ANTISERA TO GAMMA GLOBULIN AND TO ELUTED Rh ANTIBODY FOR THE DETECTION OF INCOMPLETE Rh AGGLUTININ

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ABSTRACT

The antiglobulin content in the sera of four groups of rabbits immunized with alum-precipitated human gamma globulin or with eluted anti-D were examined by two procedures, namely, the antiglobulin technique as recommended by the National Institutes of Health and the precipitin test using the serum dilution method as proposed by Martin. Each rabbit of Group I received 46.89 mg. of alum-precipitated human gamma globulin intravenously, Group II received 9.39 mg. of the globulin intravenously, Group III received 25 mg. of the globulin intraperitoneally, and Group IV received eluted anti-D intravenously.

The antiglobulin titers for halving dilutions of the anti-D ranged from 1:10240-1:160 in Group I, 1:20480-1:20 in Group II, 1:10240-0 in Group III, and 1:5120-1:160 in Group IV. The range of the mean antiglobulin titer was 1:5120-0 in Group I, 1:10240-0 in Group II, 1:10240-0 in Group III and 1:2560-1:160 in Group IV. The antiglobulin sera of Group IV gave titers only from 1:1-1:4 dilutions of the anti-D. The precipitin titers ranged from 1:640-1:320 in Group I, 1:640-1:160 in Group II and 1:640-1:160 in Group III.

The results show that potent antiglobulin sera can be prepared in rabbits with very small quantities of human gamma globulin and also with antibody recovered from incomplete anti-D sensitized erythrocytes

by the elution technique. Preliminary results of the comparisons of the anti-(eluted anti-D) sera with the antiglobulin sera on patients' incomplete anti-D sera appear to indicate that the anti-(eluted anti-D) sera are more competent for the detection of the incomplete Rh antibody.

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INTRODUCTION

In the past and in recent years, immunological and serological studies on the human serum have revealed the presence of more than one kind of isoagglutinin in some human blood groups, especially in the Rh system. These antibodies can, in general, be grouped into two broad categories. The ones that are able to agglutinate homologous red blood cells suspended in saline are known as 'saline agglutinins' or 'complete antibodies', while those that fail to do so are termed 'albumin agglutinins' or 'incomplete antibodies'. The incomplete antibodies, as they are referred to throughout this work for the sake of uniformity, are further characterized by their ability to sensitize homologous erythrocytes which may then agglutinate when suspended in plasma or other colloidal medium (9, 30, 31). This latter property is, infact, one of the means for their detection.

The presence of the incomplete antibody, a serum globulin, on the surface of the sensitized red blood cells can be demonstrated with the aid of an anti-human globulin serum. This is the basis of the antiglobulin or Coombs test, introduced by Coombs and his associates (16, 17) in 1945 for the demonstration of free incomplete Rh antibodies in sensitized patients' sera.

Subsequent investigations have proved the antiglobulin test to be the most sensitive procedure available for the detection of Rh and other blood group antibodies (36, 83) and consequently, to be of immense importance in clinical applications (66). The reliability of the method, however, is limited by the strength of the anti-globulin serum or Coombs reagent, which, after the removal of all agglutinable antibodies for normal human erythrocytes, should contain a high concentration of anti-

human globulin antibody (38).

Since the development of the Coombs test, whole human serum and different serum fractions of various degrees of purity have been reported for the production of antiglobulin serum in rabbits (15, 23, 33, 66, 68, 88). Different injection routes or combinations of them have also been used. However, the investigators have been concerned mainly with the potency of the antiserum, the technique of the test and its clinical applications, and little effort has been directed towards establishing the activity of the antiserum on a sound immunological basis.

Accordingly, an investigation was undertaken in order to study the presence, specificity and activity of the antibodies in the sera of rabbits injected with various serum fractions. This thesis, which constitutes the preliminary report of the project, describes the quantitative evaluation of samples of anti-human gamma globulin sera prepared by routine procedures, and also reports a process using eluted Rh antibody as antigen for the production of potent Coombs antisera.

HISTORICAL

As early as 1905, certain disorders in pregnancy where no incompatibility in the ABO blood groups could be detected were attributed to an immunological reaction. According to Ottenberg (58), Dienst had postulated a relationship between isoimmunization by the fetus and toxemias of pregnancy. Independent findings by McQuarrie (49) in 1923 also proposed a similar relationship. A more significant contribution, however, was made in 1938 by Darrow (27), who suggested that the fetal red cells or some component of them might be, by the process of isoimmunization, the cause of erythroblastosis fetalis.

In 1939 Levine and Stetson (45) described a case of a woman who had delivered a stillborn fetus of eight months' gestation and had a severe reaction after transfusion of 500 ml. of her husband's blood. These workers ascribed the reaction to the isoimmunization of the woman by the fetus to an antigen inherited from the father.

The following year, Landsteiner and Wiener (42) reported the finding of an antibody in the sera of rabbits injected with the blood of the monkey Macacus rhesus which agglutinated not only the monkey red cells but also the erythrocytes of about 85 per cent of humans tested. Since it also occurred in rhesus monkey blood cells, the agglutinogen responsible for the production of this antibody was named the Rh antigen, and the individuals possessing it were designated as Rh positive. Wiener and Peters (84), using the anti-Rh serum prepared by Landsteiner and Wiener (42), demonstrated that certain hemolytic reactions following transfusions of blood compatible in the

ABO groups were due to incompatibility in the Rh system. The serum of the patient who had had a transfusion reaction in 1939 (45) was then found also to contain the Rh antibody. In addition, several additional instances of transfusion reaction associated with isoimmunization to the Rh factor during pregnancy were reported by Levine and Katzin (43). Finally in 1941, Levine and his associates (44, 46) established that erythroblastosis fetalis and its related disorders were due to Rh blood group incompatibility between mother and child.

Since the discovery of the Rh system, two theories regarding its genetics and nomenclature have been proposed by Fisher and Wiener respectively (62). For the sake of simplicity, the Fisher system is employed in this work. In short, there are three Rh factors D, C, E and three corresponding Hr factors d, c, e, each of which is detected by reactions with specific antibodies. The D factor, or the first Rh antigen discovered, is most important clinically as it explains about 99 per cent of all cases of intragroup hemolytic disease. The differentiation of homozygous and heterozygous Rh-positive individuals depends on genetic principles involving three closely or completely linked sets of blood factors, D-d, C-c, and E-e.

