1963

Sensitization of rabbits to the Rh factor and desensitization with ribonuclei acid derivatives

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SENSITIZATION OF RABBITS TO THE Rh FACTOR AND DESENSITIZATION WITH RIBONUCLEIC ACID DERIVATIVES

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Submitted in Partial Fulfillment for the
Degree of Doctor of Medicine

College of Medicine, University of Nebraska

April 1, 1963

Omaha, Nebraska

TABLE OF CONTENTS

Introduction

Ottenberg in 1923 discussed a view on the pathogenesis of erythroblastosis fetalis based on an antigen-antibody reaction, but he made no attempt to present experimental data to support his therory.(1) In 1938 Darrow reviewed several theories on the pathogenesis of erythroblastosis fetalis. In his paper he suggested that the most probable cause of this disease was an antigen-antibody reaction based on differences in the maternal and fetal blood constituents.(2)

In 1940 Landsteiner and Wiener described an agglutinable factor in human blood which was recognized by reacting it with sera for Rhesus blood. They designated this agglutinable factor as Rh to indicate that Rhesus blood had been used for the production of the sera.(3) It was later found that this factor was present in the blood of about 85% of the white individuals tested.(4)

It was found that the Rh factor was of clinical importance when Wiener and Peters obtained blood samples from patients who had hemolytic reactions after they recieved repeated transfusions of blood of the proper ABO group. These patients had serum containing anti-Rh isoagglutinins while their blood cells did not con-

tain this factor.(5)

In 1939 Levine and Stetson discribed a hemolytic reaction which had occured in 1937 following a transfusion of apparently compatible blood in a woman after she gave birth to a stillborn. The transfusion was followed by an immediate severe reaction resulting in jaundice, anuria, and ultimate recovery. They theorized that the patient had been immunized by an antigen from the fetus which was inherited from the father. Later it was shown that the patient's blood contained atypical agglutinins which agglutinated approximately 80% of Group O Bloods. Three years following this reaction they were able to show that the patient was Rh negative and the husband Rh Positive.(6) It was not until 1941 that Levine et. al. presented findings in the form of a case history which established the significance of the role of iso-immunization of the mother by factors originating from the fetus as the etiology of erythroblastosis fetalis. They were able to show that of the cases of erythroblastosis fetalis investigated 93% resulted from iso-immunization of the Rh negative mother by the Rh factor present in the red blood cells of the fetus. They also pointed out the value of the agglutination test for the Rh factor in the diagnosis of

erythroblastosis fetalis.(7)

Properties Of The Rh Antigen And Antibody

Antigen-Antibody Dynamics

The dynamics of the antigen-antibody reaction within the Rh system were extensively studied by Evans et. al. They were mainly interested in whether or not the reaction conforms to a mass action equation. If the concept of mass action is applicable, the amount of antibody on the cell would be directly related to the concentration of the antibody in the medium until saturation of the receptors occurs. In all instances of their test situations the degree of transfer of antibody from sensitized group A Rh(D) positive cells to normal group B Rh (D) positive cells was directly related to the concentration of antibody to which the donor cells were originally exposed. It appears that the transfer of anti-D antibody from sensitized to normal cells presented in their work is consistent with the postulate that the reaction between antibody and fixed cell antigen is reversible and in general conforms to a mass action equilibrium relationship. They also were able to show that incubation time, temperature, pH and the presence of colloidal substances all affected the transfer of antibody to some degree.(8)

Chemical Properties Of Rh-Antibodies

Concerning chemical properties, Cann et. al. have shown by electrophoretic studies that Rh antibodies are found not only in the gamma-globulin fraction of human sera but also within the Beta and Alpha globulins. The major part of the Rh blocking antibody was found to be localized in a gamma-globulin fraction of low mobility, while those antibodies detected by the Coombs test were broadly distributed through the globulins.(9) In 1959 Abelson and Rawson discribed a simple method for separations of Rh saline agglutinins from other antibodies by fractionation of Rh antiserum on anion cellulose exchange columns.(10)

Prevention Of Erythroblastosis Fetalis

The Chain Of Pathogenesis

The pathogenesis of Rh hemolytic disease can be divided into three phases. First, the mother is Rh negative and the father Rh positive and because of inheritance the fetus obtains Rh positive red cells as an antigen which the mother does not have. Second, these Rh positive red cells acting as an antigen enter the maternal circulation and stimulate antibody formation. Thirdly, the antibodies which were formed in the mother's circulation cross the placenta and enter into the fetal circulation causing destruction of its red cells.(11)

Prevention Of Incompatible Marriages

In order to prevent erythroblastosis, many methods have been suggested to break the above chain. It is apparent that the only way to break up the first stage would be to prevent incompatible marriages. Because of our present behavioral patterns this would be most difficult, and we therefore must look toward the latter two phases for attempts at preventing erythroblastosis fetalis.

Preventing Sensitization

Finn used the procedure for detecting fetal red cells in smears of maternal blood to confirm the hypothesis that antigenic stimulation of the mother results from the passage of fetal red blood cells across a probable defect in the placental barrier. If ABO incompatibility exists the fetal red cells are rapidly destroyed by antibodies against A or B in the maternal circulation. Finn postulated that if Rh positive but ABO compatible cells were in contact with antibodies against the Rh antigen, then the fetal cell would also be destroyed. He has shown this to be the case by injecting Rh negative male volunteers with Rh positive red cells and following this thirty minutes later with an intravenous injection of anti-D serum. Approximately 50% of the foreign cells were eliminated within two days. Many of the surviving cells were also coated with antibody which probably would block their antigenic properties.(12)

The above method of preventing sensitization may have two pitfalls. First, if the cells are destroyed by hemolysis due to the introduced anti-D, the antigen will not just "go up in smoke". These products by nature of their incompatibility would enter the reticu-

lo-endothelial system and stimulate antibody formation. The second pitfall is that the anti-sera used for injection contains both saline and albumin agglutinins. The saline antibody is a large molecule of molecular weight 1,000,000, while the albumin antibody has a molecular weight of about 160,000. It has been shown that the albumin but not the saline antibodies cross the placental barrier. Therefore, if the anti-D were to be given to a pregnant female the contained albumin agglutinins may harm the fetus.(12) Finn is well aware of the above problems but states that the fetal cells that are rapidly removed are probably phagocytized and broken down before they could initiate sensitization.

