SPECIES SPECIFICITY IN THE INHIBITION OF ANTIGLOBULIN SERA

A TECHNIQUE FOR THE IDENTIFICATION OF HUMAN AND ANIMAL BLOODS

BY

A. C. ALLISON* AND J. A. MORTON

From the Clinical Pathology Laboratory, Radcliffe Infirmary, Oxford

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In forensic medical practice it is sometimes necessary to determine whether a stain is blood and whether it is of human or animal origin. The recognition of blood is comparatively easy, since a number of sensitive biochemical tests for blood are available (Glaister, 1945).

To establish whether the stain is human blood is much less easy. For this purpose the precipitin technique is in general use (Schiff and Boyd, 1942; Uhlenhuth and Seiffert, 1930). This test depends upon the fact that the proteins of human serum or whole blood, even in dilute solutions, will be precipitated by a homologous, species-specific antiserum.

The precipitin technique is excellent in principle, but in practice it suffers from certain disadvantages. Specific antisera are not easily prepared (Proom, 1943), and the results of precipitin tests on related species must be interpreted with great caution, as Landsteiner (1945) and others have emphasized. In order to ensure that an antiserum is specific in its action, it must be absorbed with blood of all the animals which may be involved in medico-legal cases, i.e., all the common domestic animals, and, if necessary, monkeys and apes. This, again, is a difficult procedure; in practice each antiserum must be tested for specificity before use and in use it must be carefully controlled. Moreover, the results of precipitin tests are sometimes equivocal because of the turbidity of the antigen extracts, which may persist after centrifugation and filtering. The delicacy of the precipitin reaction and the difficulty of ensuring that results are uniformly reliable are reflected in the enormous number of writings on the subject (references in Landsteiner, 1945; Schiff and Boyd, 1942; Uhlenhuth, 1937). Hence there is a real need for another test which can replace, or at least check, the precipitin reaction.

The purpose of the present communication is to describe a straightforward serological technique for the identification of human blood which makes use of reagents available in every well-equipped haematological laboratory. The test is not difficult to apply, requires only a very small quantity of antigen, and appears to be highly specific.

The principle of the test is as follows: if human serum or whole blood is added to anti-human globulin serum (as used in the Coombs test) the latter will be deprived of its power to agglutinate red cells sensitized with incomplete Rh antibody. Serum or blood from other animals, however, will have no such potent inhibitory effect. The same principle can be used in identifying other animal bloods, but for this purpose specially prepared antisera are required.

Technique

A 4% suspension of O Rh-positive red cells in saline is sensitized by incubation for half an hour at 37° with an equal volume of incomplete anti-D serum of high titre. The cells are then washed three times in a large volume of saline and resuspended to make up an approximately 4% suspension in saline.

Double dilutions of anti-human globulin serum are prepared, the initial and final dilutions depending on its potency. Usually an initial dilution of 1 in 10 is satisfactory; the final dilution should leave at least the last tube in the control row unagglutinated. One drop of each of the different dilutions of the serum should be placed in as many rows of serological tubes as required. A control tube containing a drop of saline is then added to each row.

A drop of saline is added to each tube in the first row. To the other rows of tubes are added single drops of various test solutions, each solution requiring one row. The tubes are shaken and left for half an hour at room temperature to inhibit the anti-globulin serum. Probably a shorter time will suffice.

To each tube one drop of sensitized red cell suspension is added. Agglutination is read after half an hour at room temperature. It is usually obvious

* Staines Medical Research Fellow of Exeter College, Oxford, in receipt of a grant from the Medical Research Council.

macroscopically, and end-points are sharp, but any doubtful tubes should be checked microscopically.

Provided that the same sera and the same individual's blood are used under standardized conditions, there should not be more than one tube variation from experiment to experiment.

Observations

Identification of Human Blood.—The inhibition of the indirect Coombs test by various dilutions of human serum is shown in Table I.

It is apparent that even a 1 in 6,400 dilution of human serum inhibits the agglutination in three tubes, while a 1 in 100 to 1 in 200 dilution inhibits agglutination in all tubes. Within the range of dilution 1 in 400 to 1 in 6,400 the inhibitory effect is almost exactly inversely proportional to the dilution of human serum. Hence in other tests 1 in 100 dilutions of serum or whole blood were used.

