The Rhesus Antibody Anti-E in Pregnancy and Blood Transfusion

I. The Frequency of Occurrence of Pure Anti-E. Report of 12 Cases

II. The Demonstration by Pure Anti-E Serum of Dosage Effect of the Antigen E in the Presence or Absence of the Antigen D

By R. H. MALONE, M.D. AND J. DUNSFORD

I. THE FREQUENCY OF OCCURRENCE OF PURE ANTI-E

REPORT OF 12 CASES

IT HAS BEEN the practice in this laboratory to search for Rh antibodies in the blood of all Rh negative multigravidae submitted for investigation, whether or not the patient's history suggested the possibility of immunization by pregnancy or transfusion, and to test Rh negative primigravidae only when there was a history of transfusion or injection of blood. Since September 1948, however, tests for antibodies have, in addition, been carried out on all Rh-positive women with a history suggesting the possibility of Rh immunization. This search for Rh antibodies in selected Rh-positive as well as in Rh-negative women resulted in the discovery, in 1949, of 9 cases of immunization due to pregnancy or blood transfusion in which anti-E was the only Rh antibody present. They are reported briefly here together with 3 others, 2 found in 1948 and another, which is of some historical interest, found in 1945.

One case was a male aged 60 (Case 10) and one a female aged 64 (Case 11) both immunized by transfusion. The remaining 10 were women of child-bearing age. Of these 10, 4 were immunized by pregnancy (Cases 3, 5, 9 and 12) and 3 others (Cases 2, 6 and 7) by transfusion. In the remaining 3 cases (1, 4 and 8) the evidence was inconclusive; in 2 the infants were illegitimate and in the third case the husband's genotype was not known. Six of the 10 mothers were Rh positive; 3, CDe/CDe and 3, CDe/cde; the remaining 4 were Rh negative, cDe/cde. Eleven of the 12 anti-E sera were saline-agglutinating and only one albumin-agglutinating.


ANTI-E IN PREGNANCY AND BLOOD TRANSFUSION


E/E = 32 and against E/e cells = 2. Remarks: direct Coombs test on infant = positive. Mother's serum × infant's cells = negative. Immunization probably due to pregnancy but infant, possessing antigen E, was apparently unaffected.


**Discussion**

Case No. 1 is of some historical interest as being the third example of anti-E reported in this country, the first (K) and second (J) having been reported in 1943 by Race et al. The patient had a miscarriage in 1945 and a sample of serum taken one week later and sent to the Galton Serum Unit, Cambridge, was reported in the rather quaint idiom of the time as "rather weak anti-Rh" thirty per cent reading." The cells were identified as: "positive with anti-Rh and Serum 96: negative with St." (Serum 96 = anti-C; St = anti-c.)

Case No. 8 illustrates the use of Rh serology in forensic medicine. The absence of the E factor in both husband and wife and its presence in the child would, quite apart from the ABO discrepancy, have established nonpaternity.

Case No. 9 is an example of a rare mating—father, edE/cde and mother, cde/cde—with immunization of the latter by the E antigen in a cde/cde fetus. Such an event must be extremely rare and, as far as we know, only one other case has been reported.²

Cases 3 and 12 illustrate another rare occurrence. In both cases the mothers were Rh negative (cde/cde) and the infants D positive (cDE/cde) yet pure anti-E was formed to the exclusion of anti-D. Two similar cases have been found in this laboratory—mother cde/cde, infant CDe/cde—in which anti-C was formed without anti-D. These are exceptions to the rule that anti-D is formed in preference to anti-C or anti-E in an Rh negative person exposed to immunization by the D antigen in fetal or transfused blood.

Certain points of interest arise. (1) How frequently does pure anti-E occur in cases where Rh antibodies are detected?

Immunization to the antigen E has been considered to be exceptional. Van Loghem and Hart³ consider immunization to the antigen E alone during preg-
nancy "very exceptional" and Wiener\textsuperscript{4} considers immunization by transfusion to be rare "because of the poor antigenicity of the factor Rh".