Strengthening The Placental Barrier

Recently Jacobs reported on the use of bioflavonoids to prevent capillary fragility at the placental barrier thereby decreasing the possibility of leaking fetal blood into the maternal circulation. His preliminary report listed a total of 103 patients; 71 of whom did not receive bioflavonoids and 32 who did. The former group produced a total of 22 surviving infants or 30%, whereas the latter group produced a total of 24 surviving infants or 75%.(13) Vitamine C has also been used to

strengthen the placental barrier.(14)

The first knowledge of a fraction of human red blood cells which would inhibit the agglutinating properties of anti-Rh serum was reported in 1947 by Carter.(15) This substance was later described in detail and called "Rh hapten" (16). A hapten when combined with a carrier (protein) will stimulate antibody production and can react specifically with the antibody formed by it, but in the absence of a carrier it cannot produce antibodies, although it may be able to neutralize Rh antibodies. In 1951 Carter reported on the use of this hapten therapeutically in 135 cases over a period of 4 years. Their results appeared good and out of 135 cases the 53 per cent who previously had lost erythroblastotic babies had normal infants after treatment with the Rh hapten during pregnancy. They felt that if the Rh hapten could be used properly fetal deaths due to the Rh factor could be eliminated.(17)

Desensitization With Rh Hapten

McDonald attempted to sensitize rabbits to human Rh positive (D) type O blood, but he later was unable to desensitize them by using small quantities of antigen subcutaneously. He used large doses intravenously in

an attempt to suppress antibody production but this also failed. The subcutaneous injection produced only an increasing titer even when large doses of antigen were given. He thought that the antigen was probably inactivated before it could enter the circulation to combine with the antibody and thereby lower the titer of Rh antibodies. The intravenous injections were also unsucessful because very large doses only resulted in a rapidly increasing titer.(18) McDonald's work does not substantiate the work of Talmage who showed that as long as free antigen was present in the serum no antibodies were free in the serum.(19)

Suppression Of Antibody Production With Steroids

Steroids have been recommended in the prophylaxis of erythroblastosis fetalis. When given during pregnancy they are supposed to reduce Rh antibody production in the mother or interfere with the fetal blood cells. To date the results are contradictory.(20)

Specific Inhibition Of Rh Antibodies

Early Speculation

Since the isolation and purification of the ABO and Lewis blood group substances, workers began to speculate that specific inhibition of an antibody by a chem-

ical substance may be indicative of a chemical similarity between the chemical substance and the antigen for which the antibody is specific.(21)

Structural Information About Blood Group Substances

Techniques for obtaining structural information about blood group substances were introducted by Watkins and Morgan.(22) These workers showed that hog and human H substances would inhibit hemagglutination of type O cells by eel-O serum (H). Subsequently they showed that simple sugars would also exert similar effects.(23)

Inhibition Of Rh Antibodies With Ribonucleic Acid Derivatives

Using the same techniques as did Watkins and Morgan, Hackel and others undertook an investigation to elucidate the structure and immunological properties of the Rh and Lutheran blood group antigens. They prepared two percent solutions of 60 chemical substances and all these were tested against anti-D, anti-C, anti-E, anti-Lu^a and anti-Lu^b. Only four of the 60 reagents tested for inhibition had any effect on the Rh antisera. Repeated trials of inhibition with these four chemicals produced consistent positive results. All four of the inhibitors were derivatives of ribonucleic acids. They are cytidine sulfate, adenylic acid, uridylic acid, and

cytidylic acid. These four chemicals were also tested against anti-A, anti-B, anti-M, anti-N anti-He, anti-P and anti-Fy^a but none of the acids would inhibit these antibodies. It was therefore thought that the D, C, and E antigens of the Rh series and the Lu^a andLu^b antigens owe some part of their specificity to ribonucleic acids or ribonucleic acid like-substances. These chemical substances have definate "antigenic" specificity because they failed to inhibit the other antisera mentioned above.(21)

Using An Enzyme To Verify The Inhibition Qualities Of The Ribonucleic Acids.

The hypothesis mentioned in the preceding paragraph was further tested by treating erythrocytes with an enzyme, ribonuclease. If any of the antigenic specificity was due to the ribonucleic acid derivatives then the enzyme should remove these sites and render the cells nonagglutinable, while the enzyme should not affect the cell sites which have no antigenic specificity. Packed cells were incubated for varying times with the enzyme ribonuclease, and controls were also set up using saline instead of ribonuclease. After preparation of the cells they were titrated with various antisera. There were no appreciable differences in the titration values between the cell treated with the enzyme and the saline

controls when titrated with anti-A, anti-B, anti-M, anti-N, anti-P and anti-I. However, in titration with anti-Rh and anti-Lutheran antibodies the degree of agglutinability of the treated cells was less than the untreated saline controls. These results substantiate the previous findings of Hackel whereby he was able to neutralize anti-Rh and anti-Lutheran antibodies with four derivatives of ribonucleic acids.(24)

Further Research On the Ribonucleic Acids By Italian Investigators.

Ricci and others in Italy decided to experiment further with Hackel's concept of a possible chemical similarity between RNA derivatives and the Rh antigen for which the Rh antibody is specific. These workers wanted to examine other antisera of the Rh system (anti-c and anti-e) to determine the nature of the inhibition and to determine whether this inhibition is explained by the hypothesis that the antigenic properties are of similar chemical structure, due to some type of reaction on the surface of the RBC or due to some type of weaker union. They also wanted to know precisely the optimum concentration at which the neutralization is obtained. They tested the inhibitory power of several chemicals but found only adenylic acid and uridylic acid would inhibit anti-D. Anti-c was inhibited by only adenylic

acid and anti-D and anti-C were inhibited by adenosine-mono-phosphoric acid.

