

A TANNED CELL HAEMAGGLUTINATION TEST FOR THE DETECTION
OF HEPATITIS-ASSOCIATED-ANTIGEN (Au-Ag) AND ANTIBODY (ANTI-Au).

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SUMMARY

A tanned cell haemagglutination technique using inactivated Au-Ag to sensitise human 'O' Rh negative erythrocytes is described. The method may be used to detect Au-Ag, by haemagglutination inhibition (H.I.) and anti-Au, by direct haemagglutination (H.A.). Sensitivity and specificity are demonstrated and its application to mass donor screening is considered.

INTRODUCTION

Although not all post transfusion jaundice is caused by hepatitis B virus, transmission of hepatitis B is one of the most serious complications of the use of blood and blood products. This situation is likely to persist until all donated blood is screened for Australia antigen (Synonyms; Au-Ag, HAA, HB_e Ag) by a rapid, specific and sensitive test system. The techniques currently used in most Transfusion Services are counter electrophoresis (CIEOP) (Prince and Burke, 1970) which lacks sensitivity and latex agglutination (Leach and Ruck, 1971) which is both rapid and sensitive, but lacks specificity (Hopkins and Das, 1973, Ziegenfuss, 1972, Burrell et.al., 1972). The radio-immune techniques (Lander et.al., 1971, Ling and Overby, 1972) are extremely sensitive research tools, but are costly and not yet applicable to the rapid mass screening demanded by the Blood Transfusion Service.

Most laboratories have experience of some form of haemagglutination, and many have found it amenable to automation. In 1969 Juji and Yokichi described a haemagglutination test using formalinised erythrocytes coated with anti-Au (Synonyms; Anti-HAA, HB Ab). Unfortunately, they observed non-specific agglutination, and a tendency for sensitised cells to lyse within a few days. In 1970, Vyas and Shulman published details of a haemagglutination technique using chromic chloride as a coupling agent. This very sensitive technique has gained acceptance in many research laboratories since it compares favourably with CIEOP, (Shaffer et al., 1972). When considering the logistics of its application to routine donor screening, limitations have arisen due to the high degree of antigen purification required (Vyas et al 1972), and great variability observed between different batches of cells (Reesink and Duimel, 1972). Furthermore, it is desirable to use inactivated antigen for coating erythrocytes which will subsequently be used as a routine test reagent. In our experience the outcome of coupling inactivated Au-Ag to human erythrocytes by chromic chloride has proved very unpredictable. This communication describes a tanned cell haemagglutination technique, incorporating inactivated antigen, which we believe goes a long way towards filling the Au-Ag screening requirements of the Blood Transfusion Service.

MATERIALS AND METHODS

Standard /

Standard Sera:

Known Au-Ag positive sera, used as controls, were derived from panels obtained from the NIH (Bethesda), American National Red Cross, Blood Research Laboratory, and apparently healthy carriers found locally. Sera from 6 healthy males (39-64 years), repeatedly tested serologically and electron microscopically, were used as negative controls. Known specific anti-Au capable of reacting with both ad⁺ and ay⁺ antigens (Le Bouvier 1971), were used in the inhibition reaction to detect Au-Ag.

Test Material:

Samples were obtained from selected donors, known contacts of Au-Ag, renal dialysis patients and a subject involved in an 'accident' with Au-Ag positive blood, who subsequently received hyper-immune anti-Au IgG prepared by the Scottish National Plasma Fractionation Centre, Edinburgh. Au-Ag positive and Au-Ag negative normal sera were coded and included among the test samples.

Phosphate Buffered Saline (PBS):

This was prepared from 0.15M Na₂ HPO₄, 0.15M KH₂PO₄ and 0.15M NaCl. Two batches were made, one adjusted to pH 6.4 and the other to pH 7.2. Cell diluent (CD) consisting of PBS 7.2 containing 1% normal rabbit serum (previously inactivated at 56°C for 25 minutes and absorbed with washed packed red cells from the batch being sensitised) was used to suspend the sensitised cells. This was also used to prepare a 1/10 and 1/20 dilution of normal human serum. /

serum. Diluting media were stored at 4°C.

Preparation of Antigen:

Au-Ag positive serum collected from apparently healthy carriers was inactivated using B-propiolactone and ultraviolet light according to Lo Grippo (Lo Grippo and Hartman, 1958, and Lo Grippo et. al., 1971). The inactivated material was pelleted at 120,000g for 4 hours in a M.S.E. Superspeed 65 centrifuge, using an 8 x 25 ml angle head, washed twice in sterile physiological saline and finally resuspended in a small volume of PBS/6.4 and adjusted to give an end point titre of 1/128 by latex agglutination (Pfizer Ltd., Kent, Batch No. L13/4, Leach and Ruck, 1971), or a titre of 1/4 by CIEOP (Das and Hopkins 1971), against a commercial anti-Au serum (Batch T2160C) supplied by Hoechst Pharmaceuticals (Hounslow). Au-Ag, thus prepared and still containing some serum proteins was stored at -20°C until required, when it was thawed and heated at 60°C for 30 minutes prior to sensitisation. /pH

Tanning and Sensitisation Procedure:

