Reaction of Cold Agglutinins with I Antigen
Solubilized From Human Red Cells

By Wendell F. Rosse and Peter K. Lauf

The antigens reacting with cold-agglutinin antibodies and present in the red cell membranes of human red cells were found in the water-phase when the washed membranes were extracted with n-butanol. The presence of these antigens was demonstrated by agglutination inhibition, complement fixation, and antibody inhibition as determined by the CI fixation and transfer test. Although antigen and antibody were not able to react at 37°C when the antigen was present in the intact cell, after solubilization, antigen and antibody reacted equally well at 37°C and at 0°C. The amount of anti-I inhibiting activity present in extracts from adult and cord cells was roughly the same, although the amount of antibody fixed to the intact cord cells was less with cord cells.

Cold agglutinins are macroglobulin antibodies which agglutinate human red cells best at reduced temperatures. As the temperature at which the reaction takes place is increased from 0°C, less and less antibody is bound to the cell until at 37°C, very little or no antibody is bound. The reason for the increased binding of antibody in the cold is not fully understood. It has been suggested that the reduction in temperature alters the conformation of the antibody to permit it to react with the antigen. On the other hand, we have previously shown that the change is probably not in the antibody but, rather in the antigen or in the red cell, since an increase in temperature brought about a greater decrease in the binding of the antibody for some cells than for other cells.

All cold agglutinins can be shown to react with all human red cells but they differ in the strength of the reaction with different cells. Most cold agglutinins react more strongly with red cells from adult donors than with red cells from newborn infants. These antibodies are said to be anti-I and the antigen with which they react the I antigen. Those antibodies which react more strongly with the cells of newborns than with the cells of adults are said to be anti-i and the antigen with which they react, the i antigen.

The reactions of cold agglutinins with their antigens have been studied.
with the antigen in place on the red cell membranes. In order to determine the role of the membrane in the reactions of the cold agglutinins and their antigens, we have extracted red cell membranes with butanol. This treatment solubilizes, in the water phase, the antigens reacting with cold agglutinins. When the I antigen is removed from its cellular environment, it reacts with antibody as well as 37°C as at 0°C. The amount of the I antigen extracted from the cells of adults and of newborns appears to be about the same although anti-I reacts more strongly with adult cells than cord cells. These findings suggest that the placement of the antigen in the membrane largely determines its reactivity with antibody.

**Materials and Methods**

**Cells from Adult Donors ("Adult Cells")**

Blood was removed from normal donors by aseptic venipuncture; blood was stored in an equal volume of Alsever's solution at 4°C until ready for use. At the time of use, the cells were washed three times in Veronal-buffered saline pH 7.4 (VBS). A standard suspension of cells, used in the following studies unless otherwise noted, contained 2.2 x 10⁸ red cells per ml. and was made according to the directions given in reference 8.

**Cells from Newborn Infants ("Cord Cells")**

Blood was collected from the cut end of the umbilical cord at the time of delivery. The cells were stored aseptically in equal volumes of Alsever's solution and were treated in the same manner as the adult cells.

**Cold Agglutinins**

Blood of patients with cold agglutinin syndrome was collected in warmed syringes and the cells were separated from the serum at 37°C. In some instances, plasmapheresis was performed at 37°C and the plasma was retained. The serum or plasma was subsequently heated to 56°C for 30 minutes and stored at −20°C until use. The antisera Step. (anti-I), Ho (anti-i) and McD (anti-i) were kindly supplied by Dr. and Mrs. John Crookston, Toronto, Ontario, Canada. Serum containing Donath-Landsteiner antibody was obtained from a patient who had hyperglobulinemia and autoimmune paroxysmal cold hemoglobinuria.

**Eluates**

Eluates of the red cells of patients with warm agglutinin hemolytic anemia were made according to the method of Landsteiner.9

**Extraction Solubilization of the Membranes with n-Butanol**

The method used was that of Poulik and Lauf.10 Red cells were washed three times in VBS. The packed cells were lysed with 3-4 volumes of 20 mM Tris buffer, pH 7.6. The stromata were washed in the same buffer until white and were made into a suspension containing 3 mg/ml. by weight. This suspension was dialyzed against distilled water overnight and was extracted with n-butanol at 0°C using three parts of butanol to four parts of stroma suspension. The extraction was carried out by shaking the mixture briefly at −2°C, allowing it to stand for 30 minutes and centrifuging it at 0°C. The lower water phase was carefully removed and lyophilized. The resulting material was light tan and flaky.

