The Variability of Hemolysis in the Cold Agglutinin Syndrome

By Wendell F. Rosse and Judith P. Adams

The amount of lysis effected by cold agglutinins is directly related to the ability of the antibody to initiate complement activation. This ability is modified by the concentration of antibody, its thermal amplitude (the highest temperature at which the antibody will react with the cell), the degree to which antibody fixation is modified by the presence of complement components (particularly C3) on the membrane, and the degree to which antibody, once fixed, is able to fix the components of complement. In vitro measurement of these factors correlates with the rate of hemolysis in vivo.

IN THE COLD AGGLUTININ syndrome, the amount of hemolysis in vivo, and hence, the degree of anemia, is highly variable. Little or no hemolysis occurs in some patients; in others, the patient is severely anemic and requires transfusion. These differences may result from differences in the concentration of antibody in the serum since, in a given patient, the amount of hemolysis generally parallels the amount of antibody present. The ambient temperature and the maximal temperature at which the antibody is able to react with the red cell may also be important in determining the rate of hemolysis; if this maximal temperature is near 37°C, the interaction of antibody and red cell is more likely to occur at the usual ambient temperature, and therefore, more hemolysis is likely to result from the same amount of serum antibody. Since hemolysis in this syndrome is mediated in large part by the actions of the components of the complement system, the concentration of these proteins in the serum may also play a role in determining the degree of hemolysis.

In this article we present the results of investigations to determine the characteristics of the antibody and its interaction with the red cell and with complement, which are important in determining the rate of hemolysis in patients with the chronic cold agglutinin syndrome. We have found that hemolysis is, in general, directly related to the ability of the antibody to initiate the complement reaction, at least through the first few steps, since the “efficiency” of the terminal steps (C3 and C5–C9) in effecting lysis was the same for all antibodies studied. Several reasons for differences in the ability of various antibodies to initiate complement sequences were defined. These were related to the relative degree of hemolysis present clinically.

MATERIALS AND METHODS

Patients

The antibodies of six patients with the chronic cold agglutinin syndrome were analyzed; all antibodies had “anti-I” specificity as all agglutinated adult red cells to a greater degree than cord cells. The clinical course of these patients is summarized in Table I. They have been classified into three groups according to the degree of hemolytic anemia. The mean red cell lifespan was determined by the method of Logue et al.

Serum

Blood of patients with cold agglutinin syndrome was drawn in warm syringes and allowed to clot at 37°C. The serum was removed, heated to 56°C for 30 min, and stored at -90°C until use (1–24 mo). The specificity of the antibody was determined by agglutination of fresh group O cord red cells and red cells from group O adult donors. With all sera, greater agglutination was seen with normal adult red cells than with cord red cells, indicating anti-I specificity.

Normal serum with a cold agglutinin titer of <1/10 was used as a source of complement. Blood was drawn into cooled test tubes, which were then centrifuged and the plasma immediately removed. After clotting, the fibrin was removed, and the serum was stored at -90°C until use.

Red Cells

Blood from normal donors or patients with paroxysmal nocturnal hemoglobinuria (PNH) was drawn into sterile Alsever’s solution and stored at 4°C until use. At that time, the cells were washed and standard suspensions containing 2.2 x 10⁶ cells/ml were made according to Rosse and Dacie. The PNH cells used consisted of greater than 70% markedly sensitive PNH III cells, as determined by the complement lysis sensitivity test.

Purification and Labeling of the Antibody

The cold agglutinins were purified by sequential adsorption and elution from normal red cells. The elution at 37°C was carried out for 1 hr; all antibody was eluted under these conditions as shown by studies with 125I-labeled antibody. When labeling with 125I was needed, the technique of Helmcamp et al. was used as outlined in the same reference. More than 95% of 125I was affixed to protein as shown by precipitation with trichloracetic acid. More than 98% of the protein was specifically bindable to red blood cells as cold agglutinin. All cold agglutinins were IgMk by Ouchterlony analysis using specific antisera.

Complement Components

Functionally pure first component of complement (C1) was made from guinea pig serum according to the method of Borsos and
Rapp.\textsuperscript{12} Purified third component of complement (C3) was made from human serum by the method of Nilsson et al.\textsuperscript{13} Antibody to the third component of complement was made in rabbits according to the method of Rosse et al.\textsuperscript{9}

**Agglutination**

Agglutination tests were performed using equal volumes of antibody-containing solution diluted in twofold falling dilutions with veronal-buffered saline (VBS) and a standard suspension of normal type O red cells incubated overnight at 4°C. Agglutination was scored 4+ (complete) to 1+ (detectable only in the microscope) according to standard criteria. The titer is the highest dilution showing detectable agglutination read microscopically on cold slides.