Human 'O' Rh negative blood was collected in heparin, washed three times in physiological saline and packed by centrifuging at 800g for 5 minutes. The packed cells were then resuspended in PBS 7.2 to give a suspension of 6.6% (V/V) to which was added an equal volume of 1:10,000 tannic acid (W/V) freshly prepared in the same buffer. The mixture was incubated in a 37°C water bath for 15 minutes, shaking gently every 3 minutes. The tanned cells were washed /

pH

washed once in an equal volume of PBS 6.4 and resuspended in the same buffer to a 4% cell suspension, which was divided into two aliquots. Au-Ag was added to one aliquot in the ratio of 1 volume Au-Ag to 4 volumes of cells, mixed and incubated for 1 hour at room temperature (20°C) on a rotostat (Luckham Ltd., Sussex) to ensure thorough mixing. The remaining aliquot of cells was sensitised in the same way, with 1/125 dilution (in PBS pH 6.4) of pooled (Au-Ag and Anti-Au negative) normal human plasma for use as a plasma sensitised cell control. The sensitised cells were washed three times in equal volumes of CD then made up to a 1.5% suspension in the same diluent and left at 4°C overnight. Next morning the supernatant was replaced with fresh CD and the cells stored at 4°C in small aliquots.

Test Procedure:

Two systems were employed. One used disposable U-bottomed microtitre plates (Flow Laboratories, Irvine) into which reagents were dispensed as drops from a pasteur pipette - referred to as the microtitre system (M.T.S.). In the other system Terasaki microtest tissue culture trays with lids (Bio Cult Laboratories, Paisley) were used. Reagents were dispensed in 5 µl volumes using an eppendorf pipette with disposable tips (Alderman and Co. Ltd., London). This was designated the mini-microtitre system (M.M.T.S.). In M.T.S. 0.75% sensitised cells were used, while a similar degree of sensitivity was obtained in M.M.T.S. with 0.3% sensitised cells.

(i) /

(i) M.T.S.

For detection of anti-Au by direct haemagglutination (HA), the test serum was diluted $\frac{1}{2}$ - $\frac{1}{4}$ in CD and mixed with an equal volume of sensitised cells. The plate was covered to diminish evaporation, and incubated at 37°C for 1 hour. Antibody-containing wells showed positive agglutination characterised by a smooth 'mat' of cells, while absence of antibody was characterised by a 'button' of cells at the bottom of the well (Fig. 1). Once confirmed, the test serum was further diluted and the end point titre, the lowest concentration of antiserum producing complete agglutination, was obtained and defined as 1 HA unit. Controls included a) virus control = sensitised cells + CD, b) positive control = sensitised cells + known anti-Au and c) negative control = sensitised cells + normal serum.

Au-Ag was detected by a two stage haemagglutination inhibition reaction (HI). The test serum was diluted $\frac{1}{2}$ - $\frac{1}{4}$ in CD and mixed with an equal volume of standard anti-Au containing 4 HA units of antibody activity and the plate incubated at 37°C for 30 minutes. Sensitised cells were then added, the plate mixed and reincubated at 37°C for a further hour. Agglutination indicated that no antigen was present. Controls included a) positive control = Anti-Au + known Au-Ag + sensitised cells and b) negative control = anti-Au + normal serum + sensitised cells.

(ii) M.M.T.S.

For /

For detection of anti-Au, test serum was diluted 1/10 in 1/20 normal serum (see PBS) and added to an equal volume of sensitised cells, mixed on a rotostat at room temperature (20°C) for 15 minutes and then transferred to a 37°C incubator for a further 15 minutes. The tray was then spun for 10-15 seconds at No. 1 speed (0-25g) in a bench centrifuge equipped with a serological head (Griffin and George, Middlesex). Finally the tray was inclined at an angle of 60-70 degrees, at room temperature and read against a light background at 5 minute intervals until control reactions were complete. If no agglutination occurs the cells slide down to the lower part of the base of the wells. (Fig. 2).

For detection of Au-Ag, the test serum was diluted $\frac{1}{4}$ in 1/10 normal serum (see PBS) mixed with an equal volume of anti-Au containing 4 HA units for 15 minutes on a rotostat at room temperature, followed by incubation at 37°C. Sensitised cells (5 μ l) were then added to each well, and the two-phase incubation procedure repeated. The tray was centrifuged and read as described. Controls for HA and HI were the same as for M.T.S.

Confirmation:

Positive reactions for anti-Au were confirmed by a) retesting the sample with plasma sensitised cells and b) using the test sera as anti-Au in an inhibition reaction with known Au-Ag positive controls and normal sera.

Positive /

Positive Au-Ag reactions were confirmed by the ability of the sample to inhibit the titre of known anti-Au sera, when compared with normal serum.

Determination of Sensitivity:

The ability of HI to detect Au-Ag was compared with immuno diffusion (ID), electrophoresis (Das et.al,1971, Hopkins and Das, 1972), latex agglutination (Leach and Ruck,1971) using two commercial reagents (Pfizer Ltd., and Hoechst Pharmaceuticals), immune electron microscopy (Kelen et.al,1971), radio-immuno-assay (RIA) using reagents from Abbot Laboratories (Kent), and radio-immuno precipitation (RIP) performed at the Bacteriology Department, Edinburgh University Medical School.

The sensitivity of HA was measured by comparing Anti-Au titres with CIEOP and also by its ability to detect circulating Anti-Au after administration of hyperimmune gammaglobulin to 'accident cases'.

Specificity:

This was investigated by coding known Au-Ag positive and Au-Ag negative sera and distributing them randomly among the test samples. All positive results were repeated for confirmation.

RESULTS

Figure 3 shows partially purified Au-Ag prior to sensitisation, while Fig. 4 shows a sensitised cell with Au-Ag bound to its surface. /

surface. No free Au-Ag can be seen in the supernatant surrounding the cell.