At the time of use, this material was resolubilized in VBS. After resolubilization, the material was centrifuged at 50,000 x g for 30 minutes to remove any insoluble particles. Once reconstituted, the material was not refrozen.

**Agglutination Tests**

Agglutination tests were performed by preparing serial two-fold dilutions of serum (0.05
Table 1.—The Inhibitory Effect of Water-Phase Material on Agglutination of Human Cells by Anti-I

<table>
<thead>
<tr>
<th>Serum</th>
<th>Initial Dilution</th>
<th>Water-Phase Material</th>
<th>Dilution of 1:16</th>
<th>Initial Dilution</th>
<th>Dilution of 1:32</th>
<th>Dilution of 1:64</th>
<th>Dilution of 1:125</th>
<th>Dilution of 1:256</th>
<th>Dilution of 1:512</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.G.</td>
<td>1:10 Absent</td>
<td>4*</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>±</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T.Bu.</td>
<td>1:100 Absent</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>±</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Step.</td>
<td>1:50 Absent</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>V.L.</td>
<td>1:100 Absent</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>±</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>±</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Agglutination was scored as follows: 4, all cells in large clumps; 3, most cells in large clumps; 2, some free cells and many small clumps; 1, many free cells with small clumps (read microscopically); ±, few small clumps (microscopically).

ml.) to which was added water-phase material (0.5 mg. in 0.1 ml.) and group O red cells from adult donors (0.05 ml., 2.2 X 10⁸ cells/ml.). In control titrations, VBS was substituted for water-phase material. The amount of agglutination was determined microscopically on cold slides after incubation for 2 hours at 0°C and was graded 4+ (complete agglutination to ± (a very few small clumps).

Complement Fixation Tests

Complement fixation was performed according to the quantitative method outlined by Mayer.⁷ Water-phase material was boiled for 5 minutes to remove anticomplementary activity and graded quantities of VBS were mixed with 0.25 ml. of 1:100 dilution of serum Step, containing anti-I and 0.25 ml. of guinea pig serum containing 35 CH₅₀ units. After reaction at either 0°C or 37°C for 2 hours, the material was centrifuged at 45,000 X g for 20 minutes and the supernatant solution was diluted in order to assay the complement remaining by the 50 per cent lysis method.⁷ Controls consisting of water-phase material alone, 0.25 ml. of water-phase material plus 0.5 ml. of VBS; 0.25 ml. of antiserum alone plus 0.5 ml. of VBS; and 0.25 ml. of guinea pig serum plus 0.5 ml. of VBS were assayed at the same time. The number of units of complement fixed by the antigen-antibody reaction was determined by subtraction.

C₁ Fixation and Transfer Test

The C₁ fixation and transfer test of Borsos and Rapp,¹¹ as modified by Rosse and Sherwood,⁴ was used to assay the amount of antibody adsorbed to human red cells. The details of this method are given in reference 4. In this test, antibody, cells and functionally purified first component of complement (C₁) are reacted together. The excess unfixed C₁ is removed by washing and the amount of C₁ fixed to the cells is determined by transferring it to sensitized sheep cells containing the fourth component of complement (EAC₄). Excess C₂ and excess C₃-C₉ are added. Under these conditions, the amount of lysis is a function of the amount of C₁ transferred; this amount can be calculated in molecular terms. Since only one molecule of antibody is required to fix a molecule of C₁,²⁴ the assay gives a relative, minimal estimate of the amount of antibody on the cells.

RESULTS

The presence of the antigen reacting with anti-I was demonstrated in water-phase material by several methods.
Agglutination Inhibition

Agglutination inhibition tests were performed as outlined above on four sera containing anti-I. The results are shown in Table 1. In each instance, agglutination was decreased in the presence of water-phase material.

Complement Fixation

When increasing concentrations of water-phase material were added to a given concentration of anti-I, complement was fixed in direct proportion to the amount of water-phase material present (Fig. 1). Less than 5 per cent of the complement was fixed by either antibody or antigen alone.

