A STUDY OF THE PRODUCTION OF RH' ANTI SERUM IN THE NEW ZEALAND WHITE RABBIT

by

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INTRODUCTION

Since the seventeenth century, difficulty has arisen from blood transfusions, due to some unknown factors.

For some years prior to 1900, many research workers in the field of bacteriology had been engaged in an extension of the knowledge that the animal body would react with, or show an immune response to, a wide variety of foreign materials and agents, called "antigens". This response could be studied experimentally by the occurance or development of mysterious properties, or substances in the blood stream and fluids of the body which would react with the antigens--to which the term "antibodies" was given. These antibodies manifested themselves differently in their reactions against material foreign to them--to antigenic substances.

In 1900, searching for individual differences in human blood, Landsteiner (Race and Sanger, 13) selected the simplest technique possible, namely, to mix the serum of one individual with the red cells of others. By this method, he found that the blood fell into three blood groups. The fourth and rarest group, AB, was not discovered until the following year by DeCastello and Sturli (Andresen, 1).

Discovery of the agglutinogens M, N, and P came next but have proved of little importance for the selection of donors for blood transfusions, because isoantibodies for M, N, and P occur only rarely in human sera.

Intragroup reactions seemed to indicate that there were more factors involved. In 1940, Landsteiner and Wiener (Race and Sanger, 13; Race, 14) discovered the Rh factor and they, along with others, explained that these unexpected reactions might be due to the Rh factors. After much work, very

potent anti-Rh sera of human origin have been produced and made available to the public.

Prior to discovery of the Rh factor, all blood typing was directed toward the use of anti-A and anti-B serums for the selection of donors for patients belonging to a particular blood group. Soon after doctors became aware of the fact that the Rh factor was important as a cause of severe transfusion reactions in their patients, it was also shown that the Rh factor was involved in hemolytic disease of newborn infants.....thus discovery of the Rh or rhesus blood factor has supplied the key to two medical mysteries, namely, the cause of hemolytic transfusion reactions in patients receiving blood of their own A-B-O group, and the cause of an obscure blood disease of newborn infants known as erythroblastosis fetalis.....hence the wide use of Rh typing serums in present day obstetrical and blood banking practices.

The blood group substances are not yet as individual as finger prints, but their inheritance is known much more precisely. The groups are determined by antigens present on the red blood cells; and an antigen is recognized when the cells carrying it are clumped, or agglutinated, by a serum containing the corresponding antibody.

At the present time a minimum of six Rh factors are recognized, giving rise, theoretically, to six anti-sera, by means of which it is possible to recognize eight distinct Rh blood types. Some of these anti sera are readily obtained, other much needed ones are difficult to prepare.

After observing the lengthy process of producing the anti-rh' sera of human origin (sometimes requiring injections over a period of a year or more to secure a titer sufficient for testing) and reading many articles and reviews of previous work, the author wondered if--by injecting the New Zealand white rabbit with the cells of an individual whose type was that of rh'--a

suitable anti-serum might be produced in a shorter time.

The source of material for this study was secured from the Topeka Blood Bank, Sharp and Dohme, Inc., American Hospital Supply Corporation (Dade Reagents, Inc.), and Mr. John Shaffer of Topeka, Kansas.

PRESENT STATUS OF THE RH FACTOR

The Rh or rhesus factor obtained its name when the first antisera were prepared by Landsteiner and Wiener (Wiener, 18) in 1940, by injecting into experimental animals blood of rhesus monkeys. At present, antiserums of human origin are usually used, either from patients who have had intragroup hemolytic reactions, or from mothers of erythroblastotic infants, or from normal male individuals immunized by repeated injections of Rh positive blood.

Of the human Rh antisera, the one corresponding to the original anti-rhesus serum, reacting with the bloods of approximately 85 percent of Caucasians, has been designated as anti-Rho. For the other two antisera has been selected the small letter designations anti-rh' for the one giving 70 percent positive reactions, and anti-rh'' for the one giving 30 percent positive reactions, to indicate that these two antisera are on an equal plane and to distinguish them from the more important anti-Rho serum.

Levine and Javert (Wiener, 28) demonstrated that Rh-negative blood contains a special agglutinogen, designated Hr by them because of its apparently reciprocal relation to Rh. Race and Taylor (Wiener, 28) independently discovered an agglutinogen which they called St, and showed that St was related to rh' as M is related to N. Wiener has proved that Hr and St are identical, so that the designation St has been abandoned. Since the Hr

antigen first found is reciprocally related to rh', Wiener suggested that it be designated as hr'.