Pooled Au-Ag containing both ad⁺ and ay⁺ subtypes was titred and compared with a variety of other techniques (Fig. 5). It is clear that HI has a similar degree of sensitivity to the radio-immuno methods, and is considerably more sensitive than most of the techniques commonly used in routine laboratories for blood donor or patient screening.

HA is capable of detecting very low levels of anti-Au as compared with CIEOP (Fig. 6). This was further evidenced by its successful application in monitoring the levels of passively administered hyperimmune anti-Au, (details of dose and inoculation schedule to be published elsewhere) in a number of cases where CIEOP has consistently failed to detect any antibody. Fig. 7 shows the levels of anti-Au detected in one such case. No antibody could be detected prior to administration of the gammaglobulin, while circulating anti-Au could be detected within 48 hours after injection and persisted up to 15 weeks later.

Figure 8 shows the results obtained when HAA positive and negative sera were coded and tested by a variety of techniques. HI detected all positive samples as did CIEOP and RIA, and gave no false positive reaction, while one batch of latex, ID and IEM failed to detect several of the positive (Au-Ag) sera, and latex agglutination from both commercial sources gave false positive reactions. /

reactions. The results shown in Table I confirm the specificity of the haemagglutination method, showing complete agreement with CIEOP when sera from selected donors and dialysis patients were tested for Au-Ag and anti-Au. In two of these patients low levels of antibody capable of agglutinating antigen sensitised cells were, in fact, due to reactions against serum protein(s) as demonstrated by agglutination of plasma sensitised cells. This antibody did not inhibit the reactivity of purified ad⁺ or ay⁺ antigens whereas a known anti-Au (diluted to a similarly low level) did have an inhibitory effect under the same circumstances.

DISCUSSION

The technique described in this report provides a simple, rapid, economic and highly sensitive test within the scope of most routine laboratories. Preparation of reagents is straight forward, and the use of inactivated antigen reduces the risk from accidental exposure to personnel involved in routine screening. The use of a 0.75% cell suspension (M.T.S.) allows a reasonable incubation time, removes the need to centrifuge plates and facilitates interpretation of results by personnel receiving only a short period of instruction, while retaining a sensitivity of the same order as that of the radio-immuno techniques. The M.M.T.S. on the other hand, reduces the volume of reagents considerably although it then becomes advisable to centrifuge the trays to obtain the best definition between /

between positive and negative results. The apparent absence of variation amongst different batches of cells eliminates any dependence upon a select group of red cell donors and reflects the stability of the technique. The extreme sensitivity suggests that antisera may be used at least 100 - 1,000 times more dilute than for immunodiffusion or counter-electrophoresis, thereby alleviating problems arising from expense or shortage of reagents. A shelf life of three weeks is acceptable if routine sensitisation is performed fortnightly. Storage of fresh cells in liquid nitrogen and the use of glutaraldehyde fixed cells are at present under investigation in this laboratory with a view to increasing the shelf life of the reagents. It remains to be seen whether or not tanned cells sensitised with HAA will prove suitable for application to an autoanalyser.

Experience in a variety of other biological systems, including hormone estimation, where the tanned cell haemagglutination system has been used extensively, have shown that non specific inhibition can occur in the assay system despite the use of specific antibody (Stavisky and Ingraham, 1964). However, the specificity of the present technique for detection of Au-Ag seems good as no false positive inhibition reactions have occurred amongst the number of samples so far tested. A prozone effect was noted in HA when testing high titre antibody such as hyperimmune sera; this was eliminated by using a starting dilution of test sera $1/4$ in M.T.S. and /

and 1/10 in M.M.T.S. For the detection of antigen it was found that diluting the test samples 1/4 in normal serum (1/10) enhanced sensitivity of the M.M.T.S. and led to a more effective 'settling' in the wells. Increasing the cell content in the M.M.T.S. reduced sensitivity and enhanced prozoning.

It is probable that a proportion of the normal, apparently healthy, population may show positive reactions due to low levels of antibody against plasma proteins when screened by this sensitive method. Brumelhuis et al (1971) found 13 such reactions in 30,000 samples tested by the Netherland Red Cross Transfusion Service using the less sensitive technique of immunodiffusion. Patients, particularly multitransfused patients, are likely to produce such reactions due to the development of iso-precipitins, (Blumberg, 1964, Langgenhuysen, 1971). One possible way of circumventing this problem is to use highly purified Au-Ag for coating the cells. However, when 'purified' Au-Ag was used in the haemagglutination method of Vyas and Shulman (1970), as modified by Prince et.al. (1971), the sensitised cells were agglutinated by commercial anti-whole human sera and an anti-human IgG (Prince, 1972). This finding may indicate the presence of anti-HAA in the commercial antisera, but is more likely to represent contaminating serum protein(s) in the 'purified' antigen. Whether the contaminant(s) is a part of the Au-Ag moiety (Millman et. al., 1971), or plasma protein, is not known. However, the practical conclusions from these observations are that any /

any serum giving a positive reaction, particularly for antibody, must be retested using plasma sensitised cells, and further confirmed by the means outlined in this paper. Fig. 9 represents the situation which may arise in a multitransfused patient possessing antibodies to both HAA and certain plasma proteins (Verucci et.al,1971). Further investigation of this phenomenon is required, but such results are of interest in the light of the high incidence of apparently low levels of anti-HAA in multi-transfused patients reported by Lander et.al, (1971) and Levy and Hawrasiak, (1972).

