Removal of HLA-A,B Antigens From Platelets

By Neil Blumberg, Debra Masel, Theodor Mayer, Paul Horan, and Joanna Heal

Recent evidence suggests that HLA-A,B antigens, present on human platelets, are acquired from plasma. Using hypertonic acid chloroquine (200 mg/ml, pH 5.0, 1 hr, 4°C), we demonstrate elution of most or all HLA-A2 and HLA-B7,40 immunoreactive material from platelets. Corre-
sponding antigens on human lymphocytes were not affected by this treatment. These findings are consistent with the hypothesis that HLA-A,B antigens are adsorbed onto platelet surfaces.

AN UNDERSTANDING of the structure of human platelet membrane antigens is of both biologic and clinical interest. HLA-A,B antigens and those of the ABH system are present on platelets. However, adsorption of exogenous soluble HLA-A,B antigens and ABH substances onto platelets has been demonstrated in vitro. This raises the question of whether these antigens are integral membrane proteins, as has been shown for the HLA-A,B antigens of lymphocytes, or are adsorbed onto the platelet surface.

Nondestructive removal of HLA-A,B antigens from platelets should be feasible if they are indeed adsorbed from plasma. The antimalarial drug, chloroquine, is known to dissociate immune complexes and to remove antibody from red cell surfaces. Because unpublished data from our laboratory demonstrated that chloroquine treatment removed passively adsorbed Lewis blood group antigens from red cells, we attempted to similarly dissociate HLA-A,B antigens from platelets.

MATERIALS AND METHODS

Detection of HLA-A,B Antigens on Platelets

Human platelets from normal blood donors of known HLA-A,B phenotype were collected in EDTA vacuum tubes (Sherwood, St. Louis, MO). These were washed three times (2,500 g/10 mm) with phosphate-buffered saline EDTA solution (PBS-EDTA: 3.0 g/liter NaCl, 3.75 g/liter Na2HPO4, and 8.2 g/liter NaCl at pH 7.0) and resuspended at a count of 350,000/d. Murine monoclonal anti-A2 and anti-B7,40 antibodies (Atlantic Antibodies, Scarborough, ME) were used to detect these HLA antigens on platelets by employing an enzyme-linked antiglobulin test. Affinity-purified anti-mouse IgG coupled to alkaline phosphatase (Sigma, St. Louis, MO) was used to detect monoclonal anti-HLA antibody bound to platelets. Bound anti-HLA was measured as endpoint optical density at 405 nm (OD405). Measurements were made in microtiter plates (Immulon II) using a through-the-plate spectrophotometer (micro ELISA Reader, Dynatech, Alexandria, VA).

Chloroquine Treatment of Platelets and Lymphocytes

To determine if chloroquine removed HLA-A,B antigens, washed platelets obtained from 7 ml of EDTA whole blood were incubated (4°C, 1 hr) either with 4 vol of chloroquine diphosphate (200 mg/ml, pH 5.0, osmolarity 980 mosmole; Gamma Biologicals, Houston, TX) dissolved in PBS-EDTA, or with PBS-EDTA alone. Resultant pH is 5.1 and osmolarity is 840 mosmole. After washing and adjusting platelet counts to 350,000/μl, 0.2 ml of platelets was incubated with 50 μl of murine anti-A2 or anti-B7.40 (1:100 dilution with PBS in 1% bovine serum albumin [BSA; Sigma]) for 1 hr at 37°C. Platelets were washed, resuspended in 0.2 ml PBS-BSA, and then incubated for 1 hr at 37°C with 0.2 ml of anti-mouse IgG coupled to alkaline phosphatase (1:500 in PBS-BSA). After further washing, 0.2 ml of substrate solution (paranitrophenyl phosphate: 1 mg/ml in 5.3 g/liter Na2CO3, 0.2 g/liter MgCl2 · 6H2O, pH 9.8; Sigma) was added to the platelet pellets and color development was allowed to proceed for 1 hr. Reactions were stopped by addition of 0.1 ml of 1 M NaOH. After centrifugation, 0.2 ml of each reaction product was transferred to a well of a microtiter plate and OD405 was measured. Absorbance at a wavelength of 405 nm (OD405), 0.4 ml at 1,000/μl was incubated with 4 vol of chloroquine solution or PBS-EDTA at 4°C for 1 hr.

