Quantitative Hemagglutination Studies in the Rh Blood Group System. I. The Assay of the Anti-D (Rh\(_d\)) Agglutinin

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Quantitative hemagglutination methods have been used to characterize the antigens and antibodies in the ABO blood group system and to investigate the thermodynamics of their reactions.\(^1\)\(^2\) The recognized limitations of currently used serological methods\(^6\) make extension of these precise, reproducible technics to the investigation of other blood groups desirable. It is the purpose of this report to describe the application of Wilkie and Becker's quantitative hemagglutination method\(^2\) to the assay of the Anti-D (Rh\(_d\)) agglutinin.

**Materials and Methods**

The glassware, preparation of buffered saline (pH 7.3), standard test cell suspensions, and dilution of antisera have been previously described.\(^5\)\(^4\) The probable genotype of the donor of the cell used throughout this study was 0,R\(_d\)R\(_d\) (cDE/cDE). The cell suspension used contained 28,000 ± 1000 cells/mm\(^3\).

**Source of Antisera:** Saline agglutinating and incomplete anti-D (Rh\(_d\)) sera were supplied by the Baltimore Rh Typing Laboratories, the Ortho Research Foundation, Raritan, New Jersey, and the Philadelphia Serum Exchange. The specificity of all sera was confirmed with a commercial panel of 0 cells.\(^*\) Five-tenths to 1 ml aliquots of sera, which had not been inactivated at 56\(\degree\) C., were kept frozen at -20\(\degree\) C., and thawed only once before use.

**Enzyme Treatment of Erythrocytes:** Enzyme modified cells were used in the study of incomplete anti-D sera. Cells were altered with trypsin by Race and Sanger's modification of Morton and Pickles technic,\(^7\) with two minor changes: (1) Trypsin\(^R\) (Crystalline Trypsin, Armour Laboratories) was dissolved in 0.001 N instead of 0.05 N HCl. In this concentration of acid the frozen enzyme stock retained its activity for a longer period of time. (2) Cells were exposed to trypsin for 30 instead of 20 minutes. It will be shown that this change resulted in more reproducible agglutination of enzyme treated cells by incomplete antibody.

**Method of Assay:** In the quantitative hemagglutination assay,\(^2\) a standard cell suspension is incubated with twofold dilutions of antiserum until equilibrium of free cells, antibody, and agglutinated cells is reached. At that time, the number of free cells are counted on U. S. Bureau of Standards Certified Hemocytometers and the percentage of agglutinated cells calculated. In the ABO system a sigmoidal relationship between the percentage of agglutinated cells and the logarithm of antiserum concentration has been reported.\(^2\) The sigmoid is transformed to linearity by means of probability units (probits).\(^8\)\(^1\)\(^2\) This log-probit regression line forms the basis of interpretation of the assay.

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ASSAY OF ANTI-D(RH) AGGLUTININ

EXPERIMENTAL PROCEDURES AND RESULTS

Since the presence of equilibrium is essential for the reproducible assay, as well as for any subsequent thermodynamic analysis of data,1 experiments were designed to establish reversibility in the D-anti-D system. In the first experiment reversal of agglutination by temperature was investigated with the R2R2 cell and saline agglutinating anti-D serum. A cell-antibody mixture was incubated at 37° C. until a stable level of agglutination was attained. The temperature was then lowered to 4° C. with a fall in cell agglutination from 48 to 24 per cent. When the temperature of incubation was returned to 37° C. the original level of agglutination was restored.

The presence of a reversible equilibrium was confirmed by showing that an approach from two directions led to the same level of agglutination (fig. 1). The lower curve illustrates the gradual increase in agglutination in a cell-antibody mixture maintained at 37° C. on a 10 rpm rotator for a four-hour period. When equilibrium is reached, no further increase in agglutination occurs. The upper curve represents dispersion of cell aggregates. These cells had been incubated for four hours at 37° C., centrifuged at 500 G. for 1 minute, and then placed on the rotator. Centrifugation resulted in cell aggregation above the equilibrium level of agglutination. Agitation on the rotator liberated cells from aggregates, and in time the percentage of agglutination decreased to the same plateau reached by aggregation. Because great variability was found in the dissociation rate of over-agglutinated cells, the more predictable aggregation method was used in this study.

