Preparation and Standardization of an Australia Antigen Antibody of Equine Origin

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Received for publication 8 March 1971

A horse has been immunized with Australia antigen (Au/SH) purified 20-fold by a procedure employing gel filtration of Cohn fraction IV derived from an Au/SH-positive human plasma pool. Hyperimmunization was initiated by the intramuscular injection of 20 ml of a mixture of equal parts of purified Au/SH and complete Freund's adjuvant. The 20-ml volume was divided into four 5-ml doses, two of which were administered on each side of the horse's neck. Booster doses of antigen alone were given as follows: 10 ml intravenously 30 days later and 5 ml intramuscularly on each of days 77 and 205. Au/SH antibody formed readily, beginning on day 17, and was demonstrated by the agar gel double-diffusion technique and the complement fixation test during the subsequent 6 months. Antihuman plasma protein antibodies were effectively removed from the horse serum by one absorption with 1 to 3 volumes of normal human plasma. Abrupt rises in anticomplementary activity observed shortly after the third and fourth antigen injections, when the horse had developed elevated and steady levels of Au/SH antibody, could possibly be due to formation of antigen-antibody complexes. After optimal conditions were determined, an Au/SH antibody reagent pool which met official requirements was prepared. It was found equally suitable for the agar gel double-diffusion, complement fixation, and counterimmunoelectrophoresis test procedures.

We have prepared Au/SH antibody in goats, sheep, and in one horse. Of the three, the last has provided the most useful and economic source of an antibody equally suitable for the micro-Ouchterlony agar gel double-diffusion technique (AG), the CF test, and the counterimmunoelectrophoresis method (CEP; reference 9). The results obtained in the horse are reported here.

MATERIALS AND METHODS

Purification of Au/SH. Antigen preparations 1272-6 and 1272-17B that were used to hyperimmunize the horse were both derived from a single 14-liter pool of plasmas from human Au/SH carriers. These had been identified in a prison population studied earlier for the presence of Au/SH (5). The AG titers of individual plasmas against a reference antiserum (KK, see below) ranged from undiluted to 1:32, and the titer of the pool was 1:8.

The antigen was isolated from Cohn fraction IV of the plasma pool, since fraction IV of Au/SH-positive plasmas has been shown to contain large amounts of Au/SH (12).

Au/SH was purified by means of gel filtration of fraction IV on 6% agarose columns equilibrated with citrate-glycine-NaCl buffer adjusted to pH 7.5 with NaOH. The antigen was eluted with the same buffer
just after the breakthrough peak and before α-macroglobulin. The column fractions containing the antigen were concentrated by ultrafiltration.

The two purified antigen preparations used to hyperimmunize the horse had AG titers of 1:100 (preparation 1272-6) and 1:256 (preparation 1272-17B) when titrated against a house reference human antibody (serum KK).

Serological assays. (i) The AG technique as modified by Prince (10) was used, except that protamine was omitted after it was found nonessential to the reaction (5). Standard microscope slides are layered with 3.0 ml of 0.9% agarose, and a seven-wall pattern is cut in the gel. The pattern consists of a center well surrounded by six peripheral wells, each 3 mm in diameter and 3 mm apart. For antigen testing, the antiserum is placed in the center well; conversely, for detecting antibody to Au/SH, the latter is placed in the center well. Test sera or plasmas are not inactivated before testing. For routine testing, each pattern includes a positive control reagent (antigen or antiseraum) in the top and bottom outer wells. Incubation is in humidified chambers at room temperature (22 to 25 C). Generally, precipitin lines are recorded after 1 or 2 days. (ii) The CF test was performed as described earlier (5). Briefly, 0.1 ml each of antigen, antibody, hemolysin, and sheep cell suspension and 0.2 ml of complement containing 2 exact units are used. Complement titers are determined in the presence of 2 to 4 units of antigen when the antibody is titrated or in the presence of 4 units of antibody when the antigen is titrated. All sera and plasmas, whether absorbed or not, are inactivated at 56 C for 30 min before testing. Antigen, antibody, and complement mixtures are incubated overnight at 4 C. After addition of sensitized sheep erythrocytes, the mixtures are further incubated in a water bath at 37 C until the complement and hemolysin controls are completely clear (10 to 20 min). Hemolysis not greater than 2+ is recorded as the titer end point.

