

I. DEVELOPMENT OF BLOOD GROUP ANTIBODIES
FOLLOWING ROUTINE BLOOD TRANSFUSIONS.

II. STUDIES ON THE AGGLUTINABILITY OF
STORED RED BLOOD CELLS.

by

Bansi Badan Tribedi, M.B.B.S., D.T.M. (Calcutta)

from

Departments of Regional Blood Transfusion Centre
and Clinical Laboratory, Royal Infirmary, Edinburgh.

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of Edinburgh, in fulfilment of the requirements
for the degree of Doctor of Philosophy.

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I. DEVELOPMENT OF BLOOD GROUP ANTIBODIES
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C O N T E N T S.

INTRODUCTION

1. General	1.
2. Problem	21.

<u>MATERIALS AND METHODS</u>	23.
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<u>RESULTS</u>	38.
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<u>DISCUSSION</u>	60.
-------------------	----	----	----	----	----	----	-----

<u>SUMMARY</u>	93.
----------------	----	----	----	----	----	----	-----

Introduction

Blood transfusion has become a comparatively safe procedure and is now regarded as invaluable in modern medicine and surgery. The discovery of blood groups, improved methods of collection, storage and preservation of blood as well as recent developments in cross-matching techniques have all helped to reduce the risks attached to the transfusion of blood.

In earlier days, the main disadvantage was lack of knowledge of -

- (1) Aseptic technique
- (2) Blood groups (compatibility) and their immunological relationships
- (3) Coagulation mechanism
- (4) Methods of preservation and storage of blood (blood banking)

These problems have been gradually solved by several workers in this field and as a result there has been a marked increase in the number of transfusions. This has confronted present day workers with a further problem, namely iso-immunisation which may be defined as immunisation of an individual by antigens from the same species.

Blood Transfusion Before the Discovery of the

ABO Groups.

The/

The first authenticated transfusion in man was probably performed by Denis (1667) who successfully transfused the blood of a lamb into a youth. His second and third transfusions were also carried out seemingly without any adverse effect, but the patient of his fourth attempted transfusion died and he was charged with murder. Following this incident, transfusion was prohibited and as a result, interest in transfusion ceased for a century and a half when Blundell(1818) a London physiologist and obstetrician revived the procedure. He successfully transfused blood to three patients with post-partum haemorrhage and advocated transfusion in the treatment of acute haemorrhage. He also insisted that human blood was more effective than that of animal origin. Blundell's work led to an understanding of the rationale of transfusion. At the beginning of the last quarter of the nineteenth century, several British and American workers (Winants 1872, Barnes 1874, Gradle 1874, Sittel 1874, Hotz 1875) practiced transfusion again with animal blood and without any due regard to the previously established fundamental principles regarding the indications for blood transfusion and met with the inevitable severe reactions. Because of these fatal reactions many/

many of which were due to incompatibility, blood transfusion practice was once more practically abandoned during the last quarter of the nineteenth century.

Discovery of the ABO Blood Groups and Their
Application in Blood Transfusion

Landsteiner (1900, 1901) first discovered the agglutination of the red cells of some individuals by the sera of others and on the basis of such reactions he classified human erythrocytes into three different and distinct groups A, B, and O. Von Decastello and Sturli (1902) discovered the fourth group AB. These epochal discoveries helped to eliminate incompatibility, one of the most difficult problems so far encountered. Ottenberg (1911) first applied this knowledge of ABO blood groups in transfusion practice.

Discovery of Anticoagulants and Preservatives
and the Operation of Blood Banks

Although the main cause of fatal reactions had been found as a result of the discovery of the ABO blood groups and methods of prevention had been demonstrated, inconvenience due to coagulation and infection still persisted. To avoid coagulation, Carrel (1907) and Bernheim (1909) tried transfusion by bringing the blood in direct contact/

contact with the intima of the blood vessels by arterio-venous anastomosis. This process was difficult and thus failed to gain popularity and as a result few transfusions were carried out in comparison with the need.

In 1914, sodium citrate was first introduced as an anti-coagulant by Hustin (1914) and very soon afterwards by Agote (1915), Lewisohn (1915) and Weil (1915) and thus it was possible to separate the collection of blood from its injection, which permitted for the first time, the so-called 'delayed transfusion' (DeGwin et al. 1949). This procedure soon gained popularity and was used extensively in World War I.

Rous and Turner (1916) published their pioneer work on the using of dextrose as a preservative of blood for transfusion and, making use of their findings, Robertson (1918) operated the first blood bank in the casualty clearing stations of the British Army.

After World War I, the principle of blood bank had been temporarily forgotten until the third decade of the present century while several Russian investigators (Skundina and Barenboim, Skundina, Rusakov and Ginsberg, cited by Kilduffe and DeBakey, 1942; Yudin 1936) revived the procedure using cadaver blood.

In/

In 1932, Skundina and Barenboim demonstrated that when cadavers were preserved at a temperature of 1 to 2° above zero, the blood in the cadaver vessels preserved its "living properties" for six to seven hours. Following these studies, a variety of methods for the collection, preservation and storage of blood for transfusion have been developed.

During the Spanish Civil war in 1936, the Loyalist forces developed the transportation of preserved blood (Jorda 1939). The present form of the Blood Transfusion Services has developed during and after World War II.

Aseptic Controls and Discovery of Pyrogens.

Gradual development of aseptic techniques, methods of sterilisation and closed systems of withdrawal and transfusion of blood (Kolmer 1924, Patton 1938, Sharpe 1939) have considerably minimized the hazards due to infection. Seibert (1923) first observed that a heat stable bacterial product (pyrogen) was responsible for some of the febrile reactions following even ABO compatible blood transfusion. It was however found that these reactions could be avoided if certain precautions were taken.

Following the discoveries of the ABO groups, anticoagulants and preservatives and the development of aseptic techniques and bacterial control, blood transfusions/

transfusions were practiced more frequently and the risk as a result of iso-immunisation then became evident.

Iso-immunisation in Man and Animals

The phenomenon of iso-immunisation was first discovered by Ehrlich and Morgenroth (1900). They observed that if the haemolysed blood of one goat were injected to other goats, the sera of the injected goats were characterised by the presence of immune antibody (haemolysin). By this method of immunisation, they were able to prove that the red cells of individual animals within the same species were of different serological properties. Iso-immunisation has since been practiced in many other animals by the same procedure and remarkable specificity of red cells has been observed. Although the discovery of the ABO blood groups coincided with the discovery of iso-immunisation, the findings in animals could not be at first correlated with the findings in man. In animals, every individual was characterised by serological specificity of its red cells whereas in man it was limited to only four main blood groups and subdivisions of group A, first discovered by von Dungern and Hirsfeld (1911). Iso-immunisation in human beings was first reported by Deinst (1905) who observed rising of anti-A and anti-B/

anti-B agglutinins in recently delivered women of group O after the birth of infants belonging to group A or B.

The phenomenon of iso-immunisation could not be explained successfully in man by repeated transfusions even at later periods when the transfusions were carried out in greater numbers. Although it was suspected on clinical grounds that patients receiving repeated transfusions showed a higher incidence of severe reactions, the actual proof of iso-immunisation - the demonstration of atypical immune antibodies - could not be supplied until lately. Landsteiner and Levine (1927) used a new immuno-logical procedure namely, hetero-immunisation and discovered the M, N, and P agglutinogens by immunising rabbits with human blood cells. These discoveries served to bridge the gap between the findings in man and animal and emphasised the analogy of individuality of human erythrocytes.

Discovery of Rh. factor and its Role in Transfusion and Haemolytic Disease of the Newborn.

During the period of 1921-40 a number of cases were reported where haemolytic transfusion reactions had occurred despite the transfusion of blood of correct ABO groups (Unger 1921, Landsteiner et al. 1928, Zacho 1936, Culbertson and Ratcliffe 1936, Neter 1936, Levine and Stetson 1939)./

Levine & Stetson 1939). In some of these cases it was possible to demonstrate the presence of atypical antibodies. Most of these atypical antibodies were unrelated to ABO groups or MN factors though some designated as G, H, K, Q, (Andresen 1935) were probably associated with P agglutinogen. To demonstrate additional factors by immunisation of animals Landsteiner and Wiener(1937) injected rabbits with Rhesus Monkey red cells which produced anti-M antibodies. Continuing this line of work Landsteiner and Wiener(1940) immunised rabbits and later guinea-pigs with the blood of the monkey *Macacus rhesus* and found that some of the antisera thus produced agglutinated the red cells of 85% of white people. Those that were agglutinated they called Rhesus positive and the remaining 15% Rhesus negative and to the new factor they applied the symbol Rh.

The discovery of the Rh factor not only explained the cause of haemolytic transfusion reaction but also helped in understanding the aetiology of haemolytic disease of the newborn, which was hitherto obscure though some investigators suggested that it might be the result of an antigen antibody reaction. Darrow(1938) in describing the pathogenesis of congenital haemolytic disease (haemolytic/

(haemolytic disease of the newborn), had postulated the iso-immunisation of the mother to foetal haemoglobin and thought that this led to the passage of resulting antibodies across the placental barrier into the blood stream of the foetus. Levine and Stetson(1939) published an interesting case report of a multipara group O woman who delivered a 33 week stillbirth. For post-partum haemorrhage, she was transfused with 500 ml. of her husband's blood (also of group O). Immediately following the transfusion, she had a severe reaction. Using a more sensitive method it was found that her serum contained an antibody which agglutinated the red cells of her husband and those of 80 out of 104 group O donors. From this observation the authors deduced "that the mother has been immunised by a foetal antigen inherited from the father and had received blood against which she had already formed antibody". This observation slightly differed from that of Darrow(1938) because it mentioned the possibility of immunising the mother by a 'new' blood factor present in the foetus and inherited from the father.

Levine and Katzin(1940) reported several additional instances of transfusion reactions associated/

associated with iso-immunisation to the Rh factor during pregnancy and were able to show that the case of Levine and Stetson(1939) was the result of Rh -immunisation. Wiener and Peters(1940) using anti-Rh -rabbit serum prepared by Landsteiner and Wiener(1940) established that four cases of haemolytic reactions following repeated transfusions of ABO compatible blood, were due to incompatibility in the Rh system. In three of these cases the sera were shown to contain an agglutinin for the Rh-positive human erythrocytes which corresponded in agglutinating properties to the anti-Rh-rabbit sera of Landsteiner and Wiener. Levine, Katzin and Burnham(1941) and Levine, Katzin, Burnham and Vogel(1941) were then able to demonstrate that Rh-immunisation was of importance in the aetiology of erythroblastosis foetalis (haemolytic disease of the newborn) and of some stillbirths and miscarriages. In a statistical study, Levine and his associates(1941) found that 91 of 111 mothers of erythroblastotic infants were Rh-negative as compared with only 15% Rh-negative in the population at large, and that 100% of 66 fathers and 58 affected infants were Rh-positive as compared with 85% Rh-positive individuals in the population. Of the Rh-negative mothers about 50%/

50% showed anti-Rh agglutinins when tested two months after delivery of an infant with erythroblastosis foetalis. It thus appeared that about 90% of the cases of erythroblastosis foetalis could be accounted for on the basis of iso-immunisation due to the Rh factor.

Varieties of Rh

The importance of the Rh factor was soon realised and several American and British workers carried out a great number of investigations and demonstrated that all anti-Rh sera were not identical in every respect. Levine(1942) showed that one serum which agglutinated 87% of bloods among the population in New York contained two agglutinins which he designated Rh₁ and Rh₂. Working with new testing sera, Wiener(1944a) found six principle genes which he called rh, Rh₀, Rh', Rh'', Rh₁, Rh₂. Of these genes, Rh₀ is the one responsible for the antigen detected by the original rabbit and guinea pig anti-Rh sera. The six genes in the series give rise to six antigens producing six antisera - anti-Rh₀, anti-Rh', anti-Rh'', anti-Rh₁, anti-Rh₂ and anti-Hr (Levine 1941). The latter (Rh reversed) was used by Wiener to signify sera which reacted with Rh-negative bloods i.e. (rh/rh etc.).

The British Classification of Rhesus Blood Groups

About/

About the same time as Wiener(1944a) in America was reclassifying the Rhesus blood groups, Race and Taylor(1943) and Race, Taylor, Boorman and Dodd(1945) in this country using a strong anti-Hr serum had produced a similar classification of the Rh genes. Fisher (cited by Race 1944) then noticed that if a gene was positive with anti-Hr serum it was negative with anti-Rh' serum. He supposed that the antigens responsible for these reactions were allelomorphic genes which he labelled c and C respectively. He further suggested that the anti-Rh₀ serum was produced by a gene D which had an allelomorph d and that anti-Rh" serum was the result of a gene E with an allelomorph e. This theory presupposed the existence of three loci on the chromosome which are responsible for the Rh characteristics. This new scheme using the letters Cc, Dd, and Ee instead of Rh₀ etc. was published by Race(1944) and is known as the Fisher-Race classification.

Now the Rh chromosomes could be combined in eight different ways namely, CDe, cDE, cde, cDe, cdE, Cde, CDE, CdE.

The main difference between Wiener's and Fisher's theory may be summarised in the words of Mourant(1957) that each of the Rh antigens "reacts specifically/

specifically with a single well-defined antibody or haemoagglutinin. While antigenically these entities are distinct, genetically they are invariably transmitted in groups of at least two and usually of three or more. Wiener regards each genetically combined group of antigens as a single antigen and considers that it is transmitted by a single gene, whereas Fisher and Race regard each elementary antigen as the product, probably, of a separate gene."

Since it is awkward to speak of "big C, big D, little e", certain short symbols for the various combinations of elementary genes are widely used. These short symbols, Wiener's and Fisher's nomenclatures of the Rh gene combinations and their frequencies for an English population (Race, Mourant et al. 1948) are shown in the following table

Short symbol	Gene combinations		Frequency
	Wiener	Fisher	
R ₁	Rh ₁	CDe	0.4076
r	rh	cde	0.3886
R ₂	Rh ₂	cDE	0.1411
R ₀	Rh ₀	cDe	0.0257
R''	Rh''	cdE	0.0119
R'	Rh'	Cde	0.0098
R _z	Rh _z	CDE	rare
R _y	Rh _y	CdE	rare

Fisher's and Wiener's terminology of the antibodies corresponding to the six elementary Rh antigens are given below

Fisher's terminology	Wiener's terminology
anti-C	anti-rh'
anti-D	anti-Rh ₀
anti-E	anti-rh''
anti-c	anti-hr'
anti-d	anti-Hr ₀
anti-e	anti-hr''

Fisher's nomenclature will be primarily followed throughout the text unless otherwise stated. (Where the term Rh-positive or Rh-negative has been used, it should always be regarded as "D-positive" or "D-negative" respectively).

The/

The discovery of anti-e by Mourant(1945) supported Fisher's hypothesis. Anti-d was first reported by Diamond(1946) and subsequently by Hill and Haberman(1948) and since it had been found mixed with other antibodies and also for its infrequent occurrence, Wiener(1950) expressed doubt about its existence.

Rosenfield and his associates(1953) discovered a new Rh antigen f with the sera of a haemophilic who had received 35 previous transfusions. Race and Sanger(1954) suggested that "anti-f and the antigen it determined would easily and comfortably fit in with what was already known of the Rh system if only a fourth series of allelomorphic antigen were postulated". After the discovery of three pairs of elementary antigens Dd, Cc, Ee, (excluding the recently discovered f antigen) several other allelomorphs have been reported by different workers e.g. C^w (Callender & Race,1946), c^v, C^u (Race and his co-workers,1948), C^x (Stratton and Renton,1954) as third, fourth, fifth and sixth allelororphs respectively at the loci of Cc; D^u (Stratton,1946) as the third antigen allelomorphic to Dd; E^u(Ceppellini et al.(1950) and e^x (Gilbey,1950) as the third and fourth allelomorphs to Ee locus.

Of these allelomorphs, only C^w and D^u are important/

important. There are therefore, three sets of important alleles or antigens $CC^w c$, $D^u d$, Ee ; the various genes excluding D^u occur in 12 different combinations on each chromosome and these chromosomes are in order of frequency CDe , cde , cDE , cDe , C^wDe , cdE , Cde , CDE , C^wde , CdE , C^wDE , C^wdE . Since each cell in adult organism has two such chromosomes there are $\frac{12(12+1)}{2}=78$ possible genotypes of which 26 major types can be recognised.

Discovery of Methods for Detection of Incomplete Antibodies.

During the first four years of Rh work, the workers in this field were puzzled because, even in instances of proven haemolytic transfusion reactions or definite erythroblastosis foetalis, the serum of the affected individual failed to disclose Rh agglutinins. Diamond(1944a) first noted that a concentrated globulin preparation from an Rh antiserum was capable of inactivating an anti-Rh saline-acting antibody of high titre. He also observed a prozone effect in this preparation. These observations suggested that there might be a 'new' type of Rh antibody which not only differed from the original Rh antibody - agglutinin - but also had some inhibitory effect on the latter. Further study on this subject by Race(1944)/

Race(1944) and Wiener(1944b) explained the nature of this phenomenon. Both observers noted the 'blocking' effect of this type of antibody on saline agglutinin; Race and Wiener called it incomplete and blocking antibody respectively.

Following these observations three principle methods for the detection of incomplete antibodies were developed namely, Albumin method (Diamond and Denton,1945), Anti-human globulin method (Coombs et al. 1945) and Enzyme-treated cell method (Pickles 1946; Morton and Pickles 1947).

Discovery of New Blood Group Systems other than Rh.

During the World War II, blood transfusion had been practised to a large extent and this had led to the better understanding of its value as a therapeutic weapon. With the post-war reorganisation of the Blood Transfusion Services, blood has become more readily available and it is now being used for a variety of conditions. It has been proved to be a life saving procedure in acute haemorrhage; in some cases of blood dyscrasias it is the only method of prolonging the life of the patient. Moreover, blood transfusion has played a great role in the development of cardiac and thoracic surgery in recent years. All these factors are responsible for the enormous increase in the number of transfusions during the post-war period. The knowledge/

knowledge of the aetiology of intragroup trans-
:fusion reactions and haemolytic disease of the
newborn and the application of the newly discovered
and more sensitive methods (especially Coombs'
technique) have led to the discovery of several
new blood factors in recent years.

Callender and Race(1946) found the Lutheran
antibody in the serum of a patient suffering from
lupus erythematosus diffusus who had had several
transfusions. Coombs, Mourant and Race(1946)
reported the first example of anti-Kell in the
serum of a mother of a child suffering from
haemolytic disease. The authors designated the
antigen recognised by this antibody as Kell
factor (K). Levine et al.(1949) first reported
the reciprocal factor Cellano(k). This was
recognised by the discovery of an antibody in the
serum of a mother of a child with mild haemolytic
disease which could not be accounted for by the
other blood factors. Only 5 of the 2,495 persons
tested were found to be Cellano negative; four of
these were retested with anti-Kell serum and found
to be Kell-positive. These findings established
the reciprocal relationship between these two factors.
Mourant(1946) first discovered Lewis factor Le^a,
detectable by an antiserum anti-Le^a, which agglutinated
about 20% of the Caucasian bloods. Andresen(1948)
reported the antiserum L₂ which is now known as
anti-Le^b and/

and recognised the antigen Le^b .

Sanger and Race(1947) described anti-S and showed that it was from an antigen associated with M and N factors. An s factor allelomorphic to S was first reported by Levine et al.(1951). The antibodies anti-S and anti-s (recognising S and s factors respectively) though related to the MN system, have been found only in human serum. Rare instances of erythrocytes showing very weak M or N agglutinogens have been reported suggesting the presence of the subgroups N_2 , M_2 , M_C , but these are of little clinical importance (Crome,1935; Friedenreich and Lauridsen,1938; Dunsford, Ikin and Mourant,1953.) The very high incidence factor U has recently been found to be related to the Ss system (Greenwalt et al.1954). Erythrocytes of individuals who lack U are therefore uu homozygous, do not react with anti-S or anti-s; the symbol S^u has been proposed.

