Amino acid substitutions in platelet membrane glycoproteins result in alloantigens. Identifying these polymorphisms is important in alloimmune-mediated platelet disorders. Immunophenotyping platelet antigens can be limited by the unavailability of specific antisera. The goal of this work was to identify human platelet antigen genotypes in individuals using a technique that would circumvent the limitations of immunophenotyping and be clinically applicable. We have successfully applied the reverse dot-blot (RDB) technique to the genotyping of the five major human platelet alloantigen systems. Allele-specific oligonucleotides (ASOs) representing each allele of these alloantigens were covalently linked to a filter. Biotinylated oligonucleotides flanking the polymorphic sequences in genomic DNA were used to amplify the major platelet alloantigens. We now report that this procedure can be performed accurately and rapidly (<24 hours) on both blood and fetal amniocytes. This approach should greatly complement the immunophenotyping data and, in some cases, may replace it. Moreover, this approach will allow the development of screening programs for the identification of fetuses at risk for thrombocytopenia.

**MATERIALS AND METHODS**

*Reagents*

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was from Pierce (Rockford, IL). Restriction endonucleases were from New England Biolabs, Inc (Beverly, MA). Taq polymerase was from Perkin Elmer (Norwalk, CT). Streptavidin-horseradish peroxidase conjugate was from Boehringer Mannheim (Indianapolis, IN). 3,3',5,5' Tetramethylbenzidine (TMB) and hydrogen peroxide were from Sigma (St Louis, MO).

*Oligonucleotides*

Polymerase chain reaction (PCR) oligonucleotides were designed from published genomic DNA sequences24 and synthesized on an Applied Biosystems (Foster City, CA) DNA synthesizer. Amino-linked allele-specific oligonucleotides (ASOs) were 15 to 18 bp in length and differed by the single nucleotide that has been shown to cause the polymorphic amino acid.

The current method for determining an individual’s platelet alloantigen phenotype is by immunophenotyping, whereas antibody detection is dependent on measuring bound antibody to a panel of typed platelets. These methods both require the availability of two crucial reagents: well-characterized typing sera and a panel of phenotyped donors. Relatively few laboratories have adequate typing sera available for the many specificities required to characterize the identity of different platelet alloantibodies. A major problem with the identification of alloantibodies can be the presence of coexisting HLA antibodies. Finally, there are times when the thrombocytopenia is so severe at presentation that it is not possible to obtain enough platelets to serologically type.

The DNA polymorphisms responsible for each of the five major platelet alloantigen systems have now been identified and are summarized in Table 1. In all cases, a single nucleotide substitution results in an amino acid change that, in turn, alters the antigenicity of the glycoprotein. Genotyping is not limited by any of the problems associated with platelet immunophenotyping. We have therefore applied the reverse dot-blot (RDB) technique to the genotyping of genomic DNA by the polymerase chain reaction (PCR), and these products were hybridized to the filters containing the ASOs. Reactivity was detected with a chromogenic substrate. This nonradioactive methodology identifies all possible genotypes in a well-defined control group of individuals and requires only two PCR reactions per patient sample. RDB analysis was used to successfully genotype women and family members with neonatal alloimmune thrombocytopenia and with posttransfusion purpura and to prenatally genotype the amniocytes from a fetus at risk for thrombocytopenia. The RDB methodology is specific, sensitive, and rapid and should enhance our ability to accurately diagnose disorders of alloimmune platelet destruction.

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amniocytes were initially resuspended in DNA. Samples were kindly provided to us: Pen and Penbm blood by Gail Teramino (Puget Sound Blood Center, Seattle, WA); and Ko DNA by Janice McFarland (Milwaukee, WI); and KO DNA by Gal Teramino (Puget Sound Blood Center, Seattle, WA). Whole blood (0.5 mL) was added to 1 mL PLB, and this process was repeated two times. The cells were centrifuged for 20 seconds at 13,000g, the pellet was resuspended in 1 mL Tris [pH 8.3], 2.5 mmol/L MgCl₂, 0.45% Tween 20, 1% Triton X-100, 0.1 mg/mL gelatin, 0.45% NP-40, 0.45% Tween 20) plus 4 mg/mL proteinase K and incubated at 55°C for 10 minutes. Multiple filters from different individuals can be washed in one tube. The wash buffer is poured off and the filters are incubated with 5 μL of streptavidin-horseradish peroxidase conjugate in 20 mL of 2× SSC/0.1% SDS for 15 minutes at room temperature. The strips are then washed three times for 2 minutes in 2× SSC/0.1% SDS and two times in 0.1 mol/L NaCitrates, pH 5.0, for 2 minutes. Color is detected with 10 μL H₂O₂ added to 20 mL of 0.1 mg/mL TMB in 0.1 mol/L NaCitrates, pH 5.0. Color was recorded by photography and by photocopying. This procedure is diagrammed in Fig 1.