Detection of HLA-A2 Eluted From Platelets by Chloroquine

Supernatants from platelets treated with 200 mg/ml chloroquine were tested for the presence of eluted HLA-A2 antigen by a solid-phase enzyme-immunoassay. To remove chloroquine, supernatant eluates (1.0 ml) from chloroquine-treated A2-positive and A2-negative platelets were passed through a 1 x 25 cm G-25 Sephadex (Pharmacia) column, equilibrated with microtiter plate coating buffer (1.6 g/liter Na2CO3, 2.93 g/liter NaHCO3, pH 9.8). After the void volume was collected, 5 0.5-ml fractions were collected and incubated in microtiter plate wells (Immulon II), each in 5 0.1-ml aliquots (37°C for 2 hr, then 4°C for 14 hr). Plates were washed (Minitwash, Dynatech) (PBS-EDTA-1% BSA), incubated with 0.2 ml of 1:200 mouse monoclonal anti-A2 for 30 min at 37°C, washed, and incubated for 1 hr at room temperature with 0.2 ml of anti-mouse IgG coupled to alkaline phosphatase (1:500). After a final wash, 0.2 ml of substrate solution was added and the OD405 measured at 1–1.5 hr. Mean results of the 5 wells were used for each column fraction. A ratio of the OD405 for the A2-positive eluate to
the OD, for the A2-negative eluate was computed for each fraction in 3 separate experiments. A ratio of 1 or less would indicate no detectable A2 antigen in the chloroquine eluate from the A2-positive platelets.

RESULTS

Table 1 shows the results of anti-A2 binding to A2-positive and -negative platelets before and after chloroquine treatment. Chloroquine treatment reduced the binding of anti-A2 to A2-positive platelets to the level of binding to A2-negative platelets. Similar results with anti-B7,40 and B40-positive and -negative platelets were obtained (Table 2). Results of testing chloroquine eluates from A2-positive and -negative platelets after passage over G-25 Sephadex to remove chloroquine are shown in Fig. 1. A2 immunoreactive material was present only in eluates from A2-positive platelets.

Table 3 displays the results of anti-A2 binding to A2-positive and -negative lymphocytes and platelets before and after chloroquine (50 mg/ml) treatment. Chloroquine at this concentration significantly decreased binding of anti-A2 to A2-positive platelets, but not to the level of that of A2-negative platelets, as 200 mg/dl chloroquine had done. Chloroquine treatment slightly increased binding of anti-A2 to A2-positive lymphocytes, but the ratio of binding, A2-positives:A2-negatives, was virtually identical with (2.20) or without (2.26) chloroquine treatment. In contrast, the ratio of binding A2-positives:A2-negatives for platelets was 2.40 before chloroquine treatment and decreased to 1.55 afterward.

Studies using 200 mg/ml chloroquine eluting solutions at pH 6 or 7 and hypertonic (980 mosmole) saline solutions without chloroquine (pH 5.0) demonstrated that the presence of chloroquine at the more acid pH was necessary for elution. Chloroquine alone at isotonic concentrations (80 mg/ml; pH 5.0) eluted only small amounts of HLA-A,B antigens from platelets. Binding of anti-P11 antibody to P11-positive platelets was unaffected by chloroquine pretreatment of the platelets (data not shown). Thus, the P11 antigen does not appear to be removed from platelets by chloroquine.

DISCUSSION

Our data suggest that hypertonic acid chloroquine treatment rapidly removed HLA-A,B immunoreactive material from human platelets, but not from lymphocytes. It is theoretically possible that the monoclonal antibodies we used preferentially recognized adsorbed HLA-A,B antigens compared to intrinsic ones. However, the demonstration that HLA-A,B antigens are detectable on lymphocytes before and after chloroquine treatment suggests that this is unlikely to be the case. We have not performed similar studies using polyclonal anti-HLA antiserum to see if similar results are obtained.