The CEP assay was based on the method of Pesendorfer et al. (9), as modified by Gocke and Howe (3) and by Alter et al. (personal communication). Lantern slides (8 by 10 cm) are coated with 10 ml of filtered 1% Bio-Rad agarose dissolved in tris(hydroxymethyl)aminomethane (0.01 M)-ethylenediaminetetraacetic acid (0.001 M)-NaCl (0.01 M) buffer adjusted to pH 7.6. Wells 5 mm in diameter are cut with an interwell edge-to-edge distance of 3 mm. Electrophoresis with tap water cooling for 1.5 hr at 40 ma (approximately 75 v) results in rather long precipitin lines from samples containing Au/SH. Scoring is difficult when smaller wells are used.

Reference antigens and antisera. Reference preparations used in testing and standardization of the horse serum samples and antibody pools were as follows. (i) Our house reference Au/SH consisted of a human plasma (790416) selected from among several carriers for suitability in both the AG and CF tests: its AG titer against reference KK antibody was 1:32, and its CF titer against reference antiserum CL in repeated tests varied in the narrow range of 1:512 to 1:1,024. This house reference is stored at −20 C and has been used for almost 2 years with no apparent change in titer or specificity.

 Provisional or interim reference antigens were also received from the Division of Biologics Standards (DBS), National Institutes of Health, and from the Research Resources Branch (RRB), National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. (by courtesy of Robert J. Byrne). The DBS antigens consisted of a strongly positive (DBS 4+) human serum and a weakly positive (DBS 2+) human serum and of an Au/SH-negative human serum (DBS-Neg.). The RRB antigen was an Au/SH-positive human serum (V801-001-027). More recently, a Reference Hepatitis-Associated Antigen (Australia Antigen) Panel was obtained from the DBS (offered by DBS to all licensed manufacturers, DBS memo dated 10 December 1970). This consisted of six sera: two positive for Au/SH by three test procedures, AG, CF, and CEP (DBS-lot 1 and DBS-lot 2); a third positive by two of the three procedures, CF and CEP (DBS-lot 3); and three negative by all tests (DBS-lot 4, DBS-lot 5, and DBS-lot 6). (ii) Three Au/SH antibody-positive sera have served as house references for the past 2 years: these are KK, CL, and DF. All three are from hemophilia patients. KK is anticomplementary and is therefore used mainly in the AG test (AG titer 1:4). CL and DF are not anticomplementary (AC). CL is reserved for the CF test (CF titer 1:16), whereas DF is used in CEP tests (AG titer, 1:8; CF titer, 1:64).

Interim reference antisera were also received from the DBS and RRB. The DBS preparation is of human origin (DBS-RS, human), whereas that from the RRB is from immunized guinea pigs (RRB-RS, GP).

(iii) Identities of house reference antigen and antisera were first established by testing against reference reagents kindly provided by A. M. Prince, New York Blood Center, New York, N.Y., and by A. G. Redeker, Los Angeles County-University of Southern California Medical Center, Los Angeles.

Identity of house reference antigen 790416 was confirmed by replicate testing by the AG procedure against DBS-RS, human and RRB-RS, GP. In every instance, unequivocal lines of identity were obtained with antigens DBS 4+, DBS 2+, and RRB-V801-001-027 and not with DBS-Neg.

Similarly, identities of our house reference antisera KK, CL, and DF were confirmed by obtaining lines of identity between them and DBS-RS, human and RRB-RS, GP.

RESULTS

Hyperimmunization and bleeding schedules of the horse. The horse used in this study was a 12-year-old gelding quarter horse in good health, weighing about 500 kg (1,000 lb). Its hyperimmunization with purified Au/SH proceeded as follows.

