Prenatal Diagnosis of Neonatal Alloimmune Thrombocytopenia Using Allele-Specific Oligonucleotide Probes


The prediction of neonatal alloimmune thrombocytopenia (NATP) in affected families has, in the past, been based on information about gene frequencies of the antigen systems involved, parental phenotyping, and fetal platelet counts. We explored the feasibility of allele-specific oligonucleotide probe typing for Pi antigens to determine the risk of second or subsequent fetuses in families where one infant had a diagnosis of anti-Pi-mediated NATP. A total of eight families at risk for delivering an affected fetus were studied with both serologic and oligonucleotide typing. The correlation between serologic and oligonucleotide Pi types was 100%.

Similarly, in an additional eight families not at risk for Pi-mediated NATP, serologic and oligonucleotide typing maintained a perfect correlation. DNA isolated from fetal leukocytes as well as fetal amniocytes was successfully typed using this technology. Oligonucleotide-based typing of fetuses at risk for NATP whose fathers are heterozygous for the Pi antigen allows early recognition of affected fetuses so that prenatal therapy of mothers can be instituted if necessary. When fetuses are found to be unaffected, invasive, and/or expensive, prenatal interventions can be avoided. © 1991 by The American Society of Hematology.

NEONATAL ALLOIMMUNE thrombocytopenia (NATP) affects approximately 1 in 5,000 live births and is characterized by marked thrombocytopenia in the fetus and neonate developing before or shortly after birth. Platelet destruction is caused by a maternal antibody directed against a fetal platelet antigen inherited from the father and lacking on the mother's platelets. The most commonly implicated platelet antigen in this disease is PiA, accounting for 41% to 83% of all cases.

The prognosis in NATP is generally good, but mortality and morbidity remain significant. Initially, the reported overall mortality was between 12% and 14%. A more recent review found a mortality rate of 9%. Intracranial hemorrhage is the most devastating complication of NATP and is estimated to occur in between 15% and 28% of all affected neonates. Although originally thought to be a result of birth trauma related to vaginal delivery, intracranial hemorrhage occurring prenatally is now recognized in between 2% and 9% of cases. In utero intracranial hemorrhage is particularly worrisome because it cannot be avoided by elective cesarean section.

The mechanism of platelet destruction in NATP is similar to that of the red blood cell (RBC) destruction in hemolytic disease of the newborn. But unlike erythroblastosis fetalis, which occurs in second or subsequent pregnancies in response to alloimmunization of the mother by fetal RBCs late in the first pregnancy, between 20% and 59% of NATP cases occur in primiparous. Transplacental movement of the immunizing antigen, eg, PiA, occurs as early as 17 weeks of gestation. In turn, maternal IgG is produced and crosses the placenta and coats fetal platelets, ultimately causing their destruction, often in first pregnancies.

Determination of platelet phenotypes of pregnant women is not routinely performed and, therefore, NATP in first-born infants cannot be anticipated. However, several techniques have been used to predict second or subsequent affected siblings. The gene frequencies of the antigen systems involved in NATP can be used to calculate the probability that subsequent offspring have the incompatible antigen. Prenatal maternal serum tests for platelet-specific alloantibodies have been monitored in an attempt to predict the occurrence and severity of NATP in siblings. Finally, percutaneous umbilical vein blood sampling of the fetus for platelet counts and platelet typing or fetal scalp vein blood sampling for platelet count have been used to diagnose subsequent fetuses who are thrombocytopenic and/or possess the incompatible antigen. Although each of these techniques is helpful to some extent in predicting affected fetuses, each is associated with some level of uncertainty and/or risk to the fetus.

Recent investigation in our laboratory of the molecular basis of the PiA alloantigen system has shown that a single nucleic acid base substitution in the gene encoding for glycoprotein IIIa (GPIIIa) differentiates the PiA antigen from its allele, PiA. Of the 762 amino acids that form the GPIIIa molecule, a Leu to Pro amino acid polymorphism resulting from this base substitution appears to be solely responsible for the formation of the PiA and PiB antigenic determinants. The alloantigenic polymorphism in the PiA system was defined using the polymerase chain reaction (PCR) for the amplification and subsequent analysis of platelet-specific mRNA sequences from individuals of known PiA phenotypes. Subsequently, taking advantage of the known sequence for the genomic DNA encoding for GPIIIa, we have further adapted this technique to amplify the polymorphic region from genomic GPIIIa DNA from peripheral blood leukocytes and from amniocytes. In the present study, allele-specific oligonucleotide hybridization analysis of amplified DNA from fetal tissue was used to...
differentiate the PI^A_1 and PI^A_2 forms of genomic GPIII DNA to detect fetuses at risk for anti-PIA^-mediated NATP.

