THE DEMONSTRATION AND CHARACTERIZATION OF THE ANTI-d AGGLUTININ AND ANTIGEN PREDICTED BY FISHER AND RACE

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WHEN the three-linked-genes theory of inheritance of the CDE cde (Rh-Hr) blood antigens was proposed by Fisher and Race (1944), some interesting correlations and remarkable predictions were made. On the basis of this theory, new blood antigens designated d and e were postulated in addition to the C, c, D and E which were then recognized. This is easily understood when it is noted that the red cell antigens C and c determined by their respective genes had a reciprocal relationship similar to the M and N antigens. In fact, Levine had earlier described this relationship between C (Rh') and the antigen c (Hr') determined by his original Hr serum. Since D (Rh₀) and E (Rh") had no such antithetical relationship to C and c or to each other, separate closely linked loci D and E were assigned to the genes determining these antigens. Because it seemed logical that D and E should likewise have antigens related to them in the same manner as c to C, Fisher predicted that the genes which he designated d and e, should be found.

Following these predictions, Mourant in 1945 reported the finding of a serum which detected an antigen having the specificity required for e. The e antigen having the same antithetical relation to E as c to C indicated that the genes E and e were allelomorphic (i.e. occurring interchangeably at the same locus in the chromosome). This serum, therefore, could be used to determine heterozygosity at the E locus. Although the Fisher-Race theory did not specifically predict more than two allelomorphic genes for each of the three loci, Callender Race and Paykoc in 1946 discovered a third allelomorph at the C locus which they designated C⁺. This was followed by the finding of an additional allelomorph at the D locus by Stratton in 1946 designated as D⁺. The finding of these two additional allelomorphs was quite compatible with modern concepts of genetics and constituted further evidence for the Fisher-Race theory.

During this period, unfortunately, the predicted anti-d serum remained undiscovered although it had become apparent that this serum would be the most important one for determining heterozygosity of husbands of iso-immunized women. At one time it was thought that the serum reported by Levine and Waller had a specificity suggestive of anti-d. However, this serum was proved to be identical in specificity to the St serum of Race and Taylor (anti-c). At the International Hematology and Rh Conference in Dallas, Texas, 1946, Diamond indicated that he
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had found a serum which contained weak antibodies against the d antigen in addition to anti-c.

The present report is concerned with the demonstration of the anti-d agglutinin predicted by Fisher and proof of its specificity. This evidence was obtained by the following: (1) Differential agglutination of key red cells of known genotype, (2) quantitative hemolytic studies on such key erythrocytes and (3) determination of specificity of the agglutinin by its reaction with 100 random blood samples.

CASE REPORT

Female, age 26, quadroon, gave a history of having received several transfusions in 1938 while being treated for Paget's disease of the breast. No untoward reactions were recalled. Three previous pregnancies terminated in the delivery of normal infants in 1941, 1943 and 1945. On November 25, 1946 after an apparently normal pregnancy, a female infant weighing 5 lbs. 13 oz. and showing no gross abnormality was delivered at the Charity Hospital, New Orleans. However, typical erythroblastosis developed with marked icterus at eighteen hours. On the second day a hemoglobin determination showed 7.5 Gm. and

| Table 1 |
|---|---|---|---|---|---|
| **Blood From** | **Group** | **Rh antisera** | **Mother's serum** |
| | | (D) (RhA) | (C) (Rh') | (E) (Rh") | Saline | Albumin |
| Father | O | + | - | - | + | + |
| Child 1 | O | + | - | - | + | + |
| Child 2 | A | + | + | - | + | + |
| Mother | A | + | + | - | - | - |

Subsequently the baby's blood was tested

Baby | O | + | + | - | + | + |

on the third day, 448 erythroblasts per 100 leukocytes were counted in the infant's blood. The first transfusion of 70 cc. Group O Rh negative blood was given on the day after birth. An additional four transfusions were given as required, the last on the tenth day consisting of 50 cc. of Rh positive blood. The baby was discharged on the twentieth day and has developed normally since that time.

Although the Rh positive erythroblastotic infant was initially given the usual Rh negative transfusions one of the authors (B. W. E.) noted that the mother was Rh positive and suggested the possibility of a rare intragroup isoimmunization. More complete serologic studies were undertaken in the Serologic Research Laboratory of the Southern Baptist Hospital. These studies by one of us (J. W. D.) as given in table 1, showed that the mother's erythrocytes were Group A and contained the C and D antigens but not the E. The father, of Group O, had D but no C or E in the red cells. The infant (and the other two children) gave Group O reactions and their red cells contained antigens D and C but not E. The mother's serum agglutinated the erythrocytes of the father and all three children. Tested against 11 random bloods (13 Rh Positive and 9 Rh Negative) the agglutinins of this serum exhibited no relationship to the M and N types. However, all Rh negative bloods were agglutinated and all but three of the Rh positive blood samples likewise showed agglutination. One additional positive blood showed agglutination in albumin but not in saline suspension. Substantial blood samples were collected from the patient in December 1946 and in March 1947. Serum from these collections were kept frozen at minus 10 C. During the period from December 1946 until May 1947 samples of serum from this case were submitted to many laboratories. All workers agreed that the serum contained an exceptionally strong anti-c (Hr") antibody and many suggested also the possibility of an anti-d antibody. Definite proof of the anti-d however was not forthcoming apparently due to a lack of rare test cells definitely known to be of genotypes negative for c but positive for d.
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METHODS

