Recovery of Blood Group Antibodies from Erythrocyte Powder

By Felix Milgrom, Carlos Orellana and Miguel Layrisse

In a previous paper, experiments on the application of erythrocyte powder in serologic laboratories were described. It was shown that erythrocyte powder may be successfully used for (1) absorption of useless and disturbing antibodies from test sera, (2) complement fixation test with strong group antibodies, and (3) preparation of “pure” antibodies by elution from the sensitized powder.

The aim of the present investigation is to determine whether normal auto-antibodies could be recovered from unsensitized erythrocyte powder and to examine some of the properties of anti-A, anti-B and anti-Rh antibodies recovered from the sensitized powder.

Methods and Materials

The methods used have already been described elsewhere. We shall summarize them briefly.

Preparation of erythrocyte powder.—Red cells from citrated blood were washed with saline and hemolized with distilled water. The stromata were lyophilized and pulverized in a mortar. At the beginning of the experiments the stromata were dried in Petri dishes at 37°C., scraped away and pulverized. The lyophilized preparation which we obtained later proved to be more active and thus was used most often. With both methods, about 1500 mg. of powder were obtained from 200 ml. of packed red cells.

Sensitization of erythrocyte powder.—The proper amount of erythrocyte powder was weighed out and washed twice with saline. The powder was then suspended, at first in a small volume of serum (about 1 ml.), and all lumps were crushed with a glass rod; thereafter, the remaining volume of serum was added.

In most experiments with anti-A and anti-B antibodies, a 100 mg. quantity of powder was sensitized with 200 ml. of serum. In the majority of the experiments with anti-D (anti-Rho) antibody, 50 mg. of powder were sensitized with 10 ml. of the serum. The sensitization with anti-A and anti-B antibody was performed during 2 hours at room temperature, and 2 hours at 37°C. with anti-D, (anti-Rh0) antibody. During sensitization the vessels were shaken every 5 minutes.

Elution.—Before elution, the powder was washed 3 times with saline and suspended in 15 to 30 drops of saline or AB serum. The tubes were placed for 5–7 minutes in a water bath at 56°C., and centrifuged for about 3 minutes in half liter bottles filled with water heated to 56°C. The temperature drop during centrifugation never exceeded 4°C.

Results

Experiment 1. Elution of the Unsensitized Erythrocyte Powder

These experiments were intended to elucidate whether there is some physiologic sensitization of red cells with autoantibodies.
The physiologic production of autoantibodies is postulated by Bernstein's theory, which states that every human being produces both anti-A and anti-B antibodies. The regularity of appearance of these antibodies, expressed by Landsteiner's rule, is explained by the absorption of incompatible antibodies (autoantibodies) by red cells, that is, anti-A by A and AB cells, anti-B by B and AB cells. Bernstein's theory and the opposing theory of Furuhata are presented in Table 1.

The physiologic production of red cell autoantibodies was shown experimentally by Hirszfeld and Lille-Szyszkwicz in colostrum, by Milgrom and Wozinczko in pleural effusion, and by Milgrom, Wozniczko and Dudziak in the fluid of artificially produced blisters. The above-mentioned authors supposed that the autoantibodies reaching the circulation are absorbed by the red cells.

Clear evidence of the correctness of Bernstein's theory could be furnished by the demonstration of autoantibodies coating the red cells. In spite of efforts along this line, including those of the present authors, no one has succeeded in recovering autoantibodies from the red cells of normal human beings. Nevertheless, it can be supposed that the experiments failed because physiologic sensitization of red cells is very weak. For this reason we have tried to recover the autoantibodies from the powder of dried cells. The cell volume is considerably reduced by drying, making it possible to elute about 20 ml of packed cells with only 1 ml of saline.

Twenty-five samples of erythrocyte powder of all ABO blood groups were tested. The amount of eluted powder was in some experiments as great as 200 mg. The elution was performed with saline and with AB serum. The antibodies were tested on slides and in tubes with A, B and O cells suspended in saline and AB serum. Indirect Coombs' tests were performed in almost every case. In no instance could any antibody be detected in the eluate. In the control experiments it was shown that the procedure of powder preparation does not destroy the anti-D, anti-Rh antibody with which the red cells were sensitized in vitro.

