The Amount of Blood Group A Substance on Platelets is Proportional to the Amount in the Plasma

By John G. Kelton, Colleen Hamid, Sylvia Aker, and Morris A. Blajchman

To investigate whether platelet ABO antigens are intrinsic to platelets or adsorbed from plasma, the amount of A-antigen on washed platelets was determined and related to the Lewis and secretor phenotypes, as these genes control the amount A, B, or H antigen present in plasma. The A-antigen on platelets was quantitated using a platelet–erythrocyte mixed agglutination assay in 28 A1 individuals of different Lewis and secretor phenotype. There was a direct correlation between the agglutination score and the predicted amount of A-antigen in the plasma. The highest agglutination scores were obtained with platelets from Le(a−b−) secretors, and the lowest score with platelets from Le(a−b+) nonsecretors. The quantity of A-substance on platelets from A1 individuals was significantly higher than from A2 individuals of the same Lewis and secretor phenotype (n = 17; p < 0.01). Group 0 platelets were incubated with A-plasma in order to determine the rate of uptake of A-substance from plasma. Within 40 min of incubation, the 0 platelets had reached 80% of the amount present after 24 hr of incubation. When 0 platelets were incubated with plasma for A donors of selected Lewis and secretor phenotypes, the agglutination scores of washed platelets was directly proportional to the amount of A-antigen in the plasma, (r = 0.8, n = 14; p < 0.01). Platelets from group 0 donors were infused into an A1, Le(a−b+) thrombocytopenic patient. Two hours after infusion, platelets obtained from the patient gave similar agglutination scores as platelets from A1, Le(a−b+) donors. These studies indicate that A-substance is rapidly adsorbed onto platelets both in vitro and in vivo and are consistent with the hypothesis that extrinsically adsorbed antigens are primarily responsible for the ABO type of platelets.

MATERIALS AND METHODS

Preparation of Platelets

Twenty milliliters of whole blood from healthy volunteers of known blood group was drawn into either 0.3 ml of 10% EDTA or ACD (5:1, v:v). Platelet-rich plasma was isolated by centrifugation at 2230 g for 10 min at 22°C, and washed twice with 0.015M EDTA, then once with 0.9% sodium chloride (pH 6.8). The platelets were then resuspended in 0.9% sodium chloride. To define the optimal test platelet count, varying concentration of platelets (20,000–1000 x 10^3/µl) were tested.

Determination of Blood Group, Lewis, and Secretor Phenotype of the Platelet Donor

The ABO group, A subtype, Lewis, and secretor phenotype of the platelet or plasma donor were determined using commercial antisera and standard techniques. The secretor phenotype of Le(a−b−) individuals was determined using saliva inhibition tests.

Preparation of A-Plasma for Adsorption Studies

Plasma from donors of known phenotype was collected into ACD (5:1, v:v). After centrifugation, the platelet-poor plasma was inactivated (56°C for 30 min) and filtered through a 1.2-µ filter.

Preparation of Test Antiserum

Commercial anti-A, anti-B, and anti-AB antisera was absorbed for 60 min at 22°C with washed platelets from group 0 donors. The platelets were then separated by centrifugation at 3000 g for 15 min, and the antisera aliquoted into plastic tubes and stored at −70°C until use.

Quantitation of Blood Group Substance on the Platelet Surface

Platelet–Erythrocyte Mixed Agglutination

The method used was a modification of a previously described technique. In brief, a fixed “optimal” concentration at washed test
platelets were incubated with increasing titers of antiserum. After washing the test platelets, the antiserum bound to these platelets was assessed by the addition of appropriate indicator red cells. One-hundred microliters of a 3 times washed test platelet suspension (final concentration 400,000/μl) was incubated at 22°C for 30 min with 500 μl of antiserum.

In all studies the concentration of platelets was fixed (400,000/μl) and the dilution of antisera ranged from neat to 1:512, with doubling dilutions between these two ranges. Previous studies showing a 1:512 dilution of antisera, did not result in any platelet samples causing erythrocyte agglutination. Following the incubation, the platelets were washed with 0.9% sodium chloride (pH 6.8) using a serofuge (Clay Adams, Parsippany, N. J.) at 3000 g for 3 min. Ten microliters of a 4% suspension of washed erythrocytes (A, or B) was added to the platelet suspensions. The red-cell–platelet–erythrocyte agglutination was determined by examining a drop of this mixture microscopically and scored as shown in Table I using a modification of a previously reported method.19

Platelet agglutination often occurred after incubation with antiserum, but the “score” was not obtained from these agglutinates, but after assessing the number of erythrocyte agglutinates according to the criteria used in Table I.