In order to study precisely the action of inhibition due to adenosine-monophosphoric acid on antisera anti-D and anti-C they proceded in the following manner. They first incubated the adenosine-monophosphoric acid for one hour at 37 degrees centigrade with the indicator cells (D positive and C positive). Before the addition of the anti-C and anti-D sera they washed the indicator cells several times with physiologic saline to remove the quantity of substance attached to the RBC's. Then the indicator cells were incubated with the anti-C and anti-D and the results showed the same agglutination results as the positive controls. They therefore showed that the inhibiting effect stops after the RBC's have been washed in saline. They believe that the inhibition is elective on the RBC of complete type (of the structure B_2M globulin which have been shown to be susceptible to weaker union with antigen). The complete type react with the antigen without attaching the complement. They found that a greater concentration of the acid is necessary to obtain the desired effect.(25) It appears that they used a greater concentration of acid solutions than did Hackel.

Experimental Work

Repeating The Work Of Hackel And Others

The work of Hackel and others was repeated and expanded. Using similar techniques we were able to show that cytidine sulfate in concentration from 2% to 15% would not only neutralized the antibodies in standard antisera (Ortho saline tube test anti-D) but it would also neutralized albumin type antibodies in a patients serum containing albumin type antibodies.

The saline anti-D and the patients serum were tested as follows. First serial dilutions of saline anti-D were made in saline. For the saline anti-D tests one drop of each dilution was mixed with a drop of saline in the case of the control test and one drop of the acid (cytidine hemisulfate) in case of the neutralizing tests and them both test series were incubated at 37 degrees centigrade for 30 minutes. Then one drop of 2% saline suspended type O Rh positive cells were added to each dilution and the results read microscopically according to the Race Scale (Table III Page 27) after incubating one hour at 37 degrees centigrade. The patients serum that was tested contained only albumin antibodies, therefore serial dilutions were made in AB serum. For the albumin tests one drop of each dilution was added

to one drop of AB serum for the control test and to one drop of acid (cytidine hemisulfate) in place of the one drop of AB serum for the neutralization tests. These two series were incubated at 37 degrees centigrade for 30 minutes and then one drop of 2% type O Rh positive cells (suspended in AB serum) was added to each dilution and incubated for one hour at 37 degrees centigrade. The results of the tests are tabulated below in Table I.

Table I

Test Identification

Test # 1---Saline control for the Ortho saline anti-D
Test # 2---Neutralization of Ortho saline anti-D with 2% cytidine hemisulfate
Test # 3---Neutralization of Ortho saline anti-D with 4% cytidine hemisulfate
Test # 4---Neutralization of Ortho saline anti-D with 15% cytidine hemisulfate
Test # 5---Albumin control for serum from a patient
Test # 6---Neutralization of albumin antibodies present in Test #5 with 15% solution of cytidine hemisulfate
Test # 7---Albumin control for Pooled serum containing albumin antibodies
Test # 8---Neutralization of albumin antibodies present in Test # 7 with 5% solution of cytidine hemisulfate
Test # 9---Neutralization of albumin antibodies present in Test #7 with 10% cytidine hemisulfate
Test #10---Neutralization of albumin antibodies present in Test #7 with 15% cytidine hemisulfate

Test Results

	Test #1 Sal.	Test #2 Sal.	Test #3 Sal.	Test #4 Sal.	Test #5 Alb.
1/1	8	8	5	2	8
1/2	8	5	3	0	8
1/4	3	3	3	0	8
1/8	3	2	2	0	5
1/16	2	2	0	0	3
1/32	2	0	0	0	2
1/64	0	0	0	0	2
Total	26	20	13	2	36

	Test #6 Alb.	Test #7 Alb.	Test #8 Alb.	Test #9 Alb.	Test #10 Alb.
1/1	5	10	8	8	8
1/2	3	8	8	5	5
1/4	3	5	5	3	3
1/8	2	3	3	3	3
1/16	0	2	2	2	2
1/32	0	2	2	0	0
1/64	0	0	0	0	0
Total	13	30	28	21	21

The results suggest that the 2% and 5% concentration of cytidine hemisulfate have less effect on the antibodies present than do the higher concentrations. The higher concentration seems more effective in neutralizing the antibodies. The differences in the neutralizing power of the different concentrations of the acid suggests a dose response.

The Discovery Of Two New Neutralizing Chemicals

In search for more chemicals which would inhibit Rh antibodies we tested 10% solutions of several chemicals such as citric acid, succinic acid, D^{+}-glucosamine, pyridoxal-PO_4, and glucuronic acid. None of

these chemicals were tested in the past by other investigators. Of these chemicals both D^+-glucosamine and pyridoxal-PO_4 showed inhibitory activity.

The above tests were set up as follows. The Ortho saline tube test anti-D, the Ortho modified (albumin) tube test anti-D, and a patient's serum containing albumin antibodies were each diluted out to a dilution of 1/512. Then one drop of each dilution from each of the three sources of anti-D was mixed with one drop of either glucuronic acid, D^+-glucosamine or pyridoxal Phosphate. A control was also set up containing one drop of saline or one drop of bovine albumin in place of the chemicals depending on whether we were testing for saline or albumin antibodies. Then each of the saline titers received one drop of saline and each albumin titer received one drop of bovine albumin. These mixtures were then incubated at 37 degrees centigrade for one hour to see whether these chemicals would neutralize the antibodies present. After the hour, one drop of 2% type O Rh positive cells was added and incubated at 37 degrees centigrade for 30 minutes longer. At the end of the thirty minutes the agglutination in each tube was read microscopically and scored according to the Race Scale (Table III Page 27) (26). The data for the results of the effect of Glucuronic acid, D^+-glucosamine, and pyridoxal

phosphate on Rh antibodies can be found in Table II.