The Incomplete Rh Agglutinin

For years following the finding of the Rh factor as the cause of erythroblastosis fetalis and transfusion reactions, investigators were puzzled by the apparent absence of demonstrable anti-Rh agglutinins in cases where the history and Rh grouping strongly suggest immunization. It was not until 1944 that this question was resolved. Race (61), in a study of mixtures of different types of human anti-Rh

sera, reported the existence of an 'incomplete' Rh antibody which combines with homologous red cells in vitro but does not agglutinate them. Independently and in the same month, Wiener (78) also announced the recognition of a similar antibody which he called the 'blocking' antibody. Since then this antibody has also been variously termed 'univalent' (78), 'hyperimmune' (29), 'glutinin' (78) and 'cryptagglutinoid' (37). Later in 1945, Diamond and Abelson (29) claimed that a phenomenon identical to that described by Race (61) had earlier been observed to have repeatedly occurred in their pooled sera, anti-C and anti-D, prepared for globulin fractionation. They had attributed the phenomenon to an 'inhibitor substance' present in some sera of the mixtures without realizing the significance of their observation.

The interest in the reports of these findings soon revealed examples of earlier work showing the same phenomenon. Coca et al (14), in their study of the rabbit anti-Pfeiffer bacillus system in 1921, and Pappenheimer (59) in his study of the horse anti-egg albumin system in 1940, had observed very much the same evidence. In fact, the Pappenheimer had employed in his paper, the terms "incomplete" and "monovalent" to describe the antibody.

Subsequent investigations have thrown more light on the nature and characteristics of the incomplete antibody. Race (61) reported that varying the salt concentrations or the hydrogen ion concentrations failed to produce agglutination of the coated cell suspensions.

Sanger (67), on the basis of her findings, gave support to the

concept that there was a common receptor on the surface of the Rh positive red cell for the complete and the incomplete Rh antibodies. This worker suggested that sufficient incomplete antibody for all the receptors would prevent agglutination even in the presence of large amounts of complete Rh serum, while on the other hand, insufficient amount of the incomplete antibody would result in agglutination of the red cells when complete Rh serum was added.

Coombs and Race (19) in 1945 observed that when antigen was added to a mixture of complete and incomplete antibody, the latter was preferentially adsorbed. These authors also found that the incomplete antibody would not pass through a collodion ultra-filter known to be impermeable to proteins of molecular weight 30,000. Electrophoretic measurements showed that surface charge on red blood cells produced by the complete and the incomplete anti-Rh was of the same order. It appeared to these workers that the incomplete antibody was protein in nature.

In accordance with this concept, Coombs and Mourant (15) in 1947 proposed that the incomplete Rh antibody was mainly in the gamma globulin fraction of the serum. On the other hand, Hill and Haberman (37), on the basis of electrophoretic studies of serum fractions containing predominantly one or another given 'order' of anti-Rh activity, suggested that nonagglutinating antibody may be beta globulin. However, the studies by Dacie (26), Renton (64), Sturgeon and Brown (72) and the recent work of Cutbush et al (23) in 1955 and Vaughan and Waller (76) in 1957 have re-emphasized the

gamma globulin nature of incomplete Rh antibody.

The effect of heat on serum containing incomplete antibody was studied by Diamond and Abelson (29). The incomplete antibody was found to be more thermostable than the corresponding complete Rh antibody. Prolonged heating at 56° C and exposure to merthiolate solution over a period of time inactivated the complete agglutinin but had slight or no effect on the incomplete antibody. Cell sensitization by incomplete antibody was found to occur at 4° C and room temperature but the reaction was most favorable at 37° C. These investigators also confirmed the protein nature of this antibody.

The conversion of complete anti-Rh to the corresponding incomplete antibody was attempted by Boyd (5, 6). Photo-oxidation was shown to inactivate the complete antibody but did not produce incomplete anti-Rh, and high pressure had the same effect. Incomplete anti-Rh sera withstood pressures which inactivated agglutinating anti-Rh, anti-A, anti-B, but not higher pressure. This observation conforms with the concept that incomplete antibodies are significantly more stable than the agglutinating antibodies.

Conclusive experimental data dealing directly with the possibility of differences in molecular size of Rh antibodies is still lacking. However, Wiener, on the basis of findings that the incomplete antibody is able to traverse the placenta more readily than the complete antibody (79, 81, 82) and assuming that this process is a simple diffusion, has concluded that the incomplete antibody is a smaller molecule. This observation appears to be related to that of

Baar (1) who, noting that incomplete anti-Rh could be found in cord blood of infants suffering from hemolytic disease much more frequently than agglutinating anti-Rh, suggested that the former antibody had greater powers of permeating the placenta.

Results from a recent attempt by Campbell, Sturgeon and Vinograd (10) to separate complete and incomplete Rh antibodies by ultracentrifugation showed that Rh saline agglutinins sedimented more completely and at a faster rate than the "complete type of antibodies. This may be an indication that incomplete antibodies have a lower molecular weight than the saline agglutinating antibodies.

Tests for Incomplete Rh Agglutinin

Tests for the detection of the incomplete Rh agglutinin have not been lacking in spite of the relatively short period of time that has elapsed since its recognition. The "blocking" test of Wiener (78) was perhaps the first of a variety of techniques that have been introduced for this purpose. The principle of this test is based on the original discovery of Wiener (78) that Rh (D) positive erythrocytes coated by the homologous incomplete antibody would no longer be able to be agglutinated by an anti-Rh (D) serum which agglutinate D positive cells in saline.

The open slide test was described by Diamond and Abelson (28). These investigators showed that incomplete anti-Rh would agglutinate Rh positive cells on a slide, provided a heavy cell suspension (50%)

in a serum or plasma medium was used. The method was regarded by these workers to be more sensitive and convenient than the blocking test, and with a lighter (2-5%) cell suspension, it could also be adopted for tube use.

