Studies of Incomplete Antibodies

I. Effect of Papain on Red Cells

By KIMMO AHO AND CHARLES L. CHRISTIAN

The treatment of red cells with some proteolytic enzymes has proved to be a suitable technic for the detection of a variety of blood group antibodies which coat but do not agglutinate erythrocytes in physiologic saline. The mechanism of this enzyme effect is not well understood. There is evidence that the antibodies revealed by the enzyme technic are not univalent. Possible bases of this enzyme effect have been discussed, and the question of enzymatic alterations of the erythrocyte surface antigens has been recently reviewed.

Enzyme treatment increases the titers of many agglutinating antibodies, but in some systems—e.g., with mononucleosis agglutinins and sheep cells, the titers are lower due to a release of the corresponding receptors from the cells. In the present work, it is shown that a variety of agglutinating antibodies agglutinate papain-treated cells in higher titers than nontreated cells, and evidence is presented that the titer increase is not due to a separate type of incomplete antibody. Rather, it is suggested that a smaller number of antibody molecules are required for agglutination of papain-treated cells because of altered surface properties of erythrocytes.

Materials and Methods

Two individual rabbit antisera against sheep red cells were obtained by intravenous immunization twice a week during a 5-week period. Pooled hamster antisera against sheep cells and rabbit cells were produced by intraperitoneal immunization twice a week over a 5-week period. A human antiserum against sheep red cells was obtained after several intramuscular injections. A sample of human anti-A antiserum, produced by immunizing with the corresponding purified blood group substance, was provided by Dr. Elvin Kabat. In addition to these immune antisera, three samples of cold-agglutinin positive human sera (two of these provided by Dr. Vincent Butler), Bacto Phytohemagglutinin M and a seed extract from Dolichos biflorus, highly specific for the blood group A1 (obtained from S. B. Penick and Company) were used in agglutination experiments.

Samples of pooled rabbit anti-C and anti-G isoantibody sera were obtained from Dr. A. Kellner. Two samples of individual anti-G antisera were provided by Dr. C. Cohen, and

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*In the present work, Kellner's nomenclature (G and g) is used for the rabbit isoantibodies because of its simplicity.
one individual anti-G serum was produced according to Kellner's method. Incomplete anti-Rh antiserum Ni originated from isoimmunization during pregnancy. Duck antirabbit serum was produced by several intramuscular injections of rabbit \( \delta \)-globulin with Freund's adjuvants. Rabbit anti-human serum was a commercial product of Knickerbocker Biologicals, Inc.

Preparations of \( \delta \)G antibodies were obtained by DEAE cellulose chromatography (fallthrough fraction=0.02 M phosphate buffer, pH 8.0). Preparations of 7S and 19S antibodies were obtained by sucrose gradient ultracentrifugation in a gradient of 10 to 40 per cent sucrose at 35,000 rpm in a Spinco Model L centrifuge for 16 hours. The low molecular weight fractions were collected by aspiration from the top of the gradient.

The papain-treatment of cells was performed by standard technics. One volume of 1 per cent cysteine-activated papain (Winthrop) in 1/15 M phosphate buffer, pH 5.4, was added to 9 volumes of 0.85 per cent saline. Two volumes of this solution were added to 1 volume of thrice-washed packed cells and incubated at 37 C. for 30 minutes. The cells were then washed 3 times with pH 7.2 buffered saline.

The agglutination experiments were done by making twofold dilution series of sera or protein preparations. 0.2 ml. volumes of each dilution were distributed into parallel rows of tubes, and 0.2 ml. of 0.5 per cent suspensions of papain-treated or nontreated red cells were added into the appropriate tubes. The reactions were read with the naked eye after 4 hours at room temperature and sometimes again after 16 hours at 4 C. In tests for cold agglutinins, the reactions were read after 16 hours at 4 C.

