HEMOLOGY OF RH IMMUNE GLOBULINS: EVIDENCE FOR A POSSIBLE THIRD ORDER OF ANTIBODIES INCAPABLE OF AGGLUTINATION OR BLOCKING

By Joseph M. Hill, M.D., Sol Haberman, Ph.D., and Frances Jones, B.A.

The possibility of a third order of reactivity of Rh antibodies, characterized by their failure to act either as classical agglutinins or true blocking antibodies has been suggested in earlier publications. In this paper, additional evidence is presented characterizing immune globulins of this third order of reactivity. In such a characterization of antibodies or immune globulins we might bear in mind that antibodies may be classified and characterized according to their response to a variety of tests and procedures. For example, antibodies may be classified according to their specificity or their mode of action. They have also been designated according to their stability under the influence of heat, whether they occur naturally or only in response to given antigens, the time of their response in respect to the immunization curve, and in many other ways.

In the Rh field the study of antibodies has been particularly interesting. Initially, studies were concerned chiefly with the specificity of the different antibodies observed utilizing the classical method of agglutination of red cells in saline suspension. For example, in the original report of Levine and Stetson in 1939, the irregular isoagglutinin which they described had a specificity of approximately 80 per cent which was suggestively close to the specificity of 84 per cent which Landsteiner and Wiener found when their anti-rhesus serum was used to test human erythrocytes. Different specificities for various human anti-Rh sera was noted in 1941 by Levine when he described specificities of 73 per cent, 85 per cent and 87 per cent when tested with random human red cells. In the same year Landsteiner and Wiener also noted human sera containing agglutinins for red cells having different specificities. These and the subsequent studies of Rh antibody specificity have proved to be of utmost importance in the working out of the relationship of the different Rh antigens and in the formation of sub-type classifications. However, the fascinating story of the discovery and use of sera of different specificity to unravel the intricate relationships of the different Rh antigens has been told by Doctor Race in his paper today. We are much more concerned here with properties of the Rh antibody affecting its mode of action.

In the early work with the Rh antibody the classical agglutination test utilizing a saline suspension of red cells was employed. It was early noted that demonstration of Rh antibodies in cases of erythroblastosis was often lacking. Furthermore, clinical correlation of severity of the disease with antibody titres was often very poor or completely lacking. Intensive investigations to improve methods of Rh testing stimulated by the necessity to demonstrate agglutinins more consistently in connection with transfusion incompatibility and erythroblastosis led

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to a very important advance in our knowledge of antibodies. In 1944 the separate reports of Race and Wiener described an incomplete or blocking antibody often present in cases of Rh isoimmunization which could specifically adsorb and completely saturate the Rh antigen of the test erythrocytes without causing apparent agglutination. The blocking test, as it was called by Wiener, took advantage of the fact that Rh positive cells treated with such sera would no longer agglutinate when anti-Rh serum of known potency was added. A somewhat different approach was made to this same problem by Diamond when he found that some Rh antisera seemed to contain an inhibitor substance which resulted in a lowering of potency when other higher titred anti-Rh sera were pooled with such sera. The development of the blocking test technic was an important contribution because it enabled us to demonstrate clearly this new kind of antibody by a convenient and practical method. Earlier workers had detected the presence of antibodies or antibody-like material in sera having a similar effect, but lacking the blocking test were unable to differentiate clearly and identify this antibody. Eisenberg and Volk, for example, used the term "agglutinoid" to designate what they considered a modified agglutinin possessing the power to combine with but not to flocculate the agglutinogen.

The blocking technic, of course, provided an additional method for the detection of antibodies in cases of Rh isoimmunization. In our experience the method proved somewhat disappointing for this purpose because it increased only slightly the percentage detection of antibodies in cases of erythroblastosis. As a method of studying agglutination phenomena and for the characterization of antibodies, however, the blocking test has been exceedingly useful. The evident exhibition of first-stage antibody reaction without subsequent agglutination has led to the conclusion by many investigators that the blocking antibody is "univalent" in nature as opposed to the "bivalent" agglutinin. These findings served to explain the zone phenomenon seen in some anti-Rh sera, and suggested that zone phenomena generally might be accounted for by the presence of blocking antibody. We feel the importance of the blocking test and the blocking antibody which it determines should not be obscured by interpretations given to the results obtained with other and later methods which will also demonstrate antibodies of blocking type. These methods, such as Diamond's albumin test, Wiener's conglutination test, etc. are considerably less specific than the blocking method in that they determine not only the blocking antibody but also the classical (saline) agglutinin as well as a certain portion of what we are designating third order antibodies in this paper.

In 1944 Chown presented a method of Rh testing of remarkable simplicity and high sensitivity. In this method a heavy suspension of red cells or whole blood is in contact with the typing serum within a capillary held at an angle of 45°. The red cells gradually fall through the zone of the agglutinin containing serum and agglutinate into very clear bands and large aggregates as the serum acts in the presence of capillary forces. We introduced Doctor Chown's technic into our laboratory as a routine procedure early in 1945 and have used it with growing enthusiasm ever since. We were most interested when we found that this method,
as we used it, was capable of detecting antibodies which would not agglutinate erythrocytes in saline in the test tube. Still more interesting was the fact that many sera which gave positive results with the blocking test would not agglutinate Rh positive red cells in the capillary. (It should be noted here that in our use of the Chown method we have employed a saline diluted serum, the usual ratio being 0.1 or 0.2 cc. of original serum to 1 cc. of saline solution.) The meaning of this peculiar discrepancy, of course, did not become clear until later. It is obvious however, that Chown’s test in addition to the blocking and classical agglutinating methods helped to characterize antibodies further.

