THE RH FACTOR

A CLINICAL AND FUNDAMENTAL STUDY OF ITS SIGNIFICANCE IN ISO- AND AUTO-HAEMOLYTIC ANAEMIAS

By

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Chapter I

INTRODUCTION

The association of haemolytic disease of the newborn with blood group differences between the mother and her unborn child was first recognized by Levine and Stetson (1939) in a patient who gave birth to a stillborn macerated fetus. They established that the mother had been immunized with an antigen which the infant had inherited from the father. It was subsequently confirmed by Landsteiner and Wiener (1940) that the antibody described by Levine and Stetson was identical to the antibody they had obtained when rabbits were injected with Rhesus monkey red cells. The blood group factor associated with this haemolytic condition was thereafter called the Rhesus (Rh) factor.

It is now well established that Rh-immunization is the result of transplacental passage of fetal Rh-positive red cells into Rh-negative mothers. The effect of this process of immunization on the clinical condition of the infant starts when the maternal Rh-antibody enters the infant's circulation through the placenta. The antibody becomes attached to the fetal Rh-positive red cells and shortens their life span. The degree of severity of this haemolytic process depends on the rate of fetal red cell destruction induced by the antibody and the ability of the fetus to compensate by increased red cell production. If the rate of red cell destruction during gestation is greater than its production through extramedullary erythropoietic activity then anaemia results and may be followed

by intrauterine death. Should the affected fetus survive the hostile in utero environment complications associated with hyperbilirubinaemia may develop which can lead to severe brain damage (kernicterus).

In white American, British and South African populations haemolytic disease of the newborn due to Rhesus incompatibility occurs in one out of 150 full-term pregnancies (Levine, Vogel and Rosenfield, 1953; Vos, 1967). Approximately 70 per cent of infants born with Rh haemolytic disease will be severely enough affected to require treatment (Kelsall, Vos and Kirk, 1958). Universal acceptance of the necessity for routine Rh-typing of all pregnant women, coupled with detailed antenatal serological investigations has now greatly simplified the problem of anticipating Rh haemolytic disease. However, for the obstetrician and paediatrician there remains the issue of individual prognosis and case management and it is with this respect that a multitude of intriguing and often unanswerable questions can be provided by experienced immunohaematologists.

During the past decade significant advances have been made towards the prevention of Rh haemolytic disease. Based on the fundamental concept that passive immunity can prevent active immunity (Smith, 1909), investigators in England and the United States were able to show that the simple injection of Rh antibodies into Rh-negative mothers can also suppress Rh immunization (Finn, Clarke, Conohoe, McConnell, Sheppard, Lehane and Kulke, 1961; Freda, Gorman and Pollack, 1963). The efficacy of this

approach has now been firmly established (Clarke, 1967; Pollack, Gorman, Hager, Freda and Tripodi, 1968).

In spite of these advances in the prevention of Rh immunization, the risk of Rh-negative mothers becoming immunized to the Rhesus antigen is still high. Conditions influencing the continuous occurrence of Rh immunization are related to several factors among which the failure of the mother to receive passive therapy during and after pregnancy is probably the main cause. There is also a good deal of evidence to suggest that the suppression of Rh immunization produced by anti-Rh is antigen-specific (Mollison, 1972). This means that the administration of specific anti-D prevents the formation of anti-D but not the formation of other types of antibodies which are likely to cause haemolytic disease of the newborn, e.g. anti-C, anti-E, anti-Kell and Fy^a and anti-Jk^a. Therefore, haemolytic disease of the newborn remains a significant clinical problem.

In the antenatal diagnosis of Rh haemolytic disease it is important that the presence of placenta-permeable antibodies is first recognized in the maternal serum. From then on it can generally be expected that the severity of the disease process <u>in utero</u> will be related to the intensity of antibody formation in the mother. Under this stimulus destruction of the unborn child's Rh-positive red cells leads to the formation of various end products including the toxic unconjugated bilirubin which has an affinity for certain nerve cells in the brain.

Quantitative tests for the determination of unconjugated bilirubin in liquor amnii will therefore directly reflect the intensity of fetal red cell destruction taking place *in utero*. The ability to predict the severity of Rh haemolytic disease by examining the amniotic fluid has been confirmed by Bevis (1956), Mackay (1961), Liley, (1961) and Freda, (1964).

Although prognosis by amniotic fluid studies appears to be simple, a considerable number of complications can occur following the practise of inserting a needle into the uterus (amniocentesis). The most common hazards are: (1) damage to fetal blood vessels on the surface of the placenta, which has been reported to occur in nearly 15 per cent of patients (Zilliacus and Ericksson, 1958); this complication has induced early separation of the placenta causing fetal death in a number of instances (Mayer, 1963); (2) if the placenta is traversed at the puncture, fetal Rh-incompatible cells invariably enter the maternal circulation to intensify Rh-antibody production. Walker and Jennison (1962) reported a post-amniocentesis antibody rise in 41 per cent of mothers. Thus in those cases where fetal blood has been aspirated by amniocentesis the chances of increasing Rh-immunization and intensifying the haemolytic process *in utero* are considerably greater. It has therefore been accepted that amniocentesis should be carried out only when the maternal antibody value is raised above a certain critical level (Queenan, 1966; Kubli, 1966; Vos, 1969).

A major problem in the application of Rh antibody determinations is the selection of suitable serological methods. In a study concerning the in vitro reactivities of Rh antibodies Hill and Soules (1957) showed that three orders of antibodies are clearly recognizable by different testing procedures: (1) the "agglutinin"; this is an Rh antibody best demonstrated by its ability to agglutinate cells in a medium of physiological saline; (2) the "agglutinoid", which can be demonstrated by the blocking test and indirect anti-human globulin test but not by any enzyme test, and (3) the "cryptagglutinoid", which is detectable by the indirect anti-human globulin test, albumin and other colloids and by enzyme-treated cells. A fourth order of Rh antibody, the "papain type" which was described by Dodd and Eeles (1961) is detectable solely by enzyme techniques.

The relationship between the various orders of Rh antibodies to the general immunization process has established that Rh antibodies measured by the indirect anti-human globulin test are of far greater prognostic significance than those determined by the albumin or enzyme tests (Vos, 1958; Tovey and Valaes, 1959; Zeitlin and Boorman, 1963; Jacobs, 1962; Hubinot, 1961).

A disturbing complication of the indirect anti-human globulin test is that considerable variation in results may be obtained when the same method is used by other workers in different laboratories (Goldsmith, Mourant and Bangham, 1967). Thus it is difficult to compare the res-

ults obtained by one laboratory with those of another. Although this lack of agreement may reflect variations in individual standards of measuring Rh antibody activity, several other causes also cannot be ignored. For instance, it has now become clear that Rh antigens may occupy a greater, or lesser, area of the red cell membrane, depending upon the zygosity of the Rh antigen and its quantitative distribution in family members (Rochine and Hughes-Jones, 1965; Masouredis, Dupuy and Elliott, 1967).

In view of the difficulties in assessing the clinical conditions of this disease by serological means, extensive investigations were carried out to examine the relationship of several methods of Rh antibody determination. (1) the standardized indirect anti-human globulin titration test (Vos and Kirk, 1958); (2) the partial absorption test (Vos, 1958) and (3) the Rh antibody inhibition test (Vos, 1969). It was hoped that these studies would provide valuable information for the evaluation of different methods in the diagnosis of the severity of Rh haemolytic disease of the newborn. The development of satisfactory testing procedures was of fundamental importance to the progress of the entire study.

A notable achievement resulting from these investigations has been the acknowledgement that the partial absorption test, described in Chapter VII, has been recommended as a testing procedure by the Council on Immunohaematology of the American Society of Clinical Pathologists.

PART ONE

THE INDIRECT ANTIGLOBULIN TITRE

Chapter II

A CRITICAL EVALUATION OF THE DIRECT AND INDIRECT ANTI-HUMAN GLOBULIN TITRATION TESTS

1. INTRODUCTION

Since the discovery of Rh haemolytic disease of the newborn by Levine and Stetson (1939) and Landsteiner and Wiener (1940), investigators have encountered many difficulties in predicting the severity of the disease before birth. To evaluate the condition of the infant by clinical signs only can be most misleading and clinicians are obliged to look to the laboratory for information to clarify an otherwise obscure clinical picture.

After much trial and error we adopted the antiglobulin test of Coombs, Mourant and Race (1945) as a routine procedure for the determination of the severity of Rh haemolytic disease. Both the direct and indirect methods were employed and standardized in every detail (Vos and Kirk, 1958). In Coombs et al's description of the use of rabbit anti-human globulin serum for detecting the sensitization of red blood cells by Rh antibodies, they emphasized the necessity for several washings of the sensitized cells prior to adding the antiglobulin reagent. In many laboratories investigators wash the sensitized cells by adding an excess (10 to 20 vol.) of 0.85 per cent saline solution, inverting the tube to resuspend the cells, centrifuging and removing the supernatant saline solution and then repeating the procedure until three washings have been completed.

Serologists are aware that simple resuspension of cells in a large volume of physiological saline solution is inadequate for reproducible end points in titrations with antiglobulin reagents. We found that other laboratories using the same test serum and the same antiglobulin serum often obtained titration values that were less than our own; in some instances the discrepancies were startling. A technique was therefore devised to eliminate these differences and to make titration values obtained in various laboratories more comparable. This is particularly important when maternal Rh antibody titres are used as a guide for the induction of labour and the treatment of the newborn infant (Kelsall and Vos, 1955; Kelsall, Vos, Kirk and Shield, 1957).

Initially our improved washing procedure consisted of using a Pasteur pipette to withdraw a suspension of sensitized cells out of the test tube and then vigorously expelling the cells back into the tube. This was repeated 10 to 15 times to ensure thorough washing. Although this procedure yielded consistent end-points in all of the titrations it was tedious and time-consuming. A simple machine was therefore designed to provide a means of shaking a rack of tubes at a constant speed. The results obtained show that sensitized cells that are washed by means of mechanical agitation yield consistently higher titration values than do cells that are washed by means of simple resuspension in a large volume of physiological saline.

2. THE CELL-WASHING MACHINE

A rack-holder is mounted on a rocker arm that is rigidly coupled through an eccentric to a 1/20-h.p. electric motor than can run at 1 000 rpm. The reciprocal motion moves the rack-holder horizontally with a maximal displacement of 1.3cm. The light aluminium rack carries 20 tubes (0.5 by 5.0 cm). The tubes are closed with small rubber stoppers and are seated on sponge-rubber cushions; they are held rigidly in position by a hinged plate that folds forward against the rubber stoppers when the rack is mounted on the rack-holder. In order to facilitate rapid placement and removal the rack is held on the rack-holder by means of a keyed slide. When removed it may be fitted into a stand on the operator's bench. Fig.1 is an illustration of the cell-washing machine with a rack mounted on the rack-holder.

3. TECHNIQUE OF ANTIGLOBULIN TITRATION

Indirect antiglobulin titration - The specifically reactive test cells are washed mechanically three times in 10 vol of 0.85 per cent saline solution and then resuspended in a sufficient amount of physiological saline solution to yield a 10 per cent suspension of cells. 0.4 ml of the undiluted serum to be tested is pipetted into the first and second serial test tubes. To the second and to each succeeding tube, 0.4 ml of 0.85 per cent saline is added. Serial doubling dilutions are then prepared in the usual manner. To each of the tubes 0.4 ml of the 10 per cent suspension is added and the tubes incubated at 37° C for 1 hr. (We have adopted the convention of calling the dil-

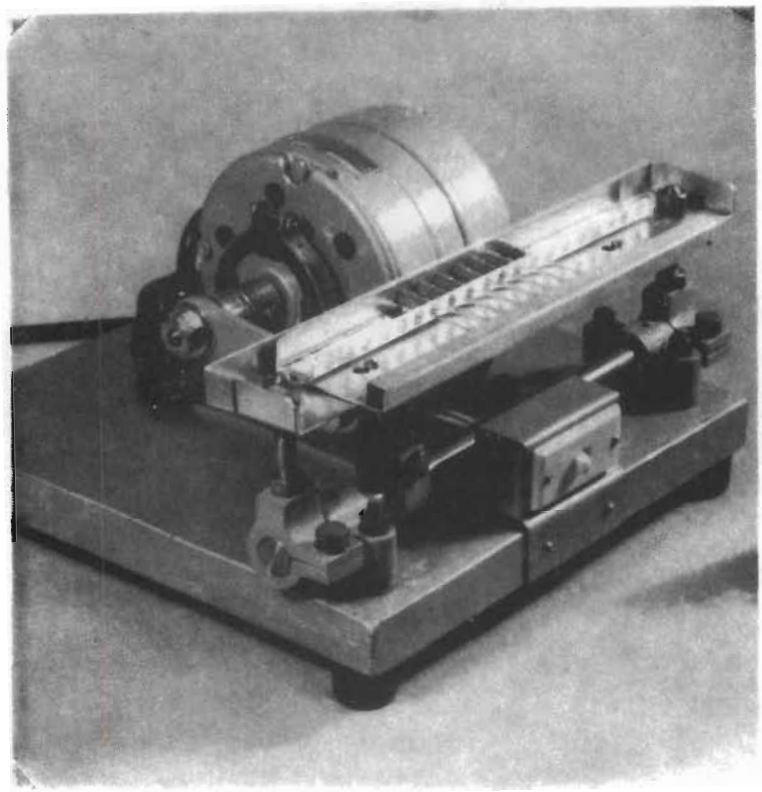

Figure 1: Photograph of cell-washing machine, with the rack for tubes mounted on the rack holder.

ution in the first tube 1:2).

After incubation the sensitized cells are washed three times by mechanical shaking for 30 sec. each time with 20 vol. of 0.85 per cent physiological saline. After removal of the supernatant fluid from the final washing two drops of anti-human globulin reagent (known to yield optimal reactions with Rh-sensitized cells) are added to the washed sensitized cells in the test tube. This suspension is then pipetted onto a glass slide so that a pool of cells approximately one inch in diameter is formed. The slide is then placed in a moist chamber at room temperature. After being left undisturbed for 10 minutes the slide is gently tilted forward and the agglutination read against a well-illuminated ground-glass screen using a 5 x magnification head fitting eyepiece. As a control, unsensitized group O, Rh-positive cells are tested in parallel with the sensitized cells.

Direct antiglobulin titration - Adult or infant cells sensitized in vivo are washed mechanically three times in 20 vol. of 0.85 per cent saline solution. After removing the supernatant saline from the final washing, two drops of anti-human globulin reagent are added to the washed cells in the test tube. This suspension is then pipetted onto a glass slide. The results are read as described for the indirect antiglobulin titration test. Group O, Rh-positive nonsensitized red blood cells are used as controls.

4. COMPARISON OF THE EFFECTIVENESS OF TWO CELL WASHING PROCEDURES

Indirect antiglobulin titrations - Seven Rh antisera were titrated by the indirect antiglobulin technique and two procedures were used for washing the sensitized cells. In the first series the cells were washed three times by adding 10 vol. of 0.85 per cent saline solution and then inverting the tube in order to resuspend the cells. In the other series the cells were washed three times by mechanical agitation on the cell-washing machine for 30 sec. The results in Table 1 indicate that in all instances the end point of the titration was higher for the mechanically washed cells. In cases 2, 3, 5 and 6 the prozone observed in the lower dilutions of the manually washed cells was not present when the cells were washed mechanically.

Direct antiglobulin titrations - A similar set of results for the direct antiglobulin test was obtained using the same two methods of washing cells (Table 2). Cases 1 to 4 were red cells obtained from the umbilical cords of infants born to Rh-immunized mothers. Cases 5 and 6 were adult cells that were sensitized with known Rh antisera. In case 5 the Rh antiserum had an indirect antiglobulin titre of 1:128; case 6 a titre of 1:4096. The mechanically washed cells yielded higher end points in four out of six cases. In five cases a conspicuous prozone was observed in the lower dilutions for the manually washed cells; this was absent when the cells were washed mechanically.

TABLE 1

Comparison of indirect antiglobulin titrations using two methods of washing sensitized cells

Case Number	Specificity of antibody	Method of Washing	DILUTION OF TEST SERUM									
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
1	anti-D	A	+++	+++	+++	++	++	+	+	W	0	0
		B	++++	++++	++++	+++	+++	+++	++	+	W	Tr
2	anti-C	A	+	++	+++	+	W	Tr	0	0	0	0
		B	++++	+++	+++	+++	+++	++	+	W	0	0
3	anti-D	A	++	+++	+++	++	+	+	W	Tr	0	0
		B	++++	+++	+++	+++	+++	++	+	W	Tr	0
4	anti-D	A	+	+	W	Tr	0	0	0	0	0	0
		B	+++	+++	++	++	+	W	Tr	0	0	0
5	anti-C + anti-D	A	0	W	++	+++	+++	++	+	W	Tr	0
		B	++++	++++	++++	++++	+++	+++	+++	++	+	W
6	anti-D	A	++	+++	++++	+++	++	++	+	W	Tr	0
		B	++++	++++	++++	+++	+++	+++	++	+	W	Tr
7	anti-D	A	+++	++	+	W	Tr	Tr	0	0	0	0
		B	++++	+++	+++	++	+	W	Tr	0	0	0

* Sensitized cells were washed 3 times in 20 vol. of 0.85 per cent saline solution

A - cells resuspended in physiological saline solution by manually inverting the tube

B - cells shaken in physiological saline solution for 30 sec. each wash on the cell-washing machine.

W = weak; Tr = trace.

TABLE 2

Comparison of direct antiglobulin titration using two methods of washing sensitized cells

Case Number	Method Of Washing	DILUTION OF ANTIGLOBULIN SERUM												
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:8192
1	A	+++	+++	++	+	Tr	Tr	Tr	?Tr	0	0	0	0	0
	B	++++	++++	++++	+++	+++	++	+	W	Tr	0	0	0	0
2	A	++	+++	+++	+	W	Tr	Tr	0	0	0	0	0	0
	B	++++	++++	++++	+++	+++	++	+	W	Tr	0	0	0	0
3	A	++	+++	++++	+++	+++	++	+	+	W	Tr	Tr	?Tr	0
	B	++++	++++	++++	++++	++++	+++	+++	++	+	+	W	Tr	0
4	A	+	+++	++++	++++	+++	++	+	W	W	Tr	Tr	?Tr	0
	B	++++	++++	++++	++++	++++	++++	++++	+++	+++	++	+	W	Tr
5	A	+++	++++	+++	+	Tr	Tr	Tr	0	0	0	0	0	0
	B	++++	++++	++++	++++	+++	++	+	W	Tr	0	0	0	0
6	A	0	0	+	+++	++++	++++	+++	++	+	W	Tr	Tr	Tr
	B	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	+	W

* Sensitized cells were washed 3 times in 20 vol. of 0,85 per cent physiologic saline solution:

A - cells resuspended in physiologic saline solution by means of manually inverting the tube;

B - cells shaken in physiologic saline solution for 30 sec. each wash on the cell-washing machine.

W = weak; Tr = trace.

5. HAEMOLYSIS AND ELUTION OF ANTIBODIES BY MECHANICAL WASHING

The amount of cellular damage caused by the mechanical washing procedure was studied by measuring the percentage of red cell haemolysis. Adult and cord-blood cells that were stored as heparinized blood at 4° C and then washed mechanically for 60 sec. with 0.85 per cent saline solution showed no measurable haemolysis when used within 24 hours of collection. The amount of haemolysis was 0.1 per cent for cells stored between 24 to 48 hours and it increased to 1.0 per cent for cells that were stored at 4° C for more than 72 hours.

The possibility that antibodies may be released from sensitized cells during the washing procedure was also studied. Adult cells that were sensitized with various Rh antisera were washed mechanically three times and the supernatant fluid from the washings was pooled. The wash liquor was then dialysed against gum acacia and the contents of the bag reconstituted to the original volume of serum by addition of A_1B serum. No unabsorbed antibody was detected in the wash-liquor when the ratio of cells to serum (10% suspension of cells) removed all the Rh antibodies from the original serum. When an excess of antibody was present in the original serum it was invariably noted that similar excesses could also be found in the wash-liquor, although at a lower concentration than in the original serum. It is in such instances that a prozone is frequently observed in direct or indirect antiglobulin titrations when the cells are washed manually.

It is important to note however that even though antibodies are detectable in the wash-liquor from these cells when they are mechanically washed, the end point in the titration when using the anti-human globulin test was always considerably higher than by the manual method.

6. DISCUSSION

Although investigators have emphasized the importance of cell washing to free Rh-sensitized cells from contamination with human serum prior to adding the anti-human globulin reagent, little direction has been given as to the precise method to be used. Our studies have revealed that great differences in end-point can occur in the direct and indirect antiglobulin test when two different methods are used for washing the sensitized cells. The mechanical procedure for washing red blood cells for the anti-human globulin test has the advantage of (1) yielding higher titration values and (2) enabling persons to obtain constant and reliable results, even when they are using the technique for the first time. If the directions for performing anti-human globulin titrations are followed as outlined, workers in various laboratories should obtain identical values. This is particularly important if the anti-human globulin test for the quantitation of Rh antibodies in the maternal serum is to be used as a prognostic aid in the management of such patients.

An observation of special interest with regard to anti-human globulin titrations is that the prozone phenomenon which can be observed when using manual procedures is

absent when the sensitized cells have been vigorously washed mechanically. A prozone is obtained with Rh antibodies only when the sensitized cells do not absorb all of the antibody from the serum. If the ratio of cells to serum is changed in such a manner that no excess antibody remains, a prozone is not formed when the antiglobulin reagent is added.

The results of experiments in which the wash-liquor from mechanically washed, sensitized cells was reconstituted to the original volume of serum, by means of dialysis, demonstrated that in instances where excess antibody was present in the original serum, antibody was also removed from the cells by the washing procedure. This suggests that additional antibody is coated loosely around the cells in these instances; this loosely bound antibody, if not removed by vigorous washing, interferes with the antiglobulin reaction. The interference occurs (1) when this loosely bound Rh-antibody is present in excess, as in the lower dilutions of the indirect antiglobulin reaction, or (2) when the antiglobulin antibody is in excess, as in the lower dilutions of the direct titration with antiglobulin. The fact that the end point of the anti-human globulin titre is higher when the cells are washed mechanically than when they are washed manually (even in instances where free Rh-antibody is detected in the wash liquor), indicates that loosely bound antibody may enhance a prozone phenomenon. The warning given by Coombs and his coworkers (1945) against too much washing because of the theoretical possibility that antibodies may be washed from the cells does not appear to apply when dealing with Rh-antibodies.

Chapter III

AN EVALUATION OF THE FREQUENCY OF INCREASED RH-ANTIBODY STIMULATION DURING PREGNANCY BY THE INDIRECT ANTIGLOBULIN TEST.

1. INTRODUCTION

Since the discovery that Rh-negative mothers can be protected against Rh immunization by the passive administration of anti-D gammaglobulin (Finn, Clarke, Donohoe, McConnell, Sheppard, Lehane and Kulke, 1961; Freda, Gorman and Pollack, 1964), considerable attention has been focused on the incidence and effect of feto-maternal bleeds occurring at delivery. Evidence that fetal red cells can find their way into the maternal circulation during pregnancy indicates that antigenic stimuli for Rh-antibody formation can be expected before, as well as after, the delivery of an Rh-positive infant (Taylor and Kullman, 1961; Robinson, Williams, Jakobowicz, Moore and Silberman, 1966).

Even though investigators employ different methods for the antenatal detection of fetal red cells there is close agreement that the frequency of positive findings increases during the final months of pregnancy. While not every feto-maternal bleed necessarily constitutes an active process of iso-immunization (because individual differences in the mechanism of antibody formation can be expected), it nevertheless raises the problem that antenatal haemorrhage from the fetus to the mother can initiate or intensify antibody stimulation. Antenatal variations in antibody specificity, nature of immunoglobulin involved, or simple

antibody titre differences can therefore be considered as evidence that renewed fetal bleeds have taken place.

Results of this investigation show that Rh-antibody follow-up studies performed throughout pregnancy can often clearly reveal the frequency of transplacental haemorrhage and its effect on fetal wastage. Although past observations have indicated that the initial development of Rh immunization occurs as a result of the trauma of parturition, we have found that it can also occur as a consequence of fetal bleeds during pregnancy.

2. MATERIALS AND METHODS

Rh-immunized mothers in this analysis were examined from the first trimester of pregnancy. In no instance were mothers included in whom the production of Rh antibodies might have been the result of previous Rh-incompatible blood transfusions and only Rh-incompatible mother-infant combinations were examined.

For the evaluation of Rh-antibody titres the standardized method of indirect antiglobulin titration was used (Chapter II). Since the frequency of antenatal episodes of Rh immunization is known to differ in Rh-negative mothers who become immunized by fetal Rh-positive red cells, the question of when and how often these mothers do show increased episodes of antibody production was important. To determine these variations the investigator must first resolve the accuracy of his indirect antiglobulin titration procedure. Table 3 shows that within the range of one dilution tube nearly 20% of inaccurate res-

TABLE 3

Accuracy in evaluation of Rh-antibody titres by standardized indirect antiglobulin method when determined by 2 investigators (blind test)

134 Samples of Rh Antibodies Examined				
Titre differences	First investigator % error	Second investigator % error	Average variation % error	Accuracy in predicting increased episodes of Rh immunization when variations exceed
3 dilution tubes	0.0	0.0	0.0	100%
2 dilution tubes	6.7	5.9	6.3	93.7%
1 dilution tube	15.6	17.9	16.7	83.3%

ults can be recorded for the same samples of blood when tested by two investigators. For a 2-dilution-tube difference the percentage of error is narrowed to 5%. The absence of titration inaccuracies exceeding three dilution tubes is significant in that an observed increase of this magnitude can confidently be regarded as a renewed episode of immunization. Assuming that titre differences of three dilution tubes or more can also imply that transplacental passage of Rh-incompatible fetal red cells has taken place, then it should also be possible to assess how often such occurrences generally take place during pregnancy.

3. RESULTS AND DISCUSSION

Table 4 records an analysis of 818 Rh-negative mothers who were either already immunized before the present pregnancy or became immunized at some stage during the present pregnancy. The findings for different gestation periods clearly show that a significantly greater frequency of episodes of increased Rh-antibody production occurs after 32 weeks gestation. Considering that this indicates increased fetal red cell leakage across the placental barrier, the results appear to be in agreement with the findings of Betke (1966) who also found a greater percentage of fetal red cells in the maternal circulation towards the end of pregnancy.

Table 5 records a similar investigation of the 818 Rh-immunized mothers, comparing those who were immunized before the present pregnancy with those who were not. Of importance here was the observation that the frequency of

TABLE 4

Analysis of frequency of antenatal episodes of Rh-immunization observed among 818 Rh-immunized mothers examined at various stages of pregnancy

Gestation range	Total number of mothers examined	Episodes of Rh immunization observed*	
		No.	%
25 weeks or less	818	0	0.0%
26-28 weeks	818	53	6.4
29-31 weeks	805	106	13.1
32-34 weeks	773	166	21.4
35-37 weeks	713	254	35.6
38-40 weeks	289	167	57.7

* Represents Rh-antibody titre increase of 3 dilution tubes or more

TABLE 5

Comparative analysis of frequency of antenatal episodes of Rh immunization observed among mothers not immunized and those already immunized before present pregnancy

Gestation range	Mothers not immunized before present pregnancy			Mothers already immunized before present pregnancy			Chi-square values (Yates correction applied)
	Total No. of mothers	Episodes confirmed * No.	%	Total No. of mothers	Episodes confirmed * No.	%	
25 weeks or less	208	0		610	0		
26-28 weeks	208	27	12.9	610	26	4.2	$X^2(1)$ 16.449 P = 0.001
29-31 weeks	208	43	20.6	597	63	10.5	$X^2(1)$ 9.454 P = 0.01
32-34 weeks	208	62	29.8	565	104	18.4	$X^2(1)$ 6.771 P = 0.01
35-37 weeks	200	87	43.5	513	167	32.5	$X^2(1)$ 3.160 n.s.
38-40 weeks	167	109	65.2	122	58	47.5	$X^2(1)$ 2.178 n.s.
Average for all gestations	199	54	27.1	502	69	13.7	X^2 11.838 P = 0.001

* Represents Rh-antibody titre increase of 3 dilution tubes or more.

raised episodes of immunization was significantly greater among mothers in whom Rh antibodies were detected for the first time. This increase appears to be closely associated with the duration of pregnancy. Mothers not immunized before the present pregnancy recorded a significantly greater frequency of episodes of immunization before 35 weeks of pregnancy than the Rh-negative mothers who were already immunized.

It would be unrealistic to assume that such differences are directly influenced by significant variations in the incidence of transplacental bleeds occurring between mothers immunized and not immunized before the present pregnancy, and the most likely explanation for the decreased occurrence of episodes of immunization is that the mother who is already immunized has a remarkable ability to conceal the recognition of the fetal Rh-positive antigen as a potential stimulus. Such a hypothesis seems quite acceptable when we consider that these mothers can only be the recipients of fetal Rh-positive red cells which were already sensitized <u>in utero</u> by placenta-permeable antibodies, whereas mothers who were not immunized before the present pregnancy were more often exposed to fetal red cells possessing the full expression of the Rh antigen. As such, the mechanism of Rh-antibody stimulation is comparable with the studies of Stern, Goodman and Berger (1961). They showed that sensitized Rh-positive red cells are far less antigenic than unsensitized red cells and these findings led to the evaluation of the prevention of Rh immunization by the injection of anti-D immunoglobulin.

If the indication of apparent suppression of increased antibody production is an accurate deduction of the experimental findings of Stern et al. (1961) it can be seen that this protection is not constant throughout pregnancy. In fact, it appears to decrease among the pre-immunized mothers as pregnancy progresses towards term. Since only small transplacental bleeds are expected before 30 weeks of pregnancy, with more profuse bleeds generally occurring after this time (Betke, 1966), it is possible that a breakdown in protection is induced by the introduction of significant variations in the amount of Rh-positive red cells crossing the placental barrier after 30 weeks' gestation. This situation bears a striking resemblance to the inability of anti-D gammaglobulin to secure complete protection against rhesus immunization when massive feto-maternal bleeds take place (Dudok de Wit and Borst-Eilers, 1968; Woodrow, Bowley, Gilliver and Strong, 1968).

These developments suggest that there are at least two ways in which the intensity of increased Rh-antibody production can be stimulated during pregnancy. One is the result of imperfectly understood defects of the placenta, which allow greater quantities of fetal red cells into the maternal circulation, while the other is undoubtedly closely associated with external version, (Vos, 1967; Robinson, Williams, Jakobowicz, Moore and Silberman, 1966), the repeated application of abdominal amniocentesis, (Zipursky, Pollock, Chown and Israels, 1963), or certain modes of obstetric management during the third stage of labour (Morrison, 1967).

The results so far presented show that fetal red cells often cross the placental barrier to stimulate increased Rh-antibody production in the mother. Not yet firmly established, however, is the effect of these episodes of immunization on the clinical manifestation of Rh-haemolytic disease. Table 6 details an analysis of 208 mothers who for the first time showed Rh antibodies during an Rh-incompatible pregnancy. It is interesting to note that fetal wastage (combined stillbirths and neonatal deaths) can be anticipated in the first immunizing pregnancy. Thus, the tendency to consider all primary immunization cases as mildly affected infants, who generally do not require further treatment, will involve a certain amount of risk unless constant follow-up studies are carried out to measure the intensity of the haemolytic process in utero.

Compared with the values of fetal wastage observed among the already immunized series of mothers (Table 7), it is significant that fetal losses among the primary immunization cases consistently took place after 35 weeks of pregnancy when Rh-antibody stimulation appeared to be most intense. This type of pattern suggests that the time during which an Rh-positive fetus is exposed to Rh-antibodies is of some importance in determining the severity of the haemolytic process in utero.