Sensitization to the Hr factors can cause clinical complications similar to those produced by Rh sensitization, namely, intragroup hemolytic transfusion reactions and erythroblastosis fetalis. However, the Hr factors are far less antigenic than the Rh factors, so that such cases are quite rare. Yet Hr antisera are necessary to resolve clinical problems caused by sensitization to factors other than Rh, and for the selection of Hr-negative donors to be used for transfusing patients sensitized to one of the Hr factors. Another important application of the Hr antisera is for a presumptive test for heterozygosity or homozygosity of Rh-positive husbands of Rh-negative prospective mothers who are known to be sensitized to the Rh factor. Still another application is in disputed paternity cases, since the Rh-Hr test considerably increase the chances of excluding a falsely accused man. Thus at present, three Rh positive factors (agglutinogens) are recognized, namely, Rho, rh' and rh''.

Wiener's designation	Fisher's designation
rh'	C
Rho	D
rh''	E

Likewise, there are three Rh negative factors: hr', Hro, and hr''.

Wiener's designation	Fisher's designation
hr'	c
Hro	d
hr''	e

It is perhaps unfortunate that two systems of designating the Rh factors are presently in use but it is not impossible to learn both classifications. Workers will find it helpful to use the British or Fisher type of nomenclature when talking about the possibilities of immunization to the Rh factors

and in talking about which genes an individual may have inherited from his parents.

The Rh factors as well as the A and B factors are inherited characteristics of an individual just as the color of one's eyes or the shape of one's face, etc. An individual gets a pair of allelic genes--one gene from each parent each carrying one Rh and Hr blood factors; these groupings remain with him unchanged during his lifetime. If the individual has children, his children receive one of his two genes to go along with one of the mother's genes and thus characteristics are passed from one generation to another.

It is a biological law that an individual who possesses a blood factor lacks the capacity to produce antibodies for that particular blood factor. On the other hand, an individual who does not have a particular blood factor may produce antibodies for that factor.

How are antibodies produced in individuals? It is a well known fact that typhoid vaccine is given to individuals to stimulate antibodies which will act upon typhoid bacilli if the individual is later exposed to them. The antibodies are produced in an individual's system so that they will protect that individual when the foreign bacteria attempt to get a foothold in the individual's body. This is what is meant by immunization. This is why vaccines are given. Now with blood, the same thing happens. For some reason, presently unknown, the (D) factor in blood is a more potent producer of antibodies in an individual than the (C), (E), (c), (d), or (e). This is why it is so common to find persons immunized to the (D) factor. It is probable that more than half of the individuals who lack the (D) factor will produce anti-D antibody following the transfusion of (D) cells into them. Exactly why the other fifty percent do not become immunized is not known.

The rh' (C), and the rh'' (E) factors potentially can and occasionally

do produce antibodies in individuals lacking them. As will be seen from the chart below, the large majority of all persons who lack the (C), (D), and (E) factors are true rh-negative (cde).

Table 1. Reactions with Rh anti-serum.

Rh blood type	Anti-Rho(D)	Anti-rh'(C)	Anti-rh''(E)	Distribution in white pop. in U.S.	
Rho		-	-	cDe	2.0
Rho'			-	CDe	53.0
Rho''		-		cDE	15.0
Rho'Rho''				CDE	15.0
rh'	-		-	Cde	1.5
rh''	-	-		cdE	0.5
rh'rh''	-			CdE	negligible
rh-negative or $Hr_o'Hr_o''$	-	-	-	cde	13.0
	85.0	69.5	30.5		100.0%

In Asiatics, American Indians, and peoples in the Pacific, the CDe and cDE types predominate. Among Negroes, cDe is the most common. CDe and cDE are the predominating types found in the peoples of Europe.

Essential difference between agglutination and conglutination is as follows:

Rh positive red cells	Anti-Rh agglutinins (bivalent antibodies)		Agglutination
Rh positive red cells	Anti-Rh blockers (univalent antibodies)	Conglutinin (X Protein)	Conglutination

Agglutination proceeds in a single step. Each molecule of agglutinin being bivalent (in the chemical sense) links two red cells together, and in this way clumping of cells occurs in any isotonic medium. For the agglutination test, the most satisfactory medium is saline solution.