In 1945 Diamond and Abelson described a slide test which proved to be a very sensitive test for recognition of antibodies and paved the way for the “conglutination” and albumin tests. In these later methods the importance of the suspension medium or fluid components of the test were recognized. By the use of albumin or serum for preparing erythrocyte suspensions, zone phenomena and blocking effects were largely eliminated from the agglutination test for antibodies. These methods not only detected the classical saline agglutinins and the antibodies determined by the blocking test but also, we believe, detected antibodies not shown by the other two earlier methods. Unfortunately the term blocking antibody has been extended to cover all of the antibodies detected by these newer tests employing colloidal suspension media.

An entirely different method for the specific detection of antibodies was the use of anti-human globulin serum as described by Coombs, Mourant and Race. This new approach shifts the emphasis from the specificity of agglutination to the more basic phenomenon of specificity of antibody adsorption. As a result the detection of antibodies does not depend upon their ability to function as agglutinins or by hapten saturation (blocking) but solely by their ability specifically to adsorb on the test erythrocytes. The anti-human globulin serum acts by developing an observable agglutination of red cells which retained the specifically adsorbed antibody (human immune globulin). The specificity of the test is determined by the choice of the proper antigen, in this case known Rh positive erythrocytes. For convenience we have used the term “developing test” in referring to this method of Coombs, Mourant and Race because it develops an observable reaction in a manner analogous to the development of a photographic image. This test promised to be a particularly useful serologic tool to demonstrate any antibody for which a suitable specific antigen could be provided which would produce visible clumping.

Coombs, Mourant and Race found that the treatment of an anti-C serum with heat (56°C) caused it to become nonreactive to the agglutinating, blocking and conglutinating tests while its ability to adsorb as demonstrated by the developing test was still present. These workers considered the method a more sensitive technic for the detection of weak and incomplete (blocking) antibodies. This method of antibody detection was added to our routine procedures shortly after its publication. As in the case of the Chown capillary technic we found interesting discrepancies in the saline agglutinin and blocking antibody titres as compared to the new method. As reported in our earlier papers, cases were observed in which antibody titres as high as 1/2048 were found by the “developing” (Coombs) test...
while the agglutinating titre (1/2) and blocking titre were minimal. Extensive experience with the developing test on a large routine service made us feel confident that such discrepancies were well beyond the 1 or 2 tube greater sensitivity of this method. On the basis of such findings, with the addition of some experimental results, we suggested the possibility of a third order of reactivity of immune globulins.

Since these preliminary studies strongly suggested that the Rh antibody could exist in a form incapable of agglutination or blocking and yet gave evidence of its antibody nature by specific adsorption and hemolytic activity, further investigations were undertaken. These were concerned with (1) the establishment of the validity and accuracy of the new methods such as developing test titrations and hemolysis quantitation (2) demonstration of antibody characteristics exhibited by the proposed third order immune globulins, such as specific adsorption and hemolysis, and (3) demonstration of differences from previously described antibodies which characterized the proposed new group of third order antibodies.*

METHODS

**Agglutination:** Studies of agglutination were carried out by the several methods described below. All antibodies were titrated by serial dilution. For routine purposes 1 cc. serologic pipets calibrated to 0.01 cc. were used to prepare the serum dilutions. One drop of each dilution was placed in 7 mm. (inside diameter) tubes by means of a capillary pipet with a tip approximately 1 mm. in diameter. In every instance this transference of a drop of serum was started at the highest dilutions to avoid error. A similar capillary pipet was used to add 1 drop of a 2 per cent suspension of the test erythrocytes (suspension of 3 or 4 Rh positive bloods including different sub groups). After thorough mixing of the cells and serum the tubes were placed in a water bath at 37°C for 1 hour. The tubes were then centrifuged at 750 r.p.m. for 1 minute and observed for agglutination macroscopically and microscopically. In the case of very high titres (1:10,000 or over) the routine technic was checked by a more accurate method of dilution in which several Kahn type pipets were used per test to avoid carry over of antibodies.

**Special Agglutination Tests:** Four tests, namely the Diamond slide and albumin tests, the Wiener "conglutination" method and the Chown capillary test were used to effect agglutination when blocking antibodies or zone phenomena interfered with agglutination by the standard test tube technic. These tests were used as described by the authors.

**Blocking Test:** The method described by Wiener was employed to show the presence of blocking antibodies. However, after the observation was made as originally recommended, we have obtained a sharper differentiation in the titration of these antibodies by centrifuging the tubes and again observing for agglutination.

**Developing Test:** To develop observable agglutination of otherwise non-agglutinating Rh antibodies and immune globulin or "cryptagglutinoids," anti-human globulin serum was used as originally described by Coombs, Mourant and Race. In this method one drop of the serum to be tested was mixed with one drop of a 2 per cent suspension of type O Rh positive erythrocytes in a Kahn type tube. This was incubated for one hour at 37°C and observed for agglutination. If no clumping was observed the cells were washed three times in saline solution by centrifugation to remove unadsorbed globulins. After the third wash, 1 drop of anti-human globulin serum was added to the sedimented cells after the

* Recently we have proposed the term cryptagglutinoid to designate the antibody which neither blocks nor agglutinates saline suspensions of erythrocytes. The manuscript has been changed by substitution of this new term for the older and less descriptive designation used in the original presentation of this paper.
hemolytic Rh immune globulins

Saline had been removed. The cells were suspended and incubated for one hour at 37°C. At the end of this second incubation the tube was centrifuged at 500 r.p.m. for one minute and observed for clumping. Agglutination indicated the presence of "cryptagglutinoids." These tests were performed routinely on all cases of jaundice of the newborn and on all Rh negative mothers and their children.

