Production of Potent Anti-Australia Antigen Sera of High Specificity and Sensitivity in Goats

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Potent antisera of high specificity and sensitivity were produced, in goats, to purified Australia antigen (Au). The antigen was prepared by one of three methods: (i) pelleting, low pH treatment, isopycnic centrifugation two times in CsCl, and rate zonal centrifugation in sucrose; (ii) same as procedure i, with the exception of the low pH treatment; or (iii) twice banding in CsCl by using a BXIV batch-type zonal centrifuge rotor with subsequent preparative Pevikon electrophoresis. The goat anti-Au sera contained high levels of precipitating antibody as tested by immunodiffusion in agar gel and discontinuous counterimmunoelectrophoresis (DCIE) as well as specific complement-fixing antibody and could be used for routine screening of sera for Au without prior adsorption with Au-negative normal human serum (NHS). Identification of 66 of 70 positive specimens (94.3%) in a panel of 98 coded sera (49 duplicates) with 100% reproducibility was made by using one of the goat anti-Au sera at a dilution of 1:16 in the DCIE method. No false positives were recorded. Low levels of antibody against NHS components were effectively removed by a single adsorption with glutaraldehyde cross-linked NHS.

Since the original observations that a unique antigen, termed Australia antigen (Au), was closely associated with long-incubation-type viral hepatitis type B (2, 7, 13), a number of reports have appeared on methodology applicable to the detection of Au. The techniques (8) include immunodiffusion in agar gel (AGD), complement-fixation (CF), counterimmunoelectrophoresis (CIE), passive hemagglutination (PHA), radioimmunoassay (RIA), immune electron microscopy and immunofluorescence. Effective utilization of these procedures is dependent on the availability of high-titered anti-Au sera. Initially, antisera were obtained from multiply transfused hemophiliac patients. However, the availability of this source has been limited, and, in most cases, because of low titer, these sera could be used only at low dilutions for routine screening of Au.

More recently, several reports have appeared on the production of anti-Au antibody in rabbits, guinea pigs, mice, and horses (3, 10, 12, 14), which have been of limited value for one or more of the following reasons: limited quantity, low titers, anticomplementary activity or the requirement for extensive adsorption with normal human serum (NHS).

This report is concerned with: (i) procedures to purify Au effectively, (ii) production of high-titer antisera in goats, and (iii) adsorption of low levels of antibody directed toward NHS components by using insoluble immunoadsorbents.

MATERIALS AND METHODS

Purification of Au. High-titer Au-positive plasma (CF titer 1:5,120) was obtained from an anicteric volunteer blood donor and purified by three different procedures, as follows (Fig. 1).