In 1949, 37,972 antenatal cases (i.e., not previously tested) were examined and 250 Rhesus antibodies of various types were found. Included in these 250 Rhesus antibodies were 7 of the 12 cases of pure anti-E reported here; that is to say, pure anti-E occurred in 2.8 per cent of all cases of Rh immunization encountered for the first time during the year. This frequency is much higher than in published figures: Rice and Watson,\textsuperscript{5} 0.2 per cent; Pickles,\textsuperscript{6} less than 0.5 per cent; Medical Research Council Memorandum No. 19,\textsuperscript{7} 1 per cent and we have been able to find only 10 cases of pure anti-E reported in the literature since its discovery in 1943.

The high proportion of cases with pure anti-E in this series may be a matter of chance but, on the other hand, it may be accounted for, at least in part, by the routine search for Rh antibodies in the serum of Rh-positive women whose history suggests the possibility of immunization, by the procedure and technics employed as a routine in this laboratory and by the fact that the red cells of four of the members of our staff possess double doses of the antigen E, the importance of which is noted below.

(2) The differentiation of E/E from E/e cells by the use of "genotyping"\textsuperscript{*} anti-E sera.

In February 1948 a serum (No. 72) containing complete anti-E and incomplete anti-D obtained from a pregnant woman was sent from this Centre to the Blood Group Reference Laboratory, Lister Institute, for confirmation of antibody content. This was confirmed and Dr. R. R. Race then made the interesting observation that the serum differentiated R\textsubscript{2}R\textsubscript{2} from R\textsubscript{2}r cells on titration. On examination in this laboratory in April 1948, when the patient was 16 weeks pregnant, the following titers were obtained: serum 72 (Connerton) \textit{vs.} CDe/cDE cells in saline, 2; cDE/cde cells in saline, 4; cdE/cdE cells in saline 64.

The genotypes of the family were: husband A, CDe/cDE, wife A, ede/cde; infant A, cDE/cde.

We have not been able to trace any reference in the literature to studies on the "genotyping"\textsuperscript{*} property of anti-E sera but a large number of tests carried out with the serum from Case 12 and a smaller number with the sera from Cases 4, 6, 8 and 11 in this series proved that these sera could be employed to distinguish E/E from E/e cells. In fact all of the 10 anti-E sera tested were capable of showing differences in reaction between cells containing a double and a single dose of the antigen E although with some of them the differences were slight.

The importance of this finding is obvious since anti-E serum is almost unobtainable. Another point which was disclosed is the necessity for using E/E cells when testing an unknown serum for the presence of anti-E because weak or negative reactions may occur if cells containing a single dose of the antigen E are used for the tests (see Cases 3, 6 and 11). The results of further investigations into the genotyping property of anti-E sera are reported in part II of this paper.

\textsuperscript{*} The term "genotyping" is used to describe an anti-serum capable of revealing dosage effect of an antigen by titration.
II. The Demonstration by Pure Anti-E Serum of Dosage Effect of the Antigen E in the Presence or Absence of the Antigen D

Dosage effect in blood grouping was first reported by Landsteiner and Levine in their work on the M and N antigens when they found MM and NN cells to give stronger reactions with anti-M and anti-N sera respectively than did MN cells. More recently Boorman, Dodd and Gilbey demonstrated this effect with human anti-O serum in testing O/O and A/O cells. Race, Taylor, Boorman and Dodd were the first to report dosage effect of the Rhesus antigen— and Race and Sanger state: "The effect is given by other anti-Rh sera, notably anti-e (anti-hr") and anti-C*, occasionally by anti-E (anti-rh") and to a very much less extent by anti-D (anti-Rhα) and anti-C (anti-rh")"

In 1948 Race reported to us that one of our anti-E sera (Sheff. 72, Conn) could differentiate E/E from E/e cells by titration. Since then each anti-E found in this laboratory has been tested for this property and all of the 9 examples of pure anti-E found in 1949 possessed it to some degree and 5 of them to a marked degree.

Table 1. Typical Reactions of a Genotyping Anti-E Serum

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Serum dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>E/E control (cdE/cdE)</td>
<td>V ++ ++ ++ + + GW W -- --</td>
</tr>
<tr>
<td>E/e control (cdE/cde)</td>
<td>+ ( + ) GW W -- -- -- -- -- --</td>
</tr>
<tr>
<td>Test Cell No. 1</td>
<td>+ ( + ) GW W -- -- -- -- -- --</td>
</tr>
<tr>
<td>Test Cell No. 2</td>
<td>V ++ ++ ++ + + ( + ) GW W -- --</td>
</tr>
</tbody>
</table>

Test cell No. 1 is E/e and test cell No. 2 E/E.

