Quantitative Immunology of Immune Hemolytic Anemia

I. THE FIXATION OF C1 BY AUTOIMMUNE ANTIBODY AND HETEROLOGOUS ANTI-IgG ANTIBODY

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ABSTRACT The fixation of the first component of complement (C1) by rabbit and goat anti-IgG antibodies reacting with auto- or isoimmune antibodies attached to red cells has been investigated. Two molecules of the rabbit IgG anti-IgG were required to fix a single molecule of C1, whereas only one molecule of goat IgM anti-IgG was required. The relationship between the number of auto- or isoimmune antibody molecules attached to the red cells and the amount of C1 fixed by anti-IgG was determined by the concentration of anti-IgG. A concentration of anti-IgG was found such that the number of molecules of C1 fixed was directly proportional to the concentration of auto- or isoimmune antibody. By this method a sensitive, reproducible minimum estimate of the amount of cell-bound and serum antibody could be made.

INTRODUCTION

The factors involved in the destruction of red cells in patients with immune hemolytic anemia have been difficult to assess because of the difficulty in obtaining quantitative data about the immunological reactions which take place at the red cell surface. These reactions involve antibodies of different types, antigens of different specificities, and, in some instances but not others, the fixation of complement (1). Recently we have investigated the relationship of the type and quantity of the antibody to the fixation of the first component of complement in vitro and thus to the initiation of the complement sequence (2). Most autoimmune antibodies which react with the red cell at body temperature are IgG in type and require two molecules of antibody in a specific geometrical relationship for the fixation of a molecule of C1. The antibodies which react with the red cell only at reduced temperature are usually IgM (cold agglutinins) and require only one molecule of antibody to fix a molecule of C1. This difference in temperature of reaction and in ability to fix C' probably determines differences in the mechanism of lysis of the cells.

Since the warm agglutinin antibodies are bound to the cell during its circulation, and since little or no complement may be fixed, the presence of antibody at the cell surface is probably the main determinant marking the cell for destruction. The relationship between the amount of antibody at the cell surface and its destruction has not been well studied, chiefly because the methods of determining the amount of bound antibody have been crude.

In recent years, methods of quantitating immune reactions have become available through increased knowledge of the complement system. Borsos and Rapp first quantitated immune reactions by measuring the amount of the first component of complement fixed by antibody-antigen reactions (3). We have applied this system to the study of the human antibodies and have found, as did Borsos and Rapp, that the amount of C1 fixed depended upon the type of antibody as well as its concentration at the red cell surface (2). In this system, the relative amount of IgM antibody on the cell may be measured directly since a single antibody molecule is able to fix a molecule of C1. However, the relative concentration of cell-bound IgG may not be directly measured since two molecules of antibody are needed to fix a molecule of C1, leaving many single antibody molecules which do not fix C1.

In the present paper, we present a method for the detection and quantitation of the amount of IgG antibody on the red cell surface using quantitative complement techniques and heterologous anti-immunoglobulin antisera. The complex relationship between the concentration of primary antibody, anti-antibody, and the amount of C1 fixed are presented in order to derive

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a method for quantitating the direct and indirect anti-
globulin test in patients with immune hemolytic anemia
due to IgG antibody.

METHODS

Patient material

The antibodies used in this study were from patients found
to have autoimmune hemolytic anemia by the presence of
hemolytic anemia and a positive direct Coombs test due to the
presence of IgG antibody on their red cells. A brief clinical
summary is given in the second paper of this series (4).

Red cells. Blood was taken by aseptic venipuncture from pa-
thents and donors and was placed immediately in an equal
volume of Alsever's solution made according to the formula
given in reference 5. At the time of use, the red cells were
washed three times in Veronal-buffered saline and the con-
centration of the suspension was adjusted to contain 2.2
× 10^11 red blood cells per ml according to the method given
in reference 6.

Antibodies. Antibody-rich eluates were made from the red
cells of patients with immune hemolytic anemia by either the
method of Rubin (7) or of Landsteiner and Miller (8). When
whole serum was used as a source of antibody, it was heated for
30 min at 56°C before use.

Heterologous antibodies against human
immunoglobulins—(anti-antibody)

Rabbit anti-IgG was obtained by the injection of purified
human IgG into rabbits, with Freund's adjuvant, or from the
Ortho Pharmaceutical Corp., Raritan, N. J. 2 ml of these an-
sera was fractionated on a Sephadex G-200 column. The anti-
IgG activity in each case was found in the peak containing
IgG immunoglobulins.