Cutbush, Mollison and Parkin(1950) discovered the Duffy (Fy) blood group and the antibody was found in a man suffering from haemophilia who had had several blood transfusions. The Fy^a antigen is present in about 65% of the population. The reciprocal factor Fy^b was recognised by Ikin et al. (1951); a woman was found two days after delivery to have an antibody for her husband's and child's erythrocytes/

erythrocytes which did not correspond to any known blood group antigens; no evidence of haemolytic disease was found in the child and in studies with a larger panel of red cells of various individuals its reciprocal(allelic) relation to Fy^a was discovered. Allen, Diamond and Niedziela(1951) discovered Kidd blood group. The antibody was detected in the serum of Mrs. Kidd, a multiparous woman whose sixth child was the first in the family to suffer from haemolytic disease. The antigen recognised by this antibody is known as Jk^a . The antithetical antibody Jk^b was first found by Plaut and his associates(1953). It was present in a woman who had a rigor following a transfusion after a miscarriage. She had had one previous transfusion after her second delivery.

In addition to the nine blood group systems, a number of additional blood factors have been reported, but these are not clinically important because the antigens by which they are recognised are nearly always absent(private family antigen) or nearly always present(public antigen).

The total number of different blood types may be calculated by permutation and combination of the recognisable major types of nine main blood group systems as follows:

Blood group system/

Blood group system	Major types that can be recognised
ABO	6
Rh	26
MNSs	9
Lewis	3
Lutheran	2
Kell	3
Duffy	3
Kidd	3
P	2

Total major types $6 \times 26 \times 9 \times 3 \times 2 \times 3$
 $\times 3 \times 3 \times 2 = 454,896$

These figures do not include D^u , c^v , c^u , C^x , M_2 , N_2 , M_c , et. nor the distinctions made by anti-d and the new antisera anti-f. They also do not include the 'private family' or 'public' antigens.

Problem

In practice only a few of the blood factors, mentioned above, are taken into account in the cross matching tests for routine blood transfusion and therefore the chance of giving 'exactly homologous' blood is very remote.

It is important to find out the actual incidence of immunisation against blood factors which are not routinely screened because such immunisations do not usually give rise to immediate complication, and doubt has been expressed in many quarters that blood transfusion is practiced more frequently nowadays than is actually needed without due regard to the possibility/

possibility of remote consequences.

So far the incidence of iso-immunisation due to blood transfusion has been studied mostly on selected cases giving rise to transfusion reactions or cross matching difficulties. From the results of such investigations it is difficult to assess the actual figure of iso-immunisation because, after blood transfusion antibodies may develop without giving rise to any reaction, and these antibodies are likely to remain undetected unless the patient requires further blood transfusion in the future, and even then if proper screening tests are not done.

The present study is therefore an attempt to find out the actual incidence of iso-immunisation after routine blood transfusions by examining the pre- and post-transfusion samples of the recipients against a number of clinically important blood group antigens. Another purpose was to compare the different methods for the detection of blood group antibodies and also to see the effect of blood transfusion on so-called naturally occurring irregular antibodies e.g. anti-M, anti-P, anti-Lewis, etc.

MATERIALS AND METHODS

Saline

Sterile physiological saline (0.9 gm% sodium chloride), hereafter referred to as saline.

Saline-Citrate Mixture

A mixture of 2 parts of saline and one part of 3.8% sodium citrate solution.

Albumin Solution

30% solution of bovine albumin (Armour Laboratories).

A 20% working solution was made by taking 2 ml. of 30% bovine albumin solution and adding to it 1 ml. of saline-citrate mixture.

Phosphate Buffer

M/10 phosphate buffer, p^H 7.7.

Proteolytic Enzymes

(A) Crystalline Trypsin (Armour Laboratories), Batch No. 15347.

A stock solution of 1% crystalline trypsin was made in N/20 hydrochloric acid.

(B) Powdered Pepsin (B.D.H.) Batch No. 439808/560601.

Coombs' Serum

Anti-human globulin serum - prepared from rabbit.

The stock serum was diluted with saline to its previously determined working strength each day, and any portion of this diluted serum remaining/

remaining after each days work, was discarded. When not in use, the stock reagent and working solution were kept frozen at -20°C .

Blood Group Anti-sera

(1) Standard anti-A, anti-B and saline anti-D sera.

(2) Anti-C, -E, -c, -e, -M, -N, -Le^a, -Le^b, -K, and anti-Fy^a.

When the presence of an antibody was detected in a patient's serum the recipient's and Donor's cells (when available) were further tested against these antibodies to obtain as complete a genotype as possible. All these antisera when not in use, were kept frozen at -20°C .

Standard Test Cells

Group O, R_1R_1 , R_1R_2 , and rr cells, selected also to include as many as possible of the clinically important blood group antigens, obtained each day by finger prick from donor panel.

The cells were collected into graduated centrifuge tubes containing saline-citrate mixture, centrifuged at 3000 rpm. for 10 minutes, and the supernatant fluid replaced with saline; the washing was repeated three times.

Saline and Albumin Suspension of Test Cells

2% suspensions were made by adding 0.1 ml. of the previously washed packed cells to 5 ml. of each/

each media and they were used in saline and albumin methods respectively.

When not in use, these cell suspensions were stored at 4°C. and any portion left after each days work was discarded.

RECIPIENT'S BLOOD

Pre-Transfusion Samples

These included all blood specimens sent to the Regional Blood Transfusion Centre for routine compatibility tests from 4 surgical, 3 medical and 3 gynaecological wards. All the specimens received from these wards were tested for the presence of antibodies and are classed as Pre-transfusion samples whether the patient had had previous transfusions or not. After withdrawal, these specimens were kept at 4°C. until tested - usually 24 to 36 hours later.

A portion of the cells and serum was removed by means of a pasteur pipette from the patient's clotted specimen, transferred to another tube and centrifuged. The serum was separated and tested for the presence of antibody while the cells were washed three times in saline and were used for determination of ABO groups, Rh type and in autoagglutination control. A portion of the cells was preserved in glycerol-citrate mixture at/
at/

at -20°C . for determination of the genotype when required (because, in case of incompatible transfusion, it is difficult to determine the recipient's genotype from the post-transfusion sample). The method for freezing red cell in presence of glycerol is discussed in Part II.

Post-Transfusion Samples

(A) First Examination - 10 to 14 days after the patient had received a blood transfusion, a sample of about 10 ml. of blood was withdrawn using a dry sterile syringe. The blood was allowed to clot at room temperature for one hour and the tube was then centrifuged and the serum separated and tested for the presence of antibody.

(B) Second Examination - In about half of the cases, (the patient being still in the hospital) it was possible to take another sample of blood between 26th and 35th day following transfusion. These samples were obtained and tested as described under first examination.

Detection of Antibodies

An initial screening test for the detection of antibodies was carried out on each serum using the following methods:

- 1) Saline
- 2) Albumin
- 3) Indirect anti-human globulin (Coombs' test)
- 4) Enzyme/

4) Enzyme treated cell

(A) Trypsin

(B) Papain

Methods 1, 2, 3 and 4A are the same as recommended in MRC Memo., No. 27.

The method 4B using papain treated cells is a simple modified method described later.

Saline Method

0.02 ml. of a 2% suspension of each type of standard test cells were placed separately in three 3" x $\frac{5}{8}$ " test tubes. Equal volumes of serum under test were added to each tube and the contents mixed by gentle shaking. Three sets of tests were set up at three different temperatures, 4°C., room temperature and 37°C. After two hours incubation the results were read macroscopically and confirmed by reading under the low power of a microscope. For microscopical examination a small amount of sedimented cell was transferred on to a clean slide with the stem of a pasteur pipette to give an even film.

Controls with D-positive and D-negative cells against weak saline anti-D serum were set up and their results were read before the tests. In addition, autoagglutinin controls were put up with patient's own cells and serum.

Albumin Method

Tests/

Tests were carried out and read in a similar manner to that described in case of saline method, except that the red cells were suspended in 20% bovine albumin solution instead of in saline.

Indirect Anti-human Globulin Method

Sensitization of cells: One volume (0.02) of a 50% saline suspension of washed standard test cells was placed into three separate test tubes (3"x $\frac{3}{8}$ ") and five volumes of serum under test added to each. The tubes were then incubated for two hours at 37°C.

Washing of sensitized cells: After two hours incubation, the cells were washed four times in excess of saline and finally resuspended to give an approximately 10% saline suspension.

Treatment of washed sensitized cells with anti-human globulin serum: One drop of the 10% cell suspension was added to one drop of diluted working solution of Coombs' serum on a chemically clean slide; and mixed by gently rotating the slide. After one minute, the slide was inspected for agglutination by gently tilting it backward and forward; if no agglutination was observed the slide was replaced on the bench until the control test showed weak agglutination of the red cells (-usually 5 to 7 minutes).

Controls -

In/

In parallel to the tests, three sets of positive and negative controls were put up, using known weak incomplete anti-D, -K and anti-Fy^a sera.

For sensitization, the cells of the control series were incubated at the same temperature and for the same period of time as the tests.

Special Precautions

In order to avoid false positive and false negative results, the following precautions were taken.

As anti-human globulin combines with human globulin at any state not only with antibody globulin fixed to the red cells, meticulous care was taken to avoid its contamination with even minute trace of human serum. In diluting the serum care was taken so that the apparatus used was not contaminated in any way. Every time freshly prepared pasteur pipettes were used for diluting anti-human globulin serum and delivery of washed sensitized cells.

The microscopic slides used for the final stage of the test were scrubbed well with soap and hot water and then rinsed thoroughly under running water. Finally they were dried with clean linen not used for any other purpose.

Direct Anti-human Globulin Method

Direct anti-human globulin tests were done on red cells of all post transfusion samples.

A portion of the recipient's red cells was thoroughly washed four times in large volumes of saline. One drop of the 10% saline suspension of the washed cells was mixed on a chemically clean slide with a drop of suitably diluted anti-human globulin serum, left for a minute and then gently rocked and observed to see if any agglutination occurred. As controls, cells known to be unsensitized and cells sensitized with known weak anti-D, anti-K and anti-Fy^a were also washed and treated with anti-human globulin serum. The observation for agglutination was continued for approximately 7 minutes with occasional gentle rocking of the slide.

Trypsin-Treated Cell Method

On the day of use, one part of stock solution of crystalline trypsin was diluted with nine parts of M/10 phosphate buffer p^H 7.7. For trypsination of cells, 4 volumes of diluted trypsin solution were added separately to one volume of each type of packed standard test cells which have been washed three times. The tubes were incubated in the water bath at 37°C. After one hour incubation the cells were washed once and resuspended in saline to give approximately a 5% suspension.

To avoid non-specific cold agglutinin reaction, the/

the cell suspension and the serum under test were placed separately at 37°C. for 10 minutes. Then equal volumes of serum and cell suspension were mixed and incubated in the water bath at 37°C. for further one hour and the results were read as in the case of the saline method. Controls with D-positive and D-negative cells against weak incomplete anti-D sera were put up in identical conditions as the tests, and the results of controls were read before those of the tests.

The activity of the stock trypsin solution was found to be quite satisfactory up to eight weeks after which slight deterioration in its activity was observed and therefore fresh stock solutions were prepared at the end of that period.

Papain Method

The following simplified papain method was used by Tribedi and Crosbie (an unpublished observation) in an investigation in which they tested 1,116 random blood samples for Rh(D) typing, 540 sera of antenatal cases for the detection of antibodies and 84 specimens involving 240 bottles of blood for compatibility testing and found that the results obtained by this papain method were better than those by albumin method and compared favourably with those of indirect Coombs' technique.

During/

During the present investigation it was found that when red cells were treated with saline suspension of papain without the addition of an activator or buffer, and incubated with an anti-serum for a few minutes at room temperature, good agglutination was observed. Preliminary tests were carried out to determine the strength of papain solution and time of exposure of the red cells to the action of papain which would best suit the required conditions namely, a simple technique which would give the maximum specific agglutination in a time which compared favourably with the existing standard methods. From the results of these tests it was decided to use a solution of 1% papain and incubate the red cells (10% saline suspension) for a minimum time of 5 minutes. It was found that non-specific agglutination appeared after 2 to 4 hours when the cells were exposed to papain solution, but they retained their specificity for 24 hours if washed once within that period. A washed cell suspension was therefore prepared by adding 1% papain solution to a 10% suspension of test cells, incubating at room temperature for 5 to 10 minutes and washing once with saline. It should be mentioned in this connection that when cell suspensions were to be used soon after the addition/

addition of papain, as in Rh (D) typing and cross-matching tests, unwashed papain treated cells were found to give satisfactory results.

The activity of the papain solution stored at 4°C. remained satisfactory up to 24 hours after preparation, but after this period it began to deteriorate rapidly. Fresh papain solution was therefore prepared daily by distributing 0.1 mg. of powdered papain into separate stoppered tubes, and each day, 10 ml. of saline was added to one tube. The tube was then shaken to obtain an even suspension and centrifuged to get rid of particulate matters. The supernatant was transferred to a fresh tube and kept at 4°C. when not in use. This constituted the working papain solution for 24 hours.

Technique

0.02 ml. of 10% suspension of papainised standard test cells were placed separately in three 3"x $\frac{3}{8}$ " test tubes and equal volumes of patients serum added to each tube. The contents were mixed by shaking and incubated at 37°C. for one hour. The results were read macroscopically by tilting the tubes and confirmed by microscopic reading. (After addition of the serum to the papainised cell, agglutination was observed within 10 minutes but for the sake of convenience, 1 hour

1 hour incubation period was selected for the present series).

The following controls were set up with the tests.

1. Positive control

Equal volumes of 10% suspension of papainised group O, CDe/CDe cells in saline and weak incomplete anti-D.

2. Negative control

Equal volumes of 10% suspension of papainised group O, cde/cde cells in saline and weak incomplete anti-D.

Titration

When any antibody was found it was titrated against appropriate cells by using a doubling dilution method and the results are expressed in terms of arbitrary scale as recommended in MRC memo., No.27.

The last tube giving at least a (+) reaction (clumps of 8 to 12 cells) is recorded as the titre.

Identification of Antibodies

When an antibody was detected in recipient's serum, the following methods were used for its identification.

1. Genotyping of the recipient's cells as fully as possible.
2. Genotyping of the donor's cells suspected to contain/

contain the offending antigen.

3. Testing the serum against a panel of cells, some containing and some without the antigen against which the antibody was suspected.
4. Absorption and Elution Techniques.

Genotyping of the Recipient's and Donor's Cells

The cells of the recipient and donor (when available) were genotyped with regard to the antigens of the ABO, MNS, Rhesus, Lewis, Kell and Duffy blood group systems. With the exceptions of anti-A, anti-B and anti-D, all the sera used were rare and therefore used in small quantities. Pasteur pipettes were marked to deliver 0.008 ml. and used for both sera and cells. Saline method was carried out in cases of all antisera except anti-Kell and anti-Duffy in which Coombs' method was used. For the tests of A, B, O, M, N, Le^a and Le^b antigens the tubes were incubated at room temperature and in all other cases, at 37°C.

For Rhesus blood factors five antisera anti-D, -C, -E, -c, and anti-e were used and the probable genotype was determined from the expected frequency table.

Testing the serum against a panel of cells

When an antibody was detected, its specificity could be 'guessed' /

'guessed' from the nature of its reaction (e.g. thermal amplitude, whether active only by Coombs' technique etc.) and from the genotypes of the recipient's and donor's red cells. However, the specificity of the antiserum was confirmed by testing it against as many as possible group O cells some of which contained the suspected antigen and the rest without it.

Absorption and Elution Techniques

In one case (Case No. 267, see later) two antibodies with different specificities were detected and both of them were active at 37°C. For isolation of these antibodies the following procedure was used:

One ml. of washed packed cells (containing the appropriate antigen) and 1 ml. of serum under test were put in a test tube and capped; this was then incubated at 37°C. for 24 hours. The supernatant was removed and tested for the presence of antibody with specially selected cells to see whether the absorption was complete or not. The red cells were washed three times in large volumes of saline, previously chilled at 4°C. The last washing was retained and tested against red cells with corresponding antigens to show that the washing had removed all uncombined antibody. After the last washing, all supernatant saline was/

was removed, 0.5 ml. of fresh saline added and the contents mixed by shaking. The tube was then placed in a water bath at 56°C. for 5 minutes and agitated at frequent intervals. To avoid cooling, the tube was spun quickly in centrifuge bucket containing water at 56°C. The supernatant - the eluate, was removed immediately and the tests with the eluate were carried out as soon as possible.

RESULTS/

RESULTS

Pre-transfusion Series

Screening tests for the detection of anti-bodies were carried out on the sera of 712 patients using the test cells and methods described above.

The following antibodies were detected.

<u>Type of antibody</u>	<u>Number of cases</u>
1. Non-specific cold agglutinins	569
2. Specific irregular cold agglutinins	
(a) Anti-Lewis	11
(b) Anti-P	2
3. anti-D	2

Non-specific cold agglutinins

The non-specific cold agglutinins, detected in the sera of 569 patients (aproximately 79% of all cases) were active only at 4°C. Every serum showing a cold agglutinin, reacted with the patient's own cells as well as with those in the cell panel, and the reactions were reversible. Titrations for cold agglutinins were carried out against patient's own saline suspended cells and in three cases the titres were especially high as follows:

<u>Case Number</u>	<u>Diagnosis</u>	<u>Titre</u>
14	Haemolytic anaemia	1:256
51	Multiple myeloma	1:128
129	Chronic lymphatic leukaemia	1:128

In/

In the remaining cases, the cold agglutinin titres varied from 1:4 to 1:32; the higher end points (within this range) being obtained in group A, B, and AB as compared to those in group O individuals.

Anti-Lewis antibodies

Of the 11 anti-Lewis antibodies detected, 8 were anti-Le^a, 2 anti-Le^b and one was a mixture of anti-Le^a and anti-Le^b. All these antibodies were active at room temperature and only one serum containing anti-Le^a haemolysed trypsinised Le(a+) cells at 37°C.

Seven of these patients with anti-Lewis antibodies in their sera were males - two of whom had been transfused with blood before, while five had no history of transfusion or injection of blood. Only two of the four female patients had incurred the risk of immunisation, one had been pregnant and the other had a history of pregnancy and previous transfusion.

Anti-P antibodies

Neither of the two male patients who had anti-P antibodies in their sera had ever been transfused or injected with blood. The antibodies in both the cases were active at room temperature.

Anti-D antibodies

The/

The anti-D antibodies were detected in the sera of one male and one female patient both of whom were Rh negative. The male patient had received two pints of whole blood seven years previously without any adverse effect. Although the Rh type of the transfused blood had not been recorded, it may be assumed that at least one of the donors was Rh(D) positive. The female patient had never been transfused or injected with blood and none of her two children suffered from haemolytic disease of the newborn. Blood samples from the children could not be obtained but as her husband was Rh(D) positive, it may be assumed that the pregnancy was the cause of immunisation in her case.

The antibodies in both instances were of the incomplete type and were detectable by albumin, indirect Coombs' test and enzyme treated cell techniques. The titres obtained in pre- and post-transfusion samples are compared in Table VI (see later).

Post-transfusion Series

Out of the 712 cases of the pre-transfusion series, only 389 patients received blood transfusions during the present investigation. Seven of these transfused patients died and 30 cases were discharged before the time for taking the/

the post-transfusion samples were due, leaving in all 302 patients whose sera could be examined after transfusion and these comprise the post-transfusion series.

A short account of the patients comprising the post-transfusion series

The distributions of ABO groups and Rh types of the patients in this series are shown in Table I.

The occurrence of both ABO groups and Rh types are well in agreement with those expected in this country. The age of the patients in this series ranged from 5 to 82 years.

Table I

ABO Group	Rh(D)positive	Rh(d)negative	Total	Percentage
O	116	22	138	45.7%
A	106	21	127	42.1%
B	25	3	28	9.3%
AB	8	1	9	2.9%
Total	255 (84%)	47 (16%)	302	100%

The cases were not specially selected but since one of the medical wards is associated with a haematological research unit, a relatively high proportion of patients suffering from blood dyscrasias has been included.

The history of previous transfusion, pregnancy and distribution of sex of the patients in this series are shown in Table II.