Table II

Test Results

	(1) Ortho Control		Glucuronic Acid	
	Sal.	Alb.	Sal.	Alb.
1/1	5	5	5	5
1/2	5	5	5	5
1/4	3	5	3	5
1/8	2	5	2	5
1/16	0	5	0	5
1/32	0	5	0	3
1/64	0	0	0	2
1/128	0	0	0	0
1/256	0	0	0	0
1/512	0	0	0	0
Total	15	30	15	30

	(3) D -glucosamine		(4) Pyridoxal Phosphate	
	Sal.	Alb.	Sal.	Alb.
1/1	0	2	0	5
1/2	0	2	0	3
1/4	0	2	0	2
1/8	0	2	0	0
1/16	0	2	0	0
1/32	0	0	0	0
1/64	0	0	0	0
1/128	0	0	0	0
1/256	0	0	0	0
1/512	0	0	0	0
Total	0	10	0	10

	(5) Patients Control		(6) Glucuronic Acid	
	Sal.	Alb.	Sal.	Alb.
1/1	0	8	0	8
1/2	0	8	0	8
1/4	0	5	0	5
1/8	0	5	0	5
1/16	0	3	0	2
1/32	0	2	0	2
1/64	0	2	0	2
1/128	0	2	0	2
1/256	0	0	0	0
1/512	0	0	0	0
	0	35	0	34

Test Results Continued

	(7) D -glucosamine		(8) Pyridoxal Phosphate	
	Sal.	Alb.	Sal.	Alb.
1/1	0	3	0	0
1/2	0	2	0	0
1/4	0	2	0	0
1/8	0	2	0	0
1/16	0	0	0	0
1/32	0	0	0	0
1/64	0	0	0	0
1/128	0	0	0	0
1/256	0	0	0	0
1/512	0	0	0	0
Total	0	9	0	0

The Ortho control titer scores (Test 1) indicate marked agglutination for both the saline and albumin titers. The control for the patients serum (Test 5) shows marked agglutination for only the albumin titer, therefore no saline antibodies were present. It appears that the glucuronic acid had no effect on the standard Ortho saline and albumin antibodies (Test 2) or the albumin type antibodies in the patients serum (Test 6), while the D^{+}-glucosamine and the pyridoxal phosphate neutralized most of the antibodies present as shown by the marked reduction in the titer scores (Tests 3,4,7 and 8). The results with succinic acid and citric acid were not placed in the table because their titers were comparable to the titer for glucuronic acid (Tests 2 and 6) both of which showed no neutralization.

In scoring the above agglutination results a peculiar phenomenon was noted. When scoring the saline titer for the patient's serum where we used D^{+}-glucosamine and pyridoxal phosphate, the indicator cells retained their red color even though the media was slightly yellow-brown due to the presence of the chemicals. These saline titers for the patients serum contained no Rh antibodies. But when scoring the albumin titers for agglutination it was noted that the cells were colored yellow-brown. It was in these albumin titers that we obtained neutralization due to the presence of the two chemicals. We feel that the differences in color (red) of the cells in the saline titer in absence of antibodies and the color (yellow-brown) of the cells in the albumin titer in presence of antibodies has a significant meaning. In our opinion the cell color (yellow-brown) in the albumin titer indicated a reaction between the chemical, the Rh antibody and the cell. In some way the chemical played a part in the prevention of agglutination.

We hypothesize that the antibody has two reactive groups. One reactive group combines with the antigen on the cell and the other combines with other cell-antibody combinations thereby causing agglutination.

We feel that the D -glucosamine and pyridoxal phosphate combine with that part of the antibody which reacts with other cell-antibody groups thereby preventing agglutination.

A Promising Area Of Research

The recent work of Hackel in the elucidation of the structure and immunological properties of the Rh antigen and his discovery of four derivatives of ribonucleic acids which would neutralize the Rh antibodies in vitro made us aware of a promising area of research in an attempt to find a way to prevent erythroblastosis. Therefore, it might be possible for one to use one of the nucleic acid derivatives to lower the Rh titers which have developed in Rh negative mothers sensitized by a Rh positive fetus. It would not have been practical to begin our investigation by giving these acids parenterally to patients with an Rh titer since we had no awareness of their possible toxicity.

We felt it was necessary to try these acids in some animal in which we had stimulated the formation of Rh antibodies. With knowledge of McDonald's work on the sensitization of rabbits to the Rh factor we decided to use rabbits to see whether the ribonucleic acids would neutralized Rh antibodies in vivo. The

procedure for sensitization would be similar to that used by Scherer (27).

The Problem Of Sensitizing Rabbits

Sensitizing rabbits with human blood cells is a blind procedure, because it is difficult to obtain a relatively pure antigen for injection. Therefore one sensitizes to a group of factors. Also one is confronted with the problem of trying to demonstrate that you are actually dealing with the particular Rh antibody rather than other factors. Therefore we have two problems; first, the preparation of the wanted antigen and second, the problem of proving the identity of the antibody formed against the sensitizing antigen.

Fifteen rabbits of the same breed were obtained and divided into three groups. The first two groups contained six each and the third group contained only three rabbits. A titer was first determined on all fifteen rabbits using as the detector cell the same cells that were to be used in sensitizing the rabbits in groups I and II. If different Rh positive cells would be used each time a set of titers was to be performed, differences in their agglutinability might affect the results obtained. The initial titer would show whether the rabbit's serum contained agglutinins

or conglutinins against the human test cell. The rabbits were bled by nicking the marginal ear vein with a razor blade after causing vasodilitation with xyleen or after several flicks of the finger. After nicking the vein the blood would drop easily into a test tube. Approximately 10 ml of blood was taken with each bleeding and this supplied sufficient serum for analysis of antibody content.

Preparation Of The Sensitizing Substances

Belkin and Weiner demonstrated that suspensions of cell stromata retained their antigenic properties (28). Therefore we prepared cells for group I in a similar fashion. The type O genotype Dce/dcE red blood cells to be injected into rabbits 1-6 of Group I were obtained from a hospital employee. Our blood source needed to be reliable because samples of blood would be utilized over a period of several weeks. The same cells that were used for sensitization were also used as the indicator cells in determining the Rh titers on the rabbits. The type O genotype Dce/dcE red blood cells to be injected into rabbit 1-6 were washed in isotonic saline until a clear supernatent was obtained. The cells were then washed in distilled water to lyse the cell wall. When the supernatant was light pink after

three to four washings the centrifuged ghost cells were mixed with saline to provide a dilution of about 1/10.

