The Blood Group A Genotype Determines the Level of Expression of the Blood Group A on Platelets But Not the Anti-B Isotiter

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**Introduction**

Platelets express ABH antigens on several platelet glycolipids and glycoproteins, such as glycoprotein (GP) IIb/IIIa, GPIb/IX and CD109 [1–3]. Earlier studies suggested that ABH-active glycolipids are of extrinsic origin (i.e., absorbed from plasma) [4, 5]. More recent data indicate that they are mainly endogenous, as ABH-active glycolipids can also be found on platelets from Le (a+b–) individuals who lack plasma ABH glycosphingolipids [6]. The A antigen can be clearly detected in donors of the A1 phenotype, while A2 individuals carry little to no A antigen on their platelet surfaces [7]. However, even among A1 individuals, antigen expression varies greatly from one individual to the next. Thus, A1 donors can be classified into low or high expressers [8]. The frequency of the high-expressor phenomenon in an A1 population is about 2–7% [8, 9].

Antigen density on platelets and donor isoagglutinin concentration in the associated plasma may become clinically relevant when platelets are transfused across blood group barriers. However, the transfusion of ABO-incompatible platelets is common [10]. The ABO incompatibility was considered of minor concern regarding transfusion efficacy, but ABO incompatibility can be responsible for lower platelet count increments, decreased platelet function and risk of platelet refractoriness, followed by higher rates of alloimmunization [10–14]. Further, hemolytic transfusion reactions may occur with ABO-minor-incompatible transfusions [3, 10, 15]. Although severe complications from the latter seem rare, mild to moderate hemolysis may be underreported [16].

To date, only few investigations related the genotype with the level of expression of the blood group A on platelets [17]. We therefore examined the role of platelet donors’ ABO genotype on platelet A antigen surface density and on the isoagglutinin concentration.
Material and Methods

Donor Population

The study was approved by the Ethics Committee of the Medical University of Vienna, and informed consent was obtained from all participants. We obtained platelet aliquots from subsequent donations from 103 group A apheresis donors at the Department of Blood Group Serology and Transfusion Medicine at Vienna General Hospital, and the Viennese Red Cross. Samples of 32 group O apheresis donors served as negative controls for flow cytometry testing. Aliquots from whole blood samples were used for Lewis determinations and genotyping, and for the determination of levels of isoaagglutinins.

Detection of ABH Expression by Flow Cytometry

Optimal conditions were found by titration experiments. We contrasted different cell counts, antibody concentrations, and washed versus unwashed platelets to achieve a display of a clear-cut platelet population on the flow cytometer. Based on the results from these experiments, the reagents were used as follows: i) anti-A reagent (BRIC 145 phycocerythrin(PE)-labeled; IBGRL Research Products, Bristol, UK), diluted 1:8 in phosphate-buffered saline (PBS) according to the manufacturer’s instructions, and further diluted 1:2 in PBS, ii) anti-B reagent (BRIC 1 PE-labeled; IBGRL Research Products), diluted 1:2 from a 1:10 dilution in PBS, iii) anti-H reagent (BRIC 198 PE-labelled, IBGRL Research Products), diluted 1:2 from a 1:10 PBS dilution. Testing washed and unwashed platelets yielded similar results; therefore, we used unwashed platelets only.

A platelet aliquot was obtained from each concentrate about 1 h after apheresis when concentrates have rested for 1 h. Concentrations were contained 35 or 44% plasma and 65 or 56% platelet additive solution (MacoPharma, Mouvaux, France). The median volume was 405 ml (range 250–505 ml), the platelet concentration was median 1,604 × 10^3/µl (range 668–2,700 × 10^3/µl). Aliquots were run consisting of 94 °C for 20 s, 61 °C for 60 s, and 72 °C for 30 s. Allophycocyanin (APC)-labeled anti-A and anti-B antibodies as a negative control, anti-H antibodies, and an isotype control. The latter, to confirm an approximate 50% drop in fluorescence intensity, anti-B antibodies as a negative control, anti-H antibodies, and an isotype control. After incubation in the dark at room temperature for 20 min, samples were re-suspended in 500 µl HEPEs-buffered paraformaldehyde.