In Table II the effect of 1 in 100 dilutions of human and animal sera upon the indirect Coombs reaction is shown. The animal sera had previously been absorbed free of agglutinins for human red cells.

The human serum has inhibited agglutination completely as before. Fowl serum has had no effect upon the reaction at all, while the sera of common domestic animals have inhibited the reaction only very slightly, each in a single tube. Even the serum from a monkey (*Macaca*) and a baboon (*Papio*) has inhibited agglutination only slightly, in two tubes.

Thus it is clear that with the anti-human globulin inhibition technique human serum can be distinguished readily from similar dilutions of the sera of other animals. The same results can be obtained with dilutions of whole blood.

The inhibitory reaction is not only species specific but is given only by blood; in other words, it is organ specific. In Table III the inhibitory effects of a 1 in 100 dilution of human serum and 1 in 50 dilution of human bodily secretions and excretions upon the agglutinating system are shown.

Again, the serum has inactivated the anti-human globulin while urine has had no effect at all and saliva and semen have had only a very slight

TABLE I
HUMAN CELLS SENSITIZED WITH INCOMPLETE Rh ANTIBODY

Dilutions of Human Serum	Titre of Anti-human Globulin in Saline									Saline
	10	20	40	80	160	320	640	1,280	2,560	
None	++++	++++	++++	++++	+++	++	++	+	—	—
1/6,400	++++	++++	++++	+++	++	—	—	—	—	—
1/3,200	++++	++++	+++	++	—	—	—	—	—	—
1/1,600	++++	+++	++	—	—	—	—	—	—	—
1/800	++++	++	—	—	—	—	—	—	—	—
1/400	+++	+	—	—	—	—	—	—	—	—
1/200	—	—	—	—	—	—	—	—	—	—
1/100	—	—	—	—	—	—	—	—	—	—

++++ Very strong or complete agglutination. +++ Strong agglutination. ++ Moderate agglutination. + Weak agglutination, read microscopically.

TABLE II
HUMAN CELLS SENSITIZED WITH INCOMPLETE Rh ANTIBODY

Inhibitor (1/100)	Titre of Anti-human Globulin in Saline									Saline
	10	20	40	80	160	320	640	1,280	2,560	
None	++++	++++	++++	++++	+++	++	++	+	—	—
Human serum ..	—	—	—	—	—	—	—	—	—	—
Monkey (<i>Macaca</i>) serum	++++	++++	++++	+++	++	+	—	—	—	—
Ox serum	++++	++++	++++	++++	+++	++	+	—	—	—
Sheep	++++	++++	++++	++++	+++	++	+	—	—	—
Horse	++++	++++	++++	++++	+++	++	+	—	—	—
Rabbit	++++	++++	++++	+++	++	+	—	—	—	—
Dog	++++	++++	++++	++++	+++	++	+	—	—	—
Cat	++++	++++	++++	++++	+++	++	+	—	—	—
Fowl	++++	++++	++++	++++	+++	++	+	—	—	—

inhibitory effect, each in a single tube. An aqueous extract of faeces has produced slightly more inhibition, which may be attributable to globulin liberated from degenerating cells. Milk taken from mothers early in the puerperium produces further inhibition, presumably because of its globulin content, including antibodies. (The Rh antibodies themselves are sometimes secreted in breast milk, Witebsky and Heide, 1943.) The inhibitory effect of serum upon the agglutination, however, is considerably more potent than that of milk, so that in practice there should be no difficulty in identifying serum or blood in an extract.

Identification of Animal Bloods.—The application of the same principle in the identification of animal bloods can be illustrated conveniently by the use of sheep cells sensitized with amboceptor and an inactivated rabbit anti-horse precipitating serum. In this case the incomplete antibody is replaced by amboceptor (horse haemolysin for sheep cells as used in the Wassermann reaction. Sheep cells sensitized by amboceptor will not be lysed in the absence of complement, but they are strongly agglutinated by

anti-horse serum. The inhibitory effect of 1 in 100 dilutions of horse and other mammalian sera upon this system is shown in Table IV.

