A Dinucleotide Deletion in Exon 4 of the PI\textsuperscript{A2} Allelic Form of Glycoprotein IIIa: Implications for the Correlation of Serologic Versus Genotypic Analysis of Human Platelet Alloantigens

By Bjorn Skogen, Ronggang Wang, Janice G. McFarland, and Peter J. Newman

Platelets from a patient with a suspected case of posttransfusion purpura were subjected to alloantigen phenotyping and found to express the PI\textsuperscript{A2}, but not the PI\textsuperscript{A1} allele, form of human platelet membrane glycoprotein (GP) IIIa on the platelet surface. However, genotyping showed unambiguously that the patient carried the genes for both of these GPIIa alleles. Based on these results, we postulated that the PI\textsuperscript{A2} allele was silent, i.e., that this patient was a carrier for Glanzmann thrombasthenia (GT). Quantitative analysis of GPIIb-IIIa surface expression showed only 20,000 GPIIb-IIIa receptors/platelet, approximately half of the value obtained with control platelets. Southern blot analysis showed no large deletions or insertions within the GPIIa gene, and amplification of all 14 exons encoding PI\textsubscript{IIa} resulted in the production of normal sized polymerase chain reaction (PCR) products in all cases. DNA-sequence analysis showed an AG dinucleotide deletion affecting codons 210 and 211 within exon 4 of the GPIIa gene, leading to a change in reading frame and the creation of a stop codon 38 nucleotides downstream. The predicted truncated protein consists of only the first 223 of the normal 782 amino acids, thus accounting for the failure to express the PI\textsuperscript{A2} allele on the platelet surface.

While encountered only rarely, carriers of either GT or Bernard Soulier syndrome that are at the same time heterozygous for human platelet alloantigenic epitopes found on GPIb, GPIIb, or GPIIa have the possibility to give discrepant results when comparing genotypic versus phenotypic analysis. In such situations, the combination of serologic and DNA-based evaluation contributes complementary and beneficial diagnostic information than either one alone are able to provide.

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MATERIALS AND METHODS

Case report. P.D.J. was a 57-year-old man with no family history of PTP or NATP who underwent coronary artery bypass surgery and developed thrombocytopenia and deep venous thrombosis of the leg in the postoperative period. The preoperative platelet count had been normal and within a week of surgery, it had fallen to 24,000/\,\mu\textnormal{L.} The differential diagnosis included PTP and/or heparin-induced thrombocytopenia. The evaluation for PTP included testing the serum for platelet specific antibody and the platelets and genomic DNA for platelet antigen phenotype and genotype. No platelet-specific

From the Department of Immunology and Transfusion Medicine, University Hospital of Tromsø, Tromsø, Norway; Blood Research Institute, The Blood Center of Southeastern Wisconsin, Milwaukee, WI; and the Departments of Cellular Biology, Pathology, Medicine, and Pharmacology, Medical College of Wisconsin, Milwaukee, WI. Submitted April 23, 1996; accepted July 12, 1996.

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alloantibody was identified in the patient's serum and the results of platelet phenotyping and genotyping studies are discussed below. A heparin-dependent platelet aggregation test was positive, consistent with the presence of a heparin-dependent antibody in the patient's serum. The clinical diagnosis of heparin-induced thrombocytopenia was made.