The findings presented in Tables 6 and 7 show that a significant percentage of fetal wastage can be attributed to the occurrence of renewed episodes of Rh-antibody stimulation. This implies that the protective factor against increased Rh-antibody formation in the already immunized

mother can be reversed through the reactivities of progressively large fetal bleeds, which in turn intensify the haemolytic process.

TABLE 6

Evaluation of incidence of fetal wastage (stillbirths and neonatal deaths) in relation to antenatal episodes of increased Rh-antibody production observed for 208 mothers who were not immunized before present pregnancy

Gestation range	Total No. of mothers observed	ANTENATAL EPISODES OF IMMUNIZATION							
		Observed*				Not observed			
				Fetal wastage				Fetal wastage	
		No.	%	No.	%	No.	%	No.	%
25 weeks or less	208	0	0	0	0	208	100.0	0	0
26-28 weeks	208	27	12.9	0	0	181	87.1	0	0
29-31 weeks	208	43	20.6	0	0	165	73.4	0	0
32-34 weeks	208	62	29.8	0	0	146	70.2	0	0
35-37 weeks	200	87	43.5	2	2.3	113	56.5	0	0
38-40 weeks	167	109	65.2	5	4.6	58	34.8	1	1.7

* Represents Rh-antibody titre increase of 3 dilution tubes or more

TABLE 7

Evaluation of incidence of fetal wastage (stillbirths and neonatal deaths) in relation to antenatal episodes of increased Rh-antibody production observed for 610 mothers who were already immunized before present pregnancy

Gestation range	Total No. of mothers observed	ANTENATAL EPISODES OF IMMUNIZATION							
		Observed*				Not observed			
				Fetal wastage				Fetal wastage	
		No.	%	No.	%	No.	%	No.	%
25 weeks or less	610	0	0	0	0	610	100.0	0	0
26-28 weeks	610	26	4.2	10	38.4	584	95.8	3	0.5
29-31 weeks	597	63	10.5	27	42.7	534	89.5	5	1.0
32.34 weeks	565	104	18.4	26	25.0	461	81.6	8	1.7
35-37 weeks	513	167	32.5	49	29.3	346	67.5	21	6.0
38-40 weeks	122	58	47.5	8	13.7	64	52.5	3	4.6

* Represents Rh-antibody titre increase of 3 dilution tubes or more

Chapter IV

THE EFFECT OF EXTERNAL VERSION ON INCREASED RH-ANTIBODY STIMULATION DURING PREGNANCY

External version is known to be associated with increased transplacental haemorrhage (Robinson et al. 1966). It was therefore important to investigate the potential hazards of this procedure in Rh haemolytic disease.

To determine the frequency and intensity of antenatal Rh-immunization as a result of external manipulation we examined two separate hospital recording systems. They comprised laboratory estimations and clinical follow-up reports which when combined revealed those patients who were subjected to external version and those who were not. The fact that this information was not routinely requested by the investigator before this study commenced, or during the time that the Rh-negative mothers were examined for Rh antibodies, shows that bias was avoided in evaluating the effect of external version on antenatal immunization.

Only Rh-negative mothers examined from the first trimester of pregnancy were included so that the frequency of the first occurrence of Rh antibodies or subsequent increases in Rh antibody production could be measured.

Of the 227 cases selected 119 formed the external version series and 108 the control series. Only Rh-incompatible mother-child combinations were used. The indirect antiglobulin titre values of maternal Rh antibodies were divided into three groups: (i) those with values of 1:64

or less; (ii) 1:128, 1:256 and 1:512 and (iii) 1:1024 or more. The evaluation of Rh antibody titre differences recorded in the first trimester of pregnancy and before delivery would then indicate the intensity of Rh-immunization for that pregnancy.

Table 8 details the occurrence of episodes of Rh-immunization among the external version and control series of mothers. It shows a higher percentage of cases with initial production of Rh antibodies in the external version series (43.7% compared with 26.8%). Although the number of cases is small the difference is significant (X^2 (1) = 7.046; P = <0.01).

When the overall frequency of antenatal Rh-immunization is examined it is evident that the version cases not only show more frequent involvement in the secondary production of Rh-antibodies (among those already immunized) but also appear to produce higher titres than the control series (X^2 (1) = 17.025; P = < 0.001).

To assess whether the results in Table 8 may have been obtained by chance selection it was necessary to evaluate the frequency of primary evidence of episodes of Rh-immunization in a random series of hospital clinic admissions. The phrase 'primary evidence of Rh-immunization' refers to the observation that Rh-antibodies were detected for the first time at some stage during the second half of pregnancy among mothers who were confirmed not to have had Rh antibodies during the first half of the present pregnancy. Table 9 details this analysis. Of an unselected series of

TABLE 8

Comparison of the frequency of antenatal episodes of Rh immunization in Rh negative mothers on whom external version was carried out after 32 weeks gestation, and Rh negative mothers on whom such manipulations were not done

Maternal Rh antibody value during:		External version series: 119 mothers						Control series 108 mothers					
First trimester	At delivery of pregnancy	Positive episodes of immunization			No episodes of immunization			Positive episodes of immunization			No episodes of immunization		
		No.	Total	%	No.	Total	%	No.	Total	%	No.	Total	%
Negative	1:64 or less	20						16					
	1:128-1:512	13	52	43.7				8	29	26.8			
	1:1024 or more	19						5					
1:64 or less	1:64 or less				7						13		
	1:128-1:512	8	18	15.1		7	5.8	10	14	12.9		13	12.0
	1:1024 or more	10						4					
1:128-1:512	1:128-1:512		17	14.3	16	16	13.4		7	6.4	29		
	1:1024 or more	17						7				29	26.8
1:1024 or more	1:1024 or more				9	9	7.5				16	16	14.8
	Totals:		87	73.1		32	26.9		50	46.2		58	53.8

TABLE 9

The frequency of external version and antenatal immunization by the Rh factor observed in 6380 consecutive admissions for pregnancy

			Number and percentage of Rh negative mothers immunized by Rh factor	Rate of Rh immunization in total number of pregnancies
6380 consecutive pregnancy admissions	5260 Rh positive (82.5%)	External version performed 492 (9.5%)		1 in 151 pregnancies
		No external version performed 4768		
	1120 Rh negative (17.5%)	External version performed 113 (100.0%)	9 (7.9%)	
		No external version performed 1007	33 (3.2%)	

6 380 consecutive admissions, 5 260 mothers were classified as Rh positive (82.5%) and 1 120 as Rh negative (17.5%). The frequency of external version carried out after 32 weeks gestation did not differ significantly between Rh positive (9.5%) and Rh-negative (10.0%) mothers.

Of the 1 120 Rh-negative mothers tested routinely on an average of 2.8 occasions during the first half of pregnancy and on an average of 4.3 occasions during the second half of pregnancy, 42 (3.7%) produced Rh antibodies before the birth of their infants. This represents a rate of one case of Rh immunization out of every 151 random pregnancies. It was found that 7.9% of the external version series produced Rh antibodies compared with only 3.2% of the controls. For the total number of Rh-negative mothers examined the difference is significant (χ^2 (1) = 6.186; P = < 0.02). This observation indicates that the results presented in Table 8 are not due to chance selection and that the higher frequency of antibody production in patients subjected to external version is real.

The antibody-producing response in Rh negative volunteers deliberately transfused with Rh positive blood is known to vary significantly (Wiener, 1949; Waller 1949). Mollison (1956) suggests that 'some volunteers are readily sensitized, others are difficult to sensitize and some, perhaps, cannot be sensitized at all'. We have found further evidence to indicate that this situation also exists with respect to antenatal Rh immunization. Thus, whilst it has been shown that Rh antibody production is increased among those mothers who received external manipulation, a far

greater percentage (92.2%) (Table 9) of the Rh-negative female population at risk consistently fail to become immunized.

That Rh immunization cannot always be induced even among 'high risk' cases as a result of the delivery of an Rh-positive infant was also established by Woodrow et al. (1965). Even among their selected Rh-negative mothers who consistently had Rh incompatible fetal red cells in the maternal circulation, a larger percentage always failed to become immunized. It therefore seems evident that Rh immunization involves more than the single event of receiving Rh-incompatible blood, either by deliberate transfusions or by disruption of the placental circulation. The distinction between those who can be readily immunized and those who can not is a matter for future study. The susceptible class of Rh-negative mothers who have the ability to develop Rh-antibodies could be considered as a separate group capable of ready sensitization to primary exposure and also to subsequent episodes of immunization. This appears evident from the results we have obtained. In these mothers the intensity of Rh antibody production was significantly increased by the possible disruption of the placental circulation as a direct result of external version. A similar effect was also reported by Woodrow et al. (1965) with respect to transplacental haemorrhage as a result of delivery. In their studies a significantly greater percentage of mothers produced Rh antibodies during the post-natal period when transplacental haemorrhage had been evident.

While there may be differences of opinion with regard to the risk of increasing antenatal immunization by external version the possibility of intensifying the haemolytic process in the child as a consequence of this procedure cannot be ignored. Therefore, the policy of limiting all antenatal manipulations which might disrupt the placental circulation should be strictly maintained.

Chapter V

THE INFLUENCE OF ABO BLOOD GROUP INCOMPATIBILITY ON RH IMMUNIZATION

1. INTRODUCTION

Since Levine (1943) first observed the influence of the ABO blood group system and its relationship to Rh immunization during pregnancy, other investigators have confirmed that Rh-immunization in pregnancy is significantly less in ABO incompatible matings than in ABO compatible matings. This deficiency had been interpreted as being due to ABO compatible pregnancies providing a more favourable condition for the survival of Rh positive fetal red cells in the Rh negative mother (Levine, 1958). In ABO incompatible conceptions it is assumed that the presence of anti-A and anti-B in the maternal serum has an inhibiting effect on the production of Rh antibodies by the transplacental passage of fetal Rh positive red cells of blood groups A or B.

Because the prognosis of Rh-haemolytic disease is closely related to the maternal Rh antibody titre during pregnancy, the frequency of ABO incompatibility in relation to the maternal Rh antibody titre was examined using a standardized indirect antiglobulin technique, as described in Chapter II.

2. MATERIALS AND METHODS

The obstetric records of 836 Rh-immunized mothers were available for examination. Of these, 25 were rejected where previous transfusion of Rh-positive blood might

have caused Rh-sensitization. Another 80 Rh-immunized mothers were rejected since the paternal blood groups and Rhesus genotype were unknown. 85 unaffected Rh-negative infants compatible with their mother's Rh type were excluded. Forty-three Rh-immunized mothers found during the postnatal period were also rejected for two important reasons: (1) the maternal Rh-antibody titre could differ considerably from the antenatal value due to episodes of immunization induced by the trauma of parturition, and (2) the postnatal cases were initially diagnosed by the clinical manifestation of Rh-haemolytic disease and not by routine antenatal testing. This would increase the number of moderately and severely affected Rh cases in the random sample, while mildly affected Rh-positive infants without clinical signs of Rh haemolytic disease are not referred to the laboratory. A total of 603 mother, father, and infant combinations among Rh-immunized families remained as suitable for study.

In some instances when blood from stillborn infants could not be examined for their ABO and Rh status by the conventional method of testing, the ability of the red cell stroma to inhibit the appropriate antiserum was used to establish these factors.

For the evaluation of ABO compatible and incompatible matings in the control series 214 families with histories of normal deliveries were examined. Mothers with obstetric histories of spontaneous abortion were not included. As these cases were drawn from the same hospital they represented a population similar to that under study.

The second group of control cases used for the evaluation of ABO incompatible combinations was composed of Rh-negative mothers and their Rh-positive infants in whom the absence of Rh-sensitization was confirmed during antenatal testing and by the direct Coombs test on the infant's red cells at birth. 548 mother-infant combinations were examined in this category. The mean number of pregnancies experienced by these mothers was not significantly different from that in the mothers producing Rh antibodies (3.2 pregnancies per mother compared with 3.8 pregnancies per mother in the Rh-immunized series).

3. RESULTS

Table 10 compares the frequency of ABO compatible and incompatible combinations in Rh-immunized father-mother and mother-child combinations with a similar combination of non-immunized cases. As expected, the overall occurrence of ABO incompatible matings in the Rh-immunized families was lower than the values observed in the control series.

The distribution of ABO incompatible combinations in the control series (31.6%) compared with the value (32.8%) reported by Reepmaker, Nijenhuis and van Loghem (1962) who examined 1 210 Rh-negative non-immunized mothers and their husbands. The 24.0 per cent incidence of ABO incompatible matings recorded in the current series of 603 Rh-immunized families also compares favourably with the reported incidence of 24.7 per cent by Levine (1943). However, it differs from the value of 18.5 per cent reported by Reep-

TABLE 10

Combinations	Rh immunized families father-mother combinations		Families with normal deliveries father-mother combinations		Rh immunized mother and Rh positive child combinations		Rh negative non-immunized and Rh positive child combinations	
	No.	%	No.	%	No.	%	No.	%
ABO compatible								
O x O	153	25.3	40	18.6	199	33.0	180	32.8
O x A	117	19.4	54	25.2	67	11.2	48	8.8
O x B	34	6.1	8	3.7	24	3.9	20	3.7
O x AB	12	1.9	3	1.4				
A x A	118	19.5	33	15.4	180	29.9	154	28.1
A x AB	13	2.1	2	0.9	15	2.4	6	1.1
B x B	8	1.3	3	1.4	27	4.4	23	4.2
B x AB	2	0.3	2	0.9	8	1.4	4	0.7
AB x AB	1	0.1	2	0.9	5	0.8	4	0.7
	458	76.0	147	68.4	525	87.0	439	80.1
ABO incompatible								
A x O	86	14.4	32	15.0	50	8.3	64	11.8
B x O	17	2.8	12	5.8	9	1.5	12	2.2
AB x O	2	0.3	2	0.9				
AB x A	5	0.8	1	0.4	7	1.2	8	1.4
AB x B	1	0.1	1	0.4	4	0.7	8	1.4
A x B	17	2.8	10	4.8	5	0.8	11	2.0
B x A	17	2.8	9	4.3	3	0.5	6	1.1
	145	24.0	67	31.6	78	13.0	109	19.9
Totals:	603		214		603		548	

maker, Nijenhuis and van Loghem (1962) on 1 742 families immunized by the Rh factor.

The differences in the distribution of ABO incompatible combinations between mother and infant among the Rh-immunized series are smaller than the corresponding values observed for mother-infant combinations in the non-immunized Rh-negative mothers. The 13.0 per cent frequency of mother-child ABO incompatibility noted in the present Rh-immunized series is practically the same as the value of 12.6 per cent reported by Levine in 1958 but it differs considerably from the 7.3 per cent frequency reported by Reepmaker (1955) on 1 608 Rh-immunized mothers.

Table 11 tabulates the distribution of the 603 Rh-immunized families, classified according to the highest observed Rh-antibody titre, into three groups: (1) those with titres of 1:64 or less; (2) those with titres of 1:128, 1:256 and 1:512 and (3) those with titres of 1:1024 or more. The percentage of ABO compatible and incompatible matings is recorded for each Rh-antibody titre group together with the outcome of the last pregnancy.

The significant relationship between the maternal Rh-antibody titre value and the severity of Rh-haemolytic disease is compatible with the observations reported by Kelsall et al. (1957) and Kelsall, Vos and Kirk (1958).

Although the overall percentage of ABO incompatible matings in the 603 families with Rh-immunization is 24.0 per cent, it is interesting that this percentage varies from as high as 34.7 per cent in mothers producing Rh

TABLE 11

Distribution of ABO compatible and incompatible combinations in Rh negative mothers with various titres of Rh antibodies

Maternal Rh antibody titre at term*	Classification of infant**	ABO compatible matings		ABO incompatible matings		Total
		No.	%	No.	%	
Group 1	Livebirths	63	64.8	34	35.2	97
1:64 or less	Died	Nil		Nil		Nil
	Stillbirths	1		Nil		1
	All cases	64	65.3	34	34.7	98
Group 2	Livebirths	199	73.7	71	26.3	270
1:128-1:512	Died	7	70.0	3	30.3	10
	Stillbirths	12	80.0	3	20.0	15
	All cases	218	73.9	77	26.1	295
Group 3	Livebirths	92	84.4	17	15.6	109
1:1024 or more	Died	27	84.4	5	15.6	32
	Stillbirths	57	82.6	12	17.4	69
	All cases	176	83.8	34	16.2	210
All cases combined	Livebirths	354	74.3	122	25.7	476
	Died	34	80.9	8	19.1	42
	Stillbirths	70	82.3	15	17.7	85
	All cases	458	75.9	145	24.1	603

* Tested by the standardized indirect antiglobulin technique (Vos and Kirk 1958).

** Last born infants.

antibodies with titres of 1:64 or less, down to 16.2 per cent in those producing Rh-antibodies with titres of 1:1024 or more.

When the distribution of ABO compatible and incompatible matings among Rh-immunized mothers with low Rh-antibody titres (1:64 or less) was compared with those producing higher titres (1:1024 or more) it was confirmed that the distribution of ABO incompatibility in these two groups differed significantly (X^2 = 12.24; P = 0.001).

Table 12 compares the same three groups of Rh-antibody titre values with the frequency of ABO incompatible combinations of the last born Rh-positive child. For ease of comparison this table also shows the ABO incompatibility of father-mother combinations as recorded in Table 11. The results of these two sets of combinations detail the mangitude of selection in Rh-antibody titre values, and, whilst ABO incompatibility between fathers and mothers appears twice as great (34.7% as opposed to 16.2%) in the lower Rh-antibody titre group (1:64 or less) when compared with the results observed in the higher titre group (1:1024 or more), this difference is at least five times greater (26.5% as opposed to 3.7%) when the actual ABO incompatibility status of the mother and child is taken into account. The 26.5 per cent value of mother-child ABO incompatible combination observed in the lower titre group of Rh-immunized mothers is also greater than the values recorded for the control series of non-immunized mother-child combinations, which was 19.9 per cent.

TABLE 12

The relationship of maternal Rh antibody titre values to ABO incompatible combinations, zygosity of the father's Rh genotype and mean number of pregnancies

Maternal Rh antibody titre at term and total number of mothers examined	Frequency of father-mother ABO incompatible combinations		Frequency of mother-child ABO incompatible combinations		Father's D antigen zygosity in relation to ABO compatible and incompatible matings								Total and mean no. of pregs. by Rh immunized mother	
					ABO compatible				ABO incompatible					
					D/D		D/d		D/D		D/d			
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	Mean value
Group 1														
1:64 or less (98 mothers)	34	34.7	26	26.5	31	46.9	35	53.1	15	46.8	17	53.2	352	3.6
Group 2														
1:128-1:512 (295 mothers)	77	26.1	42	14.2	117	53.6	101	46.4	44	57.1	33	42.9	1121	3.8
Group 3														
1:1024 or more (210 mothers)	34	16.2	10	4.7	112	64.3	62	35.7	28	77.7	8	22.3	882	4.2
All cases combined: (603 mothers)	145	24.1	78	13.0	260	56.7	198	43.3	87	60.0	58	40.0	2355	3.8

When the distribution of mother-child ABO incompatibility was compared between mothers with low Rh antibody titre values (1:64 or less) and those with higher titre values (1:1024 or more) it was confirmed that mother-child ABO incompatibility differed significantly in the two categories of Rh-immunized mothers (χ^2 = 20.31; P = 0.001).

The same table also compares the relationship of maternal Rh-antibody titre values with the father's D antigen zygosity status. These results were obtained by the conventional use of specific D,C,E,c and e antisera, while the genotypes were assigned on the basis of reactions against these sera, using the table of most frequent occurrence (Race and Sanger, 1962). The zygosity distribution among ABO compatible and incompatible matings does not appear to differ in mothers with Rh antibody titres of 1:64 or less (ABO compatible 46.9% D/D and 53.1% D/d; ABO incompatible 46.8% D/D and 53.2% D/d), but marked variations were seen in those with Rh antibody titres of 1:1024 or more (ABO compatible 64.3% D/D and 35.7% D/d, ABO incompatible 77.7% D/D and 22.3% D/d) to implicate homozygosity (D/D) alone as a most suitable condition for the production of high titre Rh antibodies even among ABO incompatible matings.

The overall distribution of homozygous D/D fathers also differed significantly in the low titre group of Rh-immunized mothers (1:64 or less) when compared with the frequency of their occurrence in the high-titred group of mothers (1:1024 or more) (χ^2 = 10.06; P = 0.001).

When the influence of the mean number of pregnancies on

the variations in Rh-antibody titre was studied (Table 12) it was noted that there was a slight increase in pregnancies where the maternal Rh-antibody titre was 1:1024 or more. These higher values are most probably associated with the mother's increased desire to give birth to a live-born child, particularly in a category where the fetal wastage due to stillbirth and neonatal death are significantly greater than among the lower titre group of mothers (Table 11). The distribution of parity does not otherwise appear to be associated with the intensity of Rh-antibody titre values in the mother.

When the total number of pregnancies among the low titre group of Rh-immunized mothers was compared with the numbers of pregnancies observed in the high titre group of mothers, no significant deviations were observed ($X^2 = 2.77$; $P = 0.09$).

Rh-immunized mothers with previous histories of spontaneous abortion were also investigated in relation to their maternal Rh-antibody titre value (Table 13). Of the 603 mothers in this series, a total of 60 (9.9%) had a history of early abortion. It was interesting to find that the greater percentage of mothers with previous history of abortion (22.4%) fell into the lowest Rh-antibody titre group (1:64 or less). This was different from the Rh-immunized mothers with antibody titre values of 1:1024 or more, where only 6.1% of mothers had experienced one or more abortion.

A differential study of the frequency of abortion in relation to ABO compatible and incompatible matings among

TABLE 13

Incidence of Rh immunized mothers with history of abortion in relation to ABO compatible and incompatible matings

Maternal Rh antibody titre at term	ABO compatible matings				ABO incompatible matings				All cases combined		
	Families without history of abortion		Mothers with history of abortion		Families without history of abortion		Mothers with history of abortion		Number of families	Mothers with history of abortion	
	No.	%	No.	%	No.	%	No.	%		No.	%
Group 1											
1:64 or less	52	81.2	12	18.1	24	70.6	10	29.4	98	22	22.4
Group 2	198	90.8	20	9.2	72	93.5	5	6.5	295	25	8.4
1:128-1:512											
Group 3											
1:1024 or more	164	93.1	12	6.9	33	97.0	1	3.0	210	13	6.1
All cases combined:	414	90.4	44	9.6	129	88.9	16	11.1	603	60	9.9

Rh-immunized mothers showed that the occurrence of abortion appeared to be the same in both combinations.

The less frequent occurrence of abortions in the higher Rh-antibody titre category of mothers primarily suggests that the intense production of Rh-antibodies may have a suppressing effect on the synthesis of antibodies associated with the aetiology of abortion.

For ABO compatible father-mother combinations the frequency of spontaneous aborters among the low titre group of Rh-immunized mothers differed significantly from the high titre group ($X^2 = 6.16$; $P = 0.013$). This difference was also significant for the ABO incompatible matings ($X^2 = 6.94$: $P = 0.009$). However, in the overall occurrence of aborters between ABO compatible and incompatible combinations, the difference was not significant ($X^2 = 0.18$; $P = 0.7$).

Table 14 shows the distribution of mother-father and mother-child ABO incompatible combinations in relation to their maternal Rh-antibody titre value. These results are tabulated to show that the frequencies of ABO incompatibility in the two series do not differ significantly from each other. The random frequency of ABO incompatible mother-child combinations observed among non-immunized mothers is included in the same table to show the degree of variation existing for the two series examined in the same population.

The rather remarkable differences observed in the distribution of ABO incompatible mother-child combinations

TABLE 14

Rh antibody titre values for ABO incompatible combinations of Rh immunized families

Maternal Rh antibody value at term	Mother-father ABO incompatible combinations		Mother-child ABO incompatible combinations		Non-immunized (control) series ABO incompatible combinations	
	Present series	Previous series	Present series	Previous series	Mother-father	Mother-child
Group 1						
1:64 or less	43 = 39.0%	34 = 34.7%	32 = 29.0%	26 = 26.5%		
Group 2						
1:128-1:512	59 = 24.0%	77 = 26.1%	35 = 14.2%	42 = 14.2%		
Group 3					31.6%	19.9%
1:1024 or more	24 = 14.2%	34 = 16.2%	14 = 8.4%	10 = 4.7%		
All cases:	126 = 24.2%	145 = 24.0%	81 = 15.5%	78 = 13.0%		

between the group (i) series of Rh-immunized mothers (29%) and the group (iii) series (8.4%) demonstrates clearly the effect on maternal Rh antibody production when immunization is induced by both ABO and Rh-incompatible fetal red cells.

Table 15 evaluates the number and percentage of ABO compatible and ABO incompatible mother-child combinations among Rh incompatible conceptions followed throughout the antenatal period for the behaviour pattern of Rh immunization. In 195 cases the first evidence for the production of Rh antibodies was observed during the second or third trimester of pregnancy in patients known not to be sensitized in the first trimester. A total of 30, or 15.3 per cent of the pregnancies resulted in the delivery of ABO incompatible infants.

These findings confirm that ABO incompatibility does not prevent Rhesus negative mothers from producing Rh antibodies. Considering that the random frequency of ABO incompatibility in non-immunized mothers observed in the same population area is only 19.9 per cent (Table 14), the much higher percentage recorded among Rh-immunized mothers with Rh antibody titre values of 1:64 or less, either during the initial stage of Rh antibody production (26.5%) or in mothers who were already immunized prior to the present pregnancy (32.6%) suggests that combined ABO and Rh incompatibility may in fact enhance the mother's potentiality to become immunized. If this is so, then the present explanation for the much lower frequency of ABO incompatibility observed among the high titred (1:1024 or

TABLE 15

Maternal Rh antibody titre values during the first and third trimesters of pregnancy in relation to the frequency of mother-child ABO compatible and incompatible combinations

Maternal Rh antibody values during:		Mother-child ABO blood group combination			
First trimester of pregnancy	Third trimester of pregnancy	ABO compatible	ABO incompatible		Total
Negative	1:64 or less	47 = 73.5%	17 = 26.5%	64	
	1:128-1:512	78 = 88.7%	10 = 11.3%	88	195
	1:1024 or more	40 = 93.0%	3 = 7.0%	43	
1:64 or less	1:64 or less	31 = 67.4%	15 = 32.6%	46	
	1:128-1:512	47 = 85.5%	8 = 14.5%	55	125
	1:1024 or more	24 = 100.0%	0 = 0.0%	24	
1:128-1:512	1:128-1:512	85 = 83.4%	17 = 16.6%	102	150
	1:1024 or more	43 = 89.6%	5 = 10.4%	48	
1:1024 or more	1:1024 or more	44 = 88.0%	6 = 12.0%	50	50
Totals		439 = 84.5%	81 = 15.5%		520

more) Rh-immunized mothers cannot be accepted as the result of direct interaction of maternal anti-A or anti-B antibodies on ABO and Rh-incompatible fetal red cells.

One possible explanation is that when the maternal antibody-forming mechanism is called on to produce two different types of antibodies (anti-A or anti-B which are generally associated with haemolysin production and anti-Rh which is not), it may do so but with reduced ability to form high titred Rh-antibodies.

The possibility that Rh-immunized mothers producing high titred Rh-antibodies possess the ability to select at the cervical level conceptions which are ABO compatible rather than ABO incompatible, as postulated among classical infertility conditions by Behrnman, Buettner-Janusch, Heglar, Gershowitz and Tew (1960) and Vos (1965), must remain in doubt until the incidence of immune anti-A or anti-B haemolysins can be assessed among Rh-immunized mothers.

Levine's hypothesis (1943) that ABO incompatible Rh-positive fetal red cells are more likely to be eliminated from the maternal circulation before mothers can respond to the production of Rh-antibodies is not compatible with the findings in this analysis. However, our observations are in agreement with others (Nevahlinna and Vainio (1956), Murray, Knox and Walker (1965) that fetal ABO incompatibility among Rh-immunized mothers affords a significant degree of protection against the occurrence of severe manifestations of Rh-haemolytic disease in the newborn.

This protection appears to be based upon the mother's inability to produce higher titres of Rh-antibodies in approximately 80 per cent of the ABO incompatible combinations examined.

4. DISCUSSION

This investigation has shown that differences in the distribution of ABO incompatible matings not only occur between Rh-immunized families and the control series, but also can be expected when the maternal response to the production of Rh antibodies is taken into account. These differences range from the normal random distribution of ABO incompatible matings where the maternal Rh-antibody titre value is 1:64 or less (26.5%) down to values frequently reported by others when the Rh antibody titre is 1:1024 or more (12.0%).

ABO incompatibility among Rh immunized families has been reported to range between 24.7% (Levine, 1953) and 13.0% (Stern, Davidsohn and Masaitis, 1956). There has been no information to indicate that such wide variations may in some instances be influenced by the inclusion of a greater proportion of severe cases of Rh incompatibility with mothers possessing higher Rh-antibody titres than would normally be expected in a random selection of Rh-immunized mothers. This situation can occur when the Rh-immunized families used for this type of investigation are composed of a large number of Rh cases which are brought to the attention of the investigator because of the obvious clinical manifestation of Rh-haemolytic dis-

ease, while mild forms of Rh-disease in infants not requiring treatment are not included. The use of Rh-immunized families detected by routine antenatal testing alone would therefore appear to be the only approach that would ensure that the normal distribution of mild and severe forms of Rh-haemolytic conditions are included in an analysis of this nature. By observing this rule of selection we found that the frequency of father-mother and mother-child ABO incompatible combinations did not differ significantly in mothers producing low-titred Rh-antibodies (1:64 or less) when compared with the distribution of these combinations in non-immunized mothers.

Where the Rh-immunized mothers produced high titred (1:1024 or more) Rh antibodies the frequency of mother-child ABO incompatibility differed significantly from the random results (4.7% as opposed to 19.9%). The wide variations of mother-child ABO incompatible combinations reported by Levine (1958) of 12.6% and Lucia and Hunt (1950) of 4.4% can most likely be attributed to the marked differences in the maternal Rh antibody titres of the mothers investigated in the two series.

The influence of the father's D antigen zygosity on maternal Rh antibody titres showed the expected higher frequency of D/D matings in mothers with Rh antibody titres of 1:1024 or more, particularly among the ABO incompatible matings (ABO compatible: D/D, 64.3% and D/d, 35.7%; ABO incompatible: D/D, 77.7% and D/d, 22.3%), suggesting that group A or B homozygous D/D fathers among

the incompatible combinations possess a greater potentiality to induce the formation of high titre Rh-antibodies than heterozygous D/d fathers of the same blood groups.

The effect of parity on the maternal Rh-antibody titre response does not appear to be significant and confirms the general observation that mothers with only two previous pregnancies can sometimes possess extremely high Rh antibody titres while those with at least six pregnancies may possess lower Rh-antibody values.

The belief that ABO incompatible matings in Rh-haemolytic disease are a sound protection against Rh-immunization was only confirmed in mothers with high titre Rh-antibodies. The normal frequency of ABO incompatible matings found in either father-mother or mother-child combinations among those producing lower values (1:64 or less) of Rh-antibodies provided evidence to suggest that the transplacental passage of fetal ABO incompatible Rh-positive red cells may not necessarily prevent the mother from being immunized. The protective mechanism assumed by ABO incompatibility does not appear to be of any significance in this category of Rh-immunized mothers.

Chapter VI

THE EVALUATION OF THE INDIRECT ANTI-HUMAN GLOBULIN TITRE AS A PROGNOSTIC INDEX

1. RH ANTIBODY LEVELS IN THE MATERNAL SERUM

A most important factor observed from comparing Rh antibody titres with the clinical severity of the disease has been the wide variations of results that different investigators obtain. This is probably caused from lack of standardization in technique and methods adopted. Of the several methods known to determine the presence of Rh antibodies (saline test, enzyme test, albumin replacement test and anti-human globulin test), we have found the indirect antiglobulin test to be the most dependable (Vos, 1969).