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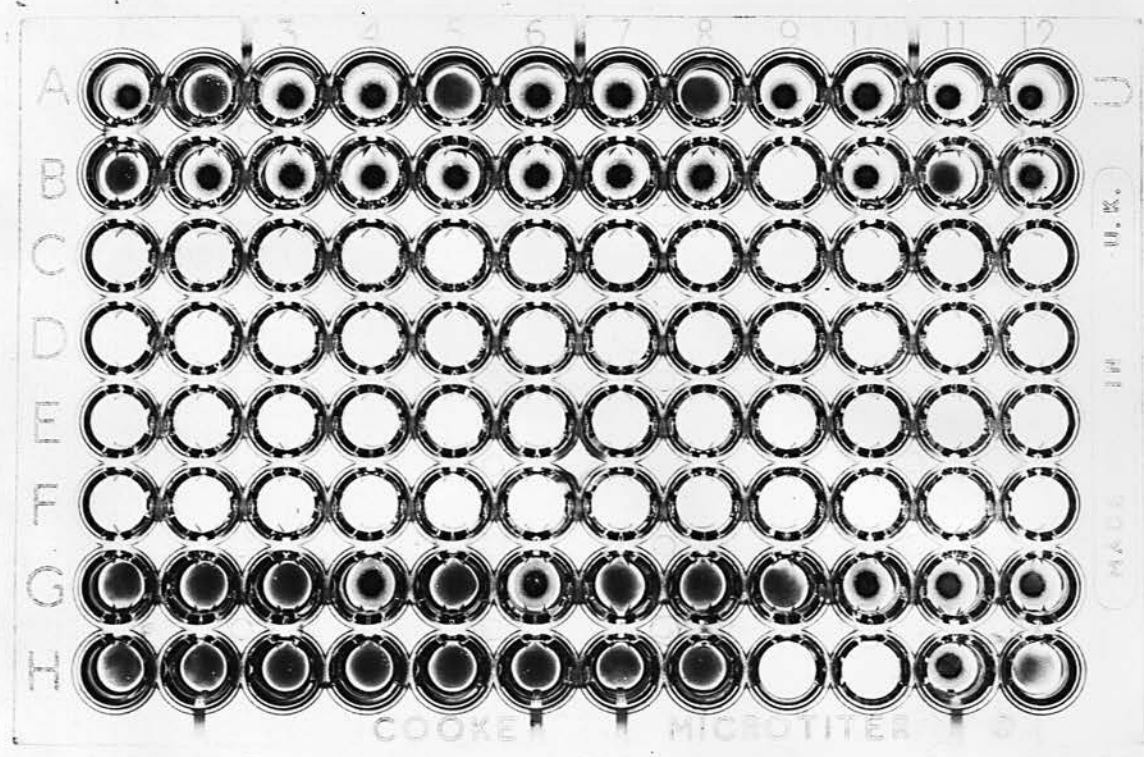


Fig. 1. Appearance of positive and negative haemagglutination patterns in microtitre system. (M.T.S.)