Procedure 1. Au was pelleted by centrifugation of 120 ml of plasma at 30,000 rev/min for 18 hr in a Spinco fixed-angle type 30 rotor. The pelleted material was resuspended in 2 to 5 ml of 0.15 M NaCl and sonically treated in a Raytheon 10 kc sonic oscillator (Waltham, Mass.) at 9 amps rf for 45 sec. Potassium phthalate buffer (0.05 M, pH 2.4) was then added, and the pH was readjusted to 2.4 with 0.1 N HCl. After incubating for 1 hr at room temperature, the acid-treated material was repelleted for 5 hr in a type 30 rotor as described above, resuspended in saline, sonically treated at 25,000 cycles per sec for 10 sec in a Branson probe-type sonifier (Plainview, N.Y.), clarified at 10,000 rev/min for 10 min, and layered over a 1.10 to 1.40 g/cm³ discontinuous CsCl gradient in 0.15 M NaCl. After the initial isopycnic centrifugation step in a Spinco SW41 rotor at 38,000 rev/min for 20 hours at 10 C, two major bands were observed. The more dense band represented denatured serum protein material, was off-white in color and rubbery in consistency, and formed a partial plug in the tube at a density of approximately 1.28 g/cm³. This
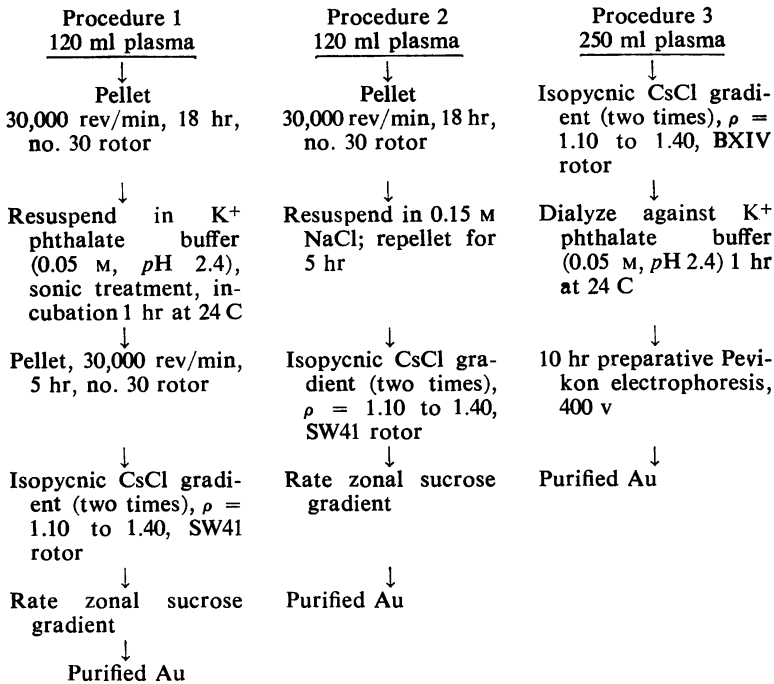
Procedure 1	Procedure 2	Procedure 3
120 ml plasma	120 ml plasma	250 ml plasma
↓	↓	↓
Pellet 30,000 rev/min, 18 hr, no. 30 rotor	Pellet 30,000 rev/min, 18 hr, no. 30 rotor	Isopycnic CsCl gradient (two times), ρ = 1.10 to 1.40, BXIV rotor
↓	↓	↓
Resuspend in K+ phthalate buffer (0.05 M, pH 2.4), sonic treatment, incubation 1 hr at 24 C	Resuspend in 0.15 M NaCl; repellet for 5 hr	Dialyze against K+ phthalate buffer (0.05 M, pH 2.4) 1 hr at 24 C
↓	↓	↓
Pellet, 30,000 rev/min, 5 hr, no. 30 rotor	Isopycnic CsCl gradient (two times), ρ = 1.10 to 1.40, SW41 rotor	10 hr preparative Pevikon electrophoresis, 400 v
↓	↓	↓
Isopycnic CsCl gradient (two times), ρ = 1.10 to 1.40, SW41 rotor	Rate zonal sucrose gradient	Purified Au
↓	↓	
Rate zonal sucrose gradient	Purified Au	
↓		
Purified Au		

FIG. 1. *Procedures used for purification of Au.*

necessitated removal of the less dense Au-positive band by side puncture. After dialysis against 0.15 M NaCl, a second CsCl isopycnic banding was performed, and the material banding at or near a density of 1.21 g/cm³ was removed. Final purification of the saline-dialyzed preparation was accomplished by rate zonal centrifugation in a preformed linear sucrose gradient (10 to 30%, w/w, in 0.15 M NaCl) in an SW41 rotor for 5 hr at 30,000 rev/min and 10 C. Fractions were collected by bottom puncture, Au activity was determined by CF, and the peak fractions were pooled.

Procedure 2. Au was purified as described for procedure 1, with the exception that the acid treatment after the first pelleting was omitted (Fig. 1).

Procedure 3. Au-positive serum (250 ml) was processed in a Spinco BXIV preparative rotor in a preformed CsCl gradient. Fractions having a density of 1.18 to 1.22 g/cm³ were pooled and recycled in a similar gradient. The Au-positive fractions were dialyzed against water to remove the CsCl and then against 0.05 M potassium phthalate buffer, pH 2.4, for 1 hr at 4 C. The Au was further purified by preparative Pevikon zonal electrophoresis with 0.05 M potassium phthalate buffer and electrophoresis for 10 hr at 400 v (4 C). Under these conditions, Au-positive material migrated toward the anode, 5 to 7 cm away from the origin, whereas the detectable protein as measured by the Folin reaction was present in fractions migrating 13 to 24 cm away from the origin. Electron microscope examination of representative negatively stained preparations obtained by each of the three methods yielded 22 ± 2 nm particles free of background debris (Fig. 2). The particle count

ranged from 10^{13} to 10^{14} per ml with a protein concentration of 70 to 200 μg/ml, as determined by the Folin method (9) with crystalline bovine albumin as a standard. About 9 to 12 ml of purified material was obtained from procedures 1 and 2 and 16 to 20 ml from method 3.