Using the standardized indirect anti-human globulin test (described in Chapter II), the relationship between the Rh antibody titre of the mother at delivery and fetal wastage was examined. 429 infants were studied where Rh antibodies were detected in the mother's serum. All cases were included in which an antiglobulin titration of the mother's serum was carried out either on the day of birth, or within two or three days prior to birth, and where the pregnancy continued for at least 20 weeks. 56 (13.0%) of the 429 infants were Rh-negative. Of the remaining 373 Rh-incompatible infants, 65 (17.4%) were stillborn and 308 (82.6%) were liveborn. Thirty-four of the liveborn infants (9.1% of the incompatible infants) subsequently died, 20 of them despite prompt and adequate exchange-transfusion. The other 14 were untreated, having died

before exchange-transfusion could be started. Four of these untreated infants were of 30 weeks or less gestation. Table 16 shows the distribution of these cases by the indirect antiglobulin titre of the mother's serum at term. The fetal wastage from all causes rises from 1.7 per cent where the maternal titre is 1:64 or less to 63.4 per cent where the titre is 1:2048 or more. Infants born to mothers with titres of 1:64 or less, with the exception of four cases, received no treatment.

Almost all of the fetal wastage in this series occurred in cases where the maternal antiglobulin titre at term was 1:512 or more. The remaining cases, although indicating that stillbirth or neonatal death may occur where the maternal titre is lower than 1:512, have to be considered in the light of the actual causes of death. Of the four stillbirths in the lower-titre categories, two at least were not due primarily to Rh-incompatibility, one being a mongol and the other an anencephalic monster. There were six neonatal deaths in the same titre categories. One of these was of only 29 weeks' gestation, was untreated, and died primarily of prematurity; another died at the age of four days with an obstruction of the duodenum, whilst one died at home aged three weeks, the cause of death being septicaemia. Thus of the 10 cases where a stillbirth or a neonatal death resulted if the mother had an antiglobulin titre at term of less than 1:512, only five were due primarily to Rh-incompatibility. This means that in this series 95 per cent of the fetal wastage due to Rh-incompatibility occurred in mothers with an indirect antiglobulin titre at

TABLE 16

Distribution of 373 cases of incompatible infants born to Rh-immunized mothers by maternal indirect antiglobulin titre at term

Maternal titre at term	No. of cases	Liveborn Rh+ infants		Stillbirths	% Foetal wastage
		Survived	Died		
1:64 or less	60	59	0	1*	1.7
1:128	30	28	1	1**	6.7
1:256	60	53	5	2	11.7
1:512	70	62	4	4	11.4
1:1024 ..	82	46	9	27	43.9
1:2048 or more	71	26	15	30	63.4
All titres:	373	274 (73.5%)	34 (9.1%)	65 (17.4%)	

* Mongol ** Anencephalic monster

term of 1:512 or more.

Although there is a close relationship between the maternal indirect antiglobulin titre at term and the chance of having a successful outcome of pregnancy, this by itself does not mean that early induction of labour should be carried out if the maternal titre at 36 weeks is 1:512, because it could remain at this level during the next four weeks. The relationship between maternal indirect antiglobulin titre at delivery and outcome, as shown in Table 16, indicates the chance of successful outcome in such cases to be greater than 90 per cent. However, the picture obtained from considering the relationship of a single maternal indirect antiglobulin titration at term and the chance of survival can be most misleading. It does not indicate whether the titre determined at the time of delivery has just arrived at that level, perhaps quite suddenly from a low or zero value, or whether the fetus has been exposed to the same quantity of antibody for a considerably longer length of time.

In 222 Rh-immunized mothers with Rh-incompatible pregnancies, antibody titrations were carried out weekly or fortnightly during the last 10 weeks of pregnancy. This made possible the calculation of a 'titre index' to give some measure of the effect of the titre level and the duration of the response on the severity of the disease in the affected child. The titre index is calculated by multiplying the titre code number by the number of weeks and summing for the total 10-weeks period. The code numbers used range from 1, for a titre of 1:16 or less, 2,

for 1:32, 3, for 1:64, etc., to 9, for a titre of 1:4096 or more. Thus a mother who has maintained a titre of 1:1024 over the whole 10-weeks period will have a titre index of 7x10 = 70. On the other hand, a mother who has a titre of 1:1024 at term but started the 10-weeks period with a titre of 1:64 and rose progressively each two weeks up to this titre will have an index of 2x3 + 2x4 + 2x5 + 2x6 + 2x7 = 50.

The distribution of the 222 cases in four categories of titre index where this information is available is given in Table 17. Where the titre index is 40 or less, as it was in 87 of the cases (39%), the chance of death is only 3.4 per cent. The one stillbirth was the mongol referred to previously. If the titre index is between 41 and 60 the chance of death is 23.9 per cent and from 61-70 it is 59 per cent. If the titre index is above 70, as it was in 25 of the cases, the chance of death is 80 per cent.

On the basis of these results it is possible to estimate the increase in severity which will occur if a pregnancy is allowed to continue for any particular length of time. For example, if a mother has reached 36 weeks and has a titre of 1:1024 and during the previous six weeks her titre score has reached 40, then even if her titre remains unchanged she will accumulate 4x7 = 28 points to give a final titre index of 68 if the pregnancy continues to full term. This means that there is almost a 50 per cent chance that the infant will be stillborn, or, if liveborn, will be very seriously affected, with a 30 per

TABLE 17

Relationship between maternal indirect antiglobulin index and outcome of pregnancies of Rh-sensitized mothers

See text for method of calculating the index

Indirect antiglobulin index	No. of cases	Liveborn Rh+ infants		Stillbirths	% Foetal wastage
		Survived	Died		
40 or less	87	84	2	1*	3.4
41-60 ..	71	54	8	9	23.9
61-70 ..	39	16	3	20	59.0
71 and over	25	5	4	16	80.0
All indexes	222	159	17	46	28.4

* Mongol

cent chance of death. In some of the liveborn cases death will occur before exchange-transfusion can be started. It is clear, therefore, that accurate knowledge of the maternal antibody level and its behaviour from the 26th week of pregnancy onward enables one to predict before birth the degree of severity of the disease in the affected child with a high degree of accuracy.

2. THE RELATIONSHIP OF MATERNAL INDIRECT ANTIGLOBULIN TITRE, CORD-BLOOD HAEMOGLOBIN AND RETICULOCYTE PERCENTAGE IN THE PROGNOSIS OF RH HAEMOLYTIC DISEASE

This series comprises 218 consecutive pregnant women in whom anti-Rh antibodies were detected by the indirect antiglobulin technique. 24 women gave birth to Rh-negative infants. In 35 cases the pregnancy ended in a stillbirth and in the remaining 159 cases an Rh-positive infant was born alive. The cells of these infants when examined immediately after birth gave a direct Coombs positive result in each case.

Values for cord-blood haemoglobin were determined using an MRC grey wedge photometer. Statistical analysis revealed no significant difference between different observers determining haemoglobin by this method. Reticulocyte percentages were determined according to the technique of Steensma (1955). Of the total of 159 Rh-positive infants of immunized mothers born alive in the present series, estimations of cord-blood haemoglobin were made in 147 and reticulocyte percentage in 145.

In an attempt to increase the prognostic value of the indirect antiglobulin titre, this titre was correlated

with the value for cord-blood haemoglobin of liveborn Rh-positive infants. The results for the 147 cases for which both sets of information were available and for the various treatment categories are shown in Figure 2. When the value for cord-blood haemoglobin results are plotted against the logarithm of the indirect antiglobulin titre a significant correlation is evident ($r = -0.612$).

Figure 2 shows that all but three of the 19 deaths occurred among babies born to mothers whose indirect antiglobulin titre was 1:1024 or higher. These 16 deaths represent 30.5 per cent of all the cases in this category. Similar observations were also made for babies born with cord-blood haemoglobin values of 10 gm/100 ml or less. There were 17 deaths in this category, representing 40.5 per cent of the cases. It is clear, then, that although the maternal indirect antiglobulin titre at delivery is highly correlated with values for cord-blood haemoglobin, it is not an ideal indicator for forecasting the severity of the disease. However, because the antiglobulin titre of the maternal serum is determined before birth it is a valuable guide for the management of the case. Under these circumstances a slight loss in efficiency may be more than compensated by the practical value of inducing labour at the earliest time consistent with safety factors involved in premature birth if the mother's titre is rising toward the level of 1:1024.

Pickles (1949) considers that the number of immature cell types present during the first day of life is of considerable prognostic significance in cases of haemoly-

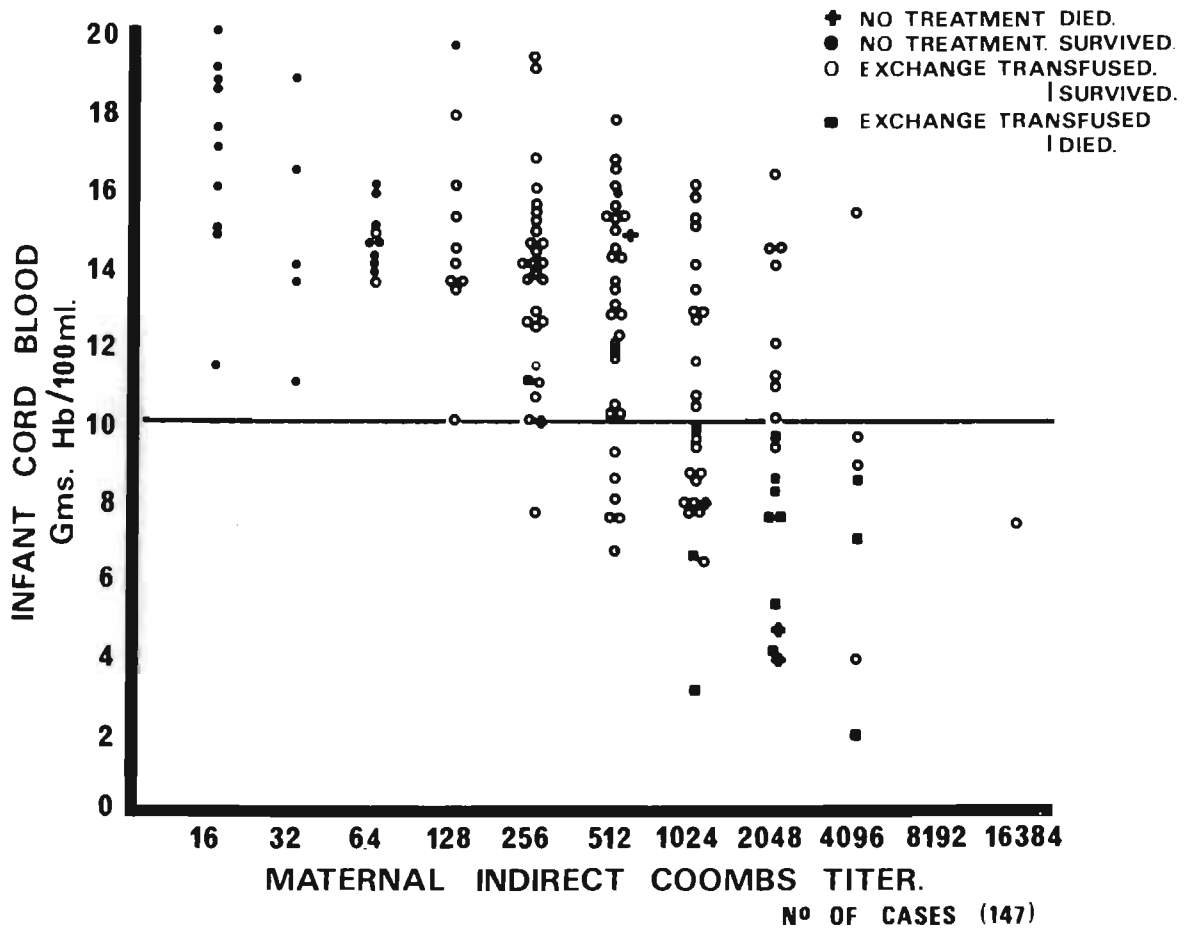

Figure 2: The distribution of 147 cases of 'incompatible' infants born alive to immunized mothers, by values for cord-blood haemoglobin and the maternal antiglobulin titre at term.

tic disease of the newborn. Figure 3 reveals, as expected, an inverse correlation which is highly significant between haemoglobin values and reticulocyte percentage in the cord bloods of 145 Rh-positive infants born alive to Rh-immunized mothers. The data in Figure 3 is best fitted by a non-linear curve, the equation for which is $Hb = 16.429 - 0.299X + 0.002X^2$ where X is the reticulocyte percentage.

In this series, 17 of the 19 deaths had values for cord-blood haemoglobin of 10 gm/100 ml or less. But of these deaths 15 occurred among the 25 cases with a reticulocyte percentage of 28 or higher. It is clear that reticulocyte counts can be used in conjunction with values for cord-blood haemoglobin to give a good index of the chance of survival. Used alone, however, the reticulocyte percentage is not quite such a good prognostic index as cord blood haemoglobin. If a discrimination is based on a reticulocyte percentage of 20 or higher, which includes 16 of the 19 deaths in the series, and is therefore comparable with a discrimination based on an indirect antiglobulin titre of 1:1024 or higher, the number of deaths represents 37 per cent of the cases in the category. However, reticulocyte percentages, like values for cord-blood haemoglobin, suffer from the disadvantage that their use for prognostic purposes must wait upon the birth of the child.

Mollison and Cutbush (1951) correlated values for cord-blood haemoglobin in their series with concentration of bilirubin. They concluded that predictions based on values for cord-blood haemoglobin and concentration of bili-

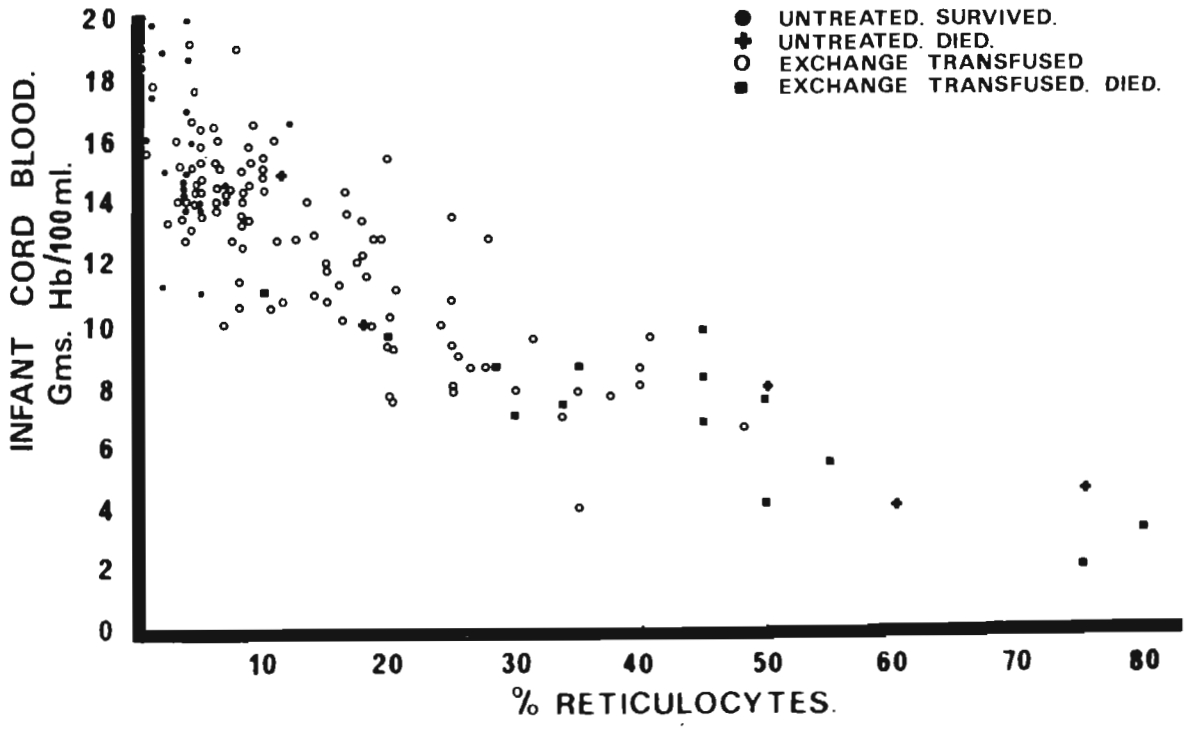

Figure 3: The distribution of 145 cases of 'incompatible' infants born alive to immunized mothers, by values for cord-blood haemoglobin and reticulocyte percentage.

rubin combined are not significantly better than those based on haemoglobin values alone. Our data confirmed this conclusion and this particular analysis was not carried further.

It is clear that considered separately the indirect antiglobulin titre of the maternal serum at term is not quite so good a prognostic tool as the values for cord-blood haemoglobin or the reticulocyte percentage. However, it does possess the real advantage of giving an antenatal prognosis of severity and can therefore be used as a guide for timing the induction of labour. Once the child is born prognosis can be based on a combination of antibody titre, cord-blood haemoglobin and reticulocyte percentage. The distribution of cases in Figure 2 shows that a high maternal antibody titre with a low value for cord-blood haemoglobin is far more serious than a similar titre with a haemoglobin value above 10 gm/100 ml. It is also obvious from the distribution of cases in Figure 3 that the prognosis is serious if the child is born with a reticulocyte percentage above 20.

Table 16 shows that 93 per cent of infants survive when the indirect antiglobulin titre of the maternal serum is less than 1:128. As the titre increases so also does the number of stillbirths and neonatal deaths in the treated and untreated cases.

PART TWO

THE PARTIAL ABSORPTION TEST

Chapter VII

THE PARTIAL ABSORPTION (PA) TEST FOR TITRATING RH ANTIBODIES

1. INTRODUCTION

The main reason for carrying out serological tests on the Rh-immunized mother during pregnancy is to provide the obstetrician with a reliable indication of the severity of the haemolytic process in the fetus. Ideally he would like to know before the infant is born whether it will be mildly or severely affected; whether if the pregnancy is allowed to continue to its natural term the fetus will be liveborn or stillborn, and whether, if the father has been shown to be a heterozygote the fetus is Rh-positive or Rh-negative.

Previous studies (Part I, Chapter VI), have shown that the titre of Rh-antibodies in the maternal serum, when determined by the indirect antiglobulin method, is highly correlated with the severity of the haemolytic process in the child. An indirect antiglobulin titre at term of 1:64 or less is of no pathological significance and in such cases almost all infants survive without treatment.

As the indirect antiglobulin titre increases, so does the severity of the disease, and even though the infant may be born alive and exchange-transfused, the chance of survival is less. The majority of stillbirths due to Rh incompatibility are associated with maternal indirect antiglobulin titres of 1:1024 or more. The indirect antiglobulin titre has therefore been found to be a valuable guide

for determining whether early induction of labour is necessary. However, the indirect antiglobulin titre alone can sometimes give rise to misleading predictions. In some cases an antiglobulin titre of 1:1024 may be associated with cord haemoglobin values well within the normal range in the newborn infant. At the other extreme, the same titre may be associated with a moribund infant having a haemoglobin of 4 grammes/100 millilitres and a reticulocyte count of 50%; or, even more misleading, a mother may have an indirect antiglobulin titre of 1:1024 throughout pregnancy, only to be induced and delivered of an Rh-negative infant. A similar serological picture in another case may result in a macerated stillborn fetus at 35 weeks. Detailed studies carried out to investigate these discrepancies revealed that if the newborn infant has no, or little free Rh antibody in circulation, the case is always mild; whereas the most severe cases are associated with high indirect antiglobulin titres of free Rh antibody in the infant's serum. Attention was focussed, therefore, on the absorbing capacity of adult non-sensitized Rh-positive cells toward maternal sera from mild and severe cases. It was found that when one volume of maternal serum, obtained on the day of delivery, was incubated with one volume of adult Rh-positive cells, the amount of free antibody left behind in the serum was almost always identical with the amount found in one volume of the cord-serum of the infant. Further observations showed that it was not necessary to use intact Rh-positive cells for carrying out this test, as identical results were obtained if one volume of a sus-

pension of haemolysed adult Rh-positive cells was substituted for the intact cells.

On the basis of these findings we developed a new antibody test which, when used in conjunction with the indirect antiglobulin test, enables a much more accurate prognosis of the outcome of pregnancy to be made. The test is referred to as the partial absorption (PA) test. The relationship of this test to other tests of Rh antibody determinations currently in use, and its behaviour during pregnancy was investigated.

2. MATERIALS AND METHODS

Antibody titrations - Indirect antiglobulin titrations were carried out as described in Chapter II. The blocking and conglutination tests were performed using the method of Wiener and Hurst (1947) and Wiener, Hurst and Sonn (1947). Albumin titrations followed the procedure of Diamond and Denton (1945).

Preparation of standard haemolysed cell suspensions - Heparinized or oxalated blood was collected from the arm veins of healthy Group O, CDe/CDe (R_1R_1) or Group O, CDe/cDE (R_1R_2) donors. The cells were packed and washed three times in large volumes of physiological saline, the samples pooled and the cells finally packed by centrifuging at 3 000 rpm for 30 minutes. An accurate 25 per cent saline suspension of the packed cells was made and this was divided into two-millilitre samples in screw-cap McCartney bottles ready for daily use. This standard suspension of cells was haemolysed by freezing to -20° C for 48 hours.

These haemolysed cell suspensions may be stored for at least twelve months at this temperature without detectable loss of absorbing capacity.

Procedure for the partial absorption (PA) test - The standard haemolysed cell suspension was thawed to room temperature and shaken thoroughly before use. 0,4 millilitre of this suspension was added to 0,4 millilitre of a 1:4 saline dilution of the maternal serum in a small test tube. The two solutions were mixed by shaking and left at room temperature for 15 minutes. The free Rh antibody remaining in the mixture was titrated in doubling dilutions using the indirect antiglobulin technique. The first tube in this titration was read as 1:16, since this is the dilution at this stage of the original maternal serum. Partial absorption titres lower than 1:16 have been found to be of no significance and were scored as negative.

3. RESULTS

The partial absorption titre of the maternal serum and the free Rh antibody in the infant's serum.

In Table 18 the PA titre of the maternal serum at the time of delivery is compared with the titre of free antibody in the infant's cord blood as determined by the indirect antiglobulin method. A total of 187 cases was available where the infant was liveborn, Rh-positive, and in which both titrations had been carried out. In all these cases the infant's cells gave a positive direct response to the Coombs test at the time of birth. In 166 of the cases (89%) the PA titre of the maternal serum was either identical with, or within one tube dilution of the

TABLE 18

Comparison of maternal partial absorption titre at the time of birth with titre of free antibody in the cord serum

Tube dilutions of maternal titre compared with cord serum titre[1]	Number	Percentage
-3	1	0.5
-2	6	3.2
-1	29	15.5
0	109	58.3
+1	28	15.0
+2	4	2.1
+3	7	3.7
+4	3	1.6
All cases	187	99.9

1 "0" = maternal PA titre identical with indirect antiglobulin titre of cord serum.

-1,-2,-3 = maternal PA titre one, two or three tube dilutions below indirect antiglobulin titre of cord serum.

+1,+2,+3,+4 = maternal PA titre one, two, three or four tube dilutions above indirect antiglobulin titre of cord serum.

titre of free Rh antibody in the cord serum. In a further 10 cases the two titration values did not differ by more than two dilution tubes.

Of the 11 cases in Table 18 where the maternal PA titre differs from the free antibody titre in the cord-serum by more than two dilution tubes, one mother was grossly oedematous, and five infants were oedematous at birth; simple dilution may account for part at least of the differences. In the five cases in which oedema was not present in the infant no satisfactory explanation can be offered for the discrepant results.

Despite the wide range of clinical conditions in the infants in this series, the PA titre of the maternal serum at the time of delivery in 90 per cent of the cases was within the limits of one tube dilution, the same as the free antibody titre of the infant's cord serum. This fact suggests that in the majority of cases the PA titre may be used during the course of pregnancy as a reliable indicator of the amount of free antibody in the fetal circulation.

Comparison of the partial absorption test with conglutination, albumin, blocking and indirect antiglobulin tests

Cases were chosen to compare the PA titre with the blocking, conglutination, albumin and indirect antiglobulin titres as determined by standard techniques. The results of ten cases are given in Table 19. No correlation was observed between the PA titre and the plasma conglutination or albumin titres. There is a correlation, however, between the PA titre and either the blocking

TABLE 19

Comparative tests on maternal Rh sera

Case Number	Partial Absorption titre	Blocking titre	Indirect antiglobulin titre	Plasma congl-utination titre	Albumin titre
302	1:2048	1:64	1:4096	1:16 tr	1:1024
213	1:1024	1:64	1:4096	1:64 tr	1:256
273	1:512	1:64	1:2048	1:256tr	1:512
261	1:256	1:4	1:4096	1:1024	1:512
342	1:256	1:8	1:2048	1:128	1:512
267	1:256	1:4	1:1024	1:512	1:4096
231	Negative	Negative	1:512	1:1024	1:2048
278	Negative	Negative	1:512	1:256	1:4096
274	Negative	Negative	1:256	1:64	1:2048
340	1:8	Negative	1:128	1:1024	1:512

or the indirect antiglobulin titre. These relationships were then examined more thoroughly on a larger series of cases. Table 20 shows the results for 42 samples of maternal sera examined. There is a significant positive correlation between both PA titre and blocking titre, and PA titre and indirect antiglobulin titre, but the correlation coefficient ($r = 0.90$) for the former is slightly higher than for the latter ($r = 0.83$).

Another interesting observation was that although the PA titre was correlated with the blocking titre, the method was more sensitive than the blocking test. In some cases indirect PA titres as high as 1:256 were recorded even though the results of the blocking tests were still negative. In this series of cases the highest blocking titre observed was 1:128.

Studies carried out to compare the PA titre with methods of determining Rh-antibody by various enzyme tests (ficin, papain and bromelin), failed to show a positive correlation between these two procedures.

Behaviour of the partial absorption titre during pregnancy

Examination of detailed case histories in which the maternal PA titre was determined at regular intervals throughout pregnancy revealed certain basic patterns in the PA titre response associated with clinical outcome, ranging from mildly affected infants to grossly hydropic stillborn infants. Since the pattern of response is sometimes more informative than a single titration value obtained near term, typical examples are described.

TABLE 20

Partial absorption titre, blocking titre and indirect antiglobulin titre for various maternal sera

Number	Partial Absorption titre	Blocking titre	Indirect antiglob-ulin titre	Number	Partial absorption titre	Blocking titre	Indirect antiglob-ulin titre
1	Negative	Negative	1:512	22	1:32	Negative	1:512
2	Negative	Negative	1:512	23	1:256	Negative	1:4096
3	Negative	Negative	1:128	24	1:64	Negative	1:256
4	Negative	Negative	1:256	25	1:128	Negative	1:2048
5	Negative	Negative	1:128	26	1:128	1:2	1:1024
6	1:16	Negative	1:512	27	1:256	1:4	1:4096
7	1:8	Negative	1:128	28	1:256	1:4	1:1024
8	1:8	Negative	1:512	29	1:256	1:4	1:1024
9	1:16	Negative	1:1024	30	1:256	1:4	1:2048
10	1:4	Negative	1:256	31	1:128	1:4	1:512
11	1:32	Negative	1:512	32	1:256	1:8	1:2048
12	1:16	Negative	1:256	33	1:512	1:16	1:2048
13	1:8	Negative	1:512	34	1:256	1:16	1:4096
14	1:4	Negative	1:512	35	1:512	1:16	1:1024
15	1:4	Negative	1:128	36	1:512	1:32	1:2048
16	1:4	Negative	1:512	37	1:1024	1:32	1:2048
17	1:32	Negative	1:512	38	1:2048	1:64	1:4096
18	1:16	Negative	1:512	39	1:1024	1:64	1:4096
19	1:32	Negative	1:1024	40	1:512	1:64	1:2048
20	1:64	Negative	1:2048	41	1:1024	1:64	1:4096
21	1:32	Negative	1:128	42	1:4096	1:128	1:8192

(a) Stillbirths - In nearly all cases of stillbirth due to Rh-incompatibility the indirect antiglobulin titre at the time of delivery was 1:1024 or more and was maintained at a high level for at least 10 weeks before delivery (Part I, Chapter VI). The PA titre in these cases can remain at a relatively low level throughout the greater part of pregnancy but may then rise rapidly. If death occurs *in utero* the PA titre may start to fall again, though the indirect antiglobulin titre generally remains steady (*vide infra*). A high indirect antiglobulin titre maintained throughout pregnancy does not necessarily mean that the fetus will be seriously affected. But if a high indirect antiglobulin titre is associated with a high PA titre throughout pregnancy and the fetus is Rh-positive, the outcome is invariably fatal.

(b) Severely affected liveborn infants - These are nearly always associated with a maternal indirect antiglobulin titre of 1:1024 or higher, but the PA titre is often low early in pregnancy and shows a sudden rise, often to a high level shortly before birth.

(c) Moderately affected infants - One of the puzzling features of previous work on the relationship between maternal antibody levels and severity of the disease in the infant has been the occurrence of relatively high antibody levels in the maternal serum throughout pregnancies which have terminated with the birth of a moderately affected infant. In typical cases the indirect antiglobulin titre of the maternal serum may persist at a moderately high level of 1:512 throughout the greater part of pregnancy,

but the PA titre remains low, at 1:16 or less, and does not rise appreciably near term. Another category of moderately affected infants can be distinguished in those cases in which the indirect antiglobulin titre is low or zero at the commencement of pregnancy, but rises to a high value during the third trimester. The PA titre may show a parallel rise. In these cases, however, the rise in antibody level occurs too late to seriously affect the infant before birth.

(d) Rh-negative infants - In families in which the father is heterozygous D/d it is important to be able to distinguish an Rh-negative fetus from an Rh-positive fetus if a correct prognosis is to be made before birth. At present there is no certain way by which this may be done. The behaviour of the PA titre during pregnancy, however, is a somewhat better guide than the behaviour of the indirect antiglobulin titre alone. If the indirect antiglobulin titre remains high through the last four months of pregnancy, as a result of antibodies from a previous pregnancy, but the PA titre remains negative or very low, it is an indication that no fresh immunization has taken place. In general the PA titre responds more rapidly than the indirect antiglobulin titre to a new episode of immunization and it tends to fall again to low levels before the next pregnancy commences. Persistently low PA titres throughout pregnancy are therefore more frequent when the fetus is Rh-negative and is failing to stimulate the production of more Rh-antibody.

(e) The effect of death in utero and maternal oedema on the PA titre - In some cases a fall in the PA titre may occur as pregnancy progresses. This may be due to three causes: (a) an Rh-negative fetus, (b) death in utero and (c) maternal oedema. Both (b) and (c) indicate the remarkable sensitivity of the PA titre as an index of what is occurring in the mother and the fetus. Sometimes, in severe cases of Rh-immunization, the fetal heart stops several weeks before parturition. If the PA titre is high when death occurs it shows a fall in about one-half of the cases. In a series of 20 stillbirths in which the PA titre was determined at regular intervals through the last ten weeks of pregnancy, eight cases showed a marked decline of two or more dilution tubes in the PA titre before the end of pregnancy. In each of these cases a badly macerated fetus was born. In six of the remaining 12 cases the fetus was again macerated but there was no detectable fall in the PA titre before delivery. It is important to note that although the PA titre fell in nearly half of the 20 stillbirth cases, in no single case did we observe the indirect antiglobulin titre to show a similar fall.

4. DISCUSSION

A number of attempts have been made to analyse the complexity of the maternal antibody response to Rh antigens by both physico-chemical and serological means (Cann et al. 1952; Krieger and Williams, 1955; Wiener and Gordon, 1953; Greenwalt and Wagner, 1955). So far no one has found a relatively simple test which, when applied to

the maternal serum antenatally, can predict with high probability the severity of the haemolytic process in the child and its chances of being stillborn, or, if born alive, of living with or without exchange-transfusions.