This paper reports investigations primarily designed to demonstrate the practical value of anti-E sera in detecting single or double dosage of the antigen E. In the course of these experiments, however, it was found that the presence of the antigen D affected the E antigen-antibody reaction in such a manner as to suggest that the agglutinability of cells possessing both E and D depends upon a quantitative relationship between the two antigens.

Technics

To determine whether or not an anti-E serum is suitable for recognising dosage of the antigen E it is titrated in precipitin tubes in serial two-fold dilutions against selected E/E and E/e cells. If the differences in titer extend over a range of two or more tubes the serum is considered to be suitable for routine use.

In using the selected serum with unknown cells, parallel titrations in serial two-fold dilutions are carried out against selected E/E and E/e cells as well as against the test cells; and for greater accuracy each of the cell suspensions and each serial dilution of the serum is prepared separately in a volume sufficiently large for all the tests required. The selected cells used as controls should be of type cDE/cDE with cDE/cDE, or cdE/cde with cdE/cdE for reasons which will become apparent later.

The experiments detailed below were carried out with selected saline-agglutinating anti-E sera against 2 per cent saline suspensions of cells.

A protocol showing typical reactions of a genotyping anti-E serum is given in table 1.
ANTI-E IN PREGNANCY AND BLOOD TRANSFUSION

Antenatal Cases

In 37 cases where the mothers were Rhesus negative (cde/cde) and the fathers Rhesus positive (D and E positive) the latter were tested by titration with a genotyping anti-E serum, with the following results:

<table>
<thead>
<tr>
<th>No. of cases</th>
<th>Fathers</th>
<th>Infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>6</td>
<td>E/E</td>
</tr>
<tr>
<td>(b)</td>
<td>18</td>
<td>E/e</td>
</tr>
<tr>
<td>(c)</td>
<td>13</td>
<td>E/e</td>
</tr>
</tbody>
</table>

The blood of the infants was not tested by titration with a genotyping anti-E serum, nor was that of the fathers tested with anti-e serum. The absence of Rh-negative infants in group (a) and the high proportion of Rh negatives in groups (b) and (c) combined led us to undertake further family studies.

Family studies

Seventeen other families were studied in which the genotypes of parents and children were determined by means of the anti-sera C, D, E, c and e and controlled by tests carried out with anti-e and a genotyping anti-E serum. In every case a cell diagnosed as E/E or E/e by the latter was proved to be such by tests with anti-e and by the genotype of one or both parents.

Three examples of these families are given below:

Case No. 53791

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[Diagram showing family tree with genotypes: cdE/cde, CDe/Cde, Cde/cdE, CDe/cdE]
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The propositus, sister and mother gave single dose (E/e) reactions with a genotyping anti-E serum. These results were confirmed by positive reactions with anti-e, the father being E negative.
Case No. 106966

The propositus, a member of the laboratory staff, has been tested with all the genotyping anti-E sera found and has given results consistent with a single dose of E with all of these sera. His father was E negative.

Case No. 86790

The mother and one brother gave a double dose reaction (E/E) with a genotyping anti-E serum, while the propositus and her other brother gave the single dose effect (E/e). This was confirmed by the father giving a positive and the mother a negative reaction with anti-e.

It would appear from these observations that the E antigen shows dosage effect which can generally be disclosed by selected anti-E sera and that in exceptional cases only should it be necessary to resort to family studies or the use of the very rare anti-e serum in order to differentiate E/E from E/e cells. This is of particular importance in determining the zygosity of the husbands of women immunized by pregnancy and where research work might demand the use of anti-e.

* Titration with a genotyping anti-E serum indicated the genotype to be cDE/cdE. For single dose of d see table 4.
Reactions of Cells of Various Genotypes

It was observed that some anti-E sera while reacting strongly with E/E failed to react with E/e cells, in particular cells of type CDe/cDE, and reactions with other anti-E sera were almost invariably weaker with this type than with type cDE/cde even though both were single dose (E/e) cells. The impression was also gained that cells of type cdE/cde reacted more strongly than either of the above types.