It is evident that the horse serum has inhibited the agglutination completely, while the other sera have had little or no such effect. This system will detect horse serum or blood in high dilution. In several species incomplete antibodies which render cells agglutinable by homologous precipitating antisera are known. Haemolysins which sensitize red cells in the absence of complement can be prepared in many mammals. In all Forssman-negative animals Forssman antibodies can be produced; these antibodies agglutinate appropriate cells in low dilutions, but in higher dilutions they merely sensitize the cells so that they are strongly agglutinable by homologous precipitating sera. Hence there is no reason why anti-globulin inhibition should not be used for the identification of any given series of animal bloods.

Cross Reactions of Anti-globulin Sera.—Simmons (1946) reported that Rh-sensitized human red cells are agglutinated by precipitins active against the serum proteins of many mammals, but not by

TABLE III
HUMAN CELLS SENSITIZED WITH INCOMPLETE Rh ANTIBODY

Dilutions of Human Body Secretions	Titre of Anti-human Globulin in Saline									Saline
	10	20	40	80	160	320	640	1,280	2,560	
None	++++	++++	++++	++++	+++	++	++	+	—	—
Serum 1/100	—	—	—	—	—	—	—	—	—	—
Milk 1/50	++++	++++	++++	+++	++	—	—	—	—	—
Faeces ?	++++	++++	++++	+++	++	+	—	—	—	—
Saliva 1/50	++++	++++	++++	+++	++	++	+	—	—	—
Semen 1/50	++++	++++	++++	+++	++	+	+	—	—	—
Urine 1/50	++++	++++	++++	++++	+++	+++	++	+	—	—

TABLE IV
SHEEP CELLS SENSITIZED WITH AMBOCEPTOR

Inhibitor	Titre of Anti-horse Serum R58 in Saline							Saline
	5	10	20	40	80	160	320	
Saline	+++	+++	+++	++	++	+	—	—
Horse serum 1/100 ..	—	—	—	—	—	—	—	—
Sheep .. 1/100 ..	+++	+++	++	++	+	—	—	—
Ox .. 1/100 ..	+++	+++	++	++	+	—	—	—
Pig .. 1/100 ..	+++	+++	++	++	+	—	—	—
Rabbit .. 1/100 ..	+++	+++	++	++	+	—	—	—
Monkey,, 1/100 ..	+++	+++	+++	++	++	—	—	—
Human ,, 1/100 ..	+++	+++	+++	++	++	+	—	—

anti-fowl serum. This cross agglutination by rabbit anti-horse serum is illustrated in Table V, together with the inhibitory effect of different mammalian sera upon the reaction.

TABLE V
HUMAN CELLS SENSITIZED WITH INCOMPLETE Rh ANTIBODY

Inhibitor	Titre Rabbit Anti-horse Serum in Saline				
	1	2	4	8	Saline
Saline	+++	++	+	—	—
Human serum 1/100 ..	—	—	—	—	—
Horse „ 1/100 ..	—	—	—	—	—
Sheep „ 1/100 ..	++	+	—	—	—
Ox „ 1/100 ..	++	+	—	—	—

The anti-horse serum has quite definitely agglutinated the Rh-sensitized human red cells, but not in a dilution higher than 1 in 4. Thus, the cross reaction is weak. If allowance is made for the relative precipitating potencies of the different sera, it is possible to deduce that the agglutination of Rh-sensitized red cells by anti-human globulin is approximately 40 times as powerful as that produced by anti-horse serum. Very similar results have been obtained with anti-sheep and anti-ox sera.

That the reaction shown in Table V was not inhibited by sheep serum and ox serum indicates that the latter do not share the antigenic features common to horse serum and human serum (through which the cross reaction must be mediated). The inference can be drawn that there is no single series of mammalian antigens common to all, but rather a complex mosaic of antigens with some slight overlap between species.

In view of the weakness of these cross reactions it is not surprising that animal sera bring about little inhibition of the anti-human globulin test.