**Measurement of Antibody Attachment**

Equal volumes of the standard cell suspension and of a solution of radiolabeled antibody were mixed and incubated at 0°C for 60 min. The tubes were centrifuged and the supernatant fluid removed. The cells were washed once in ice-cold VBS, and the radioactivity adherent to the cells was measured in a well-type gamma scintillation counter. The proportion of the added antibody that was adherent to the cells was calculated.

**Lysis of cells**

Lysis of normal or PNH cells was carried out in two steps. In the first, equal volumes (usually 0.2 ml) of a standard suspension of red cells, a solution of antibody, and of a dilution of human serum as a source of complement were incubated together for 30 min at a known temperature. In the second stage, the mixture was incubated at 37°C for 1 hr. At the end of the incubation, 4 ml of ice-cold VBS was added and the cells removed by centrifugation. The optical density of the supernatant fluid at 412 nm was determined in a spectrophotometer, and the percentage of cells lysed was determined using appropriate controls.

**Fixation of C1**

The fixation of C1 was determined by the method of Borsos and Rapp\textsuperscript{14} as modified by Rosse and Sherwood.\textsuperscript{15} This was related to the amount of antibody fixed to the cell by the use of radiolabeled antibody in duplicated experiments.

**Fixation of C3**

The fixation of C3 to the membrane was determined using 2 methods: (1) the C1 fixation and transfer method of Borsos and Rapp, using anti-C3\textsuperscript{2} and (2) a modification of the quantitative antibody adsorption technique of Borsos and Leonard\textsuperscript{16} as described by Rosse et al.\textsuperscript{9}

**Kinetics of Antibody and C3 Fixation**

To determine the rate and extent of antibody and C3 fixation during reactions between anti-I\textsubscript{a}, cells and complement at different temperatures, mixtures of equal quantities of known concentrations of radiolabeled anti-I\textsubscript{a}, human serum as a source of complement, and a standard suspension of red cells was incubated at the starting temperature for 15 min in a variable-temperature water bath. A sample was removed, the temperature was increased at the rate of 1°C/min, and samples were removed at intervals of time. One aliquot was placed on a Whatman filter, washed once with ice-cold VBS, and the amount of radioactivity present on the red cells was calculated. A second aliquot was washed and the amount of C3 present on the membranes of the red cells was determined by the quantitative antiglobulin consumption test.

**Inhibition of Antibody Adsorption by Complement**

Cells coated with complement components were made according to the methods outlined by Logue et al.\textsuperscript{2} using anti-I (Step.). These cells were incubated with equal volumes of radiolabeled antibody at 4°C for 1 hr, and the adsorption of antibody was determined at noted above. This was compared to the adsorption of antibody to cells that had not been coated with complement components.

**RESULTS**

**Agglutination Titers**

The agglutination titers of the sera at designated temperatures are shown in Table 2. The relative agglutination titers of serum do not correspond in all instances to the relative in vivo hemolytic rates. The agglutination titer of the purified antibodies is nearly the same for the same concentration of antibody.

**Lysis**

The lysis of PNH cells in the bithermic reaction with the first phase at 0°C as a function of antibody concentration is shown in Fig. 1. Normal cells are very difficult to hemolyze by most examples of anti-I\textsubscript{a} despite high concentrations of antibody, whereas PNH cells are readily lysed by these antibodies. Anti-I (Viv.), (Tur.), (Cass.), and (Gill.) effect about the same amount of lysis. Anti-I (Step.) effects much more lysis for a given amount of antibody in the reaction mixture and anti-I (Per.) effects considerably less, even when the concentration of antibody was...
markedly increased. To ascertain the importance of the temperature of incubation during the first phase of bithermic reaction, PNH red cells (2.2 x 10^7 suspended in 0.5 ml), purified antibodies (0.5 ml), and human serum (0.5 ml of a 1:2 dilution in VBS) as a source of complement were incubated for 30 min at a temperature varying from 0°C to 37°C, then incubated at 37°C for a further 30 min. The amount of hemolysis was measured (Fig. 2).

Inhibition of Lysis by Anti-I (Per.)