Hemolytic Activity of Rh Serum as Demonstrated in Vitro: Three-tenths cc. of anti-Rh serum was added to 3 cc. of heparinized freshly drawn Rh positive whole blood. All the blood used in the experiment was drawn into a dry 50 cc. syringe through a No. 19 needle, and after the removal of the needle was placed in a sterile 115 cc. flask containing 0.2 cc. of sterile heparin solution. This was done in order to keep the mechanically produced hemolysis to a minimum, and also to eliminate any possible hemolytic effect due to bacterial contamination. A set of 6 tubes was used for the test and 6 for the controls. As a rule serum of the highest available titre was used. The final effective titres were as indicated in the results of the experiment. The reagent blank and a suitable control were set up in order to obtain the net hemolysis due to the Rh serum alone. For the first blank, 0.3 cc. of anti-Rh serum and 3 cc. normal saline were used to make the final volume equivalent to that in the test. This blank represented the hemoglobin present in the anti-Rh serum which was used in the experiment. The second blank consisting of 3 cc. of saline and 0.3 cc. normal serum was set up to measure the amount of hemoglobin present in the normal serum required in the controls.

Since normal heparinized blood becomes slowly and progressively hemolyzed on standing in vitro it was necessary to use a control as follows: to 3 cc. of the same heparinized blood which was used in the experiment was added 0.3 cc. of the normal serum whose hemoglobin was determined in the second blank.

For the test 3 cc. of the heparinized blood were immediately added to each of 6 sterile tubes containing 0.3 cc. of anti-Rh serum. For the control set, 6 similar sterile tubes were used containing 0.3 cc. of normal serum in place of the anti-Rh serum. A tube from each of the series was immediately centrifuged and the supernatant cell-free serum was analyzed for its hemoglobin content. A modification of the Bing and Baker* test for hemoglobin was used. The remaining tubes of both test and control were incubated at 37°C. and at 2, 6, 12, 30 and 48 hours a tube from each series was removed, centrifuged and the hemoglobin content of the supernatant plasma determined. A Lumetron single-cell photoelectric colorimeter using narrow band filters and a high sensitivity double reflecting galvanometer was used to determine the color produced by the benzidine reaction.

Net hemolysis was calculated in mg. of hemoglobin from the formula \( H = H_1 - b_1 = H_2 - b_2 \) = net hemolysis.

Where

\[ H_1 = \text{mg. per cent of hemoglobin in supernatant of tubes containing blood and antiserum (experiment)} \]

\[ H_2 = \text{mg. per cent of hemoglobin in supernatant of tubes containing blood and normal serum.} \]

\[ b_1 = \text{mg. cent. of hemoglobin in first blank (Rh serum + saline).} \]

\[ b_2 = \text{mg. cent. of hemoglobin in second blank (normal serum + saline).} \]

Electrophoretic Studies: Electrophoretic analysis and fractionations were made in a Tiselius apparatus using the optical arrangement of Longsworth for observing boundaries. The instrument used was one assembled by Dr. Dan H. Moore of Columbia University. Patterns were made on 11 cc. aliquots of serum in a double sectioned cell at 0.5°C. Prior to analysis, the serum was diluted 1:4 with 0.02 M. sodium phosphate, ph 7.4, containing 0.85 per cent sodium chloride, and then dialyzed for 24 hours (at ice box temperature) against a large volume of buffer prepared in the same way. This same buffer was used to fill the apparatus.

After electrophoresis, the components of the serum were distributed in layers throughout the length of both arms of the cell. Separation of these fractions was accomplished by carefully noting the position of the boundaries, then removing the successive layers by means of capillary pipets, care being taken to avoid disturbing the boundaries.

* In later experiments to be reported elsewhere, guinea pig complement was also added to both the test and control series in order to provide an excess of complement and produce maximum hemolysis. By this technique much more complete hemolysis was observed, for example approximately 1 gram of hemoglobin per 100 cc. of supernatant plasma was attained in some instances.
RESULTS

The application of the agglutination, blocking and developing tests to the routine services of the blood bank and maternity unit, as well as to the experimental production of hyperimmune anti-Rh sera, resulted in the demonstration of antibody patterns that are deemed worthy of report. These results, along with the studies on the electrophoretic separation of Rh immune globulins and the quantitative demonstration of hemolytic activity of the agglutinating and "crypt-agglutinoid" antibodies, are described below.

Case 1: A type O Rh negative female gave birth to a severely jaundiced infant. The past history showed that the mother had received 1 transfusion and had had 2 miscarriages. The type O Rh (D) positive infant's erythrocytes gave a 4+ developing test. The antibody pattern presented in table 1 was found on examination of the mother's serum on the day of birth. In this instance we have completely negative agglutination and blocking tests. Nevertheless by the developing test this serum was able to sensitize Rh (D) positive erythrocytes up to a dilution of 1/512.

<table>
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<tr>
<td>Develop.</td>
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<td>Case 2 Aggt.</td>
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<tr>
<td>Develop.</td>
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<td>Case 3 Aggt.</td>
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<tr>
<td>Block.</td>
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<tr>
<td>Develop.</td>
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</table>

Case 2: A type O Rh negative mother delivered a stillborn macerated infant at an estimated 8 months gestation. Two years before this pregnancy the mother had received 1 blood transfusion following an appendectomy. The serum collected on the day of delivery showed the antibody pattern presented in table 1. The condition of the macerated infant precluded any studies of the antibodies in its serum. In this case antibodies could be demonstrated by all 3 methods. The results suggested a mixture of antibodies. The titre by the developing test seemed sufficiently greater (6 tubes) to definitely suggest an excess of antibodies not revealed by the other 2 methods.

Case 3: The mother of a severely jaundiced infant whose erythrocytes gave a 4 plus developing test presented an unusual past history. Her first child died shortly after birth of a disease, supposedly erythroblastosis. During this pregnancy the mother had received small intramuscular injections of the husband's blood with the purpose of preventing erythroblastosis. Shortly before delivery the patient was transferred from another city to Baylor Hospital. The pregnancy terminated in the birth of an extremely jaundiced infant which was treated with Rh negative blood transfusions and subsequently recovered. At birth the infant's erythrocytes gave a 4 plus developing test. The eluate from the cord blood showed neither agglutinins nor blocking antibodies, but a titre of 1/16 was found, by the developing test. The mother's serum taken on the day of birth showed the antibody pattern given in table 1. In this case the titre of antibodies demonstrated by the developing test is greatly in excess of the blocking
antibody while agglutinins are practically absent. Apparently here the antibodies are a mixture of the blocking variety and cryptagglutinoids.