MATERIALS AND METHODS

Sample Selection

Aliquots of blood samples from families sent to The Platelet Antibody Laboratory of The Blood Center of Southeastern Wisconsin, Milwaukee, for serologic evaluation for NATP were referred for allele-specific oligonucleotide hybridization analysis of PI^A system antigens. In addition, if the mothers were undergoing percutaneous umbilical venous blood sampling for determination of fetal platelet count and/or serologic phenotyping, residual fetal blood cells were referred for this analysis after plaerets had been removed from the samples. In cases in which mothers were undergoing amniocentesis for other indications, a small amount of either fresh amniotic fluid and/or cultured amniocytes was requested for oligonucleotide-based typing. Samples of parental blood and amniotic fluid were received from four families not at risk for NATP, but in whom amniocentesis was performed for other reasons. Blood samples from 14 normal individuals who had undergone PI^A system serologic phenotyping were studied to validate the genomic DNA-based phenotyping. The protocol for this typing study of blood and amniocytes was approved by the human subject research review committee of The Blood Center.

Serologic Phenotyping and Antibody Detection

Platelets were isolated from parental and fetal blood samples and the PI^A phenotypes were determined using an antigen-capture enzyme-linked immunosorbent assay (ACE). In some cases, insufficient fetal platelets were available for ACE typing, and typing was performed using the platelet suspension immunofluorescence test. Well-characterized alloantisera or eluates derived from posttransfusion purpura plasma were used in assays for PI^A (plasma) and PI^A_2 (eluate) serologic platelet typing. Platelet-specific antibody was detected in maternal serum using the ACE.

Isolation and Amplification of Genomic DNA

Genomic DNA was isolated from peripheral blood leukocytes from as little as 1 mL 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. DNA from fetal blood was isolated using proteinase K, supersaturated NaCl, Na acetate, and ethanol precipitation. DNA from cultured or fresh amniocytes was isolated using proteinase K treatment, super saturated NaCl, and phenol-chloroform extraction techniques.

One 25-base and one 26-base oligonucleotide primer (Table 1) flanking a 247-bp region of the gene for GPIIIa containing the allelic polymorphism were synthesized on a Gene Assembler (Pharmacia Fine Chemicals, Piscataway, NJ). Amplification was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) programmed to permit denaturation at 98°C for 15 seconds, followed by final incubation at 72°C for 7 minutes to allow completion of strand synthesis. PCR samples were electrophoresed on a 1.6% agarose gel (Bethesda Research Laboratories, Life Technologies, Inc, Gaithersburg, MD) for qualitative analysis of amplified DNA. If amplified DNA was present, the PCR samples were studied with allele-specific oligonucleotide hybridization and immunologic detection.

Allele-Specific Oligonucleotide Hybridization and Immunologic Detection

Amplified DNA, 100 ng, was applied to nylon membranes using a dot-blot apparatus (Magnagraph, Fisher no. NJ4HY00010; Fisher Scientific Corp, Itasca, IL) and hybridized to each of two 13-mer oligonucleotide probes differing only at the central base with specificity for the PI^A_1 or PI^A_2 sequence (Table 1). Unknown samples were always blotted with control DNA derived from known homozygous PI^A_1 and PI^A_2 individuals as well as known heterozygous PI^A_1/IA_2 DNA. The oligonucleotide probes were end-labeled with digoxigenin-11-deoxyuridine-triphosphate and were immunologically detected using alkaline phosphatase-conjugated polyclonal sheep antidigoxigenin Fab fragments (Boehringer Mannheim, Indianapolis, IN) and nitro blue tetrazolium with 5-bromo-4-chloro-3-indoly phosphate as substrate.

RESULTS

A total of 16 families were studied to determine the PI^A phenotypes of family members. In eight families the mother was known to lack the PI^A_1 antigen; in the remaining eight, the mother was positive for PI^A_1. In 12 of the families a previous neonate(s) had been diagnosed with NATP. Samples from four families who had no history of NATP and 14 normal individuals previously serologically typed for PI^A antigens were also studied.

Case Histories

Case 1. NR was a 24-year-old woman whose first pregnancy was complicated by fetal in utero intracranial hemorrhage at 35 weeks gestation. The fetus was delivered and found to have massive intracranial hemorrhage and a platelet count less than 30,000/L. The infant died after 48 hours. Subsequent serologic evaluation showed that the mother was PI^A_1 homozygous (PI^A^-negative) with a strong anti-PIA^- antibody in her serum. Her husband was heterozygous for PI^A_1 and PI^A_2. During the second pregnancy, which began 2 months later, she underwent percutaneous umbilical venous blood sampling and amniocentesis at 20 weeks gestation. The fetal platelet count was normal and fetal and parental blood was submitted for serologic phenotyping and referred for allele-specific oligonucleotide hybridization analysis (Table 2). Samples of fresh and cultured amniotic fluid were also available for analysis (Fig 1). Because the fetal platelet phenotype was PI^A_1/IA_2, the preg-