Immediately before the initial injection, a 2-liter volume of blood was drawn to provide a preimmunization serum sample for future testing. The initial injection consisted of 10 ml of prepara-
tion 1272-6 mixed with 10 ml of complete Freund's adjuvant (Difco), blended vigorously to yield a stable emulsion. The mixture was administered intramuscularly in the lateral cervical region of the neck, a 5-ml volume in each of two sites, on each side of the neck. This initial series of four 5-ml injections was followed by only minimal discomfort to the horse, although indurated nodules formed at the injection sites and remained palpable for several weeks.

Thirty days later, a second injection of purified antigen was given to the horse (10 ml of preparation 1272-17B, intravenously into the jugular vein). Within minutes after delivery of the antigen, signs of anaphylactic shock became apparent: the horse went down on its side and had labored breathing. It was treated at once with epinephrine intravenously and intramuscularly, with a corticosteroid preparation (Cortone, Merck Sharp and Dohme) intramuscularly, and with an antihistaminic agent (Benadryl, Parke, Davis & Co.) parenterally. The anaphylaxis symptoms subsided promptly, but the antihistamine injection was repeated after 6 hr. No other systemic incidents occurred after two additional booster injections of antigen administered during the following 6 months, as described below. Because of the anaphylaxis episode just described, intramuscular inoculation was substituted for the intravenous route for further injections of purified Au/SH. A 5-ml booster of preparation 1272-17B was injected 37 days after the intravenous dose, and another 5-ml injection of the same antigen was given 128 days later or 205 days after the initial inoculation. There were no untoward reactions, and no further emergency treatment was required.

The bleeding schedule of the horse over a period of 223 days is as indicated in Figure 1. Beginning 10 days after the initial four injections, a 20-ml blood sample was taken weekly for 6 weeks. When the samples indicated satisfactory response to Au/SH stimulation, 6-liter volumes of blood were aseptically withdrawn at weekly intervals. The serum was separated, clarified by centrifugation, and stored at 20°C without further processing. Small samples were retained from each 6-liter bleeding for testing, as described below.

Testing of weekly horse serum samples by AG and CF. Samples of serum were initially tested by AG against antigen 790416 by using an "in-well" absorption technique (8). In this procedure, the test serum is introduced into the well in which normal human plasma has previously been placed. In this manner, absorption of anti-human plasma protein antibodies present in the horse serum would take place at the periphery of the well, allowing the Au/SH antibody to migrate.

![Graph](https://via.placeholder.com/150)

**Fig 1.** Anticomplementary (AC) activity and anti-Au/SH antibody titers of the hyperimmunized horse by agar gel and complement fixation (CF).
further. In later tests, blood samples were absorbed with human plasma shown to be free of Au/SH and anticomplementary activity. The samples were tested individually as they became available and later retitrated in a single test by AG, with antigen 790416. Two to four units of this antigen was also used in titrations of the absorbed samples by CF, at which time the sera were also checked for anticomplementary activity. Results of the simultaneous tests are presented in Fig. 1. Although absorption of the samples resulted in their dilution by a factor of two or three in most instances, titers are expressed in terms of the dilutions of the absorbed sera and dilution due to absorption is ignored.

The earliest positive AG titer was on the 17-day sample. By the 30th day, when the intravenous booster was administered, the titer had reached the level of 1:16. It was 1:32 1 week later and, with few exceptions, remained at that level until day 153. Decrease in the AG titer to 1:16 during the subsequent 5 weeks may have been more apparent than real, since it again was 1:32 on day 202, 3 days before the last booster. It did not increase beyond 1:32 during the 2 weeks that followed the latter.

The CF antibody also first became measurable on the 17-day sample (titer 1:64). Its titer rose to 1:512 1 week after the 30-day booster and then levelled off at 1:128 until day 70, with one exception on day 48 (titer 1:256). On day 77, when the first intramuscular booster was given, the titer had decreased to 1:64. It cannot be ascertained from the data whether omission of the 77-day booster would have resulted in gradually decreasing titers. In any case, the titer rose abruptly to 1:512 on the week after the booster, and decreased stepwise to 1:16 to 1:32 over the subsequent 120 days. The same abrupt return to 1:512 was noted 11 days after the 205-day intramuscular booster, followed again by a gradual decrease.