To determine if the presence of anti-I (Per.) inhibited the lysis obtained with other examples of anti-I, purified anti-I (Tur.) was mixed with purified anti-I (Per.), PNH red cells, and serum as a source of complement at 0°C for 15 min, then at 37°C for 60 min. The amount of lysis obtained when anti-I (Tur.) was mixed with anti-I (Per.) was less than that obtained with the anti-I (Tur.) (Fig. 3). The lysis obtained by an example of anti-P (Donath-Landsteiner antibody) was not diminished by the presence of anti-I (Per.) (data not shown). The amount of inhibition by anti-I (Per.) of lysis obtained by anti-I (Tur.) was increased by prolongation of the incubation period at 0°C.

The Fixation of the First Component of Complement (C1)

In duplicate reaction mixtures, the amount of radio-labeled anti-I adsorbed to the membrane was compared to the amount of C1 fixed (Fig. 4). The temperature was maintained at 4°C throughout. The amount of C1 fixed by a given amount of anti-I (Viv.),

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**Table 2. Agglutination Studies of Cold Agglutination Antibodies**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Temperature of Reaction</th>
<th>0°C</th>
<th>15°C</th>
<th>30°C</th>
<th>37°C</th>
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<tbody>
<tr>
<td>Step.</td>
<td>102,400*</td>
<td>51,200</td>
<td>800</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Gill.</td>
<td>12,800</td>
<td>6,400</td>
<td>3,200</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Cass.</td>
<td>3,200</td>
<td>400</td>
<td>&lt;100</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Tur.</td>
<td>25,600</td>
<td>1,600</td>
<td>&lt;100</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Viv.</td>
<td>51,200</td>
<td>12,800</td>
<td>&lt;100</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Per.</td>
<td>25,600</td>
<td>3,200</td>
<td>&lt;100</td>
<td>0</td>
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<table>
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<tr>
<th>Purified Antibody</th>
<th>Minimum Agglutinating Concentration (MAC) (µg/ml)</th>
<th>Fraction Affixed to Red Cell (% of MAC)</th>
<th>Molecules/Red Cell at Minimum Agglutination Concentration</th>
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*Reciprocal of dilution of serum giving microscopic agglutination.

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**Fig. 1.** The lysis of complement-sensitive red cells by purified antibody from six patients with cold agglutinin syndrome in the presence of a relative excess of complement. The amount of purified antibody in the reaction mixture is shown on the abscissa.

**Fig. 2.** The lysis of PNH III cells by purified antibody in the presence of a relative excess of human complement in a bithermic reaction in which the temperature of the first incubation of 30 min is shown on the abscissa. All tests were incubated at 37°C during a second incubation of 30 min. All antibodies were used in the same concentration (25 µg/ml) except (Step.) (2.5 µg/ml).
The amount of anti-I adsorbed at 4°C related to the fixation of C3 after warming to 37°C. C3 was measured by the ability of anti-C3 to fix C1 by the C1 fixation and transfer method. (Tur.), and (Step.) was equal, whereas the amount fixed by anti-I (Per.) was less.

The Fixation of the Third Component of Complement (C3)

In duplicate reaction mixtures, the amount of radio-labeled anti-I adsorbed to the membrane of the PNH red cells at 4°C was compared to the amount of C3 fixed to the membrane from serum after warming to 37°C, as determined by the C1 fixation and transfer test (Fig. 5). For a given amount of bound antibody, more C3 was fixed by anti-I (Step.) and (Gill.).

The Kinetics of C3 Fixation During Temperature Change

To demonstrate the temperature relationships between the binding of antibody and its ability to fix the third component of complement, anti-I (Step), (Viv.), (Per.) and (Tur.) were mixed with red cells and serum as a source of complement at 0°C for 15 min, and the kinetics of antibody dissociation and C3 fixation were determined as the temperature of the mixture was increased (Fig. 6). The third component of complement is not fixed at 0°C but is only fixed during the warming stage. The amount of antibody adsorbed at higher temperatures and the amount of C3 fixed was greater for anti-I (Step.) than for anti-I (Tur.) or (Viv.). Anti-I (Per.) fixed little C3.