TABLE II

			127 patients with history of pregnancy		
Sex	No history of transfusion or pregnancy	58 patients with history of previous transfusion		Pregnancy alone	Total
		Transfusion alone	Transfusion & pregnancy		
Female	58	15	16	111	200
Male	75	27	-	-	102
	133	42	16	111	302

The high incidence of females (200) is due in part to the inclusion of patients from three gynaecological wards; 127 female patients were parous - 29 primipara and the rest multipara as shown in Fig. 1. (P.44)

It will be seen in the above table that 133 patients (75 male and 58 female) had no history suggestive of immunisation prior to the present transfusion.

58 patients (27 male and 31 female) had history of previous blood transfusions, but the amount of blood each patient had received, could not be ascertained. The number of, and the approximate intervals between the transfusions in these 58 cases are shown in Fig. 2. (P.44)

It will be seen that 6 patients had only one transfusion, 15 received 2 or 3 at short intervals while 12 received the same number but the intervals between them were longer. In 25 cases of multiple transfusions (more than three) the intervals were variable.

Present Transfusion

Altogether, during the present investigation, 916 bottles of whole blood (each bottle containing 110 ml. of ACD solution and 400 ml. of blood) and 58 bottles of packed cells were transfused. The duration of storage of whole blood before transfusion varied from the date of collection to 21 days. The maximum amount of blood given to a single patient was 17 bottles, while the majority of the patients received 2 to 4 bottles of blood as shown in Fig. 3. (P.44)

14 cases received 2 to 3 transfusions at intervals of 1 or 2 days and their blood samples were obtained on the 10th to the 14th day following the last transfusion. Several transfusions were given to three patients of aplastic anaemia at intervals of 3 to 4 months. Although the sera of these three patients were tested after every transfusion, (3 or 4 times during this study period), each was considered as one case in present investigation.

There/

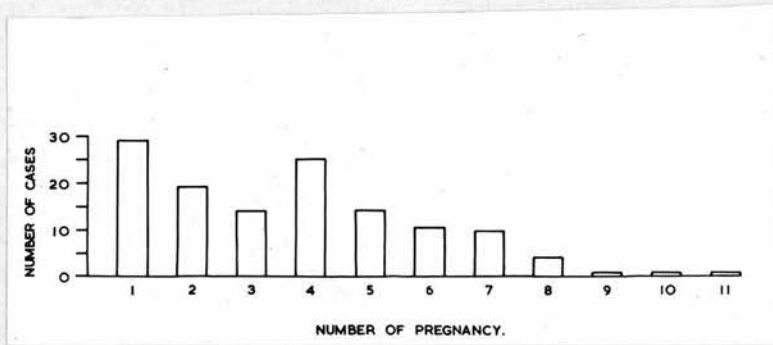


Fig.1, shows the parity of 127 patients.

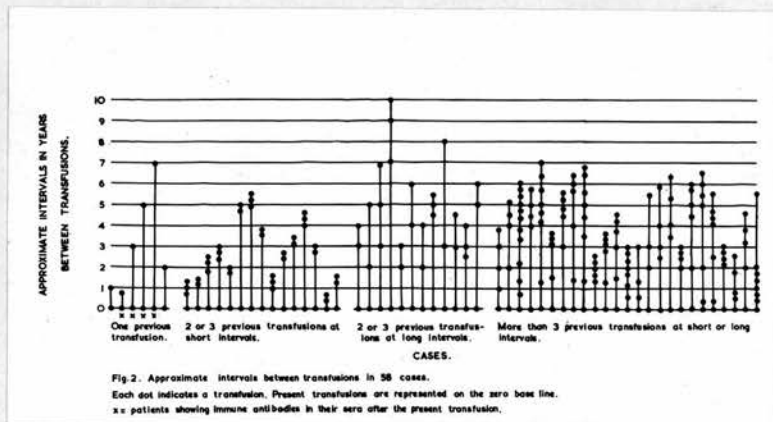


Fig.2, shows the number of and intervals between previous transfusions. Transfusions during the present observation are represented on the 0-base line.

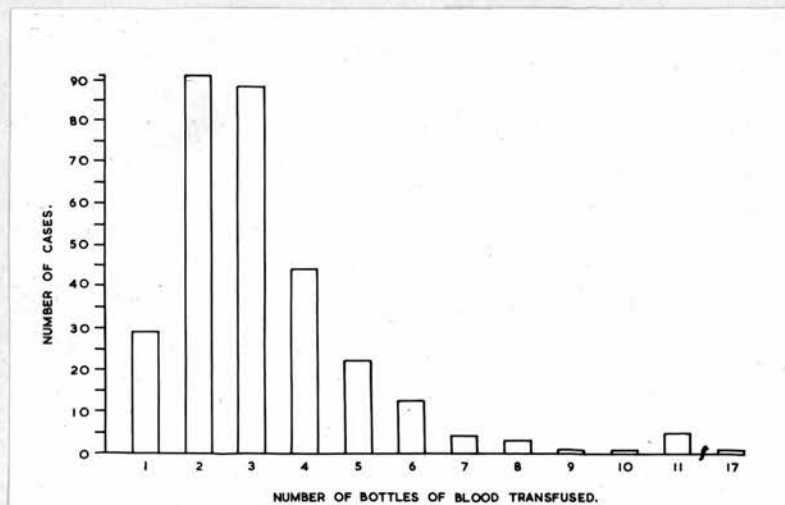


Fig.3, shows the number of bottles of blood transfused to 302 patients during the present investigation.

There were symptoms and signs suggestive of haemolytic reactions as a result of the present transfusion in three cases, but in the serum of only one of these patients could an immune antibody be detected (Case 281 - see later).

Six immune antibodies which could not be found in the pre-transfusion samples, were detected in each of six patient's serum during the screening of 302 post-transfusion samples. These are shown in Table III. (P.46)

Case 3.

A woman aged 45, was admitted to the gynaecological ward with menorrhagia. She had four normal pregnancies but had never been transfused or injected with blood. On examination, her haemoglobin was found to be 7.4 gm.% and two bottles of whole blood were transfused, followed three days later by a further two bottles. No obvious ill-effects were observed after transfusion. In the pre-transfusion sample no antibody was detected; neither was there any difficulty in the cross matching test.

Preliminary screening of the post-transfusion sample (10th day following the last transfusion) showed agglutination with standard cell No. 2 (Table IV) by Coombs' technique only. (The test was repeated at a later date with papain treated cells and the patient's serum which had been stored/

TABLE III.

Case No.	Age	Sex	Diagnosis	History of previous transfusion	History of pregnancy	ABO Gp.	Rh(D) type	Present transfusion	Antibodies detected
3.	45	Female ^M	Menorrhagia	Nil	4 + 0	O	+ve	4 bottles, 2 bottles each time at an interval of 3 days.	Anti-K
14.	28	Female	Haemolytic anaemia	2 bottles 9 months previously	Nil	O	+ve	5 bottles	Anti-o
152.	20	Male	Cholecystitis	Nil	—	A	+ve	3 bottles	Anti-o
211.	65	Male	Peptic ulcer	2 bottles 5 years previously	—	O	+ve	5 bottles during operation, 2 bottles 5 days later.	Anti-Lo ^b
267.	30	Male	Peptic ulcer	2 bottles 7 years previously.	—	O	-ve	3 bottles	Anti-Fy ^a (Anti-D)
281.	49	Female	Appendicitis	2 bottles 3 years previously	6 + 1	O	-ve	4 bottles	Anti-Fy ^a

Table III. Type of immune antibodies detected for the first time in the post-transfusion specimens of six patients with a short history in each case. Antibody in parenthesis indicates that it was detected in pre-transfusion sample.

^MHusband of this patient was Kell-negative.

TABLE IV.

Standard test cells	Case No. 3	Case No. 14	Case No. 152	Case No. 211	Case No. 267	Case No. 281
	S A IC T P	S A IC T P	S A IC T P	S A IC T P	S A IC T P	S A IC T P
No. 1. O, R ₁ R ₁ (CDe/CDe), MNS, P, Kk, Le (a-b+), Fy(a-)	- - - - -	- + + + +	- - - - -	+ + + + +	- + + + +	- - - - -
No. 2. O, R ₁ R ₂ (CDe/CDe), Ms, p, K, Le (a+b-), Fy(a-)	- - + - + ^M	- + + + +	- - + - +	- - - - -	- + + + +	- - - - -
No. 3. O, rr (cde/cde), Ms, P, Kk, Le (a-b+), Fy(a+)	- - - - -	- + + + +	- - + - +	+ + + + +	- - + - +	- - + - +

Table IV. S, A, IC, T, and P indicate saline, albumin, indirect Coombs', trypsin and papain methods respectively.

+ = Agglutination
- = No agglutination

^MTest with papain treated cells was done later using the frozen serum.

stored at $-20^{\circ}\text{C}.$, and the result was positive).

The blood groups and genotypes of the patient, husband and four donors and the reaction of her serum with donors' cells (by Coombs' method) are shown below.

	Genotype	Reaction with patient's serum by indirect Coombs' method
Patient	O, CDe/cde, MN, kk, Le(a-b+), Fy(a+)	
Husband	A, CDe/CDe, N, kk, Le(a-b-), Fy(a+)	
Donors 1.	O, CDe/cde, MN, kk, Le(a-b-), Fy(a-)	Negative
2.	O, CDe/cde, N, K, Le(a-b+), Fy(a+)	Positive
3.	O, CDe/CDe, MN, kk, Le(a-b+), Fy(a+)	Negative
4.	O, CDe/cde, M, kk, Le(a+b-), Fy(a+)	Negative

It was found that the patient's serum agglutinated the red cells of donor 2. From the genotypes and the reactions of the patient's serum with standard cells and donor's erythrocytes, it appeared that the antibody was anti-Kell.

For confirmation, 10 group O, Kell-negative and 4 group O, Kell-positive cells were tested against patient's serum by indirect Coombs' method and the reactions are shown below.

Red cells	Reactions with Patient's serum		
	Positive	Negative	
Kell-positive	4	0	4
Kell-negative	0	10	10
	4	10	14

The/

The 'exact' probability of the antibody being anti-Kell = 1,001 to 1.

the titre against standard cell number 2. by indirect Coombs' method was 1:32.

Case 14.

An unmarried woman, aged 28, was admitted to the medical ward as a case of haemolytic anaemia with 6.5 gm.% haemoglobin. 9 months prior to the present admission, she had a transfusion of two bottles of group O, D-positive blood without any apparent ill effect.

On admission, a direct Coombs' test on her red cells was negative; no immune antibody could be demonstrated in her serum but the non-specific cold agglutinin titre was high (1:256). Five bottles of blood were transfused and there was no untoward effect.

On the 13th day following the present transfusion, her serum was found to agglutinate all three standard cells (Table IV) as well as her own cells, by the albumin, indirect Coombs', trypsin and papain methods. The direct Coombs' test was now found to be positive. It was noted that reactions against standard cell Nos. 1 and 3 were much stronger than that with No. 2. The genotype of the patient was -

O, CDe/cde, MN, kk, Le(a+b-), Fy(a+).

From/

From the reactions of the serum with the standard cells it appeared that the antibody might be anti-e, showing a 'dosage effect'.

The titres of this antibody against e/e and E/e cells by different methods were as follows:

Cells	Albumin method	Indirect Coombs' method	Trypsin method	Papain method
e/E	1:4	1:32	1:16	1:64
e/e	1:8	1:128	1:128	1:512

The specificity of the antibody was confirmed by testing the sera against a panel of 53 group O cells, ten of which were Rh-negative (cde/cde) and 43 were Rh(D)positive. All the Rh-negative and 41 Rh-positive bloods were agglutinated by the sera. The two Rh-positive bloods which gave negative reactions were cDE/cDE.

Note:

This is a case of auto-immunisation because the patient herself was 'e' positive. The negative result on the pre-transfusion sample may probably be due to the quiescent stage of the disease at that period of examination.

Case 152

A man aged 20, suffering from cholecystitis, was admitted to the surgical ward for operation. He had never been transfused or injected with blood.

Anti-Le^a/

Anti-Le^a antibody was found in the pre-transfusion sample but no immune antibody could be detected.

On the 11th day following the transfusion of three pints of group A, D-positive blood an antibody was detected in his serum which reacted against standard cell Nos. 2 and 3 (Table IV) by indirect Coombs' and papain methods. The patient's genotype was -

A, CDe/CDe, N, K, Le(a-b-), Fy(a+).

From these reactions against the standard cells and the genotype of the patient, it was suspected that the antibody might be anti-c or anti-s. On testing the sera against c-positive but s-negative cells a positive reaction was obtained. The specificity of the antibody anti-c was further confirmed by testing the serum against 7 c-positive (4, c/c and 3, C/c) and 5 c-negative (C/C) cells with the following results:

Red cells	Reactions with patient's serum		
	Positive	Negative	
c-positive	7	0	7
c-negative	0	5	5
	7	5	12

'Exact' probability = 792 to 1.

Naturally occurring anti-Le^a did not interfere with the tests, as it did not show any reaction at 37°C., (although it was active at room temperature).

On/

On the 26th day following the transfusion, another fresh sample was tested but no appreciable change in the antibody titre was observed; the results of titrations on both days are shown below.

	By indirect Coombs' method	By papain method
Titre on 11th day following transfusion	1:32	1:128
Titre of 26th day following transfusion	1:32	1:64

Case 211

A man aged 65, was admitted to the surgical ward with haematemesis, and subsequently diagnosed as a case of perforated peptic ulcer. He had had a transfusion of two bottles of blood about 5 years ago without any adverse effect. On admission, no antibody could be detected in the pre-transfusion sample. During the operation, 5 bottles of group O, D-positive blood were transfused followed five days later by another two bottles.

On the 10th day following the last transfusion his serum agglutinated standard cell Nos. 1 and 3 by all the methods used as shown in Table IV. Patient's genotype was - O, CDe/CDe, N, kk, Le(a-b-), Fy(a+).

From the reactions against the standard cells, it appeared that the antibody was either anti-P or anti-Le^b. The serum was tested against P-positive but /



but Le^b-negative cells and no agglutination was observed. Blood samples from three of the donors (of present transfusion) were available and tested against patient's post-transfusion serum. The results are shown below.

Donor	Genotype	Reaction with patient's serum
1.	O, CDe/cde, MN, kk, Le(a-b+), Fy(a-)	Positive
2.	O, CDe/cde, N, kk, Le(a-b+), Fy(a+)	Positive
3.	O, CDe/CDe, N, kk, Le(a-b-), Fy(a+)	Negative

The post transfusion sample agglutinated the cells of donors 1 and 2.

Finally the serum was tested against 13 group O cells with known Lewis group and the results were as follows:

Red cells	Reaction with patient's serum	
	Positive	Negative
Le(a+b-)	0	6
Le(a-b-)	0	2
Le(a-b+)	5	0

'Exact' probability for $\frac{0}{5} \Big| \frac{8}{0} = 1287$ to 1.

The/

The specificity of the antibody as anti-Le^b was thus confirmed.

The titres of the antibody against group O, Le(a-b+) cells by different methods were as follows:

Saline	Albumin	Indirect Coombs'	Trypsin	Papain
1:8	1:16	1:64	1:32	1:64

Case 267

A 30 year old man, suffering from peptic ulcer was admitted to the surgical ward for gastrectomy. He had had a transfusion of 2 bottles of blood 7 years previously and in his pre-transfusion sample (in relation to the present transfusion) anti-D antibody was detected. This case has already been referred to, in the pre-transfusion series.

During operation, 3 bottles of group A, D-negative whole blood were transfused and there was no adverse reaction. On the 12th day following the present transfusion, an additional immune antibody was demonstrated in the patient's serum in addition to the rising of anti-D titre (see later). The patient's serum agglutinated standard cell Nos. 1 and 2 by albumin, indirect Coombs', trypsin and papain method and No. 3 by indirect Coombs' and papain methods (see Table IV). The genotype of the patient was - A, cde/cde, MN, kk, Le(a-b+), Fy(a-). Blood samples from two of the donors (of the present transfusion) were obtained/

obtained and tested against patient's serum with the following results:

Donor	Genotype	Reaction with patient's serum
1.	A, cde/cde, M, kk, Le(a-b-), Fy(a-)	Negative
2.	A, cde/cde, N, kk, Le(a-b+), Fy(a+)	Positive

The patient's serum agglutinated the cells of donor 2. From the nature of these reactions it appeared that the new antibody was anti-Fy^a.

The antibody was absorbed by treating the serum with D-negative, Fy^a-positive group O cells and finally eluted. The eluate was tested with 7 Fy^a-positive and 4 Fy^a-negative group O cells by indirect Coombs' and Papain methods and all the Fy^a-positive cells were found to be agglutinated while no reaction was observed with any of the Fy^a-negative cells.

Case 281

A married woman aged 49, was admitted to the surgical ward with appendicitis. She had six pregnancies and between the third and fourth a miscarriage. She had a transfusion of two bottles of group O, D-negative blood three years previously/

previously and there was no reaction.

On admission, no antibody could be detected in her serum. During the operation, four bottles of group O, D-negative blood were transfused and a few hours after completion of the transfusion she complained of feeling cold and had a slight rigor. The following morning she passed reddish brown urine which contained urobilin; the serum bilirubin content was 5 mg.%. Her condition settled within 24 hours and recovery was uneventful.

The compatibility test was repeated with the pre-transfusion serum and was still found to be negative, neither could any antibody be demonstrated in the immediate post-transfusion sample.

On the 10 day following the present transfusion, her serum agglutinated the standard cell No. 3 (Table IV) at first by the papain method only. The indirect Coombs' test was repeated but the result was negative. Using different dilutions of Coombs' serum the test was positive as shown below:

Red Cells	Dilutions of Coombs' serum						
	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Rh(D) sensitized cells	-	-	-	+	+++	+++	++
Standard Cell No. 3 sensitized with the serum of Case. 281	+	++	-	-	-	-	-

The/

The genotype of the patient was - O, cde/cde, MN, K, Le(a+b-), Fy(a-).

From the patient's genotype and the reaction of her serum with the standard cells it appeared that the antibody might be anti-Fy^a. The specificity of the anti-serum was confirmed by testing it against 7 Fy^a-positive and 6 Fy^a-negative group O cells with the following results:

Red cells	Reactions with patient's serum		
	Positive	Negative	
Fy(a+)	7	0	7
Fy(a-)	0	6	6
	7	6	13

'Exact' probability = 1716 to 1.

Note:

It has been found in this case that the dilution of Coombs' serum optimum for the detection of Rh-antibodies is not the same as for anti-Fy^a.

Effect of blood transfusion on the antibodies detected in patient's sera prior to the present transfusion

277 patients with non-specific cold agglutinins in their sera (detected in the pre-transfusion samples) received blood transfusion during the present investigation. In only one case (No. 14, Table III, a case of haemolytic anaemia) was there a rise in cold agglutinin titre in the post-transfusion sample. The titre in this instance, rose to /

to 1:1024 from its pre-transfusion level of 1:256.

Seven out of 11 patients who had anti-Lewis antibodies in their sera, were transfused, six with one or two bottles of Lewis incompatible and one with one bottle of Lewis compatible blood.

The Lewis blood groups of these 7 patients and those of their donors are shown in Table V in which anti-Lewis iso-agglutinin titres of pre- and post-transfusion samples have been compared. It will be seen that there was no appreciable change in titres after the transfusion. It was also observed that there was no change in thermal amplitude of these agglutinins and there was no reaction in any of these cases following transfusion.

Table V - (page 59).

Anti-D

Two bottles of D-negative blood were transfused to each of the two patients showing anti-D antibodies in pre-transfusion samples. One of these cases (Case 267, Table III) developed anti-Fy^a as a result of present transfusion and has already been described. In both cases a rise in anti-D titre in post transfusion samples was observed and this is shown in Table VI (page 59).

It/

It was thought that this rise of antibody titres might be due to weakly reacting D antigen and therefore all four donors' cells were tested for D^u by indirect Coombs' method, but all of them were found to be D^u-negative.

In 148 cases including case No.152 (Table(III)) it was possible to obtain samples again on or about the 4th week following transfusions. No antibody other than those found in previous examinations, was detected, nor was there any change in titre of anti-c antibody (in Case 152).