**DNA Samples and Preparation**

Whole blood from normal volunteers and patients with thrombocytopenia and cultured amniocytes from a fetus at risk for neonatal thrombocytopenia were used to isolate genomic DNA. A number of samples were kindly provided to us: Pen and Penbm blood by Prof. Y. Shibata (Tokyo, Japan); Ko DNA by Janice McFarland (Milwaukee, WI); and Ko DNA by Gal Teramino (Puget Sound Blood Center, Seattle, WA). Whole blood (0.5 mL) was added to an equal volume of PCR lysis buffer (PLB; 0.32 mol/L sucrose, 10 mmol/L Tris [pH 7.5], 1% Triton X-100, 5 mmol/L MgCl₂). Fetal amniocytes were initially resuspended in 1 mL of PLB. Samples were centrifuged for 20 seconds at 13,000g, the pellet was resuspended in 1 mL PLB, and this process was repeated two times. The pellet was resuspended in 0.5 mL of SIB (50 mmol/L KCl, 10 mmol/L Tris [pH 8.3], 2.5 mmol/L MgCl₂, 0.1 mg/mL gelatin, 0.45% NP-40, 0.45% Tween 20) plus 4 mg/mL proteinase K and incubated at 65°C for 1 hour. The sample was then boiled for 10 minutes to inactivate the protease K and stored at -20°C. Twenty-five microliters of lyase was equivalent to approximately 1 μg of genomic DNA.

**RDB Procedure**

**Membrane preparation.** Biodyne C membranes (Pall Bioprocess, East Hills, NY) were rinsed in 0.1 N HCl, rinsed in water, incubated in 10% (0.52 mol/L) EDTA for 15 minutes, at room temperature, rinsed in water and allowed to air dry. Amino-linked ASOs were diluted to 5 pmol/μL with 0.5 mol/L NaHCO₃/Na₂CO₃ buffer, pH 8.4, and 2 μL was carefully applied to a filter and allowed to dry for 15 minutes. Membranes were then rinsed once in 0.1 N NaOH for 1 minute, rinsed three times in water, and allowed to dry. This procedure covalently linked each ASO to the filter. We have successfully used filters that were prepared 2.5 months earlier (stored at room temperature), but they may be used for at least 6 months (Jeff Wold, personal communication, October 1993).

**Hybridizing and washing.** Two hundred nanograms of genomic DNA was amplified in a 50 μL PCR reaction with the biotinylated primers, as previously described. 15 In all cases, positive control DNAs for each antigen system were used. For each set of PCR primers, a negative control consisting of no template DNA in the PCR reaction was always performed to assure no products were amplified from potentially contaminating DNA. In some cases, two or three primer pairs were used in a single PCR reaction. Twenty-five to thirty microliters of each PCR reaction from a single individual was added to 15 mL 2× SSC (1× SSC = 150 mmol/L NaCl/15 mmol/L trisodium citrate, pH 7.0)/0.1% sodium dodecyl sulfate (SDS) in a polypropylene tube. The membrane is added, the tube is boiled for 5 minutes to denature the PCR products, and the tube is immediately transferred to a shaking 45°C water bath for 1 hour or more. Filters are washed in 40 mL of 0.5× SSC/0.1% SDS at 42°C for 10 minutes. Multiple filters from different individuals can be washed in one tube. The wash buffer is poured off and the filters are incubated with 5 μL of streptavidin-horseradish peroxidase conjugate in 20 mL of 2× SSC/0.1% SDS for 15 minutes at room temperature. The strips are then washed three times for 2 minutes in 2× SSC/0.1% SDS and two times in 0.1 mol/L NaCitrates, pH 5.0, for 2 minutes. Color is detected with 10 μL H₂O₂ added to 20 mL of 0.1 mg/mL TMB in 0.1 mol/L NaCitrates, pH 5.0. Color was recorded by photography and by photocopying. This procedure is diagrammed in Fig 1.

