Quantitative Analysis of Platelet Surface HLA by W6/32 Anti-HLA Monoclonal Antibody

By K.J. Kao, Daniel J. Cook, and Juan C. Scornik

Class I molecules of human major histocompatibility complex (HLA) are the most important antigenic system in determining the survival of transfused platelets in alloimmunized patients. Platelets with reduced expression of a specific type of HLA antigen may escape specific anti-HLA antibody-mediated destruction. By using 125I-labeled Fab fragments of W6/32 anti-HLA monoclonal antibody and competitive protein binding assays, we measured the range of total HLA concentrations on platelets. In 12 individuals examined, the mean number of HLA-A, B, and C molecules per platelet was 81,587 ± 20,016 (mean ± SD); its range was between 54,782 to 116,185 molecules per platelet. After treatment with chloroquine, 79.9 ± 7.0% (mean ± SD, n = 6) of HLA antigens were removed from platelets as determined by binding of 125I-W6/32 Fab.

The existence of HLA antigens on platelets was first demonstrated with platelet complement-fixation tests in the 1960s1-5 and was later confirmed with the purification of HLA antigens from platelets.6,7 Clinically, HLA is the most important antigen system involved in determining survival of transfused platelets in alloimmunized patients.8-13

Liebert and Aster and co-workers14-18 used an inhibition of lymphocytotoxicity test to show that several B-locus HLA antigens of platelets are variably expressed among different individuals. A threefold variation of HLA-A2 on platelets was also reported by Svejgaard.19 HLA-C antigens are only weakly expressed on human platelets20 and do not seem to play any significant role in the alloimmunization to platelets.21 In addition, platelets with reduced expression of HLA-B12 may escape destruction and survive normally in recipients whose plasma contains antibodies against HLA-B12.22,23 These observations suggest that quantitative difference of HLA antigen expression may have a potential clinical importance and stress the need for more rigorous studies to improve methods of quantitation over serological techniques relied upon by previous investigations.14-19

Recently, Blumberg and colleagues24 reported that acid hypertonic chloroquine can elute a portion of HLA antigens on platelets. The presence of HLA antigens in the chloroquine eluate of platelets could be demonstrated to contain HLA antigens similar in mol wts to intact class I molecules by an immunoblotting technique. These data suggest that 70% to 80% of platelet HLA antigens are adsorbed and that such HLA antigens are not proteolytic products of integral membrane class I molecules. The origin of the adsorbed platelet HLA-antigens remains to be determined.

Isolation of W6/32 anti-HLA monoclonal antibody. The W6/32 hybridoma, obtained from the American Type Culture Collection (Rockville, Md) was originally developed and had been shown to recognize a common monomorphic determinant of HLA-A, B, and C molecules by Barnstable and colleagues.25 The W6/32 hybridoma was maintained in RPMI 1640 culture medium supplemented with 10% FCS, gentamycin (0.5 mg/mL), penicillin (100 U/mL), streptomycin (100 μg/mL), and fungizone (0.25 μg/mL). W6/32 anti-HLA monoclonal antibody was purified from culture medium by protein A-Sepharose CL-4B affinity column chromatography.26 Protein concentration and homogeneity of the purified W6/32 monoclonal IgG antibody were determined by the assay of Lowry and co-workers27 and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)28 respectively.

Preparation of Fab fragments of W6/32 monoclonal antibody. The Fab fragments of W6/32 anti-HLA monoclonal antibody (W6/32-Fab) were prepared by papain digestion as described by Porter.29 In brief, 20 mg of W6/32 antibody in 5 mL of 0.01 mol/L of potassium phosphate buffer, pH 6.5, containing 2 mmol/L of EDTA and 0.01 mol/L of L-cysteine were incubated with 0.4 mg mercuripapain at 37°C for 4 hours. The papain digest of W6/32 antibody was dialyzed with 0.02 mol/L of Tris-HCl, pH 8.0, containing 0.025 mol/L of NaCl at 4°C for 10 hours and then

Materials and Methods

Materials. RPMI 1640 medium, L-cysteine, antibiotic-antimycotic solution, and fetal calf serum (FCS) were purchased from GIBCO Laboratory (Grand Island, NY). Protein A-Sepharose CL 4B, mercuripapain, bovine serum albumin (BSA), nitroblue tetrazolium (NBT), 5-Bromo-4-chloro-3-indolyl phosphate-p-toluidin salt (BCIP), rabbit anti-mouse IgG alkaline phosphatase conjugate, and chloroquine diphasphate were obtained from Sigma (St Louis). Na212I and solid-phase lactoperoxidase were the products of Amersham Co (Arlington Heights, Ill) and P-L, Biochemicals (Milwaukee), respectively. Fluorescein-labeled sheep anti-mouse IgG F(ab')2 fragments and mouse IgG were obtained from Cooperbiomedical, Inc (Malvern, Pa). Goat anti-human IgG F(ab')2 fragments were from Kallestad Laboratory (Austin, Tex). Diethylaminoethyl (DEAE) afi-gel blue and nitrocelullose paper with 0.45-μm pore size were purchased from Biorad Co (Richmond, Calif). All other chemical reagents were analytical grade. Mouse anti-influenza virus type A monoclonal antibody was a generous gift from Dr. P.A. Klein, Department of Pathology, University of Florida.