Table V/

Table V. The effects of transfusions of compatible and incompatible blood (of Lewis group) on the naturally occurring anti-Lewis antibodies.

Case No. 178 received Lewis compatible blood.

Case No.	Patient's Lewis group	Antibodies in patient's serum	Donor's Lewis group	Amount of blood transfused	Pre-transfusion titre	Post-transfusion titre
41	Le(a-b-)	anti-Le ^a	Le(a-b+)	2 ^x	1:16	1:16
80	Le(a-b-)	anti-Le ^a	Le(a+b-)	2 ^x	1:32	1:16
178	Le(a-b-)	anti-Le ^a	Le(a-b+)	1 ^x	1:4	1:8
202	Le(a-b-)	anti-Le ^a & anti-Le ^b	Le(a+b-)	1 ^x	1:8	1:8
249	Le(a-b-)	anti-Le ^b	Le(a-b+)	2 ^x	1:16	1:32
286	Le(a-b-)	anti-Le ^a	Le(a+b-)	2 ^x	1:4	1:8
300	Le(a-b-)	anti-Le ^b	Le(a-b-)	1 ^x	1:4	1:4

(^x bottles.)

Table VI. The effects of transfusion of D-negative blood on anti-D titres in two cases who were previously immunised.

Case No.	Samples	Methods			
		Albumin	Indirect Coombs'	Trypsin	Papain
267.	Pre-transfusion	1:4	1:32	1:16	1:64
	Post-transfusion	1:32	1:1024	1:512	1:1024
271.	Pre-transfusion	1:2	1:8	1:4	1:8
	Post-transfusion	1:32	1:512	1:512	1:512

DISCUSSION

Antigenicity of different blood factors and their
role in iso-immunisation

Advances made by several workers during the last few years in the methods of detection of iso-antibodies and their application to the study of iso-immunisation have led to a great development in blood group serology. Iso-immunisation as a result of blood transfusion, may occur if the transfused blood contains an antigen which the recipient lacks. Haemolytic transfusion reactions may be avoided by proper cross matching tests but the methods used at present are not sufficient to prevent iso-immunisation due to one or other of the new blood factors. As there are no corresponding natural antibodies against the majority of these antigens blood which appears to be suitable may not be homologous with regard to all these new blood factors. Thus, it is evident that the incidence of iso-immunisation against certain blood factors (which are not at present routinely screened in the cross matching test) will steadily rise with the increasing number of transfusions. If immunisation occurs against a common or multiple blood factors, there will be difficulty in obtaining suitable blood if required for/

for such recipients in the future. Moreover, iso-immunisation of female patients in this way before or during the childbearing period may cause haemolytic disease of the newborn in any future pregnancy.

Under these circumstances, it would be desirable to prevent such immunisation entirely, but this could only be achieved by giving "exactly" homologous blood, which is impracticable due to multiplicity of blood group antigens. Fortunately however, all the blood factors are not equally antigenic and a selection can be made. For this purpose it is necessary to know which are the more antigenic ones and the actual risk involved if a particular antigen is not screened.

The most reliable method of determining the antigenicity of a blood factor is the experimental immunisation of human volunteers or clinically the antigenicity may be assessed from the relative proportions of different blood group antibodies which occur as a result of incompatible transfusion and/or pregnancy.

The present status of our knowledge about the main blood group systems is well explained in the survey of Mollison (1956) as shown in Table VII.

Table VII /

Table VII

The main blood group systems and their clinical importance (Mollison 1956)			
System	Occurrence of antibodies in persons who have never been pregnant or transfused or injected with human blood	Known to have caused haemolytic transfusion reaction	Known to have caused haemolytic disease of the newborn
ABO	Regular	Yes (common)	Yes (mild form common)
Rh	Extremely rare	Yes (common)	Yes (common)
MNS	Rare	Yes (rare)	Yes (very rare)
P	Occasional	Yes (very rare)	No
Lutheran	Extremely rare	Yes (very rare)	No
Kell	Not yet described	Yes (occasional)	Yes (very rare)
Lewis	Occasional	Yes (rare)	No
Duffy	Extremely rare	Yes (rare)	Doubtful
Kidd	Not yet described	Yes (very rare)	Doubtful

On the clinical and experimental evidence, all the factors of the nine main blood group systems may be classified into two broad divisions -

- (1) those which give rise to iso-immunisation more readily e.g. A and B antigens of the ABO groups and D antigen of the Rh system.
- (2) those which give rise to iso-immunisation less frequently e.g. C, E, c, d, e and f antigens of the Rh system and all the antigens of MNS, P, Lutheran, Kell, Lewis, Duffy and Kidd systems.

Blood factors giving rise to iso-immunisation

more readily

ABO system: The ABO groups are by far the most dangerous in blood transfusion practice, because of the constant presence of natural iso-agglutinins in this system. The method of detection and prevention of complications due to ABO blood groups is however relatively simple and because of this, difficulties should not arise except from clerical and technical errors. Where ABO incompatible blood has inadvertently been transfused, it has been reported (Thalhimer,1921; Wiener,1941; Mollison and Young,1941; Boorman, Dodd and Mollison,1945; Wallace,1950) that there was a rise of iso-agglutinin titres in every case and the immune characteristics of the iso-agglutinins in such cases have been demonstrated. It has also been noticed by some of the authors (Wiener, 1941; Mollison and Young,1941; Wallace,1950) that there may be an initial fall in the titre immediately after an incompatible transfusion followed by a steady rise reaching a peak some time during the second week. Haemolytic disease of the newborn, though of milder degree, may also occur due to ABO incompatibility. These observations suggest that A and B factors of the ABO system act as strong antigens. Whether this action/

action is due to strong antigenicity of these factors or due to previous stimulation of the recipients against these group specific substances is not known. However the current reports (Hubinont,1950; Wiener,1951a) suggest that iso-agglutinins occur as a consequence of immunisation with blood group A and B substances, which are widely distributed in nature so that contact with them as they occur in foods, inhaled materials, danders, dusts, micro-organisms etc., is almost universal.

D antigen of the Rh system: It is not usual for naturally occurring Rh antibodies to be present in human sera (although occasionally reported e.g. anti-D by Grove-Rasmussen and Levine,1954; anti-E by Stratton,1953 and Race and Sanger,1954). So the detection and prevention of immunisation against Rh factors are rather complicated. With the wide application of screening of the D antigen during recent years, sensitization against this antigen, as a result of blood transfusion, has considerably diminished (and no case of such sensitisation as a result of transfusion during the present investigation, was detected). However, sufficient information about the antigenicity of the D factor may be obtained from the many reports published in the literature since its discovery/.

discovery. Although the clinical importance of the Rh(D) factor was realised just after its discovery, precautions against transfusion of D-positive blood to D-negative persons could not be undertaken immediately, because anti-Rh (anti-D) sera were not easily available and moreover, the Blood Transfusion Services were not so well organised at that time. As a result, during the earlier days, even after the discovery of Rh(D) factor, there were a certain number of cases of Rh(D) incompatible blood transfusions. Reports have appeared on retrospective examinations of some of these cases. At first there was no agreement among the various workers about the incidence of sensitization due to D-antigen. Wiener(1945a, 1945b, 1946a) stated that between 1 in 50 and 1 in 25 Rh-negative persons who are exposed to Rh(D) antigens become immunised. He further stated that the incidence of sensitization due to Rh-positive pregnancy and by transfusion is about the same. Unger(1946) observed that when repeated transfusions of the Rh-positive blood are given to Rh-negative patients the response to the Rh(D) antigen develops in approximately 1 in 25 patients. DeGwin(1945) in a series of 5,386 consecutive blood transfusions, found that 6.6% of the Rh-negative patients were immunised against Rh(D) factor/.

Rh(D) factor. Diamond(1946) on the other hand, demonstrated anti-D antibodies in 50% of the Rh-negative individuals in a series of 2,500 servicemen who had transfusions under war-time conditions without regard to Rh type. Ross (quoted by Hattersley,1947) in a smaller series found anti-D antibodies in nearly 50% of Rh-negative bloods. In a similar survey, Hattersley(1947) examined the blood of groups of servicemen who had at some time in the past received transfusions presumably without the benefit of Rh typing, and he found 55% of the Rh-negative people immunised. Pickles in an unpublished observation, cited by Mollison(1956) demonstrated anti-D antibody in 50% of the Rh-negative individuals after a single transfusion. The discrepancies in these results may be explained by the fact that in some of the earlier investigations, methods for detection of incomplete antibodies had not been included.

In immunisation following pregnancy, the D antigen is responsible for approximately 90% of cases of haemolytic disease of the newborn.

In addition to the above findings about the antigenicity of the D factor, further evidence may be accumulated from the work of several authors (Diamond,1944b, Hill and his associates,1945, Gold and Turcanu,1947, Wiener,1949) who carried out/

out experiments by injecting small amounts of D-positive blood into D-negative volunteers. Different authors adopted different courses of injections and found that individuals vary widely in responding to antibody formation. In reviewing the results of experimental immunisation against D antigen, Mollison(1951) concluded that "in order to provoke the formation of anti-Rh with the least possible number of injections an interval of four months should be left between the first and second injection; ten days after the second injection almost 40% of the subjects may be expected to produce antibody". The results of the present study support this observation (c.f. Cases 211, 267 and 281, Table III).

Experimental immunisation cannot be compared directly with sensitization as a result of routine blood transfusion because, (1) the condition or disease for which patients are admitted to hospital may influence the result (c.f. Case 14, Table III), (2) the intervals between the injections of blood into volunteers can be controlled, (3) the amount and age of the blood can also be controlled according to the need in case of voluntary immunisation whereas in routine transfusions this is not practicable. However, it may be concluded from the above clinical and experimental/

experimental findings that approximately 50% of the people may produce anti-D after a single transfusion and it has generally been accepted that in addition to ABO groups, the D antigen of the Rh system must be considered in all cross matching tests.

Blood factors giving rise to iso-immunisation
less frequently

While the importance of antigenicity of D factor has been established beyond doubt, no definite conclusions have been reached about the relative antigenicity of the other factors of the Rh system and other new blood factors. Originally it was thought that sensitization due to these so-called 'less antigenic' factors was very rare and Wiener(1954) stated that sensitization due to C, E, c, e, Fy, K, S etc., are the nature of 'medical curiosities'. Following the publications of numerous reports on cases of iso-immunisation against these blood factors by several workers during recent years, it has been found that these antigens are not so 'weak' as they were originally thought to be. In a series resulting mainly from transfusions Speiser and Kolbl(1952) detected 92 cases with anti-D antibodies and 40 with antibodies other than pure anti-D. In similar series of Vogel(1954) and that of Kellner(1955) the proportion/

proportion of pure anti-D to antisera other than pure anti-D (excluding anti-A, -B, -O or -H, unidentified antibodies, high titre cold agglutinins and panagglutinins) was 95:105 and 24:37 respectively. These latter antibodies included anti-C, - (in addition to anti-D+anti-C and anti-D+anti-E), -C^W, -c, -e, -K, -Fya, -Fyb, -Jka, -M, -N, -S, -Lea, -Leb and anti-P. Levine et al(1956) in reviewing the results of these series and comparing them with that of their own (a series comprised of partially selected samples) stated that anti-c, anti-E and anti-Kell can no longer be considered rare antibodies. As for anti-Kell this is confirmed by the findings of Grove-Rasmussen et al. (1954) and Bowley(1955) who reported 36 and 28 cases respectively during the last few years. Malone and Dunsford (1951) reported 12 cases of pure anti-E as a result of pregnancy and transfusion. Anti-c sera is rather a common finding and it is one of the four main Rh antibodies that are used in routine genotyping. However, Levine and his associates (1956) found 177 atypical antibodies (other than anti-D, -CD and anti-DE) and 43 of them were anti-c. The antigenicity of C and E in relation to D factor may be understood from the observations of Race (MRC Memo., No.27) that most Rh-positive bloods belong to CDe/cde or CDe/CDe, /

CDe/CDe, but those individuals of type cde/cde who form anti-Rh 60% form anti-D, and anti-D+anti-C occurs together in about 30% of all anti-Rh sera and anti-D+anti-E in about 2%

A limited number of investigations have been carried out to immunise volunteers against blood factors other than D. Van Loghem(1947) was able to produce anti-C and anti-E without anti-D in Rh-negative (cde/cde) volunteers by repeated injections of Cde/cde and cdE/cde cells respectively. Jones et al.(1954) tried to produce anti-C and anti-E in suitable Rh-positive recipients by giving injections every third week, but amongst a total of 32 volunteers injected over a period of one and a half years not one produced the desired antibody. On the other hand Johns et al. (1954) succeeded in producing anti-c in 2 out of 9 volunteers who were given repeated injections of c-positive blood over a period of 10 months. These authors also gave e-positive blood to a volunteer of the probable genotype cDE/cDE; after six years anti-e was found for the first time. Van Loghem and his associates(1953) pointed out that the order of antigenicity within the Rh system to be $D > E > C$ and $d < e < c$. Race and Sanger(1954) attempted to immunise volunteers against a number of blood group antigens. Most of/

of the volunteers were given 4 injections of 1 ml. of blood at 3 months intervals; none of them produced antibody and these results are given below.

21 MS/MS volunteers failed to produce anti-s

34 CDe/cDE volunteers failed to produce anti-d

18 candidates failed to produce anti-Fy^a

13 candidates failed to produce anti-Fy^b

16 candidates failed to produce anti-Jk^a

12 candidates failed to produce anti-Jk^b

From the above findings it can be stated that though volunteers are immunised with difficulty by injection of these 'less antigenic' blood factors, sensitization due to these antigens as a result of blood transfusion is not rare. Although the antibodies against most of these factors have been found to give rise to haemolytic transfusion reaction and/or haemolytic disease of the newborn, only the C and E antigens (in addition to D antigen of the Rh system) are screened in the cross matching tests at present and that only in selected cases viz. when the recipients are Rh-negative. This procedure of screening of C and E antigens only partially prevents the possible immunisation against these antigens since in D-positive recipients these antigens are not routinely checked and therefore, for example, CDe/CDe/

CDe/CDe and CDe/cde recipients may be transfused with cDE/cDE or cDE/cde blood or vice versa and anti-E or anti-C may be developed as the case may be. Thus some of the 85% of the D-positive population are exposed to the risk of immunisation against these antigens. Surprisingly enough, no anti-C or anti-E was found in the present investigation. It seems from the present observation as well as from that of Van Loghem(1947) and Jones et al.(1954) (stated above) that Rh-negative persons (cde/cde) are more prone to form anti-C and anti-E than suitable Rh-positive persons.

Expected Incidence

The most reliable method for determination of the incidence of iso-immunisation due to blood group antigens which are not at present routinely screened, would be to genotype fully the donors' and recipients' cells. The exact rate of 'incompatible' transfusions in relation to various blood factors would then be known, and then the recipient's sera be examined to detect complete and incomplete antibodies after an appropriate interval. This procedure unfortunately, is not at present practicable. Alternatively, by making use of the following gene frequency table (Race and Sanger 1954) the chances of incompatible transfusion regarding these blood factors may be calculated/

calculated.

Gene Frequency Table (Race and Sanger, 1954)

Blood group system

Rh	C-0.4198	D-0.5897	E-0.1554
	c-0.5673	d-0.4103	e-0.8446
	C ^w -0.0129		
MNSs	M-0.5316	S-0.3274	
	N-0.4684	s-0.6726	
P	P-0.4901		
	p-0.5099		
Lutheran	Lu ^a -0.0400		
	Lu ^b -0.9600		
Kell	K-0.0450		
	k-0.9550		
Lewis	Le(a b-)-0.2238		
	Le(a-b)-0.7161		
	Le(a-b-)-0.0601		
Duffy	Fy ^a -0.4143		
	Fy ^b -0.5857		
Kidd	Jk ^a -0.4982		
	Jk ^b -0.5018		

If for example, the D factor of the Rh-system is not taken into account in the compatibility test, the incidence, in all transfusions given, of D-negative people getting D-positive blood may be calculated thus:

(Frequency/

(Frequency of dd) x frequency of gene D, or $(0.41)^2 \times 0.59 = 10\%$ (approximately).

If iso-immunisation would occur in every case of incompatible transfusion, its incidence due to various factors could be calculated in a similar way and it would be approximately as follows:

C-13%, κ -11%, c-10%, d-14%, e-2.5%, K-4.5%,
k-0.2%, Fy^a -14%, Fy^b -10%, M-12%, N-13%, S-15%,
s-7%, Lu^a -4%, Lu^b -0.2%, Jk^a -12.5%, Jk^b -12.5%.

The above calculated values of the incidence of iso-immunisation are based on the transfusion from a single donor and the incidence will be proportionately high with the number of donors used for each recipient.

Observed Incidence

In the present study an attempt was made to detect all possible cases of immunisation due to C,c,D,d, κ ,e,M,N,S,s,P,K,k, Fy^a , Le^a and Le^b blood factors and therefore test cells containing all these antigens were used during the whole investigation. In the limited series of 302 post-transfusion samples, one example each of anti-c, -e, -K, and anti- Le^b and two of anti- Fy^a have been found and none of these antibodies could be detected in the pre-transfusion samples of the recipients.

If it is assumed that all the blood factors are/

are equally antigenic and using the established fact that 50% of the D-negative individuals become sensitised (thus 50% people being susceptible) one could expect that sensitization due to other blood factors would occur in approximately 50% of the calculated values (this will be more if the patient is transfused with blood from more than one donor, and the risk due to pregnancy is considered). However, the observed incidence is far below the figure that could be theoretically expected. This difference between the observed and expected incidence can not be explained by the weakness of the antigens, because in susceptible persons a single transfusion may produce antibodies against so-called 'weak' antigens. This fact has been observed in the present study; two patients (Cases 3 and 152) developed anti-K and anti-c respectively due to the present transfusion and neither of them had any previous history, suggestive of immunisation. On the other hand, there were three cases of aplastic anaemia who had multiple transfusions (an 'ideal condition' for the development of antibodies) but none of them produced any antibody.

Thus it appears that some other factors may possibly be responsible for these discrepancies between the results of expected and observed incidence/

incidence of iso-immunisation.

These factors are:

(1) Those suggested by various workers e.g.

(a) Hereditary (Hirszfeld, 1926) or

K-factor (Wiener, 1946b).

(b) Nutritional (Cannon et al. 1943).

(c) Hormonal and nutritional (Unger
and Wiener, 1945).

(2) That appropriate administration of an

antigen in embryonic life results in an

acquired specific tolerance to that

antigen so that in later life the

individual will not produce antibody to

it. In favour of this view are the facts
that -

(a) Buxton (1954), and Horowitz and Owen
(1954) were able to induce specific tolerance to
antigen by injecting it in animals at a particular
stage of embryonic life.

(b) In relation to the question of why
only some of the women of Rh-negative type who
produce Rh-positive offspring are immunised by the
D antigen, Owen et al. (1954) in an examination of
serological data found that the Rh status of the
woman's mother was significant. Rh-negative
women who were married to Rh-positive husbands
and had experienced three pregnancies were divided
into/

into those who had Rh-positive and Rh-negative mothers. Amongst 29 who had Rh-positive mothers, 25 had not produced antibody (86%), while 22 with Rh-negative mothers only 9 had failed to produce antibody (41%). The difference is significant and suggests that many of the first group had been conditioned to tolerate D antigen by exposure to it in utero.

(3) That the results of the present study may not be a true representation of the incidence of iso-immunisation as a result of transfusion because,

(a) The time of appearance of immune antibodies is variable and in some patients there may be a considerable delay before agglutinins become evident. As a general rule, antibodies develop during the second week following the antigenic stimuli, but Pickles (cited by Mollison, 1956) observed that the best time for examination of the sera for the presence of antibody was 20 weeks after transfusion. However in approximately half of the cases in the present series, post-transfusion samples were examined for the second time on the fourth week following transfusion but no antibody was detected which could not be found in the earlier examination of the post-transfusion samples (on the second week).