C1 Fixation and Transfer Tests

The inhibition of antibody by water-phase material was demonstrated by incubation of equal volumes of a solution containing antibody and a solution of water-phase material at 0°C. After centrifugation at 45,000 × g., 0.1 ml. of the supernatant fluid was mixed with 0.1 ml. of a standard suspension of group O adult red cells and 0.3 ml. of a solution containing C1 derived from guinea pig serum by the method of Borsos and Rapp. After incubation at
REACTION OF COLD AGGLUTININS WITH I ANTIGEN

Table 2.—Comparison of the Fixation of Anti-I by Intact Cells with the Inhibition of Antibody Activity Water-Phase Material

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Fixation of Antibody to intact Cells (Molecules Cl^\text{fixe}d/cell)</th>
<th>Per Cent Inhibition of Antibody Activity by Water Phase Material Source of Water Phase Material</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adult I</td>
<td>Cord i</td>
</tr>
<tr>
<td>Anti I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V.L.</td>
<td>6500*</td>
<td>4200</td>
</tr>
<tr>
<td>T.Bu.</td>
<td>4900</td>
<td>2500</td>
</tr>
<tr>
<td>Step. †</td>
<td>2100</td>
<td>600</td>
</tr>
<tr>
<td>M.G.</td>
<td>650</td>
<td>190</td>
</tr>
<tr>
<td>Anti i</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ho †</td>
<td>100</td>
<td>2500</td>
</tr>
<tr>
<td>McD †</td>
<td>280</td>
<td>1150</td>
</tr>
<tr>
<td>Donath-Landsteiner</td>
<td>3440</td>
<td></td>
</tr>
<tr>
<td>J.J. §</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warm-reacting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-D</td>
<td>840</td>
<td>—</td>
</tr>
<tr>
<td>J.T. §</td>
<td>550</td>
<td>—</td>
</tr>
<tr>
<td>R.F. §</td>
<td>540</td>
<td>—</td>
</tr>
</tbody>
</table>

* All results expressed as molecules of Cia fixed per red cell antibody.
† Expressed as per cent inhibition of a standard amount of antibody.
‡ Kindly supplied by Dr. and Mrs. John Crookston, Toronto, Ontario, Canada.
§ Antibodies from patients with autoimmune hemolytic anemia.

0°C for 60 minutes, the excess Cl was removed by washing in isotonic Veronal-sucrose buffer of reduced ionic strength ($\mu = 0.06$) and the number of molecules of Cl fixed was assayed. The relationship between the amount of water-phase material used in the inhibition step and the percentage of the antibody activity inhibited is shown in Fig. 2.

The supernatant fluid from the overnight incubation of water-phase material and antibody was assayed in two ways: in one series, 0.1 ml. of supernatant fluid and 0.1 ml. standard cell suspension of red cells from adult donors were incubated for 1 hour at 0°C; following incubation, the cell-antibody aggregates were washed free of residual water-phase material with 8 ml. of VBS. In the second series, the same incubation mixture was made but the water-phase material was not removed by washing. To both series, 0.3 ml. of Cl were added and the amount of Cl fixed to the cells was determined. No difference in the amount of Cl fixed by residual antibody was observed.

By these two tests, water-phase material was not directly inhibitory to Cl and, therefore, the inhibition of Cl-fixing activity of anti-I by water-phase material was due to antibody inhibition.

Other evidence of the specificity of the inhibition of anti-I by water-phase material was obtained. Sheep red cells, which do not contain the I antigen, were extracted with butanol and the water-phase material (5 mg./ml.) was reacted with anti-I. No inhibition of antibody was observed when residual antibody activity was tested with red cells from adult donors (Table 2). This
suggests that the inhibition of anti-I by water-phase material was not due to nonspecific factors generated by the extraction procedure.

When water-phase material was reacted with anti-i, Donath-Landsteiner antibody, two autoimmune warm-reacting antibodies and isoimmune anti-D, inhibition of the anti-I, anti-i and Donath-Landsteiner but not of the autoimmune and isoimmune antibodies was observed (See Table 2). This suggests that the reduction of anti-I activity upon reaction with water-phase material was not due to a nonspecific destruction of antibody.

The Effect of Temperature on Antigen-Antibody Reaction

Cold agglutinins characteristically do not react with intact red cells when the temperature at which the reaction takes place is 37°C or above. This was demonstrated with antibody Step., used in the present experiments by performing antibody inhibition studies at 37°C and at 0°C, using graded concentrations of intact red cells from adult donors. The amount of antibody remaining after absorption was determined by the Cl fixation and transfer test at 0°C, using papainized red cells from adult donors. As shown in Fig. 3, the amount of anti-I fixed at 0°C was proportional to the number of red cells present in the reaction mixture, whereas the anti-I fixed at 37°C was negligible, regardless of the concentration of red cells added.