In conglutination sera, the antibody combines with the red cells but without clumping them. In the presence of whole plasma or serum, however, clumping will occur because plasma and serum contain the so-called protein or conglutinin which is absorbed onto the sensitized cells, thus bringing about clumping. Conglutinin is relatively thermostable but sensitive to even slight dilution by aqueous solutions. Conglutinin or X protein seems to be a colloidal complex of albumin, globulin, and phospholipid into which it readily dissociates upon dilution.

"A" substance is commercially isolated as precipitate from a tryptic digest of hog gastric mucin. "B" substance from the digest of the glandular portion of horse gastric mucosa. In 10 ml. of a mixture of both substances, there are 7 mgm. of "A" and 5 mgm. of "B" substance. This product is used for the following processes:

1-neutralization of anti-A and anti-B agglutinins in universal donor blood.
2-neutralization of group specific antibodies in the method of antibody determination and titration.
3-neutralization of anti-Rh typing serums in the manufacturing process.
4-injection to stimulate the production of anti-A and anti-B agglutinins.

In the field of immunology there were few laboratory procedures which were capable of yielding information of such clinical accuracy as did the Rh antibody titrations (1, 2, 3). For example, the presence of low-titer agglutinins for pathogenic organisms such as typhoid, brucella or tularemia, often may be demonstrated in the serums of individuals suffering with a disease, and yet no other confirmatory evidence of the disease can be obtained.

On the other hand, individuals whose serums contain Rh antibodies almost invariably give a history of events, as transfusions or pregnancies, which account for the presence of the antibody. The opportunity to furnish such

valuable laboratory information to obstetricians and pediatricians make it imperative to use accurate laboratory procedures in Rh antibody titrations.

LABORATORY PROCEDURES

In testing serums for Rh antibodies, the following procedures are used:

Serum

Specimens of blood (5 cc or more) to be tested are collected in clean, dry Wasserman tubes.

(1) Centrifuge and separate serum from clot.

(2) Make a 1-3 dilution of the serum using one part serum and two parts 30 percent bovine albumin (Armour).

Red Cells

Various cells should be tested with serums of known specificity in order to select the desired Rh types. It is important to select Group O individuals who may be always readily available. Rho'Rho'' (CDE) or Rho' (CDe) and Rho'' (cDE) cells may be used, but if antibody is found to be present, pure Rho (cDe), rh' (Cde), and rh'' (cdE) cells must be used to determine the specificity of the serum antibody.

(1) Cells for antibody testing are collected in potassium ammonium oxalate, using one drop of oxalate, (2 mg.) per 1 cc of blood.

(2) Wash 1 cc freshly drawn oxalated blood with sterile 0.85 percent saline in centrifuge tube. Aspirate supernatant and add 0.2 cc of

packed washed cells to 10 cc of saline for agglutinating titrations and 0.2 cc of washed cells to 10 cc of 20 percent bovine albumin for "blocking" antibody titrations.

Slide Titration Method

(1) Prepare a serum dilution using one part of serum and two parts of 30 percent bovine albumin.

(2) Place one drop of the 1:3 dilution of serum in center of glass slide.

(3) On the same slide place two drops of Rh specific cells.

(4) With a wooden applicator mix thoroughly in circular motion in both directions. Cover most of the slide with the mixture.

(5) After the serum and cells are thoroughly mixed put slide on the plate of a warm (45-50°) viewing box and start a stop watch.

(6) Rock the viewing box and observe slide for two minutes or until complete reaction (agglutination) takes place if less than two minutes. Observe for two minutes as after that time drying takes place.

(7) The cells in the mixture will remain dispersed if no antibody is present. If antibody is present, agglutination will appear in less than two minutes and usually before one minute.

(8) Note and record appearance time of agglutination and completion time. If not complete (4) in two minutes note degree of completeness expressed as 1, 2, or 3.

(9) If 1:3 dilution gives an avid reaction and completes in two minutes or less, then make a 1:10 dilution using one part serum and nine parts of 30 percent bovine albumin. Mix thoroughly and test in same manner

as the 1:3 dilution. If 1:10 dilution gives a strong reaction, make a 1:100 dilution of the serum using one part of the 1:10 dilution and nine parts of albumin and test on slide with same cells. If a 3 or 4 reaction is still obtained then make up a 1:1000 dilution using one part of 1:100 dilution and nine parts of albumin, and test.

(10) These preliminary tests on the slide determine how far to carry the test tube titration.

