We recently examined a case of refractoriness to HLA-matched, ABO-incompatible platelet transfusions. The transfused platelets that were rapidly cleared from the circulation of the recipient expressed an amount of B antigen more than 20 times that expressed by the blood group B platelets that were successfully transfused to the recipient. These observations led us to conduct enzyme-linked immunosorbent assay (ELISA) and immunoblotting studies of the amount of blood A and B antigens expressed on the surface of platelets from randomly selected donors. The donors were clearly classified, according to the amount of A or B antigen expressed on their platelets, into two phenotypes, the high-expression and low-expression phenotypes. By ELISA, 7% of the examined donors were determined as belonging to the high-expression phenotype of either A or B antigen. The high-expression phenotype was independent of secretor phenotype. In transfusion activity assay, a donor with high expression of B had increased B transfusion activity in her serum, which suggested that the high-expression phenotype might be under the control of the glycosyltransferase gene. Family studies showed a dominant inheritance pattern of the high-expression phenotype. This report provides evidence that the expression of ABH antigens on platelets is genetically determined and that the ABO group should be given some attention in platelet transfusions.

**Materials and Methods**

**Antibodies.** Murine MoAbs, anti-A and anti-B IgM, were purchased from Ortho Diagnostics (Raritan, NJ). S22 (anti-GPIIb), S222 (anti-GPIIb), and S221 (anti-GPIIIa) were purchased from Cosmobi K K (Tokyo, Japan). 4B4 (anti-GPIIIa) was purchased from Coulter Immunology (Hialeah, FL). Rabbit polyclonal anti-human platelet antibody was purchased from Dakopatts (Copenhagen, Denmark). Fluorescein isothiocyanate (FITC)-labeled goat anti-human Ig antibody was obtained from Tago Inc (Burlingame, CA). Human serum containing only anti-A and anti-B IgG at the same titer of 1:512 was selected in our laboratory.

**Platelet Preparations.** Seven to 20 mL of whole blood was drawn from each donor into one seventh volume of acid-citrate-dextran (ACD-A) or citrate-phosphate-dextran (CPD), and stored at 4°C for 1 to 3 days. Platelet-rich plasma was first prepared by centrifugation of the anticoagulated whole blood at 250g for 10 minutes and then further centrifuged at 750g for 10 minutes to form platelet pellets. The platelets were washed three times with Ringer’s citrate-dextran (RCD) buffer, pH 6.5,15 containing 20 ng/mL prostaglandin E1 (PGE1) (Sigma Chemical Co, St Louis, MO). The washed platelets were resuspended in 1% ammonium oxalate and adjusted to 15 minutes at 4°C to lyse residual RBCs and then centrifuged at 750g for 10 minutes. The obtained RBC-free platelets were further washed twice with RCD buffer and then subjected to studies using flow cytometry or to solubilization in 10

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mmol/L phosphate-buffered saline (PBS), pH 7.2, containing 1% Triton X-100 (Wako Pure Chemical Industries, Osaka, Japan) at a concentration of 4 × 10^4/mL. The solubilized platelets were centrifuged at 11,000g for 20 minutes at 4°C to remove insoluble materials, and the supernatant was used in enzyme-linked immunosorbent assay (ELISA) and immunoblotting. Protein concentration of each platelet lysate was determined by the method of Markwell et al., using bovine serum albumin as the standard.

**ELISA.** Flat-bottom wells of microtiter plates (MaxiSorp F16; Nunc, Roskilde, Denmark) were coated with 50 μL of the rabbit polyclonal antihuman platelet antibody (Dakopatts) in 50 mmol/L carbonate buffer, pH 9.6, at a concentration of 25 μg/mL and incubated at 4°C overnight. The microtiter plates were then washed twice with 0.05% Tween 20 in PBS (TPBS) and then filled with 5% skim milk in TPBS. After blocking for 1 hour at room temperature, the microtiter plates were washed twice with TPBS, and then 50 μL/well of the platelet lysate was added at a protein concentration of 15 or 20 μg/mL. After 1 hour of incubation, the microtiter plates were washed four times with TPBS, and then the murine monoclonal anti-A or anti-B IgM antibody (Bethyl Laboratories, Montgomery, AL) diluted 1:200 in PBS was added to each well of the plates and further incubated for 1 hour at room temperature. After washing, the microtiter plates were reacted with biotinylated goat antimouse IgM (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) diluted 1:200 in TPBS and then incubated for 1 hour and then with avidin-biotin–horseradish peroxidase complex at a dilution of 1:100 in TPBS at room temperature for 30 minutes. After washing, 50 μL of peroxidase substrate solution (0.1% o-phenylenediamine in 50 mmol/L carbonate buffer, pH 5.6, containing 0.0075% hydrogen peroxide) was added to each well and allowed to stand for 15 minutes in the dark for color development. The reaction was terminated by an addition of 2N sulfuric acid, and the absorbance at 492 nm of each well was measured with an ER-8000 microplate reader (Sanko Junyaku Co, Tokyo, Japan).