Assessment of Au purity. Each preparation of purified Au was examined for contaminating NHS proteins by radioimmunoelectrophoresis (RIE) performed by the method previously described (4). Briefly, a sample of each of the preparations used for immunization of goats was iodinated (6), with the assumption that remaining serum proteins would label with the same efficiency as Au. Equal volumes of ^{125}I-Au were mixed with either Au-positive serum or NHS. These preparations were subjected to electrophoresis, and precipitin bands developed with either anti-Au or anti-NHS sera. The line observed on the exposed X-ray film (Fig. 3) corresponded to the stained precipitin line observed between Au and anti-Au. In contrast, anti-NHS serum failed to coprecipitate any radioactive material. In addition, purified labeled Au contained no detectable NHS components when analyzed by the radioimmunoassay (RIA-DA) technique (6). Further evaluation of the three different purified Au antigen preparations is presented below by analyzing the antibody response of goats hyperimmunized with each.

Immunization of goats. Two goats each were immunized with Au purified by each of the above methods. Equal volumes of purified Au (35 to 50 μg of protein per goat per immunization) and complete Freund's adjuvant were emulsified and inoculated intramuscularly into both flanks and both shoulders
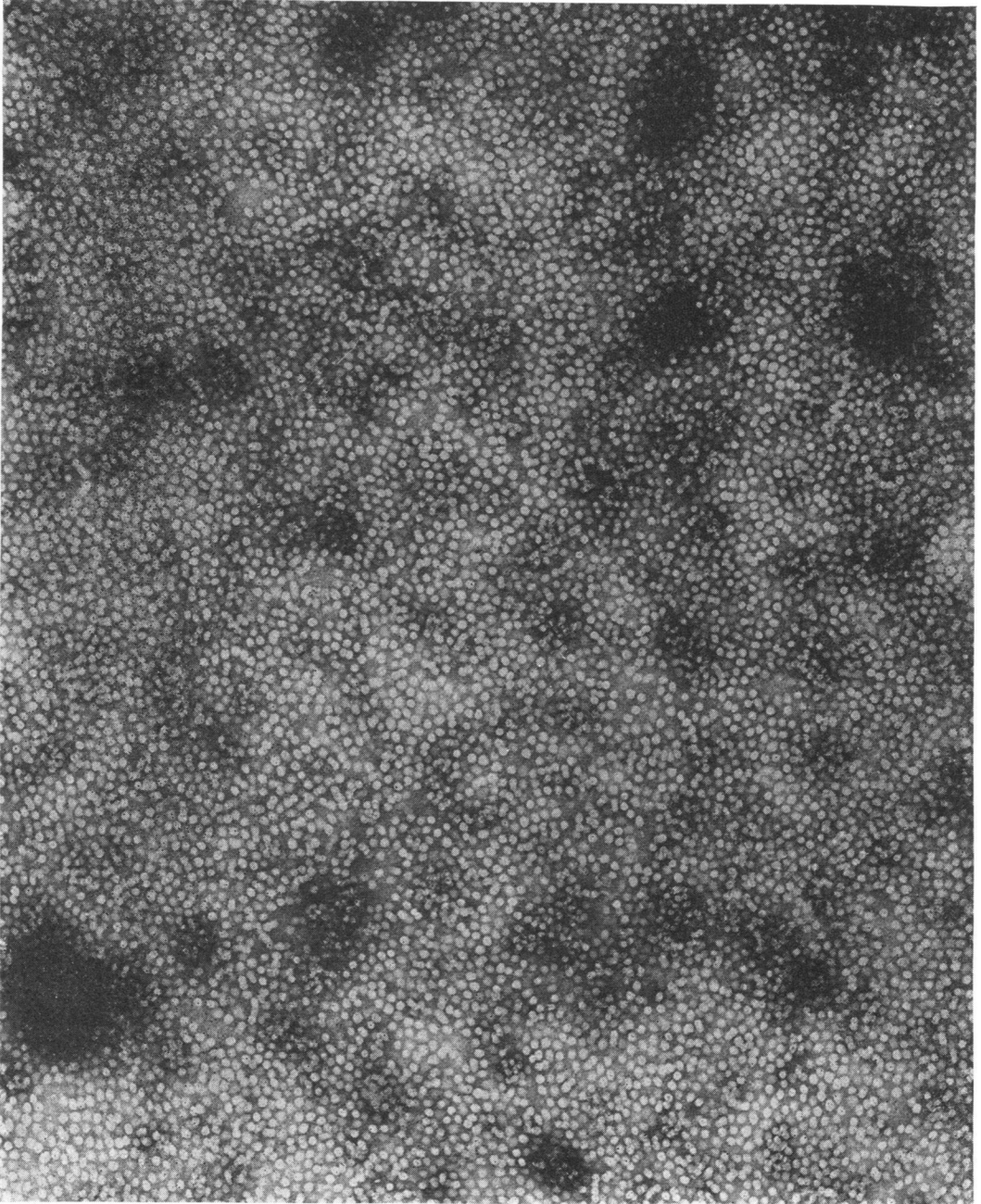
FIG. 2. *Electron micrograph of purified Au particles prepared by procedure 1, Fig. 1, stained with potassium phosphotungstate* ($\times$ 56,000).