(b)/

(b) It is doubtful whether all the anti-bodies can be detected by methods used at present. In three cases, symptoms and signs, suggestive of haemolytic reactions developed following blood transfusion during the present investigation but no antibody could be detected in the sera of two of these cases even on repeated examinations. However, the post-transfusion samples in these cases could not be tested against fresh donors' cells and that may be the cause of failure in detection of any antibody (see part II).

The third case (no. 281, Table III) developing a haemolytic reaction, had a transfusion of blood three years back and although no antibody could be detected in her pre-transfusion serum (in relation to the present transfusion) it is obvious that the antibody developed as a result of previous transfusion (in view of the haemolytic reaction due to present transfusion) and the antibody being in a "latent state" could not be detected by the methods used.

Although the incidence of iso-immunisation (with regard to blood factors that could be included in the test panel) in the present series is much less than what could be theoretically expected, it is higher than most of the results obtained by different workers during routine blood bank/

bank work dealing with investigations on transfusion reaction and cross matching difficulties.

However, in an investigation similar to the present one, Callender and Paykoc(1946) examined the post-transfusion samples 10 to 14 days following transfusion in 100 patients and found that two persons were sensitized. One of them was Rh-negative and was immunised against D factor while the other was Rh-positive and was immunised against several blood factors. This is an incidence of 1% immunisation against blood factors other than D, but this would only be found if the blood samples are examined in each case after routine transfusion. These authors could not apply Coombs' test in their investigation, and in that case it may be expected that the incidence would be higher.

Kilduffe and DeBaakey(1942) compiled the results of 43,284 blood transfusions by 18 different groups of workers. There were 1.8 haemolytic reactions in every 1000 transfusions. Speiser and Kolbl(1952) found atypical antibodies (including anti-D, -CD, -ED) in the sera of 132 individuals resulting mainly from transfusion during 1948-51 (the number of cases investigated not mentioned). Kellner(1955) detected 76 cases of iso-immunisation due to various blood factors in/
in/

in a series of 14,755 consecutive blood transfusions. More recently, Wallace(1956) reported 13 immune antibodies specific for antigens other than A, B and Rh(D) in a series of 13,000 recipients.

Most of these results cannot be compared with that of the present study because (1) the incidence of haemolytic transfusion reaction is not the same as that of iso-immunisation, since every case of immunisation may not necessarily give rise to transfusion reaction. (2) in some of the series, the samples were partially selected and included cases of sensitization due to pregnancy. However, if these results are analysed and only the blood factors against which antibodies were expected to be found in the post-transfusion samples of the present series (i.e. blood factors contained in the standard cells excluding the D antigen) are taken into account, the incidence of iso-immunisation will be 0.1 to 0.2 per cent. On this basis, in the present material of 302 post-transfusion samples, six cases of iso-immunisation were detected - an incidence of 2% - which is significantly higher than those reported above, and this observation indicates that much higher incidence would be obtained if post-transfusion samples are examined systematically rather than in response to a specific indication (e.g. reaction/

reaction or technical difficulty during compatibility testing).

Of these six cases, case 14 (Table III), whose serum contained anti-e, suffered from autoimmune haemolytic anaemia while in the remaining five, sensitisation occurred as a result of transfusions. In two of the latter cases (Nos. 3 and 152) the present stimulation appeared to be the sole cause of immunisation because, one of them a male patient, had never been transfused and the other a female patient, who developed anti-K antibody, had no history of previous transfusion and although she was parous, her husband was Kell-negative. The remaining three cases (Nos. 211, 267, and 281) had previous transfusions which may have produced susceptibility to immunisation without producing detectable antibodies (which may be the probable explanation of not finding any antibody in their pre-transfusion samples).

The relation of immunisation in these six cases to previous transfusions and/or pregnancy are summarised in the following table.

	No history of previous stimulation	Previous history of transfusion alone	History of pregnancy alone	History of trans-fusion and pregnancy	Total
Number of pre- and post-trans-fusion samples examined	133	42	111	16	302
Number of cases in which immune antibodies were detected for the first time	2	2 (1)	0	1	6

The figure in parenthesis indicates case 14.
(auto-immunisation)

It should be noted that these six cases do not include case 271., who developed anti-D as a result of pregnancy. This case is discussed under anamnestic reaction (see later).

If the occurrence of iso-immunisation is compared among the patients with a previous history of pregnancy and/or transfusions and among those without such history, it will be found that three out of 169 of the former group (1.8%) and two out of 133 of the latter group (1.5%) were immunised. This difference is not of much significance and it is of interest to note that the majority of the former group consisted of women with history of pregnancy alone. If however, one compares the incidence among those who had previous transfusion and those who had not, it will be found that three (5%) (excluding case 14) of the 58 previously transfused patients (including 16 who had pregnancy also) and two (0.8%) of the 244 patients who had no history of transfusion were immunised. These results are shown in the following table.

	Cases with no history of previous transfusion	Cases with history of previous transfusion
Total number of cases	244	58
Number of cases immunised	2	3
Percentage of immunisation	0.8%	5%

Such/

Such a difference is highly significant and confirms the previously established fact that the incidence of immunisation will be much higher among the previously transfused patients than among those who had never been transfused. This observation in particular, is of importance because the number of hospital-admission of patients for whom blood is requisitioned more than once, is constantly increasing - a fact which is emphasised by the present observation that 58 (approximately 20%) of the total 302 patients, actually transfused, had received blood at a previous admission.

The above findings also support the observation of Mollison (MRC Memo., No.27) that transfusion is a better stimulus than pregnancy.

Although it was noticed that iso-immunisation occurs more commonly among previously transfused patients, it is of interest that the present study does not confirm the common belief that patients receiving multiple spaced transfusions are more prone to be immunised. Of the 58 previously transfused patients six had only one previous transfusion and four of them were sensitized (see Fig.2) and contrary to the expectation, no antibody could be detected in the sera of the remaining 52 patients although all of them had more than one transfusion and several had multiple (more/

(more than three) spaced transfusions. This observation suggests that individual susceptibility is more important than the multiplicity and spacing of transfusions in determining the incidence of iso-immunisation in case of routine blood transfusion in hospital practice.

Since it is not practicable to screen all the antigens involved in causing iso-immunisation nor to determine the individual susceptibility at present, it may be concluded in view of the above findings that -

(1) blood transfusion should be practiced only when properly indicated, keeping in mind that the chance of immunisation is greater in previously transfused patients; (2) consideration should be given to the advisability of introducing a routine screening for antibodies (a) after the second transfusion in patients who are likely to require blood in future, (b) after transfusion in women who are likely to have further pregnancies.

Anamnestic Reaction

A non-specific rise was observed in two cases (Nos. 267 and 271) where the anti-D titres increased following the transfusion of D-negative blood. Opinion varies as to the effect of non-specific stimuli on the titre of antibodies.

van Loghem(1948) used triplo and tetra-vaccines as/

as non-specific stimuli and considered that suitable volunteers for immunisation to Rh could be selected by choosing those who gave clinical reactions to preliminary injection of vaccine. Wiener(1951b) on the other hand, was unable to confirm the phenomenon of anamnestic rise of antibody titre, as in eleven cases of previously immunised Rh-negative mothers he could not find any rise of antibody(anti-D) titre as a result of pregnancy with an Rh-negative foetus. Cochrane and his co-workers(1950) however, were able to demonstrate the rise in anti-Kell antibody in a mother following the birth of a Kell-negative baby, the mother was previously sensitized by transfusion of Kell-positive blood.

It seems from the above findings that in some cases at least, there may be a rise in antibody titre by non-specific stimuli and this point should be taken into consideration in all cases of sensitized pregnant women requiring transfusion, since the non-specific rise in the antibody titre even after compatible blood transfusion may precipitate or aggravate the haemolytic condition in the foetus.

Persistence of antibody

Once developed, anti-D antibodies persist in the circulation for very long periods; for example, Stratton(1955) reported a case in which anti-D/

anti-D was found in a woman 38 years after her last pregnancy. In the present series, anti-D was detected in the sera of a man (Case 867) after 7 years following the transfusion of 2 bottles of blood. The persistence of antibodies is of importance because, if an antibody other than anti-D develops it may also remain in the circulation for some considerable time and the use of Rh-negative group O blood without cross matching test (as sometimes done in case of extreme emergency) may not be safe.

'Dosage effect'

The serum of case 14, containing anti-e antibody showed 'dosage effect'. Such effects have also been reported in cases of, anti-M by Landsteiner and Levine(1928), anti-E by Malone and Dunsford(1951), anti-S by Levine et al.(1952) and anti-Fy^a by Race and Sanger(1954). These observations suggest that care should be taken in selecting test cell panels, since some of the antisera, particularly with low titres may give rise to false negative results with heterozygous cells (in relation to the antigen concerned).

Specific irregular cold agglutinins

From the results of present investigations as well as from those of other workers, it seems that anti-Lewis (particularly anti-Le^a) and anti-P antibodies/

antibodies are more common findings in the human sera than anti-M and anti-N. In the present series of 712 samples no anti-M or anti-N was detected while anti-Lewis antibodies were found in approximately 1.6% of the cases examined - an incidence higher than previously reported results. Kissmeyer-Nielsen and his co-workers(1955) found 58 anti-Lewis antibodies in a series of 17,000 cases - an incidence of 1 in 300. The frequency of anti-Lewis antibodies in the present material may be caused by combination of two factors:

(1) antigenic structure of test cells - Le(a+) and Le(b+) cells having been used during the whole investigation, (2) and testing all the sera at room temperature. (In Kissmeyer-Nielsen and his co-workers' series 1,200 samples were tested at room temperature). Anti-Le^a is far commoner than anti-Le^b as noted by Pettenkofer and Hoffbauer (1954) and this has also been observed in the present series that the proportion of these two antibodies was 8:2.

Although some of these patients with anti-Lewis antibodies in their sera had histories suggestive of previous immunisation, it seemed from the nature of reaction that all the antibodies (with the exception of immune anti-Le^b which developed in the sera of case 211. following/

following the present transfusion) were of spontaneous origin. Only one of these antibodies caused haemolysis of specific red cells on trypsin treatment, a finding at variance with that of Rosenfield and Vogel(1951); the low potency of most of the anti-Lewis antibodies in the recent series may be the cause of difference between these findings. It is a common experience that anti-A and anti-B titres rise after ABO incompatible transfusion and one could therefore expect that such a rise would occur in cases of incompatible transfusions in relation to Lewis blood groups. On the contrary, none of the six patients with anti-Lewis antibodies in their sera who received Lewis incompatible blood showed any rise in antibody titre - which indicates that the titres of all types of naturally occurring antibodies do not rise following specific stimulation. It was also noted that none of these patients suffered from any reaction following the transfusion.

Anti-P antibodies were detected in the sera of only two patients - a low incidence since, Henningsen(1949) found that if anti-P is looked for with a suitably sensitive technique, anti-P antibody could be detected in the sera of P-negative persons with a regularity almost approaching that of agglutinins of the ABO system. However/

However, no special sensitive technique (as recommended by Henningsen) was used to detect anti-P, but it can be stated from the results of the present observation that anti-Lewis antibodies are more readily detected than anti-P.

Although the naturally occurring anti-Lewis and anti-P antibodies are not clinically important, they may give rise to confusion in cross matching tests and this point should always be considered in carrying out these tests particularly when they are done at room temperature.

Non-specific cold agglutinins

Non-specific cold agglutinins were found in about 79% of the pre-transfusion samples. The majority of the patients with cold agglutinins in their sera received blood transfusions during the present investigation but only one case, a case of haemolytic anaemia, (Case 14.) showed any rise of cold agglutinin titre in the post-transfusion sample. No correlation between the presence of low titre non-specific cold agglutinin and disease, age or sex of the patient or previous history suggestive of immunisation was observed. However, it was noted that the cold agglutinin titres in group A, B, and AB persons were comparatively higher than those in group O persons - a similar observation made by Mollison(1956).

Relative/

Relative merits of different methods used for
detection of incomplete antibodies

Of the methods used for the detection of incomplete antibodies the albumin method was found to be least sensitive. Moreover, it failed to detect antibodies not only of the Rh system (anti-c, Case 152) but also anti-Kell (Case 3) and anti-Fy^a (Cases 267 and 281).

Trypsin-treated cells are said to be very suitable as far as the antibodies of the Rh system are concerned and Rosenfield and Vogel(1951) and Wiener and Katz(1951) reported that they did not observe an instance of "Rh-Hr sensitization" that had not been detectable by trypsin-treated cells. However, it was rather unusual that an anti-e antibody (Case 14) in the present series could not be detected by trypsin-treated cells and it seems possible that on very rare occasions trypsin-treated cells may fail to detect some of the antibodies of the Rh system.

The importance of the indirect Coombs' test for the detection of incomplete antibodies is well established and this has been demonstrated in the present series that anti-K (Case 3), anti-c (Case 152) and anti-Fy^a (Cases 267 and 281) could not have been detected if the indirect Coombs' technique had not been used. It should be noted in/

in this connection that the dilution of anti-human globulin sera which is optimum for the detection of Rh antibodies may not necessarily be always the same as for the other blood group antibodies (c.f. Case 281) and it is therefore important to put up controls with other blood group factors (e.g. Kell, Duffy) in addition to D factor in carrying out Coombs' technique.

Although the results of the simplified papain method compared favourably with those by indirect Coombs' technique, it is difficult to draw any conclusion from the results of the present series (as well as from those of Tribedi and Crosbie's series in which the same batch of papain was used) because one batch of papain was used during this investigation and it is known that the activity of different batches of papain may vary. However, since the papain method used in this investigation is simple, sensitive and less time-consuming, further trial with different batches of papain may be carried out by other workers to confirm the results of the present findings.

In view of the above observations, it may be concluded that until the perfection of some simple method (e.g. Papain method), Coombs' test must be used to detect incomplete blood group antibodies if sensitizations due to various blood factors are to be detected.

SUMMARY/

SUMMARY

An investigation has been carried out to observe the incidence of iso-immunisation as a result of routine blood transfusion. By the use of various methods and standard cells containing most of the clinically important blood factors, an attempt has been made to detect as many as possible of the cases of blood group sensitivity resulting from transfusions covered by cross matching tests at present in use.

To establish that an antibody detected in post-transfusion sample, was not present in the patient's serum prior to the present transfusion, a pre-transfusion sample of every prospective recipient was screened.

In examining the sera of 712 such recipients, non-specific cold agglutinins were detected in 79% of cases. Eleven patients showed anti-Lewis and two anti-P antibodies in their sera - all of which seemed to be naturally occurring. Some of the patients with anti-Lewis antibody in their sera received Lewis incompatible blood transfusions during the present observation but there was no reaction or rise in anti-Lewis titre in any of these cases following transfusions.

The significance of these antibodies in carrying out cross matching tests has been stressed.

Six/

Six of the 302 patients who received blood transfusions during the present investigation developed immune antibodies of different specificities - an incidence of approximately 2% which is much higher than the published figures obtained during the course of routine blood bank work dealing with the investigations of transfusion reactions and cross matching difficulties.

As expected, it was found that the incidence of immunisation among previously transfused patients is much higher than among those who had no previous transfusion. It was also noted that individual susceptibility is more important than the number and spacing of transfusions in determining the incidence of iso-immunisation.

Importance of these findings in relation to the increasing number of "repeat" cases in routine transfusions in hospital practice has been discussed.

An observation in the present study that transfusion is a better stimulus than pregnancy supports the similar finding of previous workers.

It has also been found that every case of blood group sensitivity cannot be detected by the methods used at present.

In two cases an anamnestic rise in antibody titre was observed - a fact which may be of importance/

importance in the transfusion of pregnant women. The relative merits of different methods for the detection of incomplete antibodies have been compared. It was noted that the optimum dilution of the Coombs' serum for the detection of different incomplete antibodies may vary.

A simple papain method which has been found to give satisfactory results, comparable to those by Coombs' test, is described.

BIBLIOGRAPHY.

- Agote, L. (1915). An. Inst. modelo de clín, méd., 1; 25
- Allen, F.H., Diamond, L.K., and Niedziela, B. (1951). Nature Lond., 163; 482
- Andresen, P.H. (1935). Ztschr. f. Immun. u. exper. Therap., 85; 227
- Andresen, P.H. (1948). Acta path. microbiol. scand., 25; 728
- Barnes, R. (1874). Lancet, 1; 6
- Bernheim, B. (1909). Ann. Surg., 50; 786
- Blundell, J. (1818). Med. Chir. Tr. Lond., 9; 56
- Boorman, Kathleen E., Dodd, Barbara E and Mollison, P.L. (1945). J. Path.
Bact., 57; 157
- Bowley, C.G. (1955). Proc. Fifth Int. Cong. Haem., P 65
- Buxton, A. (1954). J. gen. Microbiol. 10; 398
- Callender, Sheila T., and Paykoc, Z.V. (1946), Brit. med. J., 1; 119
- Callender, Sheila T., and Race, R.R. (1946). Ann. Eugen, Lond., 13; 102
- Cannon, P.R., Chase, W.E. and Wissler, R.W. (1943) J. Immunol., 47; 133
- Carrel, A. (1907). Bull. Johns Hopk., Hosp., 18; 18
- Cepellini, R., Ikin, Elizabeth W., and Mourant, A.E. (1950). Boll. Ist Siero
Milanese, 29; 123
- Cochrane, J.B., Malone, R.H., and Dunsford, I. (1950). Brit. med. J. ii 1203
- Coombs, R.R.A., Mourant, A.E., and Race, R.R. (1945). Brit. J. exp. Path.,
26; 255
- Coombs, R.R.A., Mourant, A.E., and Race, R.R. (1946). Lancet, 1; 264
- Crome, W. (1935). Dtsch. Ztschr. gerichtl. Med. 21; 167
- Culbertson, C.G., and Ratcliffe, A.W. (1936). Amer. J. med. Sci., 192; 471
- Cutbush, Marie, Mollison, P.L., and Parkin, Dorothy M. (1950). Nature, Lond.,
165; 188

- Darrow, Ruth (1938). Arch. Path., 25; 376
- Decastello, A. v., and Sturli, A. (1902). Munchen. med Wehnschr., P 1090
- DeGwin, E.L. (1945). J. Lab. clin. Med., 30; 99
- DeGwin, E.L., Hardin, R.C., and Alsever, J.B. (1949). Blood Transfusion,
1st. Ed., W.B. Saunders Co. Philadelphia and London
- Denis, J.B. (1667). Philosoph. Trans. Royal Soc., Lond., 2; 453
- Dienst, A. (1905), Zbl. Gynak., 29; 353 and 651
- Diamond, L.K. (1944a) Progress report to Committee on Medical Research of
the Office of Scientific Research and Development.
OEM. cmr. 384. Jan. 1st.
- Diamond, L.K. (1944b) Progress report to Committee on Medical Research of
the Office of Scientific Research and Development.
OEM. cmr. 384. Nov. 1st.
- Diamond, L.K., (1946) Paper read at Rh and Haematology Congress, Dallas,
Cited by Race and Sanger (1954)
- Diamond, L.K., and Denton, R.I. (1945). J. Lab. clin. Med. 30; 821
- Dungern, E. V., and Hirszfeld, L. (1911) Z. Immunforsch., 8; 526
- Dunsford, I., Ikin, Elizabeth W., and Mourant, A.E. (1953). Nature, Lond.,
172; 688
- Ehrlich, P., and Morgenroth, J. (1900). Berlin klin. Wochschr. 453
- Fisher, R.A., cited by Race R.R. (1944). Nature, Lond. 153; 771
- Friedenreich V., and Lauridsen, A. (1938). Acta. path. microbiol. scand.,
supp. 38; 155
- Gilbey, B.E. (1950). Brit. J. exp. Path. 31; 695

- Gold, E., and Turcanu, A. (1945-47). Arch. Roum Path. expér., 1-4; 218
- Gradle, H. (1874). Med. Exam. Chicago, 16; 294
- Greenwalt, T.J., Sasaki, T., Sanger, Ruth, Sneath, J., and Race, R.R. (1954).
Proc. Natl. Acad. Sci. (U.S.) 40; 1126
- Grove-Rasmussen, M., Dreisler, N., and Shaw, R.S. (1954). Cited by Levine
etal (1956).
- Grove-Rasmussen, M., and Levine P (1954). Amer. J. Clin. Path. 24; 145
- Hattersley, P.G. (1947). J. Lab. clin. Med. 32; 423
- Hanningsen, K. (1949). Acta path. scand., 26; 639
- Hill, J.M., and Haberman, S. (1948). Nature, Lond. 161; 688
- Hill, J.M., Haberman, S., and Orozco, A.V. (1945). J. Amer. med. Ass. 128; 944
- Hirszfeld, L. (1926). Erg. d. Hyg., 8; 367
- Horowitz, N.H., and Owen R.D. (1954). Annu. Rev. Physiol., 16; 81
- Hotz, F.C. (1875). Med. Exam. Chicago 16; 20
- Hubinont, P.O. (1950). Rev. Haematol. 5; 441
- Hustin, A. (1914). J. méd. de Brux. 12; 436
- Ikin, Elizabeth, W., Mourant, A.E., Pettenkofer, H.J., and Blumenthal, G. (1951)
Nature, Lond., 168; 1077
- Jones, A.R., Diamond, L.K., and Allen, F.H., jr. (1954). New Engl. J. Med.
250; 283 and 324
- Jorda, F.D., (1939). Lancet, 1; 773
- Kellner, A. (1955). Obstet. & Gynec. 5; 499
- Kilduffe, R.A., and DeBakey, M. (1942). The Blood Bank and the Technique and
Therapeutics of Transfusion, C.V. Mosby Co., St. Louis.