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loaded on a DEAE affi-gel blue column (1.5 × 10 cm) to separate Fab fragments from Fc fragments. The Fab fragments were recovered from the protein peak in the flowthrough and further purified with a protein A-Sepharose CL 4B column chromatography (1.5 × 3 cm) described above. 

Preparation of washed platelets. Only freshly drawn venous blood from healthy volunteer donors was used. Blood was anticoagulated with 1/6 vol of 3.8% sodium citrate. Platelets were isolated from 18 mL of the blood by differential centrifugation. The isolated platelets were washed twice with 0.02 mol/L of Tris-HCl, pH 7.4, containing 0.15 mol/L of NaCl and 1 mmol/L of EDTA (Tris-saline-EDTA) as described, and finally suspended in 0.02 mol/L of Tris-HCl, pH 7.4, containing 0.15 mol/L of NaCl and 0.2% bovine serum albumin (BSA) (Tris-saline BSA). The platelet concentrations were enumerated by an S-plus Coulter counter. The contamination of WBCs was always <0.03%. 

Chloroquine elution of platelet HLA. The chloroquine solution was prepared as reported by Blumberg and co-workers. In brief, 20 g of chloroquine diprophosphate was dissolved in 100 mL of 0.01 mol/L of sodium phosphate buffer, pH 7.4, containing 0.1 mol/L of NaCl. The pH was then adjusted to 5.0 ± 0.1 by 5 N NaOH. Four volumes of chloroquine diprophosphate were mixed with 1 vol of washed platelets (3 × 10^10 cells/mL) suspended in Tris-saline without BSA and incubated at 25°C for 60 minutes. After incubation, chloroquine-treated platelets were collected by centrifugation at 2,200 g for 10 minutes, washed twice with Tris-saline EDTA, and suspended in Tris-saline BSA for later assay.

Quantitative assay for platelet HLA-A, B, and C antigens. The concentration of total HLA-A, B, and C antigens on platelets was determined by using [125I]-labeled Fab fragments of W6/32 anti-HLA monoclonal antibody (125I-W6/32 Fab) and a competitive protein binding assay. The isolated W6/32 Fab was labeled with 125I by solid-phase lactoperoxidase method as described previously. The specific radioactivity of 125I-Fab to washed platelets were determined by incubating 5 × 10^9 platelets and specific amounts of 125I-W6/32 Fab in 250 μL of Tris-saline-BSA with or without a 160-fold excess of unlabeled W6/32 Fab. After 2 hours of incubation at room temperature, the supernatant of washed platelets was collected by centrifugation at 2,200 g for 10 minutes, washed twice with Tris-saline EDTA, and suspended in Tris-saline BSA for later assay.

RESULTS

SDS-PAGE and Western blot. SDS-PAGE was performed according to the procedure of Laemmli. The supernatant of washed platelet suspension (1 × 10^9 cells/mL) that was treated with 4 vol of chloroquine was collected by centrifugation at 4,000 g for 10 minutes, and dialyzed extensively with Tris-saline. The supernatant was then concentrated 75-fold with Aquacide II and Amicon B15 concentrator and was mixed with an equal volume of sample buffer for SDS-PAGE. Control washed platelets and chloroquine-treated washed platelets were solubilized by incubating 1 × 10^9 platelets with 0.3 mL of 0.5% Nonidet-P40 in PBS and containing 1 mmol/L phenylmethylsulfonyl-fluoride and 1% epsilon-amino-n-caproic acid for 15 minutes on ice. Insoluble cellular debris was removed by centrifugation at 10,000 g for 5 minutes. The solubilized platelets were concentrated threefold with an Amicon B15 concentrator and mixed with an equal volume of sample buffer for SDS-PAGE.

Western blot was performed by following the procedure of Tobin and co-workers in a Trans-Blot cell (Bio-Rad, Richmond, Calif). The blotting process was conducted at constant voltage of 40 V for 4.5 hours. The nitrocellulose blot was blocked with 5% nonfat milk made in PBS containing 0.05% sodium azide and incubated sequentially with W6/32 anti-HLA antibody (20 μg/mL), alkaline phosphatase conjugate of rabbit anti-mouse IgG (1:1,000 dilution), and alkaline phosphatase substrate, which contains 0.1 mg/mL of NBT and 0.05 mg/mL of BCIP as described.