Studies of the serum of this case begun at The William Buchanan Blood Center in May 1947 were designed to establish the identity of antibodies present by the methods described below. Two technics were employed to demonstrate agglutination with the key test erythrocytes of known genotype: (1) The classic test tube method9 using 2 per cent saline suspension of red cells and (2) the developing test10 employing the Coombs11 antihuman globulin serum. Since the serum from the December bleeding appeared to contain two antibodies differing in reactivity as well as specificity, these two tests were especially useful. The antibody shown to have the specificity of anti-d reacted with both tests while the other antibody with the specificity characteristic of anti-c, reacted with the developing test, but not with the tube method using saline erythrocyte suspension.

The specificity of the two antibody components of the patients serum was also determined by means of an estimation of their hemolytic action against selected erythrocytes. These studies of hemolytic activity of the serum were made by the quantitative hemolytic technic previously described.10, 12-14 This method established the net hemolysis resulting from the action of the antibodies present against the corresponding antigens of proper red cell suspensions under the standard optimum conditions over a period of forty-eight hours. Special advantage was taken of the observation made in earlier experiments15, 16 that the degree of hemolysis was related to the number of antigen antibody combinations available with a given cell and serum. The fact that the two antibodies were present in about equal strength made it possible to interpret the results very clearly.

Although the agglutination and hemolytic tests appeared to establish the specificity of the two antibodies in the patient's serum, it was considered desirable to further check the per cent specificity of the agglutinin component (anti-d) by agglutination of random samples to compare with the specificity of 65 per cent predicted by the calculations of Fisher.1, 19, 20 In so testing the erythrocytes from 100 random blood samples, the tube method using saline suspension of red cells was employed to bring out the activity of the agglutinin (anti-d) but not the cryp tagglutinoind10 (anti-c) which was inactive against saline suspensions of erythrocytes. As a further precaution, these blood samples were subjected to parallel tests with anti C, Cw, c, D, E, e and P. These tests were performed with the Chown17 capillary technic because of its great economy of serum, especially important with such rare sera as anti-Cw and anti-c. All the sera so employed were known to be pure and free of other human erythrocyte antibodies.

One of the most important features of this study was the necessity of having available erythrocytes of suitable and definitely known antigenic composition. For example, the test cell of genotype CDe/Cde was of crucial importance in that it could be used to detect anti-d in the presence of the anti-c cryptagglutinoind found in this serum, without fear that the agglutination might be due to a weak anti-c agglutinin component. Since anti-d serum was not available prior to this case, it was necessary to do family studies to establish the absolute genotype particularly with reference to heterozygosity at the D locus. While individuals of this genotype should not be too rare (about 6.97 per 1000), they would be overlooked and considered as CDe/CDc unless studied in families to show segregation of the Cde chromosome. Fortunately, the genotype CDe/Cde was established in such a family while doing genetic studies with sub-type sera on the random blood samples from the pilot tubes of blood units collected for the Texas City disaster. Of course, the very much rarer (6.4 per 100,000) Cde/Cde could be detected serologically and used to good advantage but because of its rarity was not found in this large series of approximately 1000 blood samples.

RESULTS

The results of agglutination tests using key erythrocytes designed to differentiate Rh antibodies of different specificities are shown in table 2. The reactions with the

* Cryp tagglutinoind. Term proposed to designate those antibodies which neither agglutinate saline suspensions of erythrocytes nor give positive blocking tests (saturation of Rh antigen without agglutination in saline). Such antibodies may be detectable by such technics as the capillary, albumin and serum tests and are uniformly detected by the developing test using a sensitive anti-human globulin serum.
cells shown in this table give strong evidence that an agglutinin of specificity different from anti-C, c, C*, D, E or e was contained in the serum of the patient reported in addition to the anti-c cryptagglutinoid shown by the developing test. The positive reaction with the cell CDe/Cde appears to rule in an anti-d agglutinin but does not entirely exclude the possibility of a rare antibody corresponding to an antigen determined by a rare or undiscovered allelomorphic gene of the Rh system or even

**Table 2.**

| Genotype    | Patient's serum || R.B.C. in Saline | Developing test* |
|-------------|-----------------|------------------|------------------|
| C*De/CDe   |                 |                  | rules out anti-C*, C, D and e. |
| cde/cde    |                 |                  | rules in anti-c and/or anti-d. |
| cDE/cDE    |                 |                  | rules out anti-E agglutinin. |
| Gde/CDe    |                 |                  | rules in anti-d agglutinin. |

* Identical results were obtained with albumin suspension of R.B.C.