- Kissmeyer-Nielsen, F., Bastrup-Maden, Kristen and Stenderup, A. (1955).
Dan. med. Bull. 2; 202
- Kolmer, J.A. (1924). Quoted from Kilduffe and DeBakey (1942)
- Landsteiner, K. (1901). Wien. klin. Wschr. 14; 1132
- Landsteiner, K. (1900). Zbl. Bakt. 27; 357
- Landsteiner, K., and Levine, P. (1927). Proc. Soc. exp. Biol. N.Y., 24; 600 and 9
- Landsteiner, K., and Levine, P. (1928). J. exp. Med. 47; 757
- Landsteiner, K., Levine, P., and Janes, M.L. (1928) Proc. Soc. exp. Biol.
Med. 25; 672
- Landsteiner, K., and Wiener, A.S. (1937). J. Immunol., 33; 19
- Landsteiner, K., and Wiener, A.S. (1940). Proc. Soc. exp. Biol. N.Y., 43; 223
- Levine, P. (1941)., cited by Claireux, Albert E. (1949) Thesis for the degree
of M.D., Edinburgh Univ.
- Levine, P. (1942)., Proc. N.Y. State A. Pub. Health Lab., 22; 42
- Levine, P., Baker, M., Wigod, M., and Ponder, R. (1949). Science, 109; 464
- Levine, P., Ferraro, L.R., and Koch, E. (1952). Blood 7; 1030
- Levine, P., and Katzin, E.M., (1940). Proc. Soc. exp. Biol. Med. 45; 343
- Levine, P., Katzin, E.M., and Burnham, L. (1941). J. Amer. med. Ass. 116; 825
- Levine, P., Katzin, E.M., Burnham, L., and Vogel, P. (1941). Amer. J. Obst.
Gynec. 42; 925
- Levine, P., Kuhmichel, A.B., Wigod, M., and Koch, E. (1951). Proc. Sci.
exp. Biol. N.Y. 78; 218
- Levine, P., Robinson, E., Stroup, M., McGee, R., and Bushnell, L.N. (1956).
Blood, 11; 1097
- Levine, P., Stetson, R.E. (1939), J. Amer. med. Ass. 113; 126
- Levine, P., Vogel, P., Katzin, E.M., and Burnham, L. (1941). Science, 94; 371
- Lewisohn, R. (1915). Med. Record 87; 141

- Malone, R.H., and Dunsford, I. (1951). *Blood*, 6; 1135
- Mollison, P.L. (1951). *Blood Transfusion in Clinical Medicine*, 1st edn.
Blackwell Scientific Publications Ltd., Oxford
- Mollison, P.L. (1956). *Blood Transfusion in Clinical Medicine*, 2nd edn.
Blackwell Scientific Publications Ltd., Oxford
- Mollison, P.L., and Young, I.M. (1941). *Lancet*, ii; 635
- Morton, J.A., and Pickles, M.M. (1947). *Nature*, Lond. 159; 779
- Mourant, A.E. (1945). *Nature*, Lond. 155; 542
- Mourant, A.E. (1946). *Nature*, Lond. 158; 237
- Mourant, A.E. (1957). *Brit. med. J.* August, 24, P461
- Neter, E. (1936). *J. Immunol.*, 30; 255
- Ottenberg, R. (1911). *J. exp. Med.* 13; 425
- Owen, R.D., Wood, H.R., Foord, A.G. Sturgeon, P., and Baldwin, L.G. (1954).
Proc. Nat. Acad. Sci., Wash. 40; 420
- Patton, P.B. (1938). *Amer. J. clin. Path. Techn. Suppl.*, 8; 178
- Pettenkofer, H.J., and Hoffbauer, H. (1954). *Zbl. Gynak.* 15; 576
- Pickles, M.M. (1946). *Nature*, Lond. 158; 880
- Plaut, G., Ikin, E.W., Mourant, A.E., Sanger, Ruth and Race, R.R. (1953).
Nature, Lond. 171; 431
- Race, R.R. (1944). *Nature*, Lond., 153; 771
- Race, R.R., Mourant, A.E., Lawler, S.D., and Sanger, R. (1948). *Blood*, 3; 689
- Race, R.R., and Taylor, G.L., Boorman, K.E., and Dodd, B.E. (1943). *Nature*
Lond., 152; 563

- Race, R.R., and Taylor, G.L. (1943). *Nature, Lond.* 152; 300
- Race, R.R., Sanger, Ruth (1954). *Blood Groups in Man*. 2nd edn Blackwell Scientific Publications Ltd. Oxford
- Race, R.R., Sanger, Ruth, and Lawler, S.D. (1948). *Nature, Lond.*, 161; 316
- Robertson, O.H. (1918). *Brit. med. J.* i; 691
- Rosenfield, R.E., and Vogel, P. (1951). *Trans. N.Y. Acad. Sci.*, 13; 213
- Rosenfield, R.E., Vogel, P., Gibbel, N., Sanger, R., and Race, R.R. (1953). *Brit. med. J.* i; 975
- Rous, P., Turner, J.R. (1916). *J. exp. Med.* 23; 219
- Sanger, Ruth, and Race, R.R. (1947). *Nature, Lond.* 160; 505
- Seibert, F.B. (1923). *Amer. J. Physiol.* 67; 90
- Sharpe, D.C. (1939). *J. Iowa M. Soc.* 29; 489
- Sittel, T. (1874). *Clin. Cincin.* 7; 157
- Speiser, P., and Kolbl, H. (1952). *Wien klin. Wchnschr.* 64; 133
- Stratton, F., (1946). *Nature, Lond.*, 158; 25
- Stratton, F. (1953). *Vox Sanguinis*, 3; 43
- Stratton, F. (1955). *Brit. med. J.*, i; 201
- Stratton, F., and Renton, P.H. (1954). *Brit. med. J.*, i; 962
- Thalhimer, W. (1921). *J. Amer. med Ass.* 76; 1745
- Unger, L.J. (1921). *J. Amer. med. Ass.* 76; 9
- Unger, L.J. (1946). *J. Lab. clin. Med.*, 31; 793
- Unger, L.J., and Wiener, A.S. (1945). *Amer. J. clin. Path.*, 15; 280
- Van Loghem, J.J. (1947). *Brit. med. J.*, ii; 958

- Van Loghem, J.J. (1948). Brit. med. J., ii; 326
- Van Loghem, J J., Harkink, H., and van der Hart, Mia (1953). Vox Sang. 3; 22
- Vogel, P. (1954). Bull N.Y. Acad. Med., 30; 657
- Wallace, J. (1950). Thesis for the degree of M.D. Edinburgh Univ.
- Wallace, J. (1956). Vox Sang., 1, 216
- Weil, R. (1915). J. Amer. med Ass., 64; 425
- Wiener, A.S. (1941). Arch. Path., 32; 227
- Wiener, A.S. (1944a). Amer. J. clin. Path. 14; 52
- Wiener, A.S. (1944b). Proc. Soc. exp. Biol. N.Y., 56; 173
- Wiener, A.S. (1945a). Amer. J. clin Path., 15; 106
- Wiener, A.S. (1945b). J. Lab. clin Med., 30; 957
- Wiener, A.S. (1946a). Amer. J. clin. Path., 16; 477
- Wiener, A.S. (1946b). Amer. J. Dis. Child, 71; 14
- Wiener, A.S. (1949). Proc. Soc. exp. Biol. N.Y., 70; 576
- Wiener, A.S., (1950). Letter to the Editor, Blood, 5; 693
- Wiener, A.S. (1951a). J. Immunol., 66; 287
- Wiener, A.S. (1951b). Blood, 6; 522
- Wiener, A.S. (1954). N.Y. State J. Med. 54; 3071
- Wiener, A.S., and Katz. L. (1951). J. Immunol. 66; 51
- Wiener, A.S., and Peters, H.R. (1940). Ann. Intern. Med. 13; 2306
- Winants, J.E. (1872). Amer. J. med. Sci. 63; 108
- Yudin, S.S. (1936). J. Amer. med. Ass., 106; 997
- Zacho, A. (1936). Ztschr. f. Rassenphysiol. 8; 1

II. STUDIES ON THE AGGLUTINABILITY OF
STORED RED BLOOD CELLS.

C O N T E N T S.

<u>INTRODUCTION</u>	1.
<u>MATERIALS AND METHODS</u>	7.
<u>RESULTS</u>	12.
<u>DISCUSSION</u>	28.
<u>SUMMARY</u>	34.

Introduction.

With the discovery of various blood group systems, a test cell panel is becoming an increasingly important necessity in the research laboratories dealing with blood group serology. A test cell panel is also an important tool of the equipment required in routine blood bank work to solve the increasingly frequent problems of transfusions in patients with blood group sensitivity. As erythrocytes lose their agglutinating property with storage, it has been necessary to draw fresh blood samples from the donors at frequent intervals to maintain such a cell panel.

The recent discovery of a method of preserving cells by freezing in presence of glycerol has been of great value, as it has permitted laboratory storage of blood group antigens for prolonged periods. Preservation of cells in frozen condition followed the discovery of Polge, Smith and Parkes(1949), that spermatozoa survived freezing for long periods if treated with glycerol. This discovery was extended by Smith(1950) who observed that in rabbit and human blood diluted with equal parts of 30% glycerol-saline, and frozen at $-79^{\circ}\text{C}.$, the red cells did not undergo the haemolysis usually/

usually caused by freezing and thawing. Human red cells so treated can be freed from glycerol and recovered in apparent normal condition in yields of 80 to 90% of their original number (Sloviter, 1951). Mollison and Sloviter (1951) first used this method for transfusion studies in man and they found no significant effect on the post-transfusion survival of red cells stored at -79°C . for two to three hours. Subsequent work on the storage of red cells in the frozen state was reviewed by Mollison, Sloviter and Chaplin (1952) who described the results of experiments in which red cells were stored at -15°C . or -79°C . for six months and then used for transfusion. Krijnen and his collaborators (1952) using this method observed the effects of such freezing at -79°C . on the agglutinability of the red cell antigens (A, B, M, N, S, C, D, E, c, Le^{a} , Le^{b} , Fy^{a}) and found no change during a storage period of four months. The method has since been elaborated and rapidly developed by several workers and it has now reached the stage of considerable practical use for preserving red cell samples. Chaplin, Cutbush, Crawford and Mollison (1954) preserved red cells in glycerol-citrate mixture at -20°C . and observed that the red cell antigens (A, B, D, c, E, M, N, S, Le^{a} , Le^{b} , Lu^{a} , Fy^{a} , K) reacted specifically,

specifically with blood group sera even after a year although the reactions were only slightly diminished. Grove-Rasmussen et al.(1953) used both glycerol-citrate and glycerol-lactate solutions as advocated by Brown and Hardin(1953) and preserved the cells at -16°C . to -18°C . and found no change in agglutinability in a wide range of blood group antigens during a 20 week storage period.

Thus it is well established that the red cells frozen in glycerol maintain their specific agglutination reaction (with respect to almost all the blood group antigens) for prolonged periods. In the present method therefore, glycerol-treated frozen cells were used as controls.

While all the workers agree on the stability of specific agglutination reaction of the red cells stored in frozen condition, opinions vary to such effect on their storage in citrate solution at 4° - 6°C .

Hattersley and Fawcett(1948) used ACD and Alsever's solutions and found them suitable for preserving whole blood of various antigenic types for approximately seven weeks. Wall and his associates(1950) used modified Alsever's solution and they did not find any decrease in agglutinability/

agglutinability (with the exception of A₂ cells) within two months. Simmons and his co-workers(1951) used a glucose-citrate solution in distilled water (modified Rous-Turner solution) in their anthropological studies and were able to preserve agglutinability in their blood samples for more than six months. Nijenhuis(1953) on the other hand, used Simmons' solution and observed that the agglutinability of the red cells deteriorated after a storage period of only one and a half weeks. Kooptzoff(1954) stored blood in four different fluids at 4° -6°C. and examined them at three day intervals up to 21 days and found that agglutinability of all the red cell antigens (A, B and D) decreased on storage in all the solutions studied.

The above results are summarised in the following Table/

Authors	Preserving fluids	Antigens studied	Agglutinability of stored antigens
Hattersley and Fawcett(1948)	ACD and Alsever's solutions	A, B, D	Maintained up to seven weeks
Wall and his associates(1950)	Modified Alsever's solution	A ₁ , A ₂ , B, D	Maintained up to two months (except A ₂)
Simmons and his co-workers(1951)	Modified Rous-Turner solution	A ₁ ,A ₂ ,B,M,N,S, Lea,Le ^b ,D,C,E, c,e	Maintained up to six months
Nijenhuis: (1953)	Modified Rous-Turner solution	A ₁ ,B,C,D,E,c, M,N,S,P,K,Fy ^a	Decreased after one and half week
Koepzoff: (1954)	<ol style="list-style-type: none"> 1. ACD solution 2. Rous-Turner solution 3. ACD solution containing penicillin and streptomycin 4. A mixture of sodium citrate, penicillin and streptomycin in solid state 	A, B, D	Decreased within 21 days in all the solutions used

Crawford, Cutbush and Mollison(1954) compared the agglutinability of red cells from clotted samples, and those preserved in ACD solution, and frozen in glycerol at -20°C., and in view of their findings, recommended that if red cells are to be kept for more than two weeks, it is best to preserve them in frozen condition at -20°C. in presence of glycerol.

The/

The question of stability of agglutination reaction of red cells preserved in ACD solution is of importance because, blood samples stored in such solution are often used for grouping and cross matching tests. Moreover when old cells stored in ACD solution are used for the detection of cases of blood group sensitivity, antibodies may be missed - a probable explanation of the cause of failure to detect antibodies in some cases of apparent haemolytic reactions (see Part I).

Under the circumstances, it is worth studying the problem in more detail and investigations were therefore undertaken to observe the loss of agglutinability on storage under blood bank conditions using high titre antibodies against a number of red cell antigens.

MATERIALS AND METHODS/

MATERIALS AND METHODS

BLOOD SAMPLES

First Series:

Blood from donors with previous history of malaria were selected as they are not used for transfusion purposes. 400 ml. of blood was withdrawn in the usual way in MRC bottles each containing 110 ml. of ACD solution (Dextrose - 3 gm., Trisodium citrate - 2.7 gm., Distilled water - 100 ml.) and stored at 4° - 6°C.

Second Series:

In this series specimens were obtained during routine withdrawal of blood from the donors in specially sterilized one ounce vials with strict aseptic precautions. 10 ml. of blood was withdrawn in each vial containing 2.8 ml. of ACD solution. The proportion of blood and ACD solution was therefore the same as in case of withdrawal of blood for transfusion purposes. These samples were also stored at 4° - 6°C.

Glycerol-citrate Mixture:

It was prepared by mixing 4 volumes of glycerol and 6 volumes of 5% trisodium citrate solution.

Methods used for freezing the cells in glycerol-citrate mixture and their recovery afterwards/

afterwards are those of Mollison, described by Race and Sanger(1954).

Freezing of Cells in Glycerol-citrate Mixture:

Donor's blood (First Series), stored at 4° - 6°C. were usually obtained from the Blood Transfusion Service on the third day following collection. On receiving the bottles, viscaps were removed and 20 ml. of blood was withdrawn from each bottle with aseptic precautions with a syringe, by piercing the rubber diaphragm. After replacing the viscap the bottle containing the remaining portion of blood was immediately removed to the cold storage room (4° - 6°C.). 10 ml. of blood from the syringe was placed in each of the two sterile test tubes and centrifuged at 3,000 rpm. for 30 minutes. The supernatant plasma was removed as completely as possible and one ml. of packed cell was placed in each of 8 sterile test tubes. (The remaining portion of the blood was used to find out the initial titre as stated below). To each of eight test tubes containing packed red cells one ml. of glycerol-citrate mixture was added drop by drop with continuous agitation. The tubes were then stoppered and frozen at -20°C.

Recovery of Cells:

Four solutions containing respectively 16%.
8%,/

8%, 4% and 2% glycerol (w/v) in 3% citrate solution were prepared. On every fourth day one tube of blood was thawed by placing it in a water-bath at 37°C. and then centrifuged at 3,000 rpm. for 10 minutes. The supernatant fluid was replaced by a solution (about ten times the volume of the packed cells) containing 16% glycerol in 3% sodium citrate. The solution and red cells were mixed and then immediately centrifuged again, supernatant fluid removed and the whole process repeated with 8%, 4% and 2% glycerol in 3% citrate solution. Finally, the cells were washed with saline.

Titration:

First Series:

On the third day following collection of blood, titration was done on cells left after preparation of glycerol-citrate-cell mixture (initial titre). The cells were washed twice and a 2% suspension was made in saline. Thereafter on every fourth day (up to 35th day) titrations were carried out on thawed and washed cells (from frozen samples) making a final 2% suspension in saline. In parallel to this, similar titrations on cells taken from the original bottle on each occasion were also done.

For/

For this purpose one ml. of blood was withdrawn from each bottle in the same way as described above and centrifuged, supernatant plasma was removed, the cells were washed twice and a 2% suspension was made in saline.

Titration were carried out in tubes using the doubling dilution method. In case of A_1 , A_2 and B antigens the tubes were incubated at room temperature for 30 minutes. For the D agglutino-gen, saline acting anti-D serum was used and the tubes were incubated at 37°C. for two hours. The results were read microscopically and the last tube giving clumps of at least 8 to 12 cells was recorded as the titre (MRC Memo, No.27). To simplify the Tables, titres are expressed in tubes (units) instead of in actual figures and the tube number representing actual titres are as follows:

Tube number	1	2	3	4	5	6	7	8	&c
Serum dilution (titres)	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	

For Kell(K) and Duffy(Fy^a) antigens blood specimens were obtained on the day of withdrawal and the initial titres were done on them on the same day. Thereafter for these two antigens, titres/

titres were done on every alternate day and the indirect Coombs' method was employed, as the anti-Kell and anti-Duffy sera used were of incomplete type.

The same batch of anti-sera, respective of each blood group was used throughout the tests.

Second Series:

Blood samples in this series were preserved only in ACD solution and examined on the day of collection. A second titration was done on the 35th day to observe the deviation from the initial titre. On each occasion one ml. of blood was withdrawn from each vial, washed and a 2% suspension made in saline as described above. The same doubling dilution method was used for titration. In addition to saline anti-D, an incomplete anti-D serum was used: in this case the titration was carried out by papain method.

The batch of saline anti-D sera used for this series was different from that used in the case of the first series.

Every day before titration, the specificity of reaction of each sample of stored red blood cells was checked with control sera. All the anti-sera used in this investigation were specific in reaction.

RESULTS/

RESULTS

Erythrocytes frozen at -20°C . in presence of glycerol-citrate mixture did not show any loss of agglutinating property of the antigens tested up to 35 days. On the other hand, varying degrees of loss of agglutinability were observed on red cells preserved in ACD solution at $4^{\circ} - 6^{\circ}\text{C}$.