The antiglobulin consumption test was performed by the dilution/absorption technic. Appropriate amounts of cell suspensions, usually corresponding to 0.1 ml. of packed cells, were incubated with the antisera for 90 minutes at 37 C. The sensitized cells were washed 4 times with a large volume of buffered saline and suspended in a 2.5 ml. volume. From this, eight 0.25 ml. aliquots were taken and mixed with equal volumes of the last 8 dilutions of anti-\( \delta \)-globulin serum preceding the final titer. The anti-\( \delta \)-globulin serum was absorbed with nonsensitized cells before use. After 15 minutes at 37 C., the cells were centrifuged and the supernatants were tested for the remaining anti-\( \delta \)-globulin activity. Sensitized Rh-positive cells were used as the detector system when human antibodies were measured. Rabbit G-positive cells, sensitized with the corresponding isoantibodies and sometimes sheep cells sensitized with one-third of the minimum agglutinating dose of rabbit antisheep cell antibodies, were used when rabbit antibodies were measured. The consumption results were recorded in half dilution steps based on the strength of agglutination in the last reactive dilution. The basic consumption of the nonsensitized cells did not exceed half a titer step, indicating that there was no appreciable nonspecific binding of the \( \delta \) G globulin onto autologous cells. In certain instances, additional controls were made with washed cells and isoologous normal serum. The antiglobulin consumption titer refers to the difference between reactions given by the test sample and the anti-\( \delta \)-globulin serum which was absorbed only with nontreated cells.

Preparations of polynylpyrrolidone (PVP) obtained from A. H. Thomas and Company and Antara Chemicals (Type NP-K 60) were dissolved in buffered saline and tested for hemagglutination at various concentrations with the same technics described above.

**RESULTS**

Agglutination experiments were performed with purified antibody fractions of several typical agglutinating antibodies using both papain-treated and nontreated cells. In addition, 3 cold agglutinin-positive sera and 2 phytohemagglutinin preparations were used as agglutinators. The results are shown in Table 1. It is seen that the \( \delta \)G antibodies agglutinated papain-treated cells in a uniform manner at about 16-fold higher dilutions than nontreated cells. Macroglobulin antibodies agglutinated the papain-treated cells at an average of 4-fold higher dilutions. With phytohemagglutinins the papain-effect was the greatest, about 64-fold.
Table 1.—Titer Enhancement of Different Antibodies with Papain-Treatment of Cells

<table>
<thead>
<tr>
<th>Source of Antiserum</th>
<th>Red Cells</th>
<th>Antibody Fraction</th>
<th>Titer Enhancement with Papain-Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Sheep</td>
<td>$\delta G$</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$7S^2$</td>
<td>8-16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$19S^2$</td>
<td>2-4</td>
</tr>
<tr>
<td>Human</td>
<td>Group A1</td>
<td>$7S$</td>
<td>8-16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$19S$</td>
<td>2-4</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Sheep</td>
<td>$\delta G$</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$7S$</td>
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<td>Rabbit</td>
<td>Sheep</td>
<td>$\delta G$</td>
<td>16</td>
</tr>
<tr>
<td>Hamster</td>
<td>Sheep</td>
<td>$\delta G$</td>
<td>16</td>
</tr>
<tr>
<td>Hamster</td>
<td>Rabbit</td>
<td>$\delta G$</td>
<td>8</td>
</tr>
<tr>
<td>Cold Aggl.</td>
<td>Ja</td>
<td>serum</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>El</td>
<td>Group O</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Cr</td>
<td>serum</td>
<td>8</td>
</tr>
<tr>
<td>Phytohemaggl.</td>
<td>Sheep</td>
<td>—</td>
<td>128</td>
</tr>
<tr>
<td>Bacto M</td>
<td>Group O</td>
<td>—</td>
<td>32</td>
</tr>
<tr>
<td>Dolichus bifl.</td>
<td>Group A1</td>
<td>—</td>
<td>64</td>
</tr>
</tbody>
</table>

$^1$ Obtained by DEAE-cellulose chromatography.

The effect of papain-treatment upon the reaction of $\delta G$ and $\delta M$ antibodies is also illustrated in Figure 1. This shows the titers of zone centrifugation fractions of the anti-A serum and the papain-effect in each fraction. It is seen that the titer increase with $\delta M$ antibodies was 2- to 4-fold and with $\delta G$ antibodies 8- to 16-fold.

The rabbit isoantibodies possessed somewhat different properties. In the anti-G system, only very low or no saline agglutinating activity was noted, but papain-treated cells were agglutinated in high titers. Anti-g antibodies always demonstrated some saline agglutination. The pooled anti-g serum was subjected to zone centrifugation. Two low-titer saline agglutinating peaks could be noted, one in the bottom fractions and the other in the region corresponding to the $7S$ fraction. The $19S$ antibodies agglutinated papain-treated cells in 4-fold higher dilutions than nontreated cells, whereas with the $7S$ antibodies the titer increase was 64- to 128-fold. The $8G$ fraction of anti-g antiserum obtained by DEAE-cellulose chromatography also showed some saline agglutinating activity and a 64- to 128-fold titer increase when papain-treated cells were used.