Assay Procedure for Saline Agglutinins: Test tubes, containing 0.5 ml. of cell suspension and 0.5 ml. of antiserum dilution, were placed in a 37° C. water bath and inverted at 30 minute intervals. The cell-antibody mixture remained in the water bath from 2 to 4 hours, depending on the antiserum used. The length of incubation for each serum was established by the interval required for the cell-antibody system to reach equilibrium, as determined by a stable free cell count. This period, obviously, does not only represent time

![Fig. 1.—Equilibrium time study in the D-anti-D system comparing the aggregation and dispersion methods.](image)
of sensitization, but also includes the second stage of the agglutination reaction. After the incubation period the tubes were placed on the 10 rpm rotator at 37°C, where they remained for 30 minutes to allow adequate mixing. Hemocytometers and capillary tubing used to fill the counting chambers were also maintained at 37°C. The cell counting procedure employed in this study has been described.1,4

Survival of Test Cells During Course of Assay: Cell survival under test conditions was determined on cells stored for five days. Five-tenths of a milliliter of cell suspension was added to each of 20 tubes containing an equal volume of buffered saline. Duplicate counts were done on each tube before and after a 4½ hour period which included all manipulations in the assay procedure described above. A decrease of 2.1 per cent in the original count of 14,800 cells/ccm. was found, making it apparent that over this length of time cell loss does not constitute a limitation to the assay. However, a six-hour interval was considered maximal for completion of all experimental procedures, since with longer periods cell loss and bacterial contamination became a problem.

Assay of Saline Agglutinins: The percentages of R,a cells agglutinated by a wide range of twofold concentrations of anti-D (Rh0) serum was determined with the quantitative hemagglutination technic described above. When the logarithms of these concentrations were plotted against percentage of agglutinated cells a sigmoid curve was obtained (fig. 2). This sigmoid was transformed to a linear curve by plotting the probits corresponding to the percentages of cells agglutinated against logs of antiserum concentration (fig. 2). Linearity was confirmed by statistical testing of the data. The range of linearity for the serum, shown in figure 2, extended from 15 to 95 per cent agglutination. With six different saline agglutinating anti-D (Rh0) sera the upper limit of linearity varied from 68 to 98 per cent agglutination. Differ-

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Fig. 2.—Titration curve of an anti-D serum and its transformation by means of probits.
Fig. 3.—Comparison of the agglutination curve of four saline agglutinating anti-D sera and a standard R_{cDE} (cDE/cDE) cell.
ences in this range are illustrated for two anti-D sera in figure 3; as can be seen, serum 2794 deviates from linearity at a higher level of agglutination than serum 2681. Slope and position of the log-probit regression line characterize the D-anti-D reaction. The agglutination response per unit change in serum concentration is measured by the slope. Since linearity of the regression line has been established, interpolation of a 50 per cent hemagglutination endpoint (HD₀) is a valid measure of the potency of the antiserum. The HD₀ has been defined as the antiserum concentration (ml./ml.) in the test volume that agglutinates 50 per cent of the standard cell suspension. Since significant daily fluctuations in the HD₀'s of a given serum occur, concomitant testing of a standard reference serum with an unknown serum is essential. This permits expression as a relative potency ratio of the strength of test serum in terms of that of the standard (HD₀ standard/HD₀ test).