After the introduction of the slide test, various other methods employing the use of colloid media were soon developed. The procedures are essentially the same as the ordinary saline agglutination test except for the suspension media. To distinguish these tests from agglutination in an ordinary saline diluent, the term 'conglutination' was applied by Wiener (80), on account of the resemblance of the reactions to the conglutinin phenomenon observed particularly in tests with bovine sera. This name, however, has been found objectionable by some workers on grounds of priority in connection with a different phenomenon. The use of cord sera and normal adult sera as suspension media was reported by Witebsky et al (87). Comparative studies of the efficiency of various fluids as suspension media by Diamond and Denton (31) and Cameron and Diamond (9) recommended the use of 20 per cent bovine albumin as the best choice among the materials tested. In connection with these findings, McCulloch (48) suggested that on account of the variations in the efficiency of different preparations of albumin, the activating protein might not be albumin, but some contaminating protein (alpha globulin) associated with it. However, conclusive evidence to confirm this statement is still lacking.

Of a totally different nature is another group of tests characterised by the use of enzymes. The basic principle involves,

instead of a change in the suspension media, the alteration of the surface of the red cells by enzyme activity, thus rendering them agglutinable by their homologous incomplete antibody in saline. The use of trypsin for this purpose was first made by Morton and Pickles (53). Subsequent studies (39, 54) have shown that other enzymes such as papain could be substituted for trypsin.

In 1945, a new approach to the investigation of the nature and the detection of the presence of the incomplete antibody was introduced by the antiglobulin test of Coombs, Mourant and Race (16, 17, 18). The test, also designated as the Coombs or developing test (35), is based on the principle that red cells coated with incomplete Rh antibodies and washed free from serum are agglutinated when exposed to the serum of a rabbit immunized against human globulin or whole human serum. As with the other incomplete agglutinin tests, the Coombs technique can be carried out on slides (16), glass tiles (32a), test tubes (57) or capillary tubes (13).

This important finding soon brought to light an earlier investigation (51) by Moreschi in 1908. This worker reported that a strong agglutination was obtained when a goat anti-rabbit globulin serum was added to a suspension of rabbit erythrocytes sensitized with goat serum containing rabbit hemagglutinins. Both the sensitivity and the possible implications of the test were discussed in some detail by this author, although he did not realize that he was detecting the simultaneous presence of incomplete antibodies rather than the complete agglutinins.

Since the development of the antiglobulin test, several

modifications have been conceived by investigators. Unger (74) introduced the Coombs test on trypsin-treated erythrocytes as an even more delicate test for Rh antibodies. Titers obtained with this modified technique were shown to be approximately twice as high as those obtained with the Coombs test or the trypsin-treated erythrocytes test. Various other enzymes can also be used as substitutes for trypsin (39) and it was suggested that the method should be used to detect antibodies present in concentrations below the threshold of other methods (74).

Another modification known as the multiple Coombs test also promises to be of value. Erythrocytes coated with human antibody and rabbit antibody are washed and treated with a reagent capable of detecting rabbit antibody, for instance, a chicken anti-rabbit gamma globulin serum (65). The process can be repeated so as to build up a lattice of alternate antibody globulin and gamma globulin. In this manner, the capacity to detect antibodies which cannot be shown by a single Coombs test is substantially increased, and in addition, any sub-surface antibody receptor on the erythrocytes can be demonstrated by agglutination.

The essential feature of the antiglobulin augmentation test of Sturgeon (71) is the detection of incomplete antibodies in a saline agglutinating serum. The cell-antibody agglutinates are washed three times and re-examined by the routine antiglobulin titration. Enhancement of agglutination with the addition of the antiglobulin serum indicates the presence of incomplete antibodies. The

technique was reported to be simple, reliable and capable of detecting, in saline agglutinating sera of high titer, incomplete antibodies that are not revealed by routine immunological methods.

The antiglobulin test has proved of great clinical importance both in its direct and indirect forms. The direct test, which measures cells sensitized in vivo, is an invaluable diagnostic tool for the hemolytic disease of the newborn (86). The indirect test, which measures cells sensitized in vitro, plays an important role in such clinical applications as the diagnosis of Rh-Hr sensitization in pregnancy, detection of sensitization from blood transfusions, Rh typing, evaluation of efficiency of replacement transfusion in treatment of erythroblastosis fetalis (66) and distinguishing of certain cases of idiopathic acquired hemolytic anemia from other hemolytic processes (4).

A considerable contribution to the knowledge of serology and bacteriology has been made by the antiglobulin test. It has led to the recognition of the existence of the Kell system (18) of blood groups, and to a more reliable method for the study of antibodies such as anti-MN, Ss, Jk^a, Jk^b, Jobbins, Fy^a, Be^a, Mi and presumably the entire Rh system (36). Wiener and Gordon (83) have employed the test as a rough measure of the degree of coating with antibody and of the number of sites of a blood group antigen on the erythrocytes. It has also been used to show increased titers in the serum of patients with specific bacterial infections as well as to detect natural agglutinins in human sera against certain bacteria. Among these infections were:

typhoid (52, 69), bacillary dysentry (52), Q-fever (20), brucellosis (77, 85) and tuberculosis (56, 73). In most cases, enhancement of the reaction was shown by increase of titer from one to three tubes when the antiglobulin test was used (34).

The Anti-Human Globulin (Coombs) Serum

Under standard conditions, the reliability of the antiglobulin test would depend mainly on the potency of the antiglobulin serum. In the original work (17), rabbit anti-human serum, rabbit anti-human pseudoglobulin and rabbit anti-human globulin were reported to be reliable for this test. Since then, various serum fractions and procedures were employed for immunization in order to improve the potency of the serum.

Simmons (68) undertook a study on rabbit antisera prepared with the serum of various animals. Sera from pig, dog, cat, goat, horse and ox but not fowl were found to be satisfactory antigens for preparation of Coombs antisera. The serum antigens for rabbit immunization were treated either by alcohol precipitation or the alum precipitation method of Proom (60).

Coombs and Mourant (15) investigated the preparation of antisera against unaltered human serum, alum-precipitated human serum and various serum fractions. Among the antigens employed, alum-precipitated human serum was found to produce the most potent Coombs reagent.

Wootton (88) reported that repeated intramuscular injections of alum-precipitated globulin into rabbits followed by one intraperitoneal and one intravenous injection of human serum yielded a highly potent reagent.

Emerson, Franklin and Lowell (33) employed an adjuvant containing mineral oil, an emulsifying agent and heat-killed Mycobacterium butyricum with the normal human serum. The procedure consisted of one or more subcutaneous injections followed by periodic injections of serum alone. The findings suggest this to be an effective method of producing rabbit antiglobulin serum.