Lubinski and Portnuff pointed out that heating Rh positive cells to 56 degrees centigrade made Rh positive cells suspended in saline Rh negative (29). It was also shown by Murray and Clark that the injection of this preparation into guinea pigs resulted in the production of anti-D sera (30). Hubinont confirmed the above work and also demonstrated an ^{Rh} substance in the supernatant saline by inhibition studies (31). This finding may suggest that specificity of the Rh substance may be imparted by a special side-chain or group which can be removed by heating. We used this method of preparation of the sensitizing substance for rabbits 7-18 because it may produce a more pure Rh substance and at the same time eliminate some of the unwanted sensitizing substances.

The type O genotype Dce/dcE red blood cells to be injected into rabbits number 7-12 were prepared by washing approximately 10cc of blood in isotonic saline until a clear supernatant was obtained. An equal amount of saline was added and adjusted to pH 7.2 by adding dilute KOH. The cells were then heated for seventeen minutes at 56 degrees centigrade. The cells were centrifuged and the supernatant was mixed with enough saline to make a 10% solution.

Type O, genotype dce/dce (Rh negative) red blood cells were used to inject rabbits number 13, 14, and 15. These three rabbits were to be used as controls. The cells were prepared in exactly the same manner as the cells for group I, the only difference being that the cells were Rh negative rather than Rh positive. By comparing the sensitization results of this group with group I and II we would have more information to determine whether we were building up a titer to an Rh substance or to other human red blood cell antigens.

The Method Of Sensitization

The rabbits were given two series of three daily subcutaneous injections, separated by a four day period. Rabbits 1-6 were given the ghost cell preparation and rabbits 7-12 were given the heated preparation. Rabbits 13,14, and 15 were also given the same series of subcutaneous injections but with a ghost cell preparation of O negative cells.

The rabbits were bled five times. The first bleeding and titer was done before the initial injection so that we could have a base line titer. The second bleeding and titer was done just before the beginning of the second series of three injections. The third bleeding and titer was done on the seventeenth day after the initial

injection of the first series. The fourth bleeding and titer was done on the twenty-ninth day after the initial injection. The fifth bleeding and titer was done on the fortieth day after the initial injection.

The Determination Of Antibody Production

Saline and albumin titers were performed in the usual manner in order to be very exact in the tabulation of the differences in degree of agglutination between different titers. The degree of agglutination was ranked according to the Race Scale which allots numbers to different degrees of agglutination determined microscopically (26). The Race Scale is described in Table III.

Table III

Agglutination clearly visible to naked eye	= 10
Very large agglutinates seen microscopically	= 8
Large agglutinates seen microscopically	= 5
Small agglutinates seen microscopically	= 3
The smallest definite agglutinates	= 2
No agglutination and cells evenly distributed	= 0

The Method Of Desensitization

When a significant titer had been developed the desensitization procedure was begun. Rabbits 1 and 2

were given subcutaneous injections of 1cc of 10% D^{+}-glucosamine HCl prepared in a Tris buffer for seven days beginning on the thirty-third day after the initial sensitizing dose of antigen. The rabbits were bled and a titer determined on the fortieth day after the initial injection of the sensitizing substance or twenty-three days after the last sensitizing dose. Rabbits 3 and 4 were given 1cc of 10% cytidine hemisulfate prepared in Tris buffer for seven days and were bled in the same manner as rabbits 1 and 2. Rabbits 5 and 6 received Tris buffer only for seven days and then were bled in the same fashion as above. Rabbits 7 and 8 were given the same acid injections as were rabbits 1 and 2 (the only difference being that the rabbits were sensitized with a different preparation). Rabbits 9 and 10 were given the same acid injections as rabbits 3 and 4, rabbits 11 and 12 the same as rabbits 5 and 6, rabbit 13 the same as rabbit 1, rabbit 14 the same as rabbit 3, and rabbit 15 the same as rabbit 5.

Experimental Data

Example Of How The Data Was Tabulated

The fifteen rabbits were bled five times, therefore a total of seventy-five Rh titers were performed for this project. Three examples were picked at random and appear in Table IV to give the reader an idea of how the Rh titer data was originally tabulated. In Table V a tabulated list appears with the scores of each saline and albumin titer for each rabbit. The Race Scale was used to indicate the degree of aggultination. The total of each titer is thought to be a significant indication of the antibody content of the serum tested. For example the initial titer done on the serum of rabbit number 2 before it was sensitized had a sum total of 8 for the albumin titer. After injecting the rabbit with 1cc of the cell stromata preparation for three days and then four days later the sum total of 15 was obtained for the albumin titer. It is felt that this increase of 7 points indicates a rise in the agglutinating power of the rabbit serum due to antibodies whose formation was stimulated by the antigens in the cell stromata injected. Later we will indicate our belief that the antibodies formed as indicated by the increase in the sum of degrees of agglutination for each titer were actually Rh antibodies.

Table IV

Test Results

	Rabbit 4 Titer 1		Rabbit 2 Titer 3		Rabbit 7 Titer 4	
	Sal.	Alb.	Sal.	Alb.	Sal.	Alb.
1/1	0	3	3	2	@	2
1/2	0	2	5	0	@	0
1/4	0	0	8	5	5	3
1/8	0	0	5	5	8	8
1/16	0	0	3	5	5	5
1/32	0	0	3	3	5	5
1/64	0	0	3	2	2	5
1/128	0	0	2	2	0	5
1/256	0	0	0	0	0	3
1/512	0	0	0	0	0	2
Total	0	5	32	24	25	38

In the dilutions of 1/1 and 1/2 of titer number 4 of rabbit number 7 the @ entries mean that in these two tubes the cells were completely hemolyzed. This was also found in several of the other titers but it never extended beyond the dilution of 1/2. Probably in these cases the hemolysins present were in quantities sufficient to destroy the red blood cells before the agglutinating antibodies present could cause agglutination. Because we were uncertain what agglutination values to assign in these cases we elected to assign a zero value each time this phenomena was detected.