A₁ and A₂ antigens

18 group A₁ and three group A₂ bloods were preserved both in ACD solution and glycerol-citrate mixture (GCM). The results of titrations on these bloods against anti-A serum are shown in Tables I and II(a).

Table I/

Table I

Sp. No.	Days of storage									
		3	7	11	15	19	23	27	31	35
1.	ACD	11	11	11	10	10	10	10	9	9
	GCM		11	11	10	10	10	11	10	11
2.	ACD	10	10	10	10	9	10	10	9	8
	GCM		10	10	10	10	10	10	9	10
3.	ACD	12	12	12	11	11	11	10	11	10
	GCM		12	12	12	11	12	11	11	11
4.	ACD	10	10	10	10	9	9	9	9	8
	GCM		10	10	10	11	10	10	9	10
5.	ACD	10	10	10	10	9	9	9	9	8
	GCM		10	10	10	11	10	10	9	10
6.	ACD	11	11	10	10	10	10	9	9	8
	GCM		10	10	10	10	10	9	10	10
7.	ACD	10	10	10	10	9	9	9	9	9
	GCM		10	10	10	10	10	9	10	9
8.	ACD	11	11	11	10	10	10	10	10	8
	GCM		11	12	11	11	11	11	11	11
9.	ACD	10	10	11	9	9	9	8	8	8
	GCM		10	10	10	10	10	10	9	10
10.	ACD	11	11	11	10	10	10	11	9	9
	GCM		11	11	11	11	12	11	11	11
11.	ACD	10	10	9	9	9	10	9	8	8
	GCM		10	10	10	10	10	10	9	10
12.	ACD	10	10	10	10	10	9	8	7	7
	GCM		10	10	10	10	10	10	10	10

Table I/

Table I (Contd.)

Sp.No.		Days of storage								
		3	7	11	15	19	23	27	31	35
13.	ACD	12	12	11	11	10	10	9	9	8
	GCM		12	12	12	12	12	11	12	11
14.	ACD	11	11	10	10	12	10	10	9	9
	GCM		11	11	11	11	11	10	10	11
15.	ACD	11	11	10	10	10	8	8	8	7
	GCM		11	11	11	11	11	11	11	11
16.	ACD	10	10	10	9	9	8	8	7	6
	GCM		10	10	10	11	9	9	9	10
17.	ACD	10	10	9	9	8	8	7	7	7
	GCM		10	10	10	10	10	11	10	10
18.	ACD	10	9	9	9	8	9	8	8	8
	GCM		10	10	10	10	10	10	10	10

Table I. Titres on A₁ cells on different dates against anti-A serum .

Titres are expressed in units(tubes).

ACD and GCM indicate cells preserved in ACD solution at 4° to 6°C., and cells frozen at -20°C. in glycerol-citrate mixture, respectively

Table II (a)/

Sp.no.		Days of Storage								
		3	7	11	15	19	23	27	31	35
1.	ACD	8	7	6	6	5	5	4	4	3
	GCM		8	8	8	8	9	8	8	8
2.	ACD	10	8	7	6	5	4	3	3	2
	GCM		10	9	9	10	10	9	10	10
3.	ACD	8	6	5	5	4	4	3	2	2
	GCM		8	9	8	8	8	9	8	8

Table II(a). The titres on A₂ cells with anti-A sera. See legend Table I.

The deviations of the titres from the controls (initial titres) are shown in Table II(b). A unit of deviation represents one two-fold (1 tube) dilution of anti-sera. One unit deviation is the commonly accepted error of the pipette dilution method. End point titre changes of more than 1 unit thus indicate changes in specific agglutinability of the stored red cells.

Sp.no.		Days of Storage							
		7	11	15	19	23	27	31	35
1.	ACD	-1	-2	-2	-3	-3	-4	-4	-5
	GCM		0	0	0	+1	0	0	0
2.	ACD	-2	-3	-4	-5	-6	-7	-7	-8
	GCM	0	-1	-1	0	0	-1	0	0
3.	ACD	-2	-3	-3	-4	-4	-5	-6	-6
	GCM	0	+1	0	0	0	+1	0	0

Table II(b). Deviations from the controls (initial titres) of the titres on A₂ cells in Table II(a).

None of the 21 group A cells (18 group A₁ and three group A₂) preserved in glycerol-citrate mixture showed a deviation beyond one unit (Fig.1.) which is within the error of the pipette dilution method.

In the case of erythrocytes preserved in ACD solution, all the 18 group A₁ bloods showed only a small loss in agglutinability (maximum loss in titre: 4 tubes), whereas three A₂ bloods showed a rapid and marked loss of agglutinating power as shown in Fig.2.

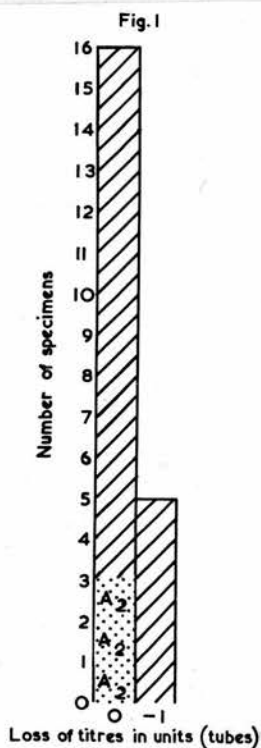


Fig.1. Loss of titres against anti-A serum on 35th day by 18 group A₁ and 3 group A₂ cells frozen in GCM at -20°C.

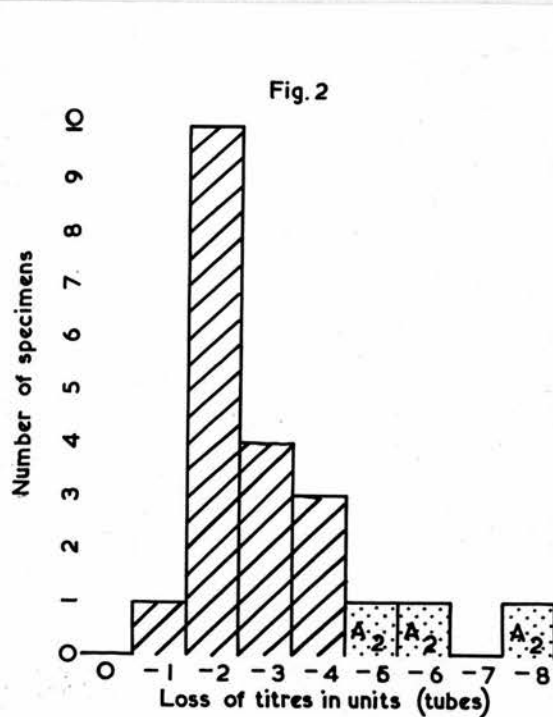
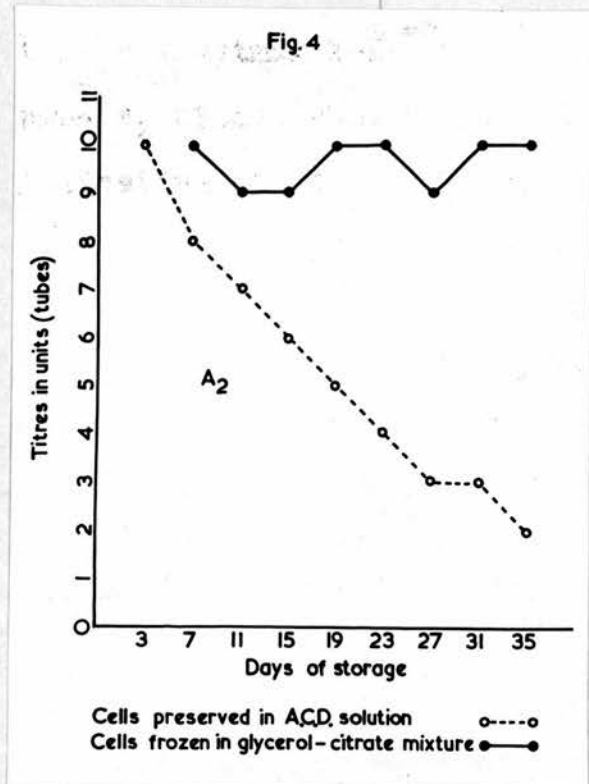
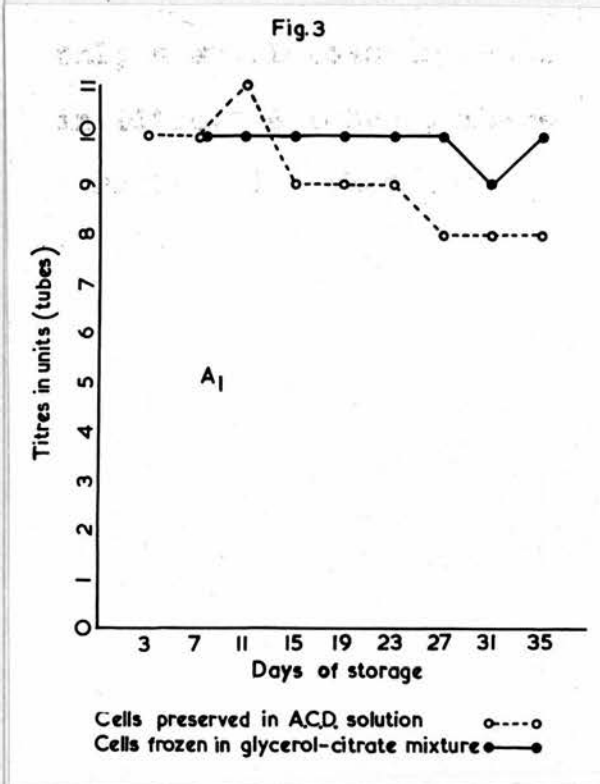


Fig.2. Loss of titres against anti-A serum on 35th day by 18 group A₁ and 3 group A₂ cells frozen in ACD solution at 4° -6°C.

The loss of agglutinability against anti-A serum of A_1 and A_2 cells preserved by two methods has been compared in Figures 3 and 4., one typical example being taken from each group.



Figs. 3 and 4. The difference in loss of agglutinability against anti-A serum of A_1 (Specimen No.9, Table I) and A_2 cells (Specimen No.2, Table II²(a)) preserved in ACD solution and glycerol-citrate mixture.

The loss of titre in case of A_1 antigens was only two tubes on 35th day where as in case of A_2 antigens it was 8 tubes. In glycerol-treated frozen samples there was no loss of titre in either case.

B antigen

Titres on B cells against anti-B serum on different dates are tabulated in Table III. No appreciable loss of titres was observed within the study period in cells frozen in glycerol-citrate mixture. On the other hand all seven group B bloods, preserved in ACD solution, lost their agglutinating property to a certain extent.

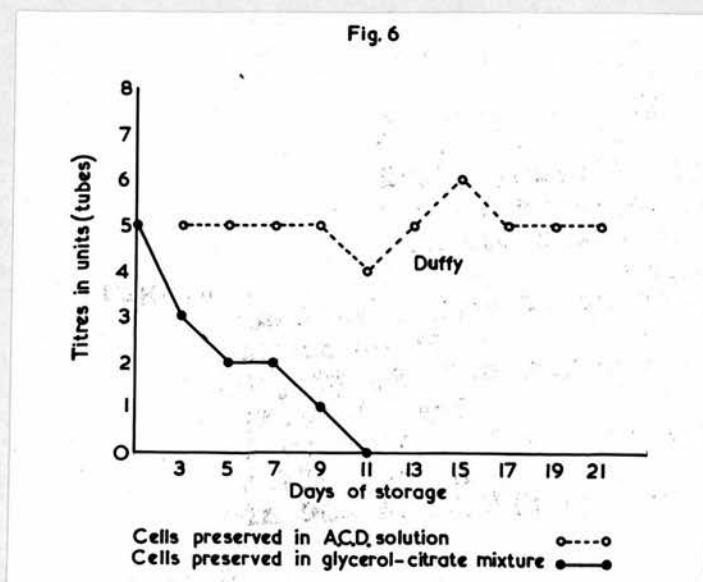
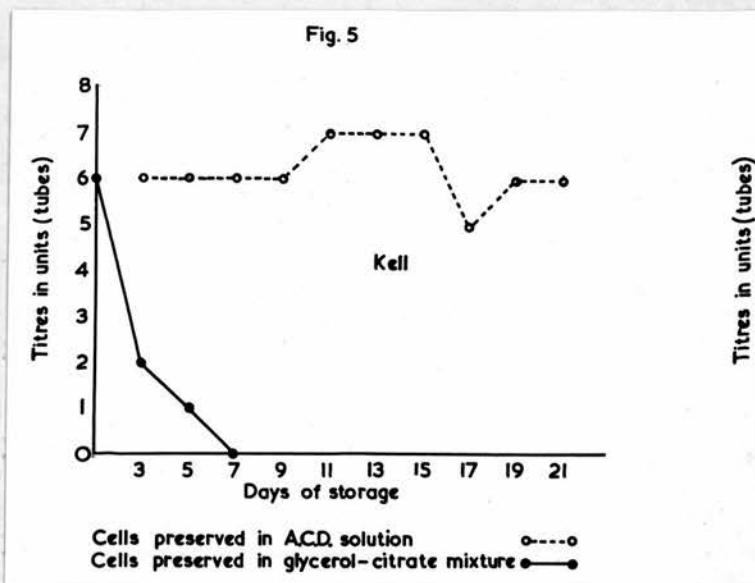
Table III

Sp.no.		Days of storage								
		3	7	11	15	19	23	27	31	35
1.	ACD	10	10	10	9	9	9	9	8	8
	GCM		10	10	10	10	10	10	10	10
2.	ACD	9	8	8	8	8	8	8	7	7
	GCM		9	9	9	9	9	9	9	9
3.	ACD	9	9	9	8	8	8	8	8	7
	GCM		9	9	10	9	9	9	9	9
4.	ACD	8	8	7	7	7	6	6	6	5
	GCM		8	9	8	7	8	8	7	7
5.	ACD	10	10	9	9	9	9	8	8	8
	GCM		10	10	10	11	9	10	11	9
6.	ACD	9	9	8	8	8	8	7	7	7
	GCM		9	8	9	9	9	9	10	9
7.	ACD	10	9	9	9	8	8	8	8	8
	GCM		10	10	10	10	9	10	10	10

Table III. Titres on B cells with anti-B sera. See legend Table I.

Kell and Duffy antigens

Only one sample each for Kell(K) and Duffy(Fy^a) antigens was preserved by the above methods. Neither antigen, preserved in ACD, could be detected on the 7th and 11th day respectively (Figures 5 and 6) although they gave as strong reactions as initial titres even up to 21 days when they were preserved in glycerol-citrate mixture.



Figures 5 and 6, Titres on cells preserved by two methods in cases of Kell (K) and Duffy(Fy^a) antigens.

D antigen

First Series:

The results of titrations with anti-D serum on 19 D-positive bloods preserved by two methods are shown in Table IV. Ten of these samples (nos. 1,2,4,5,8,9,10,11,14 and 19, Table IV) preserved in ACD solution showed marked loss of agglutinability while the remaining 9 bloods were more stable

There appeared to be a distinct division between these two types, suggesting the existence of two varieties of D antigen - a stable and an unstable one. This division into two groups is illustrated in Figure 7.

Table IV/.

Table IV

Sp.No.	ABO group		Days of storage								
			3	7	11	15	19	23	27	31	35
1.	0	ACD	8	7	7	6	4	4	3	2	1
		GCM		8	8	8	8	9	8	8	7
2.	0	ACD	9	8	7	6	4	3	3	2	2
		GCM		9	9	9	9	8	9	9	8
3.	0	ACD	8	8	8	7	7	7	7	6	6
		GCM		8	8	8	7	8	8	9	8
4.	0	ACD	10	8	8	7	6	4	3	2	2
		GCM		10	10	11	10	10	9	9	10
5.	0	ACD	9	7	6	4	4	3	2	1	1
		GCM		9	9	8	9	8	8	9	8
6.	0	ACD	9	9	8	8	9	8	8	8	7
		GCM		9	9	9	9	9	9	9	9
7.	0	ACD	7	7	7	7	7	6	6	6	6
		GCM		7	7	7	7	7	7	7	7
8.	A ₁	ACD	9	7	6	4	2	2	1	1	1
		GCM		9	9	9	9	8	9	9	9
9.	A ₁	ACD	8	6	5	5	4	3	3	2	2
		GCM		8	8	7	8	8	7	8	7
10.	A ₁	ACD	9	7	5	4	3	3	2	1	1
		GCM		9	9	9	8	9	8	8	9
11.	A ₂	ACD	9	8	7	6	4	4	2	2	2
		GCM		9	9	8	9	9	9	9	9
12.	A ₂	ACD	8	8	8	8	7	7	7	8	7
		GCM		8	9	8	8	7	8	8	8

Table IV/

Table IV (Contd.)

Sp.No.	ABO group		Days of storage								
			3	7	11	15	19	23	27	31	35
13.	B	ACD	9	9	9	8	8	8	8	8	8
		GCM		10	10	10	10	9	10	9	10
14.	B	ACD	10	8	7	7	5	4	3	2	2
		GCM		10	10	10	11	9	10	9	10
15.	B	ACD	9	9	9	8	8	9	9	8	8
		GCM		9	9	9	9	9	9	9	9
16.	A ₂	ACD	9	8	8	8	7	8	8	7	8
		GCM		9	9	9	9	8	9	9	9
17.	A ₁	ACD	9	9	9	8	8	8	9	9	8
		GCM		8	8	8	8	8	8	8	8
18.	A ₁ B	ACD	8	8	8	7	7	7	7	7	7
		GCM		8	8	8	8	8	8	8	8
19.	A ₁ B	ACD	8	6	4	3	3	3	2	1	1
		GCM		8	8	8	8	7	8	8	8

Table IV. Titres on D antigens (of 19 D-positive bloods in the first series) on different dates. See legend, Table I.

No loss/

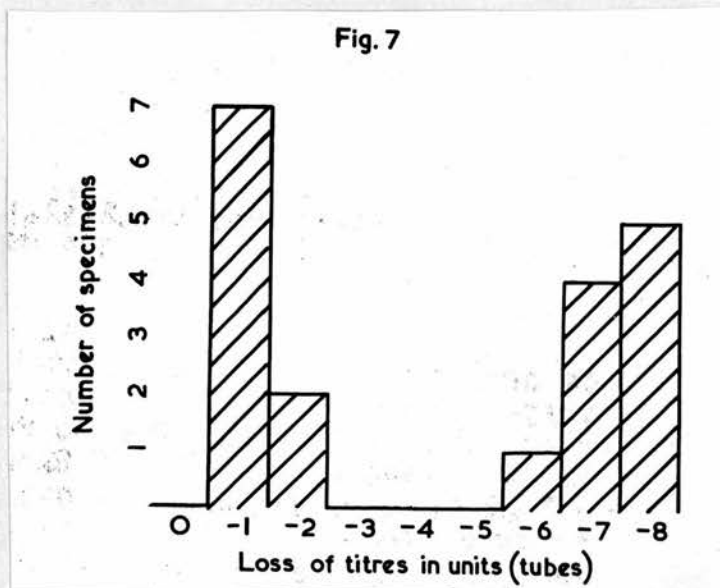


Fig.7. Loss of titres against anti-D serum on 35th day by 19 D-positive bloods (First Series) preserved in ACD solution at 4 -6°C.

No loss of agglutinability of D antigens was observed in any of the 19 D-positive bloods frozen in glycerol-citrate mixture as shown in Table IV and Fig.8.