The referred standard curve was obtained as described by Kumpel, using purified mouse IgM (Sigma Chemical Co) serially diluted at concentrations ranging from 1 to 100 ng/mL. Briefly, microtiter plates were coated with 50 μL/well of goat-antimouse IgM antibody (Bethyl Laboratories, Montgomery, AL) diluted 1:200 in carbonate buffer, pH 9.6, blocked with 5% skim milk, and incubated with serially diluted mouse IgM. The amount of captured mouse IgM was quantitated using the Vectastain ABC kit described above, and the absorbance was plotted against the concentration of IgM.

**Flow cytometry.** One hundred microliters of platelet suspension (10^8/mL) in saline was mixed with an equal volume of a blood group O donor that contained both anti-A and anti-B at a titer of 1:512 and incubated at room temperature for 30 minutes. After washing with saline, the sensitized platelets were incubated with 100 μL of FITC-labeled goat-antihuman Ig antibody diluted 1:100 in PBS at room temperature for 30 minutes. The platelets were then washed once with saline and resuspended in 1.2 mL of PBS. The platelets were analyzed on a flow cytometer (Cytomation; Ortho Diagnostics) for the intensity of fluorescence.

**Immunoblotting.** Platelet lysates prepared as above at a protein concentration of 300 μg/mL were mixed with an equal volume of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.125 mol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and bromophenol blue) and subjected to SDS-PAGE under reducing conditions using a 5% to 12% gradient slab gel. The molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were run on every SDS-PAGE. The separated proteins were electroelastically transferred to a sheet of polyvinylidene difluoride filter (Clear Blot Membrane-p; ATTO Co, Tokyo, Japan) in Tris-glycine buffer (100 mmol/L Tris, 192 mmol/L glycine, pH 9.2, containing 20% methanol at 100 mA for 2 hours). The filter was blocked with 5% skim milk in TPBS at room temperature for 1 hour and then washed three times with TPBS. After incubation with the murine monoclonal anti-A or anti-B, bound murine IgM on the filter was visualized using the Vectastain ABC kit with a substrate solution (0.05% dianisidine in PBS containing 0.0075% hydrogen peroxide).

**Immunoprecipitation.** An aliquot of washed platelets (2.2 × 10^9) was incubated with any of 100 μL each of murine antihuman platelet glycoprotein Iib, IIa, IIb, IIIa MoAb (0.2 mg/mL) or rabbit polyclonal antihuman platelet antibody diluted 1:10 in PBS for 2 hours at room temperature. After washing with PBS containing 50 mg/mL of PGE_2, the sensitized platelets were resuspended in 198 μL of TPBS and then mixed with 22 μL of 10% Triton X-100 in PBS. The solubilized platelets were centrifuged at 11,000g for 20 minutes at 4°C to remove insoluble materials. One hundred microliters of the supernatant was then incubated with 50 μL of protein G sepharose (Pharmacia Biotechnology AB, Uppsala, Sweden) suspended in TPBS at a concentration of 30% (vol/vol) for 2 hours at room temperature to adsorb the immune complex. Protein G sepharose beads were washed four times with TPBS, and the immunoprecipitated platelet proteins were visualized as the above-mentioned immunoblotting using murine anti-B and the Vectastain ABC kit.