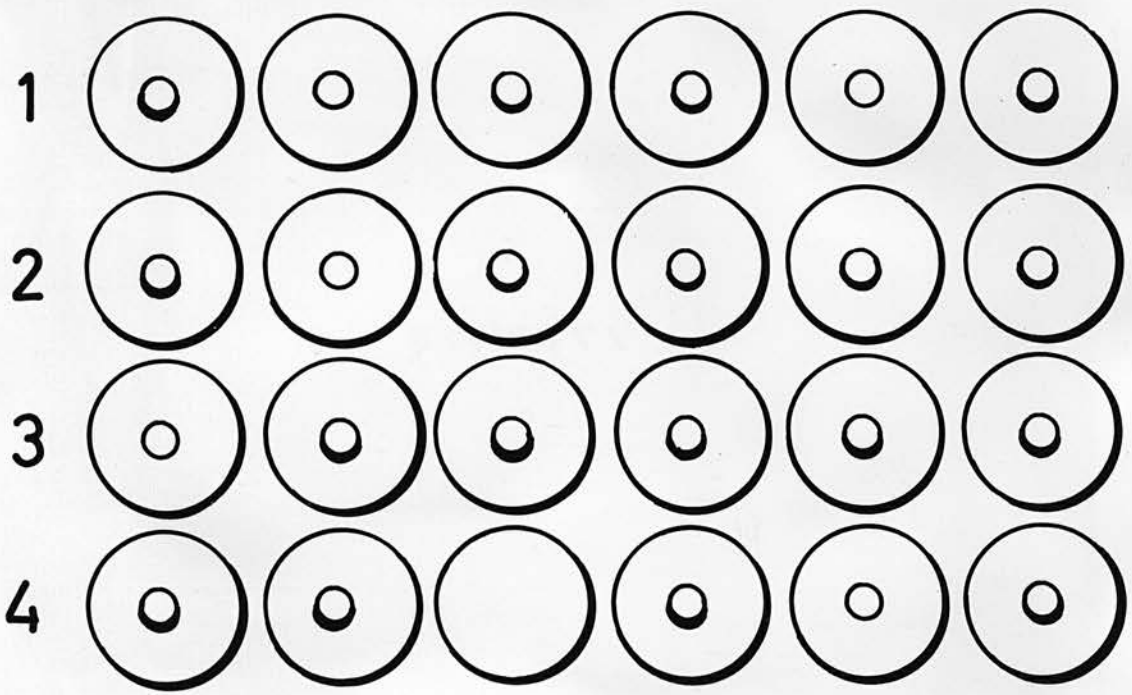
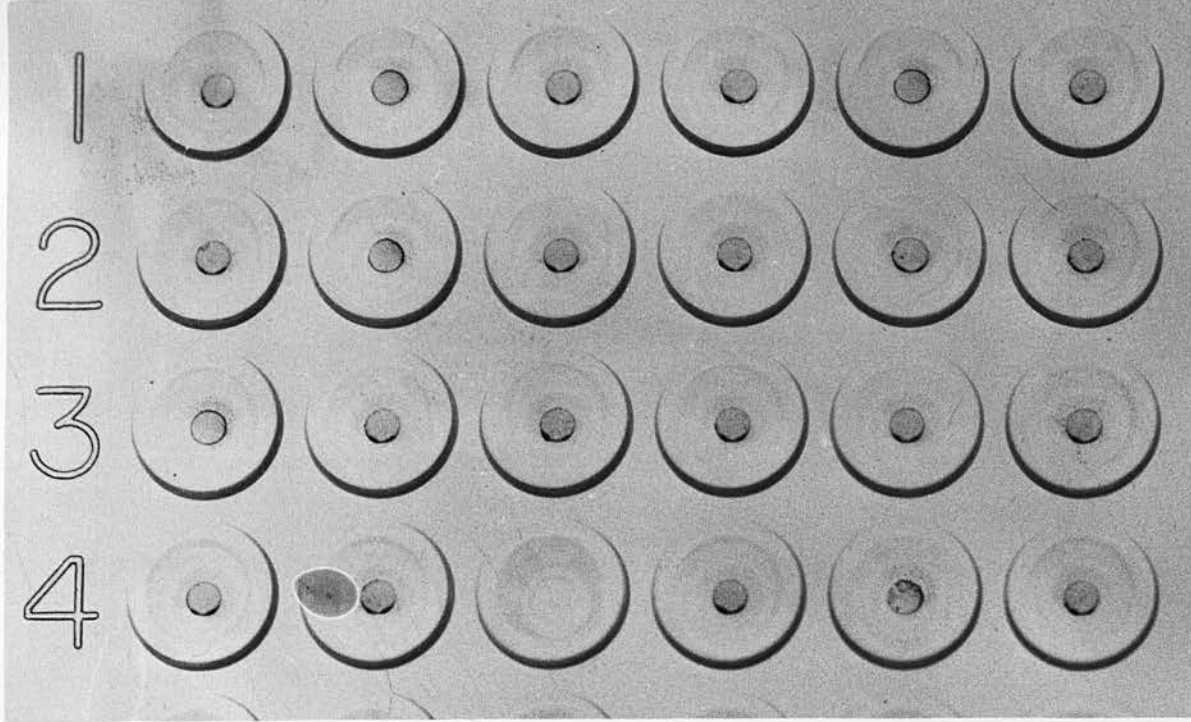


Fig. 2. Appearance of positive and negative haemagglutination patterns in mini-microtitre system (M.M.T.S.), including artists impression.

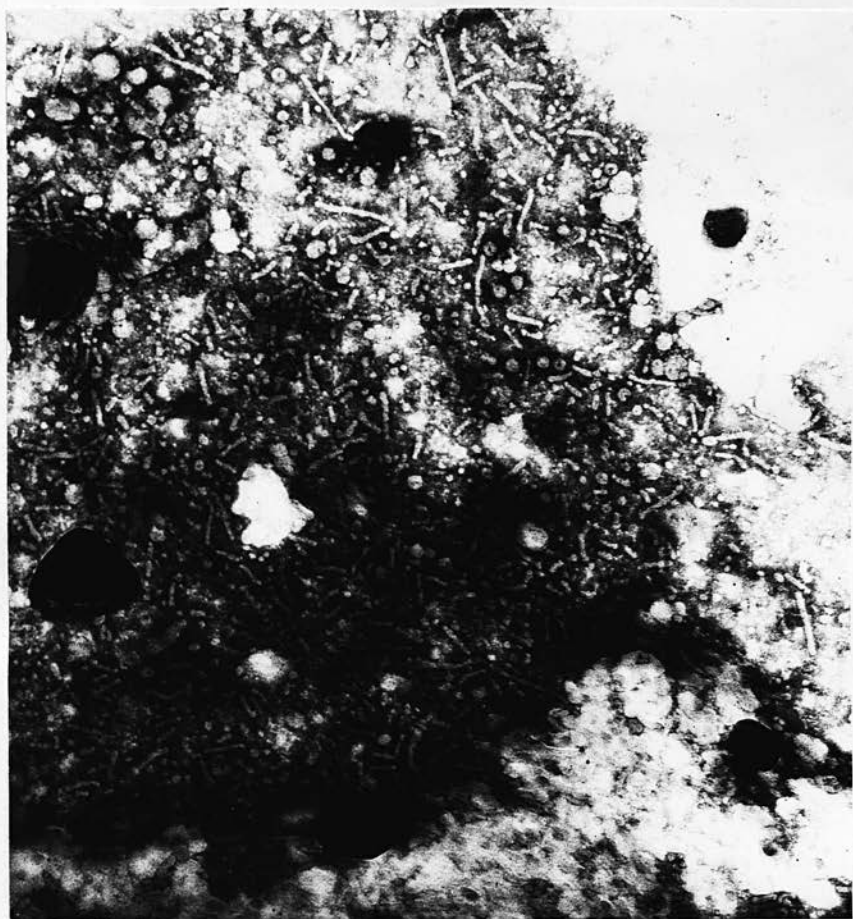


Fig. 3. Partially purified Au-Ag prior to sensitisation
(E.M. Mag. = 50,000).