**Table 3.**

<table>
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<tr>
<th>Probable Genotype</th>
<th>Incidence %</th>
<th>C</th>
<th>c</th>
<th>D</th>
<th>d</th>
<th>E</th>
<th>e</th>
<th>% Positive to Anti-d</th>
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<tr>
<td>CDe/cde</td>
<td>32</td>
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<td>32</td>
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<tr>
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<td>-</td>
<td>+</td>
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<td>-</td>
<td>+</td>
<td>15</td>
</tr>
<tr>
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<td>16</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>16</td>
</tr>
<tr>
<td>cDE/cde</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>cde/cDE</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>28</td>
</tr>
<tr>
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<td>+</td>
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<td>+</td>
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<td>1</td>
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<tr>
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<tr>
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</tr>
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<td>-</td>
<td>17</td>
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<td>-</td>
<td>+</td>
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<tr>
<td>cDe/cDE</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

100

67

an unrelated blood antigen. However, the results from the agglutination studies of 100 random bloods shown in Table 3 exclude this possibility. As further exclusion, comparisons with the Lutheran, Kell and Lewis antisera were made and no relationship was found. In this table it will be noted that 67 per cent of random bloods were agglutinated by the suspected anti-d agglutinin. This compares favorably with the 65 per cent predicted by Fisher and Race. However, if the data are examined further, it will be observed that the antigen determined by this serum (65-67 per
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cent) has the same antithetical relation to D as M has to N. In other words each specimen is positive for D or d or both but never negative for both.

In figure 1, showing the results of hemolytic tests against CDe/cde and CDe, Cde cells, we obtain a clear confirmation of the presence of both anti-d and anti-c antibodies as well as demonstrating their hemolytic activities. Due to the almost identical titre of the anti-c and d, a unitary relationship between gene, antigen determined thereby, and amount of hemoglobin released by a given strength of antibody was established. This relationship has been previously reported, and will be dealt with in detail elsewhere.

**DISCUSSION**

The evidence presented in this paper is considered reasonably complete to establish the existence of an antibody having the characteristics predicted by Fisher and Race, namely anti-d. In order to substantiate the validity of such a new antibody and the antigen detected thereby, the following criteria may be considered essential: (1) the antiserum must be shown to give specific antigen-antibody reactions,
it must be shown to differ from previously identified antibodies against human erythrocytes, (3) if more than one antibody is present in the serum it must be possible to recognize their separate effects (4) the percentage incidence (specificity) should be determined on random samples of human blood.

The specific nature of the antigen antibody reaction for both components (anti-c and anti-d) was shown by several effects. First, by the use of the developing test the antibodies were shown to be adsorbed on erythrocytes containing the c and d antigens (cDE/cDE and CDe/Cde) and did not adsorb on erythrocytes lacking these antigens (CDe/CDe) as shown in table 2. Since the red cells are washed with saline in the developing test, only immune globulins specifically adsorbed remained on the red cell and therefore constituted a demonstration of the nature of the combination of the antibodies in the patient’s serum and the corresponding antigens in the red cells. The specific antibody action of the anti-c and d components of the serum were further demonstrated by their hemolytic activity in the presence of complement as shown in figure 1. Fortunately, in this experiment through the choice of proper erythrocytes, the separate effects of the two antibodies could be observed because of the fact that they were of equivalent strength (titre). Thermal amplitude tests and studies of effect of dilutions of the serum which were done initially eliminated the possibility of panagglutinins, pseudo-agglutinins and cold agglutinins.

The results shown in table 2 indicate that the antibody component (cryptagglutinoid) demonstrable only by the developing or albumin test was identical in specificity to anti-c. The agglutinin however as shown in tables 2 and 3 did not correspond to any of the available test sera, namely anti-C, C*, c, D, E and e. Furthermore, additional tests run in parallel with the anti-Lutheran, anti-Kell and anti-Lewis serums showed no apparent correlation. Finally, comparative tests with anti-A, B and P, and the initial tests with anti-M and N done at the Southern Baptist Hospital ruled out these factors.

From the study of 100 random blood samples (table 3) a specificity of 67 per cent positive reactions was obtained. This compares with the 65 per cent specificity predicted from the calculations of Fisher. This agreement is the more significant when we note the antithetical relation between the reaction of anti-D and the agglutinin component of the serum herein described. These findings indicate allelomorphism of the gene determining the antigen (d) identified by this serum and the gene D.

It is believed that the evidence herein presented constitutes the demonstration and characterization of the d agglutinin and antigen.

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