Rosenfield, Vogel and Rosenthal (66) reported that thorough comparison failed to disclose any difference between antiglobulin sera prepared from rabbits sensitized with human gamma globulin, and rabbits sensitized with unaltered human serum. Alum-precipitated human gamma globulin was advocated by these workers for the production of antiglobulin serum.

Animals other than rabbits have been used. Hill and Haberman (36) found goats to be more desirable for this purpose. Goat anti-human globulin serum was used in parallel with rabbit anti-human globulin serum in 2,609 Coombs tests by Dunsford and Bowley (32, 32a). They reported that goat serum was in no way inferior to the rabbit product and indeed appeared to be rather more sensitive in a proportion of cases. Other advantages for preparing antisera in goats, such as ease of injection of antigen, saving of time and labor in processing a large volume of serum at one time and immunity of goats to myxomatosis were pointed out by these workers.

Elution and Antiserum to Eluate

In 1925 Landsteiner and Miller (41) introduced a technique for the recovery of antibodies from sensitized erythrocytes. According to this method, the washed erythrocytes were suspended in saline, heated at 56° C for five minutes and centrifuged, leaving a supernatant which contained the eluted antibody.

The procedure has been used successfully to recover Rh antibodies from cells of erythroblastotic infants by Carter and Loughrey (12) in 1945 and from cells of patients suffering from acquired hemolytic anemia by Crowley and Bouroncle (22) in 1956. By treatment with red cells of the appropriate antigenic structures, this method can also be applied to the separation and recovery of rare antibodies from antisera containing mixtures of antibodies (39).

Application of a somewhat similar principle to the production of antiserum was made by Murray (55). Washed, packed, Rh (D) positive red cells were suspended in saline and heated at 50° C for twenty minutes. After centrifugation, the supernatant was used for injections of guinea pigs. Antisera with titers up to 1:160 when tested against Rh (D) positive cells were obtained.

MATERIALS AND METHODS

Saline Solution.- Saline solution containing 0.85 per cent sodium chloride and 0.1 per cent sodium azide in distilled water was used as diluent and for washing cells throughout this work. Azide-free saline was used in preparing materials for injection of rabbits.

Gamma Globulin.- Human gamma globulin was kindly supplied by Dr. Bruce Chown of the Children's Hospital, Winnipeg.

Human Incomplete Anti-D Sera.- These were made available through the kindness of Dr. Bruce Chown. All sera were inactivated before use by heating at 56° C for 30 minutes.

Normal Human Erythrocytes.- Blood from a group O Rh (D) positive donor was collected aseptically in 1.2 volumes of modified Alsever's solution (7, 8), which had been autoclaved at 10 pounds for 15 minutes. The composition of the solution was as follows: dextrose 2.05 gm., sodium citrate 0.8 gm., sodium chloride 0.42 gm., distilled water 100 ml. The solution was adjusted to pH 6.1 with 10 per cent citric acid solution.

Samples of Rh (D) negative blood of group A, B and O were made available through the courtesy of Miss Catherine Anderson at the Red Cross Transfusion Service, Winnipeg.

All blood collections and samples were kept at 4° C and used for up to two weeks. An aliquot was taken at the day of testing and the cells were washed three times in saline before use.

Test Tubes.- Both the antiglobulin and the precipitin tests were carried out in 10 X 75 mm. tubes.

Preparation of Washed, Packed Erythrocytes

Red cells stored in Alsever's solution were centrifuged at 2,200 r.p.m. for 10 minutes. The sedimented cells were washed three times with six volumes of saline. Following the last washing, the cells were centrifuged at the same speed for 15 minutes, and the supernatant was discarded. The washed, packed red cells were stored at 4° C and used within one day.

Preparation of Alum-precipitated Gamma Globulin

The human gamma globulin was prepared according to Kabat's method (40). 5 ml. of 1 per cent sterile alum was added to 100 ml. of sterile saline (0.85% NaCl) solution of gamma globulin containing 2.5 mg. of gamma globulin per ml. The solution was neutralized with N/10 NaOH to maximum turbidity.

Preparation of Eluted Rh Antibody

The eluted Rh antibody was prepared by the technique of Landsteiner and Miller (41). An inactivated and undiluted incomplete anti-D serum was added to an equal volume of washed, packed, group O Rh (D) positive red cells. After two hours at 37° C, the cells were washed six times with ice cold saline and finally resuspended in an amount equal to the initial volume. The saline suspension of anti-D sensitized cells was gently agitated in a 56° C water-bath for five

minutes. The cells were quickly centrifuged with the centrifuged cup filled with water at 56° C. The supernatant containing the eluted anti-D was removed immediately and stored at -20° C until use. The eluted anti-D was prepared fresh every week for injections.

Injection of Rabbits

Four groups of adult albino rabbits were immunized. Group I were injected with alum-precipitated human gamma globulin containing 2.5 mg. per ml. intravenously daily for four days out of each week for a period of four weeks. The schedule (40) used was as follows: 3 injections of 0.5 ml.; 3 injections of 0.75 ml.; 4 injections of 1 ml.; 4 injections of 1.5 ml.; 2 injections of 2.5 ml. Each of the three rabbits received a total of 46.89 mg. of alum-precipitated human gamma globulin. The animals were bled on the fifth day after the final injection.

Group II were injected with alum-precipitated human gamma globulin containing 0.5 mg. per ml. intravenously. The injection schedule was the same as that of Group I. Each of the three rabbits in this group received a total of 9.39 mg. of the antigen, or only one-fifth of that of Group I.

Group III were injected intraperitoneally with 5 mg. of alum-precipitated human gamma globulin in 1 ml. dosage in four days intervals over a period of twenty days. Each of the five rabbits in this group received a total of 25 mg. of alum-precipitated human gamma globulin. The animals were bled on the tenth day after the final injection.

Group IV consisting of three rabbits were injected intravenously with eluted anti-D. The injection schedule used was the same as that of Group I and II.

Collection and Separation of Rabbit Antisera

The rabbits were bled either from the marginal ear veins or by cardiac puncture. The blood collected was allowed to clot at room temperature for two hours, and was then rimmed and stored at 4° C overnight. Separation of serum was performed at 4° C by centrifugation until a cell-free serum was obtained, which was stored at -20° C.