Case 4: The Rh negative patient was admitted to the hospital for a possible pelvic abscess following a laparotomy and because of a persistently low erythrocyte count (1,900,000/c.m.m.). The patient had had 2 caesarian deliveries with blood transfusions from the husband each time. During the hospital stay a transfusion was ordered. The husband offered himself as the donor. When it was found that he was Rh positive, the antibody pattern of her serum was studied. The results presented in table 2 demonstrate that isoimmunization had occurred. Subsequent Rh negative transfusions were given without incident. This antibody pattern shows a low titre of agglutinins, no blocking antibodies and a fairly high titre of immune globulins or cryptagglutinoids. There is also a peculiar zone effect in the developing test for which we have not found an adequate explanation.

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<td>-</td>
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</tr>
<tr>
<td>2</td>
<td>+</td>
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<td>4</td>
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<td>8</td>
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<tr>
<td>16</td>
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<tr>
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<td>Develop</td>
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Case 5: An Rh negative mother gave birth to a severely jaundiced infant. Six years before this pregnancy the mother had received 3 transfusions. There was no history of previous pregnancies. The infant's erythrocytes gave a 4 plus reaction to the developing test. The antibody pattern in the maternal serum on the day of birth is presented in table 2. This pattern illustrates the situation where the developing test parallels the blocking test, apparently showing that only the blocking antibody is responsible for the titre of 1:64 shown. Since this is a plus minus reaction the results are practically identical. We feel that this is the case when pure agglutinating or blocking antibodies are demonstrated by the anti-human globulin technic. A titre 1 tube higher is frequently found as compared to the agglutinating or blocking tests.

Case 6: An Rh negative mother gave birth to a slightly jaundiced infant whose erythrocytes gave a 4 plus developing test. The mother had had one previous child who was living and well. The sera collected on the day of birth and 1 month later showed the antibody patterns presented in table 2. The similarity of the antibody patterns of these studies shows a relatively similar sensitivity of the developing test and agglutination test when only 1 type of antibody is present. It is probable that these sera had only the agglutinin present.
Antibody Patterns Studied during Deliberate Hyperisoeimmunization for Rh Serum Production. In addition to the above cases, another group should be described because of significant antibody responses and patterns. This group consisted of individuals who had received small doses of Rh positive cells with the deliberate intention of producing high titre Rh typing serums. With these individuals antibody patterns and variations could be very closely followed in relation to administration of the Rh antigen.

Table 3.—Mrs. R. W. Titre of Anti-Rh Agglutinins Produced by Injection of Rh Positive Blood into Previously Isoimmunized Woman

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<tbody>
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<td>*</td>
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<tr>
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<td>416</td>
<td>1/4000</td>
<td>1/8</td>
<td>1/1000</td>
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</table>

The tests were not done in the cases marked with asterisks in tables 3 and 4.

The first of this group, Mrs. R. W., has been described in detail in an earlier publication. She was the mother of a severely icteric second child who recovered with the administration of multiple Rh negative transfusions. The original titre of the mother’s serum was 1/1280 by the agglutination reaction. Blocking and developing tests were not available at that time. In table 3 the schedule of intravenous injections of Rh positive blood is given together with the time intervals and
antibody response. The various long time intervals were due in part to the itinerant habits of the volunteer donor.

The second individual to be hyperimmunized, Mrs. O. M., was the mother of a severely icteric child who had recovered after treatment with multiple Rh negative transfusions. The original titre of the mother’s serum was 1/256 by the agglutination reaction. The results of the intentional isoimmunization program are presented in figure 1. The program was started in April, 1946, and was discontinued temporarily by an automobile accident to the volunteer. Several months later the isoimmunization was undertaken again. In this case, the agglutination reaction

failed to reach a titre suitable for the production of typing serum. However, the antibody pattern obtained is of considerable interest.

The third of this group who showed very significant antibody patterns during a course of injections of Rh positive blood to produce Rh testing serum, was a male patient, Mr. P. D., admitted in September, 1943, for the surgical treatment of polyposis of the colon and rectum. A colectomy was performed and later further surgery was done to remove the remaining rectum and anus. During this treatment the patient had been given multiple transfusions which finally resulted in a severe transfusion reaction. It was found that the patient was Rh negative and had an anti-Rh(C) titre of 1/128. Subsequent transfusions of Rh negative blood were uneventful. In March, 1946, the patient volunteered for the isoimmunization program. The results presented in figure 2 are remarkable for the extremely high titres

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**Fig. 1. Antibody Pattern Produced by Injection of Rh Positive Blood into a Previously Isoimmunized Woman**
by the developing test and especially at those points where no antibodies could be
demonstrated by the agglutination or blocking technic.

Study of Antibody Patterns during Deterioration: During the isoimmunization of
Case 1, several blood collections were made for the preparation of anti-Rh typing
serum. The third collection of 250 cc. yielded a serum with an agglutination titre

\[ \text{FIG. 2. TITRE OF ANTI-Rh AGGLUTININS PRODUCED BY INJECTION OF RH POSITIVE BLOOD INTO A MAN PREVIOUSLY SENSTIVIZED BY TRANSFUSION} \]

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</tr>
<tr>
<td>4-10-46</td>
<td>1/1024</td>
<td>1/356</td>
<td>1/2048</td>
</tr>
<tr>
<td>4-17-46</td>
<td>1/4</td>
<td>1/4</td>
<td>1/2048</td>
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of 1/8192 and a blocking reaction of 1/16. The serum was stored in a freezing
unit at 15°F. Within two days the agglutination titre began to fall. It was de-
cided not to use this serum for typing purposes. From time to time the serum was
examined for the antibody pattern as this deterioration of agglutinins progressed.
The results as presented in table 4 represent the antibody pattern found during the
deterioration of this labile serum.
In figure 3 is shown the *Electrophoresis* pattern of the serum of Mr. P. D. of the serum production program, when the blocking titre was 1/2 and the agglutination and developing titres were 1/4000. The electrophoretically separated albumin and globulin fractions were tested for the antibody pattern to determine the location of the antibodies in the serum fractions. These were found in the gamma globulin.