Flow cytometry analyses were performed on a FACSCalibur flow cytometer (BD) equipped with a 488 nm argon laser. CaliBRITE beads (BD) were used to adjust instrument settings and compensate overlaps in fluorescence expected with the use of PE and APC fluorochromes. Forward scatter versus side scatter and versus fluorescence gates were set. A total of 10,000 events per sample were gated and analyzed. Data were recorded as the mean fluorescence intensity (MFI). To standardize quantification of fluorescence intensity, BD Quantibrite™ PE beads (BD) were run with each batch of samples, to allow the conversion of MFI values to antibodies bound per cell (ABPC) using known PE-to-antibodies ratios.

Donor Isoagglutinin Concentration

Donor anti-B titer was determined by the glass plate method, the result being the reciprocal of the highest dilution at which macroscopic agglutination occurred.

Genotyping

ABO genotyping was based on SSP-PCR as previously published [18, 19]. Primers for A1, non-A1, A2, non-A2, O1, non-O1, O2, non-O2, B, and non-B were obtained from MWG-Biotech (Ebersberg, Germany). 5 µl of the appropriate primer mix were pipetted into PCR tube strips. 5 µl of a PCR master mix containing PCR buffer, MgCl₂, distilled water, a dNTP mix, Taq polymerase, and the respective DNA of the donor were then added for a total amplification volume of 10 µl. Following DNA denaturation at 94 °C for 2 min, 10 cycles were run consisting of 94 °C for 20 s and 65 °C for 60 s, succeeded by 20 cycles consisting of 94 °C for 20 s, 61 °C for 60 s, and 72 °C for 30 s. Lewis blood group typing was performed using the DiaClon Anti-Le<sup>a</sup> and Anti-Le<sub>a</sub> 1D-cards (Bio-Rad Laboratories, Hercules, CA, USA). The secretor status was further determined for Le<sub>a</sub>– individuals, using an allele-specific PCR protocol. The Se/se genotype was determined by the 428G>A and 571C>T nucleotide polymorphisms of the α1,2-fucosyltransferase 2 gene (FUT2) with primers 5ʹ-CTGATCCCTGCTCTCG-3ʹ and 5ʹ-CTGTTACTGTGGCAGCC-CAACG-3ʹ (428Gor 5ʹ-TAGGGGTGCTTCTGGCC-3ʹ and 5ʹ-CTGTTACTGTGGCAGCC-CAACG-3ʹ) (571C). The Le/le genotype, coded by the α1,3/ 4-fucosyltransferase 3 gene (FUT3), was determined by typing for 2027C>T polymorphism using the allele specific reverse primers 5ʹ-GGGAAAGGCGCATGTCA-3ʹ (2027T) and 5ʹ-GGGAAAGGCGCATGTCA-3ʹ (2027C) and 5ʹ-CTTCCGAAAAACCCAGTCCG-3ʹ as the common forward primer. The 59T-G nucleotide polymorphism was determined by the allele-specific forward primers 5ʹ-GGCTGTCTGCGGGGTC-3ʹ (59T) and 5ʹ-GGCTGTCTGCGGGGTC-3ʹ (59G) and a common reverse primer 5ʹ-TGGTAGTTGCTTCTGGTCTG-3ʹ. The PCR-products were separated by agarose gel electrophoresis and subsequently visualized via UV transillumination.

Statistical Analysis

Population statistics are described as mean and standard deviation, or median and range. The Mann-Whitney U-test and Kruskal-Wallis test for non-parametric testing as well as chi-square tests for testing nominal data were applied. Correlations were calculated with the Spearman rank test. A p value < 0.05 was considered significant.

Results

89 of the 103 group A donors were assigned an A<sub>1</sub> genotype: A<sub>1</sub>O<sub>1</sub> (60), A<sub>1</sub>A<sub>1</sub> (17), A<sub>1</sub>A<sub>2</sub> (8) and A<sub>1</sub>O<sub>2</sub> (4). Of the 14 A<sub>2</sub> donors, 13 had an A<sub>2</sub>O<sub>1</sub> genotype, and only one individual was typed A<sub>2</sub>A<sub>2</sub>. This genotype distribution is in line with previous data in the urban Viennese population.