Tabulation Of The Titer Scores

Table V

Key For Table V

1. Titer 1 score
2. 3 days of sensitization
3. 4 day period
4. Titer 2 score
5. 3 days of sensitization
6. 7 day period
7. Titer 3 score
8. 12 day period
9. Titer 4 score
10. 2 day period
11. 7 days of acid injection
12. Titer 5 score

Titer Scores

Procedure	Rabbit 1 Titers		Rabbit 2 Titers		Rabbit 3 Titers	
	Sal.	Alb.	Sal.	Alb.	Sal.	Alb.
1	8	13	8	8	4	7
2-3						
4	12	8	7	15	7	20
5-6						
7	11	15	32	24	20	25
8						
9	11	26	12	10	16	18
10-11						
12	10	16	17	9	16	19

Procedure	Rabbit 4 Titers		Rabbit 5 Titers		Rabbit 6 Titers	
	Sal.	Alb.	Sal.	Alb.	Sal.	Alb.
1.	0	5	5	2	0	2
2-3						
4	11	11	7	8	13	8
5-6						
7	20	26	8	30	19	19
8						
9	24	18	16	27	9	19
10-11						
12	13	17	10	20	11	15

Titer Scores Continued

Procedure	Rabbit 7 Titers		Rabbit 8 Titers		Rabbit 9 Titers	
	Sal.	Alb.	Sal.	Alb.	Sal.	Alb.
1	2	12	2	2	12	11
2-3						
4	28	31	8	6	11	25
5-6						
7	23	39	15	22	18	36
8						
9	25	38	12	20	14	37
10-11						
12	13	14	9	8	21	22

Procedure	Rabbit 10 Titers		Rabbit 11 Titers		Rabbit 12 Titers	
	Sal.	Alb.	Sal.	Alb.	Sal.	Alb.
1	0	4	11	10	11	13
2-3						
4	8	9	16	14	23	20
5-6						
7	28	43	11	20	38	18
8						
9	13	23	19	25	17	18
10-11						
12	2	14	10	6	6	0

Procedure	Rabbit 13 Titers		Rabbit 14 Titers		Rabbit 15 Titers	
	Sal.	Alb.	Sal.	Alb.	Sal.	Alb.
1	6	10	6	5	2	0
2-3						
4	8	16	5	13	10	14
5-6						
7	12	25	17	22	13	17
8						
9	13	15	16	30	8	9
10-11						
12	10	8	4	4	13	14

Discussion Of The Results

The Objective Of The Experiment

Before we begin to discuss the results of the data obtained we should again state the objectives. In order to build up an Rh titer in rabbits 1-6 they were injected with a cell preparation containing Rh positive stromata. A different preparation which was heated to a certain degree to obtain a supernatant rich in Rh antigen was used to inject rabbits 7-12. It was intended that the latter preparation would stimulate the formation of a higher titer than the Rh positive stromata preparation. Rabbits 13, 14, and 15 were injected with a preparation containing Rh negative stromata, and we hoped that this preparation would not stimulate a titer in these rabbits. After the production of a significant titer we then planned to reduce the titers by injecting the rabbits with compounds that were previously shown to inhibit Rh antibodies in vitro. Five of the rabbits were injected with Tris buffer to see whether this substance, which was used to buffer the above chemicals, would have any effect on the titer scores.

Evidence Suggesting The Presence Of Agglutinins Before Sensitization

Examination of the scores for the initial titers

determined on the serum indicates that the serum contained agglutinins toward the test cell (blood type O, genotype Dce/cdE) before the rabbits were sensitized to this same cell. These agglutinins are probable agglutinins toward human red blood cells that the rabbits normally possess. It was shown in 1921 by Hooker and Anderson that normal rabbit sera possess weak agglutinins for the four groups of human erythrocytes. He showed that in certain rabbits this agglutinating capacity is group-specific, being particularly marked for groups A and AB. They also found that group-specific hemolysins were developed with the agglutinins. They also noted that a few rabbits failed to produce group-specific antibodies but no cause for this individual variation was discovered. (32) We did not try to see whether these hemolysins or agglutinins were group specific.

Evidence For And Against Desensitization

The data concerning rabbits 1-6 (Table I) indicates that a titer had developed to the Rh positive cell stromata in these rabbits. In all six rabbits there was an initial rise in titer after the first series of injections except for the albumin titer in rabbit number 1 where the initial titer was higher than the second titer. In

all six rabbits the titer score continued to rise after the second series of injections of Rh positive stromata. Thirty-three days after the initial injection rabbits 1 and 2 were given 1cc of 10% D -glucosamine HCl for 7 days and the next bleeding and titer which was done after the substance was injected. The results indicate that the only titer that was reduced significantly was the albumin titer for rabbit number 1 which was reduced 10 points. The albumin titer for rabbit 2 was reduced only 1 point. The saline titer actually increased 1 point for rabbit number 2. The cytidine hemisulfate which was given to rabbit number 3 and 4 did not appear to lower their titers. The saline titer score remained the same after the acid was injected and the albumin titer score actually increased 1 point. As for rabbit number 4 the saline titer score decreased 10 points which may indicate some inhibition of antibodies while the albumin titer score was reduced only 1 point. Looking closely at the data for rabbit number 4 one can see that there was a steady increase in titer from a score of 0 to 11, to 20, then to 24 and then when the scid was injected an abrupt change in the score occurred. This could indicate neutralization of the antibodies present or possibly an error in titration. As for the albumin titer no gradual increase in titer occurred. Actually

it began to decrease 12 days before the acid was injected. Rabbits 5 and 6 were used as controls to see whether the Tris buffer used in preparation of the acid had any effect on reducing the antibody titer. The saline titer score of rabbit 5 reduced 6 points and the albumin titer score reduced 7 points after the Tris buffer was injected. This drop may indicate neutralization of some of the antibodies or it may indicate no effect and suggest the normal gradual reduction of the titer over a period of time. Added information could have been gained if we would have had several sensitized rabbits which received no injections. The titer score for rabbit 6 decreased 10 points before the rabbit was injected with the Tris buffer and then rose 2 points after injection. This result is difficult to interpret although it is likely a chance deviation.