Treatment of Antisera Before Use

The antisera were treated to remove natural hemagglutinins that may be present by heating in a 1:10 dilution of saline at 70° C in a water-bath for 60 minutes, with shaking at regular intervals. The process also served to inactivate the complement.

In some cases, the antisera were absorbed with Rh (D) negative red cells. The absorption process was carried out by heating an antiserum at 56° C for 30 minutes, diluting it 1:10 in saline and adding to an equal volume of group O Rh (D) negative packed red cells which has been washed ten times. The serum-cells mixture was allowed to stand at room temperature for 10 minutes with frequent shakings and then centrifuged at 2,200 r.p.m. for 10 minutes. The procedure was repeated until complete absorption was attained.

Antiglobulin Test

A slight modification of the technique recommended by the National Institutes of Health (57) was adopted.

Sensitization of Cells.- Eleven twofold serial dilutions of the incomplete anti-D serum were made ranging from undiluted to 1:1024. A control with saline only was included. An equal quantity of two per cent washed, packed red cells was added to each dilution of the incomplete anti-D and to the control. The suspensions were incubated at 37° C for one hour. The cells were then washed six times and finally suspended in the original volume to reconstitute a two per cent suspension.

Titration of Rabbit Antiserum.- Eleven twofold serial dilutions of the heated or absorbed rabbit antiserum were set up ranging from 1:20 to 1:20480. A saline control was included. From each dilution of the antiserum, 0.1 ml. quantities were transferred to a series of twelve tubes. 0.1 ml. of the red cells sensitized with incomplete anti-D in a 1:1 dilution was added to the first tube of each dilution of the rabbit antiserum. Similarly, 0.1 ml. of the red cells sensitized with incomplete anti-D in a 1:2 dilution was added to the second tube of each dilution and so on. Final dilution of the incomplete anti-D was 1:1024.

Reading of the Test.- All tubes were thoroughly shaken and centrifuged at 1,500 r.p.m. for two minutes. Agglutination was read macroscopically by gently dislodging the packed cells.

Precipitin Test

The procedure as proposed by Martin (47) was adopted with slight

modifications. Both the antigen, which was a saline solution of gamma globulin containing 1 mg. of gamma globulin per ml., and the antibody, which was a rabbit antiglobulin serum diluted 1:5 in saline, were filtered and centrifuged to remove any particulate matter and lipoid substance. A preliminary titration with a constant volume of serum and progressively decreasing amounts of antigen was performed. The tubes were examined for flocculation after incubation at 37° C for thirty minutes and refrigeration at 4° C overnight. The least quantity of antigen which yielded a flocculate was then used with progressively decreasing amounts of serum. The tubes were incubated and read as in the preliminary part of the test. The highest dilution of the serum which showed flocculation was taken as the endpoint.

RESULTS

ACTIVITY OF COOMBS ANTISERA

Each of the three rabbits in Group I received a total of 46.89 mg. of alum-precipitated human gamma globulin by intravenous injection. The results of the antiglobulin sera tested against red cells sensitized with serial dilutions of incomplete anti-D are recorded in Table I(a). The titers are expressed as reciprocals of the highest dilutions of antisera showing macroscopic agglutination of the test cells.

The titers of the three antisera ranged from 1:10240-160, 1:5120-0 and 1:2560-0 for the 11 serial dilutions of incomplete anti-D. The highest titer obtained in this group with a 1:1 dilution of incomplete anti-D was 1:10240 and the highest obtained with a 1:1024 dilution was 1:160. The mean titer of these antisera ranged from 1:5120 with undiluted incomplete anti-D to 0 with diluted incomplete anti-D.

In Group II, each of the three rabbits received a total of 9.39 mg. of alum-precipitated human gamma globulin by intravenous injection. The activity of the antiglobulin sera were tested as in Group I. Table I(b).

The titers of the three antisera ranged from 1:10240-20, 1:20480-0 and 1:10240-0 for the dilutions of incomplete anti-D. The highest titer obtained in this group with a 1:1 dilution of incomplete anti-D was 1:20480 and the highest obtained with a 1:1024 dilution was 1:20. The mean titer of these antisera ranged from 1:10240 with undiluted incomplete anti-D to 0 with diluted incomplete anti-D.

In Group III, each of the five rabbits received a total of 25 mg. of alum-precipitated human gamma globulin by intraperitoneal injection. Table I(c). The titers of the antiglobulin sera ranged from 1:5120 to 1:10240 with a 1:1 dilution of incomplete anti-D, and 0 to 1:1280 with a 1:4 dilution of incomplete anti-D. No titer was observed beyond the 1:4 dilution of the anti-D.

In Group IV, the antisera from three rabbits injected with eluted incomplete anti-D by intravenous route were tested. Table II. The titers of the three antisera ranged from 1:2560-1:160, 1:2560-1:80 and 1:5120-1:160 for the dilutions of incomplete anti-D. The highest titer obtained in this group with a 1:1 dilution of incomplete anti-D was 1:5120 and the highest obtained with a 1:1024 dilution was 1:160. The mean titer of these antisera ranged from 1:2560 with undiluted incomplete anti-D to 1:160 with diluted incomplete anti-D.

No agglutination was observed in the saline controls included in all the tests.

Incomplete Anti-D

The activity of antiglobulin serum #2 from Group I was tested against red cells sensitized with incomplete anti-D sera from three donors, Seepish, Goodman and Briston. Table IV. The titer of the antiglobulin serum ranged from 1:160-1:20, 1:320-1:80 and 1:5120-1:640 for the dilutions of the sera from the three donors respectively.

Eluted Incomplete Anti-D

The activity of antiglobulin sera tested against red cells sensitized with freshly prepared eluted incomplete anti-D is shown in Table V. The titer of antiserum #1 ranged from 1:160 with a 1:1 dilution of eluate to 1:40 with a 1:8 dilution of eluate. No titer was observed beyond the 1:8 dilution. The antiglobulin test was performed twice with antiglobulin serum #2. The titer of this antiserum ranged from 1:640 and 1:1280 with a 1:1 dilution of eluate to 0 and 1:40 with a 1:16 dilution of eluate respectively.

The eluted incomplete anti-D was also used to sensitize group A and group B, Rh negative red cells which were then tested with the antiglobulin sera. No agglutination was observed with all dilutions of the eluate.