The A<sub>1</sub>A<sub>1</sub> Genotype Is Associated with High A Antigen Expression

Examples of fluorescence histograms illustrating the expression of the A antigen on platelets are shown in figure 1. A wide range of A antigen densities among A donors is apparent (fig. 2). Values by their four genotypes, significant differences among subgroups were also observed (fig. 2). An overlap of ABPC levels is apparent between homozygous A<sub>1</sub>A<sub>1</sub> and heterozygous A<sub>1</sub>A<sub>2</sub> individuals. Still, the A<sub>1</sub>A<sub>1</sub> genotype is associated with a particularly high antigen expression (p < 0.0001). When comparing the flow cytometry results of A<sub>1</sub> donors grouped by their four genotypes, significant differences among subgroups were also observed (fig. 2). An overlap of ABPC levels is apparent between homozygous A<sub>1</sub>A<sub>1</sub> and heterozygous A<sub>1</sub>A<sub>2</sub> individuals. Still, the A<sub>1</sub>A<sub>1</sub> genotype is associated with a particularly high antigen expression (p < 0.0001).
platelet expression of the A antigen from A1O1 donors were not related to the plasma volume in the platelet concentrate (p > 0.05).

The Correlation of Antigen Expression Using Two Different Concentrations of Anti-A Antibody

As described above, platelets were tested for their A antigen expression using two different concentrations of the anti-A antibody: a concentrated version according to manufacturer’s instructions and a 1:2 dilution thereof. An approximate 50% drop in MFI values across all samples was seen. There was positive correlation between the two series of antibody concentrations (r = 0.97; p < 0.0001), indicating that no prozone phenomenon due to an excess of antibodies or antigens interfered with the data and that using saturated antibody concentrations were not a prerequisite for the results of the study.

A Antigen Density of Platelets from A2 versus Those from Blood Group O Donors

Several A2 donors showed an antigen density on their platelets that exceeded the background fluorescence recorded on O platelets, and differences between these two populations were significant (p = 0.007) (fig. 3). Our results included one outlier, whose anti-A values exceeded the population’s mean for A2 donors almost 2.5 times (1,056 mean antibodies bound per cell versus 421.71 mean antibodies bound per cell of the A2 donors). This donor was genotyped A2A2 (the only A2A2 individual in our study population).

The Expression of H-Antigen Was Directly Associated with the A Genotype

Like A antigen expression, H antigen expression was also associated with the donor genotype (table 1, p < 0.001). As expected, A2 individuals expressed more H antigen on their platelets than A1 donors, and H antigen density was particularly low in A1A1 donors. We also determined an H-to-A ratio, as recently published by DeLelys et al. [17]. Correspondingly, we found ratios to be lowest in A1A1 donors, followed by A1O2 < A1O1 < A2A2 < A2O1.

The Influence of the Secretor Status on Platelets’ Blood Group A Expression

The secretor status was assigned to 78 individuals. The presence of the secretor status did not influence the A antigen expression (fig. 4, p = 0.067). To rule out skewed results in smaller genotype groups, we evaluated separately the expression in the largest group. However, also in A1O1 donors the expression of A was not associated with the secretor status (p = 0.507). However, higher MFI values tended to be scored by samples from donors of the secretor status, an observation that included the three high-expresser individuals in the study population.
Donors’ Isotitter

We determined the anti-B isoagglutinin concentration in 103 group A apheresis donors (fig. 5). The median isotitter for the entire study population was 1:8. The lowest titer was 1:2, the highest titer was 1:128 – the latter in an A2O2 donor. Anti-B titers were not different among donors grouped according to their genotype (p = 0.627). Also, the three high expressers of the A antigen had similar anti-B titers as the other donors (p = 0.628).

Discussion

We showed the close association of the blood group A genotype with the quantity of the A antigen expressed on apheresis platelets.

Table 1. H antigen detection (ABPC) and H/A ratio across blood group A genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>ABPC H</th>
<th>H/A-ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1A1</td>
<td>12,164</td>
<td>3.72</td>
</tr>
<tr>
<td>A1A2</td>
<td>15,110</td>
<td>14.69</td>
</tr>
<tr>
<td>A1O1</td>
<td>22,399</td>
<td>12.40</td>
</tr>
<tr>
<td>A1O2</td>
<td>15,730</td>
<td>7.22</td>
</tr>
<tr>
<td>A2A1</td>
<td>63,826</td>
<td>85.56</td>
</tr>
<tr>
<td>A2O1</td>
<td>26,374</td>
<td>142.56</td>
</tr>
</tbody>
</table>

Fig. 4. Blood group A expression of platelets from secretors versus non-secretors. The expression of the A antigen is not different between secretors and non-secretors (p = 0.184). Outliers and extremes are marked as circles and stars, respectively.

Fig. 5. Isoagglutinin titers across group A genotypes. Titers do not differ across A genotypes (p = 0.627). Experimental design as in figure 4.