**RESULTS**

**SDS-PAGE of W6/32 monoclonal antibody and W6/32 Fab.** The mouse W6/32 anti-HLA monoclonal antibody is an IgG antibody and reacts specifically with a common determinant of HLA-A, B, and C molecules. As shown in Fig 1, the isolated W6/32 anti-HLA monoclonal antibody appears to be homogeneous and contains equal amounts of two light chains as described previously. One of the light chains is the parental myeloma type. The purified Fab fragments of W6/32 monoclonal antibody are homogeneous, as shown in Fig 1, and have a mol wt of 46 kd. After reduction with mercaptoethanol, Fab fragments also contain...
equal amounts of two intact light chains. The findings suggest that only 50% of W6/32 Fab may be functional and will react with HLA-A, B, and C molecules. This was experimentally tested by incubating 125I-W6/32 Fab (1 \mu g/mL) and platelets (2.0 \times 10^9 cells/mL) that contained an excess of HLA antigens in the presence or the absence of 150 \mu g/mL of unlabeled W6/32 Fab at 25°C for 3 hours. All the incubations were performed in duplicate. The results showed that 47% of 125I-W6/32 Fab specifically bound to platelets, thereby confirming that ~50% of the Fab fragments have HLA binding activity.

Quantitative Assay for Platelet HLA-A, B, and C Antigens. The purified W6/32 Fab was labeled with 125I and used to quantitate platelet HLA concentrations in a competitive protein binding assay.32,33 Fab fragments were used to avoid binding to platelet Fc receptors. First, the specific binding of 125I-W6/32 Fab to 5 \times 10^6 platelets was studied as a function of duration of incubation. The results showed that 2 hours of incubation is required for the binding to reach a steady state (Fig 2). Thereafter, specific binding was determined as a function of increasing concentrations of 125I-W6/32 Fab after a 2-hour incubation time. As shown in Fig 3, the specific binding of 125I-W6/32 Fab to a constant number of platelets was saturable. As expected, the nonspecific binding increased linearly with increasing concentrations of 125I-W6/32 Fab and was not saturable.34 The binding data of Fig 3 were then analyzed by a Scatchard plot38 to determine the steady-state dissociation constant (Kd) and the concentration of total platelet HLA in the incubations. The results showed that the Kd was 6.1 nmol/L and total platelet HLA concentration was 3.35 nmol/L or 100,835 molecules per platelet (Fig 4).

To demonstrate the specificity of ligand binding, human fibrinogen and IgG from human and mouse were used to compete with 125I-W6/32 Fab for binding. As shown in Fig 5, only unlabeled W6/32 Fab can compete for the binding of labeled W6/32 Fab. The results in Fig 5 were also analyzed by Scatchard plots. Similar binding affinity (Kd) and concentration of platelet HLA molecules to those determined from the binding data of Fig 3 were obtained. In these studies (Figs 3 and 5), washed platelets from the same individual were used. The results indicate that both methods are precise quantitations of platelet HLA concentration.

The specific bindings of 125I-W6/32 Fab to human lymphoblastoid cells, platelets, Daudi cells (a Burkitt's lymphoma cell line), and RBCs were also determined to demonstrate that the binding of 125I-W6/32 Fab is specific for class I HLA molecules. Specific binding per 5 million cells was observed in a lymphoblastoid cell line (28,035 cpm) and platelets (11,836 cpm) but not in RBCs (500 cpm) and Daudi cells (191 cpm). The results agree with earlier studies demonstrating that lymphoblastoid cells are rich in HLA antigens39 while HLA antigens are low or absent in Daudi cells40 and RBCs.41

The interassay variation for quantitating platelet HLA concentration by using 125I-W6/32 Fab and Scatchard analysis was also evaluated.42 HLA concentrations on platelets...
harvested from three different persons were determined individually after their immediate collection and on the second and the third day were stored in Tris-saline containing 0.05% sodium azide at 4 °C. The interassay variations for each individual were 6.3%, 6.7%, and 6.8%, respectively.

Platelet HLA concentrations in normal adults. Platelet HLA concentrations in 12 healthy adults were determined by the established binding assay. All except 1 of the 12 adults were HLA typed. Among these 12 individuals, the average number of HLA-A, B, and C molecules per platelet varied between 54,782 to 113,068 molecules per platelet (Table 1). The mean number of HLA molecules on a platelet was 81,587 ± 20,016 (mean ± SD). The results indicate that there is at least a twofold variation in platelet HLA concentration among different individuals. Because the interassay variation is 7%, the observed twofold difference most likely reflects true individual variation.