It has been reported (Davidsohn and Stern, 1948; Wiener et al. 1952) that blocking antibodies are associated with more severe manifestations of erythroblastosis in the baby, and de Kromme and Vervaat (1953) attempted to show that variations in conglutination-positive and conglutination-negative reactions can be used to explain differences in the severity of the disease.

Titration values obtained by the PA test have therefore been of importance in the understanding of the complexity of Rh-antibody production in response to the stimulus of an Rh-incompatible pregnancy. Diamond (1947) noted that severe cases of erythroblastosis were associated with the presence of free-Rh antibody in the cord serum of an affected infant. Our results show that if one volume of packed adult cells or the equivalent volume of haemolysed cells is used to absorb one volume of maternal serum the antibody left free in the serum, as measured by the indirect antiglobulin technique, can vary over a wide range of values. For instance, in cases where the indirect antiglobulin titre is 1:1024 the antibody left free in the serum can vary from zero up to, but never exceeding 1:1024. The titre of free antibody is the PA titre, and our results confirm and extend the observations of Diamond. The first conclusion to be drawn from these results is that in vivo the absorbing power of

the fetal red cells is roughly the same as that of adult red cells. Secondly, since all infants in whom the PA titre is zero are mildly affected, it would suggest that the haemolytic process in the fetus does not commence until all the antigenic sites on the red cells have been saturated with antibody, or blocked from accepting more. Work in progress indicates that at least two kinds of antigen-antibody reaction are involved in the process of absorbing Rh-antibodies on Rh-positive cells. In the first of these the antibody is bound firmly to the cell and can be released only by extracting with ether which completely alters the structure of the Rh antigen on the cell surface. In the second the antibody bound to the Rh antigen can be released simply by freezing and thawing the cell. In this way the cell is haemolysed but the antigenic sites are relatively undamaged. When cells are sensitized with an Rh serum with a zero PA titre and then thoroughly washed, no Rh-antibody is released after haemolysis caused by freezing and thawing. If, however, the cells are sensitized with an Rh serum which has a positive PA titre, the antibodies released from the cells after freezing and thawing are found to be identical with the PA titre of the original serum.

These results indicate that under conditions where the sensitizing serum has a positive PA titre, a portion of the Rh antibodies is in equilibrium with its corresponding antigen. A tentative hypothesis suggests that it is only when sensitized cells carry antibody in this manner that they are readily haemolysed in the fetal circulation. If

this interpretation is correct, then the amount of free antibody in the cord serum, as estimated by the PA technique, would be related directly to the rate of cell destruction *in vivo*. The persistence of such a process at a high rate over several weeks is almost certain to result in severe or fatal manifestations of erythroblastosis in the infant.

Chapter VIII

THE EVALUATION OF THE PARTIAL ABSORPTION TEST IN THE PROGNOSIS OF RH HAEMOLYTIC DISEASE

1. INTRODUCTION

It was shown in Chapter VI that the prognostic value of the maternal antiglobulin titre is considerably improved if titrations are carried out regularly during the last ten weeks of pregnancy. The titration values are given a code number from 1 to 9 and these are summed for each week to give a titre index. This is a measure not only of the height of the titre, but of the length of time that the titre has been operating. Although the titre index is a much better guide to the likely outcome of any particular pregnancy than is a single titre value for antibodies in the maternal serum at term, there is still a small number of cases which do not fit into the general pattern. It is believed that the explanation for some, at least, of these exceptional cases is to be found in the differing amounts of antibody which are left in the infant's serum. In Chapter VII a simple partial absorption (PA) test was described which enables an estimate of the amount of antibody in the fetal serum to be made from titrations carried out on the maternal serum. The present chapter provides a more rigorous evaluation of this test.

2. MATERIALS AND METHODS

Venous blood samples were collected from Rh-negative women as part of the routine antenatal tests. When Rh antibodies were present in the maternal serum every effort was made to obtain samples at regular intervals. During

the last three months of pregnancy samples were taken at fortnightly, or even weekly intervals, whenever possible. Samples of infant blood were obtained from the fetal end of the umbilical cord at birth. The indirect antiglobulin procedure used was described in Chapter II. The method of carrying out the partial absorption test on the maternal serum was described in Chapter VII.

The determination of cord-blood haemoglobin values and reticulocyte percentages, and the methods of carrying out exchange-transfusion have been described in detail by Kelsall, Vos, Kirk and Shield (1957).

3. RESULTS

290 Rh-immunized mothers were examined for whom the indirect antiglobulin titre and the PA titre of the maternal serum were available at the time of delivery. There were 41 liveborn Rh-negative infants. Of the remaining 249 Rh-incompatible pregnancies, 36 resulted in stillborn infants: of the 213 liveborn Rh-positive infants 21 subsequently died despite prompt and adequate exchange-transfusion.

Maternal PA titre at delivery and survival of Rh-incompatible infants - The single death among the 57 cases in which the PA titre at the time of delivery was zero was an anencephalic monster (see Table 21). There were no pathological signs that death in this case was in any way due to Rh incompatibility. In addition, one of the neonatal deaths, where the mother had a PA titre of 1:16, was due primarily to obstruction of the duodenum. Of the other deaths in the low-titre categories of 1:16 and 1:32, shown

TABLE 21

Distribution and outcome of cases of Rh-incompatible infants born to immunized mothers by partial absorption titre of the maternal serum at term

PA titre	Liveborn infants		Stillborn infants	Total No. infants	Percentage mortality
	Survived	Died			
0	56	0	1	57	1.75
1:16	40	3	1	44	9.09
1:32	17	2	1	20	15.00
1:64	25	2	3	30	16.67
1:128	10	2	3	15	33.33
1:256	25	3	7	34	26.47
1:512	12	5	8	25	52.00
1:1024	7	4	10	21	66.67
1:2048 or more	0	1	2	3	100.00
All titres	192	21	36	249	22.89

in Table 21, at least one of the neonatal deaths and both of the stillbirths were to mothers who were suffering from toxaemia of pregnancy with marked oedema. In many cases oedema associated with a stillbirth results in a fall in the PA titre before birth (Vos, 1958) and this may well have happened in the other case where the infant died very soon after birth. The fall in the PA titre reduces its usefulness as a prognostic guide in such cases, particularly if the only titration carried out is near the end of pregnancy.

It can therefore be concluded that the risk of stillbirth due primarily to Rh-incompatibility (when the maternal PA titre at birth is lower than 1:64), in the absence of marked oedema in the mother, is no greater than in normal pregnancies. For PA titres of 1:64 or more the risk of neonatal death or stillbirth is greater. In two-thirds of the cases with a maternal PA titre at birth of 1:1024 the infants were either stillborn or died soon after birth, whilst no infant has survived yet when the maternal PA titre was 1:2048.

Correlation of maternal PA titre with cord-blood haemoglobin values - It was shown in Chapter VI that the maternal indirect antiglobulin titre at term is highly correlated with the cord-blood haemoglobin values ($r = -0.62$). For 178 of the liveborn Rh-incompatible infants in the present series both cord-blood haemoglobin values and the PA titre of the maternal serum at term are available. The distribution of these values, together with the method of treatment and outcome is shown in Figure 4.

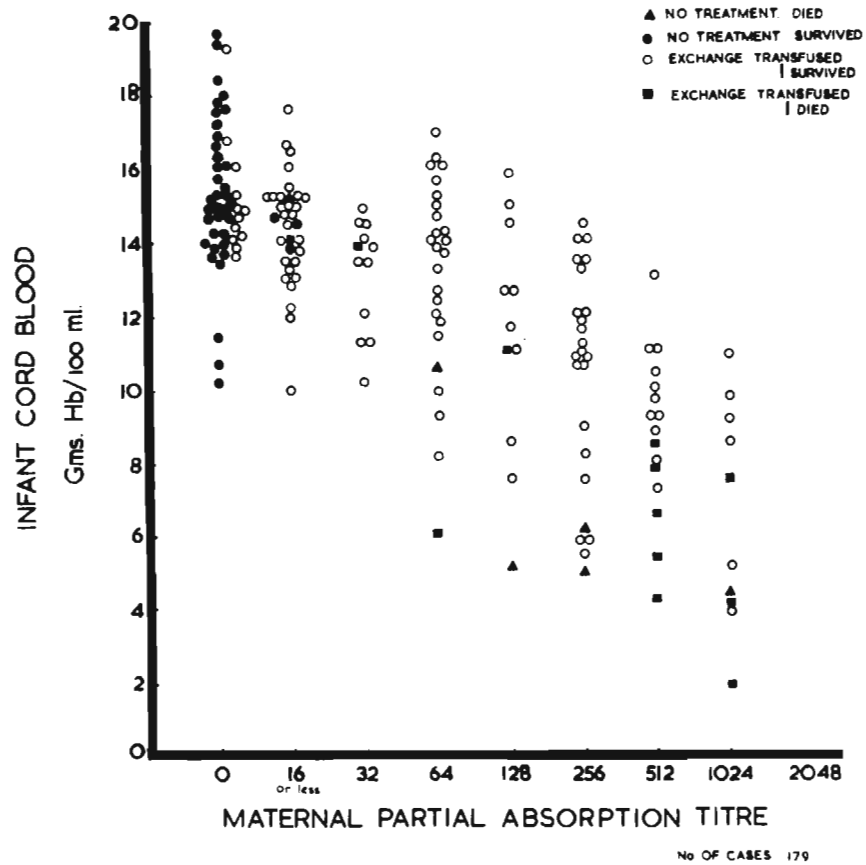

Figure 4: The distribution of 179 live-born Rh-incompatible infants by cord-blood haemoglobin value and maternal P.A. titre at the time of birth.

When this distribution is compared with the evaluation of maternal indirect antiglobulin titre at term and cord-blood haemoglobin value, as shown in Chapter VI, it is clear that the PA titre of the maternal titre gives a much better indication of the severity of the haemolytic process in the child than does the maternal indirect antiglobulin titre. This is in fact borne out by the statistical analysis which gives a correlation coefficient between the PA titre and cord-blood haemoglobin value of -0.72.

For infants compatible with their mothers for the Rh factor, the range of cord-blood haemoglobin values is from 11.0 to over 20.0 g/100ml, with a mean value of 16.24 and a standard deviation of 1.7. It might be expected therefore that for any particular level of the maternal PA titre there would be a corresponding spread of cord-blood haemoglobin values. Figure 4 shows this to be true.

Relationship between maternal indirect antiglobulin titre and partial absorption titre - If all cases are considered in which the maternal indirect antiglobulin titre at the time of birth is 1:1024, the cord-blood haemoglobin values can range from 3.0 to 17.0 g/100ml with approximately half of the cases possessing haemoglobin values above 10.0 g/100ml. (Figure 2, Chapter VI). It has been found that infants with haemoglobin values above 10.0 g/100ml. are not bad risks for exchange-transfusion. However, in cases in which the maternal indirect antiglobulin titre at birth is 1:1024 but the cord-blood haemoglobin value is below 10.0 g/100ml. the infants have a much poorer prognosis for

survival even when exchange-transfused.

For any given maternal indirect antiglobulin titre the PA titres of the maternal serum can vary over a wide range in different cases. In Table 22 the distribution of cord-blood haemoglobin values for 95 Rh-positive liveborn infants born to mothers with a maternal indirect antiglobulin titre at term of 1:512 to 1:4096 is given in relation to the difference between the indirect antiglobulin titre and the PA titre of the maternal serum. The difference in titre is expressed in dilution tube numbers. This means that for a particular indirect antiglobulin titre (1:1024 for example), the corresponding PA titre can range from the same value (difference = 0) down to 1:16 (difference = 6 dilution tubes).

From the distribution of cases in Table 22 it is clear that there is a high correlation between the difference in titre and the cord-blood haemoglobin value ($r = 0.61$). This means that the PA titre enables us to improve considerably on the prognosis based on the maternal indirect antiglobulin titre at term. Of the 95 liveborn infants recorded in Table 22, 15 subsequently died. None of these deaths occurred, however, among the 24 infants in whom the PA titre was more than three dilution tubes lower than the maternal indirect antiglobulin titre at term. Thus, a high indirect antiglobulin titre in the mother need not be considered a serious sign if the PA titre at the same time is low.

Stillbirths - There were 36 stillbirths among the 249 incompatible infants. One of these was an anencephalic

TABLE 22

Relationship between cord-blood haemoglobin values in liveborn Rh-incompatible infants and the difference between the maternal indirect antiglobulin titre and PA titre at term

Cord-blood haemoglobin value (grammes per 100 millilitres)	Difference in tube numbers between maternal indirect anti-globulin titre and PA titre at term							All titre differences
	6	5	4	3	2	1	0	
18.0 or more	-	-	-	-	-	-	-	-
16.0 to 17.9	1	-	-	-	-	-	-	1
14.0 to 15.9	1	6	4	6	3	-	-	20
12.0 to 13.9	2	3	3	4	4	3	-	19
10.0 to 11.9	-	-	4	4	10	4	-	22
8.0 to 9.9	-	-	-	3	7	4	1	15
6.0 to 7.9	-	-	-	1	5	2	0	8
4.0 to 5.9	-	-	-	1	3	4	1	9
2.0 to 3.9	-	-	-	-	-	1	0	1
All values	4	9	11	19	32	16	2	95

monster: the maternal indirect antiglobulin titre was only 1:128 and the PA titre was zero at term, so that it seems reasonable to consider that Rh incompatibility was not of aetiological significance. Of the remaining 35, 27 were associated with peak PA titres of 1:256 or more (see Table 21). It is necessary to use the peak PA titre in these cases since in many cases of stillbirth the PA titre drops rapidly after death has occurred in utero. A terminal value of the PA titre in such cases therefore does not represent the true value to which the living fetus has been exposed.

The rapid drop in the maternal PA titre which often occurs after the fetus has died means that prognosis based on a single titration value obtained near term is likely to be misleading. This probably explains why eight of the stillbirths in Table 21 were associated with PA titres of 1:128 or less. In a number of these it was possible to obtain blood samples from the mother only near term. If these cases had been titrated at regular intervals over at least the last ten weeks of pregnancy the peak values obtained would almost certainly have been significantly higher.

For 73 of the incompatible pregnancies in the present series antibody titrations have been carried out on the maternal serum before the end of the second trimester. Table 23 shows that in these cases, when the PA titre was already 1:64 or higher at the twenty-fourth week of gestation, only one out of 17 infants survived; 12 were stillborn and four died after birth. Indeed, of the children

TABLE 23

Outcome of cases in which the maternal PA titre was determined before the end of the second trimester

Outcome	Maternal PA titre at or before 24 weeks gestation									
	0	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	All titres
Liveborn-survived	28	7	6	1	0	0	0	0	0	42
Liveborn-died	2	1	2	3	1	0	0	0	0	9
Stillborn	2	3	5	1	2	5	1	2	1	22

of mothers who had a PA titre at all at the end of the second trimester, only 14 out of 43 incompatible infants survived. Clearly, the occurrence of a PA titre in the maternal serum at any level early during pregnancy is an unfavourable sign. If it is 1:64 or more the fetus almost always dies.

The Rh-negative infant - There were 41 Rh-negative infants born to mothers who had been immunized by a previous pregnancy. In the majority of these cases the indirect antiglobulin titre was of little value in helping to predict before birth whether the infant would be Rh-positive or Rh-negative. In fact, 34 of these Rh-negative infants were born to mothers with an indirect antiglobulin titre of 1:128 or more - that is, in the range which would make an exchange-transfusion necessary if the infant was Rh-positive (Table 24). Actually 25 of the pregnancies were terminated artifically, since a correct determination of the infant's genotype could not be made before birth.

The PA titres of the same cases show an entirely different distribution; 18 of the mothers had a zero PA titre at the time of birth and a further 13 had a PA titre only in the lowest dilution. In these latter cases the PA titre value of 1:16 had been maintained unchanged during the greater part of pregnancy. The constant antibody level was true also of all but two of the 10 cases with a maternal PA titre higher than 1:16. In one of the exceptional cases the PA titre fell from an early value of 1:512 to 1:128 at term. In the other case the PA titre rose from 1:64 to 1:128. This is unique in our experience

TABLE 24

Distribution of Rh-negative infants born to immunized mothers by indirect antiglobulin titre and PA titre of the maternal serum at birth

Titre	Distribution of infants	
	PA titre	Indirect antiglobulin titre
Zero	18	-
1:16	13	3
1:32	4	2
1:64	3	2
1:128	3	11
1:256	-	9
1:512	-	11
1:1024	-	3
Total	41	41

and represents the only example in which an Rh-negative infant was born to a mother who had a rise in either indirect antiglobulin or PA titre during the course of pregnancy.

The distribution of cases in Table 24 shows that the PA titre is of greater value than the maternal indirect antiglobulin titre in deciding whether a pregnancy should be allowed to terminate spontaneously. If the father is known to be heterozygous D/d, then a zero or constantly low PA titre maintained throughout pregnancy is a reasonable indication that the infant is Rh-negative. In such cases, even if the indirect antiglobulin titre is high, the pregnancy can be allowed to continue. It must be emphasized, however, that if this course is adopted,weekly checks of the PA titre are essential during the last two months. In some cases in which an incompatible infant is involved the PA titre may remain at a low level until the last few weeks, only to rise rapidly with serious consequences for the infant if labour is not induced immediately.

4. DISCUSSION

A technique for estimating indirectly the amount of Rh antibody free in the infant's serum by a partial absorption test on the maternal serum gave early indications that it would improve the reliability of prognosis based on the maternal indirect antiglobulin titre alone. The present evaluation of this test, based on a series of 290 cases has confirmed this.

The correlation between cord-blood haemoglobin value

and maternal PA titre is higher than between cord-blood haemoglobin value and the ordinary indirect antiglobulin titre. Indeed, the variability in cord-blood haemoglobin value for any particular maternal indirect antiglobulin titre is due almost entirely to the variability in the PA titre. The value of the discrimination between mild and severe cases which the use of the PA test makes possible is indicated by the fact that no deaths have occurred among cases with a maternal indirect antiglobulin titre of 1:512 or more where the maternal PA titre was more than three dilution tubes lower (Table 22).

The use of the PA test enables also a much safer prediction of an Rh-negative infant in cases in which the father is known to be heterozygous D/d. This is an important use of the test if it means that some pregnancies which would otherwise merit early induction will be allowed to continue to full term when the PA titre is zero or steady at 1:16. However, the PA test is not so valuable in predicting stillbirths unless the titrations have been carried out at regular intervals during pregnancy. This appears to be due mainly to the very marked drop in the PA titre which may take place when death occurs <u>in utero</u>. Terminal titration values obtained shortly before birth therefore mislead, since they may suggest a milder degree of involvement than is true when the pregnancy terminates with the birth of a grossly hydropic fetus. However, the records show that if the maternal PA titre at the end of the second trimester is 1:64 or more the fetus almost always dies.

PART THREE

THE RH ANTIBODY INHIBITION TEST

Chapter IX

THE SIGNIFICANCE OF THE RH ANTIBODY INHIBITION TEST IN DETERMINING THE SEVERITY OF RH HAEMOLYTIC DISEASE OF THE NEWBORN

1. INTRODUCTION

Evidence was presented in Chapter III to show that increased Rh-antibody stimulation can be expected more often towards the last few weeks of pregnancy than in early pregnancy. Since only Rh-incompatible fetal red cells entering the maternal circulation can be responsible for this increase, it is likely that stronger fetal movements during the latter part of pregnancy may also be the cause of some placental bleeds which lead to increased Rh-antibody stimulation. While it is accepted that episodes of immunization are associated with renewed antigenic stimuli, it is also known that the over-all effect of such stimuli often leads to increased fetal wastage in the form of stillbirth or neonatal death. This was confirmed by investigating a large series of Rh-immunized mothers who, during pregnancy, showed Rh-antibody titre increases of 3 dilution tubes or more, but since an analysis of this nature only showed the general effect of antibody behaviour over a large series of cases, it does not necessarily imply that the severity of Rh-haemolytic disease can be accurately predicted in each individual case by this procedure. This is obvious when it is considered that a 3-fold increase in Rh-antibody titre from a value of 1:8 to 1:64 is of a smaller magnitude than an increase which begins at 1:256 and rises to 1:4096. Also, it has been

established that Rh-antibody titrations alone do not clearly prognosticate the severity of Rh-haemolytic disease in all instances.

These findings led to the introduction of a 'titre index' system and a partial absorption test. With the former procedure some quantification can be obtained by computing the product of the maternal titre and the time during which the Rh-antibodies were present, while with the latter procedure partial antibody quantification is obtained by recording the amount of Rh-antibody not removed after one absorption by Rh-positive red cells.

A procedure of Rh-antibody quantification is now introduced which considers both the time factor and the amount of antibody to which the infant has been exposed during pregnancy. With this method it is not necessary to measure the amount of antibody by the conventional titration method, which, as shown previously, was found to lack reproducibility (Chapter II). The striking variations found by Race and Sanger (1954) in their attempts to remove Rh antibodies by repeated absorption with packed Rh-positive red cells were taken into consideration. The effectiveness of Rh-antibody absorption values was compared with conventional titration procedures with the aim of estimating the extent of accuracy for predicting the severity of Rh-haemolytic disease in individual cases.

2. MATERIALS AND METHODS

Rh-immunized mothers were examined from the first trimester of pregnancy onwards. Mothers were excluded where the production of Rh-antibodies may have been the result

of previous Rh-incompatible blood transfusions, and only Rh-incompatible mother-infant combinations were used. For the evaluation of Rh-antibody titres the standardized indirect antiglobulin titration method was used.

Rh antibody inhibition test

Preparation of standard haemolysed Rh-positive cell suspensions - Blood collected in ACD solution from a pooled source (2 or 3 donors) of group O, R_1r (CDe/cde) donors was washed three times in large volumes of physiological saline and finally packed by centrifugation at 3 000 rpm for 30 minutes. An accurate 50% saline suspension of the packed cells was made and divided into 10 ml. aliquots for daily use. This standard suspension of cells was haemolysed by freezing at -20° C for 48 hours. It may be stored for many months at this temperature without detectable loss of capacity to inhibit Rh-antibodies. Since significant differences in the inhibition of Rh-antibody can be expected when CDe/cde or cDE/cde red cells are used it is important that only CDe/cde donors are selected for the preparation of standard haemolysed test cells. The heterozygous genotype is always preferred because it not only resembles more directly the phenotype of all Rh-immunized infants' red cells but also ensures that consistent inhibition results are obtained. D^U- positive red cells should not be included in the donor pool as the antibody inhibition capacity of these red cells is quite different from that of D+ D^U bloods.

Method of testing - The standardized suspension of haemolysed Rh-positive cells was thawed at room temperature

and shaken thoroughly before use. A constant volume (0.4 ml.) of haemolysed cells was placed into six test tubes, numbered 1 to 6. To tube 1 was added one volume (0.4 ml.) of a 1:2 saline dilution of Rh antiserum (1 part undiluted serum and 1 part saline in this instance was recognized as a 1:2 dilution of serum and not 1:1); to tube 2, 1 volume (0.4 ml.) of a 1:4 saline dilution of Rh antiserum and to tubes 3,4,5 and 6 similarly increased dilutions of Rh antiserum were added (1:8, 1:16, 1:32 and 1:64). The diluted serum and haemolysed cells in each test tube were mixed and left undisturbed at room temperature for 10-15 minutes. The free Rh-antibody not inhibited by the haemolysed suspension of Rh-positive red cells was measured by adding one volume (0.4 ml.) of unhaemolysed Rh-positive red cells and left for a further 20 minutes at room temperature. The presence or absence of free Rh-antibodies in each test tube was then determined by the indirect antiglobulin test. The need to use avid broad-spectrum anti-human gammaglobulin serum must be emphasized because antiglobulin reagents of lesser quality cannot always detect free antibody on the red cells by this procedure.

Interpretation of results - Since an increasing amount of Rh antigen (in the form of haemolysed Rh-positive red cells) is actually combined with the Rh antiserum, a measurement can be made of the amount of Rh antigen that is required to inhibit the Rh antibody. From tubes 1 to 6 the ratio of serum to Rh antigen varies from 1:1 to 1:2, 1:4, 1:8, 1:16 and 1:32. The absence of Rh-antibodies in

the first tube, which represents 50% packed haemolysed cells and a 1:2 dilution of serum (1:1 ratio), indicates that 1 unit of Rh-positive red cells was required to inhibit the Rh-antibody. Should a positive indirect antiglobulin result be recorded for tube 4 and not 5, it indicates that the anti-Rh serum required 8 units of packed Rh-positive red cells to remove the antibody. The results are summarized in Table 25.

To obtain a quantitative evaluation of the amount of Rh-antibody to which the infant has been exposed over a given period of pregnancy the observed Rh-antibody inhibition unit is multiplied by the duration of pregnancy to record an 'inhibition index'. For example, an Rh-immunized mother who at 32 weeks' gestation was able to maintain a constant 'inhibition value' of 36 units will have an 'inhibition index' of 32 x 36 = 1152. In other circumstances the Rh-antibody inhibition test can reveal marked changes during pregnancy as a result of increased antibody stimulation. When this occurs the 'inhibition index' may be obtained by the procedure set out in Table 26. At least one examination must be performed before 24 weeks of pregnancy in order to obtain a baseline value. Subsequent tests may then be carried out at two-weekly or monthly intervals depending on the results obtained.

3. RESULTS AND DISCUSSION

Table 27 shows how closely the fetal wastage rate is related to the maternal Rh-antibody titre recorded at delivery. It is important to emphasize, however, that such

TABLE 25

Results of Rh-antibody inhibition test

Test tube No.	Volume of haemolysed red cells used (50% packed CDe/ cde cells)	Anti-Rh serum		Rh anti-body inhibition units
		Volume	Dilution	
1	0.4 ml	0.4 ml	1:2	1
2	0.4 ml	0.4 ml	1:4	2
3	0.4 ml	0.4 ml	1:8	4
4	0.4 ml	0.4 ml	1:16	8
5	0.4 ml	0.4 ml	1:32	16
6	0.4 ml	0.4 ml	1:64	32

TABLE 26

Calculating inhibition index

Patient examined at: (duration of pregnancy).	Observed Rh antibody inhibition unit	Progressive calculation of the inhibition index during pregnancy
15 weeks	4	15x4 = 60
23 weeks	4	23x4 = 92
28 weeks	16	27x4 +1 x 16 = 124
30 weeks	16	124+2 x 16 = 156
32 weeks	32	156+16 + 1x32 = 204
36 weeks	32	204+ 4 x 32 = 332

TABLE 27

Relationship between maternal indirect antiglobulin titre and outcome of pregnancy

Maternal Rh antibody titre at delivery	Rh negative mothers No.	Rh positive infants			Overall fetal wastage	
		Survived	Died	Stillborn	No.	%
1:64 or less	176	176	0	0	0	0.0
1:128-1:512	326	310	10	6	16	4.9
1:1024 or more	316	164	53	99	152	48.1
Total:	818	650	63	105	168	20.5

a correlation can be seen only in a combined analysis of many cases and that misleading information can easily be deduced from it if it is assumed that all Rh-immunized mothers with antibody titres of 1:1024 or more will have severely affected Rh-positive infants. The findings do, however, confirm that no fetal wastage due to Rh-immunization need be expected if a mother has an antibody titre of 1:64 or less. For practical purposes a number of investigators (Crawford, Cameron and Walker, 1966; Farrell and Davis, 1967) have therefore accepted that amniocentesis need not be performed on Rh-immunized mothers with low Rh-antibody titres.

It has often been reported that Rh-antibody titrations cannot be regarded as a reliable criterion for the prediction of the severity of Rh-haemolytic disease in individual cases. We also found this to be so by correlating the range of cord-blood haemoglobin values against the maternal Rh-antibody titres observed at birth. Since the greatest percentage of false predictions can be expected among mothers with titres of 1:1024 or more a selective analysis was carried out (Table 28). At least 40 per cent of the mothers had Rh-positive infants with cord-blood haemoglobin values greater than 10 G/100 ml. These evidently tolerated the intensity of a 1:1024 titre much better than those with cord-blood haemoglobins less than 10 G/100 ml. The last-mentioned group also experienced a high percentage of neonatal deaths.

The variations observed between the apparent clinical significance of low Rh-antibody titres (1:64 or less) and

TABLE 28

Distribution of Rh positive infants born alive to immunized mothers with indirect antiglobulin titres of 1:1024 or more by values for cord-blood haemoglobin and neonatal deaths

Infant cord-blood haemoglobin G/100 ml	Rh negative mothers		Rh positive infants		
			Survived	Died	
	No.	%	No.	No.	%
14.0	46	40.5	46	0	0.0
10.0-13.9	42		42	0	0.0
6.0- 9.9	98	59.5	71	27	27.5
5.9	31		5	26	83.8
Total:	217		164	53	24.4

lack of such correlation when the Rh-antibody titres are high (1:1024 or more) draws attention to the fact that the conventional procedure of antibody titration does not adequately measure the true quantity of Rh-antibody, particularly in the higher dilutions.

In an attempt to discover other recognizable variations between low and high-titred Rh-antibodies it was found that significant differences often existed in the capacity of Rh-positive red cells to absorb Rh-antibodies. By modifying the procedure in order to obtain a greater degree of standardization and reproducibility the Rh-antibody inhibition test was compared with the antibody titration procedure. It was found (Table 29) that serum samples with Rh-antibody titres of 1:64 or less are not generally difficult to inhibit by one unit of haemolysed Rh-positive red cells, although a few cases may require four units of haemolysed cells. By comparison, Rh-antibody titres of 1:1024 or more showed a significantly greater range of inhibition results, some requiring 2-4 units of haemolysed red cells while others required 16 units or more. Although some Rh-antibodies possessed titres of 1:1024 or more they appeared to have the same inhibition value as those with titre values of 1:64 or less. This presupposes that the haemolytic effect of some 1:1024 Rh-antibody titres is the same as some observed within the 1:64 category.

With such marked variations observed in the ability to inhibit Rh-antibodies it was important to determine the value of this test in prognosticating the severity of Rh-haemolytic disease before birth. Table 30 details an

TABLE 29

Comparative analysis between relationship of Rh antibody titres and inhibition test

Maternal indirect antiglobulin titre value	No. of mothers examined	Units of haemolyzed cells required for the inhibition of Rh antibodies						
		1	2	4	8	16	32	64
1:64 or less	56	37	13	6	-	-	-	-
1:128) 1:256) 1:512)	74	17	21	15	10	7	4	-
1:1024 or more	39	-	2	6	13	8	6	4

TABLE 30

Rh antibody inhibition index as a measure of severity of Rh haemolytic disease

Maternal Rh antibody inhibition index	No. of mothers examined	Rh positive infants			Live-births		
		Alive and well	Died	Stillborn	Exchange transfused	Not exchange transfused	Foetal wastage %
80 units or less	82	82	-	-	7	75	0.0
80 - 500 units	54	48	4	2	46	6	11.1
500 units or more	33	15	6	12	21	-	54.5

analysis of 169 Rh-immunized mothers examined throughout pregnancy. The inhibition test is expressed as a measure of 'inhibition index' which estimates the period that an Rh-incompatible infant has been exposed to maternal Rh-antibodies. By such calculations a wide range of inhibition index values were recorded which, for the purpose of this analysis, have been divided into three categories. The first category represents Rh-immunized mothers who throughout pregnancy have accumulated an Rh-antibody inhibition index of only 80 units or less. Not one of the Rh-incompatible pregnancies resulted in a stillbirth or neonatal death and more than 90 per cent of the liveborn infants did not require exchange-transfusion. From this information it is apparent that a good correlation exists between a low Rh-antibody inhibition index and a less severe haemolytic manifestation of the disease neonatally. For those mothers who had higher inhibition index values during pregnancy the effect of in utero haemolysis was most obvious when judged by the increased frequency of fetal death and the significantly raised incidence of infants who required exchange-transfusions.