Patients' Incomplete Anti-D

A comparison of the activity of the anti-(eluted anti-D) serum with the antiglobulin serum against red cells sensitized with patients' incomplete anti-D is shown in Table VI. Each patient serum was tested with an anti-(eluted anti-D) serum and an antiglobulin serum simultaneously.

The titer of the anti-(eluted anti-D) serum #12 ranged from 1:2560-1:160 and that of the antiglobulin serum #2 ranged from 1:2560-1:20 for the dilutions of incomplete anti-D serum 'Reideger'. The titer of anti-(eluted anti-D) serum #14 ranged from 1:160-0 and that of

the antiglobulin serum #1 ranged from 1:80-0 for the dilutions of incomplete anti-D serum 'Lagerway'. The titer of the anti-(eluted anti-D) serum #12 ranged from 1:80-0 and that of the antiglobulin serum #2 ranged from 1:40-0 for the dilutions of incomplete anti-D serum 'Brailsford'.

PRECIPITIN CONTENT OF ANTIGLOBULIN SERA

The precipitin content of the antiglobulin sera from Group I, II and III is shown in Table III. The titers are expressed as the reciprocals of the highest dilutions of antisera showing flocculation. The titers ranged from 1:320-1:640, 1:160-1:640 and 1:160-1:640 in the three groups respectively. No significant difference in titers was observed among the groups.

TABLE I

ACTIVITY OF ANTIGLOBULIN SERA AGAINST RED CELLS SENSITIZED WITH INCOMPLETE Rn ANTIBODY

(a) Intravenous Injection. Total of 46.89 mg. GG/Rabbit

Antiserum No.	Dilution of Incomplete Anti-D (Briston)										
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
1	10240*	5120	10240	5120	2560	1280	1280	640	320	320	160
2	5120	2560	2560	1280	1280	640	160	160	80	80	0[x]
3	2560	10240	2560	2560	1280	1280	640	160	40	40	0
Mean Titer	5120	5120	2560	2560	1280	1280	640	160	80	80	0

*Reciprocal of highest dilution of antiserum showing macroscopic agglutination of test cells expressed as titer.

[x]no observable macroscopic agglutination.

TABLE I (cont.)

(b) Intravenous Injection. Total of 9.39 mg. GG/Rabbit

Antiserum No.	Dilution of Incomplete Anti-D (Briston)										
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
4	10240*	2560	2560	1280	2560	2560	640	1280	640	160	20
5	20480	20480	5120	5120	2560	1280	640	160	640	20	0[x]
6	10240	10240	5120	1280	2560	1280	2560	640	320	320	0
Mean Titer	10240	10240	5120	1280	2560	1280	640	640	640	160	0

*Reciprocal of highest dilution of antiserum showing macroscopic agglutination of test cells expressed as titer.

[x]no observable macroscopic agglutination.

TABLE I (cont.)

(c) Intraperitoneal Injection. Total of 25 mg. GG/Rabbit

Antiserum No.	Dilution of Incomplete Anti-D (Briston)										
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
7	10240*	40	0[x]	0	0	0	0	0	0	0	0
8	10240	5120	1280	0	0	0	0	0	0	0	0
9	5120	20	0	0	0	0	0	0	0	0	0
10	5120	20	0	0	0	0	0	0	0	0	0
11	10240	2560	20	0	0	0	0	0	0	0	0
Mean Titer	10240	40	20	0	0	0	0	0	0	0	0

*Reciprocal of highest dilution of antiserum showing macroscopic agglutination of test cells expressed as titer.

[x]no observable macroscopic agglutination.

TABLE II

ACTIVITY OF ANTI-(ELUTED ANTI-D) SERA AGAINST RED CELLS SENSITIZED WITH INCOMPLETE Rh ANTIBODY

Intravenous Injection.

Antiserum No.	Dilution of Incomplete Anti-D (Briston)										
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
12	2560*	1280	2560	1280	1280	1280	1280	320	320	160	160
13	2560	5120	5120	1280	640	1280	320	320	320	320	80
14	5120	10240	20480	5120	2560	2560	1280	640	640	320	160
Mean Titer	2560	5120	5120	1280	1280	1280	1280	320	320	320	160

*Reciprocal of highest dilution of antiserum showing macroscopic agglutination of test cells expressed as titer.

TABLE III

PRECIPITIN CONTENT OF ANTIGLOBULIN SERA

(a) Intravenous Injection.

Total Amount of Gamma Globulin Received Per Rabbit 46.89 mg.		Total Amount of Gamma Globulin Received Per Rabbit 9.39 mg.	
Rabbit No.	Titer	Rabbit No.	Titer
1	640*	4	160
2	320	5	640
3	640	6	320

(b) Intraperitoneal Injection.

Total Amount of Gamma Globulin Received Per Rabbit 25 mg.			
Rabbit No.	Titer	Rabbit No.	Titer
7	320	10	320
8	320	11	640
9	160		

*Reciprocal of highest dilution of antiserum showing flocculation expressed as titer.

TABLE IV

ACTIVITY OF ANTIGLOBULIN SERUM AGAINST RED CELLS SENSITIZED WITH VARIOUS INCOMPLETE ANTI-D

Antiserum No.	Incomplete Anti-D	Dilution of Incomplete Anti-D 1:1	1:2	1:4	1:8	1:16	1:32	1:64
2	'Seepish'	- *	160**	320	160	80	80	20
2	'Goodman'	-	320	320	320	160	320	80
2	'Briston'	2560	5120	2560	1280	640	640	640

TABLE V

ACTIVITY OF ANTIGLOBULIN SERA AGAINST RED CELLS SENSITIZED WITH ELUTED INCOMPLETE ANTI-D

Antiserum No.	Dilution of Eluted Anti-D 1:1	1:2	1:4	1:8	1:16	1:32	1:64
1	160**	160	80	40	0[x]	0	0
2	640	160	160	160	0	0	0
2	1280	640	320	80	40	0	0

*Test not performed.

**Reciprocal of highest dilution of antiserum showing macroscopic agglutination of test cells expressed as titer.

[x]no observable macroscopic agglutination.