(1.0 ml/site). Injections were made on days 0, 14, and 25, and blood was drawn from the jugular vein on days 32, 39, and 56. On days 77 and 89, each of the goats was boosted intramuscularly with 35 μg of Au purified by method 1 with Freund's complete adjuvant. Approximately 500 ml of blood was obtained from each goat on days 98 and 105. All goats were reinocu-

lated on day 158 with material purified by procedure 1 and bled 10 days later (day 168).

Adsorption of antisera. Samples of goat anti-Au sera were adsorbed with an Au-negative NHS pool rendered insoluble by cross-linking with glutaraldehyde by the method described by Avrameas and Ternynck (1). Briefly, one volume of the polymerized
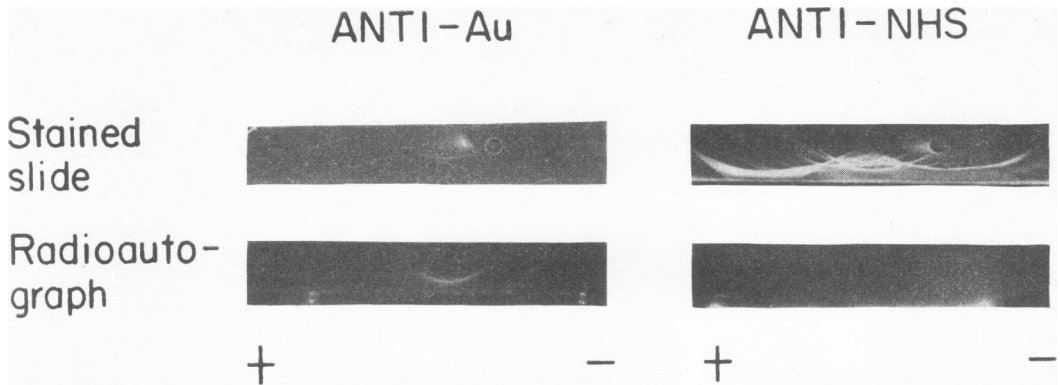
FIG. 3. *Radioimmunoelectrophoresis of purified ^{125}I-Au antigen. A mixture of Au-positive human serum and purified ^{125}I-Au was placed in each well and either goat anti-Au or goat anti-normal human serum (NHS) was placed in the trough.*

TABLE 1. *Antibody titers of goat sera after the first immunization series with purified Au[a]*

Test procedure	Reciprocal antibody titers in response to Au antigen purified by:					
	Method 1 (pH-treated)		Method 2 (untreated)		Method 3 (Pevikon-treated)	
	Goat 2324	Goat 2325	Goat 2326	Goat 2327	Goat 2328	Goat 2329
CF test						
Au/BT0037	<40	80	<40	40	40	<40
NHS[b]	<40	20	<40	40	20	<40
AC activity[c]	<40	10	<40	<10	<10	10
DCIE test						
Au/H368A	256	64	64	64	16	16
Au/Wilson	256	64	64	64	16	16
NHS	Neg	Neg	Neg	Neg	16	4

[a] Goat inoculated on days 0, 14, and 25; blood obtained on day 39.