To study these points titrations were made against a series of group O cells of known genotypes using a genotyping anti-E serum (table 2). They were carried out by one of us (I. D.) at a single sitting, in order to insure that the readings should be comparable.

As a control each of these cells was tested with anti-e and, as there appeared to be considerable differences in their agglutinability, titrations were carried out with an anti-e serum against a series of cells, as shown in table 3. Only 6 cells were tested by titration on account of the limited supply of anti-e serum available.

Several points of interest may be noted in table 2: 1) the slight variations in agglutinability of the cells of different individuals of the same genotype and, generally speaking, the much larger variations when cells of different genotypes are tested with the same anti-E serum.

2) The marked effect of the presence of a double dose as compared with a single dose of the antigen E, e.g., when comparisons are made between cell (1) and cell...
(4); between cells (2) or (3) and cells (8), (9), (10) or (11); and between cells (1), (2) or (3) and cells (5), (6) or (7).

3) The effect of the absence or presence in single or double dose of the antigen D on the agglutinability of cells containing either a single or double dose of the antigen E, e.g., when comparisons are made between cell (1) and cells (2) or (3);

### Table 3.—Titration Scores of Cells in Table 2

<table>
<thead>
<tr>
<th>Cell</th>
<th>Score*</th>
<th>Serum dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41</td>
<td>Double E: low grade D* = 41.0</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>Double E: double D = 38.5 (Avg. score)</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>Double dose of E.</td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td>Single E: no D = 28.0</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
<td>(Avg. score)</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>Single E: single D = 15.7</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>(Avg. score)</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>Single E: double D = 7.0</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>(Avg. score)</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>(Avg. score)</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>(Avg. score)</td>
</tr>
<tr>
<td>12</td>
<td>(ede/ede)</td>
<td>= 0</td>
</tr>
</tbody>
</table>

* Scores: V = 10; ++ = 8; + = 5; (+) = 3; GW = 2; W = 1.

between cell (4) and cells (5), (6) or (7); and most markedly between cells (1) or (4) and cells (8), (9), (10) or (11).

Similar effects are seen with anti-e (table 3) but they are much less noticeable, possibly on account of the low titer of the anti-e serum used.

The picture becomes clearer when the titrations shown in table 2 are scored according to the method suggested by Race and Sanger as in table 4 below.
Although it is generally believed that pure anti-E is rarely produced by blood transfusion or pregnancy and that dosage effect is only occasionally demonstrable by anti-E sera the observations recorded here do not support these beliefs. Five of the 10 anti-E sera tested possessed this property and 1 has been used routinely for genotyping the husbands of immunized women in order to conserve the much rarer anti-e.

Two points in the technic employed, which are also applicable when Rhesus antigens and antibodies other than E and anti-E are sought for, need to be emphasized:

1. In the examination of unknown sera for the presence of anti-E it is essential to use E/E cells. Indeed, as some anti-E sera have been found which do not react with E/e cells, no serum can be said to be free from anti-E unless it has been tested with E/E cells and, if possible, with a cell lacking the antigen D (R"R"). The use of CDe/cDE cells as a sort of omnibus blood for the detection of all kinds of Rhesus antibodies is not to be recommended.

2. It is essential when testing unknown cells with anti-E serum that the positive control should be a single dose (E/e) cells and preferably a very weak reactor of type CDe/cDE, e.g., Cell No. 11 of table 2. Unless this is done, CDe/cDE cells may be typed as CDe/cDe when using the 4 standard anti-sera D, C, E and c.

Again, in defining the characters of a standard anti-E serum it is not sufficient to record the titer and the technic employed in determining it. The genotype of the cells used in the titration must also be stated and the titer of the serum must be high enough to detect the weakest reactors, viz., cDE/CDe cells.

By using anti-E sera selected for the purpose and with careful technic it is clearly possible to distinguish single from double dosage of the antigen E in cells similar in respect of D content, but of greater import is the effect of the presence of the D antigen in cells possessing E.

From the titration scores (table 4) and the general picture shown in table 2 two conclusions may be drawn:

1. The agglutinability of cells possessing either a single or a double dose of the antigen E is reduced by the presence in single or double dose of the antigen D.