TABLE VI

COMPARISON OF ACTIVITY OF ANTI-(ELUTED ANTI-D) SERUM WITH ANTIGLOBULIN AGAINST RED CELLS SENSITIZED WITH PATIENTS' INCOMPLETE ANTI-D

Incomplete Anti-D	Coombs Antiserum	Dilution of Incomplete Anti-D 1:4	1:8	1:16	1:32	1:64	1:128	1:256
'Reideger'	#12	2560*	320	640	640	160	160	160
	# 2	2560	1280	1280	320	320	40	20
'Lagerway'	#14	160	80	0[x]	0	0	0	0
	# 1	80	0	0	0	0	0	0
'Brailsford'	#12	80	80	0	0	0	0	0
	# 2	40	0	0	0	0	0	0

*Reciprocal of highest dilution of antiserum showing macroscopic agglutination of test cells expressed as titer.

[x]no observable macroscopic agglutination.

DISCUSSION

The results of this investigation indicate that potent anti-globulin sera can be prepared in rabbits with very small quantities of human gamma globulin. The findings also show that antibody recovered from incomplete anti-D sensitized red cells by the elution technique can be used to produce Coombs antisera containing a concentration of antiglobulin just as high, indeed if not higher, as those prepared with gamma globulin.

For the preparation of antiglobulin sera, alum-precipitated human gamma globulin has proved satisfactory. Neither anaphylaxis nor reaction to the alum was observed in any of the rabbits used. The difference in the responses of the individual animals within each group was also so small as to be negligible.

The diluent used in all the testings throughout this work was a 0.85% saline solution with 0.1% sodium azide added as preservative. The use of the azide was based on the report by Batson et al (3) that this substance was bacteriostatic and that it did not affect the titer of anti-Rh antibody.

The removal of any natural or immune anti-human hemagglutinins present in the Coombs antisera was effected by the heat method of Menolasino and Davidsohn (50). The procedure was chosen for reasons of simplicity, convenience and time-saving as against the laborious absorption process with A, B and O type human red cells as suggested by Rosenfield et al (66). The antiglobulin sera treated by this method was found to be satisfactory for the Coombs test; special cases where absorption with blood cells were required will be discussed later.

Tests for the activity of the Coombs antisera, after the removal of all agglutinable anti-human hemagglutinins, against red cells sensitized with incomplete Rh antibody were done quantitatively so that the strength of the antisera could be fully evaluated. For this purpose, a full cross-titration as recommended by the National Institutes of Health (57) was adopted. A simple titration is inadequate since it is essential to determine how the reagents react not only with strongly sensitized cells but with weakly sensitized cells as well. This decision is based on the belief that the titer against strongly sensitized cells might not be a true expression of the antiglobulin activity because the cells are 'supersaturated' to an undetermined degree.

In order to avoid unnecessary complications in the test, special attention should be paid to certain points. It is noted that the volume of saline used in washing the cells is large in comparison with the volume of packed erythrocytes employed; this is essential to the proper performance of the test since insufficient washing may leave traces of serum proteins which react with the antiglobulin serum, thus causing a weak or false negative reading. When there is doubt as to the adequacy of the washings it is preferable to wash excessively rather than insufficiently, since antibodies do not 'wash off' in these circumstances.

Barrier and Krieger (2) reported that sensitized cells after the final washing should not be allowed to remain in contact with the saline medium for prolonged periods, even in low temperature. This was due to the gradual elution of the antibody into the saline solution thus resulting in less avid or even negative reactions.

The occasional presence of cold auto-agglutinins is known to be a possible source of false positive reaction. This can be guarded against by either using warm saline for washings or warming the sensitized cells at 37° C for one to three minutes before adding to the antiglobulin serum. Both the processes serve to elute the cold antibodies from the surface of the red cells where they might interfere with the reaction. However, neither these antibodies nor the incomplete forms described by Dacie (24, 25) was encountered throughout this work.

From a study on the factors affecting the speed of agglutination in the Coombs test, Wootton (89) reported that maximal adsorption of antibodies by incubation may take up to six hours. The adsorption curve is of the exponential type with the most rapid combination occuring in the first half hour.

Apart from matters of technique, there are three important variables in the test, namely, the cells, the sensitizing antibody and the antiglobulin antibody. Each of these is to be discussed in their order.

The variation of the agglutinability of the cells with this test is shown by the properties of the D^u antigen (63, 70). The influence of the properties of the cells on the results of the test has not been studied here, and this variable has been eliminated by using cells from only one donor.

A consideration of the sensitizing antibody can be divided into two parts, firstly, its relation with the cells in the adsorption stage and secondly, its relation with the antiglobulin antibody in the agglutinating stage of the test. The variation of the sensitizing power of different antisera is shown in Table IV. With the same antiglobulin

serum, higher titers were obtained with all dilutions of the sensitizing antibody when incomplete serum 'Bristol' was employed. By the use of the same incomplete antiserum throughout the tests, it has also been possible to eliminate the variable of the sensitizing antibody.

In the relationship of the sensitizing antibody to the anti-globulin serum as well as in the influence of the antiglobulin antibody itself on the test, the most important factor to be noted is the capacity of the antiglobulin serum to react with a wide range of human globulins. An antiserum possessing this property is spoken of as having a broad specificity while one which reacts with only one or two globulins is described as having a narrow specificity. The choice of the range of specificity would rest on the nature of the work in which the antiglobulin serum is employed.

For diagnostic use, a broad specificity to include the complete spectrum of human globulins should be more useful than a narrow one. This observation is based on the concept that the antiglobulin serum might not be equally reactive with each and every type of globulin in which antibodies are found. For such purposes, the most suitable antigen for the development of antiglobulin serum would seem, at least theoretically, to be whole human serum or some preparation containing all the globulin factors. In practice, however, such an antigen might be quite low in potency, although most of the injected animals will yield an adequate antibody level if the stimulations are continued over a sufficient period of time. A good substitute, if not a better one, can be found in human gamma globulin. In fact, it has been shown

by Kabat (38) that the strength of antiglobulin serum is directly proportional to its content of anti-human gamma globulin antibody. Alum-precipitated human serum may also be used, but it is reported that certain animals respond to the albumin rather than the globulin fraction and will fail to yield a satisfactory reagent (66).