Development of Assay Procedure for Incomplete Antibody: Enzymatic treatment with trypsin was used to render red cells agglutinable by incomplete antisera. The optimal time reported in the literature for exposure of red cells to the enzyme ranges from 10 to 60 minutes. In view of this discrepancy, the following experiment was designed to establish the relationship between time of enzyme treatment and the agglutinability of R₂R₂ cells by incomplete antibody. One volume of red blood cells was incubated at 37 C. with four volumes of 0.15 M pH 7.7 phosphate buffer, containing trypsin in a concentration of 0.5 mg./ml. Aliquots were removed from this mixture after 2, 5, 10, 20, 30 and 60 minutes of incubation. The cells were washed three times with phosphate buffered saline (pH 7.3) and then assayed with a standard incomplete anti-D serum. Parallel log-probit regression lines were found. When the reciprocal of the HD₀'s (i.e. 50 per cent endpoint dilution) from these assays was plotted against time of incubation with trypsin, the curve shown in figure 4 was obtained. It can be seen that there is increasing agglutinability of the R₂R₂ test cell in the first 20 minutes. At 30 minutes a plateau is reached, which extends to at least 60 minutes. It is apparent that greater reproducibility of the assay will be achieved when the incubation period is prolonged to the plateau. Furthermore, a cell exposed to trypsin for 30 instead of 10 minutes will detect smaller amounts of antibody. Although in a limited number of observations we did not encounter any loss of antigenic specificity after 60 minutes of enzyme treatment, it nevertheless must be remembered that this danger exists.

Aside from pre-treatment of the test cell with trypsin, the same procedure was followed with incomplete antisera as in the assay of saline agglutinating sera. Linearity was demonstrated for the log probit regression curve with incomplete anti-D sera, and the same measurements derived as in the assay of saline agglutinin.

Evaluation of the Assay: To validate the assay, the potency of 10 anti-D sera was compared with a standard reference serum against a R₂R₂ cell. Table 1 gives the results of the assays of four doubling concentrations of standard and test sera. The first five unknowns are saline agglutinating anti-D (Rh₀) sera, while the others contain incomplete antibody and were tested with enzyme treated cells.
ASSAY OF ANTI-D (Rh\(_n\)) AGGLUTININ

Fig. 4.—Effect of duration of trypsin treatment on agglutinability of an R\(_2\)R\(_2\) (cDE/cDE) cell by incomplete antibody.

*1/HD\(_{50}\) = 50 per cent endpoint dilution.

It can be seen from the second column that there was considerable day to day variation in slope of the reference serum. However, in no instance was there a significant difference between slopes of unknowns and standard sera assayed simultaneously (P > 0.05). The method of Bliss\(^{12}\) was used to calculate the HD\(_{50}\)'s and standard errors (column 3). Arithmetic potency ratios (antilog of M) which express the percentage strength of the test serum in terms of the standard, and their standard errors (S\(_{\text{M}}\)) are shown in columns 4 and 5.

**DISCUSSION**

In the experiments presented above, the application of Wilkie and Becker's quantitative hemagglutination assay to anti-D sera has been described. Comparisons of sera by this method are valid only under the following conditions:

*\(S.E. = \text{One Standard Error.}\)