Inhibitory Effect of Degraded Serum Proteins.—Hill, Reid, and Haberman (1949) have shown that heating incomplete Rh antibodies to 70° for one

hour only slightly reduces their titre. It is therefore reasonable to suppose that the inhibitory effect upon the indirect Coombs test of serum or blood heated to the same temperature will only be slightly impaired.

Heating the sera to a higher temperature, or treatment with acid or alkali, will of course denature the proteins and their inhibitory effect will be reduced, although it is not abolished altogether. In Table VI inhibition produced by serum proteins denatured with alkali is shown; NaOH was added to human serum to give a pH of 12.5 for half an hour, and the whole subsequently neutralized with HCl. The inhibitory effect upon the indirect Coombs test is clearly reduced, but it is still highly significant. It is impossible to say whether this inhibition is due to residual unchanged protein, or to a partial overlapping of the antigenic pattern of native and denatured proteins, or to both.

On the other hand, fractions produced by hydrolysis of the protein in hot N/5 HCl and by tryptic digestion have no inhibitory effect whatever. Thus the inhibition seems to depend largely upon the antigenic mosaic of serum proteins in their native state. It is extremely unlikely that in practice false results would be obtained because of the presence of small protein fractions, as might well happen in the case of some carbohydrate haptens.

Identification of Stains

With the above results in mind, the following procedure is recommended for the identification of blood stains:

An extract of the stain is made as stated in the standard works of reference on the subject (e.g., Schiff and Boyd, 1942). Extraction of a small piece of cloth for an hour at room temperature is satisfactory in most cases. A control extract from an unstained, adjacent, similar piece of material should also be taken.

The dilution of proteins in the extract is roughly estimated. For this purpose two tests are usually given: diluting the extract until stable foam is no longer formed and until it no longer gives a visible

TABLE VI
HUMAN CELLS SENSITIZED WITH INCOMPLETE Rh ANTIBODY

Inhibitor	Titre Anti-human Globulin in Saline								Saline
	10	20	40	80	160	320	640	1,280	
Saline	++++	++++	++++	+++	+++	++	++	+	—
1/100 human serum ..	—	—	—	—	—	—	—	—	—
1/100 alkali denatured human serum	+++	++	++	+	—	—	—	—	—
1/100 acid hydrolysate of serum	++++	++++	+++	+++	++	++	+	—	—
1/100 tryptic digest of serum	++++	++++	+++	+++	+++	++	+	—	—

TABLE VII
HUMAN CELLS SENSITIZED WITH INCOMPLETE Rh ANTIBODY

Inhibitor	Titre of Anti-human Globulin in Saline									Saline
	10	20	40	80	160	320	640	1,280	2,560	
None	++++	++++	++++	++++	++++	++++	++++	++++	++++	—
1/100 human blood ..	—	—	—	—	—	—	—	—	—	—
Unknown extract 1 ..	++	—	—	—	—	—	—	—	—	—
.. .. 2 ..	++++	++++	++++	++++	++++	++	+	—	—	—
Blank	++++	++++	++++	++++	++++	++	+	+	—	—

precipitate over concentrated nitric acid in the precipitin tube. Both these final dilutions are said to represent about 1 in 1,000 serum (Schiff and Boyd). These methods are inaccurate, however, as is the photometric estimate of blood pigment (usually methaemoglobin) in the extract. We have used a simple method giving reasonably reproducible results which are quite satisfactory for inhibition and precipitin tests. Drops of double dilutions of fresh human blood starting with 1 in 25, and a single drop of each extract, are allowed to fall upon a strip of filter paper. The paper is then dried (preferably in an oven at about 100° for 10 minutes), and stained in a standard solution devised for staining proteins after electrophoresis on filter paper. The staining solution is made up as follows:

Bromphenol blue	...	...	0.1%
Ethanol	...	...	100%
Saturated with HgCl ₂			

After staining, the paper is washed in three changes of 1% acetic acid which brings out the blue. The unknown can readily be compared with the standard, and the extract diluted until it corresponds approximately in a 1 in 100 dilution of human blood.* The blank is diluted to the same degree.

The diluted extracts are tested for their inhibitory effect upon the sensitized red cells-anti-globulin system set up as described in the section on technique above.