Fig. 4. Sensitized cell with Au-Ag bound to surface. No free Au-Ag visible in supernatant (E.M. Mag. = 28,000).

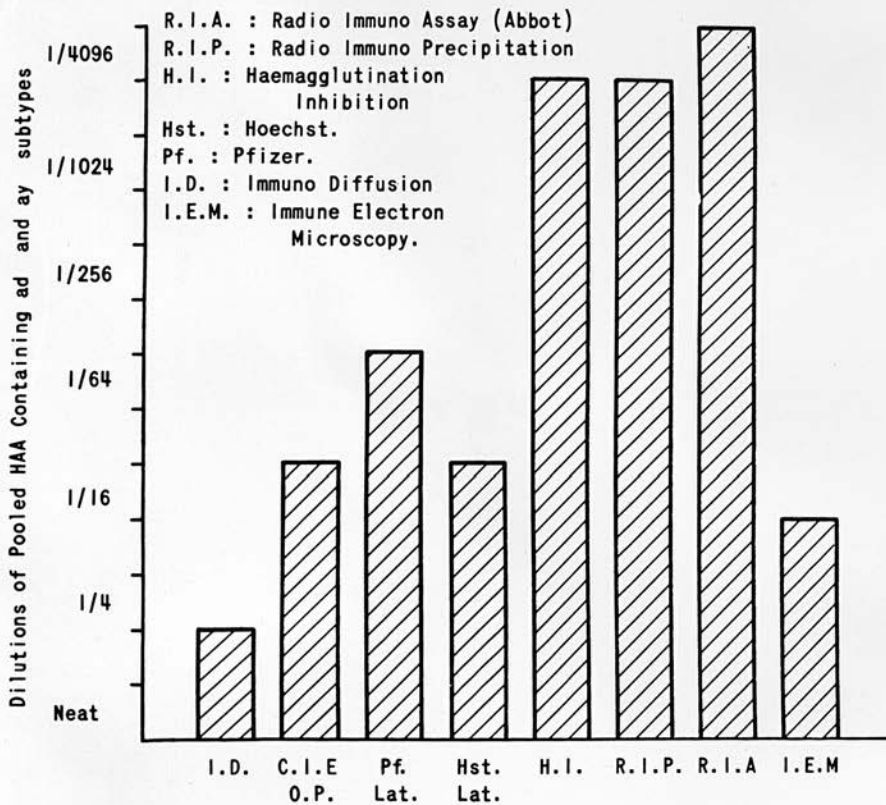


Fig. 5. Sensitivity of HI for detection of Au-Ag in comparison with other techniques.

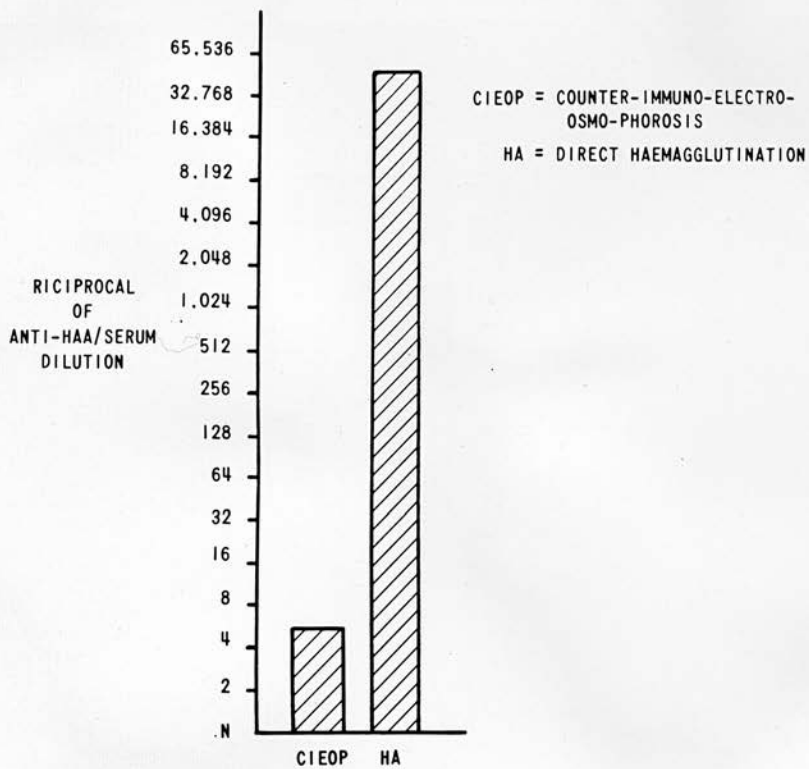


Fig. 6. Comparison of HA and CIEOP for detection of anti-Au.