Tube Titration Method

The approximate amount of antibody having been determined by slide tests, a rack of tubes are set up and numbered according to the dilution of serum in the tubes as 1, 2, 4, 8, 16, etc.

Saline and albumin titration series are made. Saline antibody will react in both saline and albumin. "Blocking" antibody will cause clumping in albumin, serum, or plasma, but not in saline.

(Example: Serum tested gives an appearance time of approximately 30 seconds with a 2 reaction on slide with 1:100 dilution, the titer should be around 2-4 x 100, or 200-400. Tubes are set up from the undilute serum to a dilution 1:512 or 10 tubes.

1:10 dilution of serum set up to 1:64

1:100 dilution of serum set up to 1:8

(1) Using a 1 cc pipette deliver to bottom of all tubes but the first 0.1 cc of 20 percent albumin for blocking titration or 0.1 cc saline for "agglutination" titrations.

(2) With 0.2 cc pipette deliver 0.1 cc of serum or serum dilution to the first tube and 0.1 cc of serum or serum dilution to the second tube.

Mix the content of the second tube three times and discard pipette. With a clean pipette transfer 0.1 cc from second tube to third tube and mix, etc. When last tube has been mixed, draw up 0.1 cc and discard.

(3) With 1 cc pipette add 0.1 cc of 20 percent cell suspension to each of the tubes. (For agglutinating titrations, use saline suspended cells. For conglutinating titrations, use albumin suspended cells.)

(4) Shake tubes well and place in 37° water bath for 30 minutes--if albumin suspended cells were used. Leave at room temperature if saline suspended cells were used.

(5) After incubation, shake tubes vigorously for one minute and spin at 2000 rpm for one minute.

(6) Shake tubes just enough to dislodge button from bottom of tube.

(7) Observe for agglutination macroscopically. Record as end point the tube containing the greatest dilution of serum which causes definite macroscopic agglutination.

RAPID ESTIMATION OF ANTIBODY BY SLIDE TEST

While much has been written concerning tube titration procedures, the value of slide titrations was less well known. The slide test was found to be rapid and reasonably accurate in the estimation of antibody titer. It had one distinct advantage over the tube test in that zone phenomena which were commonly associated with high-titered serums were not noted when whole blood was used with blocking antibody on the slide. Using the slide test for the preliminary estimation of antibody content, the limit of serial dilutions necessary to prove the titer in the tube can be estimated.

The slide method of estimating antibody titer has certain limitations.

(1) The relation of antibody titer and appearance time of agglutination must be established for a given test cell. Cells from various individuals react differently with regard to the appearance time of agglutination and completeness of reaction. (2) The technique and time involved in mixing the serum dilutions and whole blood must be relatively standard. The time lapse between mixing and the appearance of agglutination must be carefully recorded, preferably with a stop watch. (3) The serum should be titered by the tube method for confirmation.

A 30 percent solution of bovine albumin has been found to be most useful as a diluent for the serum to be tested. It is important that red blood cells from blood having a hematocrit within normal range (40-45%) be used.

METHODS OF EXAMINATION

Five cc. specimens of blood were secured from the Topeka Blood Bank. Each specimen was typed as to the A-B-O groupings and with anti-Rho sera. When a specimen failed to agglutinate, it was then tested with anti-rh' sera and anti-rh'' sera. Approximately fifty bloods were tested. A suitable rh', group O donor was found and upon his consent, specimens were drawn when necessary to insure fresh cells. The cells were collected into sterile flasks containing ACD Solution.

ACD Solution (Baxter-Alsever):

Sodium citrate, trisodium dihydric	2.1 gm------ 1.6 gm.
Citric acid, monohydric	0.66 gm------ 0.5 gm.
Dextrose, anhydrous	2.0 gm------ 1.5 gm.
Distilled water, up to	100.0 ml------75.0 ml.

Storage temperature 5-10° centigrade.

Blood Group Specific Substances A and B Solution (Sharp and Dohme, Inc.) were added at the rate of 10 cc. per 500 cc. of Group O blood before injection into the rabbits.

Three rabbits were injected with identical amounts and at the same intervals.

Injection No.1: 2.0 ml. of 10% cell suspension.
Injection No.2: 4.0 ml. of 10% cell suspension.
Injection No.3: 5.0 ml. of 10% cell suspension.
Injection No.4: 5.0 ml. of 10% cell suspension.
Injection No.5: 5.0 ml. of 20% cell suspension.
Injection No.6: 5.0 ml. of 20% cell suspension.