**Immunophenotyping**

Immunophenotyping was performed using a radiolabeled anti-globulin test described by Cines and Schreiber 26 and adapted for platelet typing. Control antisera used to type platelets have been obtained from our patients (anti-PI⁺, PI⁻, -Bak⁺, -Bak⁻, -Br⁺, -Br⁻) or supplied by Drs Y. Shibata (anti-PI⁻, -Pen⁺, -Pen⁻, -Br⁺, -Br⁻) and F. Ishida (anti-KO⁺). Exchange with other laboratories has confirmed the specificity of our antisera as typing reagents.

**Monoclonal Antibody Capture Enzyme-Linked Immunosorbert Assay (MACE)**

The MACE assay was performed according to the method of Ishida et al. 28 In this assay, normal platelets are incubated with test

<table>
<thead>
<tr>
<th>Table 1. Human Platelet Alloantigens</th>
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<tr>
<td><strong>Alloantigen</strong></td>
</tr>
<tr>
<td>PI⁺, Zwa⁺</td>
</tr>
<tr>
<td>PI⁺, Zwb⁺</td>
</tr>
<tr>
<td>Ko⁺</td>
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<tr>
<td>Ko⁻</td>
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<tr>
<td>Bak⁺, Sib⁺</td>
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<tr>
<td>Bak⁺, Sib⁻</td>
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<td>Pen⁺, Ykb⁻</td>
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<td>Br⁺, Zav⁺</td>
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<td>Br⁺, Zav⁻</td>
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<table>
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<th>Table 2. RDB Oligonucleotides</th>
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<tr>
<td><strong>Name</strong></td>
</tr>
<tr>
<td>Pi⁺</td>
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<td>PI⁻</td>
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<tr>
<td>Bak⁺</td>
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<tr>
<td>Bak⁻</td>
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<tr>
<td>Ko⁺</td>
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<tr>
<td>Ko⁻</td>
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Fig 1. Diagram of RDB procedure.
antisera and lysed in Triton X-100. After centrifugation at 12,000g, the suspension is applied to microtiter wells coated with monoclonal antibodies specific for GPIIb, GPIIa, and GPI (from Amac, Inc, Westbrook ME). The amount of human IgG in the test antisera bound to each platelet glycoprotein is measured by incubating with the F(ab')2 fraction of biotinylated goat antihuman IgG (Chemicon, Temecula, CA), washing, adding p-nitrophenylphosphate and streptavidin conjugated to alkaline phosphatase, and measuring the absorbance at 405 nm in a microplate reader (Biotek, Winooski, VT).

Establishing Panel of Controls

Control DNAs for the RDB analysis were identified by both immunophenotyping and DNA analysis or just by DNA analysis. For the DNA analysis, PCR products were either directly sequenced or restriction-digested using the enzymes Hha I, Fok I, and Mnl I, which distinguish the homozygous or heterozygous state for K0, Bak, and Br, respectively. As mentioned above, these controls were used with all analyses to eliminate false-negatives caused by reagent failure or execution problems.

RESULTS

A series of preliminary studies were performed to establish the correct conditions for this assay. The first essential aspect of this work was to develop a panel of control DNA samples from individuals representing all three combinations of alleles (2 homozygous samples and 1 heterozygous sample) for all five antigen systems. Controls were identified by both immunophenotyping and DNA analyses (either restriction endonuclease digestion or direct nucleotide sequencing of PCR products). Because there were not specific antisera available to us for all homozygous states, only those tested are shown in Fig 2. The panel of controls is shown in Fig 2 and demonstrates that the RDB results are consistent with the immunologic and nucleotide sequence information.

The second body of preliminary work was to define ASOs for detection of each of the various genotypes. Initially, we generated biotinylated PCR primers flanking the known areas of the genome that contain the polymorphism resulting in each antigen system (data not shown). We arbitrarily chose hybridization conditions that allowed the accurate detection of the two Pt alleles and then required the remaining eight ASOs to correctly hybridize under these same conditions. Specifically, we hybridized in 2× SSC/0.1% SDS at 45°C and washed in 0.5× SSC/0.1% SDS at 42°C. We then synthesized ASOs for the K0, Bak, Pen, and Br systems that specifically reacted with their corresponding amplified PCR products under these same conditions. Eventually, ASOs, hybridization, and washing conditions were determined that permitted successful genotyping in a single hybridization. These RDB results were consistent with both the immunophenotyping and the nucleotide sequence analysis (data not shown).