That our results indicate elution of HLA-A,B antigens, rather than destruction, is suggested by our ability to recover antigen in the eluates from HLA-

![Fig. 1. Binding of anti-A2 to chloroquine eluates of A2-positive and A2-negative platelets. Eluates were fractionated on Sephadex G-25 and fractions bound to microtiter plate wells. The binding of anti-A2 to fractions from an A2-positive eluate was compared to the binding to those of an A2-negative eluate by a ratio of the respective OD, for each fraction. Three experiments with 6 different individuals' platelets were performed. Mean OD, for the A2-positive eluate fractions was 0.158, 0.139, 0.177, 0.188, 0.210 and for the corresponding A2-negative eluate fractions 0.136, 0.137, 0.163, 0.080, 0.063.

Table 1. Anti-HLA-A2 Binding to A2-Positive and Negative Platelets Before and After Chloroquine Treatment

<table>
<thead>
<tr>
<th></th>
<th>Before Chloroquine</th>
<th>After Chloroquine</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>OD</td>
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<tr>
<td>A2-positive</td>
<td>46</td>
<td>0.617 ± 0.287</td>
</tr>
<tr>
<td>A2-negative</td>
<td>56</td>
<td>0.215 ± 0.075</td>
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*p = 0.5.

†p < 0.0001.

All data are OD, ± 1 SD.

Statistical significance by unpaired Student's t test.

Table 2. Anti-HLA-B7.40 Binding to B40-Positive and Negative Platelets Before and After Chloroquine Treatment

<table>
<thead>
<tr>
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<tr>
<td></td>
<td>n</td>
<td>OD</td>
</tr>
<tr>
<td>B40-positive</td>
<td>8</td>
<td>0.516 ± 0.061</td>
</tr>
<tr>
<td>B40-negative</td>
<td>8</td>
<td>0.273 ± 0.107</td>
</tr>
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*Not significantly different (p > 0.2).

†p < 0.001.

All data are OD, ± 1 SD.

Statistical significance by unpaired Student's t test.
A2-positive platelets. These data are consistent with the hypothesis that HLA-A,B antigens are associated with the platelet surface in a manner different from that of lymphocytes. A possible explanation is that they are adsorbed onto the surface of platelets, rather than being integral membrane components. It seems likely that HLA-A,B antigens are acquired from soluble plasma sources rather than from endogenous synthesis during thrombopoiesis. Proof of this hypothesis awaits detailed structural characterization of HLA-A,B antigens.

It appears that the platelet is an immunobiologic "sponge." The platelet's role in cleaning immune components from the circulation has been experimentally studied in humans.\(^\text{12}\)

The theoretical possibility of using washed platelets denuded of HLA-A,B antigens for treatment of patients refractory to platelet transfusion is attractive. However, the methods we describe are unlikely to produce hemostatically effective platelets due to the hypertonic, acid nature of the eluting solution. We have not studied the effects of chloroquine upon in vitro functions nor upon the in vivo hemostatic efficacy of platelets. If conditions can be developed for removing HLA-A,B antigens from platelets without affecting platelet function, the transfusion of such platelets to refractory recipients might be useful.

ACKNOWLEDGMENT

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REFERENCES


Table 3. Binding of Anti-HLA-A2 to A2-Positive and A-2 Negative Lymphocytes and Platelets After Chloroquine Treatment

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Before Chloroquine</th>
<th>After Chloroquine</th>
<th>Statistical Significance Before Versus After</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>OD</td>
<td>n</td>
</tr>
<tr>
<td>A2-positive platelets</td>
<td>30</td>
<td>0.484±0.193</td>
<td>33</td>
</tr>
<tr>
<td>A2-positive lymphocytes</td>
<td>13</td>
<td>1.679±0.134</td>
<td>14</td>
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<tr>
<td>A2-negative platelets</td>
<td>40</td>
<td>0.202±0.089</td>
<td>35</td>
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<tr>
<td>A2-negative lymphocytes</td>
<td>25</td>
<td>0.742±0.160</td>
<td>24</td>
</tr>
</tbody>
</table>

*\(p < 0.001\).
\(\dagger p < 0.001\).

All data on OD\(_{\text{nm}}\) ± 1 SD.

Statistical significance by unpaired Student's t test.
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