The findings in Table 30 show the close correlation between the Rh-antibody inhibition index and the final outcome of pregnancy. It was not intended to show the antenatal behaviour pattern of the inhibition test, nor the manner in which the findings should be interpreted to make an accurate prediction of the haemolytic process in each individual case. This will be more fully detailed in the next chapter. However, the observations do emphas-

ize that an inhibition index of 500 units or more is unquestionably associated with an intense process of immune Rh-antibody production which, as shown in Table 31, can be responsible for the introduction of other complicating immunological responses.

The presence of other antibodies apart from anti-Rh is not unusual and their occurrence would seem to follow the same course as documented by Nilsson (1965) with regard to the influence of multiple Rh-antibodies. When multiple Rh-antibodies are present the severity of the disease is usually greater; this is probably because the total amount of Rh-antibodies bound to the infant's red cells is, for example, greater in CDe/cde infants born to mothers with anti-C plus anti-D, than if the same mothers had produced only anti-D. It is quite possible that there is a connection between increased antibody loading on the red cells and the severity of the disease. However, the primary cause of the development of multiple antibodies appears to be directly associated with the intensity of the stimulation of the antibodies as a result of repeated episodes of placental haemorrhage.

It has been shown that antibodies other than anti-Rh are produced in response to frequent episodes of re-immunization. This complication is most often found among mothers with significantly raised Rh-antibody values (Table 31), who as a rule have more severely affected infants (Table 30), and possibly a greater incidence of placental haemorrhage. Future studies directed towards the prevention of placental haemorrhage must therefore lead to an even greater reduction in fetal wastage.

TABLE 31

Frequency of other immune red cell antibodies among mothers who were initially sensitized by the Rh factor

Maternal Rh antibody inhibition index	No. of mothers examined	Immunizing antibody		Percentage of two antibody systems observed %
		Anti-Rh alone	Anti-Rh plus others*	
80 units or less	82	82	0	0.0
80 - 500 units	54	49	5	9.2
500 units or more	33	27	6	18.1

* Rh immunization found in association with immune anti-A, B, Kell, Fy^a or Jk^a.

Chapter X

THE VALUE OF THE RH-ANTIBODY INHIBITION TEST IN RELATION TO LIQUOR AMNII STUDIES

1. INTRODUCTION

Considerable progress was made in the management of pregnancies complicated by Rh-immunization with the introduction of amniotic fluid studies (Bevis, 1950, 1952). Investigators have now confirmed the relationship of bile pigment concentration in the amniotic fluid with the intensity of Rh-haemolytic disease *in utero*. However, the spectrophotometric (Walker, 1957), chemical (Mackay and Watson, 1962) and pigment-protein ratio procedures (Morris, Murray and Ruthven, 1967) cannot always be accepted as being infallible, their shortcomings being too seldom emphasized while their advantages are simply taken for granted (Vos, 1969).

Some investigators (Pirofsky, 1965; Robertson, 1961) believe that with the introduction of liquor amnii studies nothing useful can be gained by the inclusion of maternal Rh-antibody investigations. Others (Liley, 1963; Hyslop and Whiley, 1960; Walker, 1967) emphasize that the prediction of the severity of Rh-haemolytic disease by amniotic fluid analysis can at times be most misleading. Fully endorsed cautions about relying on the predictions obtained from amniotic fluid studies alone have led investigators to believe that bile pigment levels should be used only in conjunction with maternal Rh-antibody estimations (Nilsson, 1965; Crawford, Cameron and Walker, 1966).

Since repeated abdominal paracentesis on every Rh-immunized mother can result in errors in the antenatal prediction of the severity of the disease (Zipursky, Pollock, Chown and Israels, 1963; Misenhimer, 1966), selective amniocentesis has become an accepted practice. The recording of low maternal Rh-antibody values is really not indicative of severe haemolytic manifestations of the disease.

We found that by using Rh-antibody inhibition studies we could predict far more reliably the severity of Rh-haemolytic disease, particularly when the significance of liquor amnii values appears to be in some doubt. Routine maternal antibody studies form an effective supplement to the results obtained by spectrophotometric analysis. Rh-antibody studies, can, in fact, be regarded as a test to show whether a clinically favourable forecast of severity obtained by liquor examinations at 26-30 weeks gestation continues to be the same at 34 weeks' gestation, especially when it is taken into account that more frequent episodes of re-immunization may be expected after 30 weeks of pregnancy (see Chapter III).

2. MATERIALS AND METHODS

Rh-immunized mothers were examined from the first trimester of pregnancy onwards. Those who might have produced Rh-antibodies as a result of previous Rh-incompatible blood transfusions were excluded and only Rh-incompatible mother-child combinations were studied.

To evaluate the Rh-antibody inhibition index, the

procedure described in Chapter IX was used. Rh-antibody titre values were determined by the standardized method of indirect antiglobulin titration (Chapter II).

Amniotic fluid studies - Amniotic fluid obtained by abdominal paracentesis was centrifuged immediately after collection at 4 000 rpm for 30 minutes to remove cellular elements and was then placed in a light-proof container if an immediate analysis was not carried out. Bile pigment concentration in the specimen was analysed using the spectrophotometric procedure of Fleming and Woolf (1965) and total protein value by the biuret reaction described by Morris, Murray and Ruthven (1967). Pigment protein ratio was calculated as recommended by the same authors. Serum bilirubin estimations on the infants' cord bloods were determined by the method of Powell (1954).

3. RESULTS AND DISCUSSION

In order to compare objectively the efficacy of amniotic fluid measurements with that of Rh-antibody inhibition studies, an investigation was carried out on 66 Rh-immunized mothers who had given birth to Rh-incompatible infants. In the assessment of how closely these two procedures can forecast the severity of fetal red cell destruction *in utero* it is important to remember that the Rh-antibody inhibition test determines the measured intensity of antibody effect up to the time of delivery.

The amniotic fluid studies (particularly when performed before 30 weeks of pregnancy) can only measure the intensity of fetal red cell haemolysis present at the time

the sample was obtained by amniocentesis.

To reduce such variations to a minimum, only the highest projected spectrophotometric measurement obtained from a series of amniotic studies on the same patient was used. An average of 2.4 amniocentesis samples were tested from the 66 mothers examined. Their time of collection ranged from 28 to 33 weeks of gestation and in no instance were single amniocentesis studies included if they were performed after the 33rd week of pregnancy. A detailed analysis of the serological, biochemical and haematological findings of the 66 Rh-immunized mothers and their infants is shown in the Appendix of this chapter.

To determine how closely the Rh-antibody 'inhibition index' and bilirubin protein values are related to the severity of the infant's anaemia at birth, a biserial correlation coefficient analysis was carried out for the two testing procedures against (a) the haemoglobin concentration of the infant's cord blood at birth; (b) total serum bilirubin value at birth, and (c) the severity of Rh-haemolytic disease as determined by the infant's clinical condition. Table 32 shows the results of this analysis and it can be seen that both procedures (amniotic fluid values and antibody inhibition studies) accurately predicted that an infant's cord blood haemoglobin level will be low when the bilirubin/protein ratio or Rh-antibody inhibition index values are high. A corresponding correlation is also confirmed for the ability of these two procedures to measure the overall severity of the disease. However, no real correlation was established

TABLE 32

A statistical evaluation of the accuracy in predicting the severity of Rh haemolytic disease before birth of Rh-incompatible infants by the Rh antibody inhibition test and amniotic fluid studies

Comparative analysis between		r Correlation and significance
Maternal Rh antibody inhibition index value at delivery	Infant cord-blood haemoglobin value	- .764 *** (1)
	Infant cord-blood bilirubin value	.581 ***
	Severity of Rh haemolytic disease	.847 ***
Highest bilirubin/ protein ratio values obtained between 28 and 32 weeks gestation	Infant cord-blood haemoglobin value	- .696 ***
	Infant cord-blood bilirubin value	.248 N. S.
	Severity of Rh haemolytic disease	.799 ***

*** denotes significance at .001 level.

between the bilirubin/protein ratio and the cord-blood bilirubin values, whereas a significant association between Rh-antibody inhibition results and cord-blood bilirubin values is evident.

Such variations lend support to the argument that the bilirubin/protein ratio test (done at least four weeks before the birth of the infant) is able to determine only the intensity of in utero haemolysis noted at the time of collection and does not necessarily indicate the true intensity of red cell haemolysis to be expected at birth.

As more frequent episodes of antibody stimulation can be expected as pregnancy progresses towards term, the lack of positive correlation between bilirubin/protein ratio values and cord-blood bilirubin may be influenced by the increased rate of red cell destruction seen after the amniocentesis samples were taken. The possibility that raised Rh-antibody production (induced by the disruption of fetal-placental circulation at amniocentesis), is a contributing factor cannot be ignored, and like the practice of external version (Vos, 1967) can be considered as furthering the intensity of the disease (Zipursky, Pollock, Chown and Israels, 1963).

A real measure of confidence in the prognosis of Rh-haemolytic disease can be attained when two different testing procedures are employed. However, it was found that even if a statistical correlation between the testing procedures and the severity of the disease is evident in a combined analysis of many cases, the same may not necessarily be true for some individual cases. Marked

differences can occasionally be observed due to the condition of an infant being influenced by factors other than Rh-immunization (Mollison, 1972).

With the knowledge that such variations can be expected in individual cases it appeared that the prognosis of the disease should ultimately be based on the assessment of a combination of parameters (previous history, father's Rh-genotype, Rh-antibody value and amniotic fluid results) concurrent with any additional experience gained from other statistical studies. For this reason a number of individual cases are included to show how the Rh-antibody inhibition test can be of particular significance in providing extra information about the haemolytic condition in utero.

Table 33 details the study of an Rh-immunized mother with a previous history of two normal pregnancies which were not complicated by Rh-immunization. During her third pregnancy Rh-immunization was confirmed at 12 weeks' gestation, the preliminary observations strongly suggesting that this was the direct result of postnatal immunization. A high Rh-antibody value scored at 12 weeks' gestation also served as an indication that the present pregnancy could result in a very severely affected infant. This prediction was reached by noting that the Rh-antibody inhibition index value at 26 weeks would have attained a level of at least 832 units which, as shown in Chapter IX, is dangerously high. Subsequent amniotic fluid studies clearly confirmed that the infant was indeed likely to be badly affected, indicating that only intra-uterine trans-

TABLE 33

Mrs H.L.: Age 25.
Previous obstetric history: Two infants alive and well. No stillbirths or spontaneous abortions.
Father's Rh genotype: CDe/CDe

Period of gestation	Rh antibody studies by:			Liquor amnii studies			
	Indirect antiglobulin titre	Inhibition		Optical density at .450 mu	Bilirubin µgm/ml	Protein mgm/ml	Ratio bilirubin µgm/mgm of protein
		Value	Index				
12/52	1:1024	32	384				
24/52	1:1024	32	768				
26/52	1:1024	32	832				
27/52	1:1024	32	864	0.170	2.72	4.8	0.56
29/52	1:1024	32	928				
30/52	1:1024	32	960	0.140	2.40	3.9	0.61
32/52	1:1024	32	1024				

Outcome: Spontaneous labour at 32 weeks. Macerated stillborn fetus.

fusion may have saved the infant from being stillborn at 32 weeks.

The case presented in Table 34 is very similar to the one described in Table 33, the only difference being that the comparative intensity of *in utero* haemolysis, as judged by the amniocentesis value, appeared to be more pronounced. The bilirubin/protein ratio values between this and the previous case were at least three times greater, whereas the relative Rh-antibody index value scored at delivery differed only slightly.

These variations do not necessarily imply that the two testing procedures differed in their prognostic reliability, but it does show that individual variations can be expected within certain limits. There would be reason for real concern, of course, if the two values had been significantly different. Table 35 describes a case where it was very difficult for the clinicians to decide whether intra-uterine transfusion or very early induction of labour was the better mode of treatment. This mother had a moderately low Rh-antibody inhibition index value when examined at 30 weeks' gestation and at this stage a very severely affected infant would not have been suspected. On the other hand, liquor amnii studies clearly showed that the infant was severely affected, the bilirubin/protein ratio values obtained being greater than the results recorded for the stillborn infant in Table 33. Since no other immune antibodies were detected in the maternal serum to aggravate the haemolytic process *in utero*, no measures were taken to treat the infant premat-

TABLE 34

Mrs P.K.: Age 32
Previous obstetric history: First infant died 12 days after birth. Second infant alive and well. Third infant stillborn at 33 weeks. Fourth infant Rh-affected, delivered by Caesarean section; exchange-transfused, alive and well.

Period of gestation	Rh antibody studies by:			Liquor amnii studies			
	Indirect antiglobulin titre	Inhibition Value	Inhibition Index	Optical density at 450 mu	Bilirubin µgm/ml	Protein mgm/ml	Ratio bilirubin µgm/mgm of protein
22/52	1:512	16	352				
26/52	1:512	16	416				
31/52	1:1024	32	512	1.001	10.7	6.7	1.5
33/52	1:2048	32	576	0.710	10.2	5.3	1.9
34/52	1:4096	32	608				
35/52	1:4096	32	640				

Outcome: Spontaneous labour at 35 weeks. Macerated stillborn fetus: intra-uterine death was established at 34 weeks.

TABLE 35

Mrs G.M.: Age 30.
Previous obstetric history: Three infants alive and well. No stillbirths or spontaneous abortions.
Father's genotype: CDe/cDE

Period of gestation	Rh antibody studies by:			Liquor amnii studies			
	Indirect antiglobulin titre	Inhibition Value	Inhibition Index	Optical density at .450 mu	Bilirubin µgm/ml	Protein mgm/ml	Ratio bilirubin µgm/mgm of protein
26/52	Not examined						
28/52	1:64	4	112	-	-	-	-
30/52	1:64	4	120	0.190	3.3	5.0	0.66
32/52	1:64	4	128	-	-	-	-
34/52	1:256	8	140	0.162	3.02	3.5	0.86
36/52	1:256	8	156	0.141	2.30	3.4	0.68
38/52	1:512	16	180	0.135	2.27	2.9	0.80

Outcome: Induced labour at 38/52 Infant's condition satisfactory at birth. Not oedematous; Rh positive, direct Coombs positive ++++; cord haemoglobin 10.3 gm%, reticulocytes 12%, bilirubin total 3.9 mg%, conjugated 0.3 mg%.
Two exchange-transfusions required. Infant alive and well.

urely. Follow-up studies gradually showed raised Rh-antibody formation while the amniocentesis values continued to indicate an apparently intense haemolysis in utero. At birth the clinical condition and haematological findings were in complete accord with the serological findings but disagreed with the amniocentesis values scored since 30 weeks of pregnancy.

Whilst it can be accepted that a high percentage of predictions scored by amniocentesis investigations are reliable indicators of the intensity of in utero haemolysis, the recognition of some shortcomings may help future reasoning regarding the mechanism of bilirubin entry into the amniotic sac.

Table 36 describes a case where it is again evident that the initial process of Rh-immunization occurred as a possible consequence of the trauma of parturition. In this instance Rh-antibody production was not very intense during the early stages of her second pregnancy; however, from 32 weeks gestation onwards a marked increase in Rh-antibody titre was noted, whilst the inhibition index value remained low. Amniocentesis values confirmed that the intensity of in utero haemolysis was not of clinical concern and that the postnatal course of the disease would be commensurate with such findings. It is important to remember however that should this mother conceive another Rh-positive infant soon after the present pregnancy the clinical outcome would most likely follow a course very similar to the case described in Table 34 where an already immunized mother commenced pregnancy with lethal Rh-anti-

TABLE 36

Mrs D.B.: Age 23.
Previous obstetric history: One infant alive and well. No stillbirths or spontaneous abortions.
Father's genotype: CDe/cde.

Period of gestation	Rh antibody studies by:			Liquor amnii studies			
	Indirect antiglobulin titre	Inhibition Value	Inhibition Index	Optical density at .450 mu	Bilirubin µgm/ml	Protein mgm/ml	Ratio bilirubin µgm/mgm of protein
20/52	1:64	0	0				
25/52	1:64	0	0				
28/52	1:64	0	0				
31/52	1:64	0	0	0.012	0.3	1.9	0.15
32.52	1:512	4	4				
34/52	1:1024	4	12	0.018	0.7	2.5	0.28
35/52	1:1024	16	28				
36/52	1:1024	16	44				

Outcome: Induced labour at 36 weeks. Infant in excellent condition at birth. Rh positive, direct Coombs positive ++++, cord haemoglobin 15.6 gm%, reticulocytes 2%, bilirubin total 2.1 mg% conjugated 0.1 mg% . Not exchange-transfused. Infant alive and well.

body values which resulted in stillbirth.

In an effort to help women in these circumstances to avoid pregnancies with a high risk of stillbirth, prenatal counselling and examination is advocated until the mother can be assured that the Rh-antibody value indicates that there is a good possibility of her delivering a live born child.

Finally, it must also be acknowledged that the use of liquor examinations is of real practical value in determining the presence of Rh-negative infants. Table 37 details a case where it was obvious from a combination of maternal Rh-antibody and liquor amnii values that the baby was Rh-negative. The prediction by amniocentesis of a mildly affected infant did not agree with the forecast of a very severely affected infant by Rh-antibody inhibition values. If we accept that Rh-antibodies destroy fetal red cells then the lack of haemolysis by amniocentesis studies almost certainly indicated that the infant's Rh-type had to be compatible with the mother's.

TABLE 37

Mrs R.K.: Age 27
Previous obstetric history: First infant alive and well; second infant affected by Rh-immunization, exchange-transfused, alive and well; third infant stillborn.
Father's Rh genotype: cDe/cde.

Period of gestation	Rh antibody studies by:			Liquor amnii studies			
	Indirect antiglobulin titre	Inhibition Value	Inhibition Index	Optical density at .450 mu	Bilirubin ugm/ml	Protein mgm/ml	Ratio bilirubin ugm/mgm of protein
10/52	1:2048	16	160				
25/52	1:2048	16	400				
30/52	1:2048	16	480	0.029	1.2	3.5	0.34
32/52	1:2048	16	512	0.021	0.9	4.1	0.21
35/52	1:2048	16	560				
36/52	1:2048	16	576				

Outcome: Induced delivery at 36 weeks: infant Rh-negative and unaffected.

APPENDIX

SEROLOGY, BIOCHEMISTRY AND HAEMATOLOGY OF 66 RH-IMMUNIZED MOTHERS WHO DELIVERED RH-POSITIVE INFANTS

Case No.	Maternal Rh-antibody inhibition index at delivery	Liquor amnii studies: Bilirubin µg./ml.	Liquor amnii studies: Protein mg./ml.	Liquor amnii studies: Ratio bilirubin µg./mg. of protein	Cord-blood estimations: Haemoglobin G/100 ml.	Cord-blood estimations: Bilirubin total mg./100 ml.	Time of delivery, treatment, and outcome*	Clinical evaluation of the disease†
1	450	2·5	6·4	0·39	11·8	5·4	35/52, Ex. Tr. A & W	2
2	560	3·2	3·6	0·89	—	—	Stillborn 28/52	4
3	640	12·4	6·8	1·82	—	—	Stillborn 30/52	4
4	204	1·4	5·2	0·26	16·2	2·2	36/52, A & W	1
5	620	10·7	6·7	1·56	—	—	Stillborn 35/52	4
6	570	1·9	6·3	0·31	6·4	8·6	36/52, Ex. Tr. A & W	3
7	312	2·3	5·7	0·40	11·6	3·7	35/52, Ex. Tr. A & W	3
8	36	1·1	5·4	0·20	12·3	3·3	36/52, A & W	1
9	144	1·2	3·8	0·31	12·0	3·0	36/52, Ex. Tr. A & W	2
10	896	6·2	4·9	1·26	—	—	Stillborn 31/52	4
11	206	2·9	7·1	0·40	10·8	4·8	35/52, Ex. Tr. A & W	2
12	428	1·4	3·4	0·44	9·4	3·1	36/52, Ex. Tr. A & W	3
13	68	1·2	5·8	0·20	13·6	3·4	36/52, Ex. Tr. A & W	1
14	560	1·9	3·1	0·61	10·2	2·5	35/52, Ex. Tr. A & W	2
15	488	2·4	3·8	0·63	6·8	5·2	35/52, Ex. Tr. A & W	3
16	1,120	3·9	6·2	0·63	8·0	5·7	35/52, Ex. Tr. A & W	3
17	10	1·1	3·8	0·28	17·8	2·1	40/52, A & W	1
18	72	0·8	2·8	0·28	13·3	3·1	38/52, A & W	1
19	24	0·6	2·9	0·20	15·0	3·3	40/52, A & W	1
20	180	3·3	5·0	0·66	10·3	3·9	38/52, Ex. Tr. A & W	2
21	280	1·0	2·7	0·37	9·3	2·6	36/52, Ex. Tr. A & W	3
22	320	2·0	2·8	0·71	10·7	3·8	35/52, Ex. Tr. A & W	2
23	700	12·0	7·0	1·70	—	—	Stillborn 31/52	4
24	220	1·6	5·4	0·29	15·1	2·4	37/52, Ex. Tr. A & W	1
25	800	2·0	5·8	0·34	5·1	7·2	36/52, Ex. Tr. A & W	3
26	350	2·6	6·1	0·42	10·8	2·9	36/52, Ex. Tr. A & W	3
27	54	1·3	6·3	0·20	13·8	2·7	36/52, A & W	1
28	400	2·2	5·8	0·37	11·0	5·0	36/52, Ex. Tr. A & W	2
29	600	3·0	3·4	0·88	—	—	Stillborn 30/52	4
30	820	8·4	7·3	1·15	—	—	Stillborn 29/52	4
31	840	9·7	7·2	1·34	—	—	Stillborn 34/52	4
32	150	1·5	4·8	0·31	13·4	2·6	37/52, Ex. Tr. A & W	2
33	230	2·4	5·4	0·44	11·7	4·3	36/52, Ex. Tr. A & W	2
34	454	1·6	3·8	0·42	9·1	3·7	36/52, Ex. Tr. A & W	3
35	80	0·9	4·2	0·21	14·0	2·7	37/52, Ex. Tr. A & W	1
36	540	2·0	3·1	0·64	11·9	2·9	36/52, Ex. Tr. A & W	3
37	522	2·8	4·2	0·66	7·0	5·6	35/52, Ex. Tr. A & W	3
38	980	4·2	5·8	0·72	6·7	5·0	35/52, Ex. Tr. A & W	3
39	26	1·7	7·3	0·23	15·4	2·1	38/52, A & W	1
40	92	0·5	2·4	0·20	14·3	2·4	39/52, A & W	1
41	78	0·9	4·3	0·21	13·9	3·1	40/52, A & W	1
42	200	4·1	6·8	0·60	12·0	4·8	37/52, Ex. Tr. A & W	2
43	310	1·5	4·4	0·34	10·1	3·7	36/52, Ex. Tr. A & W	2
44	420	1·9	2·4	0·79	9·4	3·9	35/52, Ex. Tr. A & W	3
45	640	3·4	3·8	0·89	—	—	Stillborn, 29/52	4
46	500	2·8	6·6	0·42	12·0	6·1	36/52, Ex. Tr. A & W	2
47	180	1·2	5·8	0·20	14·3	2·7	36/52, Ex. Tr. A & W	1
48	620	2·3	5·2	0·44	8·7	4·8	35/52, Ex. Tr. A & W	3
49	300	1·8	4·1	0·43	12·1	3·9	36/52, Ex. Tr. A & W	2
50	68	1·4	6·1	0·26	14·2	2·4	36/52, A & W	1
51	96	1·0	3·3	0·30	15·1	2·7	37/52, A & W	1
52	190	2·0	5·2	0·38	11·7	3·9	36/52, Ex. Tr. A & W	2
53	720	11·4	6·5	1·75	—	—	Stillborn, 30/52	4
54	400	1·3	4·1	0·31	10·7	4·2	36/52, Ex. Tr. A & W	3
55	72	0·8	3·8	0·21	13·7	2·1	38/52, A & W	1
56	510	1·8	3·0	0·60	12·1	3·8	36/52, Ex. Tr. A & W	2
57	480	2·3	4·3	0·53	9·7	4·9	36/52, Ex. Tr. A & W	3
58	846	4·7	7·4	0·63	8·4	5·5	35/52, Ex. Tr. A & W	3
59	42	2·1	9·4	0·22	16·3	2·0	39/52, A & W	1
60	960	5·2	4·8	1·08	—	—	Stillborn, 33/52	4
61	864	9·1	6·4	1·57	—	—	Stillborn, 30/52	4
62	78	1·3	5·4	0·24	14·7	2·4	39/52, A & W	1
63	88	0·7	3·1	0·22	16·4	2·0	40/52, A & W	1
64	210	3·9	6·3	0·61	11·8	3·9	38/52, Ex. Tr. A & W	2
65	248	1·9	4·6	0·41	12·6	3·7	37/52, Ex. Tr. A & W	2
66	310	1·7	3·2	0·53	10·7	4·3	36/52, Ex. Tr. A & W	3

* Ex. Tr. = exchange transfusion; A & W = alive and well.
† 1 = mild; 2 = moderate; 3 = severe; 4 = very severe.
For comparative statistical analysis of the Rh-antibody inhibition index against other values, scored results were divided by a factor of 1,000; for example, case 1 index of 450 = 0·45.

SUMMARY

Using a standardized antiglobulin test for the determination of Rh-antibodies it was found that Rh-immunization occurs in one or more episodes during pregnancy and that the resulting antibody titre can either be high or low. The frequency of increased antibody stimulation, as determined by a three-fold rise in the indirect antiglobulin titre, appears to be directly related to a large percentage of fetal wastage. The findings also show that a significantly greater number of episodes of increased Rh-antibody production occur after 32 weeks gestation, indicating more frequent fetal red cell leakage across the placental barrier. The practice of external manipulation for breech presentation or transverse lie, which is known to cause fetal-maternal haemorrhage, should therefore be avoided among Rh-immunized mothers. The results presented confirm that these procedures can disrupt the placental circulation and the ultimate risk involved in intensifying the haemolytic process in utero cannot be ignored.

Antenatal follow-up studies also established that the incidence and intensity of Rh-immunization can differ as a consequence of fetal-maternal ABO blood group variations. In mothers who produced lower values of Rh-antibody titres (1:64 or less) the incidence of mother-child ABO blood group incompatible combinations was generally within the normal range (20%), whilst the reverse (4.7%) was true for mothers producing high-titre Rh-antibodies (1:1024 or more). This disparity between the production of high and low Rh-antibody titres by ABO and Rh-incompatible fetal

cells suggests that significant suppression of Rh-antibody formation can result from 'double incompatibilities'.

The antibody titre of the maternal serum at term, as measured by the indirect antiglobulin technique, is not quite such a good prognostic index for forecasting the chance of survival of individual Rh-positive infants as are the values for cord-blood haemoglobin or reticulocyte percentage. However, titration values of the maternal serum have the distinct advantage of being determined before birth and can indicate the best management of the case.

Another aspect of this investigation was the assessment of a partial absorption (PA) test which enables the quantity of Rh-antibodies free in the fetal serum to be estimated by titrations carried out on the maternal serum. With this test a more accurate prognosis can be made of the outcome for the infant than if the indirect antiglobulin titre is used alone. In a study of 290 cases of infants born to Rh-immunized women it was found that the PA test could determine mildly and severely affected cases of haemolytic disease more accurately than the indirect antiglobulin titre. The correlation coefficient between the PA titre of the maternal serum at delivery and cord-blood haemoglobin for liveborn Rh-positive infants was 0.72. However, the PA titre was found to be unreliable for predicting intra-uterine death unless regular titrations were carried out from at least the twenty-fourth week of pregnancy. Follow-up studies showed that this is due to the rapid fall in PA titre observed in many cases

when the fetus has died *in utero*.

In the final study a new procedure of Rh-antibody quantification was introduced. Of importance here was the determination of the number of absorptions an Rh-antibody requires by Rh-positive red cells before it is completely removed from the serum. The Rh-antibody inhibition test was designed to consider both the time factor and the amount of antibody to which the infant is exposed during pregnancy. Extensive investigations confirmed that the application of this test, in conjunction with spectrophotometric analysis of liquor amnii, can introduce a real measure of prognostic reliability in predicting the severity of Rh-haemolytic disease before the birth of the infant. The Rh-antibody inhibition test can in fact be considered as a testing procedure for revealing whether a clinically favourable forecast by liquor amnii studies between 26-30 weeks gestation continues to be the same after 33 weeks gestation. This is particularly important when it is considered that (a) the spectrophotometric test can be completely misleading when performed after 33 weeks gestation and (b) that more frequent episodes of re-immunization can be expected from 30 weeks of pregnancy onwards. This was clearly established by finding a positive correlation between Rh-antibody inhibition index values and cord-blood bilirubin results. The absence of a similar positive correlation between amniotic fluid bilirubin values and cord-blood bilirubin indicates that liquor amnii studies cannot always predict whether increased fetal red cell destruction can be expected after the amniotic fluid

samples have been taken.

The findings presented indicate that the effect of Rh-antibodies on the fetus differs in almost every Rh-immunized mother. In some immunized pregnancies the antibody value remains the same throughout pregnancy whilst significant changes may increase fetal mortality in others. From the numerous tests performed it is also evident that the relationship between Rh-antibody titres and the severity of Rh-haemolytic disease is more complicated than has been thought up until now. In many laboratories antibodies are titrated by the simple method of diluting the serum containing the antibody. In this way it is safe to predict that Rh-immunized mothers with antibody values below a certain critical dilution (titre of less than 1:32) will generally give birth to Rh-positive infants who are only mildly affected. However, similar predictions are not possible when the antibody values are above a certain critical level. In an effort to find a much closer association between maternal Rh-antibodies and the severity of the disease the 'partial absorption test' was introduced. In terms of predicting the condition of the fetus in utero, the partial absorption test is significantly better than any other method of Rh-antibody determination based on serial dilutions of serum.

A more refined innovation of the partial absorption test is the 'Rh antibody inhibition test'. This test is based on the observation that some Rh-antibodies require many absorptions with Rh-positive red cells to remove the antibody, whilst others require only few absorptions. As

a method for predicting the severity of Rh-haemolytic disease it is more sensitive than the partial absorption test.

These studies show that Rh-antibody quantification by serological procedures may be more accurately performed by measuring the amount of Rh-antigen utilized in absorption studies than by expressing Rh-antibody reactivity in dilutions.

SECTION TWO

THE SPECIFICITY AND IMMUNOGLOBULIN CHARACTERISTICS OF AUTOANTIBODIES IN ACQUIRED HAEMOLYTIC ANAEMIA OF THE 'WARM' TYPE

Chapter XI

1. INTRODUCTION

Early in this century Widal, Abrami and Brule (1908) and Chauffard and Troisier (1908) described factors resembling autoantibodies in the serum of patients with acquired haemolytic anaemia. They were the first investigators to observe autoagglutination of patients' red cells *in vitro* and recognized it as a useful diagnostic indicator for this haematological disorder. Significant advances in the immunology of acquired haemolytic anaemias were hindered for many years because of the lack of appropriate methods for detecting red cell antibodies, especially those that could not be detected by direct *in vitro* autoagglutination. It was not until Coombs, Mourant and Race (1945) introduced the anti-human globulin test (Chapter II) for detecting Rh-isoimmunization that Loutit and Mollison (1945) discovered the significance of this test to recognize the incomplete form of autoantibodies in acquired haemolytic anaemias.

The subsequent development of other serological methods, such as the procedure for recovering antibodies from sensitized red cells (Kidd, 1949; Vos and Kelsall, 1956; Weiner, 1957) enabled investigators to describe these autoantibodies in detail. The important demonstration followed that autoantibodies can be classified into two main groups: (1) the 'warm' type of autoantibodies and (2) the 'cold' type of autoantibodies. Patients in whom these two types of autoantibodies are found fall correspondingly into two broadly distinguishable clinical types.

The 'warm' type affects all ages and the patients may suffer from chronic mild anaemia to severe acute haemolytic episodes. Jaundice due to excess unconjugated bilirubin in their blood and moderate enlargement of their spleen is common. The 'cold' type of autoantibody mainly affects elderly people and is generally a very chronic disorder in which the anaemia is seldom severe. For much of the clinical information available concerning autoimmune haemolytic anaemias we are indebted to Dacie's authoritative monograph published in 1962.