In another set of experiments, anti-I (Step.) and anti-I (Tur.) were incubated at 37°C with cells and serum and the temperature was gradually lowered. At intervals, the amount of antibody adsorbed and the amount of C3 fixed were determined. At each time interval, a sample was removed and returned to 37°C and allowed to incubate for 15 min; the amount of C3 fixed to the red cell membrane was determined. The results are shown in Fig. 7. Anti-I (Step.) is able to fix
Fig. 6. The kinetics of antibody elusion and C3 fixation during warming of a mixture of PNH red cells, ¹¹¹I-labeled anti-I, and serum as a source of complement. The temperature was changed at the rate of 1°C/min. At intervals, samples were removed and analyzed for anti-I adsorption and C3 fixation.

Fig. 7. The kinetics of the fixation of antibody and of C3 to PNH red cells during cooling (1°C/min). using a thermostated bath, of a mixture of PNH red cells, ¹¹¹I-labeled anti-I, and human serum as a source of complement. At intervals, samples were removed and the amount of C3 fixed was measured immediately and, for anti-I (Tur.), after incubation at 37°C (indicated by arrows).

Fig. 8. The relationship between the fixation of C3 from serum to PNH red cells by various examples of anti-I and the amount of lysis that is produced during the reaction. Bound C3 was measured by antiglobulin consumption.

The Relationship Between C3 Fixed and Lysis Obtained

PNH red cells were incubated with complement and different amounts of the various antibodies in a bithermic reaction (0°C → 37°C). The amount of C3 bound to the membrane and the amount of lysis obtained were determined (Fig. 8). The amount of lysis is directly related to the amount of C3 bound and is equal for all antibodies tested.

The Inhibition of Antibody Fixation by Membrane-Bound C3

Cells were prepared with different amounts of C3 affixed to the membrane surface by incubation with serum and anti-I (Step.), first at 0°C and then at 37°C. The amount of C3 fixed was estimated by the Cľ fixation and transfer test. This test fixed about 1 molecule of C1 for every 10 molecules of C3 present on the membrane.

The amount of inhibition of antibody fixed in the presence of membrane-bound C3 is demonstrated in Fig. 9. Very little inhibition of antibody binding was
found for anti-I (Step.); the binding of anti-I (Cass.) was somewhat inhibited, whereas the binding of other examples of anti-I was markedly inhibited by the presence of C3 on the membrane.

**DISCUSSION**

In patients with chronic cold agglutinin disease, the amount of hemolysis that occurs in vivo is highly variable, ranging from little or none to severe in patients requiring transfusion. The patients in the present series were divided into three groups for consideration of the causes of the differences in antibody-red-cell complement interactions, which determine the rate of hemolysis. Three patients, Step., Cass., and Gill., had marked hemolysis, such that transfusions were required even when precautions against cold ambient temperatures were taken. Two patients, Viv. and Tur., had only moderate hemolysis and did not require transfusions to maintain an adequate hematocrit (group II). Although there is a great variation, this degree of hemolysis probably is the most common for patients with chronic cold agglutinin syndrome. One patient, Per., had little hemolysis but had marked symptoms of agglutination in vivo (acrocyanosis, pain on eating cold food, etc.). We have endeavored to determine why the patients in the first group had more hemolysis than those of the second, and why the third group had less.

One variable that might explain differences in the degree of hemolysis is differences in concentration of the antibody in the serum. This is exceedingly difficult to measure directly and quantitatively. The usual method of assessing concentration is the agglutination titer. Since the agglutination achieved by a given concentration of purified antibody is not greatly different for any of the examples of anti-I from different patients, the agglutination titer of the serum is a reasonable approximation of the concentration of antibody in the serum.

Differences in agglutination titer do not explain most of the differences in the amount of hemolysis seen in the present patients. The titer of the serum of Cass. (group I) is lower than those in group II, whereas that of Per. (group III) is higher. Hence, factors other than serum concentration must be important in determination of the hemolytic rate in these patients. These factors were sought in analysis of the ability of the antibodies to interact with antigen, fix complement components, and effect lysis under different in vitro conditions.

Marked differences were found in the ability of a given amount of antibody to effect lysis of PNH red cells in a bithermic (0°C–37°C) reaction. This was shown to be due to differences in the ability of the antibody to initiate complement sequences, since the amount of C3 fixed by a given amount of antibody, a measure of the efficiency of the first steps of the sequence, varied considerably, whereas the amount of lysis obtained for a given amount of fixed C3 was the same for all antibodies. Several reasons were found to account for differences in the ability of different antibodies to initiate complement sequences on the red cell surface.