[b] Au-negative normal human serum pool.

[c] Anticomplementary activity.

NHS adsorbent was centrifuged at 2,000 rev/min for 10 min, washed in phosphate-buffered saline, pH 7.2, and recentrifuged. The pellet was resuspended with one volume of goat anti-Au serum. After 1 hr of incubation at room temperature, the immunoadsorbent material was removed by centrifugation at 10,000 rev/min for 10 min.

Antibody titrations. The concentration of antibody to Au or to NHS in the hyperimmunized goat sera was determined by a modified microtiter CF test (11) and by a discontinuous counterimmunoelectrophoresis (DCIE) technique (17) by using a two-dimensional titration. Unfractionated normal or known Au-positive sera were used as reference antigens in these tests. The specificity of the antibody activity was determined either by AGD or by immunoelectrophoresis (IE) (4). The anti-Au sera were also titrated by a radioimmunoassay procedure employing a second

antibody to separate the immuno-complexes (RIA-DA) by the methods outlined previously (6) and by the PHA technique (6, 16).

RESULTS

Antibody response to purified Au. Two goats were immunized with Au purified by each of the methods shown in Fig. 1. Sera obtained 14 days after the last injection of the first immunization schedule (day 39) were tested by CF, DCIE, and AGD, with both Au-positive and Au-negative reference sera. All six goats responded with anti-Au titers ranging from 1:16 to 1:256 as measured by DCIE (Table 1). Goats immunized with Au purified by methods 1 and 2 produced antibody of greater specificity than those goats inoculated with Au purified by method 3. The relatively low antibody activity detected by CF was unexpected, and only goat 2325 was acceptable as a CF reagent, with a titer of 1:80. Low levels of contaminating antibodies to human serum proteins in goat sera 2324 through 2327, as detected by IE, were directed mainly against normal gamma globulin, beta globulin, or albumin, whereas goat sera 2328 and 2329 reacted most strongly with normal alpha globulin components.

All six goats were reinjected on days 77 and 89 with a second preparation of Au purified by procedure 1. The antibody response as detected in sera obtained 16 days (day 105) after the last booster injection is depicted in Table 2. Each goat showed a typical anamnestic response to Au, whereas the response to contaminating NHS was decreased and even undetectable in goat 2327. All of these sera were usable as screening reagents by any of the serologic methods; for example, goat serum 2327 could be used at a dilution of 1:40 for CF, 1:16 for DCIE, and 1:8 for AGD. An anti-Au titer in excess of 1:5,000,000 and

TABLE 2. *Antibody titers of goat sera after second immunization series with purified Au[a]*

Test procedure	Reciprocal antibody titers					
	Goat 2324	Goat 2325	Goat 2326	Goat 2327	Goat 2328	Goat 2329
CF test						
Au/H-368	80	320	10	640	160	40
Au/BT0037	160	320	160	640	320	160
NHS[b]	<10	<10	<10	<10	40	<10
AC activity[c]	<10	<10	<10	<10	<10	<10
DCIE test						
Au/H-368	256	64	256	1,024	256	1,024
Au/Wilson	1,024	256	256	1,024	1,024	1,024
NHS	Undil[d]	Undil	Undil	0	4	Undil
AGD test						
Au/H-368	64	64	32	32	64	32
Au/Levy	32	16	32	32	128	32
NHS	Undil	Undil	Undil	0	Undil	Undil

[a] Goat bled on day 105 after two intramuscular booster inoculations.
[b] Au-negative normal human serum pool.
[c] Anticomplementary activity.
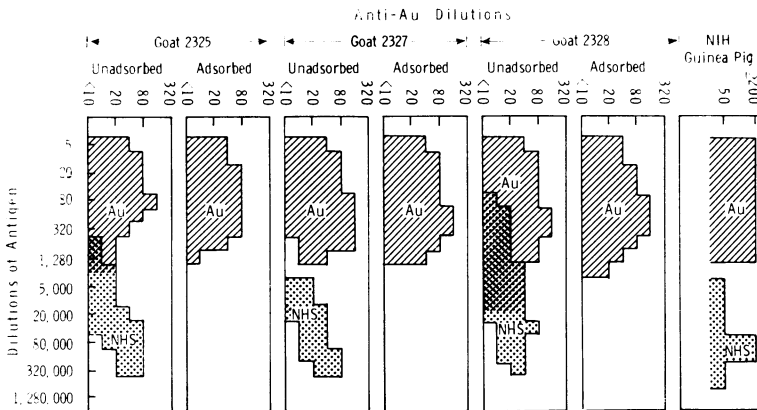[d] Positive reaction only with undiluted serum.

