The Detection and Quantification of Rh(D) Antigen Sites on Human Leukocytes

By Arthur E. Barnes, Hernando Sarasti, Hilmi Mavioglu and Wallace N. Jensen

The D antigen has been detected on the surface of Rh(D) leukocytes by a variety of indirect technics. Dausett, Colombani and Evelin used the methods of antiglobulin consumption and anti-D absorption by leukocytes. Janković and Lincoln showed fluorescence of Rh(D) leukocytes with fluorescein conjugated antihuman globulin after the exposure of leukocytes to anti-D serum. Hollman was able to inhibit Rh(D) leukocyte migration by the exposure of white cells to anti-D serum. Other studies, however, have failed to provide evidence for the presence of D antigen on Rh(D) leukocytes. This diversity of results has been found even when similar or apparently identical technics were used by different investigators. Methods of antigen detection which are dependent upon endpoint reactions or manifestations such as agglutination, precipitation or the consumption of antibody and antiglobulin are often difficult to assess. Leukocyte agglutination may occur nonspecifically from the tendency for white cells to clump together, or to other cell surfaces regardless of antigenic composition. The quantification of the precipitin reaction or of antibody consumption is complicated by the nonspecific association of serum globulins with leukocytes. All methods for the quantification of leukocyte isoantigens must either eliminate or measure the antigenic contribution of the inevitable contaminant erythrocytes and platelets.

In this study, the capacity of leukocytes to bind a specific 1131-labeled anti-D prepared by elution from D positive red cells was used to quantitatively detect D antigen in the presence of variable amounts of red cell contamination. The radiiodinated globulin associated with the Rh(D) leukocytes was quantitatively determined by means of comparison with D negative red cells and rh(d) leukocytes. The results of this study indicate that few, if any, leukocyte antigen sites are available for association with the D antibody.

Methods

Separation of Leukocytes from Whole Blood

Whole blood was drawn into siliconized 50 ml. syringes which contained 500 U.S.P.

From the Department of Medicine of the University of Pittsburgh Medical School, Pittsburgh, Pa.

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The use of the prefixes "Rh(D)" or "rh(d)" with reference to leukocytes is used to denote that such leukocytes were obtained from Rh(D) or rh(d) donors who were identified by erythrocyte Rh phenotype.

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units of heparin. Four volumes (50 to 250 ml.) of the heparinized blood were mixed with one volume of isotonic 6 per cent dextran in saline solution in siliconized glassware and the erythrocytes allowed to sediment at 37 C. After 2 to 3 hours, the supernatant fluid containing the leukocytes was gently pipetted into plastic tubes and centrifuged at 700 rpm (Gmax = 125) for 30 minutes. The sedimented white cells were resuspended in isotonic phosphate buffered (pH 7.4) NaCl, pooled in a single plastic tube and centrifuged at 700 rpm (Gmax = 125) for 20 minutes. The supernatant fluid was removed and the leukocytes resuspended in a final volume of 1.8 ml. normal isotonic phosphate buffered NaCl. The white cell count of the final suspension varied between 20,000 per mm.3 and 140,000 per mm.3 and the ratio of leukocytes to erythrocytes varied from 1:5 to 1:1.

**Preparation of the Red Cell Free Leukocyte Suspensions**

Leukocyte suspensions obtained by dextran sedimentation were further rid of red cells by osmotic lysis and centrifugation. Three volumes of distilled water were added to the red cell contaminated leukocyte suspensions. After 60 seconds, three volumes of 1.8 per cent NaCl solution were added to restore isotonicity. The white cells were then separated from the hemolysate by centrifugation at 700 rpm (Gmax = 125) for 15 minutes. The lysis procedure was twice repeated and the leukocytes were finally suspended in 1.7 ml. of buffered NaCl solution.

**Preparation and Use of the 111-1abeled anti-D Eluate**

Radiolabeled anti-D eluates (1*anti-D) were prepared and used as described in a previous publication. Triplicate 0.5 ml. aliquots of 1*anti-D were incubated with 0.5 ml. of red cell or white cell suspension in plastic tubes for 8 hours at room temperature (approximately 23 C.). After incubation, 8 ml. of buffered isotonic NaCl solution were added to each tube; the tubes were then centrifuged (96,000 Gmax) for 15 minutes and the supernatant solution which contained the unbound 1*anti-D removed. The cell pellets were radiocounted. Calculations were made from data properly corrected for background and physical decay and expressed as the percentage of the total added radioactivity which was associated with the cells—that is, bound antibody.