**Table 1.—Assay of Ten Anti-D (Rh\(_n\)) Sera**

<table>
<thead>
<tr>
<th>Anti-D (Rh(_n)) Serum</th>
<th>Test Serum Slope (S.E.*)</th>
<th>Standard Serum Slope (S.E.)</th>
<th>Test HD(_{50}) S.E.</th>
<th>Standard HD(_{50}) S.E.</th>
<th>Arithmetic Potency Ratio (Std/Unk) S.E.* of Potency Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>22005/1</td>
<td>1.55 ±0.02</td>
<td>1.68 ±0.08</td>
<td>4.99 ±0.20</td>
<td>1.04 ±0.04</td>
<td>0.2099 ±0.04</td>
</tr>
<tr>
<td>P625S</td>
<td>1.80 ±0.05</td>
<td>1.75 ±0.01</td>
<td>0.68 ±0.01</td>
<td>1.35 ±0.01</td>
<td>2.0110 ±0.02</td>
</tr>
<tr>
<td>162S</td>
<td>1.52 ±0.12</td>
<td>1.76 ±0.01</td>
<td>0.82 ±0.05</td>
<td>1.35 ±0.01</td>
<td>1.6444 ±0.01</td>
</tr>
<tr>
<td>2551S</td>
<td>1.44 ±0.01</td>
<td>1.56 ±0.01</td>
<td>2.32 ±0.21</td>
<td>1.04 ±0.04</td>
<td>0.5229 ±0.04</td>
</tr>
<tr>
<td>11005/1</td>
<td>1.61 ±0.25</td>
<td>1.47 ±0.09</td>
<td>4.18 ±0.51</td>
<td>1.27 ±0.06</td>
<td>0.3978 ±0.04</td>
</tr>
<tr>
<td>16501/1</td>
<td>1.10 ±0.06</td>
<td>1.15 ±0.07</td>
<td>15.99 ±0.62</td>
<td>14.18 ±0.67</td>
<td>0.9902 ±0.02</td>
</tr>
<tr>
<td>16201</td>
<td>1.22 ±0.08</td>
<td>1.13 ±0.07</td>
<td>0.69 ±0.35</td>
<td>14.18 ±0.67</td>
<td>19.74 ±0.02</td>
</tr>
<tr>
<td>23014</td>
<td>1.52 ±0.19</td>
<td>1.51 ±0.11</td>
<td>0.46 ±0.05</td>
<td>8.91 ±0.49</td>
<td>19.22 ±0.02</td>
</tr>
<tr>
<td>HBF14</td>
<td>1.83 ±0.24</td>
<td>1.46 ±0.12</td>
<td>1.51 ±0.15</td>
<td>0.64 ±0.04</td>
<td>0.4603 ±0.04</td>
</tr>
<tr>
<td>H11/1</td>
<td>1.24 ±0.09</td>
<td>1.38 ±0.12</td>
<td>5.25 ±0.30</td>
<td>1.83 ±0.13</td>
<td>0.3654 ±0.03</td>
</tr>
</tbody>
</table>

\(\ddagger\) = Saline agglutinating sera.

\(\ddagger\) = Incomplete sera. The first three of these were tested against one standard serum (15601), while another standard (BBF3I) was used in the last two assays.
(1) A linear relationship must exist between logarithm of anti-serum concentration and the percentage of agglutinated cells expressed as probits. (2) The log-probit regression lines of the unknown and standard sera must be parallel. Statistical analysis of data from the 10 assays of antisera with R2R2 cells, shown in Table 1, indicated that these conditions were fulfilled with these cells and sera. Thus, it is justifiable to express the agglutinating strength of an unknown serum as a ratio of standard HD50/unknown HD50. Since comparison to a reference serum minimizes the effect of daily fluctuations in HD50, the potency ratio rather than the HD50 becomes the more reliable unit of measure of antiserum strength.

The average standard error (Sm) of the log potency ratios of the 10 sera shown in Table 1 was 9.9 per cent, with a range of 3.3 to 19.9 per cent; the mean 95 per cent confidence limits of the potency ratio, calculated from the standard errors, was 29 per cent with a range of 9.3 to 52.5 per cent.

Although the precision of probit bioassay of the D-anti-D system is less than that reported in the ABO system, it is greater than that of test tube titrations. This is illustrated by sera 2794 and 2681 in Figure 3. The test tube titer for both sera was 1:256, suggesting identical potencies. Their HD50’s, however, reveal an 88 per cent difference in their potency which, while highly significant by the assay method, was not apparent from the test tube titration. The increase in reliability of data obtained with quantitative hemagglutination technics compared to that of test tube titrations stems from several differences in methodology. Greater reproducibility is attained by measuring agglutination from a stable system at equilibrium. More sensitive and objective discrimination of small changes in agglutination can be made from hemocytometer free cell counts than from the rather subjective gradations used in test tube titrations. Independent preparation of antiserum dilutions using analytical equipment and technic results in more accurate quantitation of antibody and avoids the “carry over” errors of serial dilutions. Finally, probit transformation offers the advantage that a 50 per cent endpoint (HD50) can be interpolated from the steep portion of a sigmoid curve, where small changes in antibody concentration produce the greatest response in agglutination.