To illustrate the scoring system, the result of a representative experiment is shown in Fig. 2. The score (or grade) of the reaction for each dilution of antisera was determined by using Table I, all scores added together to obtain a final numerical score for those test platelets.

The scoring of all test results was done by a single technologist who had no knowledge of the Lewis or secretor phenotype of the platelet donor.

Determination of Methodology for the Assay

The following variables were assessed in the platelet–erythrocyte mixed agglutination assay: platelet count (final concentration 10⁴–10⁵/μl); relative volume of antiserum to platelet suspension; optimal temperature of reaction (22°C and 4°C); duration of incubation of antisera with the platelet suspension (5–45 min); and the volume (10, 25, 50, and 100 μl), the concentration (2%, 4%, 6%, 8%, and 10%), and the A subtype of the indicator red cells.

The specificity of this technique was investigated by determining the ABO group of platelets from 50 donors of different ABO groups.

The sensitivity of this technique was evaluated by mixing platelets from an A₁ individual with platelets from an O individual, so the final percent of test platelets from the A₁ individual ranged from 2% to 100%.

Platelets from A₁ or A₂ individuals of selected Lewis and secretor phenotypes were scored using the platelet–erythrocyte mixed agglutination assay.

The Uptake of A-Antigen by Platelets From Group O Individuals

In Vitro Studies

In these series of experiments, platelets from group O individuals were isolated and washed twice with 0.015 M EDTA and more with 0.9% sodium chloride. The washed platelets were incubated with 7–10 ml test plasma so that the final platelet concentration was 300,000–400,000/μl. After variable incubation times (see below), the platelets were washed three times and the amount of A-antigen measured as described previously. To assess the effect of time on the uptake of A-antigen by O platelets, platelets from group O individuals were incubated with plasma from an A₁, Le(a+b−) donor for varying periods of time (5 min to 24 hr) at 22°C. The platelets were washed, and a numerical score obtained (see Table I) using the platelet–erythrocyte mixed agglutination assay.

The effect of the Lewis and secretor phenotype of the plasma donor on the uptake of A-antigen by platelets from group O donors was investigated. Platelets from O donors were incubated for 60 min at 22°C with plasma from different A₁ individuals of different Lewis and secretor phenotypes. The platelets were washed 3 times (0.9% sodium chloride, pH 6.8) and the A-antigen quantitated using the platelet–erythrocyte mixed agglutination assay.

The reversibility of the binding of A-antigen to group O platelets was also investigated. Platelets from group O donors were incubated with plasma from A₁ donors for 60 min at 22°C. The platelets were washed 3 times and then incubated with plasma from an O donor for a further 60 min at 22°C, washed, and the amount of A-antigen determined. The anti-A had been adsorbed from the O plasma by prior incubation with A₁ erythrocytes.

In Vivo Studies

A group A₁, Le(a−b+) man had acute myelogenous leukemia and was treated with standard chemotherapeutic agents. Both direct and indirect platelet-associated IgG were within the normal range, indicating that he was not alloimmunized.11,12 Two weeks later he developed a regenerative thrombocytopenia with a platelet count of 5000/μl. He was given 15 U of random group O donor platelets. Two hours after receiving the transfusion, his platelet count was 15,000/μl. The amount of A-antigen on the circulating platelets was quantitated using the platelet–erythrocyte agglutination assay.

RESULTS

Platelet–Erythrocyte Mixed Agglutination Assay

The ABO group of the platelets was identical to the ABO group of the erythrocytes for each sample from 50 platelet donors, including 28 group A, 12 group B, 8 group O, and 2 group AB.