Routine red and white cell counts were done by visual methods or by use of the Coulter cell counter. The hemoglobin was determined according to the method of Crosby.

**Estimation of 1*anti-D Bound by Red Cell Fraction**

The amount of 1*anti-D bound by the contaminant red cell fraction of the Rh(D) leukocyte preparations was estimated on the basis of a reference 1*anti-D uptake curve (fig. 1) prepared with each experiment. Varying numbers (1 to 200 x 10⁹) of Rh(D) positive red cells were diluted in 2.5 x 10⁹ D negative cells in final volumes of 1.0 ml. These were reacted with 1*anti-D using standard conditions of volume, temperature, time, and pH. Following the reaction, the amount of D antibody associated with the various quantities of D positive erythrocytes was determined. The amount of D antibody uptake contributed by the red cell fraction of the predominantly white cell suspension was obtained by reference to the D antigen dilution curves which showed the per cent 1*anti-D uptake of an equivalent amount of D positive erythrocytes from the same donor.

**Results**

The results show a specific binding of the 1*anti-D by the red cell D antigen and a lack of specific binding of the 1*anti-D by leukocytes from Rh(D)
Specificity and Sensitivity of the I*anti-D Reactivity

The rates and amounts of I*anti-D bound by 1.0 per cent suspensions of D positive and D negative red cells are shown in figure 2. The D negative red cells were associated with 3.0 per cent of the added radioactivity within 15 minutes and 4.5 per cent after 8 hours. Similar concentrations of D positive red cell suspensions bound 59 per cent of the I*anti-D at 3 hours and 61 per cent at 8 hours. These data show that a stable equilibrium of the D antigen-antibody system was reached between 3 and 8 hours of incubation.

In figure 1, D positive erythrocytes in numbers from 1 to 200 x 10⁶ cells were diluted by 2.5 x 10⁶ D negative red cells and reacted in a constant volume with excess I*anti-D. At successively lesser D antigen (D positive red cells) concentrations, lesser quantities of D antibody were associated with the cells. Of importance is the finding of I*anti-D uptake by the relatively few D positive cells in the presence of a much larger number of D negative cells. The percentage of I*anti-D bound was not demonstrably proportional to the number of cells in concentrations below 4 x 10⁶ per ml., but the ex-
Fig. 2.—Kinetics of the specific I*anti-D binding by D positive RBC. The uptake of I*anti-D by 1.0 per cent suspension of D positive RBC is shown in the upper curve and by a 1.0 per cent suspension of D negative RBC by the lower curve.

The experiment shows the ability to detect as few as $1 \times 10^6$ D positive red cells when these are mixed with 2500 times that number of D negative red cells. It should be noted that the concentration of cells is plotted in log values.

I*anti-D Binding by Rh(D) Leukocytes after Resedimentation with D Negative Erythrocytes

In an attempt to minimize the number of D positive erythrocytes which contaminated the final leukocyte suspensions, D positive red cells were co-sedimented with added D negative erythrocytes. White cell suspensions were prepared from the whole blood of four normal Rh(D) donors and to this cell suspension was added red cells from the control rh(d) donors and another white cell isolation procedure performed. Reference I*anti-D binding capacity curves were prepared with use of the red cells from the same donors to allow measurement of the antibody which was bound by the contaminant red cells. In the four experiments where the reduction of D positive red cell contamination by repeated co-sedimentation with D negative red cells was attempted, the amount of D positive red cell contamination in the final cell preparation was unknown. The total I*anti-D bound by the Rh(D) leukocyte preparations and by the rh(d) leukocyte preparations are uncorrected for red cell contamination and are presented in table 1. The uncorrected estimates of bound I*anti-D of 0.108, 0.052, 0.010 and 0.041 per cent per $10^6$ leukocytes may be compared with values of 1.00, 1.06, 1.63 and 1.08 per $10^6$ red cells obtained from the control D positive red cell dilution curve. Thus, equivalent numbers of white cells from Rh(D) donors show D antibody
Table 1.—Data from Four Paired Normal Rh(D) and rh(d) Leukocyte Preparations