Two preparations of polyvinylpyrrolidone (PVP) were dissolved in buffered saline. Papain-treated and nontreated human Group O, sheep and rabbit erythrocytes were tested for agglutination in various PVP concentrations. In each instance, the papain-treated cells were agglutinated in higher PVP dilutions than the nontreated cells. The difference was greatest (about 16-fold) when sheep cells were used and smallest (2- to 4-fold) when human Group O cells were used.
Anti-globulin consumption experiments were done to measure the δG antibody absorbed onto the cell surface under different test conditions. Figure 2 illustrates the consumption titers when the pooled anti-G serum, the pooled anti-g serum, and a nonfractionated antisheep erythrocyte serum (with most of the agglutinating activity in the 7S fraction) were used for coating non-
Table 2.—Absorption of Antibody onto Papain-Treated and Nontreated Cells

<table>
<thead>
<tr>
<th>Source of Antibody</th>
<th>Difference between Papain-Treated and Nontreated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human antisheep cell</td>
<td>No measurable difference</td>
</tr>
<tr>
<td>Human anti-Rh</td>
<td>½ tube greater consumption with papain-treated cells</td>
</tr>
<tr>
<td>Rabbit antisheep cell</td>
<td>0 1 tube smaller consumption with papain-treated cells</td>
</tr>
<tr>
<td>Rabbit anti-G</td>
<td>No measurable difference</td>
</tr>
</tbody>
</table>

1δG fraction.
2Nonfractionated serum.

Diseased cells. Under the experimental conditions employed, the consumption titers theoretically reflect log2 values of the bound antibody—i.e., a two-tube difference in titer corresponds to a 4-fold difference in the amount of bound δ-globulin. It is seen that rabbit cells sensitized with an excess of mainly incomplete isoantibody sera did not consume more anti-δ-globulin serum than sheep cells sensitized with subagglutinating amounts of agglutinating anti-sheep cell antibodies. Similar results were obtained with two individual anti-g sera and with one anti-G serum.

Table 2 summarizes comparison in the amounts of absorbed antibody between papain-treated and nontreated cells. Antisera or antibody preparations were used to sensitize cells in different amounts within a 32-fold range. With the agglutinating antisera the greatest amount of antibody corresponded to one agglutinating unit using nontreated cells, and with the incomplete antisera the smallest amount corresponded to one agglutinating unit using papain-treated cells. It appears that the amount of antibody absorbed onto papain-treated cells and nontreated cells was of the same order of magnitude.

Discussion

We have previously presented some evidence suggesting that a sufficient explanation for the incompleteness of the anti-Rh antibodies might be a limited number of Rh receptors. This was based on measurements of antibody bound onto the erythrocyte surface by sensitizing the cells with subagglutinating amounts of some human agglutinating δG antibodies and with an excess of incomplete anti-Rh antibodies. Since there are agglutinating anti-Rh antibodies and they reside in the δM fraction, an essential requisite for the thesis is that δM antibodies are more efficient in agglutinating red cells than δG antibodies. This was demonstrated for rabbit δG antibody against human Group A cells by Greenbury et al.

The present experiments add some further support to the above hypothesis regarding the nature of the incomplete antibodies—i.e., limited number of antigenic receptors. Rabbit cells sensitized with an excess of mainly incomplete rabbit isoantibodies were shown not to consume in antiglobulin consumption experiments more of antirabbit δ-globulin serum than sheep cells which were sensitized with subagglutinating amounts of corresponding typical agglutinating δG antibodies. As noted by Rockey and Kunkel and confirmed in the present work, a part of the δG rabbit isoantibodies, especially in the anti-g system, are complete. A probable explanation for this is that the number and arrangement of receptors is very close to that needed for agglutination.
Several possible bases can be considered for the agglutination of enzyme-treated cells by incomplete antibodies: (1) the number of antigen receptor sites increases after enzyme treatment; (2) different antigens are "uncovered" by enzyme action and agglutination reflects the presence of a type of antibody separate from complete agglutinins; (3) enzyme treatment removes surface materials which in a special sense prohibit the linking of two erythrocytes by an antibody with finite dimensions; and (4) enzyme treatment or the mere attachment of the enzymes to the erythrocyte surface alters the physicochemical character of the erythrocyte surface in a way that lessens inter-erythrocyte repulsion and favors agglutination.