An experiment to determine the effect of varying incubation times on the platelet–erythrocyte mixed agglutination assay using different dilutions of antiserum is illustrated in Fig. 1. Low concentrations of antiserum required longer incubation for the maximal

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**Table 1. Scoring System of Platelet–Erythrocyte Mixed Agglutination Assay**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Score</th>
<th>Microscopic Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>12</td>
<td>All red cells are agglutinated in rosette formation around platelets.</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>The majority of red cells are agglutinated in rosettes around platelets, with few free red cells.</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>Many small platelet–red-cell agglutinates, with many free red cells.</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>One or two small agglutinates per microscopic field, most red cells are not agglutinated.</td>
</tr>
<tr>
<td>wk</td>
<td>3</td>
<td>Less than one rosette per microscopic field.</td>
</tr>
<tr>
<td>wk</td>
<td>2</td>
<td>Rare platelet–erythrocyte agglutinates, less than 1/10 microscopic fields.</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>No platelet–erythrocyte agglutinates.</td>
</tr>
</tbody>
</table>
agglutination to occur. There was an inverse relationship between the agglutination score and the dilution of antiserum (Fig. 2).

The platelet-erythrocyte mixed agglutination assay could detect the presence of as few as 2% A₁ platelets in a suspension of platelets from group 0 donors. Increasing scores occurred until the mixture contained approximately 50% A₁ platelets, at which point the agglutination score reached a plateau (see Fig. 3).

The agglutination scores of 11 samples of platelets from A₁ individuals of Le(a⁻b⁺) phenotype were higher than the score of platelets from 6 A₂ donors of identical Lewis and secretor phenotype (see Fig. 4) (p < 0.01, unpaired t-test).

Platelets from 27 A₁ individuals of selected Lewis and secretor phenotype were scored using the platelet-erythrocyte mixed agglutination assay (Fig. 5), and there was a significant relationship between platelet score and the predicted amount of A-antigen in the plasma (ranked from 1 to 4) (r = 0.6, p < 0.01). The highest scores were obtained with platelets from 8 Le(a⁻b⁻) secretors. Platelets from the 2 Le(a⁻b⁻) nonsecretors gave the lowest scores. Platelets from Le(a⁻b⁺) and Le(a+b⁻) individuals gave intermediate scores.

The Uptake of A-Antigen by Platelets From Group 0 Individuals

In Vitro Studies

The uptake of group A antigen onto platelets from group 0 donors was rapid. Within 40 min, the uptake had reached a plateau (Fig. 6), and further incubations of up to 24 hr did not significantly increase the score.

The A-antigen adsorbed onto platelets from group 0 donors was not tightly bound. The agglutination score decreased by approximately 50% after 60 min of incubation with 0 plasma.

Washed platelets from group 0 individuals were incubated with plasma from 14 A₁ individuals of selected Lewis and secretor phenotypes (Fig. 7). The score of the platelets was approximately one-third lower than the score given by the donors’ own platelets (see Figs. 5 and 7). Anti-A antiserum consistently gave higher agglutination scores than anti-AB antiserum. Platelets incubated in plasma from A₁ Le(a⁻b⁻) secretors gave the highest score, whereas plasma from the A₁ Le(a⁻b⁻) nonsecretor gave the lowest score, and there was a significant relationship between the score and the predicted amount of A-antigen in the plasma (r = 0.8, p < 0.01, n = 14). Platelets incubated with plasma from Le(a⁻b⁺) donors have a higher
Fig. 3. The platelet–erythrocyte mixed agglutination assay score (ordinate) obtained with different mixtures of A₁, Le \((a - b +)\) platelets and O platelets (abscissa). A score than those incubated with Le\((a + b -)\) individuals.

**In Vivo Studies**

Assuming equal mixing of the transfused platelets with the patient’s own platelets, approximately 4% of the platelets tested were the patient’s own platelets. Platelets obtained from the patient 2 hr after the transfusion typed as A with a score of 82 using the platelet–erythrocyte mixed agglutination assay. A 4% mixture of A₁, Le\((a - b +)\) platelets in 96% O platelets gave a score of approximately 40 (see Fig. 3).

**DISCUSSION**

Although platelets can acquire A and B antigens from the plasma in vitro,\(^3\) it is uncertain whether these antigens are acquired from plasma in vivo or are intrinsically part of the platelet membrane. The present study indicates that the amount of A-antigen on the platelet surface, as assessed by the platelet–erythrocyte mixed agglutination assay, can be correlated with the amount of free antigen in the plasma.

**PHENOTYPE OF PLATELET DONOR**

Fig. 4. The platelet–erythrocyte mixed agglutination score (ordinate) of platelets obtained from 17 A₁ or A₂ individuals of the same Lewis and secretor phenotype.