Goat anti-IgG antiserum. Antiserum to purified human IgG
immunoglobulin was prepared in goats. The antiserum was
absorbed with type I and type II Bence Jones protein. On im-
munoelectrophoresis against whole human serum, a single arc
due to precipitation with IgG globulin was seen.

2 ml of the goat anti-IgG antiserum was chromatographed
on a column of Sephadex G-200. The anti-IgG activity was
predominantly in the first (IgM) peak. A small amount was
found in the second peak containing IgG immunoglobulins.

Buffers

Veronal-buffered saline (VBS). An isotonic saline buffer,
P H 7.4, was made according to the formula given in reference
5. Ca++ and Mg++ were added to concentrations of 0.015
mole/liter and 0.005 mole/liter, respectively.

Sucrose. An isotonic solution of sucrose was made accord-
ing to the formula given in reference 9. When buffers of inter-
mediate ionic strength were required, this buffer was mixed
with appropriate amounts of VBS. The usual mixture was
60% isotonic sucrose and 40% VBS; this buffer has an ionic
strength of 0.067 and will be referred to as VBS-sucrose buffer.

EDTA (ethylenediaminetetraacetate, disodium salt). An isoto-
nic solution of 0.09 M EDTA was made according to the
formula given in reference 10. When dilutions of this were
required, they were made with VBS without added calcium
and magnesium.

Complement components

C1. The first component of complement was partially puri-
from guinea pig or human serum by the method of Borsos
and Rapp (11). The solutions which were used contained
10^8–10^9 effective molecules of complement by the hemolytic
 assay of Borsos and Rapp (11). These solutions contained no
C2 or C4 as detected by hemolytic assay.

C2. The second component of complement was partially puri-
from guinea pig serum according to the method of Borsos,
and Cook (12). This method was slightly modified
in that the pH of the CM-cellulose was brought to 4.8.
All exchange resins were allowed to equilibrate with their
buffers for at least 1 wk before use. The solution that was ob-
tained contained 10^9–10^10 effective molecules of C2 by the
hemolytic assay of Borsos and Rapp (13).

Sheep cells. Sheep cells were obtained fresh at weekly in-
tervals and were collected and stored in an equal volume of
Alsever's solution which had been made according to the
formula given in reference 5. At the time of use, the sheep
cells were washed twice in 0.015 M EDTA, and three times in VBS.

Antibody. Antibody to boiled sheep red cell stroma was
made in rabbits according to the method of Mayer (5). This
antibody was IgM in immunoglobulin type and had almost no
agglutination properties.

EACr. Sensitized sheep red cells containing only the
fourth component of complement (C4) were made ac-
ording to the method of Borsos and Rapp (14). All procedures were
performed strictly at 0°C; in order to minimize the number of
ineffective C4 sites.

C–EDTA. In order to provide the other components of
complement (C3, C5–C9), guinea pig serum absorbed with
sheep red cells was diluted 1 part in 50 in 0.015 M EDTA. Be-
cause of the chelation of the cations, calcium and magnesium,
C1 and C2 present in the serum were not able to act in the
complement sequence.

C1 fixation and transfer test (C1 ft test). The fixation
and transfer test was performed according to the method
of Borsos and Rapp (3), with modification of the first stage.
Human red cells in standard suspension were sensitized with
appropriate amounts of autoimmune ("primary") antibody.
After washing with VBS-sucrose buffer, they were reacted
with an appropriate concentration of heterologous antisem
containing anti-IgG antibodies. After being washed twice, the
cells were then suspended in 0.3 ml of partially purified guinea
pig C1 which had been absorbed twice with normal human red
cells. After incubation for 30 min at 30°C, the cells were sus-
pended in VBS-sucrose buffer, centrifuged, and the pellet
carefully transferred to a second tube. The cells were washed
thrice more with VBS-sucrose buffer and were suspended in 9
ml of VBS buffer. The number of molecules of C1 contained in
this solution was then determined by the method of Borsos
and Rapp (11), and the number of molecules of C1 fixed per
red cell was calculated. The fixation of C1 by red cells alone,
red cells and primary antibody, and red cells and anti-IgG was
determined for each experiment when appropriate.

RESULTS

The fixation of C1 by antibody with and without
anti-IgG

0.1 ml aliquots of a standard suspension of cells were
sensitized with 0.1 ml volumes of different dilutions of
euate from the cells of patient L. W. To one set of
sensitized cells, 0.2 ml of rabbit anti-IgG was added; to
another, 0.2 ml of goat anti-IgG, and to a third, 0.2 ml of sucrose-VBS buffer. CI was added to all sets after incubation and the amount of CI fixed per cell was determined in the CI fixation and transfer test. The results are shown on Fig. 1. No CI is fixed in the absence of anti-IgG. The maximal fixation of CI is at a 1/4 dilution concentration of antibody L. W. with the rabbit anti-IgG. Nearly maximal amounts of CI were fixed at 1/2 dilution of antibody when the goat anti-IgG was used.