Development of anticomplementary activity seemed to be influenced by booster injections of antigen. Starting at undetectable levels on days 0 and 10, anticomplementary activity was first detected on day 17 (titer 1:4) when the Au/SH antibody first appeared. Its titer began to decrease on day 24, after a peak titer of 1:8, but rebounded to 1:16 after the first booster injection. It began to decline once again after day 48, reaching a level of 1:4 between days 62 and 77. As was the case with the CF titer, it also rose abruptly to 1:256 1 week after the second booster. Thereafter, it gradually returned to undetectable levels on day 160 and remained undetected until the 205-day booster. Then, another rapid rise to a titer of 1:128 occurred and started to decrease again on day 216.

Preparation and standardization of equine Au/SH antibody reagent. The blood sample obtained 55 days after the start of hyperimmunization of the horse was selected for preparation of a pool of Au/SH antibody reagent. The choice was made on the basis of an elevated CF titer (1:128) and a relatively low anticomplementary titer (1:8).

With a small sample of serum from this bleeding, it was determined that 3 volumes of normal human plasma was required to absorb completely antibodies against human plasma proteins in the horse serum. Consequently, a 1,400-ml portion of the pool was absorbed with 4,200 ml of human plasma. The latter was a pool of equal volumes of five normal plasmas. After 48 hr at 4 C, the precipitate that formed was removed by centrifugation at 2,000 rev/min for 10 min, and the supernatant fluid was stored at —20 C. Three days later, the fluid was thawed and passed through sterile cotton gauze to remove the small amount of fibrinogen which had aggregated. The resulting clarified preparation was designated 1250-132.

A sample of 1250-132 was diluted 1:2, 1:4, 1:5, 1:6, and 1:8 in saline, and the dilutions were tested by AG against reference 790416 and against a house panel of human plasmas which included Au/SH-positive and Au/SH-negative specimens. Parallel tests were carried out with KK antibody against the same house panel. Comparison of the results indicated that a 1:4 dilution of 1250-132 was optimal, judging by its ability (i) to detect even weakly positive samples which were missed with undiluted 1250-132, (ii) to produce a sharp precipitin line approximately midway between the antigen and antibody wells with a 1:4 dilution of antigen reference 790416, and (iii) to give clearly negative results with Au/SH-negative specimens.

A sample of 1250-132 was also repeatedly titrated by CF against 2 to 4 units of antigen reference 790416, giving Au/SH antibody titers of 1:64 to 1:128 and AC titers of 1:4 to 1:8. A typical checkerboard testing of 1250-132 is shown in Table 1, in comparison with that of house reference human antibody CL; whereas the latter had at most a titer of 1:16, 1250-132 gave an end point of 1:128.

On the basis of these AG and CF results, 4,000 ml of 1250-132 was diluted 1:4 by mixing with 12,000 ml of saline containing 3.2 g of sodium azide as preservative. The resulting preparation, designated 1250-136, represented the final Au/SH antibody reagent. It was dispensed into smaller volume containers and was stored at —20 C while its standardization (or potency testing) was being carried out in several tests, including AG, CF, and CEP.