The rabbits were injected at intervals of three to four days. (Monday and Friday). At the end of the injection period, a week was allowed to pass before approximately forty cc. of blood was removed from each rabbit (aseptically from the heart). The blood was allowed to clot; then centrifuged and the serum removed. The serum was measured into five cc. portions, placed in sterile test tubes (with screw caps), and placed in the deep freeze until time for titration determinations to be made.

Slide avidities were run on each serum using Rho' cells, rh' cells, and rh negative cells, by the following procedure:

> A serum dilution of 1 part serum to 2 parts of 30 percent bovine albumin was prepared. One drop of the dilution was placed on a clean glass slide to which was added two drops of the known Rh specific cells. After thoroughly mixing, the cells were observed for two minutes--noting the appearance and the completion time.

The titrations were run in duplicate using saline and albumin dilutions with known Rho' M positive, N negative cells, rh' cells, and rh negative M positive, N negative cells. Each titration series was carried to the 1:256 dilution.

Tube	1	2	3	4	5	6	7	8	9
Dilution	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Saline or Albumin	0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Serum	0.1	0.1	Mix and carry over 0.1 to next tube; repeat.						
2% cell Suspension	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Shake tubes 10 seconds and place in a 37 degree water bath for 30 minutes if albumin was used. Leave at room temperature if saline was used. After incubation, shake 1 minute and centrifuge tubes at 2000 rpm for 1 minute. Dislodge button from bottom of tubes and read macroscopically.

RESULTS

Serum No. 1

Slide avidity:

Rho'..... 2 plus @ 2 minutes.
rh'..... Appearance time @ 20 seconds; complete @ 2 minutes.
rh negative.... Negative @ 2 minutes.

Titration:

Saline:

Rho'	(1)	2	2	1		0	0	0	0	0
	(2)	3	2		0	0	0	0	0	0
rh'	(1)	4	4	4	3	2	0	0	0	0
	(2)	4	3	4	4	3	1		0	0
rh-		0	0	0	0	0	0	0	0	0

Albumin:

Rho'	(1)	1	-	0	0	0	0	0	0	0
	(2)	2	1		0	0	0	0	0	0
rh'	(1)	4	4	3	1		0	0	0	0
	(2)	3	4	2		0	0	0	0	0
rh-		0	0	0	0	0	0	0	0	0

Serum No. 2

Slide avidity:

Rho'..... Appearance time @ 35 seconds; 2 plus @ 2 minutes.
rh'..... Appearance time @ 15 seconds; complete @ 2 minutes.
rh negative.... Negative @ 2 minutes.

Titration:

Saline:

Rho'	(1)	4	3	2		0	0	0	0	0
	(2)	4	4	2	0	0	0	0	0	0
rh'	(1)	4	4	4	4	3	1		0	0
	(2)	4	4	3	3	2		0	0	0
rh-		0	0	0	0	0	0	0	0	0

Albumin:

Rho'	(1)	3	1		0	0	0	0	0	0
	(2)	3	2		0	0	0	0	0	0
rh'	(1)	4	4	4	3	0	0	0	0	0
	(2)	4	3	4	3	2		0	0	0
rh-		0	0	0	0	0	0	0	0	0

Serum No. 3

Slide avidity:

Rho'..... Appearance @ 30 seconds; 2 plus @ 2 minutes.
rh'..... Appearance @ 20 seconds; complete @ 2 minutes.
rh negative.... Negative @ 2 minutes.

Titration:

Saline:

Rho'	(1)	2	1		0	0	0	0	0	0
	(2)	3	1	2	1	0	0	0	0	0
rh'	(1)	4	3	4	4	2		0	0	0
	(2)	4	4	3	4	1	1		0	0
rh-		0	0	0	0	0	0	0	0	0

Albumin:

Rho'	(1)	3	1		0	0	0	0	0	0
	(2)	4	2	0	0	0	0	0	0	0
rh'	(1)	4	4	3	3	1		0	0	0
	(2)	4	3	4	2	1	1	0	0	0
rh-		0	0	0	0	0	0	0	0	0

SUMMARY

The results seemed to indicate that a suitable titer can be achieved. Agglutination occurred in dilutions up to 1:16 and 1:32 after six injections. In present day procedures of producing rh' serum from human sources, sometimes months to years are required to secure a titer sufficiently high for commercial preparations.