The number of molecules of heterologous anti-IgG required to fix a molecule of CI

Human red cells were sensitized with a high concentration of autoimmune IgG antibody (primary antibody). Aliquots of these cells were reacted with graded concentrations of rabbit anti-IgG (an IgG antibody) or goat anti-IgG (an IgM antibody). The dose-response curves at limiting concentrations of anti-IgG differed for the two classes of anti-antibody. For the IgG rabbit antibody, the curve was parabolic when the relative concentration of anti-IgG was plotted against the number of molecules of CI fixed per sensitized cell. When the logarithm of the relative concentration of anti-IgG was plotted against the logarithm of the number of CI molecules fixed, a straight line resulted, the slope of which was 2.0. This indicates that two molecules of anti-IgG in juxtaposition were required for the fixation of a single molecule of CI (Fig. 2).

For the IgM goat anti-IgG antibody, the dose-response curve was linear at limited concentrations of anti-antibody. When the logarithm of the relative concent-

![Figure 1](http://www.jci.org) The fixation of CI by different concentrations of antibody L. W. in the presence of 0.2 ml of rabbit or goat anti-IgG antibody.

![Figure 2](http://www.jci.org) The fixation of CI by graded limiting concentrations of anti-IgG reacting with antibody L. W. present in high concentration on red cells. The logarithm of the relative anti-IgG concentration is graphed against the logarithm of the number of molecules of CI fixed per red cell.

![Figure 3](http://www.jci.org) The fixation of CI by graded concentrations of goat and rabbit anti-IgG reacting with antibody L. W. present in high concentration on normal red cells.

**Fixation of CI by IgG Antibody and Anti-Antibody** 729
The effect of anti-antibody concentration on dose-response curves of primary antibody

The effect of different concentrations of anti-antibody on the amount of CI fixed by cells sensitized with graded amounts of primary antibody (L. W.) was demonstrated using both goat anti-IgG and rabbit anti-IgG. When the goat anti-IgG was used, the amount of CI fixed was proportional to the relative concentration of primary antibody used to sensitize the cells for all concentrations of the anti-IgG. The slope of the line relating the logarithm of the primary antibody concentration to the logarithm of the amount of CI fixed was about 1 regardless of the concentration of anti-antibody used (Fig. 4).

However, when rabbit anti-IgG was used, the shape of the dose-response curve, and hence the slope of the logarithmic plot of relative concentration of primary antibody used in sensitization against the amount of CI fixed by the cells depended upon the concentration of the anti-IgG (Fig. 4 and Table I). At limiting concentrations of anti-IgG (0.05 ml), the slope of the dose-response line was less than 1. As the amount of anti-IgG was increased, the total amount of CI fixed by a given dilution of primary antibody increased as did the slope of the dose-response curve; a maximum of both measurements was found at a concentration equivalent to 0.2 ml of anti-IgG. At very high concentrations of anti-IgG, both the total amount of CI fixed by a given concentration of primary antibody and the slope of the logarithmic dose-response curve decreased.

Table II

<table>
<thead>
<tr>
<th>Antibody dilution</th>
<th>Anti-IgG</th>
<th>Cells</th>
<th>Date</th>
<th>Molecules CI/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-IgG</td>
<td>0.2</td>
<td>J. P.</td>
<td>9-27-66</td>
<td>6380</td>
</tr>
<tr>
<td>Rabbit anti-IgG</td>
<td>0.2</td>
<td>J. P.</td>
<td>8-16-67</td>
<td>3850</td>
</tr>
<tr>
<td>Rabbit anti-IgG</td>
<td>0.2</td>
<td>J. P.</td>
<td>11-07-67</td>
<td>5550</td>
</tr>
<tr>
<td>Rabbit anti-IgG</td>
<td>0.2</td>
<td>J. P.</td>
<td>8-29-67</td>
<td>750</td>
</tr>
<tr>
<td>Rabbit anti-IgG</td>
<td>0.2</td>
<td>J. P.</td>
<td>9-12-67</td>
<td>620</td>
</tr>
<tr>
<td>Rabbit anti-IgG</td>
<td>0.2</td>
<td>J. P.</td>
<td>9-27-66</td>
<td>6380</td>
</tr>
<tr>
<td>Rabbit anti-IgG</td>
<td>0.2</td>
<td>J. P.</td>
<td>10-26-66</td>
<td>3850</td>
</tr>
<tr>
<td>Rabbit anti-IgG</td>
<td>0.2</td>
<td>J. P.</td>
<td>10-27-66</td>
<td>5282</td>
</tr>
</tbody>
</table>

* Number of times test performed.