Although no diversity of behavior other than potency differences were observed in the linear portion of the log-probit regression line produced by various anti-D (Rh0) sera, it is noteworthy that deviation from linearity in the upper range occurred at different levels of agglutination (Fig. 3). Upon further increase in antibody concentration, a maximal level of agglutination was reached beyond which point more antibody resulted in decreasing agglutination. This prozoning phenomenon was most pronounced with those sera which lost linearity at relatively low levels of agglutination.

Prozones are a relatively common occurrence in the Rh system and have been attributed to the blocking of antigenic sites by incomplete antibody or to excess of saline agglutinins. Either of these could prevent the formation of the intercellular lattice necessary for agglutination. Renton and Hancock concluded from experiments with mixtures of saline agglutinating and incomplete sera that these concepts were not mutually exclusive and suggested that
while very high concentration of agglutinin may cause a prozone by antibody excess, this zone will be reached more readily when some of the antigenic sites are already occupied by incomplete antibody. While we frequently observed prozones, no correlation was found between the prozone and the proportion of incomplete to saline agglutinin as determined by the relative antiglobulin and saline titers in a given serum. This is illustrated with serum 569 (fig. 3) which manifests a more marked prozone than serum 2794 despite the fact that the latter serum showed a greater extension of its saline anti-D (Rh0) titer by the antiglobulin test (see table in fig. 3). It is, of course, possible that in addition to the proportion of the two antibodies in the serum, their relative affinities for the antigen may predetermine which antibody will occupy the available antigenic sites. Serum 569 also illustrates the common finding that the more potent the antiserum the greater the tendency to form prozones. This observation would support the antibody excess hypothesis as a cause of prozoning. Quantitative hemagglutination technic was useful in the investigation of prozones since small decreases in agglutination which would not be apparent from test tube titrations are easily distinguished.

Prozones occur more frequently in the Rh than in the ABO system. This may be related to the presence of incomplete antibody in most saline anti-D sera, and to the far greater number of reactive sites reported for the A or B antigens than for the D agglutinogen. Both of these conditions would predispose to antibody excess and prozone formation in the Rh system by decreasing the number of antigenic sites available for attachment of saline agglutinating antibody.

**Summary**

The quantitative hemagglutination technic of Wilkie and Becker has been modified for the assay of saline and incomplete anti-D sera. By this method, the relative potencies of anti-D (Rh0) sera were determined with an average error of 29 per cent (95 per cent confidence limits). The effect of duration of trypsin treatment upon the cell's agglutination by incomplete antibody was investigated. The sensitivity of the technic in discerning small changes in agglutination was applied to the characterization of the prozone. The presence of a reversible equilibrium in the D-anti-D system has been established by two independent methods.

**Summario in Interlingua**

Le technica pro le studio quantitative del hemagglutination secundo Wilkie tination. This prozoning phenomenum was most pronounced with those sera e Becker esseva modificate pro le essayage de seros anti-D salin e incomplete. Per medio de iste methodo, le relative potentias de seros anti-D (Rh0) esseva determinate con un error medie de 29 pro cento (limites de confidentia, 95 pro cento). Le effecto del duration de tractamento con trypsina super le agglutination del cellula per anticorpo incomplete esseva investigate. Le sensibilitate del technica in le detection di micre alterationes del agglutination esseva utilisate in le characterisation del prozona. Le presentia de un reversibile equilibrio in le sistema D-anti-D esseva establite per duo methodos independente.
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