In a first AG test, 1250-136 was tested against
| Dilutions of house reference Au/SH antigen (790416) | House reference CL at dilutions | Equine antibody prepn 1250-132 at dilutions | Equine antibody prepn 1250-136 at dilutions |
|--------------------------------------------------|--------------------------------|--------------------------------------|--------------------------------------|
| 1:12                                            | 4 4 4 4 2 0 0 0               | 4 4 2 0 0 0                          | 4 4 2 0 0 0                          |
| 1:16                                            | 4 4 4 3 0 0 0               | 4 4 2 0 0 0                          | 4 4 2 0 0 0                          |
| 1:32                                            | 4 4 4 4 4 4 0 0               | 4 4 2 0 0 0                          | 4 4 2 0 0 0                          |
| 1:64                                            | 4 4 4 4 3 0 0               | 4 4 2 0 0 0                          | 4 4 2 0 0 0                          |
| 1:128                                           | 4 4 4 4 4 4 4 0               | 4 4 2 0 0 0                          | 4 4 2 0 0 0                          |
| 1:256                                           | 4 4 4 4 3 0 0               | 4 4 2 0 0 0                          | 4 4 2 0 0 0                          |
| 1:512                                           | 4 4 4 4 4 4 0 0               | 4 4 2 0 0 0                          | 4 4 2 0 0 0                          |
| 1:12,024                                        | 4 4 4 4 4 4 4 0 0               | 4 4 2 0 0 0                          | 4 4 2 0 0 0                          |
| 1:2,048                                         | 4 4 4 4 4 4 4 0 0               | 4 4 2 0 0 0                          | 4 4 2 0 0 0                          |
| 1:4,096                                         | 4 4 4 4 4 4 4 0 0               | 4 4 2 0 0 0                          | 4 4 2 0 0 0                          |
| 1:8,192                                         | 4 4 4 4 4 4 4 0 0               | 4 4 2 0 0 0                          | 4 4 2 0 0 0                          |
| AC activity                                     | 4 4 4 4 4 4 4 0 0               | 4 4 2 0 0 0                          | 4 4 2 0 0 0                          |

a Anticomplementary.

The house panel used above with 1250-132, as well as against reference 790416. All Au/SH-positive samples gave clear precipitin lines, and all negative specimens were unequivocally negative.

In a second AG test, 1250-136 was tested against reference 790416 along with DBS-RS, human; RRBS, GP; K; and DF. Preparation 1250-136 was placed in both the top and bottom wells of the seven-well pattern. A perfect “circle” of identity was obtained with all of these antibody preparations.

Several CF titrations of 1250-136 against 2 to 4 units of reference 790416 gave antibody titers of 1:16 to 1:32 and AC titers of 1:2. Typical checkerboard titrations of 1250-136 and of 1250-132 and the house reference human antibody CL are presented in Table 1: reagent preparation 1250-136 gave a CF titer of 1:16 and an AC titer of 1:2.

Preparation 1250-136 was tested for potency in each of two separate AG tests against the DBS panel of lots 1 to 6. As shown in Table 2, the DBS findings with AG were duplicated in every respect with 1250-136. It was established, furthermore, that DBS-lot 1 had an AG titer of 1:8 and DBS-lot 2 had a titer of 1:2. Similarly, it can be seen that the results of the two CF tests were in good accord. Moreover, it can be observed that, in addition, like the result obtained by DBS, lots 1, 2, and 3 were also positive in our tests; lot 1 had the highest antigen titer (1:1,024), lot 2 an intermediate titer (1:64 to 1:128), and lot 3 had the lowest titer (1:8). Lot 4 was found somewhat anticomplementary in both of our tests but probably negative for Au/SH, and lots 5 and 6 were negative (titers <1:4).

Finally, both 1250-132 and 1250-136 were used repeatedly in the CEP test against house reference 790416, the house reference panel of plasmas referred to above, and the DBS panel of lots 1 to 6. The observations made were as follows. (i) Results could be read in 1.5 hr. (ii) Although both 1250-132 and 1250-136 detected Au/SH-positive specimens in every instance, precipitin lines were generally sharper with 1250-132, leading us to the conclusion that, for maximum sensitivity, the CEP test requires an antibody preparation about four times as concentrated as that giving optimal precipitin lines in the AG assay. (iii) DBS lots 1, 2, and 3, reported by the DBS as Au/SH-positive in the CEP assay, were also positive in our tests, whereas lots 4, 5, and 6 were unequivocally negative. We could also distinguish a clear Au/SH potency gradient with the positive DBS lots, lot 1 giving the most intense precipitin line, lot 2 being intermediate in this regard, and lot 3 producing a faint but discernible line.

**DISCUSSION**

Reliance on hemophilia patients as a source of Au/SH antibody has been rather restrictive, as evidenced by the delay in adoption of the Au/SH test for routine screening of all blood donations. Where testing for Au/SH by CF is preferred, the restriction is further compounded by the fact that the sera of many hemophilia patients are anticomplementary.