In the interpretation of the negative results just described, the following possibilities may be considered:

1. Bernstein's theory is not correct, and there is no production of antibody, in disagreement with Landsteiner's rule.
2. The antibody which is in disagreement with Landsteiner's rule is neutralized by the corresponding antigen before it reaches the circulation.
3. The cells sensitized with autoantibody are immediately eliminated from the circulation.

The second possibility is to some extent in agreement with the findings of Milgrom, Wozniczko and Dudziak, who have obtained considerable quantities of autoantibody in the blister fluid withdrawn 1 hour after trauma but only traces of it in the fluid withdrawn after 24 hours.

Experiment 2. Elution of Anti-A and Anti-B Antibodies

The eluate of anti-A and anti-B antibodies obtained from intact cells always contains a considerable amount of hemoglobin and never shows a
ERYTHROCYTE POWDER AND BLOOD GROUP ANTIBODIES

TABLE 1.—Occurrence of Blood Group Agglutinogens and Agglutinins According to Bernstein and Furuhata

<table>
<thead>
<tr>
<th>Genes</th>
<th>Characteristic Conditions</th>
<th>Gene Domination</th>
<th>Genotypes</th>
<th>Phenotypes</th>
<th>Antibody Produced</th>
<th>Antibody Present in Circulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bernstein</td>
<td>A Agglutinogen A</td>
<td>A&gt;0</td>
<td>OO</td>
<td>anti-A, anti-B</td>
<td>anti-A, anti-B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B Agglutinogen B</td>
<td>B&gt;0</td>
<td>BO, BB</td>
<td>anti-A, anti-B</td>
<td>anti-A, anti-B</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AB</td>
<td>nil</td>
<td>nil</td>
<td></td>
</tr>
<tr>
<td>Furuhata</td>
<td>A Agglutinogen A</td>
<td>A&gt;a</td>
<td>(ab)</td>
<td>O</td>
<td>anti-A, anti-B</td>
<td>anti-A, anti-B</td>
</tr>
<tr>
<td></td>
<td>B Agglutinogen B</td>
<td>B&gt;b</td>
<td>(ab), (Ab), (Ab), (aB), (aB)</td>
<td>B</td>
<td>anti-A</td>
<td>anti-A</td>
</tr>
<tr>
<td></td>
<td>a Agglutinin Anti-A</td>
<td></td>
<td>(ab), (aB), (aB), (aB)</td>
<td></td>
<td>nil</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b Agglutinin Anti-B</td>
<td></td>
<td>(Ab), (Ab), (Ab)</td>
<td></td>
<td>nil</td>
<td></td>
</tr>
</tbody>
</table>

High titer. The eluate obtained from erythrocyte powder was water-clear and contained only about 10 mg. per cent of protein. The titer of these antibodies was in some experiments as high as 1:1000.

The described technic of elution was used to elucidate the problem of the "linkage" of anti-A and anti-B antibodies in O sera.

Hektoen and Landsteiner and Witt have shown that from A cells agglutinated by O serum, not only anti-A but also anti-B antibodies may be recovered; similarly, anti-A antibodies as well as anti-B antibodies may be eluted from B cells agglutinated by O serum.

Based on his own experiments, Milgrom advanced a theory of multispecificity of natural antibodies. This term is attributed to antibodies, of which one molecule contains receptors against different antigens. Assuming trivalence of antibodies, the author distinguished the following antibody types in the serum group O: aαa, aαo, aαo, aαβ, aαβ, aββ, aβo, aββ, Bβo, Bββ, where α designates anti-A receptor, β anti-B receptor, and o lack of receptor or some non-group receptor.

Dodd and Bird assume also that O serum contains some antibody molecules of double anti-A plus anti-B specificity.

On the other hand, Matsunaga, Wiener and Wiener et al. assume that the O serum contains besides anti-A and anti-B an additional anti-C antibody, which reacts with the C antigen shared by A, B and AB cells and lacking in O cells.