**Measurement of serum α-D-galactosidase transferase (B-transferase) activity.** Group O RBCs were incubated with an equal volume of 0.2% papain (E Merck AG, Darmstadt, Germany) in saline at 37°C

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**Table 1. Effect of ABO-Incompatible Platelet Transfusions**

<table>
<thead>
<tr>
<th>Transfusion Date</th>
<th>Donor and Blood Group</th>
<th>1 h CPI × 10^4</th>
<th>Cross Match Results</th>
<th>Expression of A/B Antigen on Platelets</th>
<th>Amount of Bound Anti-B IgM (μg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nov 21</td>
<td>O (K+)</td>
<td>3.09</td>
<td>3.40</td>
<td>0 NT</td>
<td>NT</td>
</tr>
<tr>
<td>Nov 28</td>
<td>B (Ni)</td>
<td>1.38</td>
<td>0.151</td>
<td>1:32 0</td>
<td>NT</td>
</tr>
<tr>
<td>Nov 30</td>
<td>A (Ty)</td>
<td>3.11</td>
<td>2.65</td>
<td>0 NT</td>
<td>NT</td>
</tr>
<tr>
<td>Dec 6</td>
<td>B (MiS)</td>
<td>3.52</td>
<td>2.69</td>
<td>0 NT</td>
<td>Low 0.074</td>
</tr>
<tr>
<td>After six successful transfusions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jan 17</td>
<td>A (Ta)</td>
<td>2.82</td>
<td>2.28</td>
<td>0 NT</td>
<td>Low 0.074</td>
</tr>
<tr>
<td>Jan 23</td>
<td>AB (kiN)</td>
<td>-0.64</td>
<td>-1.17</td>
<td>1:16 0</td>
<td>1.7</td>
</tr>
<tr>
<td>Jan 24</td>
<td>B (MiS)</td>
<td>3.68</td>
<td>1.061</td>
<td>0 NT</td>
<td>Low 0.074</td>
</tr>
</tbody>
</table>

Abbreviations: CPI, corrected platelet increment; MPHA, mixed passive hemagglutination test; NT, not tested.

* After absorption with B RBCs or Synsorb B.
† CPI at 48 hours after transfusion.
‡ Identified by immunoblotting.

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ABH ANTIGENS ON PLATELETS

Fig 1. Comparison of flow cytometric profiles between high-expression platelets (solid line) and low-expression platelets (dotted line). Group O platelets reacted with anti-B showed fluorescence intensity similar to that of the low-expression platelets (data not shown).

For 30 minutes to prepare sugar acceptors. After washing three times with saline, the papain-treated RBCs were suspended in saline at a concentration of 50% (vol/vol). The B-transferase activity was assayed according to the method of Yabe et al., with a few modifications. Briefly, 50 μL of 1.6 mmol/L uridine diphosphate (UDP)-D-galactose (Sumitomo-seika Co, Osaka, Japan), 100 μL of 0.1 mol/L cacodylic acid, pH 6.0, containing 0.1 mol/L MnCl₂, and 25 μL of the papain-treated RBCs were mixed with 250 μL of a serum sample. After incubation at 37°C for 5 minutes, the RBCs were washed three times with saline, and a 2% suspension of the RBCs in saline was prepared. Agglutination scores against human anti-B antibody (Dade, Miami, FL) were then recorded.

RESULTS

Studies on the refractoriness of the patient to HLA-matched platelets. The reactivity of the patient's IgG with platelets from donors NI and KN in MPHA was abolished by prior incubation with either group B RBCs or synthetic B immunoadsorbent (Synsorb B; Chembiomed, Edmonton, Canada) but not with Synsorb A (Table 1). Studies using ELISA, immunoblotting, and flow cytometry showed that the platelets from both NI and KN expressed a much larger quantity of B antigen on their surface than the others (Table 1). The platelets from donor KN bound 23 times as much anti-B antibodies in ELISA as successfully transfused platelets from donor MS did. The platelets from donor NI expressed roughly 24 times more B antigen in flow cytometry than platelets from donor MS (Fig 1). Thus, abundantly expressed B antigen on NI and KN platelets was implicated in the refractoriness in this patient.