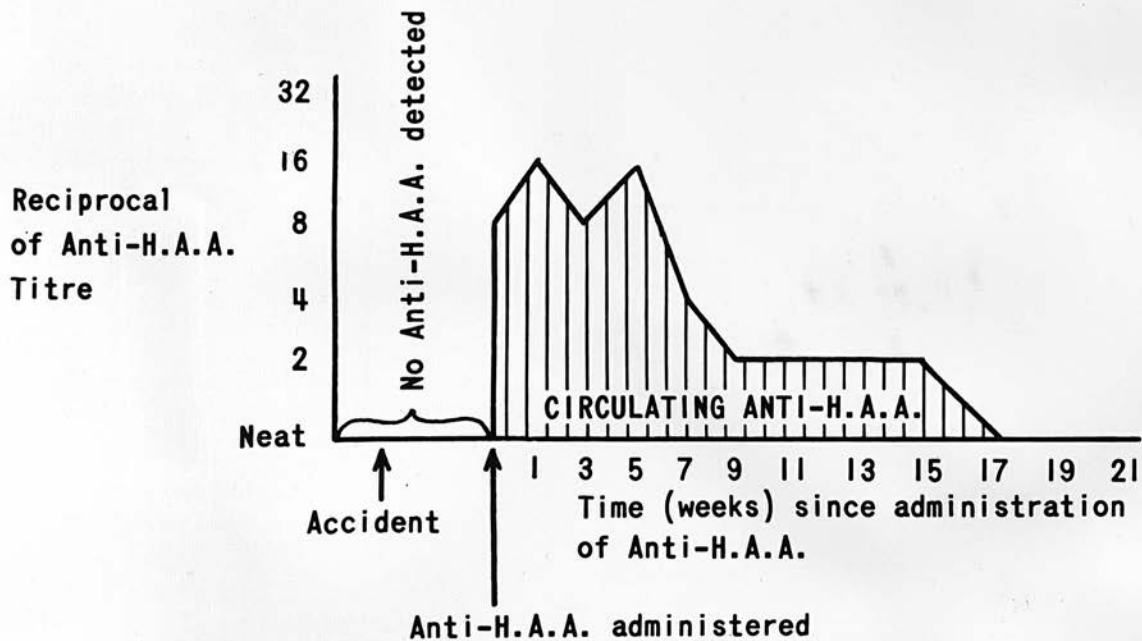


Fig. 7. Detection of hyperimmune anti-Au in accident patient by HA.

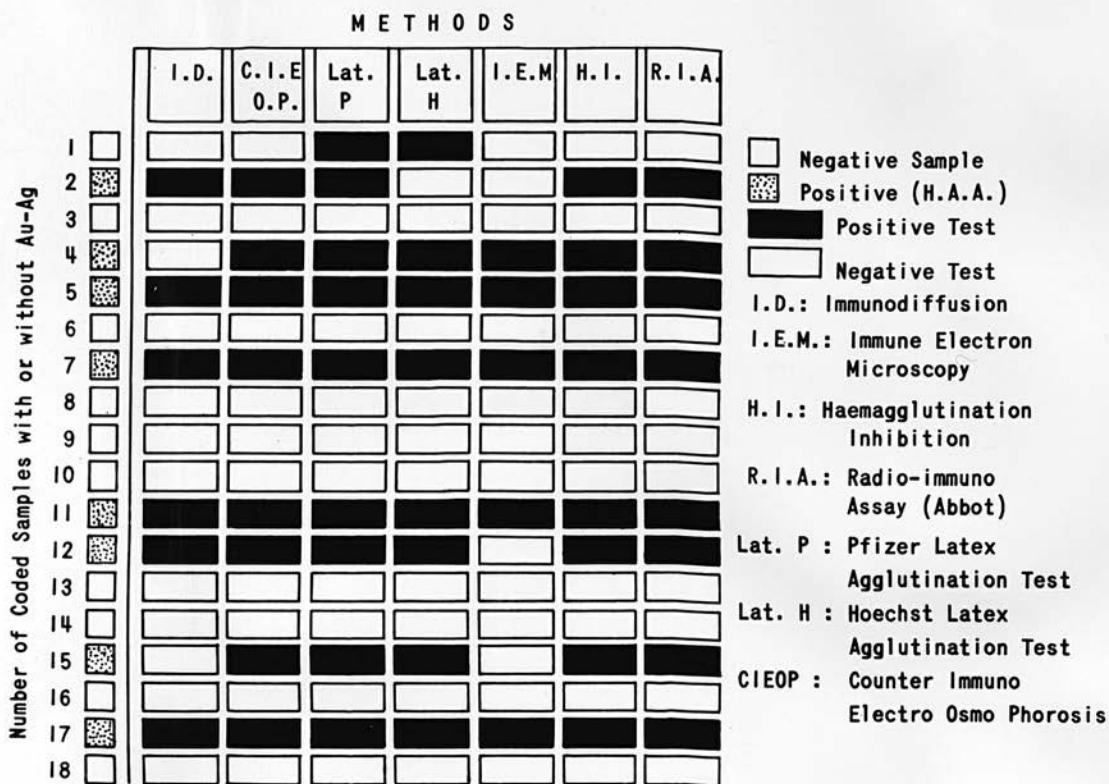


Fig. 8. Results obtained when Au-Ag positive and Au-Ag negative sera were coded and tested by a variety of techniques.