Group A erythrocyte powder was sensitized with O serum. After careful washing, the powder was eluted with saline. The eluate agglutinated A and B cells. The eluate was then neutralized with group A-specific polysaccharides, one drop of commercial polysaccharide preparation was added per 5 drops of eluate and the tubes were allowed to stay 20 minutes at room temperature. The neutralized eluate was examined in tube agglutination tests with A and B cells. Complete disappearance of anti-A activity was demonstrated; but the anti-B activity was in most experiments only slightly decreased. This experiment was repeated many times with the same results.

*Analogous experiments with the eluate of B erythrocyte powder could not be performed because of marked A activity of the commercial preparations of B polysaccharides. It should be emphasized that the commercial blood group substances used here (Sharp & Dohme) are of animal origin, and there is no evidence that they are identical with the substances of human red cells.
Similar experimental results were obtained by Dodd\textsuperscript{11} with intact red cells. 

According to the theory of C antigen\textsuperscript{12-15} the eluate from A erythrocyte powder sensitized by O serum contains anti-A and anti-C antibodies. The agglutination of B cells is explained by the action of anti-C antibody. After the addition of A polysaccharides, two events could take place: (1) neutralization of anti-A antibody only, assuming that A polysaccharides do not contain C antigen and (2) neutralization of both anti-A and anti-C antibodies, if A polysaccharides contain C antigen. In case 1, the anti-C antibody present in the eluate would agglutinate both A and B cells. In case 2, the eluate would not contain any antibodies and would react neither with A nor with B cells. In none of the cases could the results obtained in the experiments (no agglutination of A cells and agglutination of B cells) be anticipated.

The experiments described furnish additional evidence for the theory of the multispecific character of anti-A and anti-B antibodies in O sera. According to this theory,\textsuperscript{9,10,16} eluate from A erythrocyte powder sensitized with O serum contains antibodies with a receptor, that is of the types: \(\alpha\alpha\alpha, \alpha\alpha\alpha, \alpha\alpha\alpha, \alpha\alpha\beta, \alpha\beta\alpha, \alpha\beta\beta\). The anti-B activity of this eluate is explained by the presence of anti-B receptors. The addition of A polysaccharides neutralizes anti-A receptors, but anti-B receptors of multispecific antibody remain un-neutralized, explaining the persistent anti-B activity (table 2).

It should be stressed that these experiments do not exclude the presence of C antigen. However, evidence has been presented that the C antigen theory is not able to explain all the properties of anti-A and anti-B antibodies of O sera.

**Experiment 3. Elution of Anti-Rh Antibodies**

For the sensitization of group O Rh + erythrocyte powder, an incomplete anti-D (anti-Rho) serum group O was used, with a titer of 1:1024 in serum-albumin medium. No traces of anti-A or anti-B activity were shown in the eluate in the AB serum and saline.

### Table 2.—Reactions of the Eluate of A Erythrocyte Powder Sensitized with O Serum. Reactions I and II Expected According to Theories of C Antigen and Multispecificity of Natural Antibodies Respectively. III = the Observed Data

<table>
<thead>
<tr>
<th>Antibodies Present</th>
<th>Agglutination of Red Cells</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Original Eluate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I Anti-A, anti-C</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>II (\alpha\alpha\alpha, \alpha\alpha\alpha, \alpha\alpha\beta, \alpha\beta\alpha, \alpha\beta\beta) &amp; + &amp; +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Eluate Neutralized with Group A Polysaccharides</th>
<th>Agglutination of Red Cells</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>I 1| Anti-C</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>II 2| nil</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>(\alpha\beta\alpha, \alpha\beta\beta)</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*\(\alpha\) Anti-A receptor, \(\beta\) anti-B receptor, \(\alpha\) lack of receptor or non-group receptor.

\|Case 1: Group A polysaccharides do not contain C.
\|Case 2: Group A polysaccharides contain C antigen.
The eluate in AB serum had all the properties of an incomplete anti-D (anti-Rh₀) serum of group AB. It had an agglutinating titer in colloidal medium of 1:2048, and could be successfully applied for Rh testing of red cells of all ABO groups. It sensitized strongly Rh⁺ cells for the action of Coombs serum, and the careful washing of sensitized cells was required to obtain positive indirect Coombs' test.