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Reproducibility of the results

Reproducibility of the test results was investigated by repeating the test on different days, using a single concentration of autoimmune antibody, cells from the same donor, and a given concentration of anti-IgG. The results are shown on Table II.

The relationship of C1 fixation and titers to agglutination.

In two series of tubes, normal red blood cells in standard suspension (0.1 ml) were incubated with 0.1 ml of graded dilutions of serum of patient R. A. (a patient with autoimmune hemolytic anemia and chronic lymphocytic leukemia). After washing, 0.1 ml of rabbit anti-IgG was added and incubated for 30 min. The cells were centrifuged and, in one series, were resuspended with gentle shaking and the amount of agglutination was microscopically estimated. In the other series, C1 was added and the amount of C1 fixed per cell was estimated. The results are shown in Fig. 5.

The effect of complement components on the red cell membrane upon the determination of antibody concentration.

In order to determine if the presence of complement components on the cell surface altered the ability to detect IgG antibody, studies were performed in which the amount of antibody R. F. was determined when fresh serum was added to permit the addition of complement components and when the cells used were previously coated with complement components. In both instances, the amount of C1 fixed by anti-IgG was the same as when complement components were not present (see Table III).

DISCUSSION

The fixation of C1 by antibody appears to depend upon two characteristics of the antibody and its interaction with antigen. The immunoglobulin class of the antibody is important since it has been shown that a single mole-

![Figure 5](http://www.jci.org)

**Figure 5** A comparison between the amount of C1 fixed and the agglutination induced by a given concentration of rabbit anti-IgG reacting with cells sensitized with various concentrations of serum from a patient with immune hemolytic anemia.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Molecules of C1 fixed per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum R.F. + E§</td>
<td>1300</td>
</tr>
<tr>
<td>Serum R.F. + Normal serum + E</td>
<td>580</td>
</tr>
<tr>
<td>Serum R.F. + EC3†</td>
<td>600</td>
</tr>
<tr>
<td>Serum R.F. + EC3</td>
<td>210</td>
</tr>
</tbody>
</table>

* Assay of cell-bound third component of complement (C3) by anti-C3 and C1 fixation and transfer as described by Logue and Rosse. In preparation.
† Serum R. F. heated 56°C for 30 min.
§ E, normal cells.
° EC3, normal red cell to which complement components have been added by reacting with cold agglutinin and fresh serum.

The ability of IgM antibody from a variety of sources is capable of fixing a single molecule of C1, whereas a “doublet” or two molecules of IgG antibody in some particular geometric relationship appear to be necessary for the fixation of a molecule of C1 (2, 3). IgA antibodies apparently lack the site for the fixation of C1 since none has been demonstrated to do so (16).

In addition to the immunoglobulin class of the antibody, the arrangement of the antigen sites on the cell surface also appear to be important in determining the fixation of C1. Certain antibodies, especially those against antigens in the Rh system, are incapable of fixing C1 even at very high concentrations of antibody. However, when IgG antibodies of several specificities in the Rh system are present on the cell at the same time, C1 may be fixed, indicating that the lack of fixation of C1 by antibodies of any given specificity is not an intrinsic property of the antibody (2). The absence of C1 fixation is probably due to the distribution of the antigen sites such that doublet formation cannot occur.

The quantitation of antibody on the cell surface by its ability to fix C1 is simple for antibodies of the IgM class which react with antigens present in large numbers on the red cell surface. We have shown that cold agglutinins may be easily measured on the surface of red cells since there is a direct relationship between the amount of cold agglutinin present and the amount of C1 fixed (2, 17). However, quantitation of IgG antibodies by this method is very much more complex since many iso- or autoimmune antibodies do not fix C1 at all and other antibodies only at a very high concentration of antibody. It is for this reason that we have investigated the
quantitation of cell-bound IgG antibodies with the use of heterologous anti-IgG antibodies and the C1 fixation and transfer test.

The fixation of C1 by anti-antibody reacting with antibody attached to the red cell surface appear to have the same requirements as the fixation of C1 by antibody reacting directly with the red cell. From the present studies, it is apparent that a single molecule of IgM anti-IgG reacting with an IgG primary antibody molecule is sufficient for the fixation of a molecule of C1. Thus, for a given amount of primary antibody the relationship between the amount of IgM anti-IgG present and the amount of C1 fixed is always linear. On the other hand, two molecules of IgG anti-IgG, reacting with cell-bound antibody, are necessary for the fixation of a molecule of C1 since the slope of the dose-response line was 2 when the concentration of IgG anti-IgG is varied but remains limited. This result implies that the C1-fixing site of the IgG anti-IgG antibody cannot interact with the C1-fixing site of the primary IgG antibody to form a doublet. If this were the case, the amount of C1 fixed would be directly proportional to the amount of IgG anti-IgG present and the slope of the dose-response curve on log-log plot would be 1.