<table>
<thead>
<tr>
<th>Rh(D) Leukocyte Preparation</th>
<th>rh(d) Leukocyte Preparation</th>
<th>Per Cent 1anti-D Uptake</th>
<th>Per Cent 1anti-D Uptake</th>
<th>Per Cent 1anti-D Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cells x 10^6</td>
<td>Cells x 10^6</td>
<td>Cells x 10^6</td>
<td>Total</td>
<td>Cells x 10^6</td>
</tr>
<tr>
<td>76</td>
<td>15.2</td>
<td>0.260</td>
<td>153</td>
<td>14.1</td>
</tr>
<tr>
<td>73</td>
<td>30.1</td>
<td>0.412</td>
<td>55</td>
<td>19.8</td>
</tr>
<tr>
<td>130</td>
<td>15.7</td>
<td>0.120</td>
<td>100</td>
<td>11.0</td>
</tr>
<tr>
<td>50</td>
<td>10.3</td>
<td>0.206</td>
<td>32</td>
<td>5.3</td>
</tr>
</tbody>
</table>

†Net equals difference between 1anti-D uptake on Rh(D) leukocytes and on rh(d) leukocytes.

binding capacities at least 10 to 150 times less than D positive red cells from the same donors. Since D positive red cells were present in these Rh(D) white cell preparations, the net 1anti-D bound by Rh(D) leukocytes is much likely less than the numbers indicate.

Ianti-D Uptake by Leukocytes Prepared without Osmotic Lysis

Leukocyte preparations were made from 12 normal subjects and from one patient each with acute myeloblastic leukemia, acute lymphotatic and chronic lymphatic leukemia. These donors were Rh(D) positive individuals, and the preparations of leukocytes were made without use of distilled water osmotic lysis of contaminant erythrocytes. The results of the 15 experiments are presented in table 2. The number of isolated leukocytes varied from 8.1 to 940 x 10^6 cells and the number of red cells in each preparation varied from 3.8 to 136 x 10^6 cells. The mean uncorrected uptake of labeled antibody by the total cell suspensions was 35.5 ± 3.73 per cent.† Estimations of the antibody uptake which were due to red cell contamination in the cell suspension ranged from 19 to 66 per cent and the mean value of the 15 experiments was 37.8 ± 3.78 per cent. Thus, the entire cellular association of labeled antibody in these experiments could be attributed to erythrocytes. When the values for labeled antibody uptake are related to 10^6 cells, a negative value for leukocytes was obtained −0.044 ± 0.033 per cent in contrast to a value of 0.57 ± 0.11 per cent uptake for erythrocytes. These same data are plotted in figure 3 and depict a high degree of correlation (r = 0.89) which exists between the per cent of labeled antibody bound by the total cell suspension and that which was bound by the red cell constituents of the total cell suspension.

Figure 4 shows the lack of influence on antibody uptake over a 100-fold concentration of leukocytes. This scattergram shows that no correlation exists between the total number of leukocytes present in a cell suspension and the amount of labeled antibody which is associated with the white cells. The uptake of D antibody per 10^6 leukocytes varied from −0.280 to +0.110 with an average of −0.044 ± 0.033 per cent. In contrast, the uptake of 10^6 erythrocytes varied from 0.28 to 1.73 with an average of 0.57 ± 0.11 per cent. Quite wide

†Mean and standard error of mean is used throughout this paper.
ranges of antibody uptake were found for the leukocytes and for the erythrocytes, but there are no values for leukocytes which overlap with red cell uptakes. Figure 5 depicts the data seen in tables 1 and 2 and shows the ranges and mean uptakes of labeled antibody by red cells and by white cells. The average uptake of leukocytes was \(-0.02 \pm 0.028\), and the average uptake of the erythrocytes was \(0.70 \pm 0.010\) per cent per 10^6 cells. There was a wide scatter of values for red cell uptake (0.22 to 1.78 per cent), but the leukocyte values are within a more restricted range \((-0.260\) to +\(0.108\) per cent).

\(I^\text{anti-D} Uptake by Leukocytes Prepared with Osmotic Lysis\)

Three experiments were performed in which D positive and D negative red and white cell concentrations were quite similar. Sufficient cells were obtained to prepare serial dilutions of red and white cells from the same donor. After dilution of the cells, the uptake of labeled antibody was measured. The results of the three individual experiments are shown in figures 6, 7 and 8. The per cent uptake of antibody is plotted in arithmetic fashion against the log of the concentration of antigen (cells). In each experiment, the curves of antibody uptake for leukocytes are uncorrected for red cell contamination. The D positive erythrocytes show a typical curve of antigen dilution over the tenfold cell concentration from 3 to 40 x 10^6 cells. The \(I^\text{anti-D}\) uptake of the D positive red cells varied from 10 per cent at the lowest to 50 per cent at the highest cell concentrations. The D negative erythrocytes were associated with 8 to 15 per cent of the added \(I^\text{anti-D}\) over the entire range of cell concentrations. In each experiment both the Rh(D) and rh(d) leukocyte dilution curves of \(I^\text{anti-D}\) uptake were similar to the D negative red cell dilution curves. In figure 7 there is an additional amount of labeled antibody bound by
Fig. 3.—The direct correlation existing between the total I*anti-D bound to D positive red cells and the suspension of D positive leukocytes and erythrocytes.