![Electrophoretic Pattern. Davison Serum](image)

*Fig. 3. Electrophoretic Pattern. Davison Serum*

The studies of the hemolytic effects of Rh immune globulins are presented in graph form. In figure 4 is presented the comparison of the benzidine dihydrochloride modification of the method of Bing and Baker as used by Hill and Haberman and the iron thiocyanate method as described by Moore for the determination of hemoglobin. The results of these 2 methods were so nearly identical, that it was decided to use only the benzidine dihydrochloride technic in the later experiments. The third graph in figure 4 shows the comparison between the use of saline and the use of normal serum in the controls to replace the anti-Rh serum being tested for hemolytic activity. No significant difference was found between these 2 methods of preparing controls.
To prove the specificity of the hemolytic activity of anti-Rh immune globulins, the experiments were repeated on both Rh positive and Rh negative cells. These results are given in figure 5. In this experiment the hemolytic action of the "crypt-agglutinoid" antibody is studied with reference to complement. In the first graph the Rh positive and Rh negative erythrocytes were washed 3 times in saline by centrifugation and diluted to a 20 per cent suspension. To one aliquot of these cells complement was added (2 units), and no complement was added to the remainder.
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In the figure it can be seen that no significant hemolysis as compared to the controls was observed against Rh negative cells. Also, very little hemolysis occurred when no complement was added to Rh positive saline suspensions. However, when complement was added to Rh positive saline suspensions, significant net hemolysis occurred. When the above experiments were repeated with Rh positive and Rh negative whole blood, similar results were obtained. The greatest hemolysis was produced when complement was added to the mixture of Rh positive whole blood and anti-Rh antibodies. When complement was not added to the Rh positive blood, significant hemolysis occurred due to the complement already present in the fresh human blood used in the experiment. The Rh negative blood showed no net hemolysis when treated with the anti-Rh serum under the conditions of these experiments.

Further studies on the hemolytic capabilities of the "cryptagglutinoid" antibody were made in the same manner as those presented in figure 5, introducing the effect of shaking to the mixtures of Rh positive blood, complement and anti-Rh serum. The tests for the effects of shaking were performed in 25 cc. Erlenmeyer flasks which were mounted on a rotary table. The rotary travelled at a speed of 100 r.p.m. and caused the blood mixture in the flask to mix gently. The results presented in figure 6 show that the hemolytic effect of the Rh cryptagglutinoid was enhanced by complement and shaking.

To compare the effect of different titres of anti-Rh agglutinins and "cryptagglutinoids" on the same Rh positive cells (Rh0 or CDe), the following experiment was done. A pure agglutinating serum (original titre 1/32,000) was added to 3 tubes containing Rh0(CDe) blood with the intention of producing a final titre of 1/1000, 1/100 and 1/10. However, after the preparation of the dilutions a final titration showed only 1/500, 1/25 and 1/2.5. To a similar series of Rh0(CDe) blood was
added a pure "cryptagglutinoid" serum (original titre 1/16,000). In this case final
titres of 1/200, 1/100 and 1/10 resulted. It was hoped that examination of the
hemolytic activity of the preparations described would give some evidence of the
sensitivity of the developing test titration and establish the validity of titration
by this method. These mixtures were then studied in the same manner as described
in the presentation of methods. The results show that as the titres of both the
agglutinin and the "cryptagglutinoid" antibody were increased, the hemolytic
effect became more marked with relatively good correlation of titre and hemolysis.
These results presented in figure 7 indicated that the developing test was not merely
a supersensitive technic but determined titres of "cryptagglutinoid" antibodies.

![Diagram showing comparison of effect of agglutinating and cryptagglutinoids on Rh+ RBC at different titres.]

**Fig. 7. Comparison of Effect of Agglutinating and Cryptagglutinoids on Rh+ RBC at Different Titres**

**DISCUSSION**

It would seem apparent that the forces involved in antigen-antibody reactions are
so closely linked to physical chemistry that attempts to represent pictorially these
activities on the basis of the knowledge at hand is pure speculation. Such pictorial
representations as have been given in the recent past for the blocking and agglutinat-
ing antibodies seemed adequate for a short period of time. However, the
Diamond slide and albumin tests and the Wiener serum suspension tests quickly
demonstrated its inadequacy, while the new technic of Coombs, et al.15 opened up
new possibilities for study of antibodies as herein reported.

When the agglutination reaction as performed in the test tube is used as the
index of Rh sensitization it was found that many cases of clinical isoimmunization
could not be explained due to the failure to demonstrate the classical agglutinin.
Later, the discovery of the blocking antibody resulted in the demonstration of an
antibody, possibly incomplete with respect to valence. In this case the antibody
was capable of being adsorbed without producing agglutination in the test tube.
The obvious explanation appeared to be purely one of valence or incompleteness
of the antibody molecule. However, the newer evidence found by Diamond and
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Denton by varying the suspension medium of the test Rh positive erythrocytes showed that the blocking antibody could be made to produce agglutination by substituting colloids such as serum and albumin for saline solution. The reaction of Wiener, using neutral serum as the erythrocyte suspension medium for the tube test produces the same results. It would seem then, that the valence of antibodies as originally propounded by Marrack and Heidelberger and Kendall and pictorially represented by Wiener for the Rh complex does not entirely explain the findings in the Rh problem. Although the principle of valence may yet explain the blocking antibody, it remains to be shown that such a concept explains the "cryptagglutinoid" antibody.

