor blood for Au antigen, except in emergency conditions.

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† Very recently, a preliminary report of a hemagglutinin-inhibition technique for the assay of Au antigen that appears to be more sensitive than complement fixation has been published [Vyas, G. N., and N. R. Shulman, Science, 170, 332 (1970)]. Despite several inherent disadvantages (less precise quantitation, the relative instability of the sensitized cells, possible lack of reproducibility in conjugating Au antigen to erythrocytes), hemagglutination inhibition offers a convenient approach to Au antigen detection. We are currently comparing the sensitivity and reproducibility of hemagglutination inhibition with that of the radioimmunoassay.