Any reliable high titre incomplete anti-D serum and good anti-human globulin serum seems to be satisfactory. Our anti-human globulin serum is prepared in rabbits as recommended by Mollison, Mourant, and Race (1948). In the case of very powerful anti-globulin sera the initial dilution should not, of course, lie in the prozone described by Wootton (1950). The serum fraction inhibiting the anti-human globulin appears to be mainly γ globulin (Coombs and Mourant, 1947), although

*In this dilution agglutinins for human red cells should be inactive. If any are active, this will be apparent in the control tubes: then the extract must be absorbed free of agglutinins for human cells before proceeding further.

other fractions may be implicated also (see Race and Sanger, 1950).

The first row of Table VII (positive control) should show agglutination to a high titre as should the last (blank) row. The second row (human serum) should show complete, or nearly complete, inhibition of agglutination. If the unknown is human blood there will be no agglutination beyond the second tube (extract 1 in the table); otherwise, agglutination should continue to a high titre (extract 2). All the controls in the last tubes (saline+inhibitor+cells) should of course show no agglutination.

With this technique 30 blood stains on cloth and paper have been tested. Twenty of the specimens were from old forensic cases (some more than 10 years old), kindly supplied by Professor J. M. Webster, Professor Keith Simpson, and Dr. F. Camps. The stains were given code numbers, and the results subsequently checked. In 24 of the cases satisfactory extracts could be recovered: it was extremely difficult to obtain protein eluates from material dried on paper, even in alkaline solution. In 23 of the 24 cases the material was correctly identified as being of human or animal origin. In the other case the inhibition was intermediate between that normally given by human and animal bloods; this was interpreted as indicating that the blood had been partly denatured or mixed, and on reference to Professor Webster it transpired that the blood stain had been contaminated with faeces.

Hence it seems that the test should have a place in the forensic medical laboratory. The relative advantages of the precipitin and inhibition techniques for the identification of blood stains must be assessed by those who are experienced in serological forensic practice. Perhaps the two techniques should be used side by side: they may, in fact, depend to some extent upon different antigenic features, so as to be complementary, each helping to confirm the specificity of the other.

Experiments on Material from an Ancient Desiccated Cadaver

A further test was made with material obtained from a dried cadaver from the predynastic period in Egypt, which must be at least 5,000 years old, and for this valuable specimen we are indebted to the Department of Egyptology of the British Museum. Much of the skin and some of the muscles and blood vessels had been impregnated with nitrites and remained in a notably good state of preservation. A small piece of the desiccated femoral artery was powdered and dissolved in saline, a muscle fragment of approximately the same weight serving as a control. The arterial extract, which presumably contained blood at death, gave considerable inhibition of the anti-globulin system (one tube less than the unknown extract 1 in Table VII), whereas the control inhibited the reaction in only one tube. This is a remarkable observation. It has long been known that blood-group specific substances can be found in Egyptian mummies (Boyd and Boyd, 1937) and the material very similar to ours was successfully grouped by Gilbey and Lubran (1953). The blood group antigens are probably relatively simple carbohydrate haptens, however, while the inhibition of the Coombs reaction indicates that in the predynastic cadavers some of the dried blood proteins remain in or near their native state, so that their antigenic properties are but little changed. The finding was perhaps not entirely unexpected, since Uhlenhuth (1937) was able to obtain a positive precipitin reaction for blood from prehistoric mummified material.

Summary

If human serum or whole blood is added to anti-human globulin serum (as used in the Coombs test) the latter will be deprived of its power to agglutinate red cells sensitized with incomplete Rh antibody. Serum or blood from other animals,

however, will have no such potent inhibitory effect on the Coombs reaction. Hence the inhibition technique can be applied to identify human blood. Similar techniques can be used in the recognition of blood from other animals. The inhibition technique has a useful application as a check on the precipitin technique for the identification of bloods and blood fractions in forensic medical practice and elsewhere.

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Addendum

Since the above was submitted for publication we have found that Wiener, Hyman, and Handiman (1949, *Proc. Soc. exp. Biol., N.Y.*, **71**, 96) had previously described the species specificity of anti-human globulin inhibition. The application to animal bloods and denatured proteins is not mentioned in their communication.