From investigations in the present paper and in previous reports, it appears that the antibody demonstrable by the developing test differs from the classical agglutinin, and the blocking antibody. This antibody is capable of specific adsorption, without causing agglutination or blocking. However, when such antibodies are tested by the albumin and serum methods, agglutination occurs but in a somewhat lower titre than with the developing test. In one case clearly showing antibodies by the developing test no agglutination could be detected by the conglutination method. It would seem then, that the problem of antibody reactivity rather than valence alone is concerned. On this basis, 3 orders of antibody reactivity may be considered. First, specific adsorption with subsequent agglutination (classical agglutinins); second, specific adsorption with saturation of the antigen and no agglutination (blocking); and third, specific adsorption without evident saturation of the antigen and without agglutination ("cryptagglutinoids").

Evidence for such a third order of Rh antibodies differing from the agglutinating and blocking types has been presented. The evidence has been offered in the following categories: (1) Studies of antibody patterns in the serum of mothers of erythroblastotic children; (2) antibody patterns found during deliberate hyperiso-immunization for the production of Rh typing serum; (3) investigation of changes in antibody characteristics during deterioration in vitro over a period of time; (4) the demonstration of the hemolytic action of the Rh antibodies, especially the third order or "cryptagglutinin" type to establish their antibody nature, and (5) studies of electrophoretic separations of Rh antisera with a view to determining whether the agglutinating and "cryptagglutinin" antibodies were found in different protein fractions.

In examination of the sera obtained from the mothers of erythroblastotic children it was found that the test tube agglutination method failed to detect many instances of isoimmunization. Case 1 of this series demonstrates such an instance. In this case the blocking test failed to reveal the antibodies. However, when the developing test was used a hidden antibody titre of 1/512 was found. In the second case the blocking titre was 1/32 with a weak agglutinin present at 1/2 dilution. The developing test showed a titre of 1/1024, a 6 tube difference between it and the blocking test and a 10 tube difference above the agglutination reaction. The antibody pattern of Case 3 also shows these wide differences between the three

* Since presentation of this paper, 4 clear cut examples of "cryptagglutinin" antibodies demonstrable with the developing test but negative with the albumin or conglutination tests have been studied.
titration methods. In Cases 4 and 6 are presented instances where the blocking antibody was not present. In Case 4, a 7 tube difference was found between agglutination and developing reactions. The weak developing reaction at a titre of $1/4$ (zone effect) found in this case is probably due to some carry over of nonspecific globulins which could neutralize the developing serum. In Case 6, the "cryptagglutinoid" titre and the agglutination titre were almost the same, and it is believed that sera of this type represent mostly agglutinin with very little or, more likely, no third order antibody.

The use of the anti-human globulin serum of Coombs, Mourant and Race as a developing test not only detects the third order ("cryptagglutinoid") antibody but also yields visible agglutination when the blocking effect is found and when agglutinins are present as questionable or weak reactors. When this antibody pattern approach was applied routinely to the study of sera from the maternity service, an improved degree of correlation was found between titre of antibody and severity of erythroblastosis.

In the examination of the data concerned with deliberate increase of antibody titres to produce testing sera it can be seen that the agglutinins were persistent in the case of Mrs. R. W. However the blocking effect was transitory and never reached a significantly high titre, while the "cryptagglutinoid" antibody in many instances was much stronger than the agglutinin. Also, the antibody titres can be lowered by using different Rh subgroup cells in the isoimmunization series. This change in titre resembles a laglike phase in the progress of antibody production. At one time during the program, the developing ("cryptagglutinoid") titre reached $1/128,000$ and agglutination was evident at $1/32,000$ with saline suspension of erythrocytes. It should be noted that as the immunization program progressed the "cryptagglutinoid" titre dropped and no longer exceeded the agglutinin titre. This result seems to indicate that there is a degree of independence in the production of the antibodies studied.

In the case of Mrs. O. M., the blocking antibody was evident with some persistency. However, when the frequency of blood injections were increased to 2 or 3 times per week, the blocking antibodies disappeared and agglutinating antibodies became evident. Throughout the stimulation period the "cryptagglutinoid" antibody exceeded the strength of the agglutinin or blocking antibody.

In the case of Mr. P. D., it was found that several years after the original isoimmunization by transfusion, a developing ("cryptagglutinoid") titre of $1/256$ persisted while the blocking and classical agglutination tests were entirely negative. Five days after the initial injection of Rh positive blood, agglutinins and blocking antibodies appeared in the volunteer’s serum. In one series of tests in the study, the volunteer’s antibody pattern completely failed to show blocking antibodies or agglutinins, although a very high titre of "cryptagglutinoids" ($1/16,000$) was observed. When the Diamond albumin and Wiener serum tests were applied, agglutination occurred at titres of $1/4096$ and $1/1024$ respectively suggesting that a part of the "cryptagglutinoids" were sufficiently reactive to be demonstrated by these methods. In continued studies of this type similar results were observed. Furthermore, agglutination could be observed in Chown’s capillary
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test with many serums giving positive albumin tests. However, serums containing pure blocking antibodies only would not agglutinate Rh positive cells by this method. The blocking antibody appeared at irregular intervals in the isoimmunization programs and rarely attained significant strength. On the other hand, the data presented shows that the "cryptagglutinoid" antibody appeared in the patient's serum first, usually attained the highest titres, and persisted longer in the patient's blood stream.

In reviewing the data on the deliberate hyperisoimmunization of the 3 individuals presented, it can be seen that considerable variation occurred. These differences in antibody patterns can be attributed to the individual's particular response to the repeated injection of Rh positive blood. No apparent correlation could be made between the type of antibody and the phase of immunization, although the "cryptagglutinoid" type of antibody persisted longer after antigenic stimuli ceased and could often be more easily increased during the active phase of stimulation. However, the "cryptagglutinoid" antibody often appeared first while agglutinins might or might not appear later. On the other hand we had previously observed a case where agglutinin titres up to 1/100,000 were produced by the injection of Rh positive cells and at no time were blocking or "cryptagglutinoid" antibodies detected.