The thermal amplitude (the temperature within which a cold-reacting antibody can interact with the red cell) is an important determinant of the ability of the antibody to initiate the activation of complement that may lead to lysis. Of the present antibodies, (Step.) and (Gill.) have a high thermal amplitude (Table 2, Fig. 2). They both fix more C3 for a given amount of antibody fixed (Fig. 5). This is probably due to the fact that C3 and C4 are fixed only at temperatures above 10–15°C. Antibodies of higher thermal amplitude are able to react with the antigen and initiate complement sequences at a temperature at which these components can be more efficiently fixed to the membrane.

The difference in ability to fix C3 is also shown when the temperature is lowered gradually from 37°C to 0°C (Fig. 7). Anti-I (Step.) is able to fix antibody at higher temperatures and, hence, the fixation of C3 occurs at 30°C with this antibody but does not with anti-I (Tur.). The marked hemolytic syndrome of patients Step. and Gill. can thus be related, at least in part, to the increased thermal amplitude of those antibodies.

The marked hemolytic rate of patient Cass. is not explained by any of the above mechanisms. The agglutination titer of the serum is relatively low, the antibody has a relatively poor affinity and a low temperature amplitude. However, this antibody is peculiar in that its adsorption to the antigen is not markedly inhibited by the presence of C3 on the membrane surface. Evans and Bingham found that newly transfused cells were more susceptible to hemolysis in patients with the chronic cold agglutinin syndrome than cells of the patient because the patients' own cells were protected by the presence of the third component of complement on the membrane surface. The present studies show that the degree of inhibition of antibody adsorption by membrane-bound C3 varies with different antibodies and that anti-I (Cass.) and (Step.) are not markedly inhibited by the presence of C3 on the cell surface. The circulating cells of these patients could continue to add more antibody and, thus, more C3 to the surface of the cells. This would be expected to result in increased hemolysis and may explain the marked hemolysis seen in Cass.
The lysis studies with anti-I (Per.) suggest that this antibody fixed to the membrane did not fix C1 well. The agglutination titer was similar to the other antibodies at a similar protein concentration, but the amount of lysis obtained was markedly less and could not be increased by an increase in the concentration of protein. The presence of anti-I (Per.) appeared to inhibit the lysis engendered by other anti-I antibodies but not that engendered by a Donath-Landsteiner antibody. This suggested that molecules of anti-I (Per.) were able to occupy antigen sites, thus inhibiting the adsorption of other anti-I antibodies, but were not able to fix complement. Direct demonstration of the inability to fix complement was shown in the C1 fixation studies. Anti-I (Step.), (Tur.), and (Viv.) all fix about the same amount of C1 for a given amount of antibody; anti-I (Per.) fixes much less.

The reason for the decreased fixation of C1 by anti-I (Per.) is not clear. This antibody, like some other examples of anti-I,20 is cold precipitable when purified. The structural change accompanying cold precipitation might be related to its inability to fix C1. In tests of inhibition of binding of other complexes of anti-I by anti-I (Per.), preincubation of the anti-I (Per.) in cold for various periods of time before the addition of the other anti-I serum and cells led to progressive decrease in ability of anti-I (Per.) itself to induce lysis and an increase in the degree of inhibition of lysis by the other anti-I by anti-I (Per.), suggesting that the conformational change that resulted in the loss of solubility may also have resulted in the loss of ability to fix complement. On the other hand, other examples of cryoprecipitable cold agglutinins have been shown to be capable of fixing amounts of C1 comparable to anti-I (Step.) (personal observations).

Regardless of the reason, the relative inability to fix C1 probably accounts for the milder hemolytic syndrome in patient Per.; he is able to maintain a normal hematocrit, and the red cell lifespan is usually only about half that of normal. When he is subjected to cold stimulus, he demonstrates marked agglutination but relatively little increase in his hemolytic rate or in the level of plasma hemoglobin in the cooled part.

These studies demonstrate that the reasons for the differences in in vitro hemolysis by different anti-I antibodies reside largely in differences in the interaction of the antibody molecule with the red cell membrane and in its ability to fix the first components of the complement sequence. Other factors, such as the concentration of complement components and of complement inhibitors or inactivators, may also be important in determining the in vivo rate of hemolysis, but these studies show that the characteristics of antigen–antibody interaction and complement activation that can be demonstrated in vitro are useful in assessing reasons for differences in hemolytic rate in vivo.

ACKNOWLEDGMENT

We wish to thank Norma Martell for help in preparation of the manuscript and Dr. John Crookston for the provision of the serum Step.

REFERENCES


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