FIG. 4. *Two-dimensional complement-fixation test with Au-positive (lined area) and Au-negative (dotted area) human sera (NHS) versus goat or guinea pig anti-Au antibody. Adsorbed goat antisera were prepared by a single adsorption with glutaraldehyde cross-linked NHS. The guinea pig serum was tested at dilutions of 1:50 and 1:200 only.*

1:1,000,000 was obtained by using the sensitive RIA-DA and PHA procedures, respectively.

The value of the immunization procedure with the purified and concentrated antigen is emphasized in that an inoculation of 30 μg of Au purified by procedure 1 on day 168 again elicited a high-titer anti-Au response with no increase in antibody to NHS components. The anti-Au titers of sera obtained 7 days later were essentially the same as the second set of booster injections. It is noteworthy that antibody to NHS had not increased but actually had decreased in some cases. For example, anti-NHS titers in goat 2328

serum dropped in titer from 1:40 (Table 2) to 1:20 by CF and from 1:4 to undiluted by AGD between the second and third series of Au inoculations.

Specificity of goat anti-Au. A two-dimensional CF titration of representative goat anti-Au sera obtained on day 168 showed peak antibody levels of 1:80 to 1:160 with known Au-positive sera (Fig. 4) and indicated that these antisera could be used at dilutions of 1:20 or 1:40 for diagnostic CF screening. No reactivity against NHS components was observed at the optimal antibody dilution selected until an antigen dilution of

TABLE 3. *Two-dimensional DCIE titration: goat anti-Au (Day 168) versus Au Antigen (H368)*

Au antigen (H368)	Goat anti-Au (day 168)																		
	Goat 2325										Goat 2327								
	Undil	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Undil	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Undil	4+	4+	4+	4+	2+		○	○	○	○	4+	4+	4+	3+	2+	○	○	○	○
1:2	4+	4+	4+	4+	3+	2+	○	○	○	○	4+	4+	4+	4+	2+	○	○	○	○
1:4	3+	3+	3+	4+	4+	3+	+	○	○	○	3+	3+	3+	4+	3+	+	○	○	○
1:8	3+	3+	3+	3+	4+	3+	+	+	○	○	3+	2+	2+	3+	4+	2+ᵃ	+	○	○
1:16	2+	2+	2+	2+	+	3+	+	+	+	○	2+	2+	2+	2+	3+	+	+	○	○
1:32	2+	+	+	2+	+	2+	+	+	+	○	2+	+	+	+	+	+	+	○ᵃ	○ᵃ
1:64	+	+	+	+ᵃ	+ᵃ	+ᵃ	+ᵃ	+ᵃ	+	○	+	+	+	+	+	+	○ᵃ	○ᵃ	○
1:128	+	+	+ᵃ	○ᵃ	○ᵃ	○ᵃ	○ᵃ	○ᵃ	○	○	+	+	○ᵃ	○ᵃ	○ᵃ	○ᵃ	○	○ᵃ	○
1:256	+	+	○ᵃ	○ᵃ	○ᵃ	○ᵃ	○ᵃ	○ᵃ	○	○	○	○	○	○	○	○	○	○	○

ᵃ Became positive after dilution of goat antisera in undiluted normal goat (anti-Au-negative) serum.

1:1,280 to 1:5,120 or higher was reached (Fig. 4). This indicated that the anti-Au sera contained low levels of antibody to one or more major serum protein components.