Having established the specificity of the RDB for the five major platelet alloantigens, we tested its utility in clinical situations of alloimmune thrombocytopenia. We obtained blood from women who had previously delivered a baby with neonatal alloimmune thrombocytopenia and also from their husbands. Genomic DNA from these individuals was used for DNA analysis or just by DNA analysis. Having established the specificity of the RDB for the five major platelet alloantigens, we tested its utility in clinical situations of alloimmune thrombocytopenia. We obtained blood from women who had previously delivered a baby with neonatal alloimmune thrombocytopenia and also from their husbands. Genomic DNA from these individuals was used for DNA analysis or just by DNA analysis.

DISCUSSION

We have successfully applied the technique of RDB analysis to genotype the five major platelet alloantigen systems. This method is simple, rapid, and accurate and was useful in characterizing cases of NAIT and PTP. ASO hybridization has been used to detect single-base changes at a specific location in the genome. Because the ASO was labeled with 32P, this method has traditionally been confined to the research laboratory. The RDB technique is a nonradioactive PCR-based methodology for the rapid identification of specific alleles that differ by a single nucleotide. It has been successfully used for genotyping in the prenatal diagnosis of thalassemia and cystic fibrosis. It is similar to the above described ASO hybridizations except that it is "reversed,"
The ASO is fixed to the filter and the PCR products are in the hybridization solution. Because the ASOs are previously applied to the membranes, the entire procedure can be performed in 1 day (DNA extraction, 1 hour; PCR reaction, 2 to 3 hours; hybridization, 1 to 2 hours; washing and color development, 1 hour; total, 6 hours). Finally, we have been able to obtain enough DNA for these analyses from as little as 5 μL of whole blood and from fetal amniocytes.

During the course of our studies, we experienced problems with reliability of typing sera and availability of platelets, prohibiting an accurate and comprehensive immunologic characterization. The most reliable methods for detecting alloantibodies measure antibody binding to a panel of typed platelets. This measuring requires the availability of well-characterized typing sera and a panel of phenotyped donors. Well-characterized antisera is not commercially available. Therefore, laboratories must obtain their own or obtain them from other investigators. In addition, relatively few laboratories have all the typing sera required to characterize the plethora of different platelet alloantibodies. This is particularly important for a patient who is heterozygous and typing sera is only available for one of the polymorphisms. For these diallelic loci, proof of heterozygosity eliminates that antigen system as a possible stimulus for antibody formation, and the appropriate genetic counseling can be administered. Another major problem with the identification of alloantibodies can be the presence of coexisting HLA antibodies. It is also difficult to distinguish an antibody to a high-incidence platelet alloantigen from an autoantibody that also reacts with all platelets tested. Immunoblotting or the use of monoclonal glycoprotein capture assays can be used to circumvent some of these problems, but not always conclusively. Another issue complicating the identification of antibodies is the growing number of antigens (many of which are low
incidence) that have been described. Also, frequently the thrombocytopenia is so severe at presentation that it is not possible to obtain enough platelets to immunophenotype. Other problems can occur, such as low titer antibodies and the disappearance of the antibody over time. Attempts to overcome these problems have generally been handled by using combinations of assays, but equivocal results may still occur. Genotyping is not limited by these problems and would be an extremely useful complement to serologic studies. Genotype information indicates which alloantigen systems are incompatible, i.e., those for which an antibody could theoretically be generated. Nevertheless, for the reasons listed above, we believe that genotyping rather than immunophenotyping is a more accurate and reliable method for determining the antigen status of both normal and patient populations.

There are a number of effective methods that have been used to genotype patients for single nucleotide changes in genomic DNA, each with its own advantages. McFarland et al. used a nonradioactive variation of ASO hybridization to genotype families with a history of NAIT. These ASOs were identical with each other except for the single nucleotide difference at the polymorphic site, which provided a 100% correlation with serologic typing for either PI A or PI B. Simsek et al. have used another PCR-based technique to genotype 98 Dutch blood donors for PI A, Ko, and Bak. They PCR-amplified genomic DNA containing the polymorphic region, restriction-digested the products with an enzyme that cut one allele but not the other, and analyzed these products on agarose gels. More recently, Metcalfe and Waters used allele-specific primers in a PCR amplification to test individuals for their PI A genotype. Although only used for one antigen system, this method was simple, rapid, and accurate.