Fig. 4.—Lack of correlation between the amount of I*anti-D bound and leukocyte concentration.

the Rh(D) white cells when they are compared with the rh(d) suspension. This finding and the presence of a slope in the Rh(D) leukocyte curve are probably due to the failure to correct these data for red cell contamination. In this particular Rh(D) leukocyte suspension, a small but significant amount of hemoglobin was measured in the first undiluted tube. The stroma from the red cell source of this hemoglobin would account for at least 4 per cent of the plotted I*anti-D uptake. Correction for this red cell contamination would reduce the additional amount of I*anti-D bound and the slope of the Rh(D) leukocyte curve to probable insignificance.

**DISCUSSION**

Erythrocyte ABO isoantigens have been identified on leukocytes, platelets, epithelial cells, sperm and other tissues from humans.
Fig. 5.—I*anti-D uptake by WBC and RBC from 19 Rh(D) donors. The solid circles represent the I*anti-D bound by red cells. The open circles represent the I*anti-D bound by white cells corrected for the uptake by the contaminant RBC fraction. The stippled areas show one standard deviation each side of the mean.

by a variety of serologic technics. The same serologic methods used by the same investigators have failed, in most studies, to establish the presence of the Rh antigens on leukocytes. The ABO antigens, but not the Rh antigens, have been demonstrated by means of the direct agglutination of leukocytes,20 the “mixed” agglutination of leukocytes with isoantibody coated erythrocytes5,7,9 and the specific absorption of antisera with subsequent fall in antibody titers.4,6 Platelets10,22,30 and sperm31,32 also appear to lack the Rh antigens. On the other hand, some investigators have reported the detection of Rh antigens on leukocytes,1,2 platelets1,33,34 and sperm28,35 by the use of these or other technics.

Studies dependent upon the specific absorption of anti-D by Rh(D) leukocytes or the association of either labeled or unlabeled anti-human globulin by anti-D coated cells are subject to various interpretations and yield inconsistent results. Dausset, Colombani and Evelin and Janković and Lincoln concluded on the basis of various types of absorption studies that leukocyte D antigen sites were present. In contrast, other investigators4,6,8 were unable to provide evidence of anti-D absorption or antihuman globulin consumption. The evidence is less contradictory, albeit indecisive, when the capacity of leukocytes to adsorb globulin in a “nonspecific” fashion is considered. The nonspecific leukocyte adsorption of globulin was demonstrated quantitatively by means of purified radio-iodinated antihuman globulin by Anderson and Walford. Costea, Schwartz, Constantoulakis and Dameshek36 also noted nonspecific uptake of radiolabeled antihuman globulin by leukocytes which contaminated their erythrocyte preparations. The small amount
Fig. 6.—First set of I\(^{131}\)anti-D uptake curves of dilutions of paired D positive and D negative RBC and WBC. The solid circles represent red cells, and the open circles represent white cells.

of I\(^{131}\)-labeled anti-D eluate which was associated with the D negative erythrocytes and the leukocytes of rh(d) donors and Rh(D) donors in the present study and the D negative erythrocytes in the studies of Masouredis\(^37\) are, presumably, similar observations. The unattenuated and comparable levels of the anti-D uptake with serially decreased concentrations of the D negative erythrocytes, the rh(d) leukocytes and the Rh(D) leukocytes are further evidence of the nonspecific binding of anti-D as globulin.