Routine AGD testing, in which representative antiserum (goat 2325, day 105) was used, showed the development of strong precipitin lines with Au at dilutions of 1:3 to 1:27, showing a line of identity with that precipitated with a human hemophiliac serum (6). No reactivity was observed with undiluted NHS. However, a reaction was observed between undiluted goat 2325 and NHS diluted to 1:256. These contaminating antibodies were found to react with human albumin and immunoglobulin G (Fig. 5a). Similar reactivity was observed after IE of a guinea pig anti-Au (lot V801-501-058) kindly supplied by the Reference Reagents Branch, National Institute of Allergy and Infectious Diseases, NIH (Fig. 5b). A two-dimensional titration by AGD effectively illustrated the danger in accepting purity of antisera by a single line titration by using dilutions of antisera against undiluted NHS. Evidence of low levels of contaminating antibody often did not show up until the NHS was diluted above 1:36 since soluble complexes formed below this level due to antigen excess. A single adsorption of the goat antisera with glutaraldehyde cross-linked NHS abolished all reactivity to NHS components (Fig. 5d) but did not appreciably decrease the antibody titer for Au (Fig. 4, 5c). It is noteworthy that the adsorbed antisera did not become anticomplementary.

Sensitivity of goat anti-Au. A two-dimensional titration by the DCIE method using two goat antisera (2325 and 2327) against an Au-positive reference serum (H-368) is shown in Table 3. Dilution of the goat antiserum in Veronal buffer, pH 8.6 ($\mu=0.015$), indicated that the highest antigen titer was obtained by using undiluted serum or a 1:2 serum dilution. However, when the antisera were diluted in undiluted preimmune (or anti-Au-negative) goat serum, higher dilutions of goat antisera could be used while maintaining the same degree of sensitivity (Table 3). In a DCIE test against 98 coded sera (49 duplicates) supplied by the National Research Council through the Center for Disease Control, Atlanta, Ga., goat 2327 (day 105) antiserum diluted 1:16 in preimmune goat serum identified 66 of the 70 (94.3%) positive specimens with 100% reproducibility. No false positives were observed. A similar degree of sensitivity was observed by using goat 2327 antiserum (day 168) at a 1:20 dilution in the CF test in comparison with the titers obtained by using the NIH-GP reference anti-Au serum (Table 4).
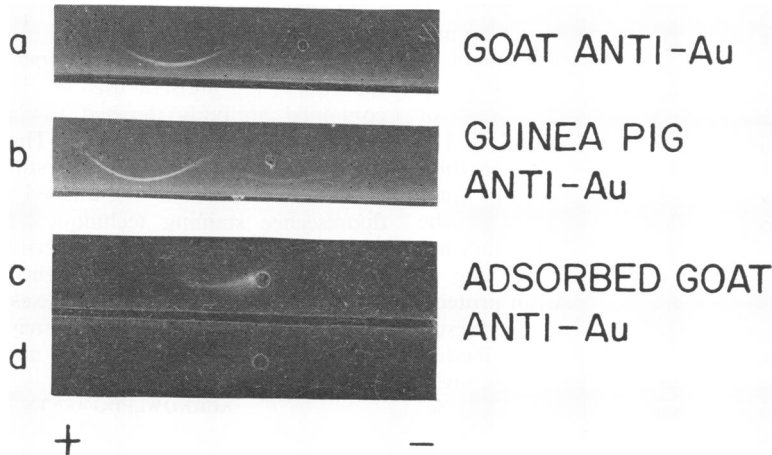
FIG. 5. *Immunoelectrophoretic analysis of anti-Au sera. Wells a, b, and d were filled with Au-negative serum (NHS) diluted 1:64, and well c contained undiluted Au-positive serum. Adsorbed and unadsorbed goat anti-Au (2324, day 105) and NIH guinea pig anti-Au sera were used in the designated troughs.*

TABLE 4. *Detection of Au by complement fixation comparing two hyperimmune sera: goat anti-Au and NIH guinea pig (GP) (lot V801-501-058)*

Specimen identification	CF titer vs. antiserum	
	Goat 2327 (1:20)	NIH-GP (1:200)
1	<8[a]	<8
2	<8	<8
3	8	16
4	16	16
5	8	16
6	64	64
7	<8	<8
8	<8	<8
9	320	320
10	640	640
11	640	640
12	640	1,280

[a] Reciprocal of serum dilution.