In order to determine the effect of temperature on the reaction of anti-I and antigen after solubilization, complement fixation tests were performed at 0°C and at 37°C using serum Step. The results are shown in Fig. 1. The amount of complement fixed by the reaction of water-phase material and anti-I was proportional to the amount of water-phase material present and was greater at 37°C than 0°C for all concentrations of water-phase material. This indicated that the antigen reacting with anti-I was able to react at 37°C when solubilized but was unable to react at that temperature when present in the red cell membrane.
REACTION OF COLD AGGLUTININS WITH I ANTIGEN

The Inhibitory Activity of Water-Phase Material from Adult I and Cord i Cells Against Anti-I and Anti-i

The specificity of the cold agglutinins (anti-I and anti-i) is denominated by their relative reactivity with red cells from adult or newborn donors. In order to determine whether these differences in reactivity are due to differences in the amount of antigen present in the membrane, membrane extracts were prepared from red cells of adult and newborn donors. The amount of inhibition of each of the antibodies by the extracts from each of the types of cells was assessed by the Cl1 fixation and transfer test, using cells from adult donors to test anti-I and from newborn donors to test anti-i. This was compared to the amount of antibody adsorbed by the intact adult and newborn cells, determined by the Cl1 fixation and transfer test. The results are shown in Table 2. Although smaller amounts of anti-I were adsorbed to newborn cells than to adult cells, the amount of inhibition of this antibody by water-phase material from newborn cells was nearly equal to that by water-phase material from adult cells. Almost no anti-i was fixed by intact adult cells, whereas water-phase material from these cells inhibited anti-i.

DISCUSSION

The present studies indicate that the I antigen can be removed from the red cell membrane in water-soluble form. The presence of the antigen in the water-phase following butanol extraction of the membranes was demonstrated by inhibition of the antibody activity and by the fixation of complement. The inhibitory activity could not be found in cells apparently lacking the antigen (sheep cells) and not all human antibodies were inhibited by the extraction of human cells, suggesting that the inhibition seen was due to specific antigen-antibody interaction. Poulik and Lauf have previously shown that this water-phase material contains up to 80 per cent of the membrane proteins, including glycoproteins, but little lipid material. The chemical nature of the material reacting with anti-I is currently under investigation.

The reaction of the antigen with its antibody are different when solubilized than when attached to the membrane. The most striking difference is the loss of temperature-dependence for antigen-antibody interaction. It has been suggested that the greater reactivity of these antibodies in the cold is due to the induction of changes in the conformation of the antibody. In previous studies, we have suggested that the effect of temperature change was not upon the antibody but was rather upon the antigen or the red cell. This conclusion was based upon the fact that the change in affinity of antibody for red cells of newborn infants (cord i cells) or of adult cells which resemble them (adult i cells) was very much greater than for cells of normal adults. Since any effect on the antibody of change in temperature would have been the same in both situations, we concluded that a change in conformation of the antigen or the red cell surface must be responsible for the characteristic cold reactivity of the antigen-antibody interaction.

The present experiments indicate that the reactivity of cold agglutinins only in the cold is due to an effect of cold upon the red cell membrane, since
the antigen-antibody reaction occurs as well at 37°C as at 0°C when the antigen is removed from the membrane. This suggests that at 37°C the antigen on the intact membrane may be "hidden" whereas at 0°C the antigen may become available for reaction with the antibody. The extraction process appears to render the antigen reactive at both temperatures.

In previous studies, we have suggested that the difference between "I" cells and "i" cells in reactivity with anti-I was not largely one of a difference in the number of I antigen sites. When the "affinity" of the antigen and antibody were increased by papainization of the cells or by cold temperature, the total amount of antibody fixed by both types of cells became nearly equal. The present studies tend to confirm that conclusion. Although much less anti-I was fixed to the intact cord cell than to the intact adult cell at 0°C, the same proportion of antibody activity was removed from solution by equal concentrations of water-phase material from adult and from cord cells.

From these studies, it would appear that the I antigen is placed in the membrane in such a way that under certain conditions the antigen is not available to react with the antibody. Solubilization of the antigen from the membrane appears to render it more reactive. Thus dependence upon the reduction in temperature and the differences in reactivity of cells from adult and newborn donors are probably largely functions of the placement of the antigen in the membrane surface.

**REFERENCES**

Reaction of Cold Agglutinins with I Antigen Solubilized From Human Red Cells

WENDELL F. ROSSE and PETER K. LAUF