**Phenotypic and genotypic analysis of human platelet alloantigens.** Platelets were isolated from EDTA-anticoagulated blood, and the PI<sup>A</sup>/PI<sup>B</sup> phenotypes were determined serologically using a modified antigen-capture enzyme-linked immunosorbent assay (MAE), as previously described. Genotype analysis of human platelet alloantigens was performed using allele-specific oligonucleotide hybridization using standard methods. Briefly, genomic DNA was isolated from peripheral blood leukocytes of sodium-EDTA anticoagulated blood by standard proteinase K treatment followed by salting out of protein with super saturated NaCl, precipitation of DNA with absolute ethanol, and spooling. Following PCR amplification of the GPIIIa gene in the region encompassing the PIA polymorphism, 100 ng amplified DNA was applied to MagnaGraph nylon transfer membranes (MSI, Westboro, MA) using a dot-blot apparatus (Fisher Scientific Corp, Itasca, IL) and hybridized with each of two 13-mer oligonucleotide probes differing only at the central base specific for the platelet allelotype sequences (T<sub>196</sub> = PIA<sup>1</sup>, C<sub>196</sub> = PIA<sup>2</sup> ) Samples from individuals with known PI<sup>A</sup> genotypes were included as controls. The oligonucleotide probes were end-labeled with digoxigenin-11-deoxyuridine-triphosphate, and were immunologically detected using alkaline phosphatase-conjugated polyclonal sheep anti-digoxigenin Fab fragments (Boehringer Mannheim, Indianapolis, IN). Antibody conjugates were detected using the nitro blue tetrazolium5-iodo-2'-deoxyuridine-triphosphate, and were immunologically detected by the suppliers. Fragments were separated by electrophoresis through 0.6% agarose and transferred to a Gene Screen Plus membrane (New England Nuclear, Boston, MA). Hybridization and washing were performed at 65°C. A full-length GPIIIa cDNA was used as probe for the genomic Southern blot analysis. The probe was radiolabeled with α<sup>32</sup>P-dCTP using random hexamers and the Klenow fragment of DNA polymerase using standard methods.

**DNA sequence analysis of the GPIIIa gene.** Thirteen sets of primers flanking all 14 exons of the GPIIIa gene were constructed based on the published genomic sequence. Exons 6 and 7 were amplified as one product. In each reaction of 50 μL, 500 ng of genomic DNA was used. Following an initial preheating step at 94°C, PCR amplification was performed for 30 cycles using a protocol of denaturation at 94°C for 1 minute, annealing at 58°C for 2 minutes, and extension at 72°C for 1 minute. After the final cycle, the sample was kept at 72°C for 10 minutes and then stored at 4°C until use. PCR-amplified DNA corresponding to exons 1-14 of GPIIIa was gel purified (GeneClean; Dianova) and used as template for PCR amplification. PCR products were also subcloned into the plasmid vector pGEM5 (Promega Corp, Madison, WI) and the inserts sequenced by the dye chain termination method, using Sequenase T7 DNA polymerase (US Biochemical Corp, Cleveland, OH).

## RESULTS

Since the inception of DNA-based genotyping for human platelet alloantigen polymorphisms, more than 7 years ago, more than 1,000 patients have been DNA typed and the results correlated with those obtained using traditional serological methods (McFarland and Newman, unpublished data, April 1996). To date, the overwhelming majority of samples have yielded identical results using either technique, i.e., the phenotypes and genotypes were concordant. Rarely, however, genotypic analysis shows the presence of an allelic form of a platelet membrane glycoprotein that is not detected on the platelet surface using well-characterized human alloantisera of defined specificity. To better understand the reasons for these occasional discrepancies, we examined in more detail the molecular basis in one such patient, P.D.J.

As shown in Table 1, platelets from patient P.D.J. strongly expressed on their surface the PI<sup>A</sup> alloantigenic determinant, whereas anti-PI<sup>A</sup> antisera failed to bind, consistent with P.D.J. being homozygous for the PI<sup>A</sup> allelic form of GPIIIa. In contrast, both allele-specific oligonucleotide hybridization

**Table 1. Surface Expression of the PI<sup>H</sup> and PI<sup>0</sup> Allelic Determinants on Patient P.D.J. Platelets as Determined by Antigen-Capture ELISA**