2. By the use of selected anti-E sera it is generally possible to distinguish E/E from E/e in cells possessing D/D, D/d or no D without using anti-e serum; and, as a corollary, to distinguish D/D from D/d in cells possessing E/E or E/e, without using anti-d serum.

A possible explanation of this phenomenon is that it is an example of epistasis, in which the action of the gene D results in the partial or complete suppression of the activity of the gene E. That this suppression does not occur during the agglutination reaction is shown by the fact that little or no change in agglutinability occurs if the D antigen is removed from the field of action by the "blocking" technic.13

A clue to the mechanism of this suppression may be found in table 4 which clearly shows that there is a quantitative relationship between the scores and the dosage of the D and E antigens present, as if the score of a particular cell represented the ratio of E to D.
The work on flower color in the garden dahlia by Scott-Moncrieff, Lawrence and others has a definite bearing on this point. Lawrence\textsuperscript{14} states: "Further investigations showed that interaction occurred in the production of all four of the pigments responsible for flower color in *Dahlia*, namely the anthocyanins, cyanin and pelargonin, the flavone apigenin and the chalcone butein. In other words, there was competition between these pigments in their parallel production from a common, limited source or intermediate, and this competition was proportional to the dosage and competitive value of the flower color genes governing the synthetic processes. When much anthocyanin was produced, there was less flavone; when much butein, less anthocyanin and/or flavone; and so on."

Again, Race and Sanger\textsuperscript{15} remark, with reference to the views of Darlington and Mather,\textsuperscript{16} "... work at present going on in our Unit has led us to the conception of a basic raw material which can well be identified with the carrier..." (from which specific antigens are derived by gene action).

Our results fit this conception, for if we suppose that there is a limited amount of basic nonspecific substance from which the specific antigens are developed by action of the genes and that the amount of a specific antigen produced depends upon gene dosage and activity, it follows that the larger the amount of D produced in a cell the less would be the amount of C or E and when both C and D are produced, as in Cells 8, 9, 10 and 11 (table 4), the amount of E would be minimal.

The common observation that CDe/cde cells are usually less agglutinable with anti-D sera than cDe/cde cells may be explained on this hypothesis by assuming that the gene C has a greater "competitive value" than the gene E and, therefore, the combination CDe will possess a smaller amount of D than the combination cDE.

If degree of agglutinability depends upon the amount of a specific antigen present in a cell and this in turn is a function of the amounts of other antigens produced in competition, it is probable that a random sample of cells possessing varying amounts of E and D could be arranged in serial order based on their titration scores. Parallel titrations with a genotyping anti-E serum would then disclose that these cells tend to fall into groups—single dose of E plus single dose of D, single E plus double D, etc.—and their genotypes might well be revealed without resorting to family studies or the use of anti-e and anti-d sera.

**Summary**

1. Twelve cases of immunization by pregnancy or blood transfusion in which the Rhesus antibody anti-E was found, to the exclusion of anti-D or any other Rh antibody are reported.

2. Two hundred and fifty Rh antibodies were discovered in this laboratory during 1949 in 37,972 antenatal cases not previously tested, i.e., 1 in 152. Seven of these were pure anti-E, i.e., 2.8 per cent. This frequency is considerably higher than any previously reported.

3. All of the anti-E sera tested possessed to some degree, and 5 of them to a marked degree, the property of distinguishing cells with a double from those with a single dose of the antigen E.
ANTI-E IN PREGNANCY AND BLOOD TRANSFUSION

4. The presence, in cells of various genotypes, of the antigen D in association with the antigen E reduced their agglutinability with the same anti-E serum in such a manner as to suggest that the agglutinability of these cells depends upon the relative quantities of the E and D antigens present.

5. By means of parallel titrations with a genotyping anti-E serum, against cells of known and unknown genotypes, it was possible to distinguish E/E from E/e cells in the presence or absence of associated D and, as a corollary, to distinguish D/D from D/d cells in the presence of associated E’, E or E’e.

6. These results fit the hypothesis that the specific Rhesus antigens C, D, and E are produced from a limited amount of “basic raw material” by the activity of the three genes: that the genes act independently and in competition and that the amount of each antigen produced is proportional to the dosage and “competitive value” of the corresponding gene.

REFERENCES


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