Rabbits 7-12 were sensitized with the heated preparation which we hoped would stimulate the production of a higher antibody titer. Examining the data grossly one sees that a few rabbits produced titers with scores in the high thirties. Titers this high were not obtained in the rabbits 1-6 or in rabbits 13-15; therefore, we may postulate that this preparation did have greater antigenicity. In rabbit 7 both the saline and albumin titer scores were markedly reduced after the injection of D -

glucosamine HCl for 7 days. Titer scores in both the saline and albumin titers had remained high on three occasions or for approximately 22 days before the preparation was injected. Then seven days later after seven injections of the preparation the saline titer score was reduced 12 points and the albumin titer 24 points. This data appears to indicate that the preparation D^{+}-glucosamine has a neutralizing effect on the antibodies present in the serum of rabbit 7. The titer scores for both the saline and albumin titers of rabbit 8 were also reduced and most markedly for the albumin titer.

Rabbits 9 and 10 were desensitized with cytidine hemisulfate. The saline titer score of rabbit 9 increased 7 points after the acid was injected while the albumin titer score was reduced 15 points, which is a marked reduction especially in the event of a rising titer. In rabbit 10 both the saline and albumin titer scores were markedly reduced after the desensitization procedure.

Rabbits 11 and 12 were given Tris buffer as a control to see whether this by itself would have an effect on the titer values. It appeared that this substance had a marked effect on the saline and albumin titers of both rabbits. These results indicate that either the Tris buffer is a better desensitizing substance or the

reduction in titer indicates an expected leveling off of the antibody content. The data may also suggest that the D^{+}-glucosamine, the cytidine hemisulfate and the Tris buffer had no effect at all on the antibodies and that the titer would have decreased if nothing had been given.

Rabbits 13, 14, and 15 were sensitized with a blood type O Rh negative cell preparation and it was hoped that since this preparation contained no Rh antigens we would see little or no rise in the titer scores of these three rabbits. The results from Table V suggest that saline and albumin titers were developed to the sensitizing substance. The scores of these titers appear to be quite similiar to the titer scores for rabbits 1-6 probable indicating that titers in both cases had developed to the same sensitizing substance. In both cases the sensitizing substance probably is the human red cell and in response to these cells the rabbits produced hemolysins and agglutinins which we were detecting in all the titers. The marked elivation of the albumin titer of rabbits 7, 9 and 10 all of which received the heated preparation indicated the presence of something not present in the rabbits 1-6, 13, 14 and 15. Within these titers we may be dealing with Rh antibodies. Also the albumin titers of these three rabbits were

markedly affected by the desensitizing substance used.

Elucidation Of The Identity Of The Agglutinating Substance.

Since we do not know exactly what type of antibody we are dealing with, we attempted to elucidate the identity of the agglutinating substance in two ways. First, we attempted to remove unwanted agglutinins and hemolysins against human type O Rh positive test cells by reacting 1 cc of serum from rabbit number 3 and rabbit number 9 with 10 cc of packed type O Rh negative (dce/dce) cells which were washed three times with normal saline and packed in a conical centrifuge tube by centrifuging at approximately 2000 rpm for 10 minutes. The 1 cc of serum from rabbit number 3 and 1 cc serum from rabbit number 9 and the packed cells were allowed to stand for one hour during which they were mixed 4-5 times by inversion of the centrifuge tubes. Then the red cells were packed and the absorbed serum removed. A titer was performed on the absorbed serum, and the results of the initial titer before absorption tabulated next to the titer after absorption in Table VI.

Table VI

Rabbit Number 3 (Titer 4)

	Before Absorption		After Absorption	
	Sal.	Alb.	Sal.	Alb.
1/1	3	5	0	0
1/2	3	3	0	0
1/4	3	3	0	0
1/8	5	3	0	0
1/16	2	2	0	0
1/32	0	2	0	0
1/64	0	0	0	0
1/128	0	0	0	0
1/256	0	0	0	0
1/512	0	0	0	0
Total	16	18	0	0

Rabbit Number 9 (Titer 4)

	Before Absorption		After Absorption	
	Sal.	Alb.	Sal.	Alb.
1/1	2	3	0	0
1/2	2	2	0	0
1/4	5	5	0	0
1/8	3	8	0	0
1/16	2	8	0	0
1/32	0	3	0	0
1/64	0	2	0	0
1/128	0	2	0	0
1/256	0	2	0	0
1/512	0	0	0	0
Total	14	35	0	0

It appears that the type O Rh negative cells removed the agglutinins and hemolysins against human cells from the serum of rabbits number 3 and 9. No Rh antibodies were left in the serum after the absorption because the titer determined on the absorbed serum gave negative results (zero score). This finding suggests

that the rabbit serum contains only hemolysins and agglutinins toward human cells unless these antibodies mask the Rh antibody in some way.

To further test the serum of these 2 rabbits for the presence of Rh antibodies, Detecto-cells were obtained from the Blood Center at Mount Sinai Medical Research Foundation. These are a panel of 9 different red blood cells each containing different antigens of known types. By reacting the absorbed rabbit serum with each of these different cells in both a saline and albumin medium one can tell which antibodies are in the serum by exclusion. The results are tabulated in Table VII.

Table VII

Non-absorbed Serum

	Rabbit Number 3 Titer 4 Swrum		Rabbit Number 9 Titer 4 Serum	
	Sal.	Alb.	Sal.	Alb.
Tube				
1.	10	10	10	10
2.	10	10	10	10
3.	10	10	10	10
4.	10	10	10	10
5.	10	10	10	10
6.	10	10	10	10
7.	10	10	10	10
8.	10	10	10	10
9.	10	10	10	10
10.	0	0	0	0

Table VII Continued

Absorbed Serum

Tube	Rabbit Number 3 Titer 4 Serum Sal.	Alb.	Rabbit Number 9 Titer 4 Serum Sal.	Alb.
1.	0	0	0	0
2.	0	0	0	0
3.	0	0	0	0
4.	0	0	0	0
5.	0	0	0	0
6.	0	0	0	0
7.	0	0	0	0
8.	0	0	0	0
9.	0	0	0	0
10.	0	0	0	0

When non-absorbed serum from rabbits 3 and 9 were tested with the panel of Detecto-cells, we observed agglutination in tubes 1-9. The tenth tube was a saline control and no agglutination was noted in this tube. This again indicates the presence of antibodies against the Detecto-cells which are human cells. Absorbed serum was also run against the Detecto-cells, and there was no agglutination in any of the ten tubes indicating that the antibodies present were removed by the absorbing Rh negative cells leaving no Rh antibodies to be detected by the Detecto-cells.