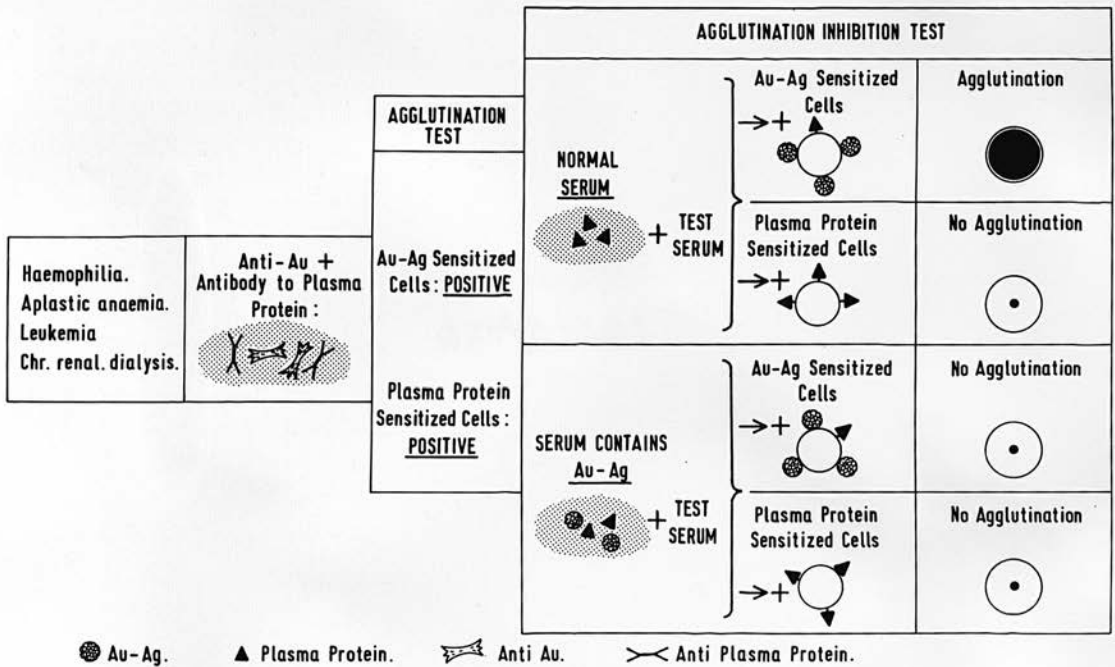


Fig. 9. Situation which may arise in a multitransfused patient possessing antibodies to both Au-Ag and certain plasma proteins.

	NO. TESTED	C. I. E. O. P.		TANNED-CELL HAEMAGGLUTINATION		
		ANTI-HAA POSITIVE	HAA POSITIVE	ANTI-HAA POSITIVE	HAA POSITIVE	'PLASMA-CELL' AGGLUTINATION
DONORS	76	4	7	4	7	0
DIALYSIS PATIENTS	14	2	0	2	0	2 *

* Different samples from these possessing anti-H.A.A.

Table I. Results obtained when sera from selected donors and dialysis patients were screened for Au-Ag and anti-Au by HI and CIEOP.

Improved sensitivity of the electrophoresis method by tannic acid for detection of Australia antigen

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Improved sensitivity of the electrophoresis method by tannic acid for detection of Australia antigen

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For detection of Australia antigen (Au-Ag) by counter-immunoelectrosmophoresis (CIEOP) staining the agarose gel plates with certain dyes has been claimed to improve the sensitivity (Combridge and Shaw, 1971). In our experience, however, a simpler and less time-consuming procedure is that of layering the gel plates with 1% freshly made tannic for 10 minutes (Alpert, Munroe, and Schur, 1970) after the routine CIEOP procedure (Das, Hopkins, Cash, and Cumming, 1971). This has resulted in a significantly increased sensitivity by improving visualization of precipitin lines.

Serial dilutions of Au-Ag containing serum and anti-Au (human origin) were set up in the test system using a 'chessboard' design. After the electrophoresis 'run' the gel plates were observed at an angle under direct light over a dark background. The results were scored as + for sharp precipitin line, \pm for weak precipitation, and - for no reaction. Table I shows that the titre of Au-Ag against the neat antiserum was 1/4, and no significant improvement was noticed when the same plate was reviewed after overnight incubation. Tannic acid was now added and the plate read after 10 minutes: the titre was now 1/16. This improvement reflects an increased sensitivity of the system as a whole; thus, before tannic acid treatment, the total number of

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positives in the 'chessboard' was 14, and after tannic acid treatment they were 21. This was further confirmed over a period of two weeks during which 212 selected specimens from patients, including drug addicts, with clotting disorders and hepatitis, as well as blood donors, some of them already known to be carriers of Au-Ag, were subjected to the procedure described above. Results show (Table II) that the number of positive samples were eight before and 12 after tannic acid treatment; the additional

During a period of five months a total of 19 423 donors were tested, 10 were found to be positive by routine procedure and no additional positive appeared, however, after tannic acid treatment of the CIEOP plates.

The mechanism by which tannic acid increases the sensitivity of the system is not clear, but from a practical point of view there is no doubt that it is capable of bringing out Au-Ag-antibody precipitin lines, especially amongst the patient's sera. The present procedure in this laboratory is to score the results immediately after the routine CIEOP 'run', the plates are washed, then treated with tannic acid, re-read and photographed immediately. All positive samples are re-investigated for identity reaction, if necessary, after concentration.

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	No. of Samples Tested			
	Blood Donor	Patient	Laboratory Staff	Total Positive
	178	27	7	
Before tannic acid	3	5	0	8
After tannic acid	4	8	0	12

Table II Number of positive specimens amongst 212 selected samples before and after tannic acid treatment of CIEOP plates

positive results consisted of three from the patient group and one from the blood donors. In view of these results the above procedure was extended to the 'routine' laboratory where every unit of blood donated was screened for the presence of Au-Ag.

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