For experimental studies, antiglobulin serum of narrow specificity would be of more value. To this end, single purified globulin fractions might prove useful antigens. In connection with this observation, it may be of interest to note that according to Cann (11), the purity of cold ethanol gamma globulin fractions vary from 57 to 89 per cent and even the electro-phoresis-convection method gives gamma globulin varying from 90 to 99 per cent purity. The human gamma globulin used in the present investigation has not been characterized and is, in all probability, impure. However, the finding that only very small quantities are required for the preparation of antiglobulin sera of good strength would prove useful in attempts to obtain specific antiglobulin sera by immunizing rabbits with purified protein fractions.

Other methods for narrowing the specificity of antisera thus rendering them capable of reacting only with certain globulins can be found in the work of various investigators. Crawford and Mollison (21) achieved this by differential absorption with cells with particular antibodies. Renton (64) also utilized a similar technique. Cutbush et al (23) prepared such sera by addition of specific fractions. These workers, employing a technique described by Boyden (7), also reported the preparation of an antiglobulin serum free of anti-gamma globulin by absorption with cells that had been treated with tannic acid and

then exposed to a solution of gamma globulin.

The precipitin content of the anti-gamma globulin sera was tested to serve as a basis for the comparison of the sera by the Coombs test. The technique selected for this purpose was the serum dilution method of Martin (47) who proposed it as a more reliable procedure for the titration of precipitin in a single antigen-antibody system than the antigen dilution technique. The latter was excluded on the basis of the observation that the smallest amount of antigen producing a visible precipitate is a function of the antigen alone. Since a fixed quantity of antigen is used in the serum dilution method, the proportions of antibody to antigen varies from great antibody excess through equivalence to inhibition by excess antigen. In order to avoid the inhibiting effect of excess antigen, the least amount of antigen capable of yielding a visible precipitate with an equivalent amount of antibody is first determined in a preliminary titration. The highest dilution of serum precipitating this amount of antigen is then taken to be the true antibody content of the serum, as there is no excess of precipitable antigen present in the mixture.

A point of special interest in this investigation is the fact that although the precipitin content of the anti-gamma globulin sera by the intravenous route correspond closely to those prepared by the intraperitoneal route (Table III), the titers of the antisera of the two groups against incomplete anti-D sensitized cells are far from agreement (Table I). The observation is more puzzling in view of the production of satisfactory antisera reported by Cutbush et al (23) in rabbits by intraperitoneal injections with a shorter course of immunization and a

smaller amount of gamma globulin. The difference may be due to the diminished avidity or combining power of the intraperitoneal antisera for the sensitized cells which may be the result of the method of preparation, the length of the immunization course, or the particular combination of both.

The results presented have demonstrated that Coombs antisera of good strength can be prepared with eluted incomplete anti-D (Table II) and the principle may prove valuable in other antigen-antibody systems. However, before the procedure can be fully adopted for routine use, a more reliable and effective technique for elution, among other factors, should be investigated. In Table V, contrary to the findings of other tests, antiglobulin #1 appears to be more potent than serum #2. Even with the same serum #2, considerable difference in range of titers is shown between two consecutive testings. Such disagreement may be attributed to the fluctuation of the amount of antibodies eluted at different times.

One other method of elution has been tried but not described in this work, namely, the elution of incomplete anti-D sensitized cells at 7° C for overnight. The test was based on an earlier report by Barrier and Krieger (2) that strongly sensitized cells left in the cold overnight gave very weak agglutination when tested on the following day. However, eluate capable of sensitizing the homologous D positive cells have not been recovered, possibly due to denaturation of the eluted antibody on prolonged contact with the saline medium.

The Coombs tests on three selected patients' incomplete anti-D sera, each performed with one antiglobulin serum in parallel with an anti-(eluted anti-D) serum, appear to indicate that the anti-(eluted anti-D) serum are more competent for the detection of the incomplete Rh antibody (Table VI). It should be

mentioned that it was necessary to absorb the anti-(eluted anti-D) sera used in these tests with O-Rh negative red cells, as the antisera gave agglutination with non-sensitized washed erythrocytes even after the heat treatment (50). No attempt for the explanation of this inconsistency has been forwarded at this stage. The patients' sera employed in the tests are chosen on the basis of low titers with antiglobulin sera but where high titers are expected clinically. The experimental data obtained so far are too scanty to provide conclusive evidence, and further testing on a larger number of patients' sera will be carried out.

Van Loghem et al (75) have reported that some high-titered antiglobulin sera may exhibit a prozone of inhibition when tested against weakly sensitized cells, possibly due to antibody excess. Such an antiserum was not encountered in this investigation, but the phenonmenon described lends itself to one more argument in favor of standardizing antiglobulin sera by the full cross-titration technique adopted throughout this work.

SUMMARY

1. The antiglobulin content in the sera of four groups of rabbits immunized with alum-precipitated human gamma globulin or with eluted incomplete anti-D were examined by the antiglobulin technique of the National Institutes of Health and the precipitin test of Martin.

2. Each rabbit of Group I received 46.89 mg. of alum-precipitated human gamma globulin intravenously, Group II received 9.39 mg. of the globulin intravenously, Group III received 25 mg. of the globulin intraperitoneally, and Group IV received eluted incomplete anti-D intravenously.

3. The antiglobulin titers for eleven halving dilutions of incomplete anti-D ranged from 1:10240-1:160 in Group I, 1:20480-1:20 in Group II, 1:10240-0 in Group III, and 1:5120-1:160 in Group IV. The range of the mean antiglobulin titer was 1:5120-0 in Group I, 1:10240-0 in Group II, 1:10240-0 in Group III and 1:2560-1:160 in Group IV. The antiglobulin sera of Group IV gave titers only from 1:1-1:4 dilutions of the anti-D.

4. The precipitin titers ranged from 1:320-1:640 in Group I, 1:160-1:640 in Group II and 1:160-1:640 in Group III.

5. The results show that potent antiglobulin sera can be prepared in rabbits with very small quantities of human gamma globulin.

6. The evidence also indicates that antibody recovered from incomplete anti-D sensitized erythrocytes by the elution technique was effective ~~as~~ antigen for the preparation of potent Coombs antisera in rabbits.

7. Preliminary results of the comparisons of the anti-(eluted anti-D) sera with the antiglobulin sera on patients' incomplete anti-D appear to indicate that the anti-(eluted anti-D) sera are more competent for the detection of the incomplete Rh antibody.

8. Certain theoretical and technical aspects of the antiglobulin test were discussed.

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