In nearly one-third of the patients with autoimmune haemolytic anaemia of the 'warm' type the disease is described as being 'secondary' because it is associated with other diseases, particularly chronic lymphocytic leukaemia, reticulosarcoma and also virus pneumonia and disseminated lupus erythematosus. Some of these 'secondary' conditions are considered to be autoimmune themselves. In the other two-thirds of patients there is no other disease specifically associated with the haemolytic condition and these are described as being 'idiopathic', meaning a disease for which no cause is so far known.

The 'warm' type of autoantibodies characteristically show maximal sensitizing activity for red cells at 37° C without lysing them, whereas the 'cold' types are only slightly or completely inactive at 37° C. They differ from the 'warm' type in their ability to lyse enzyme-treated red cells. It is significant that both the 'warm' and 'cold' autoantibodies react not only with the patient's own red cells but also with almost all other human red

cells. For this reason it was originally thought that the autoantibodies were 'non-specific' in their combining activity for red cells. The term 'non-specific' used for these autoantibodies is however not immunologically correct, since all antibodies must have a specificity of reactions.

It has since been shown that nearly all examples of autoantibodies of the 'cold' type have anti-I specificity (Wiener, Unger, Cohen and Feldman, 1956). For the 'warm' type of autoantibodies to be discussed from now on the recognition of antibody specificity is not as simple. Using the antibodies recovered from the red cells of patients with autohaemolytic anaemia of the 'warm' type, Sturgeon (1947), Kidd (1949) and Wiener, Gordon and Gallop (1953) found that these autoantibodies reacted with the red cells of man, chimpanzees and Rhesus monkeys but not with red cells of the rabbit, fowl, sheep, guinea pig or horse. In this respect the reactions seemed to parallel the behaviour of Rhesus isoantibodies. However, these observations did not help to establish the specificity of the autoantibodies. The first reported recognition of the role of Rh specificity in acquired haemolytic anaemia of the 'warm' type came from Weiner, Battey, Cleghorn, Marson and Meynell (1953) who demonstrated the presence of anti-e. Many investigators have since confirmed that other varieties of Rh-antibodies, e.g. anti-D, anti-c, anti-c+e could also be recognized as autoantibodies. Using red cells possessing the normal complement of Rh-antigen determinants, Mevli (1957) and Hollander and

Batschelet (1958) were able to show that the relative incidence of the specific Rh-antibodies found in auto-immune haemolytic anaemia of the 'warm' type corresponds well with the incidence of the frequency of the D,C,E,e and e antigens in the population.

By combining the findings of other investigators Dacie (1962) established that only 34 per cent of the 152 patients examined actually had autoantibodies possessing true 'Rh specificity'. The nature of the autoantibodies which lacked Rh specificity could apparently not be resolved by testing the antibodies against red cells with a wide range of Rh-phenotypes. The first indication that the unidentified autoantibodies do possess detectable 'Rh-related specificities' was established by Weiner (1961). He confirmed that the autoantibodies which always reacted with any combination of red cells possessing a normal complement of Rh antigens (DCe/DcE, DcE/ce, ce/ce, etc.), sometimes failed to sensitize red cells lacking the normal complement of Rh-antigens (Dc-/Dc- or D--/D--). This observation showed that specific autoantibodies directed against a high incidence antigen can appear to lack specificity unless tested against rare samples of blood from which some Rh-antigens have been lost by chromosomal deletion or suppression. With the aid of red cells lacking all recognizable Rh-antigens (Vos et al. 1961) and those lacking the Cc and Ee series of Rh-antigens (Race, Sanger and Selwyn, 1950), it was established that the so-called unidentified autoantibodies were in fact related to the Rh system (Weiner and Vos, 1963).

The discovery that a high percentage of autoantibodies do possess 'Rh-related specificities' has raised two questions: What is the significance of the source of the antigen involved in this autoimmune system, and to what extent can it provide additional information about the composition of the Rh genome?

The investigations described from here on were designed to evaluate both the specificity and the nature of the immunoglobulin involved in acquired haemolytic anaemia of the 'warm' type.

Another important fact to be considered in this disease is how the reactions of autoantibodies for Rh-antigens or Rh-related antigens could have developed. There is reason to suspect that under pathological conditions the red cell surface may be modified by some exogenous factor which in turn alters the physicochemical structure of the Rh-antigen to elicit the formation of autoantibodies. Proof of this concept has not yet been established because studies along these lines require a profound knowledge of the chemistry of Rh-antigens. Alternatively, one could explain the abnormality by postulating some defect of the patient's immunological apparatus and the emergence of 'forbidden' clones of lymphocytes which are unable to distinguish the body's own Rh-antigen from foreign Rh-antigen. This trend of thought would be in line with the immunological ideas of 'self and not self' of Burnet and Fenner (1949).

Using a number of classical serological observations as a guide, Milgrom (1969) suggested that autoantibodies to Rhesus antigens may result from the inability of these

antigens to elicit self-recognition when they are altered by certain viral or chemical agents. In this situation it was postulated that antibodies are not only produced against the affected Rh-antigen sites but also against the autoantigens. The mechanism of autoantibody formation to Rh-antigens is obviously of profound interest to the whole problem of autoimmune haemolytic anaemia. So far there is evidence available to indicate that autoantibodies with Rh-related specificities may be the result and not the cause of the disease.

Chapter XII

THE RH SYSTEM OF BLOOD GROUPS

1. INTRODUCTION

Since the blood group specificities of autoantibodies found among patients suffering from acquired haemolytic anaemia of the 'warm' type have been identified as Rh or Rh-related antibodies it is important that a definition of the Rh system from 'normal' to the 'missing' Rh antigen types is included in this study. An appreciation of the complex factors surrounding the conventional Rh system is of particular value in helping to classify the nature of Rh-related autoantibodies. These antibodies do not recognize the well-defined DCEce antigens of the Rh system and are therefore difficult to incorporate into the accepted theories of today. Indeed, very little information about the relationship of these autoantibodies to the Rh system would have been available if rare bloods lacking some or all Rh antigens had not been discovered. Thus the inclusion of the so-called 'unidentified' antibodies into the Rh system of antibodies begins with a basic perception of the present day structure of the Rh genome.

2. THE NORMAL RH GENE COMPLEX

Rh antigens are molecular configurations on the surface of red cells of which the chemical composition has not been clearly defined to date. Recent studies by Green (1968a, 1968b) do however suggest that Rh antigens are lipoproteins capable of being inactivated by certain sulfhydryl reagents by heating at 56° C and by enzymatic dig-

estion using proteases and urea. Their presence or absence on the red cells is determined by genes.

Two theories have been proposed to explain the heredity of the Rh system (Figure 5). According to Wiener and Wexler (1958) one gene at a single locus on a chromosome controls the entire Rh system, whereas the Fisher-Race theory (1944) suggests that the Rh system is determined by three closely linked genes on each chromosome. A comparison of the Wiener and Fisher-Race notations for the eight major Rh alleles is shown in Table 38. Regardless of which concept is considered to be correct, the pattern of inheritance has been shown to fit both theories. The two concepts have unfortunately led to the introduction of two nomenclatures of the Rh system. For the study presented here the Fisher Race notation has been used because most investigators regard it as a versatile representation of the genetical situation.

The expression of Rh blood groups in man is demonstrated by specific antisera which determine the presence or absence of a particular Rh antigen on the red cells. The five main antisera concerned with this expression are anti-D, anti-C, anti-E, anti-c and anti-e and they define only the presence of the corresponding Rh antigen and not the genotype. As can be seen from Fig. 5, the gene complex DCe determines the presence of antigens D,C and e on the red cells. The combinations of genes which the single chromosome carries are transmitted as a unit from mother to infant. The Rh genotype of the infant is determined by pairs of these chromosomes, one derived from the father and one

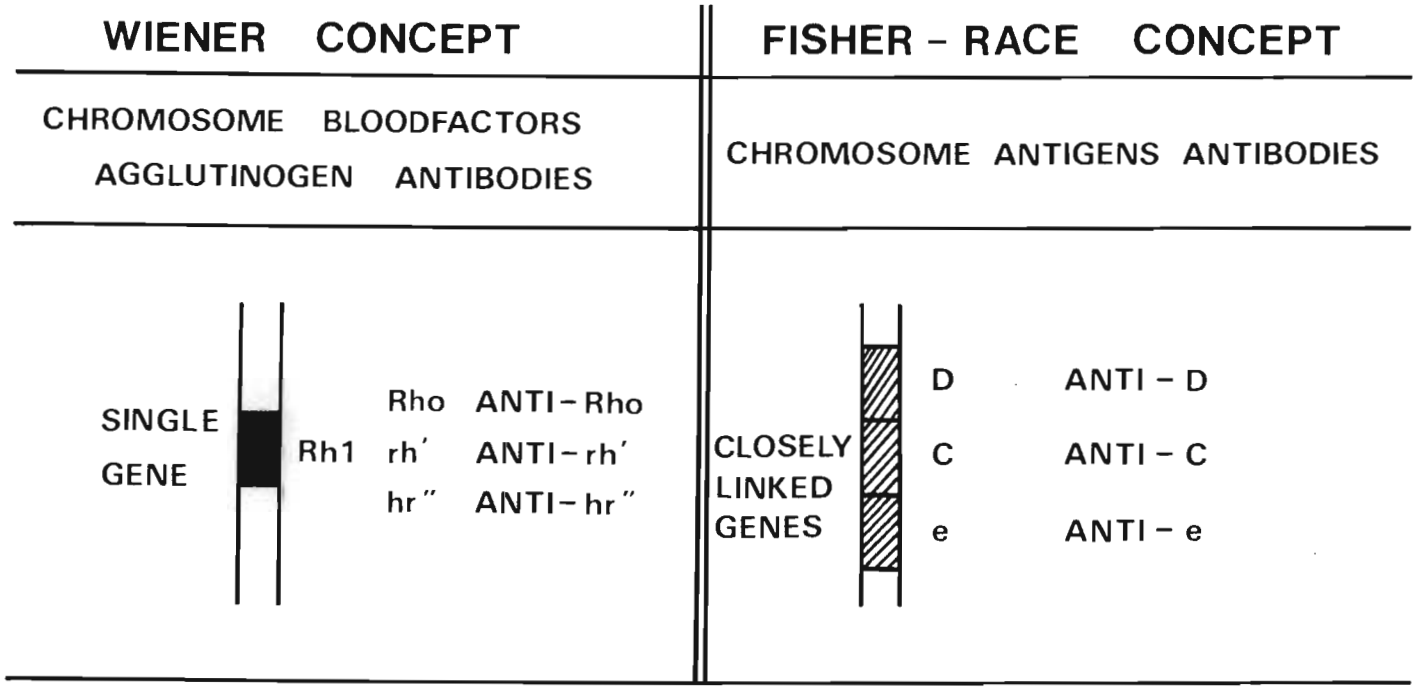

Figure 5: The Wiener and Fisher-Race concept of the Rh system.

TABLE 38

Comparison of the Fisher-Race linked gene theory and the Wiener multiple allele theory

Fisher-Race Notation		Wiener Notation		
Gene complex	Antigens	Genes	Agglutinogens	Factors
Dce	D,c,e	R^o	Rh_o	Rh_o, hr', hr''
DCe	D,C,e	R^1	Rh_1	Rh_o, rh', hr''
DcE	D,c,E	R^2	Rh_2	Rh_o, hr', rh''
DCE	D,C,E	R^z	Rh_z	Rh_o, rh', rh''
dce	c,e	r	rh	hr', hr''
dCe	C,e	r'	rh'	rh', hr''
dcE	c,E	r''	rh''	hr', rh''
dCE	C,E	r^y	rh_y	rh', rh''

from the mother during fertilization. For example, all infants of a CDe/CDe x cde/cde parental combination will be of the genotype CDe/cde because one CDe chromosome would have been received from one parent and one cde chromosome from the other parent. Of the eight basic types of Rh chromosomes recognized through extensive family studies (Table 38) each individual is known to carry a pair of these chromosomes which represents his genotype. In numerous clinical and anthropological studies carried out throughout the world the Rh gene complex has always been observed to follow a simple Mendelian pattern of inheritance. Table 39 lists some of the Rh gene complexes known to exist today with the resulting Rh antigens they possess.

3. THE MISSING RH ANTIGEN TYPES

In 1950 Race, Sanger and Selwyn described a blood which failed to react with any Rh antisera except those containing anti-D. The Rh genotype of the person concerned was described as D--/D-- and it was considered possible that the Ce and Ec portions of the Rh gene complex had been lost by deletion from the Rh chromosome. Since then several other examples of D--/D-- have been described and people whose Rh genotype appears to be DC^w-/DC^w- have been reported. In describing this particular Rh genotype Race and Sanger (1968), for the lack of absolute evidence, suggested that it could be the result of (1) a short deletion of a small piece of chromosome, or (2) a built in inhibitor which fails to express the missing antigens.

During routine testing of blood collected for anthrop-

TABLE 39

Rh gene complexes with their corresponding antigens

Gene complex	Antigens				
cDe	D	G	c	ce	e
CDe	D	G	C	Ce	e
cDE	D	G	c	cE	E
CDE	D	G	C	CE	E
cde	-	-	c	ce	e
Cde	-	G	C	Ce	e
cdE	-	-	c	cE	E
CdE	-	G	C	CE	E
$C^{w}De$	D	G	C^{w}	?	e
$cD^{u}e$	(D)	(G)	c	ce	e
cde^{s}	-	-	c	ce^{s}	e^{s}
cDe^{s}	D	G	c	ce^{s}	e^{s}
$Ccde^{s}$	-	G	C&c	ce	e^{s}
(C)d(e)	-	G	(C)	-	(e)

ological purposes from Australian aborigines from the Western Desert of Western Australia, a sample of blood was found (Vos, Vos, Kirk and Sanger, 1961) which failed to react with the following sera: 25 anti-D, 13 anti-C+D (+G), 13 anti-C (including some of the specificity anti-Ce and anti-CE), 2 anti-C^w, 10 anti-E, 1 anti-E^w, 3 anti-c+E, 14 anti-c, 14 anti-e, 2 anti-f (anti-ce) 2 anti-V (anti-ce^s), 2 anti-VS (anti-e^s), 5 sera from immunized D--/D-- persons, 1 from an immunized DC^w-/DC^w- person, 2 from immunized Dc-Dc- persons and with 7 sera from immunized Negroes who have made antibody not unlike anti-e (anti-Hr^s).

The cells of individuals with such deletions have since been named 'Rh_{null}' by Ceppellini (1964). The view that the Rh_{null} genotype is the result of suppression or deletion of that part of the chromosome bearing the Rh locus has been considered (Vos, 1961). Another possibility may be that Rh_{null} (---/---) represents basic material remaining after loss of products of the Rh genes by one or another genetic mechanism, but on the other hand, it could represent basic material on which the Rh genes deposit their products.

4. THE LW ANTIGEN AND THE RH_{null} PHENOTYPE

When Landsteiner and Wiener (1940) injected the red cells of Rhesus monkeys into rabbits they observed that the resulting antibody could, after partial absorption with human Rh negative cells, react strongly with Rh-positive red cells and weakly with Rh-negative red cells.

The strong positive reactions against Rh-positive red cells suggested that the specificity was the same as that of human anti-D. It is now accepted that the reaction of the rabbit antibody to Rhesus monkey red cells is directed against a different specificity referred to as 'LW' in honour of Landsteiner and Wiener (Levine, Celano, Wallace and Sanger, 1963). Levine has also proposed that although the genes for Rh and LW are inherited independently they may act on the same basic Rh substance to produce their respective red cell antigens.

With the discovery of the Rh_{null} (---/---) blood an excellent opportunity was provided to determine whether these red cells also carried the LW antigen. If not, the question arose of the relationship of the LW antigen to basic Rh substance.

Parallel studies were undertaken, based on three criteria: (a) absorption of guinea pig anti-LW with Rh_{null} cells, (b) elution of the antibody and (c) antigenicity of the Rh_{null} factor in guinea pigs. Immunization experiments were carried out with Rh-negative and Rh_{null} red cells. At three-day intervals each of two series of guinea pigs received two injections of 2.0 ml of the bloods. When tested ten days later five guinea pigs injected with Rh_{null} cells failed to produce anti-LW, two of these five produced an antibody identified as anti-N. Since the Rh_{null} red cells used for this study were of the genotype NN the source of this antibody stimulation is obvious. Of the four guinea pigs injected with Rh-negative red cells, three showed very weak anti-LW specificity

when absorbed with Rh-negative red cells. After absorbing these sera with the Rh_{null} cells all four gave anti-LW specificity of stronger activity than that obtained on absorption with Rh-negative red cells. In contrast to Rh-negative bloods, the Rh_{null} cells failed to yield eluates with anti-LW specificity, as shown in Table 40.

The results of our study indicate that when a gene produces an Rh antigen determinant it almost always produces LW antigen and that the LW material constitutes unaltered basic Rh substance which, by a series of mutations in man, has evolved into the complex of Rh factors as it exists today. This theory was mentioned as early as 1952 by Murray and Clark. In any event it can be concluded that the LW antigen is associated with all human blood bearing any one or more of the antigenic determinant groups of the Rh system (see Chapter XVI).

5. THE ASSOCIATION OF MULTIPLE PHENOTYPIC ABNORMALITIES WITH RH_{null}.

A most important observation with the discovery of the original Rh_{null} blood and some 15 or 20 other Rh_{null} propositi which have now been found, is the failure of some Rh_{null} bloods to react with most anti-U or anti-s reagents. Although there is no evidence for linkage between the gene loci of Rh determinants and SsU blood group systems, there may, nevertheless, be a structural relationship between the antigenic determinants in these two systems.

Examples of blood which fail to react with anti-U reagents have always been found in Negroes and have so far not been reported in Caucasians, Mongeloids, Australoids

TABLE 40

Absorption and elution studies of anti-LW using Rh-negative and Rh_{null} red cells

	Tested with							
Pooled guinea pig anti-rhesus serum 1:5	R_2R_2	R_1R_1	R_1r	R_or	rr	rr	rr	---/---
Absorbed with rr eluate from red blood cells:	+ ±	+ ±	+ +	+ +	±	±	0	0
---/---	0	0	0	0	0	0	0	0
rr	++++	++++	++++	++++	0	0	0	0

Readings were taken after incubation at room temperature for 1 hr., light centrifugation and resuspension of the sedimented red cells

or Asiatics. Only one example of a U-negative blood has so far been described in an Asiatic family (Moores, 1972), indicating that the U-negative characteristic is basically Negroid in origin.

In the determination of the U blood group status of four Rh_{null} individuals against a number of anti-U reagents (Vos, Moores and Lowe, 1971) it was shown that only one (H.A.) possessed a normal U expression of the red cells (Table 41). This suggests that the absence of a normal U expression in samples H.H., L.M., and E.N., may be due to differences in the structural composition of the Rh_{null} factor.

To provide further information on the genetic background of the Rh_{null} factor, extensive family studies have been carried out (Levine, et al. 1965; Ishimori and Hasekura, 1967). A comparative analysis of these studies revealed some remarkable variations to explain why some but not all Rh_{null} bloods possess an aberrant U blood group. For family L.M. (Table 41) it was confirmed that the Rh_{null} propositus was able to transmit to her child the full expression of the LW and CDE antigens, which she herself, being homozygous for the Rh_{null} factor, did not show. In the Japanese family the parents of the propositus (H.A., Table 41) lacked the complete expression of one set of CDE antigens on an allele, indicating that they were heterozygous for the Rh_{null} factor.

These family studies clearly revealed the existence of two mechanisms that influence the LW and CDE expressions. Among the theories proposed to explain the absence of all

TABLE 41

Red cell agglutination test of known U-positive and U-negative bloods for various antisera

SsU phenotype		Reactivity of red cells for antisera													
		Anti-LW		Anti-S			Anti-s					Anti-U			
		AHA	G.Pig	1172	159	60	385	7	Dubl.	Perth	176	959	31	Mah	128
Control	S+s+U+	4	4	4	4	4	4	4	4	2	2	4	4	3	4
Control	S-s+U+	4	4	0	0	0	4	4	4	2	2	4	4	4	4
Control	S+s-U+	4	4	4	4	3	0	0	0	0	0	4	4	4	4
2671	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
2538	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
1784	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
9484	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
5816	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
7799	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
4166	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
3836	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
3357	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
4661	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
1458	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
2439	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
Krum	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
Amich	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
Kamla	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
Sara	S-s-U-	4	4	0	0	0	0	0	0	0	0	0	0	0	0
H.A.	S-s+U+	0	0	0	0	0	4	4	4	2	2	4	3	3	4
H.H.	S-s+U-	0	0	0	0	0	4	0	4	2	0	0	0	0	0
L.M.	S-s+U-	0	0	0	0	0	4	0	4	2	0	0	0	0	0
E.N.	S-s+U-	0	0	0	0	0	4	0	4	2	0	0	0	0	0

Rh antigens on the red cells are (1) chromosomal deletions (Ishimori and Hasekura, 1967) and (2) the existence of an independent amorphic gene $X^{o}r$ which blocks the expression of the Rh genes (Levine et al., 1965). Fig.6 illustrates a tentative model for the two types of conditions that can lead to Rh_{null} expression.

It is difficult to explain why some Rh_{null} red cells (samples H.H., L.M., and E.N.) do not react with anti-U reagents. The fact that the expression of the U antigen can be altered by the same mechanism that is involved in the blocking phenomenon of the Rh precursor substance suggests that the two independent systems Rh and Ssu are influenced by a single aberration. Schmidt et al., (1967) proposed that this aberration may be due to the sequential action of genes controlling shared terminal sugar(s) which in turn has the capacity to alter the various specificities depending on the basic precursor substance involved (Watkins, 1966).

Race and Sanger (1968) made the important observation that discrepant anti-S, anti-s and anti-U results against unrelated Rh_{null} bloods were not given when saline or albumin tests could be used in place of the indirect antiglobulin test. The question thus arises whether the absence of U reactivity in some Rh_{null} bloods may be associated with a spatial membrane arrangement of the U antigen whereby the receptors are not fully exposed above the surface.

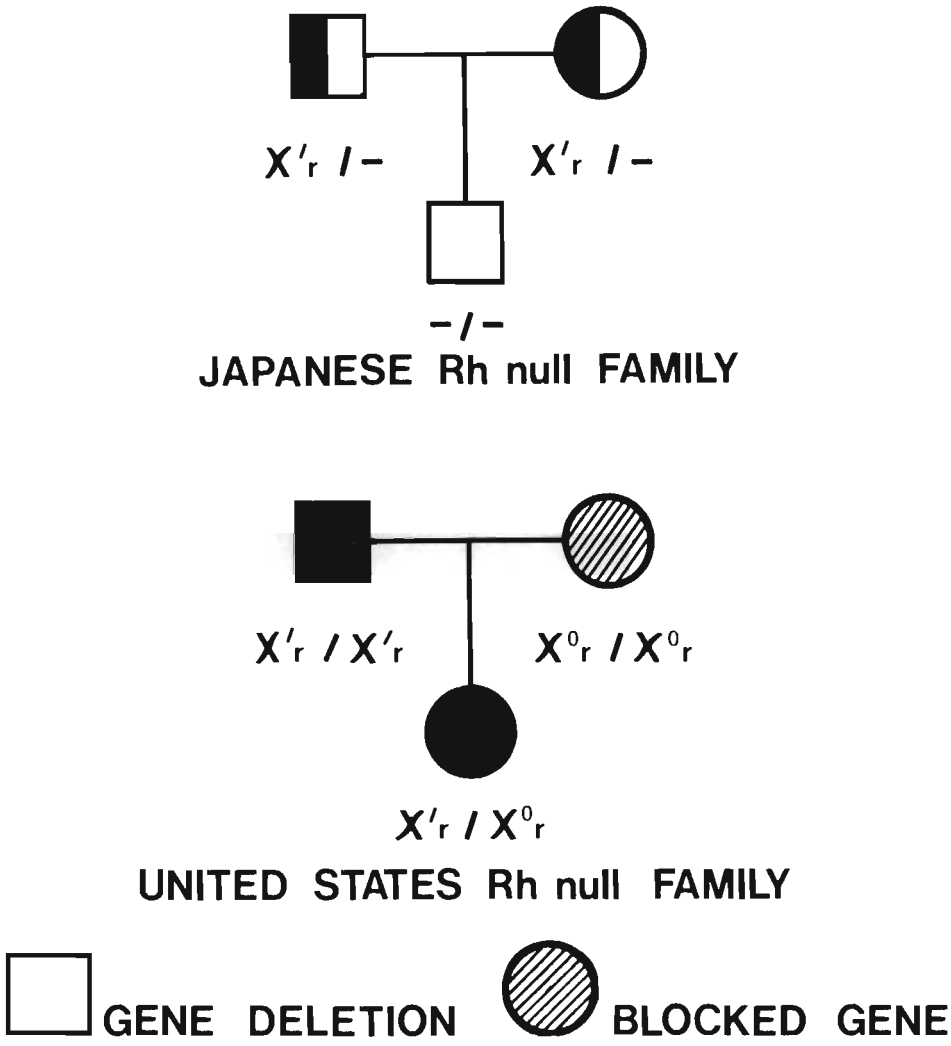

Figure 6: An illustration of two types of condition that can lead to Rh_{null} expression.

Chapter XIII

THE SPECIFICITY OF ACQUIRED HAEMOLYTIC ANAEMIA AUTOANTIBODIES

1. INTRODUCTION

If a serum contains a gammaglobulin which will react with (agglutinate, haemolyse or sensitize) certain genetically well-defined red blood cells but not others, it is said to possess an antibody, and the antibody is said to be specific for a certain property of the red cells. Thus the specificity of an antibody depends on this selective reactivity. This simple approach for determining antibody specificity is often difficult to demonstrate if the range of activity is widespread and if there are only a few cells available which lack the property against which the antibody reacts. A good many blood grouping laboratories have in their freezers sera containing antibodies against 'Public Antigens'. These sera have up to now remained unclassified because they react with all available red cells except those of the patient or donor. Nobody will dispute the antibody nature of these reagents or call them 'non-specific'. If these 'non-specific' agents could be shown to be 'specific', that is not to react with certain genetically well-defined red cells, more workers might be persuaded to regard them as true antibodies.

An important feature of the antibodies found in acquired haemolytic anaemia of the 'warm' type is their ability to react with the patient's own red cells *in vivo*. Until 1953 when Weiner, Battey, Cleghorn, Marson

and Meynell discovered that such autoantibodies possess definite 'specificity' it was generally thought that the antibodies reacted with an undetermined antigen.

Using red cells with a wide variety of well-defined Rhesus phenotypes it was established that about 30 per cent of patients with acquired haemolytic anaemia of the 'warm' type possess autoantibodies with specificities directed against several of the known Rhesus antigens on the surface of the red cells. In the other 70 per cent of patients the specificity of the autoantibodies could not be identified because they did not share the true characteristics of known Rhesus antibodies.

With an added knowledge of the genetics of the Rh system from the discovery of Rhnull cells (Vos et al, 1961) and red cells from which the Cc and Ee portions of the Rh gene complex had been lost (Race, Sanger and Selwyn, 1950) the nature of non-specific autoantibodies was re-examined.

The autoantibodies from 60 patients suffering from acquired haemolytic anaemia were tested against a variety of cells with common and very rare Rhesus genotypes.

2. MATERIALS AND METHODS

Eluates obtained from the red cells were used rather than sera because sera often do not contain all the antibodies that can be detected in eluates.

The eluates were prepared by the ether elution method of Vos and Kelsall (1956). They were tested against Cde/Cde, cdE/cdE, CdE/cde, CDE/CDE, cde/cde KK cells, one D--/D-- blood, one Dc-/Dc- sample and the original Rhnull

blood. The cell panel included the following Rhesus antigens: 'normal' cells (not deleted: nl), 'partially' deleted cells (pdl) and 'fully' deleted cells: (dl). The tests were performed with positive and negative controls. Indirect Coombs tests were done to confirm doubtful results but generally the enzyme method proved to be a valuable qualitative test. None of the eluates produced agglutination of saline-suspended cells. Most eluates agglutinated cells suspended in bovine albumin but the reactions were much better with enzyme-treated cells and the latter method was generally used.

Absorptions were done in the following way: 1 volume of packed red cells (usually unmodified) was incubated with 1 volume of eluate at 37° C for 2 hours. The supernatant was removed and kept. The red cells were then (without washing) incubated for another 2 hours with 3-4 volumes of the same fresh eluate and were then eluted.

Titrations were done using doubling dilutions of the eluates in saline and enzyme-treated cells. 66 eluates were available in quantities large enough for more detailed investigation. Six showed 'blood group specificity' and were excluded. Of the remaining 60, 10 were from 'normal' donors who were found to have 'coated' red cells. The remaining 50 eluates were from patients suffering from typical autoimmune haemolytic anaemia (warm type) of varying severity.

3. RESULTS

Weiner (1961a) established that when 'partially' del-

eted cells (D--/D--) were used together with other 'normal' red cells, the eluates fell into three groups which were called Class 1, Class 2 and Class 3. This classification depended on whether they reacted as strongly with D--/D-- cells as with 'normal' cells (Class 1), more weakly with the former (Class 2) or not at all with them (Class 3). This classification was confirmed by us, but a further subdivision was necessary to include the observed reactions obtained with completely deleted (---/---) cells. Some Class 1 eluates reacted equally strongly with ---/--- cells (1a), others reacted more weakly (1b) and the rest showed no reactivity with them (1c). Class 2 eluates could be subdivided in the same manner (2a, 2b,2c) but Class 3 eluates gave only one pattern of reactivity, i.e. they did not react with the deleted (---/---) cells at all. Table 42 shows the reactivity of the 60 eluates.

There was no correlation between the class and subdivision of the eluates with the ABO groups or Rhesus genotypes of the patients or donors. Both ABO groups and Rh genotypes showed the expected distribution. Amongst the patients there were 28 men and 24 women. Of the 'normal' donors all 10 were men (amongst the donor population from which these donors were drawn, the ratio of men to women is almost exactly 1:1).

It would appear that more women than men develop an antibody which will react with the ---/--- cell, but the differences shown in Table 43 are statistically not significant. This table also shows that of the 60 eluates examined, 32 failed to react against the ---/--- cell and

TABLE 42

Classification of 60 acquired haemolytic anaemia red cell eluates

	Class 1			Class 2			Class 3
	a	b	c	a	b	c	
Patients	13	3	10	2	6	8	8
Donors	2	2	1	1	-	3	1
Total	15	5	11	3	6	11	9

Class 1: React equally strongly against D--/D-- cells as against "normal" cells.

Class 2: React less well against D--/D-- cells than against "normal" cells

Class 3: React well against "normal" cells; do not react against D--/D-- cells.

Sub-division:

a. Eluate reacts equally well with all three types of cells

b. Eluate reacts less well with ---/--- cells than with "normal" or D--/D-- cells.

c. Eluate does not react with ---/--- cells

TABLE 43

Distribution of the various classes of eluates between male and female, patients and donors

		Class 1			Class 2			Class 3
		a	b	c	a	b	c	
Total	Male	6	3	7	2	1	10	7
	Female	8	2	5	1	5	1	2
Patients	Male	4	1	6	1	1	7	6
	Female	8	2	5	1	5	1	2
Donors		2	2	1	1	0	3	1

11 eluates showed less activity against these cells than 'normal' cells. Thus 43 (71 per cent) eluates did not react, or reacted weakly against ---/--- red cells when compared with the reactivity against 'normal' red cells. This pattern of reactivity suggested a mixture of antibodies in the 'reacting' eluates which was confirmed by absorption and re-elution experiments.