If the antiglobulin-C1 fixation system is to be of value for the estimation of the amount of autoimmune or isoimmune (primary) antibody attached to red cells, the dose-response relationship between the amount of primary antibody and the amount of C1 fixed must be established. This relationship is simple in the case of the goat IgM anti-IgG since the amount of C1 fixed is proportional to the relative concentration of primary antibody, regardless of the concentration of anti-IgG present. As the concentration of anti-IgG was increased, more C1 was fixed at all concentrations of primary antibody. However, the concentration of anti-IgG in this antiserum was limiting since a maximum fixation of C1 was not reached, even with high concentrations of the antiserum.

Because two molecules of the IgG anti-IgG are required for the fixation of a molecule of C1, the relationship between primary antibody concentration and the amount of C1 varied with the concentration of anti-IgG. Theoretically, the two anti-IgG molecules forming the C1-fixing doublet may situate on a single primary antibody molecule (a "unit" doublet) or on adjacent primary antibody molecules (an "adjacent" doublet).

When the concentration of anti-IgG is very low, the number of doublets formed does not vary directly with increases in the amount of primary antibody attached. As more primary antibody is added to the cell, more potential sites are present for the random distribution of the limited number of anti-IgG molecules. Therefore, the chances of two molecules of anti-IgG forming a doublet are diminished. This is reflected in the fact that the logarithmic plot of the slope of the dose-response curve relating relative primary antibody concentration to C1 fixed when 0.05 ml of anti-IgG is present is less than 1.

If all doublets were "unit" doublet, the amount of C1 fixed would be directly proportional to the concentration of primary antibody and the slope of the logarithmic plot of the dose-response curve would be 1. If all doublets fixing C1 were "adjacent" doublets, then the slope of the logarithmic plot of the relative concentration of primary antibody to the amount of C1 fixed would equal 2. In the present experiments, as the amount of anti-IgG is increased, the slope of the dose-response curve reaches a maximum approaching 2, suggesting that both unit and adjacent doublets were formed. The proportion of "adjacent" doublets, as measured by the degree to which the slope approaches 2, is apparently somewhat less when Rh antibodies are used. This may relate to the fact that the antigen sites for the Rh antigens are probably sparser than for most other antibodies; therefore, there would be less chance of adjacent doublets forming with these antibodies. At very high concentrations of anti-IgG, the relationship between the concentration of primary antibody and C1 fixation is difficult to explain.

In order for the measurement of C1 fixation to be of value in the estimation of antibody coating of cells, the relationship between the amount of antibody on the cell and the amount of C1 fixed must be linear (i.e. the slope of the logarithmic plot of the dose-response curve must equal 1.0). This condition obtains when 0.1 ml of IgG anti-IgG is used. It is not apparent from the data whether this relationship is due to the formation of unit doublets only.

Constantoukalis, Costea, Schwartz, and Dameshek (18) have suggested that up to seven anti-IgG molecules may react with a single IgG molecule. If this is so, then it is theoretically possible to have more than one "unit" doublet per IgG molecule. That condition, if it exists, does not alter the validity of the proportionality relationship between the amount of primary antibody present and the amount of C1 fixed at an appropriate concentration of anti-IgG.

The efficiency of this antiglobulin system in the detection of IgG antibody is difficult to assess. Using a similar technique, Borsos, Colten, Spalter, Rogentine, and Rapp estimated that 60–100% of IgG molecules were detected in certain instances and as low as 5% in other instances (19). However, these investigators did not include data concerning the effect of anti-antibody concentration on the relationship between primary antibody and the fixation of C1. The present data show that by the appropriate adjustment of this concentration of anti-IgG, a minimal but proportional estimate of the num-

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ber of IgG antibody molecules at the surface may be obtained. Clearly many antibody molecules may go undetected but valid conclusions may be drawn from these relative estimates.

The advantages of this method of measuring IgG antibody on the red cell are the precision, reproducibility, and ease with which the test can be performed. Its usefulness in following the course of patients with immune hemolytic anemia will be demonstrated in a subsequent paper (4).

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REFERENCES


Fixation of C'1 by IgG Antibody and Anti-Antibody 733