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Thereby, the presence of an A1 allele had the greatest influence on levels of the A antigen density. However, the mean for ABPC within the A1 donors varied considerably, depending on their status of zygosity. The isoagglutinin titer was not associated with the blood group A genotype.

We quantified the A antigen expression converting the MFI values to ABPC through the use of BD QuantiBRITE PE beads. Thereby, the anti-A antibody was used in two different concentrations, one just 50% of the more concentrated one. This procedure allowed us to exclude a prozone phenomenon, or an underestimation of the antigen expression. In accordance with previously published studies [6–9], we showed that the presence of an A1 allele had the greatest impact on A-antigen density. An about tenfold increased difference of A antigen expression was discernible between platelets from A1 and A2 donors. However, even among A1 platelets the antigen expression varied considerably. Platelets from homozygous A1 individuals expressed by far more A antigen than those from A2A2 or A1O1 donors. This is further substantiated by our findings that all high expressers of the A antigen were typed A1A1. Various factors leading to the high expresser phenotype have been discussed. Ogasawara et al. [8] observed an increased serum glycosyl transferase activity in group B high expressers, and similar findings were made by Curtis et al. [7], who reported a 12.6-fold increase in type-II high expressers. A mutation leading to a gain of function in the mature protein has been discussed; however, sequencing studies have shown no differences to reported normal gene sequences [7]. Mutations in the FUT1 or FUT2 gene, resulting in larger quantities of H antigen available for conversion to A antigen have also been debated, but no effective polymorphisms were detected [9]. An autosomal dominant trait was discussed, but mechanisms of inheritance have yet to be defined, as are its molecular origins [7–9].

While it has been established that group A2 platelets carry considerably less A antigen than those of an A1 phenotype [3, 6, 7, 9], their differentiation from group O platelets by flow-cytometric analyses were inconclusive. Cooling et al. [6] suggested that A2 platelets possess a Bombay-like phenotype, lacking both A and H antigen. Curtis et al. [7] found that A2 platelets could not be differentiated from O platelets via flow cytometry. Sant’Anna Gomes et al. [9] and DeLelys et al. [17] described clear differences, however. In our study, 12 samples scored anti-A MFI values similar to a group O donor, 10 of these were even A1 donors.

We see a clear association of the H antigen expression with the genotype. Recently, it was reported that this association becomes
even more apparent, when H-to-A antigen ratios are calculated [17]. Our data are in line with the previous report showing a correlation between the A and H antigen in A1A1 donors, but not in A1O donors (data not shown).

An impact of ABO matching on the prevention of clinical bleeding, and only a small effect on transfusion intervals. Blumberg et al. [24] reported an association of ABO-incompatible platelet transfusions with an increase in morbidity and mortality in cardiac surgery patients. Others, however, found no such an association [25]. Shehata et al. [26] emphasized the need of studies on the effects of ABO matching on patient outcomes such as survival and bleeding rates.

We also determined isoagglutinin concentrations as they can trigger hemolytic transfusion reactions after ABO-minor-incompatible transfusions. [3, 10, 15, 27]. Our data indicate that the concentration of isoagglutinins is not associated with the A genotype. Reported hemolytic transfusion reactions usually involve group O donors [28], but Daniel-Johnson et al. [29] also observed two severe hemolytic transfusion reactions by a high anti-B titer from a group A donor. Currently, however, there is no consensus cut-off for a 'dangerous' titer [30]. Further, antibody titers alone might not be sufficient to predict the risk of developing hemolysis after a minor-incompatible transfusion [31]. Furthermore, nowadays platelet concentrates may contain only small amounts of plasma, as the major part is substituted with platelet additive solutions.

Limitations of our study are the small sample size of certain genotypes (our study included only 1 A2A2 genotype). Second, a trend towards higher antigen density in secretor status-positive individuals may have been influenced by the naturally occurring high percentage of secretors in the Caucasian population. Third, we prepared samples for flow cytometry testing soon after apheresis, but antigen expression may differ after prolonged storage [32]. Finally, we have no clinical outcome data.

In summary, we have shown that platelets from an A1 genotype express significantly more A antigen on their platelets than A2 platelets and that A antigen expression is strongest in individuals of the A1A1 genotype, possibly rendering them less suitable for out-of-group platelet transfusions. Since we observed no differences in isoagglutinin concentrations between the genotypes, the genotype does not predict the risk of hemolytic transfusion reactions. Distinguishing between A1 and A2 donors could be a practical approach in reducing the risks associated with incompatible platelet transfusions.

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Disclosure Statement

Conflict of interest: none declared.

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