A few typical examples are given. Eluate B.A.B. (Table 44) gave the following reactions: ++++ against normal cells, ++++ against the D--/D-- cell and +++ against the ---/--- cell. When this eluate was absorbed with ---/--- cells the antibody against ---/--- was taken out but the reactivity against normal cells and D--/D-- was almost identical with the pre-absorption activity. A further absorption with D--/D-- left an antibody in the supernatant which reacted only with 'normal' cells. Re-elution of the antibodies from the absorbing red cells produced reagents which had the expected reactivity. Table 45 shows a similar absorption experiment on eluate L.A.

Non-reactivity of certain eluates against D--/D-- and/or ---/--- red cells might have been thought to be the result of quantitative rather than qualitative factors. However, the results demonstrate that the classes of the eluates obtained were independent of the titres of the eluates against 'normal' cells. Scores of different eluates against different red cells are shown in Table 46. It can be seen that the scores (or titres) against normal cells have no influence on the class or subdivision of the eluate. In Class 3, for instance, scores range from

TABLE 44

Absorption experiments - Eluate B.A.B.

	cdE/cdE	D--/D--	Cde/Cde	cde/cde	---/---	CDe/cDE
No. 1. Original eluate	3	4	4	4	3	4
No. 2. Eluate absorbed ---/---	3	3	3	3	-	3
No. 3. Eluate absorbed D--/D--	3	-	3	3	-	3
No. 4. No. 2 absorbed D--/D--	2	-	2	2	-	2
No. 5. Eluate re-eluted off ---/---	3	3	3	3	3	3
No. 6. Eluate re-eluted off D--/D--	3	3	3	3	3	3

The figures indicate degree of agglutination (0-4) of papainized cells by the eluates.

TABLE 45

Absorption experiments - Eluate L.A.

	cdE/cdE	D--/D--	Cde/Cde	cde/cde	---/---	CDe/cDE
No. 1. Original eluate	4	4	4	4	4	4
No. 2. Eluate absorbed ---/---	4	3	3	3	-	3
No. 3. Eluate absorbed D--/D--	4	-	3	3	-	3
No. 4. No. 2 absorbed D--/D--	4	-	3	3	-	3
No. 5. Eluate re-eluted off ---/---	3	2	3	3	3	2

The figures indicate degree of agglutination (0-4) of papainized cells by the eluates.

TABLE 46

Agglutination scores of eluates against enzymated cells

	"Normal" cell	D--/D--	---/---
Class 1a eluate	30	30	30
Class 1b eluate	40	40	34
Class 2c eluate	85	56	-
Class 2c eluate	59	20	-
Class 3 eluate	85	-	-
Class 3 eluate	45	-	-

45 to 85 and in other classes conditions were similar.

4. DISCUSSION

It was established that the eluates of cells from patients with AHA and from 'normal' donors contained three different types of antibodies. The red cell antigens reactive with these antibodies are listed in Table 47. The results suggest that the eluates may contain anti-nl, anti-pdl, and anti-dl, singly or in combination.

It was found that one person may form one antibody whereas another may form two or three against related antigens. This is common in people developing antibodies within the Rhesus system (e.g. anti-D plus anti-C and anti-G). It appears that all 'normal' cells contain the antigen designated as 'nl'. This may be produced by a gene on the Ee locus as it is absent from the D--/D-- and the Dc-/Dc- cells. Or it may be a 'composite' antigen whose production depends on the presence of a full complement of 'normal' genes. Its absence from the partially deleted cells could be explained by either hypothesis. The anti-pdl antibody may react with an antigen produced by a gene near the D locus, nearer in fact than the C gene, as both D--/D-- and Dc-/Dc- carry it. It is not a part of the D antigen as it can be found on D negative cells in the same strength as on D positive cells. As far as we know the 'dl' antigen has a universal distribution.

The factors determining the type of antibody which is formed in different patients are not known. The severity of the illness has apparently no bearing on this question

TABLE 47

Suggested antigenic make-up of "normal", "partially" deleted and "fully" deleted cells

"Normal" cells	nl	pdl	dl
D--/D-- or Dc-/Dc- cells		pdl	dl
---/--- cells			dl

For explanation of symbols, see text

as all types of eluates were found in all types of patients, even 'normal' donors. Similarly, the genotype of the patient or donor has no bearing on the development of a particular class of antibody. At present it is unexplained why some people form an antibody, e.g. against c or e antigens and others against the antigens described in this study, though all patients or donors who were investigated carried the latter antigens on their cells.

Investigation of antibodies contained in the eluates of patients and 'normal' donors has demonstrated three antigens within the Rh system, antigens which seem to be additional to those recognized by the commonly used Rh antisera. They have demonstrated that the 'fully deleted cell' still carries an antigen. It is not known whether this antigen is the 'ground substance' of the Rh system or part of the complex Rh system necessary for the expression of other Rh antigens.

Chapter XIV

THE SEROLOGICAL CHARACTERISTICS OF ACQUIRED HAEMOLYTIC ANAEMIA AUTOANTIBODIES

1. INTRODUCTION

The present study was designed to determine the serological characteristics of the various autoantibodies found in patients with AHA. Using normal erythrocytes and Rh_{null} erythrocytes the autoantibody specificity was studied and the reactivity defined both with the antiglobulin test and erythrocytes treated with a proteolytic enzyme (ficin). A further subject for study was the evaluation of complement components on the erythrocytes of patients with warm AHA autoantibodies. The presence of both IgG and complement components in erythrocyte eluates was defined with the antiglobulin test using monospecific antiglobulin sera (anti-IgG and anti-complement). From these results a hypothesis for the apparent discrepancy in the complement fixing properties of Rh isoantibodies and the autoantibodies found in AHA of the 'warm' type is presented.

2. MATERIALS AND METHODS

Selection of patients depended upon a positive direct antiglobulin test of the red cells and the haematological association of an autohaemolytic anaemia of the 'warm' type. Of the 24 patients on whom red cell eluate studies could be performed only 12 were available for a detailed investigation of the serum as well as the red cells.

To avoid *in vitro* sensitization of the red cells due

to cold antibodies on storage at 5° C, separation of the red cells from the defibrinated blood was performed on the same day as collection. For the preparation of eluates red cells were washed four times with 20-40-fold volumes of isotonic sodium chloride solution (0.85%) and stored at -20° C for varying periods as a 50% concentration of packed red cells in saline.

Antibody elution - To recover autoantibodies from the patient's *in vivo* sensitized red cells the ether elution procedure of Vos and Kelsall (1956) was used. The only modification to the published method was that ether was added directly to the 50% suspension of packed washed red cells. At no stage before or after the elution procedure was fresh human serum added to the suspension of haemolyzed red cells.

Antiglobulin test - The antiglobulin test was performed on a translucent white rectangular tile as described by Dacie and Lewis (1963). A 'broad spectrum' commercial anti-human serum capable of detecting IgG and complement components was used. A specific anti-IgG was made by absorbing the broad spectrum anti-human globulin serum with cells sensitized with incomplete Le^a (IgM) antibody and complement components. An 'anti-non-gamma' reagent was made by adding purified human IgG globulin to the broad spectrum serum as described by Dacie (1962). In the more recent experiments an antiserum to complement components C3 and C4 was used. Parallel experiments using the anti-complement and 'anti-non-gamma' serums showed similar reactivity to that reported previously by Harboe

et al. (1963) and Leddy and Bakemeier (1967).

Ficin test - For the enzyme test a solution of 1% commercial ficin in isotonic sodium chloride solution and buffered to pH 7.2 with Sorensen buffer was prepared. To detect antibodies one drop of this enzyme solution was added to one drop of serum or red cell eluate, followed by one drop of a 5% suspension of known red cells. At no stage were the red cells pre-treated with this enzyme for the recognition of ficin-reactive antibodies. The enzyme agglutination tests were performed on translucent tiles and left in a damp chamber at room temperature (22° C) for one hour. Agglutinations were read macroscopically with a 5 x magnification head fitting eyepiece.

Red cells used - To detect the presence of known antibodies a commercial panel of red cells was used. Antibody absorption and elution studies were performed with DCe/DcE and Rh_{null} red cells, each obtained from a single donor.

Definitions of antibody specificity - In acquired haemolytic anaemia it is not unusual to find more than one specific autoantibody. Antibodies characterized in this study were defined as detailed in Chapter XIII.

(a) Antibodies for well-defined red cell antigens: -

These antibodies show distinct specificity against any of the well-defined blood group antigens such as C,c, E,e,Kell, Fy^a, Le^a etc. Since undiluted serum or eluate sometimes reacted with all normal cells (i.e. those without Rh gene deletions), absorption studies were necessary to demonstrate such specificity.

(b) Antibodies reactive with 'normal' red cells (anti-nl): Anti-nl antibodies are defined as those that react with normal human red cells possessing the common Rh phenotypes ce/ce, DCe/ce, DcE/DcE etc. but fail to react with the fully deleted Rh_{null} red cells (---/---). Since partially deleted red cells of the phenotype D--/D-- were not available for this study, it is highly probable that some antibodies for normal red cells could also contain anti-pdl (Weiner and Vos, 1963).

(c) Antibodies reactive with Rh_{null} red cells (anti-dl): Anti-dl antibodies are defined as those reacting with Rh_{null} erythrocytes. The Rh_{null} bloods used were obtained from three sources: (1) Mrs.E.N. (the original Rh_{null} Australian Aborigine, Vos et al. 1961); (2) the Japanese Rh_{null} (Ishimori et al. 1966); (3) a white Californian Rh_{null} (P. Sturgeon, unpublished). Serum or red cell eluates reactive with normal cells and with Rh_{null} red cells were absorbed using equal volumes of serum or eluate against washed packed Rh_{null} red cells. If the antibody was completely removed by these red cells the specificity was considered to be anti-dl. If the Rh_{null} cells removed the antibody against Rh_{null} cells but left an antibody against normal cells the antibody in the serum or red cell eluate was considered to be a combination of anti-nl and anti-pdl.

Differentiation of ficin and antiglobulin-reactive autoantibodies - AHA autoantibodies can be detected by serological techniques such as the antiglobulin and ficin tests (Dacie, 1962). These techniques can also be used

to further characterize AHA autoantibodies of multiple specificity. For example, should an AHA autoantibody sensitize normal red cells by the antiglobulin as well as the ficin test, and Rh_{null} red cells by the ficin test only, then the first procedure is to absorb this serum or eluate with ficin-treated Rh_{null} red cells. Subsequent tests may then show that the absorbed serum or eluate still contains the antiglobulin-reactive antibody for normal red cells but not the ficin-reactive antibody for Rh_{null} red cells. Using this mode of testing a clear differentiation of the type of serological reactivity involved can be established.

3. RESULTS

The specificity of antibodies found in the serum and red cell eluates of 12 patients with AHA is shown in Table 48. Antibodies reactive for well-defined red cell antigens (anti-e, anti-E etc.) are at times observed in the serum. In this series such antibodies were not found in the eluates. Since antibody activity in an erythrocyte eluate more strongly implicates it in the pathogenesis of the disease than antibody activity which is present only in the serum, emphasis was placed on those antibodies present both in serum and eluates.

Investigations were carried out to record how often anti-nl and anti-dl autoantibodies could be detected as ficin or antiglobulin-reactive varieties or a combination of both.

Table 49 shows that autoantibodies detectable only by

TABLE 48

Observed frequency of "anti-n1" and "anti-d1" autoantibodies in the serum and the red cell eluates of 12 patients with acquired haemolytic anaemia.

Patients investigated	Antibody observed for "known" red cell antigens		"Anti-n1" antibody		"Anti-d1" antibody	
	Serum	Eluate	Serum	Eluate	Serum	Eluate
H. L.	+ anti-e	-	+	+	-	+
B. C.	-	-	+	+	-	-
F. R.	+ E	-	-	-	+	+
T. B.	+ Le^{bH}	-	+	+	-	-
H. N.	-	-	-	+	+	+
S. T.	-	-	+	+	-	-
W. B.	+ Fy^b	-	+	+	-	-
C. R.	-	-	+	+	+	+
L. T.	+ E	-	-	-	+	+
C. F.	+ Jk^a	-	+	+	-	-
K. P.	-	-	+	+	-	+
J. L.	-	-	+	+	-	+
Total positive	6 = 50%	0	9 = 75%	10 = 83%	4 = 33%	7 = 58%

+ denotes presence of antibody; - denotes no antibodies detected.

TABLE 49

Frequency of "anti-nl" and "anti-dl" autoantibodies observed in 12 serums and red cell eluates by the ficin test and combined ficin and antiglobulin test

Investigations on:	Antibody detected by:	No. of patients positive for:	
		"anti-nl"	"anti-dl"
Twelve serum samples	Ficin test only	4	1
	Antiglobulin test only	0	0
	Ficin as well as antiglobulin test	5	3
	Total*	9	4
Twelve red cell eluates	Ficin test only	4	0
	Antiglobulin test only	3	5
	Ficin as well as antiglobulin test	3	2
	Total*	10	7

* See also Table 48 for specificity evaluation of each individual case.

the antiglobulin test were not found in the serum but were frequently present in the red cell eluates.

Table 50 shows the frequency of anti-nl and anti-dl autoantibodies detected by the ficin and indirect antiglobulin tests on the same 24 red cell eluates. The Rh_{null} red cells from three donors gave similar results and were more often reactive by the antiglobulin test than by the ficin test. No marked differences were observed between the ability of the ficin or the antiglobulin test to recognize the autoimmune anti-nl antibodies. To determine whether this is because patients cannot produce at one time two different antibodies reactive by the same serological technique, the data were analyzed to determine how often ficin-reactive anti-nl could be found in the red cell eluates together with ficin-reactive anti-dl. Tables 51 and 52 detail this analysis for combinations possible considering the two different autoantibodies and the two testing procedures. Table 51 indicates that the red cell eluates possessing strong ficin-reactive anti-nl very seldom appeared to have strong ficin-reactive anti-dl. Table 52 shows that antiglobulin-reactive anti-nl, together with antiglobulin-reactive anti-dl was also uncommon. In contrast, Tables 51 and 52 show that strong ficin-reactive anti-nl and strong antiglobulin-reactive anti-dl, or strong antiglobulin-reactive anti-nl and strong ficin-reactive anti-nl were combinations of autoantibodies frequently recovered in eluates. The simultaneous recovery of anti-nl and anti-dl antibodies which were strongly reactive by the anti-

TABLE 50

Number of ficin and antiglobulin-reactive autoantibodies observed by examining the red cell eluates against three "normal" and three Rh_{null} samples of blood

Test cells	24 red cell eluates examined by the:			
	Ficin test		Indirect antiglobulin test	
	Positive	Negative	Positive	Negative
"Normal"				
CDe/CDe (R_1R_1)	18	6	21	3
cDE/cDE (R_2R_2)	18	6	21	3
cde/cde (rr)	18	6	21	3
Rh_{null}				
Australian (E.N.)	3	21	11	13
U.S.A. (H.H.)	3	21	11	13
Japanese	3	21	11	13

TABLE 51

An evaluation of the simultaneous occurrence of ficin-reactive "anti-nl" and ficin-reactive "anti-dl" or antiglobulin-reactive "anti-dl"

No. of red cell eluates examined for:			No. of red cell eluates showing presence of: Ficin-reactive "anti-dl"			Antiglobulin reactive "anti-dl"		
			++++ +++	++ +	-	++++ +++	++ +	-
Ficin-reactive "anti-nl"	++++) +++)	11	0	1	10	3	2	6
	++) +)	7	0	0	7	1	4	2
	-	6	2	0	4	0	1	5

++++ and +++ denote strong agglutination; ++ and + denote weak agglutination+ - denotes no agglutination

TABLE 52

An evaluation of the simultaneous occurrence of antiglobulin-reactive "anti-nl" and antiglobulin-reactive "anti-dl" or ficin-reactive "anti-nl"

No. of red cell eluates examined for:			No. of red cell eluates showing presence of:					
			Antiglobulin-reactive "anti-dl"			Ficin-reactive "anti-nl"		
			++++ +++	++ +	-	++++ +++	++ +	-
Antiglobulin reactive "anti-nl"	++++) +++)	13	0	3	10	6	2	5
	++) +)	8	1	4	3	4	3	1
	-	3	3	0	0	1	2	0

++++ and +++ denote strong agglutination; ++ and + denote weak agglutination; - denotes no agglutination

globulin test was uncommon. Thus it was distinctly unusual to demonstrate both autoantibodies by a single serological technique.

In an experiment designed to determine the frequency of complement activity in relation to a number of other parameters so far mentioned it could be shown that complement components appeared only in those red cell eluates which also revealed strong ficin-reactive autoantibodies. In addition, strong ficin-reactive autoantibodies were often strongly reactive in the indirect antiglobulin test using anti-IgG and generally possessed a high incidence of both anti-nl and anti-dl autoantibodies, the latter being uncommon among the weak ficin-reactive autoantibodies. It was also observed that the occurrence of complement components in the red cell eluates is not necessarily associated with the presence of antibodies strongly reactive in the indirect antiglobulin test using anti-IgG. Thus, using anti-complement sera nearly the same percentage of eluates reactive for the indirect antiglobulin test was found whether the eluates gave a strong or weak positive reaction with anti-IgG.

A much better correlation was therefore evident between the occurrence of complement and a strongly positive ficin test. Table 53 demonstrates that six out of eight red cell eluates containing a combination of both anti-nl and anti-dl activity possessed complement components. In contrast, none of the 16 eluates with either specific anti-nl or specific anti-dl activity (but not both) contained complement components. Thus all the red

TABLE 53

Relationship of indirect antiglobulin pattern to presence of "anti-nl" and "anti-dl" autoantibodies in 24 red cell eluates

Indirect antiglobulin test	Numbers observed	"Anti-nl"	"Anti-dl"	No. of red cell eluates	Percentage
Positive using anti-IgG only	18	++	-	9	72
	75%	++++	-	4	
		++++	++	2	11
		++++	++++	0	
		-	++++	2	17
		-	++	1	
Positive with anti-IgG and anti-complement	6	++	-	0	-
	25%	++++	-	0	
		++++	++	3	100
		++++	++++	3	
		-	++++	0	-
		-	++	0	

++++ denotes strong agglutination; ++ denotes weak agglutination;
- denotes no agglutination

cell eluates containing complement components had both anti-nl and anti-dl activity.

4. DISCUSSION

This investigation was carried out to characterize autoantibodies found in the serum and on the red cells of patients with acquired haemolytic anaemia of the 'warm' type. Antibodies of varying specificity were found including those specifically reactive against well-defined red cell antigens (anti-e, anti-E), against all normal red cells (anti-nl) and against Rhnull red cells (anti-dl). Antibodies against well-defined red cell antigens were found in the serum only and not in the red cell eluates, suggesting that in this series such antibodies were isoantibodies resulting from blood transfusion.

In contrast, anti-nl and anti-dl autoantibodies were often detected in the red cell eluates as well as in the serum; they were observed either singly or in combination (anti-nl 54%, anti-nl plus anti-dl 33%, anti-dl 13%). Such antibody specificity was best delineated using both the antiglobulin test and the ficin test. Some autoantibodies were detected only by one or the other of these techniques. This is analogous to some Rh isoantibodies since on rare occasions Rh isoantibodies can be identified only by serological techniques utilizing erythrocytes treated with proteolytic enzymes but not by the antiglobulin test (Vos *et al*. 1962; Kornstad *et al*. 1960) or conversely, only by the latter procedure but not the former (Lucia *et al*. 1960; Viessen *et al*. 1964). This confirms Race and Sanger's (1954) observation that Rh antibodies can exist which are

sharply separated from each other both by the nature of their antigenic specificity as well as by the technique needed for their demonstration. The latter distinctions are presumably explained by subtle differences in antibody molecular structure even within a given major immunoglobulin class. Hence, antibody formation in AHA could be the result of sequential immunization which closely resembles the manner in which different combinations of specific Rh-isoantibodies are produced. For example, repeated episodes of immunization by DCe cells can result in the production of Rh-antibodies commencing from anti-D alone to anti-D and separable anti-C and then to a combination of anti-D and anti-CD (anti-G)(Vos, 1960). This might be analogous to the formation of anti-nl, anti-dl and then combined anti-nl and anti-dl.

Another aspect of this study relates to the finding that AHA autoantibodies frequently fix complement to erythrocytes *in vivo* (Dacie, 1962). Red cell eluates may contain not only antibody but also components of complement which can be detected by the indirect antiglobulin test using an anticomplement reagent (Leddy, et al. 1965; Eyster *et al*. 1967; Petz, *et al*. 1968). It appears that such components of complement are fixed to the antibody and are carried with it in the eluate. Since in this study a source of fresh complement was not added during the incubation period of the indirect antiglobulin test, the antibody in the eluate did not fix complement to the red cells at that time.

Leddy and Bakemeier (1967) considered the possibility

that non-complement-fixing autoantibodies were 'Rh-related' and that complement-fixing autoantibodies were 'non-Rh-related'. In support of this suggestion they found that the eluates from AHA red cells with a positive direct antiglobulin reaction for both IgG and complement usually contained autoantibodies reactive with Rh_{null} cells whereas eluates from red cells that had a direct antiglobulin test positive for IgG only usually lacked reactivity with Rh_{null} cells. Results obtained with 24 eluates indicate, however, that anti-nl autoantibodies may be present with anti-dl autoantibodies and that when both are present complement components will also be detectable in the eluate, whereas with anti-dl autoantibodies alone complement components need not necessarily be found.

Since there is ample precedent for the fixation of complement components by multiple Rh isoantibodies (Harboe et al. 1963; Rosse, 1968) and since the absorption studies revealed multiple autoantibodies in those eluates containing complement components, there would seem to be no need to propose a 'non-Rh-related' autoantibody when complement fixation is present (Leddy et al. 1967). Hence it is preferred to hypothesize that the presence of complement components in association with a combination of anti-nl and anti-dl autoantibodies is analogous to complement fixation produced by multiple Rh isoantibodies. In considering these autoantibodies of multiple specificity the more frequent occurrence of anti-nl (Table 53) suggests that this autoantibody might indicate an early state of autoantibody production. The progressive formation of anti-nl with

anti-dl which then tends to result in the fixation of complement on the erythrocytes may well be a natural progression to a more advanced state of autoimmunization comparable with the development of Rh isoantibodies where as a rule anti-D is more often noted in the early stages of isoimmunization than anti-CD.

Chapter XV

THE IMMUNOGLOBULIN CHARACTERISTICS OF ACQUIRED HAEMOLYTIC ANAEMIA AUTOANTIBODIES

1. INTRODUCTION

The red cells of patients with acquired haemolytic anaemia of the 'warm' type are known to be coated with immunoglobulins which not only represent various specificities of autoantibodies but also structural differences in the physicochemical composition of the autoantibody (Weiner and Vos, 1963; Vos, Petz and Fudenberg, 1970; Dacie and Worlledge, 1969; Leddy, Peterson, Yeaw and Bakemeier, 1970). Therefore the activity of the antibody can sometimes only be determined by using antisera specific against a variety of immunoglobulins.

It has also been shown that some patients may form a mixture of specific autoantibodies, some components of which may react with all, or almost all, human red cells. Although such antibodies appear to lack specificity, individual specificities can often be demonstrated by selective absorption and elution studies using rare red cell antigens. In this way it was shown that the autoantibodies recovered from the red cells may possess different specificities against one or another well-defined red cell antigen closely associated with the Rh system (see Chapter XIII).

This study was designed to determine the incidence of IgG, IgM or IgA coating the red cells of 55 patients with acquired haemolytic anaemia of the 'warm' antibody type. It was important to establish whether one or more auto-

antibodies specific for each red cell antigen determinant found in erythrocyte eluates was correlated with the presence of more than one immunoglobulin class of antibody and with the presence of complement components.

2. MATERIALS AND METHODS

Fifty-five consecutive patients with autoimmune haemolytic anaemia of the 'warm' type, as defined by haematological and serological criteria (Dacie, 1962) and with strongly positive direct Coombs' tests, were studied.

Red cells were separated from defibrinated blood immediately after collection. The cells were then washed four times with 40-fold volumes of isotonic sodium chloride solution (0.85%) and stored at -20° C for varying periods as a 50% concentration of packed red cells in saline.

Reagents - Monospecific anti-IgG, anti-IgM and IgA were used. Specificity of these reagents was determined by haemagglutination techniques and not by immunoelectrophoresis. The reaction patterns of these antisera conformed with the findings of Engelfriet et al. (1968). The anti-complement serum used was prepared as described by Harboe et al. (1963). Previous experiments revealed that the antiserum was specific for complement components C3 and C4.

Red cells used - To detect known antibody systems a selected panel of red cells was used. Antibody absorption and elution studies were performed with CDe/cDE red cells, partially deleted red cells of the phenotype -D-/-D- and fully deleted Rh_{null} ---/--- red cells. The Rh_{null} bloods

used were obtained from the original Rhnull Australian Aborigine and the Japanese Rhnull.

Absorptions - Red cell eluates reactive with normal cells, partially deleted cells and fully deleted Rhnull red cells were absorbed using equal volumes of eluate against washed, packed Rhnull red cells. If all the antibody was completely removed by these red cells the specificity was considered to be 'anti-dl'. If the Rhnull cells removed the antibody against Rhnull cells but left an antibody that still reacted with normal and partially deleted red cells a second absorption was carried out using partially deleted cells. Removal of all antibody activity for partially deleted cells indicated that the eluate also contained 'anti-pdl'. If the eluate continued to show antibody activity for normal red cells the specificity was considered to be 'anti-nl'. Using this method of absorption a clear differentiation of the type of specific antibody or antibodies present could be established.

3. RESULTS

Three specific varieties of autoantibodies were recovered from the red cells of patients with acquired haemolytic anaemia. These antibodies were classified in accordance with the antigenic make-up of a number of selected red cells as shown in Table 47, (page 169).

55 red cell eluates were examined for the presence of one or more varieties of autoantibodies. As shown in Table 54, antibodies with a single specificity (anti-nl, or anti-pdl or anti-dl) were more often present (63%) in the eluates than antibodies with multiple specificities

TABLE 54

Distribution of "anti-n1", "anti-pd1" and "anti-d1" in 55 red cell eluates

Autoantibody specificity	No. observed	Percentage	
Anti-n1	24[a]	44)	
Anti-pd1	5	9)	63%
Anti-d1	6	11)	
Anti-n1 plus pd1	4[b]	7)	
Anti-n1 plus pd1 plus d1	14	26)	37%
Anti-pd1 plus d1	2	3)	
Total:	55		

a Two red cell eluates in this group disclosed antibody activity for well defined Rh antigens, one anti-e and one anti-C.

b One red cell eluate in this group possessed anti-e in addition to anti-n1 and anti-pd1.

(37%). Table 54 also shows that antibodies with 'nl' specificity were more frequent (77%) than antibodies with specificities 'anti-pdl' (46%) or'anti-dl' (40%). Three red cell eluates showed the presence of antibodies for well-defined Rh antigen in addition to 'anti-nl' or 'anti-pdl'. Table 55 describes this observation for one example (eluate S.C.). This eluate failed to react with Rh_{null} cells but not with partially deleted (-D-/-D-) cells, indicating the absence of 'anti-dl' and the presence of 'anti-pdl' (experiment 1). After absorption with -D-/-D- red cells (experiment 2) the eluate retained antibody activity for six examples of normal red cells, suggesting the presence of 'anti-nl'. Subsequent absorption of this eluate by cDE/cDE red cells (experiment 3) disclosed the presence of anti-e. The 'anti-nl' activity was absorbed onto cDE/cDE cells and could be recovered by re-elution from the sensitized cells (experiment 4).

Table 56 relates the autoantibody specificity to the results of indirect antiglobulin tests using antiglobulin reagents specific for IgG, IgM, IgA and complement. As shown, antibodies with single-blood-group specificity usually consist of a single class of immunoglobulin, but this is not true of red cell eluates containing two or three specific autoantibodies. Thus eluates of multiple specificities always contained more than one class of immunoglobulin. As further indicated in Table 56, complement components were detected in 18 of 55 red cell eluates examined (33%). All these patients had positive direct antiglobulin tests with anti-complement reagents. The

TABLE 55

Absorption experiments with red cell eluate S.C.

Expt. No.	Procedure	Test Cells 1 cDE/cDE	2 CDe/CDe	3 CDe/cDE	4 cdE/cdE	5 Cde/Cde	6 cde/cde	7 -D-/-D-	8 ---/---
1	Original eluate	4[a]	4	4	4	4	4	2	0
2	Original eluate after absorption with test cell No. 7	4	4	4	4	4	4	0	0
3	Absorbed eluate from experiment 2 reabsorbed with test cell No. 1.	0	2	1	0	2	2	0	0
4	Re-elution of test cell No. 1, after sensitization with absorbed eluate from Experiment 2	4	4	4	4	4	4	0	0

a "4", "3" and "1" denote intensity of agglutination reaction, with 0 indicating no reaction.

TABLE 56

Indirect antiglobulin reactivity of various AHA autoantibodies for specific preparations of anti-IgG, anti-IgM, anti-IgA and anti-complement

Immunoglobulin pattern of observed autoantibodies	Anti-"nl"	Anti-"pdl"	Anti-"dl"	Anti-"nl" + "pdl"	Anti-"nl" + "pdl" + "dl"	Anti-"pdl" + "dl"
IgG	18	4	2	-	-	-
IgM	4	1	3	-	-	-
IgG + IgM	2	-	1	1	-	-
IgG + IgA	-	-	-	-	1	-
IgG + IgM + C	-	-	-	3	8	2
IgG + IgM + IgA + C	-	-	-	-	3	-
IgG + IgA + C	-	-	-	-	2	-
Totals:	24	5	6	4	14	2

demonstration of complement components was associated only with antibodies of multiple immunoglobulin classes or multiple antibody specificities. In contrast, complement was not demonstrable in red cell eluates possessing only one immunoglobulin type and a single specific autoantibody. Table 57 shows that autoantibodies of a single specificity were more often of the IgG than IgM or IgA class. IgG autoantibody (and to a lesser extent IgM autoantibody) was most often observed when the autoantibody had specificity for a single red cell antigen. Multiple specificities were frequently associated with a wider range of immunoglobulin types, particularly IgM and IgA. The observed variation in the distribution of different types of immunoglobulin might perhaps be attributed to renewed episodes of antibody stimulation by reactive red cell antigens that are not coated by a specific antibody.

4. DISCUSSION

This investigation has shown that eluates of red cells from patients with acquired haemolytic anaemia contain autoantibodies of differing Rh specificities. Some are specifically reactive against well-defined red cell antigens of the Rh system (anti-e, anti-C and the like), others against all normal red cells (anti-nl), partially deleted Rh red cells (anti-pdl) and fully deleted Rh red cells (anti-dl). Autoantibodies directed against the patient's own well-defined CDEce antigens are seldom found in the red cell eluates. In contrast, anti-nl, anti-pdl and anti-dl were the predominating autoantibodies, either singly or in combination. Such antibodies

TABLE 57

Frequency of various immunoglobulin classes observed in 55 acquired haemolytic anaemia eluates

Autoantibody combinations	Immunoglobulin class of autoantibody		
	IgG	IgM	IgA
Single specificity, e.g., anti-"n1" anti-"pd1" or anti-"d1"	27	11	0
Multiple specificities, e.g. anti-"n1" + "pd1" + "d1", etc.	20	17	6
Combined single and multiple specificities	47	28	6

could be formed by a sequential process of *in vivo* immunization.

Serum complement components on the red cells seem to be associated exclusively with autoantibodies of multiple immunoglobulin classes or multiple red cell specificities. The fixation of complement on the red cells of patients with acquired haemolytic anaemia together with the progressive formation of multiple red cell antibodies may represent the natural evolution of the disease.