All of these genotyping techniques have the advantages of (1) speed, (2) circumventing the need for specific typing sera, and (3) not requiring a certain number of platelets that can often be difficult to obtain. Each technique has certain disadvantages as well, such as a large number of hybridizations, the use of restriction enzymes, and two PCR reactions for each antigen system. The use of restriction enzymes introduces a potential for error with both incomplete digestion and overdigestion of the DNA. In addition, not all polymorphisms will be amenable to this technique because they may not alter a restriction enzyme site. The advantages of the RDB over standard ASO hybridizations are twofold. (1) Once the conditions of hybridization have been established for all the ASOs, all primers can be spotted on a single filter and stored indefinitely. (2) For a given DNA sample, only one hybridization is necessary, rather than one hybridization for each ASO probe. The disadvantage of RDB, a hybridization step, is present in some other techniques, and seems counterbalanced by the lack of requirement for restriction enzymes, electrophoresis, and extra PCR reactions. In fact, we are currently genotyping the five major antigen systems with just two PCR reactions: one reaction contains two pairs of primers and the other reaction contains three pairs (multiplex PCR; data not shown). Reducing the number of PCR reactions represents a substantial savings of time and cost.

We controlled for false-positive results with this technique by performing PCR reactions using all sets of PCR primers and no template DNA. Consideration of a false-negative
A Patient | Plt Antibody Studies | DNA Sequence | Reverse Dot Blot
---|---|---|---
CC | anti-GPla Ab | | Br^b / Br^b
JC | | | Br^b / Br^a
FC | | | Br^b / Br^b

B M aa CYC JC FC

Fig 5. Prenatal RDB analysis of a woman with a known anti-GPla antibody. (A) MACE data showed that serum from CC contained an antibody directed against an epitope on GPla. When she became pregnant, the platelet alloantigen genotype of the patient and her husband (JC) was determined by RDB analysis. Similar analysis on cultured fetal amniocyte DNA (FC) indicated that the fetus was not at risk for NAIT caused by Br incompatibility. (B) To provide additional support for a Glu to Lys substitution at amino acid 505 of platelet GPlba, we digested the PCR products with the restriction enzyme Mnal, which can distinguish between homozygous Br^a (lane 2, control), homozygous Br^b (lane 3), and the heterozygous state (lane 4). Note that the analysis on the fetus was identical to that on the mother (CVC).

result is made by always amplifying our positive control DNA samples for each of the 10 alleles tested. In addition, because our PCR primers are able to amplify both alleles of all five antigen systems (Fig 2), a heterozygous genotype result would not be mistaken for a homozygous one. That is, as long as one of the two "dots" were positive, the only potential source of error concerning the negative ASO was if that ASO had inadvertently not been applied to the membrane. For this reason, whenever the putative offending antigen was detected, the analysis was repeated with a new "strip" to rule out the possibility of an error when spotting the ASO.

There are several other platelet-specific antigens that have been described and an increasing number of antibodies to these antigens have been reported. Many of these appear to be directed against private or low incidence alloantigens. The characterization of these antigens is limited by the unavailability of antisera and methods for detection and specific identification. Once the nucleotide substitution responsible for these rare polymorphisms has been identified, testing for them could easily be added to the five we have performed.

Accurate characterization of an individual’s platelet alloantigens in the immune-mediated thrombocytopenias is needed so that (1) the most appropriate therapy can be administered, (2) genetic counseling can be given, (3) factors involved in their pathogenesis can be studied, and (4) comprehensive screening programs for obstetric patients can be developed. The development of reliable antenatal testing and effective therapies for hemolytic disease of the newborn has dramatically improved the survival of fetuses at risk for Rh disease. Similar advances are needed in NAIT. The RDB is a rapid, accurate, and sensitive assay that can easily be applied to a clinical laboratory that would identify women at risk for being alloimmunized.

ACKNOWLEDGMENT

We thank Farid Chehab, Jeff Wall, and She-ping Cai for their time and help establishing this assay; H. Kim for technical assistance with the MACE assay; Y. Shibata for supplying the Pen samples; Janice McFarland for the Ko^a control DNA; Gail Teramino for the Ko^b DNA; and Sentot Santoso for providing the PCR sequences for the Br amplification before their publication.

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Rapid genotyping of the five major platelet alloantigens by reverse dot-blot hybridization

PF Bray, Y Jin and T Kickler