Preparation and Standardization of an Australia Antigen Antibody of Equine Origin

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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.

Discovery of Australia antigen (Au/SH) and its association with viral hepatitis (1, 2, 10) has stimulated great interest and activity in the study of all facets of this infection (15). In particular, in an effort to reduce the risk of post-transfusion hepatitis, it has generated considerable pressure for routine testing of all blood donations intended for transfusion, even though available techniques would detect only 25 to 30% of expected serum hepatitis carriers (14, 15). Within a brief period, there has been a proliferation of test methods advocated for the detection of Au/SH; some are claimed to be more sensitive than others (15). All of these methods, however, have depended primarily on an antibody found in the serum of some multiply transfused hemophilia patients.

In an attempt to make the supply of Au/SH antibody more dependable and, above all, more widely available, its preparation in a number of animal species was and continues to be investigated. Although success has been reported with small animals (6, 8, 11), the limited yields preclude large-scale production. Initial results in goats and donkeys were encouraging, but sera from these species were not suitable for the complement fixation (CF) test (15).

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 modi-
ified by Prince (10) was used, except that protemine
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-well 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
antisera 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 anti-
serum) in the top and bottom outer wells. Incubation
is in humidified chambers at room temperature (22 to
26 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, hemol-
ysin, and sheep cell suspension and 0.2 ml of comple-
ment 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 hemolys-
in controls are completely clear (10 to 20 min).
Hemolysis not greater than 2+ is recorded as the titer end
point.

(iii) The CEP assay was based on the method of
Peskendorfer 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(hydroxy-
methyl)aminomethane (0.01 M)-ethylenediaminetetra-
acetic acid (0.001 M)-NaCl (0.01 M) buffer adjusted to
pH 7.6. Wells 5 mm in diameter are cut with an inter-
well 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 prepara-
tions 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 antisera 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 posi-
tive (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 manufactur-
ers, 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 anti-
sera 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. Rede-
ker, 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 hyper-
immunization with purified Au/SH proceeded as
follows.

Immediately before the initial injection, a 2-liter
volume of blood was drawn to provide a pre-
immunization 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 antihuman plasma protein antibodies present in the horse serum would take place at the periphery of the well, allowing the Au/SH antibody to migrate

![Fig 1. Anticomplementary (AC) activity and anti-Au/SH antibody titers of the hyperimmunized horse by agar gel and complement fixation (CF).](http://aem.asm.org/)

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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 retitrated 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, was 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
TABLE 1. Checkerboard complement fixation testing of equine Au/SH antibody preparations 1250-132 and 1250-136 in comparison with house reference human antibody CL

<table>
<thead>
<tr>
<th>Dilutions of house reference Au/SH antigen (790416)</th>
<th>House reference CL at dilutions</th>
<th>Equine antibody prep 1250-132 at dilutions</th>
<th>Equine antibody prep 1250-136 at dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2</td>
<td>1:4</td>
<td>1:8</td>
<td>1:16</td>
</tr>
<tr>
<td>1:2</td>
<td>1:4</td>
<td>1:8</td>
<td>1:16</td>
</tr>
<tr>
<td>1:32</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>1:64</td>
<td>4</td>
<td>4</td>
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</tr>
<tr>
<td>1:128</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>1:256</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>1:512</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1:1,024</td>
<td>1</td>
<td>±</td>
<td>0</td>
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<tr>
<td>1:2,048</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1:4,096</td>
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<td>0</td>
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</tr>
<tr>
<td>1:8,192</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ACa activity</td>
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<td>0</td>
</tr>
</tbody>
</table>

*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; RRB-RS, GP; KK; 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, 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.

<table>
<thead>
<tr>
<th>Test no.</th>
<th>Antigen</th>
<th>DBS results a</th>
<th>Titers with 1250-136</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AG</td>
<td>CF</td>
</tr>
<tr>
<td>1</td>
<td>DBS-lot 1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>DBS-lot 2</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>DBS-lot 3</td>
<td>-</td>
<td>-</td>
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<td></td>
<td>DBS-lot 4</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>DBS-lot 5</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>DBS-lot 6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>House reference 790416</td>
<td>1:32</td>
<td>2,048</td>
</tr>
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<td></td>
<td>Negative control</td>
<td>-</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>2</td>
<td>DBS-lot 1</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>DBS-lot 2</td>
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<td></td>
<td>Negative control</td>
<td>-</td>
<td>&lt;1:4</td>
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</tbody>
</table>

a Titers not specified by DBS.
b Anticomplementary activity.

to the study reported possible but could not 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.

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LITERATURE CITED