The variability in multiple antibodies may reflect continuous differences in the inciting stimulus resulting from altered configuration of red cell antigenic determinants. Assuming that trapping and processing of antigen are prerequisites for the induction of antibody response it may be postulated that an adequate concentration of anti-nl on the red cell antigen site inhibits further synthesis of this antibody but not necessarily the enhancement of antibody synthesis for other unbound antigens on the immunizing red cells (anti-pdl and anti-dl). The suggestion that multiple antibody and immunoglobulin formation follows a predetermined course of development as a consequence of variability in antigen presentation is not in full agreement with the idea that the disease primarily results from an aberration of the immune mechanism. The findings suggest that the initial site of action of autoantibody development to Rhesus-antibody-implicated haemolytic anaemia (anti-nl, anti-pdl and anti-dl) results from a defect of the structural composition of the Rhesus genome which is thereafter rejected by the normal

immune mechanism. The subsequent development of additional specificities to other red blood cell antigens involving multiple immunoglobulin classes does not necessarily indicate an aberrant immune apparatus.

The observed pattern of serological and immunological findings does not support the concept that red cell autoantibodies are a homogeneous population of immunoglobulins, at least not in the same sense that myeloma globulins are products of monoclonal proliferation.

The concept that modification of an individual's red cell antigen can lead to the production of autoantibodies has been reported by others (Worlledge, Carstairs and Dacie, 1966; Mohn, Lambert, Bowman and Brason, 1965; van Loghem, 1965). It is postulated that the initial defect in most cases of autoimmune haemolytic anaemia does not involve an aberration of the antibody-forming mechanism but a modification of the Rh antigen determinant.

Chapter XVI

THE VARIOUS SPECIFICITIES OF AUTOANTIBODIES FOUND IN ACQUIRED HAEMOLYTIC ANAEMIA WITH SPECIAL REFERENCE TO THE LW FACTOR

1. INTRODUCTION

It has been established that patients with autoimmune haemolytic anaemia of the 'warm' type may form mixtures of specific autoantibodies which react with all, or almost all, human red cells (Table 54). It has also been found that acquired haemolytic anaemia of the 'warm' type can be associated with autoantibodies to blood group factors LW and U (Celano and Levine, 1967, Nugent, Coledge and Marsh, 1971), suggesting that not all autoantibodies possess specificities against 'Rh-associated antigens' (anti-nl, anti-pdl, anti-dl).

The LW antigen is almost always found in human bloods bearing any one or more of the known Rh antigen determinants (DCEce), and is always lacking from the fully deleted Rh_{null} (---/---) red cells, indicating that the LW factor is closely linked to the Rh system (see Chapter XII). It was particularly important in the present investigation to establish whether autoantibodies to the LW factor may be regarded as a possible variant of one of the 'Rh-associated antibodies' so far described. Confirmation of an association between LW and the various types of autoantibodies detailed in the previous chapters would not only further our knowledge of the LW system but would also help to place the 'nl', 'pdl' and 'dl' varieties of antigens (Table 47) more closely within the framework of

the Rh system.

Reports that autoantibodies are directed against other blood group antigens such as Kidd (van Loghem, J.J. and Hart, v.d.M., 1954), Kell (Dausset and Colombani, 1959) and Xg^a are rare.

2. MATERIALS AND METHODS

Selected AHA patients were studied because their red cell eluates had previously been shown to contain multiple antibodies. The panel of red cells used (Table 59) had been stored in a buffered glycerol citrate solution at -20° C. Frozen cells were recovered by Weiner's method (1961) using Visking dialysis tubing. In no instance did the frozen and thawed red cells react differently from fresh cells before storage.

Antiglobulin tests were performed using standard techniques and antiglobulin serum that had previously been evaluated in detail (Chapter XV).

Absorption studies on AHA eluates were carried out using equal volumes of eluate and washed packed red cells. After incubation at 37° C for one hour the red cells were washed four times in saline. Antibody elution was then performed on a 50 per cent saline suspension of the red cells. The ether elution procedure of Vos and Kelsall (1956) was used, modified only in that ether was added directly to the 50 per cent suspension of packed washed red cells. The sequence of absorption and re-elution used for the differentiation of the type of specific antibody to be recovered is detailed in the Results.

For the formation of anti-LW guinea pigs were given subcutaneous injections, at two-weekly intervals, of a 5 per cent suspension of washed LW positive cells mixed in equal volumes of Freund's complete adjuvant. This produced intense antibody responses within 8 to 10 weeks. For absorption studies, the highest dilution of guinea pig serum giving the strongest reaction with the immunizing red cells was accepted as the optimal starting dilution. Using this procedure repeated absorption of the antibody with valuable LW negative red cells was avoided. The range of antibody titre values scored was between 1:512 and 1:4096. It was found that the method of Levine and Celano (1967) in which guinea pigs were given six intraperitoneal injections of a 25 per cent suspension of LW positive cells at intervals of three to four days resulted in considerably lower responses.

3. RESULTS

Table 58 records the indirect antiglobulin titre values of eight selected AHA eluates tested against a panel of red cells capable of differentiating a variety of specific antibodies. Six of the eight eluates revealed significant differences in reactivity when they were tested against red cells of varying genotypes, suggesting that mixtures of specific autoantibodies may be involved.

Table 59 summarizes the results of antibody determinations performed on the same eluates using two different sets of panel cells. The findings show that cell panel 1 was limited in its scope for detecting anti-U and anti-LW because it lacked the required cell types to differentiate

TABLE 58

Indirect antiglobulin reactions of various AHA red cell eluates for known red cell types

Red Cell Type	Red Cell Eluates from Acquired Haemolytic Anaemia Patients							
	BN	BL	SP	CT	FR	MT	VP	BS
cDE/cDE, LW+, U+	512*	16	256	4	32	128	64	256
cde/cde, LW+, U+	256	16	256	4	32	128	64	256
CDe/CDe, LW+, U-	512	16	256	4	32	128	64	256
cDE/cde, LW+, U-	256	16	256	4	32	128	64	256
CDe/cde, LW-, U+	256	16	256	4	8	128	64	256
-D-/-D-, LW+, U+	256	16	256	4	32	128	64	32
---/---, LW-, U+	8	4	256	4	0	0	64	0
---/---, LW-, U-	0	4	256	0	0	0	64	0

* Titres are expressed as reciprocals of highest dilution giving definite positive results

TABLE 59

Specificities of autoantibodies recognized by selective absorption and elution studies using different panels of red cells.

	Antibodies in eluates from patients' red cells recognized by:	
	Panel 1	Panel 2
	CDe/CDe, LW+, U+ cDE/cDE, LW+, U+ cde/cde, LW+, U+ -D-/-D-, LW+, U+ ---/---, LW-, U-	CDe/CDe, LW+, U- -D-/-D-,LW+,U+ cDE/cDE, LW+, U+ ---/---,LW-,U+ cde/cde, LW+, U+ ---/---,LW-,U- cDE/cde, LW+, U- CDe/cde, LW-, U+
Patients		
BN	anti-pdl	anti-pdl, anti-U
BL	anti-pdl, anti-dl	anti-pdl, anti-dl, anti-LW
SP	anti-pdl, anti-dl	anti-pdl, anti-dl, anti-LW
CT	anti-pdl	anti-LW, anti-U
FR	anti-pdl	anti-pdl, anti-LW
MT	anti-nl, anti-pdl	anti-nl, anti-pdl, anti-LW
VP	anti-dl	anti-dl, anti-U
BS	anti-nl, anti-e, anti-pdl	anti-nl, anti-e, anti-LW
Observed	13 specific autoantibodies	20 specific autoantibodies

anti-U from anti-n1 and anti-pd1, and also anti-LW from anti-pd1.

Restrictions on the availability of rare cell types, particularly the CDe/cde, LW-negative, U-positive cells of Mrs Bigelow (De Veber, Clarke, Hunking and Stroup, 1971) and the Japanese Rh_{null}, LW-negative, U-positive blood (Ishimori and Hasekura, 1967) reduced the opportunity of isolating the full spectrum of autoantibodies.

Table 60 shows the results of specificity testing using red cell eluate FR. This autoantibody had previously been classified as anti-pd1 (Table 59) when a more limited panel of test cells was used. The demonstration of anti-LW specificity and the close association of this antibody with anti-pd1 was recognized when the rare CDe/cde, LW negative red cells of Mrs Bigelow were included. As shown in Table 60, step 1, the antibody in eluate FR reacted more strongly with LW positive cells than the LW negative cells of Mrs Bigelow. The eluate did not react with the two Rh_{null} LW negative red cells indicating the presence of anti-pd1, or anti-pd1 + n1 and possibly anti-LW as well. Step 2: Absorption of the antibody from the original eluate with test cell number four (-D-, LW+, U+) which has pd1, LW and U antigens but not n1, completely removed all antibody activity indicating pd1, or pd1 plus LW specificity. Step 3: The original eluate after one absorption with test cell number 3 (CDe/cde, LW-, U+) retained antibody activity for LW positive test cells only. Step 4: Re-elution from test cells number 4 (-D-, LW+) following step 2, established the presence of an antibody that sen-

TABLE 60

Absorption and elution experiments with red cell eluate FR

Step Number	Procedure	Test cells 1 cDE/cDE LW+, U+	2 CDe/CDe LW+,U-	3 CDe/cde LW-, U+	4 -D- LW+,U+	5 Rh_{null} LW-,U+	6 Rh_{null} LW-, U-
1	Original eluate	32*	32	8	32	0	0
2	Original eluate after 1 absorption with test cell No. 4.	0	0	0	0	0	0
3	Original eluate after one absorption with test cell No. 3.	4	4	0	4	0	0
4	Re-elution of antibody from test cell number 4 (step 2)	16	16	4	16	0	0

* Titres are expressed as reciprocals of highest dilution giving definite positive results in the indirect antiglobulin test

sitized LW positive red cells more strongly than the LW negative red cells of Mrs Bigelow (test cell 3). This eluate was then tested against over 200 random samples of blood to determine whether other examples of weak positive reactions similar to the weak reactions observed with Mrs Bigelow's red cells could be recognized but none were found.

Table 61 illustrates the results obtained when eluate PM, which reacted with all cells tested except Rh_{null}, was absorbed with various cells including animal cells. One absorption of the eluate with Mrs. Bigelow's CDe/cde LW negative cells removed activity for her cells completely and reduced the reaction against cde/cde LW positive and CDe/cde LW positive cells. Absorption with cde/cde LW positive cells removed the activity against Mrs Bigelow's cells and CDe/cde LW positive cells and reduced the activity against CDe/cDE LW positive cells. Absorption with CDe/cDE LW positive cells removed all antibody activity. Baboon and monkey cells also removed all antibody activity but absorption with sheep, rabbit or horse did not. These results expand the observations made in Table 60.

The data may be interpreted as follows: If Mrs Bigelow's cells represent a weak variant of LW the antibody recovered from the -D-/-D-, LW positive sensitized red cells (Table 60), step 4) can be designated as anti-LW rather than anti-pdl. This implies that Rhnull red cells would remain as the true LW negative bloods, as originally described by Levine, Celano, Vos and Morrison (1962). The ability of anti-pdl to react more strongly with LW posit-

TABLE 61

Absorption experiments with red cell eluate PM

		Reactions with test cells				
		CDe/cDE LW+	CDe/cde LW+	cde/cde LW+	CDe/cde LW-	Rh_{null} LW-
Before absorption		++++	++++	++++	+++	0
Absorbing cells	Rh_{null}, LW-	+++	+++	+++	++	0
	CDe/cde, LW-	+++	++	+	0	0
	cde/cde, LW+	+	0	0	0	0
	CDe/cDE, LW+	0	0	0	0	0
	Baboon	0	0	0	0	0
	Monkey	0	0	0	0	0
	Sheep	++++	++++	++++	++++	0
	Rabbit	++++	++++	++++	++++	0
	Horse	++++	++++	++++	++++	0

ive test cells (Table 60, step 4) and very weakly with the LW negative test cells of Mrs Bigelow, may be interpreted to imply that anti-pdl is anti-LW plus anti-LW_1 which may be compared with a group B serum with anti-A plus anti-A_1. If such an analogy exists then it might be expected that absorption of anti-LW plus LW_1 (anti-pdl) by a subgroup of LW (e.g. Mrs Bigelow) should leave unabsorbed anti-LW_1 activity and that the eluate from the absorbing red cells would contain anti-LW and react with LW positive cells. This hypothesis was confirmed by absorbing the original eluate with Mrs Bigelow's red cells (Table 60, step 3). These cells not only removed the LW activity from anti-LW plus LW_1 but also considerably reduced the strength of anti-LW_1, a feature which parallels the preparation of anti-A_1 serum which has been absorbed with group A2 cells. The results in Table 61 confirm and expand these findings as monkey and baboon cells are known to be rich in LW antigen (Levine and Celano, 1962). The results further suggest that homozygous D positive cells have more LW antigen than heterozygous D positive cells, which have more LW antigen than D negative cells.

To confirm that Mrs Bigelow does possess a weak LW antigen her cells were injected into guinea pigs. The results are shown in Table 62. The antibody formed by Mrs Bigelow's cells was exactly the same as that observed after injection of LW positive cells. In all the immunization experiments, whether Rh positive, LW negative, or Rh positive LW positive cells were used, the guinea pigs always produced two types of antibody; one that reacted

TABLE 62

Absorption experiments using guinea pig antisera to Rh positive LW-(Mrs Bigelow) cells

		Reactions with test cells				
		CDe/cDE LW+	CDe/cde LW+	cde/cde LW+	CDe/cde LW-	Rh_{null} LW-
Before absorption		++++	++++	++++	++++	++++
Absorbing cells	Rh_{null}, LW-	++++	++++	+++	++	0
	CDe/cde, LW-	++++	+++	+	0	0
	cde/cde, LW+	++	(+)	0	0	0
	CDe/cDE, LW+	0	0	0	0	0
	Baboon	0	0	0	0	0
	Monkey	0	0	0	0	0
	Sheep	++++	++++	++++	++++	++++
	Rabbit	++++	++++	++++	++++	++++
	Horse	++++	++++	++++	++++	++++

with all human cells and could be removed with one absorption with Rh_{null} cells (probably anti-dl); and an antibody that reacted exactly the same as the eluate shown in Table 61. The reactions of this antibody were apparent only after the guinea pig serum had been absorbed with Rh_{null} cells. The guinea pig antibody for Rh positive, LW positive red cells could always be completely removed by one absorption with Rh positive, LW positive cells and red cells from baboons and monkeys, suggesting that these red cells possess an abundance of LW sites.

Autoantibodies reactive for Rh_{null} cells were observed in nearly 40 per cent of 51 red cell eluates from AHA patients (Chapter XIII). They were found either singly or in combination with other Rh-related autoantibodies. The activity of autoantibodies for Rh_{null} red cells can be removed by absorption with any human or non-human primate red cells but not sheep, rabbit or horse red cells. This is illustrated in the results obtained with eluate K.P. (Table 63) where it was found that the specificity of the antibody may be directed against the 'nucleus of the Rh substance', as postulated by Wiener, Gordon and Gallop (1953). The ability of Rh_{null} cells to separate this autoantibody from other varieties of Rh-related antibodies influenced us to classify this autoantibody as anti-dl (i.e. 'deletion').

Guinea pigs injected with the original Rh_{null} red cells produced antibodies which were identical to those recovered in the eluates from the red cells of some patients with AHA (Table 63). In no instance was the production of other

TABLE 63

Absorption experiments with red cell eluate KP

		Reactions with test cells				
		CDe/cDE LW+	CDe/cde LW+	cde/cde LW+	CDe/cde LW-	Rh_{null} LW-
Before absorption		++++	++++	++++	++++	++++
Absorbing cells	Rh_{null}, LW-	0	0	0	0	0
	CDe/cde, LW-	0	0	0	0	0
	cde/cde, LW+	0	0	0	0	0
	CDe/cDE, LW+	0	0	0	0	0
	Baboon	0	0	0	0	0
	Monkey	0	0	0	0	0
	Sheep	++++	++++	++++	++++	++++
	Rabbit	++++	++++	++++	++++	++++
	Horse	++++	++++	++++	++++	++++

known varieties of Rh-related antibodies observed, e.g. anti-LW in guinea pigs injected with Rh_{null} red cells.

Table 64 shows the results obtained with a third variety of Rh-related autoantibody often recovered from the red cells of AHA patients. These antibodies do not agglutinate partially deleted -D-/-D-, LW positive red cells and Rh_{null} cells but react with all other red cells (i.e. anti-nl). Their specific activity seems to be directed towards a region of the Rh antigen representing the CcEe sequences. The antibody can be completely absorbed by Rh positive, LW negative, and Rh positive, LW positive cells but not with red cells from baboons and monkeys. In our immunization studies we were never successful in producing this type of antibody in guinea pigs. Experiments are now in progress to establish whether antibodies to the CcEe region may be more readily produced in non-human primates. Like the donor of the partially deleted -D-/-D- red cells they are known to lack these particular determinants.

4. DISCUSSION

Nugent, Coledge and Marsh (1971) and Marsh, Reid and Scott (1972) recently reported anti-U as a specific autoantibody in AHA. They also postulated that the 'Rh related' autoantibodies which often fail to be identified as specific Rh antibodies may in fact be anti-U. In our study the reactions of red cell eluates from eight AHA patients were examined against a selected combination of extremely rare types of red cells. Absorption and elution experiments showed autoantibodies with U specificity to be found

TABLE 64

Absorption experiments using a selected red cell eluate which did not react with Rh_{null} or partially deleted red cells

		Reactions with test cells					
		CDe/cDE LW+	CDe/cde LW+	cde/cde LW+	CDe/cde LW-	-D-/-D- LW-	Rh_{null} LW-
Before absorption		++++	++++	++++	++++	0	0
Absorbing cells	CDe/cde, LW-	0	0	0	0	0	0
	cde/cde, LW+	0	0	0	0	0	0
	CDe/cDE, LW+	0	0	0	0	0	0
	-D-/-D-, LW+	++++	++++	++++	++++	0	0
	Baboon	++++	++++	++++	++++	0	0
	Monkey	++++	++++	++++	++++	0	0

in some, but not all, preparations of red cell eluates. Thus, no association between anti-U activity and other 'Rh related' autoantibodies was found and the suggestion by Nugent et al. (1971) that the reported 'Rh related' antibodies may be anti-U in specificity was not confirmed.

In eight selected AHA eluates two examples of anti-nl, five anti-pdl, three anti-dl, six anti-LW, one anti-e and three anti-U autoantibodies were observed. This represents an 85 per cent incidence of antibodies with specificities related to the Rh system and a 15 per cent incidence of anti-U. Considering that almost all autoantibodies so far described are related to the Rh system, the finding of a 15 per cent incidence of anti-U lends support to the suggestion that some association may exist between Rh and U antigens (Schmidt, Lostumbo, English and Hunter, 1967).

The 'pdl' autoantibody is often found in AHA eluates and is known to react with all human red cells except Rh_{null}. Previously this antibody could not be classified as anti-LW because it sensitized Mrs Bigelow's 'LW negative' red cells. In the present study it was particularly interesting to find that Mrs Bigelow's LW negative red cells could separate anti-pdl into anti-pdl plus anti-LW (Table 60). It therefore appears that although Mrs Bigelow has been accepted to be LW negative there is a weak form of LW antigen present. The presence of LW antigen on Mrs Bigelow's red cells was also established by injecting her red cells into guinea pigs (Table 62).

It is tempting to suggest an analogy to the ABO system and postulate that Rh positive cells represent LW_1; Rh

negative cells, LW_2 (as suggested by Levine and Celano, 1967) and Mrs Bigelow, LW_3. Rh_{null} appears to be the true LW negative (? 1w). It will be interesting to see whether known Rh negative, LW negative samples represent a weaker form of LW than Mrs Bigelow, who is Rh positive. A contradiction of this analogy would be that if Mrs. Bigelow represents LW_3, comparable to A_3 in the ABO system, then the anti-LW in her serum should react with only LW_1 (i.e. Rh positive) red cells, comparable to the anti-A_1 found in the serum of an A_3 individual. However, the anti-LW reacts strongly with Rh positive (LW_1) cells and weakly with LW_2 (Rh negative) cells. It is possible that the results are simply reflecting a quantitative rather than qualitative difference. Even though anti-A_1 usually does not agglutinate A_2 cells, some workers have reported that anti-A_1 found in A_x individuals reacts with both A_1 and A_2 cells (Issitt, 1970).

We know there is a quantitative effect, as anti-A_1 can be removed by several absorptions with A_2 cells. This demonstrates that anti-A_1 does react with A_2 cells. By analogy anti-LW_1 reacts with LW_1 strongly and LW_2 weakly. The only difference between the reactions of LW_1 and those of anti-A_1 with A_1 and A_2 cells is that the LW reactions are demonstrated by strong agglutination versus weak agglutination, whereas the A_1, A_2 reactions are demonstrated by agglutination versus absorption. The results of these studies indicate that LW represents a spectrum of variations from LW_1, LW_2, LW_3 and maybe LW_4. LW varies even between homozygous D positive and heterozygous D positive individuals which adds to the evidence of the very close association between D and LW.

SUMMARY

The discovery of red cells lacking some (D--/D--) or all (---/---) Rh antigen determinants made it possible to identify not only the so-called 'non specific' auto-antibodies in patients with acquired haemolytic anaemia but also to demonstrate more clearly the possible composition of the basic structure of the Rh genome. Figure 7 represents a schematic construction of the Rh chromosome developed in the light of our observations. In this model it is suggested that the 'deletion' factor (recognized by the ability of certain AHA autoantibodies to sensitize Rh_{null} cells) represents a fundamental precursor of the Rh gene complex capable of directing the formation of specific enzymes for the subsequent development of the 'pdl' (LW, LW_1, LW_2), D, 'nl' C,c,E and e determinants. The 'dl' factor can always be found on the red cells of anthropoid apes, the 'nl' factor never, indicating the possible sequence of these factors in the evolution of modern man.

The 'pdl' factor is assumed to have been formed as a result of gene duplication, 'pdl' being a precursor of the adjacent 'dl' factor in the synthetic pathway but differing from it in possessing enzymes that will add additional specificities to it not possessed by the 'dl' factor. The subsequent appearance of the 'nl' factor adjacent to the 'pdl' factor can be accepted as a later expansion of the Rh genome which has occurred only in man. How factors 'dl', 'pdl' and 'nl' could be associated with

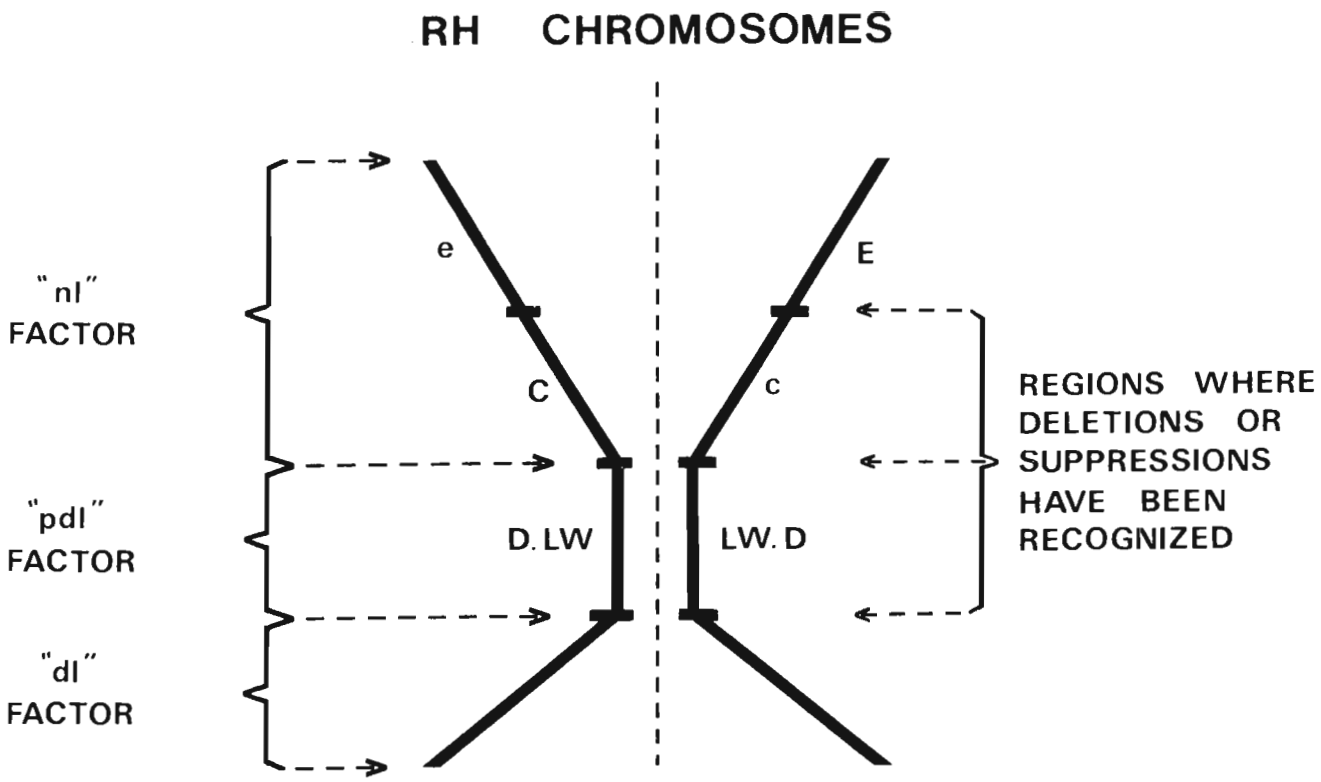

Figure 7: A model of the Rh chromosomes based on the evaluation of acquired haemolytic anaemia autoantibodies.

the D,C,E,c and e determinants is not clear. The 'dl' factor, being present on Rhnull red cells (---/---) as well as on those possessing the D,C,E,c,e determinants (Figure 8) is the least complicated part of the Rh system since it has no substance of the LW, D,C,E,c,e variety in its structure. It could therefore be considered as the primary precursor with enzymatic potential to develop secondary precursors on which the genes giving rise to the development of antigens LW, D,C,E,c and e can exert their effects.

Evidence that 'non specific' autoantibodies possess distinct blood group specificities within the Rh system also contributed to a better understanding of the serological and immunological characteristics of these autoantibodies.

In a study of many red cell eluates from patients with acquired haemolytic anaemia of the 'warm' type it was established that antibodies of varying Rh specificities can be involved in the disease. Some are specifically reactive against well-defined red cell antigens of the Rh system (anti-e, anti-C and the like), some against all normal Rh red cells (anti-nl), some against partially deleted Rh red cells (anti-pdl) and some against fully deleted Rh red cells (anti-dl). Antibodies against well-defined Rh antigens (CDEce) were not often detected in the red cell eluates. In contrast, anti-nl, anti-pdl and anti-dl autoantibodies were frequently found.

The demonstration of serum complement on patients' red cells seems to be associated only with autoantibodies

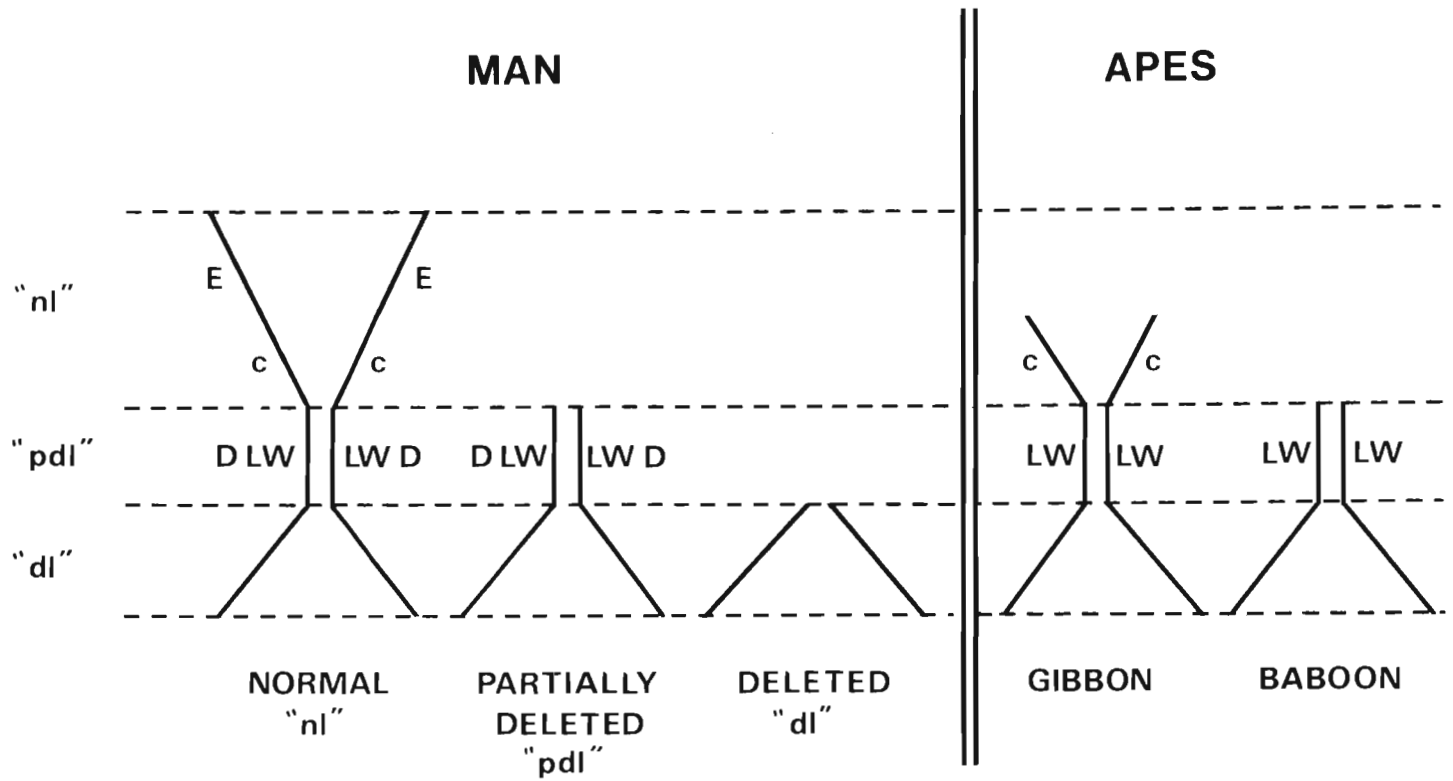

Figure 8: Rh chromosomes of man and apes and their relationship to determinants 'nl', 'pdl' and 'dl'.

of multiple immunoglobulin classes or multiple red cell specificities. The structural heterogeneity of immunoglobulin types (IgG, IgM and IgA) for the various varieties of autoantibodies (anti-nl, anti-pdl and anti-dl) can be pictured as arising from multiple antibody-forming cell lines.

The findings suggest that the initial site of autoantibody development in AHA results from a defect in the structural composition of the Rhesus genome, which is thereafter rejected by a normal immune mechanism. The subsequent development of a more generalized auto-immune abnormality involving the formation of multiple autoantibody-forming cell lines does not necessarily indicate that an aberrant immune apparatus has been established.

Additional studies using red cell eluates revealed that no direct correlation could be established between the presence of complement components and the intensity of the indirect antiglobulin test using anti-IgG serum. However, the simultaneous presence of 'anti-nl' and 'anti-dl' autoantibodies was often associated with the presence of complement components in the red cell eluates. Fixation of complement by Rh isoantibodies of multiple specificity has been reported and thus the presence of complement components in association with anti-nl and anti-dl may be analogous to complement fixation by multiple Rh isoantibodies. The formation of anti-nl with anti-dl, which tends to result in complement components being fixed by the antibody, may be considered as a natural progression of antibody synthesis associated with an advanced state

of autoimmunization.

Absorption and elution studies using rare Rh positive LW negative cells (Mrs Bigelow) showed that anti-pdl may in fact represent anti-LW plus LW_1 and that Mrs Bigelow's cells may possess a weak variant of LW. Injection of her red cells into guinea pigs produced an anti-LW that reacted similarly to the antibody produced by injecting Rh positive LW positive cells. It is suggested that normal Rh positive LW positive cells represent LW_1; Rh negative LW positive cells represent LW_2, Mrs Bigelow's cells LW_3 and that Rh_{null} cells are the only true LW negative (1w) cells.

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