The advantages of the horse for antibody production are self-evident: the animal can provide a plentiful, steady, and reliable supply of a reagent. As reported above, we succeeded in preparing an equine Au/SH antibody of good
TABLE 2. Potency testing by agar gel double-diffusion (AG) and by complement fixation (CF) of equine Au/SH antibody reagent against Division of Biologics Standards (DBS) reference antigen panel

| Test no. | Antigen          | DBS results* | Titers with 1250-136 |
|----------|------------------|--------------|----------------------|
|          |                  | AG | CF | AG | CF | AC |
| 1        | DBS-lot 1        | +  | +  | ≥1:1 | 1:512 | <1:4 |
|          | DBS-lot 2        | +  | +  | ≥1:1 | 1:64  | <1:4 |
|          | DBS-lot 3        | -  | -  | -   | 1:4   | <1:4 |
|          | DBS-lot 4        | -  | -  | -   | 1:4   | <1:4 |
|          | DBS-lot 5        | -  | -  | -   | 1:4   | <1:4 |
|          | DBS-lot 6        | -  | -  | -   | 1:4   | <1:4 |
|          | House reference 790416 | 1:32 | 1:2,048 | <1:4 |
|          | Negative control | -  | -  | <1:4 | <1:4 |
| 2        | DBS-lot 1        |   |    | ≥1:8 | 1:024 | <1:4 |
|          | DBS-lot 2        |   |    | 1:2  | 1:128 | <1:4 |
|          | DBS-lot 3        |   |    | -   | 1:8   | <1:4 |
|          | DBS-lot 4        |   |    | -   | 1:8   | 1:8  |
|          | DBS-lot 5        |   |    | -   | <1:4  | <1:4 |
|          | DBS-lot 6        |   |    | -   | <1:4  | <1:4 |
|          | House reference 790416 | 1:32 | 1:2,048 | <1:4 |
|          | Negative control | -  | -  | <1:4 | <1:4 |

* Titers not specified by DBS.

**Anticomplementary activity.

potency equally suitable for the AG, the CF, and the CEP procedures.

To avoid excessive production of antihuman plasma protein antibodies by the horse, purified Au/SH was prepared. By use of gel filtration of Cohn fraction IV of an Au/SH-positive plasma pool and based on AG titration, more than 80% of the Au/SH in the pool was recovered in preparations containing less than 5% of the protein in the solution applied to the filtration column. In addition to Au/SH, only four to six proteins could be detected in the β and α2 regions by immunoelectrophoresis. Thus, the antigen was purified approximately 20-fold by this gel filtration procedure. Further purification was possible but was found unnecessary for the hyperimmunization study reported above; moreover, any antihuman plasma protein antibodies produced by the horse were effectively absorbed by 1 to 3 volumes of normal human plasma.

Au/SH antibody appeared in the serum of the horse within a relatively short interval (17 days) after the initial administration of Au/SH. Only three additional booster injections of antigen were given over the next 205 days, and the AG antibody titers varied within narrow limits (1:16 to 1:32) during the entire period. It is not possible to say from these data whether and for how long these titers would have been maintained without the boosters.

An abrupt rise of anticomplementary activity of the horse serum followed the third and fourth antigen injections, at the time when elevated and steady Au/SH antibody levels had developed. It is possible that this rise was due to sudden formation in the horse of large quantities of antigen-antibody complexes which removed complement from circulation. This hypothesis conforms to Shulman and Barker’s explanation of the nature of anticomplementary activity in sera from hemophilia patients (13), a suggestion which received recent confirmation by Millman et al. (7), who reported direct evidence of the presence of antigen-antibody complexes in the serum of two Au/SH-positive patients, and by Gocke et al. (4), who demonstrated the presence of complexes of Au/SH, Au/SH antibody, and complement in the sera of patients with typical polyarteritis syndromes and mild hepatic damage.

ACKNOWLEDGMENTS

We thank Donald F. Cox for valuable help in injecting and bleeding of the horse and for providing expert veterinary consultation. We also express our appreciation to Burton I. Wilner and Lynde Petter for able assistance in preparing this manuscript.

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