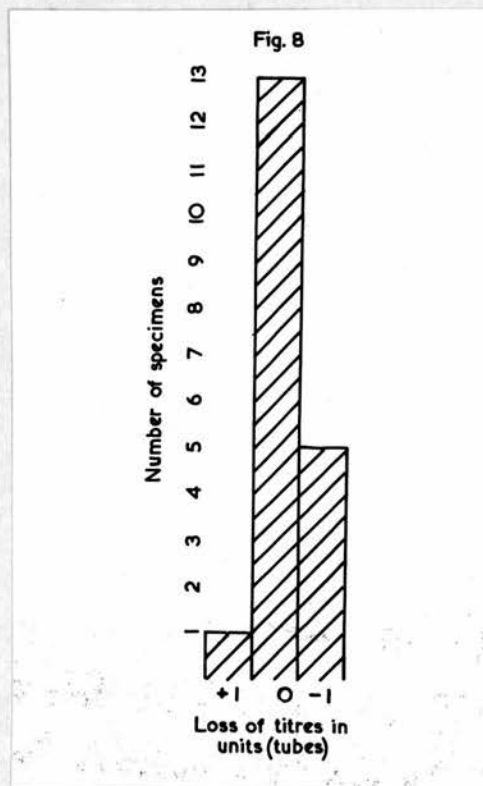


Fig.8. Loss of titres against anti-D serum on 35th day by 19 D-positive bloods frozen in glycerol-citrate mixture at -20°C.

Figure 9, a typical example from the more stable group shows the progressive loss of activity in the course of 35 days period. The final loss of titre on the sample preserved in ACD solution was only two tubes on the 35th day. In the example in Figure 10., from the less stable group the loss of the titre in the same period was 7 tubes.

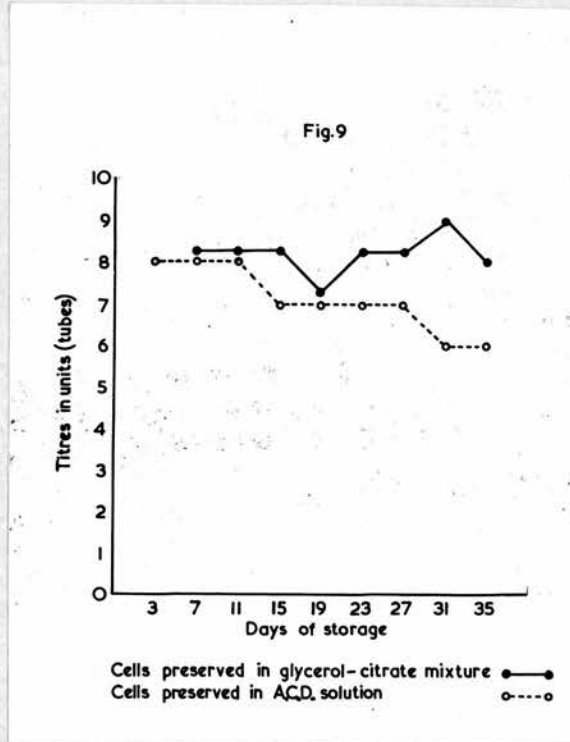


Fig.9. Titres given by one of the stable D antigens (No.3, Table IV).

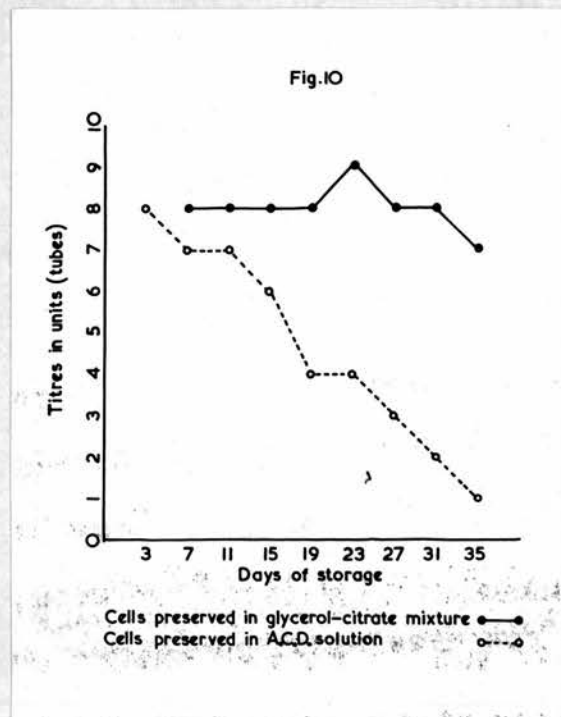


Fig.10. Titres given by one of the less stable D antigens (No1 Table IV)

Second Series:

In view of the results on D antigens in the first series, a further investigation was carried out on a second series of 28 D-positive cells to ascertain whether the division into two classes of the D agglutinogens would be maintained; and also to see if this division could be correlated with any other factor. The zygosity in relation to D factor, ABO groups, and the age and sex of the donors of this series were noted. As it was found that the red cells preserved in frozen condition do not lose agglutinability up to 35 days, the samples in the second series were stored only in ACD solution.

Results of titrations of 28 D positive cells both against saline anti-D and incomplete anti-D (by papain method) are tabulated in Table V. Deviations of the titres from the originals on 35th day are also shown.

Table V

Sp.No.	ABO Gp.	Age	Sex	Titres on		Deviation
				1st day	35th day	
20.	A ₁	34	F	S 8	6	2
				P 10	9	1
21.	B	41	M	S 9	8	1
				P 10	9	1
22.	A ₁	53	F	S 9	3	6
				P 11	6	5
23.	A ₁	32	M	S 7	7	0
				P 9	8	1
24.	B	49	F	S 8	2	6
				P 9	5	4
25.	A ₂	33	F	S 9	8	1
				P 10	9	1
26.	O	49	F	S 7	3	4
				P 9	5	4
27.	A ₁	48	M	S 8	3	5
				P 9	5	4
28.	B	26	M	S 7	6	1
				P 8	8	0
29.	A ₁	30	F	S 8	6	2
				P 9	7	2
30.	A ₁	38	M	S 9	3	6
				P 10	6	4
31.	A ₁	20	M	S 9	7	2
				P 10	9	1
32.	A ₁	20	M	S 8	3	5
				P 9	4	5
33.	A ₁	30	M	S 6	5	1
				P 8	8	0
34.	A ₂	30	M	S 9	3	6
				P 11	6	5

Table V/

Table V (Contd.)

Sp.No.	ABO group	Age	Sex	Titres on		Deviation
				1st day	35th day	
35.	A ₁	25	F	S 9	7	2
				P 10	9	1
36.	B	30	M	S 8	7	1
				P 10	8	2
37.	A ₁	40	M	S 7	6	1
				P 9	8	1
38.	O	30	M	S 8	6	2
				P 10	8	2
39.	B	31	F	S 8	3	5
				P 9	4	5
40.	O, CDe/CDe	56	M	S 7	7	0
				P 9	8	1
41.	O, CDe/cde	33	F	S 8	7	1
				P 10	10	0
42.	O, CDe/cDE	45	M	S 6	1	5
				P 9	3	6
43.	O, CDe/CDe	25	F	S 7	1	6
				P 10	4	6
44.	O, CDe/cDE	56	F	S 8	8	0
				P 10	10	0
45.	O, CDe/CDe	41	F	S 6	2	4
				P 10	6	4
46.	O, CDe/CDe	37	M	S 8	8	0
				P 10	9	1
47.	O, CDe/cde	59	M	S 7	1	6
				P 9	5	4

Table V. Titration results against anti-D sera on 28 D-positive bloods (second series) preserved in ACD solution. Titres in units (tubes) as obtained on 1st and 35th day are tabulated. Deviations of the titres from the controls (titres obtained on the first day) on 35th day and Rh genotypes of last 8 bloods are also shown.

S - - - titres against saline anti-D serum

P - - - titres against incomplete anti-D serum (by papain method)

Of 28 specimens twelve (Nos. 22, 24, 26, 27, 30, 32, 34, 39, 42, 43, 45 and 47, Table V) showed marked loss of agglutinability (4 units or more) on 35th day distinct from the remaining 16 (loss - 2 units or less). This is illustrated in Fig.11 which represents the loss in titre against saline anti-D. It was also noted that the loss in titre against saline anti-D and incomplete anti-D (by papain method) was uniform (see Table V) and a distribution figure similar to Fig.11 is obtained with the loss in titre against incomplete anti-D.

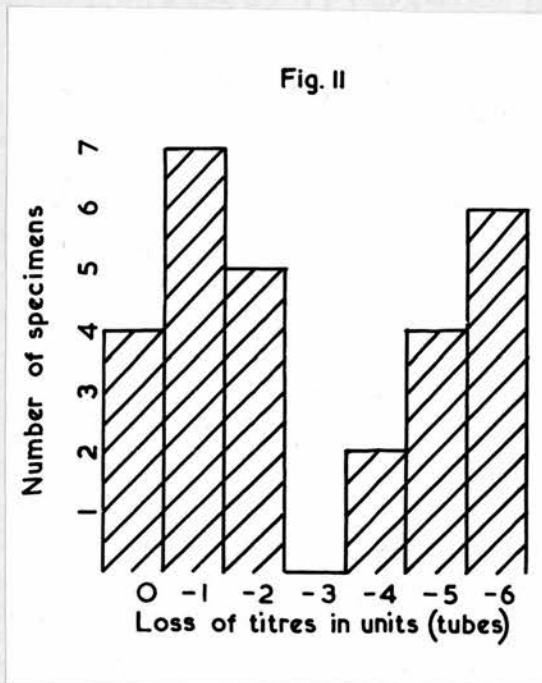


Fig.11. Loss of titres (against saline anti-D) on 35th day by 28 Rh(D) positive bloods (Second Series) preserved in ACD solution.

If the loss of titres over the period of 35 days for all D antigens (preserved in ACD solution) studied, are gathered together, the result is as shown in Fig.12 which confirms that the division of the D antigens into two classes is maintained. The distribution curve thus tends to show two maxima, one corresponding to only a slight loss of agglutinability (one tube) and the other to considerable loss (6 - 7 tubes).

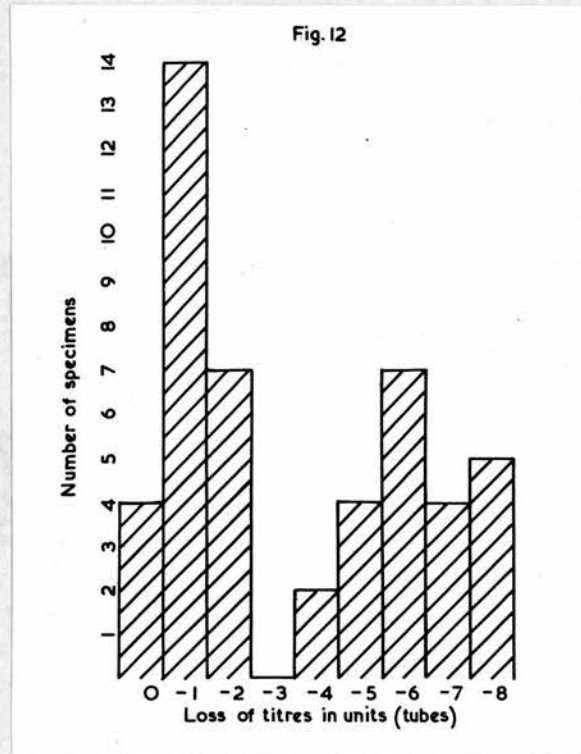


Fig.12. Loss of agglutinability against anti-D sera by 47 D-positive cells (of both series 1 and 2) preserved in ACD solution. In case of second series titres only against saline anti-D sera has been considered.

Six of the 8 samples with known Rh genotypes were homozygous and two heterozygous with relation to the D factor (Table V). Three homozygous and one heterozygous bloods lost agglutinability to a marked degree while the rest (three homozygous and one heterozygous) were more stable. This suggests that the loss of agglutinability is not related to the homo- or heterozygosity of the D factor.

The initial titres on both stable and unstable D antigens varied from 7 to 9 and 8 to 10 tubes respectively in the first series (Table IV, titres on cells preserved in ACD solution) and in both types of antigens they varied from 6 to 9 tubes in the second series (Table V, titres against saline anti-D). The loss in titres was not due to deterioration of the anti-sera was proved by (1) adequate controls and (2) the use of three different antisera. No relation was found between the stability of the antigen and the initial titre.

It was also observed that the loss of agglutinating power was not related to the age and sex (Table V) and ABO groups (Tables IV and V) of donors of the cells.

DISCUSSION

Storage of red blood cells in the frozen state in a glycerol-citrate mixture has been reported to give preservation of agglutinability. This method was therefore used as a control in the present study on storage of red cells in ACD solutions. In the frozen glycerol-citrate controls the deviation of agglutinability was found to be within the limit of error of pipette dilution titration, confirming the observations of previous workers. The controls served to verify that the anti-sera used were maintaining full titre over the period of storage of the cells. None of the A_1 , A_2 , B, D, Kell and Duffy antigens (of frozen samples) investigated, showed any loss of agglutination reaction during 35 days study period. The agglutinability of the erythrocytes preserved in ACD solution on the other hand, deteriorated within the same period and therefore, if storage of test cells is necessary it is desirable that they should be preserved in frozen state in glycerol-citrate mixture.

The degree of loss of agglutinability of the red cells preserved in ACD solution varied with different blood groups. A_1 and B antigens were stable and A_2 , Kell and Duffy were unstable. Wall et al. (1950) in their study on specific agglutinability/

agglutinability of red cells noted that A_2 agglutininogen to be least stable. The present finding that all 3 A_2 bloods showed rapid and marked deterioration of agglutinability corroborates their observation.

In the case of D antigens in the first series it was noticed that all the D positive cells did not lose their agglutinating power to the same extent when preserved in ACD solution. On the basis of loss of agglutinability they appeared to fall into two classes - one undergoing rapid loss of agglutinability and the other being comparatively stable. This is illustrated in Fig.7. This observation was confirmed by a further study on 28 D-positive cells in the second series. 12 bloods in this series also showed more marked loss of agglutinability. Taking both the series together 22 (approximately 47%) out of 47 D-positive bloods could be distinguished from the rest on the basis of greater loss of agglutinability as a result of storage (see Fig.12).

An attempt was made to correlate this division of D antigen into two groups with various factors likely to give an approximately 50% distribution. However, no correlation was found with any of the factors investigated: age, sex and ABO groups of the donors, homo- or heterozygosity of the cell with relation to the D factor or chromosome frequency, and also the initial titres.

Little/

Little previous work has been published on the variations in agglutinability of the D antigen as a result of storage. However, compared with ABO antigens it has been found by some authors that D antigen is less stable. Koopzoff(1954) noted that the D antigen lost its agglutinability at a faster rate than A and B antigens when the red cells were stored in citrate solutions at 4° - 6°C.

Nothing much is known of the reason for instability of the antigens when the cells are preserved in citrate solution. However, the observations of Wiener(1943), Nijenhuis(1953) and Koopzoff(1954) that if the concentration of the blood in the preserving fluid was low, the agglutinability of the antigens decreased at a faster rate than if the concentration of the blood was high, suggest that the citrate solution may have some direct or indirect action on the agglutinating property of the red cell antigens. It may also be possible that the loss of agglutinability is due to certain elements in the plasma e.g. enzymes or due to ultimate pH of the stored blood. These factors, however, were not studied in the present investigation.

It/

It is not known with certainty whether the agglutinability of the red cells is directly related to their antigenic power. It may be possible that on storage agglutinability may be lost as well as antigenicity. If it is so, then the above observations indicate one of the possible reasons for the finding that transfusion of incompatible blood (with the exception of ABO groups) is not necessarily followed by sensitization (see Part I). Some of the blood transfused during the second or third week of storage may lose antigenic potency and may be less likely to stimulate antibody formation or to cause reaction than comparatively fresh blood.

If on the other hand, red cells losing agglutinability, retain still their antigenic potency to stimulate antibody formation, it is of practical importance in cross matching tests for transfusion of blood. Some of the antigens (K and Fy^a in this series) may deteriorate so quickly that they may not be detectable within a few days of storage in ACD solution. Although no complete loss of agglutinability in the case of D antigen was observed in this investigation (possibly due to high titre of the anti-sera used) Renton and Stratton(1950) reported false D-negative results with some anti-D sera when testing with old specimens./

specimens. In such cases false negative results may be obtained if the bloods are cross matched after a few days of storage and specially if the antibody is very weak.

From the above findings it can be concluded that further consideration should be given to the question of deterioration of agglutination capacity in blood intended for transfusion.

The present practice is to collect a separate sample of blood at the time of withdrawal from the donor and to use the cells from this sample for compatibility (or to enter the bottle for the same purpose). The latter test is intended to confirm (1) that there has been no error in the ABO grouping of the donor or the patient, (2) to confirm that the patient's blood does not contain antibodies which would react with the donor's cells. However, since the donor's cells may have been stored up to three weeks, the possibility arises that the cell antigens may have lost their agglutinating capacity. In this case no reaction would be obtained in the compatibility tests specially in the case of very weak antibody and the 'incompatible' blood transfused. Such blood might or might not be involved in a haemolytic reaction; it is probable that the antigen would still be capable of causing further antibody response/

response. Moreover, for the same reason, the use of stored red cells (in ACD) of the donor for the investigation of transfusion reaction may give rise to false negative results.

In view of this situation, it is suggested (1) that attempt should be made to find a preservative solution for donor's blood specimens intended for compatibility testing which will preserve agglutinability for full three weeks, (2) that in the meantime, for patients who are likely to have antibodies, or who have antibodies which have not been identified, relatively fresh blood should be selected.

SUMMARY

Red cell antigens stored in ACD solution at 4° - 6°C. showed different degree of loss of agglutinating power which varied according to different blood group systems. A₁ and B antigens were found to be stable whereas A₂ was unstable. On the basis of loss of agglutinability, two types of D antigens could be recognised - one stable and the other relatively less so. Although approximately 50% of the D antigens were found to be less stable, no correlation was observed between the stability of this antigen and any of the factors studied.

Kell(K) and Duffy(Fy^a) antigens could not be detected after a storage period of a few days. The importance of such loss of specific agglutinability of the stored red blood cells in blood transfusion practice has been discussed.

It has also been noted that none of the A₁, A₂, B, D, K and Fy^a antigens investigated, showed any loss of agglutination reaction during 35 days study period, when the red cells were stored in glycerol-citrate mixture at -20°C. This finding confirms the observation of previous workers that if red blood cells are to be kept for more than a few days for serological studies, it would be better to mix them with glycerol and store at -20°C.

BIBLIOGRAPHY.

- Brown, I.W. & Hardin, H.F. (1953) Arch.Surg. 66, 267.
- Chaplin, H. jr., Crawford, Hal, Cutbush, Marie, and Mollison, P.L.
(1954) Lancet, 1, 852.
- Crawford, Hal, Cutbush, Marie, and Mollison, P.L. (1954)
Vox Sang. 4, 149.
- Grove-Rasmussen, M., Shaw, R.S., Sobky, Z. & Casna, E.M. (1953)
Vox Sang. 3, 119.
- Hattersley, P.G. & Fawcett, M.L. (1948) J.Lab.clin.Med.
33, 1177.
- Kooptzoff, O. (1954) Aust.J.exp.Biol.Med.Sci. 32, 695.
- Krijnen, H.W., Heier, Anne M. & van Loghem, J.J. (1952)
Bull.Centr.Lab.Bloedtr.Ned.Rode Kruis, 2, 285.
- Mollison, P.L. & Sloviter, H.A. (1951) Lancet, 11, 862.
- Mollison, P.L., Sloviter, H.A. & Chaplin, H. jr. (1952)
Lancet, 11, 501.
- Nijenhuis, L.E. (1953) Vox Sang. 3, 84.
- Polge, C., Smith, A.U. & Parkes, A.S. (1949) Nature,
London, 164, 666.
- Race, R.R. & Sanger, Ruth (1954) Blood Groups in Man.
2nd edn.p.282. Blackwell Scientific Publications Ltd., Oxford.
- Renton, P.H. & Stratton, F. (1950) Ann.Eugen. 15, 189.
- Simmons, R.T., Graydon, J.J. & Semple, N.M. (1951)
Med.J.Aust. 1, 425.
- Sloviter, H.A. (1951) Lancet, 1, 823.
- Smith, A.U. (1950) Lancet 11, 910.
- Wall, R.L., Jayne, M & Beauchamp, J. (1950) J.Lab.clin.Med.
36, 378.
- Wiener, A.S. (1943) Blood Groups and Transfusion, 3rd edn.
C.C. Thomas, Springfield, U.S.A.