There is some evidence suggesting an increase in antigen receptor sites, although the increased uptake of antibody may result from its more firm binding onto enzyme-treated cells. Antiglobulin consumption experiments carried out in the present work revealed a slightly increased uptake in the Rh system and no increase in the other systems tested. Due to the semiquantitative nature of the antiglobulin consumption test, the difference in the Rh system may not be significant. Definite evidence against the view that an increase in the number of available receptors is responsible for the agglutination induced by enzyme treatment is the observation that presensitized and washed nonagglutinated cells are agglutinated after subsequent enzyme treatment.

The possibility that enzyme-treated cells react with a separate type of antibody—i.e., "new" antigens are exposed, is unlikely in view of the present data. A variety of agglutinating 8G antibodies from three different species uniformly agglutinated papain-treated cells in the 8- to 16-fold higher than titers of agglutination for nontreated cells. It is difficult to understand how a separate type of antibody could be operative in agglutination experiments utilizing cold agglutinins which have properties of monoclonal antibodies. Absorption and elution studies indicate that most phytohemagglutinins are homogeneous in their serologic properties, behaving like monoclonal antibodies. The existence of a separate class of incomplete phytohemagglutinins is an unlikely explanation for the observed enhanced agglutinability of phytohemagglutinins for papain-treated cells.

It is known that enzyme treatment causes many changes in the erythrocyte surface—e.g., release of considerable amounts of sialic acid. The amount of sialic acid lost has been compared with the reduction in charge of the erythrocytes and with the reduction in the electrophoretic mobility of the cells. The recent work of Pollack et al. suggests that the agglutination of antibody-coated erythrocytes depends upon the electrokinetic potential of the surface of the cells and upon the dimensions of the antibody molecules. The value of the enzymes used in blood group serology was shown to be related to their ability to reduce the net surface charge with consequent reduction in the electrokinetic potential.

*One cannot exclude the possibility that some immune antisera contain antibodies that react with enzyme-revealed red cell antigens. Many immune antisera do contain antibodies that fail to react with enzyme-treated cells because enzyme treatment releases the corresponding antigens.
The possibility that enzyme treatment removes surface materials which
spatially interfere with the bridging of two erythrocytes by an antibody mole-
cule cannot be excluded by the current data, but the observed uniform en-
hancement of all δG antibodies regardless of the species of origin or the type
of erythrocyte used and the increased titers of the serologically homogeneous-
cold agglutinins and phytohemagglutinins make such a possibility unlikely.
This uniform behavior with respect to enhanced agglutination of enzyme-
treated cells suggests that physicochemical changes in the erythrocytes, rather
than removal of spatial interference, underlie the increased agglutinability.
Further support for this hypothesis, and evidence against the other hypotheses,
is the observation that papain treatment of cells enhances their agglutinability
by PVP.

Summary

A variety of typical agglutinating δG antibodies agglutinated papain-treated
cells in a uniform manner in about 16-fold higher dilutions than nontreated
cells. With δM antibodies the corresponding titer increase was on the average
4-fold. The papain-treatment of cells also increased the titers of cold agglu-
tinins, phytohemagglutinins and preparations of PVP. Antiglobulin consump-
tion experiments revealed that no more antibody was fixed from the same
volume of antiserum onto papain-treated than onto nontreated cells. Possible
mechanisms underlying enhanced agglutination of enzyme-treated cells were
discussed. It is suggested that the same type of antibody may react with papain-
treated and nontreated cells but that a smaller number of antibody molecules
are needed for agglutination of papain-treated cells because of altered surface
properties of treated erythrocytes.

Summario in Interlingua

Un varietate de typic agglutinante anticorpore δG agglutinava cellulas
tractate con papaina uniformemente in diltltiones plus alte per un factor de
circa 16 que illos al qual cellulas non assi tractate eseva agglutinate. Con
anticorpores δM, le correspondente augmento de titro eseva a media un
quadruplicazione. Le tractamento de cellulas con papaina augmentava etiam
le titros de cryoagglutinininas, phytohemagglutinininas, e preparationes de poly-
vinylpyrrolidona. Experimentos in le consumo de antiglobulina revelava que
le quantitate de anticorpore fixate ab le mesme volumine de antisero a cellulas
tractate con papaina non excedeva illo in le caso de cellulas nontractate.
Es commentate le mechanismos possibile del promovite agglutination de
cellulas tractate con enzyma. Es postulate que le mesme typo de anticorpore
reage con cellulas tractate e non tractate con papaina sed que un plus micre
numero de molecules anticorporee es requirite pro le agglutination de cellulas
tractate con papaina a causa del alterate proprietes del superficie in le
erthrocytos tractate.

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REFERENCES

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