Quantitation of HLA molecules on chloroquine-treated platelets. The specific bindings of 125I-W6/32 Fab to platelets pretreated with 4 vol of 20% chloroquine for 15 to 60 minutes were determined. It was noted that loss of HLA antigens reached its maximum after 45 minutes of incubation with chloroquine. The elution of platelet HLA antigens was then studied as a function of chloroquine concentration, and all platelets were incubated with chloroquine for 45 minutes. The results of these studies indicated that maximal elution was attained after incubating platelets with 4 vol of 20% chloroquine for 45 minutes. Subsequently, we performed experiments in six healthy individuals whose platelets were pretreated with 4 vol of 20% chloroquine for 60 minutes. Losses of 71.5% to 90.0% of HLA-A, B, and C molecules from the platelets were observed (Table 2). Although chloroquine treatment reduced HLA antigens, PI* antigens remained unchanged on platelets, as determined by the binding of anti-PI* antiserum and quantitative immunofluorescence flow cytometry. The mean fluorescence intensity for HLA antigen on each of the chloroquine-treated platelets was only 25.3 ± 9.3% (mean ± SD) of the control.

Western blot of platelet HLA and chloroquine eluate. To demonstrate whether the eluate of chloroquine contains HLA antigens lost from platelet surface, Western blot was used. The HLA antigens transblotted to nitrocellulose paper was detected by W6/32 monoclonal antibodies and alkaline-phosphatase conjugate of anti-mouse IgG antibodies. The results in Fig 6 show the presence of HLA antigens in the eluate of chloroquine-treated platelets with the same apparent mol wt of 45 kd as the intact membrane Class I HLA molecules. When the similar blot was incubated with control mouse anti-influenza virus type A monoclonal antibody, HLA antigens were not detected.

DISCUSSION

Although the presence of HLA antigens and variable expression of some specific types of HLA antigens on human platelets are known, the exact quantity of HLA molecules on platelets and its variability among different individuals has not been demonstrated. By using Fab fragments of W6/32 anti-HLA monoclonal antibody and competitive protein binding, a quantitative assay for determining concentration of platelet HLA-A, B, and C molecules was developed.

Table 2. Effect of Chloroquine Treatment on Platelet HLA Concentration

<table>
<thead>
<tr>
<th>Case No.</th>
<th>HLA Concentration (Molecules/per Platelet)*</th>
<th>HLA Eluted (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72,240</td>
<td>77.1</td>
</tr>
<tr>
<td>2</td>
<td>54,782</td>
<td>81.9</td>
</tr>
<tr>
<td>3</td>
<td>65,317</td>
<td>71.5</td>
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<tr>
<td>4</td>
<td>79,765</td>
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<tr>
<td>5</td>
<td>113,068</td>
<td>73.8</td>
</tr>
<tr>
<td>6</td>
<td>80,667</td>
<td>90.0</td>
</tr>
</tbody>
</table>

Mean ± SD 77,639 ± 19,861 15,928 ± 7,889 79.9 ± 7.0

*The HLA-antigen concentration on platelets were determined by 125I-W6/32 Fab binding studies and Scatchard analyses.
The saturability of binding, ligand and cellular specificity, and high binding affinity substantiate the validity of this assay.

As reported previously, the W6/32 monoclonal antibody contains equal amounts of two different light chains and reacts with a common monomorphic antigenic determinant on HLA-A, B, and C molecules. One of the light chains is parental myeloma type. Therefore, it is expected that only 50% of Fab fragments will react with HLA antigens, a fact that we confirmed experimentally. This was taken into consideration when the binding data were analyzed by Scatchard plot. The linear Scatchard plots indicated that only one class of binding sites on platelets and Fab fragments was apparently homogeneous in binding.

The results of studying platelets from 12 healthy individuals revealed that at least a twofold variation exists between the lowest and highest platelet HLA concentrations. This finding is in parallel with the reported variability for platelet HLA antigens as do intact HLA molecules. Previous-lytic products of intact membrane HLA molecules. Previously, Lalezari and Driscoll demonstrated that platelets may acquire HLA antigens from plasma. The possible contribution of plasma HLA antigens to those adsorbed on platelets remains to be determined.

In conclusion, the results of our studies indicated that human platelets contain high concentrations of HLA antigens. More important, most HLA antigens are adsorbed on platelets. Thus, further studies on the origin and the binding mechanism of adsorbed platelet HLA antigens may lead to the development of ways to manipulate platelet HLA antigens quantitatively and qualitatively. By such manipulation, one may be able to reduce the platelet immunogenicity for their use in patients with alloimmunization against HLA antigens in the future.

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