**PHENOTYPE OF A₁ PLATELET DONOR**

Fig. 5. The effect of the Lewis and secretor genes (abscissa) on the platelet–erythrocyte mixed agglutination score of platelets obtained from 27 different individuals of blood group A₁. The phenotype of the platelet donor (abscissa) is ranked in descending order from left to right according to the amount of A-antigen in the plasma.
The source of A and B antigens on circulating blood cells has been the subject of several studies in recent years. The majority of ABH antigens on erythrocytes are intrinsic to the membrane, whereas the same antigens on lymphocytes appear to be derived from the plasma. Thus, if plasma-derived A-antigen contributed to the amount of A-antigen on the platelet surface, then platelets from Le(a–b–) individuals who were secretors would have the most A-antigen on their platelets. Conversely, platelets from nonsecretors of the same Lewis phenotype would have the least, as the Lewis and secretor genes control the quantity of free antigen in plasma.

Using the platelet-erythrocyte mixed agglutination assay, the relationship between the amount of antigen on the platelets and the red cell phenotype of the platelet or plasma donor was studied. This assay was specific; the blood group of platelets from 50 individuals was identical to the ABO group of the red cells. This assay was also sensitive and could detect a mixture of 2% A, platelets in 0 platelets.

The platelet-erythrocyte mixed agglutination score was significantly higher using platelets obtained from A, individuals than A2 individuals of the same Lewis and secretor phenotype (Fig. 4). This observation is consistent with the hypothesis that adsorption of extrinsic antigens play an important role in determining the amount of A-antigen on the platelet surface, as there is more A antigen in the plasma of A, compared to A2 donors. This observation, however, also supports the alternative hypothesis that the A-antigen is intrinsic, since A, erythrocytes have more antigen than A2 erythrocytes.

When the amount of A-antigen on platelets from A, donors of different Lewis and secretor phenotypes was assessed using the platelet-erythrocyte mixed agglutination assay, the highest scores were obtained with platelets from Lewis-negative secretors and the lowest scores with Lewis-negative nonsecretors. No difference was observed in the score of platelets from donors who were Lewis-positive and either secretor-positive or secretor-negative (Fig. 5). However, a difference became apparent when 0 platelets were incubated with plasma from these individuals, and the amount of bound A-antigen estimated from the score (Fig. 7).

The method used in this report to investigate the amount of A-antigen on platelets was indirect and relatively crude. Lower quantities of A-antigen could be more readily measured than higher amounts (Fig. 3). This may explain why greater differences in the scores were observed when 0 platelets were incubated with plasma from donors of different Lewis and secretor phenotypes compared to the score of the donors own platelets. It is also possible that the lower
temperature used in the in vitro studies (22°C) could contribute to the in vitro, in vivo difference. A 5:1 ratio of antiserum to test platelets was used in all studies. Higher ratios might have demonstrated a clearer separation of the scores of platelets from donors of different Lewis and secreter phenotypes, but would have required much greater amounts of antiserum.

Blood group A antigen rapidly bound to platelets from group 0 donors both in vitro and in vivo. In vitro, approximately 80% of the maximal binding, assessed by the score, occurred within 40 min. However, the score was invariably less than that obtained using the plasma donor's own platelets. Similarly, the administration of group O platelets to an nonalloimmunized individual of blood group A,, Le(a – b +) resulted in the platelets typing as group A when tested 2 hr after transfusion. The score of these platelets was similar to the score obtained from platelets of an A,, Le(a – b +) donor.

These studies raise a number of issues. Plasma-derived A-antigen is reported to have type 1 configuration, whereas intrinsic A-antigen has a type 2 configuration. Since antiserum from O individuals (anti-AB) has greater anti-type-I specificity than antisera from B individuals, the anti-AB antiserum might have been expected to give a different score than anti-A antiserum when extrinsically derived A-antigen was measured (Fig. 7). The reason why we did not observe this is uncertain. We also observed that the amount of A-antigen in the plasma from Le(a – b –) non secreter individuals was reduced, but significant amounts were present (Fig. 7). The source of this antigen is not known.

These studies indicate that group A antigen can readily be acquired by the platelet both in vitro and in vivo, and plasma-derived antigen contributes to the amount of A-antigen on the platelet. It remains uncertain whether any group A antigen on platelets is intrinsically derived. In vitro studies also indicate that acquired A-antigen can elute from the platelet into the plasma. These observations may also explain why ABO-incompatible platelets have a reduced recovery when transfused in vivo but are not entirely removed from the circulation.

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