<table>
<thead>
<tr>
<th>Target Platelets</th>
<th>Control PI&lt;sup&gt;1&lt;/sup&gt;/PI&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Control PI&lt;sup&gt;2&lt;/sup&gt;/PI&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Patient P.D.J.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-PI&lt;sup&gt;H&lt;/sup&gt;</td>
<td>1.31</td>
<td>0.16</td>
<td>1.56</td>
</tr>
<tr>
<td>Anti-PI&lt;sup&gt;0&lt;/sup&gt;</td>
<td>0.12</td>
<td>1.26</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Numbers represent the optical density at 495 nm following a 30-minute development time of secondary antibody. Note that patient P.D.J. platelets strongly expressed the PI<sup>H</sup> alloantigenic determinant, but failed to bind anti-PI<sup>A</sup> alloantisera, indicating that he is homozygous for the PI<sup>A</sup> allelic.
analysis (data not shown), as well as sequence-specific PCR primer analysis (Fig 1) clearly demonstrated that patient P.D.J. carries both the PI^A1 and PI^A2 alleles of GPIIIa, i.e., is heterozygous for PI^A. Based on these results, we hypothesized that the P.D.J. had inherited a silent PI^n allele, effectively making him a carrier for type I Glanzmann thrombasthenia.

Platelets from type I Glanzmann heterozygotes typically express on their surface approximately 50% to 60% of normal levels of the GPIIb-IIIa complex. To determine whether P.D.J. platelets express reduced levels of surface GPIIb-IIIa, 125I-AP3 binding studies were performed using methods that have been previously described. As shown in Fig 2, whereas control platelets bound 45 ng AP3/5 × 10^6 platelets at saturation (corresponding to approximately 50,000 GPIIb-IIIa complexes/platelet), P.D.J. platelets bound only 20 ng of AP3/5 × 10^6 platelets at saturation, which corresponds to approximately 20,000 GPIIb-IIIa complexes/platelet. The observation that P.D.J.’s platelets express only half the number of GPIIb-IIIa complexes on their surface is consistent with the notion that P.D.J. is a carrier of a defective GPIIIa allele that (1) encodes a C at nucleotide 196 (and therefore genotypes as a PI^n allelic form), and (2) has a molecular defect elsewhere on the gene that prevents its synthesis and/or expression.

To identify the mutation responsible for the putative type I Glanzmann thrombasthenia carrier status in patient P.D.J., all 14 exons of the GPIIIa gene were PCR-amplified using primers corresponding to the flanking regions of the exons. All PCR products derived from P.D.J. genomic DNA were identical in size to those obtained from the normal control DNA, and genomic Southern blots were normal, as well (data not shown), indicating that the defective allele did not harbor major insertions, deletions, or gene rearrangements. However, direct automated sequence analysis of the PCR products showed a two-base deletion (nucleotides 728 and 729 of GPIIIa cDNA) within exon 4 (data not shown). To confirm this observation, the PCR product containing exon 4 was subcloned into pGEM5, and individual clones subjected to manual DNA sequence analysis. The sequence of...
18 different clones was determined, with approximately 50% of them containing the dinucleotide deletion within the codons for GPIIIa amino acids 210 and 211 (Fig 3). This frameshift results in a change in the sequence of amino acids 211-223 and, more importantly, creates a stop codon 38 nucleotides downstream, yielding a truncated protein that would, if fully transcribed and translated, contain only the first 223 of 762 total amino acids normally present in mature, wild-type GPIIIa. Thus, P.D.J. is a carrier for type I Glanzmann thrombasthenia, having one normal GPIIIa allele that encodes the PI\textsuperscript{A1} alloantigenic determinant, as well as a PI\textsuperscript{A2} allele of GPIIIa that is not expressed on the cell surface.

DISCUSSION

The human platelet plasma membrane contains over 100 proteins that can be recognized biochemically as individual discrete spots on two-dimensional sodium dodecyl sulfate (SDS) polyacrylamide gels. During the past 20 years, the biochemical basis for platelet function has advanced greatly as the structures and functions for many of these membrane components have been solved at the molecular level. With the realization that platelet membrane glycoproteins control crucial platelet functions such as platelet activation, adhesion to sites of vessel wall damage, aggregation, and clot retraction, we have also come to appreciate that many of these same glycoprotein receptors are polymorphic within the human gene pool, that is, there exist two or more functional allelic forms. The clinical consequences of these molecular variations are not unlike that found in other cellular and organ systems, in that these polymorphisms can be and are recognized as immunologic targets in a transplant setting—broadly defined. Such settings can include organ transplantation, blood transfusion, and pregnancy.