Many duplications should be run before a suitable procedure could be established. There is doubt as to whether this agglutination of the cells is due entirely to the antigens present on the known cells and the antibody produced in the sera or whether it is due to the human-rabbit element.

Agglutination due to the A, B, and O group, to the Rh factor, and to the M, N, and MN factor has been established. Since the Rho' M positive, N negative cells were agglutinated, the serum could not have contained antibodies against the N factor. Since the rh, M cells were not agglutinated, the serum could not have contained antibodies against the M factor. The P factor is not commonly found in rabbit sera. The more recently found factors were not considered.

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Discovery of the agglutinogens M, N, and P came next but have proved of little importance for the selection of donors for blood transfusions, because isoantibodies for M, N, and P occur only rarely in human sera.

Intragroup reactions seemed to indicate that there were more factors involved. Not until after 1937, Landsteiner, Wiener, and others explained that these reactions might be due to the Rh factors. After much work, very potent sera of human origin have been produced and made available to the public.

After observing the lengthy process of producing the anti-rh' sera of human origin (sometimes require injections over a period of a year or more to secure a titer sufficient for testing) and reading many articles and reviews of previous work, I wondered if---by injecting the New Zealand white rabbit with the cells of an individual whose type was that of rh'---a suitable anti-sera might be produced in a shorter time.

METHODS OF EXAMINATION

Five cc. specimens of blood were secured from the Topeka Blood Bank. Each specimen was typed as to the A-B-O groupings and with anti-Rho serum. When a specimen failed to agglutinate, it was then tested with anti-rh' serum and anti-rh'' serum. Approximately fifty bloods were tested. A suitable rh', group O donor was found and upon his consent, specimens were drawn when necessary to insure fresh cells. The cells were collected into sterile flasks containing ACD Solution. Blood Group Specific Substances A and B Solution was added at the rate of 10 cc. per 500 cc. of Group O blood before injection into the rabbits.

Three rabbits were injected with identical amounts and at the same intervals.

Injection No.1: 2.0 ml. of 10% cell suspension.
Injection No.2: 4.0 ml. of 10% cell suspension.
Injection No.3: 5.0 ml. of 10% cell suspension.
Injection No.4: 5.0 ml. of 10% cell suspension.
Injection No.5: 5.0 ml. of 20% cell suspension.
Injection No.6: 5.0 ml. of 20% cell suspension.

The rabbits were injected at intervals of three to four days. At the end of the injection period, a week was allowed to pass before approximately forty cc. of blood was removed from each rabbit (aseptically from the heart). The blood was allowed to clot; then centrifuged and the serum removed. The serum was measured into five cc. portions, placed in sterile test tubes (with screw caps), and placed in the deep freeze until time for titration determinations to be made.

Slide avidities were run on each serum using Rho' cells, rh' cells, and rh negative cells, by the following procedure:

A serum dilution of 1 part serum to 2 parts of 30 percent bovine

albumin was prepared. One drop of the dilution was placed on a clean glass slide to which was added two drops of the known Rh specific cells. After thorough mixing, the cells were observed for two minutes---noting the appearance and the completion time.

The titrations were run in duplicate using saline and albumin dilutions with known Rho' cells, rh' cells, and rh negative cells. Each titration series was carried to a dilution of 1:256.

Tube No.	1	2	3	4	5	6	7	8	9
Dilution	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Saline or Albumin	0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Serum	0.1	0.1	Mix and carry over 0.1 to next tube; repeat.						
2% cell Suspension	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Shake tubes 10 seconds and place in a 37 degree water bath for 30 minutes if albumin was used. Leave at room temperature if saline was used. After incubation, shake 1 minute and centrifuge tubes at 2000 rpm for 1 minute. Dislodge button from bottom of tubes and read macroscopically.

RESULTS

Serum No. 1

Saline:	Rho'	1:4	Albumin:	Rho'	1:2
	rh'	1:32		rh'	1:8
	rh-	0		rh-	0

Serum No. 2

Saline:	Rho'	1:4	Albumin:	Rho'	1:4
	rh'	1:16		rh'	1:16
	rh-	0		rh-	0

Serum No. 3

Saline:	Rho'	1:4	Albumin:	Rho'	1:2
	rh'	1:16		rh'	1:16
	rh-	0		rh-	0

The results seemed to indicate that a suitable titer can be achieved. Many duplications should be run before a suitable procedure could be established.