The detection of leukocyte isoantigens by means of agglutination reactions is complicated. Variable degrees of spontaneous agglutinability are well recognized and may depend upon “leukergic” states of the donor, the presence of foreign inert particles in the white cell suspension and contamination by intact or fragmented red cells.\(^11\) Bakemeier and Swisher\(^5\) studied the marked propensity of leukocytes, irrespective of phenotype, to clump around isoantibody-coated erythrocytes. In fact, clumping indistinguishable from the leukagglutination which resulted from specific antigen-antibody interaction occurred when cell stroma was added to leukocyte preparations. In the currently reported studies, the use of I\(^{131}\)-labeled antibody removes the dependence upon intercellular reactions secondary to the primary fixation of the antibody. In addition, the system allows for the quantification of the uptake of any intact red cells or stroma not eliminated by the leukocyte separation procedure. Thus, the ability to compare quantitatively the amount of D antibody bound by known quantities of D positive red cells to the measured amounts of erythrocyte contamination in the Rh(D) leukocyte preparations...
Fig. 7.—Second set of I*-anti-D uptake curves of dilutions of paired D positive and D negative RBC and WBC. The solid circles represent red cells, and the open circles represent white cells.

minimized the error of the estimation of D antibody uptake contributed by red cell sources.

The high degree of correlation between the radiolabeled anti-D uptake by the total cell (leukocytes plus contaminant erythrocytes) suspension and by numbers of erythrocytes equal to that of the contaminant red cells is consistent with the concept that little or no anti-D was specifically bound. Necessary to, and in support of, the postulated lack of leukocyte D antigen sites was the finding of slightly less than zero D antibody uptake by the leukocytes of the 19 Rh(D) donors after correction for uptake by red cell contamination and for nonspecific radioiodinated protein cell association. The slightly negative mean uptake of labeled antibody may be related to interference with the D positive erythrocyte capacity to bind antibody in the presence of high numbers of leukocytes.

Finally, as emphasized before, the lack of significant attenuation of D antibody uptake by dilution of the three Rh(D) leukocyte preparations in which red cells were lysed suggests the absence of D antigen sites on Rh(D) leukocytes. While a few leukocyte D sites may escape detection by the binding of I-131-labeled D antibody method, it seems unlikely that the ratio of erythrocyte to leukocyte D antigen sites exceeds 1 to 2500.

SUMMARY

Radiolabeled eluates of human anti-D were used to measure the capacity of leukocytes to bind the D antibody in cell suspensions prepared from 16
Fig. 8.—Third set of I\textsuperscript{*} anti-D uptake curves of dilutions of paired D positive and D negative RBC and WBC. The solid circles represent red cells, and the open circles represent white cells.

normal and 3 leukemic bloods from Rh(D) donors. The contamination of the leukocyte suspensions by D positive red cells was measured and the contribution of D antigen sites by these cells was estimated. After correction was made for the D antibody bound by the contaminant red cells, no specific binding of D antibody by Rh(D) leukocytes could be detected. Three pairs of Rh(D) and rh(d) leukocyte dilution curves of I\textsuperscript{131}-labeled anti-D uptake were compared with the uptake by D positive and D negative red cell dilutions. No significant differences among the D negative erythrocyte, the rh(d) leukocyte and the Rh(D) leukocyte curves were obtained. The results were collated with previous serologic evidence concerning the presence of ABO and Rh antigens on human leukocytes.

**Summario in Interlingua**

Radio-marcate eluatos de anti-D human esseva usate pro mesurar le capacitate de leucocytos a ligar anticorpore D in suspensiones cellular preparate ab 16 normal e 3 leukemic specimens de sanguine ab donatores Rh(D). Le contamination del suspensiones de leucocytos per erythrocytos D-positive esseva mesurate, e le contribution de sitos de antigeno anti D per iste cellulas esseva estimate. Post correction pro le anticorpore D ligate per le contaminante erythrocytos, nulle specific ligation de anticorpore D per leucocytos Rh(D) esseva detegite. Tres pares de curvas de dilution del captation, per leucocytos Rh(D) e rh(d), de anti-D marcate con I\textsuperscript{131} esseva comparate con le correspondente captation in dilutiones de erythrocytos D-positive e D-negative. Nulle significative differentias esseva obtenite inter le curvas del erythrocytos
D-negative, del leucocitos rh(d), e del leucocitos Rh(D). Le resultatos es
discutite con referentias a previe evidentia serologic concernente le presentia
de antigenos anti ABO e Rh in erythrocytos human.

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Arthur E. Barnes, M.D., Instructor in Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

Hernando Sarasti, M.D., Research Fellow in Hematology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

Hilmi Mavioglu, M.D., Research Fellow in Hematology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

Wallace N. Jensen, M.D., Associate Professor of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pa.
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ARTHUR E. BARNES, HERNANDO SARASTI, HILMI MAVIOGLU and WALLACE N. JENSEN