The eluate in saline did not agglutinate the red cells. It sensitized strongly the Rh⁺ red cells for the action of Coombs' serum. The washing of the sensitized cells could be omitted without any appreciable influence on the indirect Coombs' test.

The Coombs' test could also be performed in a way somewhat similar to the one applied by Milgrom, Dubiski and Wozniczko in their experiments on human sera with "anti-antibody." One drop of Coombs' serum was mixed with one drop of saline eluate, then immediately one drop of 3 per cent suspension of Rh⁺ red cells was added. The tubes were incubated for 30 minutes at 37 C. and for 15 minutes at room temperature. The agglutination was estimated after one minute of centrifugation. A typical example is illustrated in table 3.

A powder from dried human erythrocyte stromata was prepared to determine whether normal autoantibodies could be recovered from unsensitized erythrocyte powder and to study some of the properties of anti-A, anti-B and anti-Rh₀(D) antibodies recovered from sensitized powder.

Twenty-five samples of unsensitized erythrocyte powder of all ABO blood groups were tested. The amount of eluted powder was as great as 200 mg,
Fig. 1.—Coombs' test with Rh + red cells sensitized with: (a) anti-D serum and (b) saline eluate of anti-D antibody. Reactions with cells not washed after sensitization.

representing about 25 ml. of packed red cells. In no instance could antibody be detected in the eluates. This negative finding could be explained in three ways: by lack of antibody production, in disagreement with Landsteiner's rule; by neutralization of antibody by a corresponding antigen before it reached the circulation; or by immediate elimination of sensitized erythrocytes from the circulation.

The eluate of A erythrocyte powder sensitized with O serum agglutinated both A and B red cells. After neutralization with A polysaccharides, the anti-A antibody disappeared and anti-B remained. These experiments appear to support the theory of the multispecific character of natural antibodies.

Rh-positive erythrocyte powder was sensitized with an incomplete anti-D (anti-Rh,) serum and eluted in saline. The eluate did not agglutinate Rh-positive cells, but sensitized them for the action of Coombs' serum. The mixture of Coombs' serum with the eluate also produced agglutination of Rh-positive cells. These phenomena are explained as being due to the lack of or the very small amount of ballast proteins in the saline eluate.

SUMMARIO IN INTERLINGUA

Un pulvere de desiccate stromas de erythrocytos human esseva preparate pro determinar si normal autoanticorpore pote esser recovrate ab pulvere de non-sensibilisate erythrocytos e pro studiar certe proprietates de anticorpore anti-A, anti-B, e anti-Rh(D) recovrate ab pulvere sensibilisate.
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Vinti-cinque specimens de non-sensibilisate pulvere erythrocytic de omne gruppus de sanguine ABO esseva testate. Le quantitate de pulvere eluite esseva usque a 200 mg, i.e. le equivalente de circa 25 ml de paccate erythrocytos. In nulle caso poteva anticorpore esser detegite in le eluatos. Iste constatation negative poteva esser explicate in tres manieras: (1) Per manco del production de anticorpore, in disaccordo con le regula de Landsteiner; (2) per le neutralisation de anticorpore per un correspondente antigeno ante que illo attingeva le circulation; o (3) per le elimination immediate de sensibilisate erythrocytos ab le circulation.

Le eluato de pulvere de erythrocytos A sensibilisate con sero O agglutinava erythrocytos de gruppo A si ben como de gruppo B. Post le neutralisation con polysaccharidos de gruppo A, le anticorpore anti-A dispareva, e le anticorpore anti-B remaneva. Iste experimentos pare supportar le theoria de un character multispecific del anticorpos natural.

Pulvere de erythrocytos Rh-positive esseva sensibilisate con un sero incomplete anti-D (anti-Rh) e eluite in un solution salin. Le eluato non agglutinava cellulas Rh-positive sed sensibilisava los pro le action de sero de Coombs. Le mixtura de sero de Coombs con le eluato etiam produceva le agglutination de cellulas Rh-positive. Iste phencmenos es explicate como resultato del absentia o del presentia de solmente micrissime quantitates de proteinas ballast in le eluato salin.

REFERENCES

Recovery of Blood Group Antibodies from Erythrocyte Powder

FELIX MILGROM, CARLOS ORELLANA and MIGUEL LAYRISSE