The study of the labile serum collected from Mrs. R. W. of the immunization program showed that the "cryptagglutinoid" titre remained constant while the agglutinin deteriorated rapidly. The apparent increase in the blocking titre may have been due to the lability of the agglutinin.

The specific nature of the adsorption of the Rh "cryptagglutinoid" antibody on Rh positive erythrocytes is strongly suggestive of the destructive role this antibody must play in sensitizing the red cells for their hemolysis in vivo so characteristic of Rh transfusion reactions and erythroblastosis. Prior to our recent reports on hemolytic activity of Rh antibodies,1,2 Diamond and Abelson9 had noted hemolysis in doing their slide test. In the quantitative experiments described in this paper, we have attempted to establish the hemolytic nature of the "cryptagglutinoid" antibody detected by the developing test. It was considered important to demonstrate that the globulins shown to be specifically adsorbed on red cells were actually able to function as antibodies. It was also found that complement was essential to their hemolytic action as in the case of the Rh agglutinin. The amount of hemoglobin released in these experiments was small but significant because in each case without exception there was a definite net hemolysis as compared to controls. In terms of the sensitive chemical technic employed however, this magnitude of hemolysis was easy to detect quantitatively.

Electrophoretic pattern studies of sera containing Rh agglutinins on the one hand, and cryptagglutinoids on the other, showed no significant variation from the normal. Furthermore, when separations were made by removing the protein fractions from the electrophoresis cell and titrations of each fraction performed it was found that both agglutinins and "cryptagglutinoids" were limited almost entirely to the gamma globulin. These studies have not been sufficiently extensive to be considered other than preliminary in nature.
SUMMARY AND CONCLUSIONS

We believe that sufficient evidence exists to justify 3 classes of Rh antibodies based on their reactivity. These are the classical agglutinin, the blocking antibody, and the proposed 'cryptagglutinoid.' Sufficient distinction exists to retain the blocking antibody as determined by Wiener's original blocking test. This antibody is further characterized by failure to act in Chown's capillary technic. The identity of this antibody with its important role in reawakening investigation in this field of immunology should not be lost through inclusion in the broader group of those antibodies determined by the albumin test and similar methods. The proposed third order antibody or 'cryptagglutinoid' which is usually, but not always, detected by these more inclusive tests, and the capillary technic, appears to be of great clinical importance because of the frequency with which it appears in significant titres. Detection of isoimmunization and closer correlation of antibody titre with clinical severity of disease should be possible through study of this 'cryptagglutinoid' antibody.

REFERENCES

experiment was particularly significant where the agglutinin and the blocking antibodies fell completely, and there was no change in titre of the third order antibody. Regarding terminology, I think the analogy to me they have gone a long way toward making out a case for a third order. It struck me that the storage disappearance of agglutinins. I tried to adsorb those blocking antibodies out by the method developed by Dr. Levine and myself, that is, by adsorbing at zero temperature, and we could not demonstrate any disappearance of agglutinins. I tried to adsorb those blocking antibodies out by the method developed by Dr. Levine and myself, that is, by adsorbing at zero temperature, and we could not demonstrate any of these types of examinations, and in his electrophoresis studies, whether this serum was kept in the frozen or in the liquid state. We bledd that woman and want to bleed her again after 4 months. in the meantime she has not become pregnant again and has not received any transfusions, and she only has blocking antibodies and no agglutinins. I wonder whether you could offer an explanation of this.

Dr. Robert K. Walter: I would like to present the last 2 cases we ran into. One of them was a woman who was Rh negative, and we immunized her with a small amount of Rh positive blood. Initially she had an anti-Rh agglutinating titre of roughly 1:4 or 1:8 after the first injection and then 14 days later the titre rose to approximately 400 or 500. We gave another injection of about 2 cc. of blood and the titre rose to approximately 4,000 after 10 days. Now we concluded if we could get it up to 4,000 we could get it up higher, and injected about 5 cc. of red cells and got a blocking antibody of 4,000 and a complete disappearance of agglutinins. I tried to adsorb those blocking antibodies out by the method developed by Dr. Levine and myself, that is, by adsorbing at zero temperature, and we could not demonstrate any agglutinins with the exception of a small agglutinin titre of 1:4. That was the first case. The second case was a mother who after the birth of her erythroblastotic child had a titre of approximately 1:500. We bled that woman and want to bleed her again after 4 months. In the meantime she has not become pregnant again and has not received any transfusions, and she only has blocking antibodies and no agglutinins. I wonder whether you could offer an explanation of this.

Dr. Levine: Dr. Hill, have you noticed any special symptomatology in these infants when they have blocking antibodies exclusively? I see that you did not do any tests with titration of the antibodies with the albumin test. Now I first thought I had a third order of antibodies in the titration of the blocking antibodies in the albumin test. We found a zone. I suspected that we had another variety of blocking antibody, and I almost committed myself except that when I did the adsorption experiment I found after treatment of the serum with Rh positive blood I did not remove this zone effect. So far as my own findings are concerned I am a little bit less certain but I think as Dr. Race pointed out, the most striking observation is that on the serum which deteriorated and lost everything except your third order antibody, and I almost committed myself except that when I did the adsorption experiment I found after treatment of the serum with Rh positive blood I did not remove this zone effect. So far as my own findings are concerned I am a little bit less certain but I think as Dr. Race pointed out, the most striking observation is that on the serum which deteriorated and lost everything except your third order antibody.

Dr. Scudder: I think it is excellent that you have gone on into electrophoresis studies and have found these antibodies present in the gamma globulin. When I visited Dr. Cohn's laboratory and spoke to Dr. Diamond concerning such studies, especially those concerning the sedimentation constants, I found that such studies had not been completed as yet and I would like to ask Dr. Hill whether he has carried on any of these types of examinations, and in his electrophoresis studies, whether this serum was kept in the frozen or in the liquid state.