The first indications that the platelet surface was polymorphic appeared in the late 1950s and early 1960s, when alloantibodies that developed in patients with PTP and NATP were shown to discriminate antigen-positive from antigen-negative platelets. Since that time, serological studies have continued to provide important population frequency data for the currently recognized human platelet alloantigenic epitopes, which now number more than eight. Recent advances in molecular and cellular biology, however, have made it possible to build upon previous serologic and biochemical studies of human platelet alloantigen systems in important and exciting ways. In addition to providing a detailed basic understanding of the polymorphisms that are responsible for eliciting an alloimmune response to the platelet surface, the molecular biologic characterization of platelet membrane glycoprotein polymorphisms has enabled the development of DNA-based typing for the platelet alloantigenic epitopes, which in turn has markedly improved our ability to diagnose and manage platelet alloimmune disorders.

A variety of molecular diagnostic methods have been adopted for the determination of platelet alloantigen genotypes, including RFLP-based approaches, allelic-specific oligonucleotide hybridization techniques, and PCR reactions that employ sequence-specific PCR primers. Each of these techniques offers significant advantages over serologically-based methods, which rely on the use of (1) human alloantisera, which are often available in only limited quantities in specialized centers, and (2) patient platelets, which are often difficult to obtain in sufficient quantities from patients who are often thrombocytopenic. Nonetheless, serologic methods remain the only way to determine whether or not individuals have produced an antplatelet alloantibody, and are still the most frequently used way to determine the antigenic composition of the platelet surface.

Despite the fact that genotypic and phenotypic analysis of platelet alloantigenic epitopes almost always yield identical results, we and others have noted rare circumstances in which discordant results have been obtained. A number of explanations have been offered for such discrepant findings, including the postulated presence of rare, allelic variants of existing human platelet alloantigenic epitopes (for example,
a third allele of the PIA system (PIA\(^{33?}\)) caused by yet-to-be-described mutations at or near amino acid 33 of GPIIIa) that could interfere with current DNA typing methods. The purpose of the present investigation was to investigate whether a simpler explanation might be found in those cases in which phenotyping and genotyping do not agree.

One such example was provided by patient P.D.J., who serologically typed as a PIA\(^{AVAV}\) homozygous individual (Table 1), but who very clearly carried both the PIA\(^{A}\) as well as the PIA\(^{AV}\) allele of GPIIIa upon genotypic analysis (Fig 1). Because the PIA\(^{AV}\) allelic form of GPIIIa was not detectable using serological methods, we postulated that this allele harbored a mutation that prevented its expression, a hypothesis that was borne out by quantitating the amount of GPIIIa on the platelet surface (Fig 2), and by our finding a dinucleotide deletion in this allele that results in the production of a truncated protein (Fig 3). Thus, the PIA\(^{AV}\) allele of GPIIIa in this individual is able to be detected only by genotypic analysis, because it is silent at the protein level.

In conclusion, while encountered only rarely, carriers of Glanzmann thrombasthenia (GPIb or GPIIIa mutations) or Bernard Soulier (GPIbx, GPIby, GPV, or GPIX mutations) alleles that are at the same time heterozygous for human platelet alloantigenic epitopes carried on these same glycoproteins, have the possibility to give discrepant results when comparing genotypic versus phenotypic typing results. Clearly, the combination of serologic and DNA-based evaluation contributes complementary and beneficial diagnostic information than either one alone are able to provide. In light of these findings, it is probably not necessary to invoke more complicated explanations for discordant DNA typing versus serologic typing results. Recently proposed rare third allelic variants for human platelet alloantigen systems are unlikely to be borne out.

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