DISCUSSION

A method is described by which antisera of high specificity and sensitivity towards Au can be produced in large quantities. Purification procedures 1 and 2 yielded 800 to 2,400 μg of purified Au from 120 ml of anicteric sera which had a starting CF titer of 1:5,120. The immunization schedule which yielded peak titers of antibody with the highest degree of specificity involved an initial injection series of three biweekly intramuscular inoculations followed by two booster inoculations 6 weeks and 8 weeks later using a total of 175 μg of Au protein per goat, emulsified with complete Freund's adjuvant. Thus, 5 to 12 goats could be effectively immunized with Au derived from 120 ml of Au-positive serum, and 5 to 10 liters of high-titering anti-Au could be expected upon exsanguination of the goats. Procedure 3 could be used on a commercial basis to produce large amounts of purified antigen similar to that reported by Gerin et al. (5) by repeated zonal centrifugation, although greater contamination with NHS components occurred in this study. It is also of note that these goats have now been boosted with procedure 1-purified Au a total of four times with little loss of specificity. The specificity is such that it can be used for diagnostic purposes at dilutions at which NHS is not detectable by AGD, CIE, and CF.

The goat antisera produced in this study are suitable as diagnostic reagents by CF, since no anticomplementary activity was detected, unlike that previously observed for goat anti-Au sera (18). On the basis of the very high precipitin titers produced, a higher reactivity would have been expected by the CF reaction. This lower sensitivity in the CF test is unexplained but could be related to poor fixation of guinea pig complement due to unique species differences or to loss of specific CF antigen during purification.

The low levels of anti-NHS present in these antisera were directed toward major serum proteins as evidenced by the two-dimensional CF titrations and by IE. Since these contaminating antibodies were observed after hyperimmunization, one must conclude that NHS proteins were present in the purified preparations at levels below the sensitivity of the RIA-DA and RIA methods employed. The importance of including an NHS control in CF screening of

sera for Au is illustrated in Fig. 4, in that false positives may occur at high dilutions of human serum due to the anti-NHS antibody component in these experimental anti-Au sera. For example, serum from goat 2327, used at a dilution of 1:40, was specific for Au at dilutions of 1:5 through 1:1,280, but, at higher human serum dilutions, the reaction is no longer Au-specific since NHS components are detected. Because of the low levels of anti-NHS antibody, this would not represent a problem in diagnostic screening of sera by CF. However, testing for Au in protein-deficient media, e.g., urine or feces, may result in a false positive. Contaminating antibodies to NHS components were effectively removed by a single adsorption with insoluble glutaraldehyde cross-linked NHS.

There appear to be several advantages in the use of insoluble immunoadsorbents to produce monospecific anti-Au sera. First, the contaminating antibodies can be effectively removed so that no soluble immune complexes remain. Consequently, such an adsorption provides antisera which are not anticomplementary in the CF reaction, and the possibility of elution of contaminating antibody from these soluble complexes is avoided. A second advantage is that antisera which have high levels of contaminating antibody can be adsorbed with large amounts of cross-linked NHS without dilution of the anti-Au sera. Thirdly, the phenomenon of detecting NHS components at high antigen dilutions is eliminated.

In this study, the goat antisera were rendered monospecific with whole insolubilized NHS since the anti-NHS antibodies were of low titer and directed against major NHS components. However, selection of specific proteins to prepare water-insoluble immunoadsorbents could be made after the specificity of the contaminating antibodies was defined. For instance, rabbit anti-Au produced in this laboratory against a partially purified Au preparation contained high levels of antibody against human alpha globulins. Thus, these antisera were more effectively adsorbed with cross-linked human alpha globulin (fraction IV, Pentex Biochemicals, Kankakee, Ill.), since the amount of alpha globulin in whole serum is relatively low.

Schmidt and Lennette (15) emphasized the importance of using optimal proportions of human anti-Au sera to assay Au by either CF or AGD in that an extensive "pro-zone" of inhibitory activity was observed. This phenomenon was confirmed when these potent goat anti-Au were used as screening reagents for CF and AGD tests. The pro-zone effect was minimized as observed by DCIE testing if the goat anti-Au serum was diluted in normal goat serum. The

stabilizing effect of the normal goat serum is under investigation.

It is noteworthy that these goat antisera and the guinea pig antisera used in this study both contained antibody directed toward serum albumin and immunoglobulin G. This is of particular concern in relationship to studies in which experimental antisera are utilized for immunofluorescence staining techniques. Detection of material in the liver, renal glomeruli, and arterial walls could possibly be due to the deposition of soluble antibody-antigen complexes unrelated to Au since experimental antisera may contain low levels of anti-immunoglobulin G antibody.

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