Quantitation of ABH antigens on platelets by ELISA. Platelets from 313 donors were examined by ELISA for the amount of A and B antigens expressed on their surface. The blood groups of the donors were group A, 107; group B, 100; group AB, 106 (Fig 2). The donors were clearly classified into two phenotypes according to the results of ELISA: high-expression and low-expression phenotypes. When measured absorbance exceeded the mean +2 SD, it was classified as the high-expression phenotype. The number of high-expression phenotype donors in each blood group was group A, 6 of 107; group B, 5 of 100; group AB, 7 of 106; group AB (A expressive), 4 of 106. Totally, 7% of all the donors examined belonged to the high-expression phenotype of either A or B antigen. None of the group AB donors showed high expression of both A and B antigens at the same time.

Subsequently, secretor (Se) and nonsecretor (se) phenotypes of the donors were determined by Lewis phenotype or saliva inhibition test to see if they have some correlation to the high- and low-expression phenotypes. As summarized in Table 2, both Se and se phenotypes were observed in the high-expression phenotype donors. The population of the
Table 2. Relationship Between the Expression of Platelet A/B Antigen and Secretor Phenotype

<table>
<thead>
<tr>
<th>Expression of Platelet A/B Antigen</th>
<th>Frequency of Secretor Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Se (%)</td>
</tr>
<tr>
<td>High</td>
<td>14 (78)</td>
</tr>
<tr>
<td>Low</td>
<td>18 (82)</td>
</tr>
</tbody>
</table>

Se phenotype in the high-expression phenotype was equivalent to that in the low-expression phenotype donors. Thus, the amount of ABH antigens expressed on platelets was obviously indicated to be independent of secretor phenotype.

Measurement of B-transferase activity. Serum B-transferase activity of five high-expression donors and six low-expression phenotype donors was studied. The serum of high-expression phenotype donors gave high agglutination scores ranging from 38 to 67, whereas the serum of low-expression phenotype donors scored 0 (Table 3). Regarding transferase activity, a previous report indicated that one donor, TE, and 6 of her 12 family members had high serum B-transferase activity that was inherited in a mendelian dominant manner. In the present study, we confirmed that donor TE and three of the six members of her family really had high serum B-transferase activity and that they belonged to the high-expression phenotype (Fig 3). These observations clearly indicated that high serum glycosyltransferase activity correlated with the high expression of ABH blood group antigens on platelets.

Family studies on platelet A and B antigens. Family members of the five high-expression phenotype donors (HO, KM, KN, SS, and MN) were studied in terms of the expression of A and/or B antigen on their platelets to see if the high- and low-expression phenotypes were genetically determined (Fig 4). Totally, 8 of 16 members of the five families belonged to the high-expression phenotype. The existence of these pedigrees is consistent with the possibility that inheritance of the high-expression phenotype is autosomal dominant.

Immunoblotting and immunoprecipitation. In immunoblotting, the murine monoclonal anti-B antibody blotted several different proteins of the platelet lysates from six B high-expression phenotype donors (Fig 5). The apparent molecular weight of the major protein was 126,000 under reducing conditions. None of the proteins of the low-expresssion phenotype platelets was blotted by the antibody. Then platelets from a B high-expression phenotype donor were analyzed by immunoprecipitation using murine MoAbs against platelet glycoproteins Ib, Ia, Ib, and Ila to identify the major protein that bound murine anti-B IgM most in immunoblotting. As shown in Fig 6, glycoproteins Ib, Ia, Ib, and Ila carried B determinants and GPIIb seemed to be the most prominent among them.

The same blotting profile was obtained using the anti-A MoAb with six A high-expression phenotype donor platelets (data not shown).

DISCUSSION

With the increase in the number of refractory patients to random platelet transfusions, administration of HLA-matched but ABO-incompatible platelet transfusions has been rapidly increasing in Japan. Regarding ABO compatibility in platelet transfusions, Freireich et al. showed that there seemed to be no difference in the clinical outcome between ABO-compatible and -incompatible platelets. Duquesnoy et al. reported that ABO incompatibility reduced the recovery of transfused platelets by 23% in alloimmunized thrombocytopenic patients, and they concluded that the reduction was not serious enough to contraindicate ABO-mismatched platelet transfusions. However, several cases of platelet transfusion refractoriness caused by high-titered anti-A and anti-B antibodies have been reported by Brand et al. and Skogen et al. Although the frequency of this type of refractoriness remains unclear, it may be higher than generally anticipated. Concerning the ABO blood group, it is important to note the difference in the frequency of subgroup A2 between whites and Japanese. We have typed 51,101 donors at our blood center and only 62
Fig 4. Schematic diagram of the inheritance of the high-expression phenotype in five families. ○ and ●, A antigen high-expression phenotype; ■ and ■, B antigen high-expression phenotype; ○ and □, low-expression phenotype; ○ and ○, not tested; arrow, proposita. The blood group is indicated below each symbol.