Dr. Haberman: In response to the question asked by Dr. Scudder, we can state that the electrophoresis tests were done at 0.5°C. In one case the serum had been kept frozen before electrophoresis patterns were made. The pattern shown was made from an unfrozen serum. I believe that Dr. Hill will have something to say concerning studies done on the antibodies by us. In response to Dr. Hattersley's question concerning the production of developing serum by using the purified gamma globulin as the antigenic stimulus, theoretically it would appear that such a substance used as an antigen in rabbits would be the ideal one for the production of anti-human globulin serum. This, of course, rests on the assumption that all human antibodies are gamma globulin in nature. If, however, gamma globulin does not represent all of the human antibodies then this type of antiserum would fail to demonstrate some antibodies. The
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problem of the production of anti-human globulin serum which we have called the developing serum is not a simple one. Production of the antibodies in the rabbit is not too difficult to obtain. However, the purification of this serum is the crux of the problem. If red cells used to adsorb out the unwanted antibodies are insufficiently washed or improperly washed, some globulin will remain adsorbed on the red cells or in the saline used for the washing process. When such a red cell concentrate is used there will be sufficient globulin present to neutralize a good deal of the anti-human globulin factor. Consequently, the titre, avidity and specificity of such a serum will be definitely lowered if not completely lost. On the other hand, if red cells are washed too frequently, that is, 8, 9 or 10 times, as a precautionary measure to eliminate the presence of serum or serum proteins in the red cell pack, the red cell membrane may be damaged and release hemoglobin, which seems to be capable of acting somewhat as a globulin in affecting the serum adversely. We have found such hemoglobin to neutralize some damaged and release hemoglobin, which appears to be capable of acting somewhat as a globulin in affecting the serum adversely. We have found such hemoglobin to neutralize some damaged and release hemoglobin, which seems to be capable of acting somewhat as a globulin in affecting the serum adversely.

We have found such hemoglobin to neutralize some of the antibody, and the serum produced by overwashed cells have a titre that seems adequate and an avidity sufficiently high, but the sensitivity of the serum is greatly reduced, giving poor or very weak reactions where 4 plus or strong reactions should have been anticipated. We advise that the cells be washed 5 times in saline with all of the supernatant carefully removed after the last centrifugation and the adsorption carried on with equal quantities of serum and washed cells. Frequently it will take as many as 5 or 6 such adsorptions before the serum is sufficiently purified. We have found that adsorption in the icebox frequently removes the anti-human globulin antibody by nonspecific adherence. We prefer to do the adsorptions and purification of developing serum at room temperature where such nonspecific adsorption of desired antibodies is at a minimum. After completing the adsorption process it is wise to test the serum against some 50 bloods to be sure that all of the anti-human red cell factors have been removed. We have found that it usually takes more than a month of immunization using 1 or 3 injections intravenously each week for a sufficiently high titre of anti-human globulin antibody to be produced. Use 1 cc. intravenously for each injection. It is not unusual to have some of the rabbits fail to show human globulin antibodies of a sufficient high titre or avidity.

We have used purified globulin or whole human serum for the production of the anti-human globulin serum. The commercially available immunizing globulins such as the type used in measles prevention should not be used as the antigenic stimulus for the production of developing serum.

Dr. Hill: In answer to Dr. Scudder's question concerning further physico-chemical studies, I am sorry to say that we have not had the facilities to do ultracentrifugal studies and to determine sedimentation constants. Our electrophoretic studies, however, must be considered preliminary in nature because of the brief period of time during which such studies have been made. During these investigations we have consistently found all 3 orders of antibodies, namely, the classical agglutinins, the blocking antibodies and the third order antibodies to be in the gamma globulin fraction. In one case the gamma globulin was re-run in the electrophoresis cell with results showing a single peak in the gamma globulin with the antibody titre present in the gamma globulin being identical to that found in the original separation. Also tests for Rh antibodies which were run on the protein fractions remaining from the original separations after withdrawal of the gamma globulin failed to show evidence of Rh antibodies. It is quite obvious that we can draw no conclusions from such meager data and that many more experiments must be performed to determine this point. One difficulty in doing work of this sort is the problem of having sufficient quantities of very high titred sera containing relatively pure classical agglutinins on the one hand or third order antibodies on the other. We will, of course, be very interested to know what Dr. Diamond and Dr. Cohn will be able to discover in respect to the molecular size of these different forms of antibodies through use of the ultracentrifuge.

In answer to Dr. Levine's point concerning possible special symptomatology for infants having adsorbed different types of antibodies, that is, blocking versus agglutinating, I should like to state that we have not observed any correlation between the type of antibody and the clinical disease. Rather, any correlation apparent has been related to the titre as shown by the developing test. We feel that correlation of titre and clinical severity of the disease will be considerably improved by the use of such methods as Diamond's albumin test, or through the use of any of the methods which are capable of detecting substantially all forms of antibodies. Dr. Waller's experience with the variability of response of different patients through stimulating doses of Rh positive cells is similar to our own. Our experience leads us to believe that the most variable factor is the individual recipient rather than the method or the amount of blood given, although we do have the impression that our somewhat larger doses when given...
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at fairly frequent intervals may result in some success in raising agglutinin titres to usable levels. More impressive, however, is the fact that some individuals in whom we have undertaken further immunization by doses of Rh positive cells have shown only pure saline agglutinins even after many doses and many blood collections. In one case in particular, the titre continued to rise with no evidence of blocking or third order antibodies until a titre in excess of 100,000 was reached. On the other hand, we have also seen cases where an almost pure third order type of antibody was observed throughout the entire course of immunization with only transient very low levels of saline agglutinin or blocking antibody being present.

I should like to thank Dr. Race and his coworkers for providing us with this fine tool for the study of antibodies, namely, their anti-human globulin method which for convenience we have termed the "developing" test.
HEMOLYTIC Rh IMMUNE GLOBULINS: EVIDENCE FOR A POSSIBLE THIRD ORDER OF ANTIBODIES INCAPABLE OF AGGLUTINATION OR BLOCKING

JOSEPH M. HILL, SOL HABERMAN and FRANCES JONES

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