Summary

Twenty three years have elapsed since the identification of the Rh factor and the elucidation of its clinical importance. Methods of identification have reduced the clinical importance of this factor in blood banking, but it still remains a problem in Rh incompatible marriages. Since 1941 many attempts have been made to break-up the chain of pathogenosis. These included attempts to prevent sensitization by the intravenous use of anti-D, to find a substance which would strengthen the placental barrier and thereby prevent sensitization, and to find substances which would either render the already formed antibody nonreactive or a substance which would suppress the production of antibodies. A few of these appeared promising but to date none of them have been effective in the prevention of erythroblastosis fetalis.

A few workers began to elucidate the structure and immunological properties of the Rh factor in order to learn more about the dynamics of this substance. This work has been valuable in opening new areas of investigation. Hackel's work in this area suggests that a few RNA derivatives have the ability to neutralize Rh antibodies in vitro. We repeated his experiments and

at the same time found two other substances which were not derivatives of RNA, but would neutralize Rh antibodies. Since there were several compounds available that would neutralize Rh antibodies in vitro we set up an experimental plan whereby we could test their effect on antibodies which had developed within an animal sensitized by an Rh substance. I chose rabbits because these animals were previously used in similar experiments. In order to make this a controlled experiment we divided fifteen rabbits into three groups. The first group contained 6 rabbits, all of whom received the same sensitizing substance. After the establishment of a titer we attempted to reduce it in two of the rabbits with D^{+}-glucosamine and in two others with cytidine hemisulfate. Tris buffer was given to the remaining two rabbits, since this substance was used as a buffering agent in the preparation of the two chemical substances and acted as a control in the last two rabbits. The second group of rabbits received a heated preparation as a sensitizing substance which was supposed to be a more pure substance. The first two animals in this group received D^{+}-glucosamine, the second two cytidine hemisulfate, and the last two tris buffer. The third group consisted of only three rabbits who were sensitized with a cell preparation of type O Rh

negative cells. This group was used as a control and it was hoped that no titer would develope in these rabbits. Also as a control the first rabbit of this group received D^{+}-glucosamine, the second cytidine hemisulfate and the third Tris buffer.

After the establishment of a titer and before we began to desensitize them with the two chemical substances, we tried to elucidate the identity of the agglutinating substance present in the titers in two ways. First, we tried to remove the unwanted agglutinins and hemolysins present in the rabbits serum by reacting the serum with type O Rh negative cells. The absorbed serum was then tested for the presence of Rh antibodies with type O Rh positive cells. The second attempt at elucidation was to use "Detecto-cells" to see whetner the absorbed serum contained Rh antigens.

Conclusions

I feel that my investigative work has been of value in two areas. First, two different chemical substances were discovered which would neutralize Rh antibodies in vivo, and because of the objective finding I was able to propose a probable mechanism of action of these two chemicals. This mechanism of action appears to be different than that discribed by the Italian workers for the RNA derivatives.

No previous attempts have been made to use these substances in the neutralization of Rh antibodies in sensitized animals. In the work described we attempted to set up an experimental plan to sensitize rabbits to the Rh factor and then neutralize the resultant antibodies with these compounds. The results show that we sensitized the rabbits to some type of antigen but the absorption test and "Detecto-cells" were unable to pick-up the presence of an Rh antibody. By the use of different sensitizing methods we were able to show that the heated preparation was the better sensitizing substance. Previous reports suggested that this preparation proved to be a more pure Rh substance, and we hoped that it would produce Rh antibodies that could be detected, but we were unable to do so. It appears

that the rabbit serum tested contains hemolysins and agglutinins to human red blood cells whether they be Rh positive or Rh negative red blood cells. This concept is supported by the data obtained for rabbits 13, 14, and 15 which were sensitized to Rh negative cells and in response to these cells produced rather high titers which were similar to the titers obtained from rabbits sensitized with the Rh positive preparation. There is a possibility that we were dealing with Rh antibodies but their presence was masked by the hemolysins and agglutinins to human cells, and we were unable to detect their presence.

Because neutralization effects were noted in nearly all the rabbits, there are three possible explanations. First, these chemicals may not have specificity and will neutralize any antibody present. Second, there may be no effect and the drop in titer scores indicate only a normal decrease in titer which would decrease whether the acid was given or not. Thirdly, these chemicals may have specific neutralizing action against the Rh antigen, but the neutralization was masked by the hemolysins and agglutinins present in the rabbit serum tested. The greatest neutralization effect appears to be in the group which received the heated cell preparation, and it appears that this substance did stimulate the pro-

duction of Rh antibodies and that the marked decrease in titer indicated the specificity of the chemicals used.

I feel that a great deal of information has been gained from this project and that it provides a good basis to continue research in the area of prevention of erythroblastosis fetalis. Looking into the future I feel there are several areas which need improving.

(1) It would be advantageous to search for a more pure Rh antigenic substance.

(2) Better ways should be found to identify the presence of the Rh antibody.

(3) Intravenous injection of the chemical substance rather than sub-cutaneous injection would probably be more effective in neutralizing antibodies.

(4) Search should continue for new and better neutralizing substances.

(5) An attempt should be made to see whether the hemolysins and or agglutinins toward human cells present in rabbit serum are group specific.

(6) The supernatant solution and the cell part of the heated cell preparation should be tested with anti-sera to see whether the heating removed the Rh antigen from the cells.

(7) More controls are necessary in the future work, such as including a group of rabbits which would receive no desensitizing injections.

(8) Future data obtained by experimentation should be analized statistically.

Acknowledgement

To Doctor Warren Pearse M.D. my sincerest gratitude for the aid and criticism in the preparation of this paper. I feel indeed fortunate to have the opportunity to benefit from his expert guidance and knowledge within this field of study. I also wish to acknowledge Walter Gills for his willingness to donate his blood which was used as the sensitizing substance. To Richard Green I am also greatful for his help in the culmination of this research project.

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