Fig 5. Immunoblots of platelet lysates stained with the anti-B antibody. Lane 1, Coomassie brilliant blue G250 staining of the molecular weight standards; lanes 2 through 7, B antigen high-expression phenotype; lane 8, low-expression phenotype.

(0.12%) belonged to subgroup A₂. In contrast, more than 10% of Caucasians belonged to subgroup A₂.

The present study describes a case of refractoriness to ABO-incompatible platelet transfusions. Ten HLA-matched, ABO-incompatible platelet transfusions were successfully administered to the patient who had high-titered anti-A and anti-B antibodies (1:512). But platelets from two HLA-matched donors, NI and KN, failed to increase the patient's peripheral platelet count at all. The platelets from NI and KN were shown by ELISA and/or immunoblotting to express larger amounts of B antigen than the others and were classified as the high-expression phenotype. In flow cytometric analysis using a high-titered human anti-B antibody, the platelets from donor NI bound roughly 24 times more anti-B than the platelets from donor MS, which yielded reasonable increments in the patient. Because both NI and MS were group B and HLA-matched with the patient to the same extent, the cause of the rejection was attributed to the higher amount of B antigen expressed on NI's platelets. Although a previous report indicated that the expression of ABH antigens on platelets varied even in platelet preparations from one individual, our data clearly showed that the high-expression phenotype platelets always expressed the blood group antigen to the same extent.

It has been reported that platelets have intrinsic ABH antigens expressed on their surface as well as extrinsic, adsorbed soluble A and B antigens from plasma. The intrinsic ABH antigens expressed on platelet membrane have
been demonstrated by immunoblotting after SDS-PAGE under reducing conditions. The expression of adsorbed ABH antigens was reported to be under the control of the Se and Le genes. In the present study, we quantitated the amounts of blood group A and B antigens on the platelets of randomly selected healthy Japanese donors by ELISA and immunoblotting. The donors could be clearly classified into two phenotypes, high-expression and low-expression phenotypes, according to the amounts of A or B antigen on their platelets, as determined by the ELISA. Twenty-two of the 313 donors examined (7%) belonged to the high-expression phenotype. The relationship between the high-expression phenotype and the secretor phenotype was investigated, but no clear correlation was observed. Instead, we demonstrated that glucosyl transferase activity most significantly contributed to the expression of blood group antigens on platelets on the basis of the following observations. First, the serum glucosyl transferase activity of all five B high-expression donors was significantly higher than that of the six low-expression donors. This observation clearly indicated that high serum glucosyl transferase activity correlated well with the high-expression phenotype. Second, the results of the family studies were consistent with the possibility that the high-expression phenotype was genetically determined and co-inherited with high glycosyl transferase activity in an autosomal dominant manner.

Recently, Santoso et al. reported that platelet glycoproteins IIa, IIIa, and Ib mainly carried A and B antigens, and that GPIIa seemed to be the most prominent among them. However, in our study, GPIIb was the protein that bore the largest amount of B antigen expressed on the surface of platelets (Fig 5), whereas GPIIa seemed to bear the largest amount of B antigen on each molecule (Fig 6). Because GPIIb-IIIa is the most prominent protein expressed on platelet surface and plays a central role in platelet aggregation, antibodies bound to blood group antigens expressed on GPIIb may not only lead to destruction but also inhibit the function of the platelets.

Thus, in administration of ABO-incompatible, single-donor platelet transfusions, it will be necessary to quantitate blood group antigens expressed on the donor's platelets in addition to titering anti-A and anti-B in the recipient's plasma.

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Study on the expression of ABH antigens on platelets

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