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antisense</td>
<td>5' GTGCAACTCTGCGGACTGACTTG3' PIA_1 5'TGCCTCGGGCTCG3'</td>
</tr>
<tr>
<td>Sense</td>
<td>5'CGAGGAGGTCAGGTCGCCATAG3' PIA_2 5'TGCCCTCGGGCTCG3'</td>
</tr>
</tbody>
</table>

The antisense primer from base 366 through 342 was used to prime the synthesis of amplified DNA from genomic GPIIIa DNA. The second strand was generated by the sense primer beginning in the intron preceding exon 2 66 bases from the splice junction at base 196 during the first round of PCR. The oligonucleotide probes specific for the PI^A_1 and the PI^A_2 forms of the GPIIIa gene differed only in the central base (underlined).
nancy was managed expectantly with no prenatal treatment for fetal thrombocytopenia. Serial maternal anti-PIA' antibody titers were performed in the ACE, and anti-PIA' was detected throughout the pregnancy, presumably persisting from the previous pregnancy. At 39 weeks gestation a normal-term female infant was delivered by cesarean section for obstetrical indications. The platelet count was 300,000/μL. Follow-up examination of the infant at 2 months was completely normal.

**Case 2.** LB was a 26-year-old woman who had delivered two thrombocytopenic infants in the past (platelet counts of 12,000/μL and 20,000/μL). During her third pregnancy samples from both parents were sent for serologic evaluation, and LB was found to be PI\(^{A2}\) homozygous and the father was PI\(^{A1}\) homozygous. She underwent percutaneous umbilical vein blood sampling at 27 weeks and the fetus was found to have a platelet count of 124,000/μL. Fetal blood was referred for serologic and DNA-based platelet phenotyping, and both confirmed the obligate heterozygous status of the fetus (Fig 1). Weekly prenatal intravenous immunoglobulin (IVlg) (Biotest Pharma GmbH, Frankfurt, Germany) was begun at a dose of 1.0 g/kg from the 33rd to the 38th week of pregnancy. Maternal anti-PIA' antibody was detected 10 weeks before delivery. At 37 1/2 weeks gestation, a second fetal blood sampling was performed and the fetal platelet count had increased to 262,000/μL. Labor was induced and a normal-term male infant was delivered. The infant's platelet count on the day of delivery was 289,000/μL and 2 days later the platelet count was 287,000/μL.

**Case 3.** KS, a 33-year-old woman whose first pregnancy resulted in a severely thrombocytopenic infant (platelet count 2,000/μL), was studied after that delivery and found to be PI\(^{A2}\) homozygous with a potent anti-PIA' antibody in her serum. Platelets from her husband were subsequently phenotyped and found to be heterozygous for PI\(^{A1/2}\). At 32 weeks gestation in her second pregnancy she underwent fetal blood sampling for fetal platelet phenotyping and platelet count. The fetal platelet count was 265,000/μL and the fetus was determined to be PI\(^{A2}\) homozygous by both serologic and DNA-based platelet phenotyping (Fig 1). Maternal serum was positive for anti-PIA' at this time. The pregnancy was managed expectantly, without prenatal treatment, and at 37 1/2 weeks gestation a term 2,841-g infant was delivered vaginally. The platelet count after birth was 378,000/μL.

**Other families.** Five additional families at risk for PI\(^{A1}\)-mediated NATP were studied with both serologic and DNA-based platelet phenotyping of parents and fetuses (Table 2). Both typing methods showed the same phenotypes in each case. Of particular note were the four families (nos. 1, 3, 5, and 8) with heterozygous fathers in which the fetal type was determined to be PI\(^{A1/2}\). Anti-PIA' was shown in maternal serum at some point in pregnancy in three of these four cases. In each case, prenatal therapy for the mother was withheld and the neonate did not have thrombocytopenia. Case no. 6, in which the father was homozygous PI\(^{A1/2}\) and the fetus an obligate heterozygote, was interesting in that thrombocytopenia detected at 19 weeks was
Fig 1. Three representative family studies from cases 1 (A), 2 (B), and 3 (C). Each strip containing “dots” of amplified DNA was hybridized with the nonradioactive probe corresponding to the \( \text{Pi}^{A1} \) (A1 probe) or the \( \text{Pi}^{A2} \) (A2 probe) allelic sequence. Fa, father; Mo, mother; PBL, peripheral blood leukocytes; A1, \( \text{Pi}^{A1} \) homozygote; A2, \( \text{Pi}^{A2} \) homozygote; Ht, \( \text{Pi}^{A1/A2} \) heterozygote.
unresponsive to IVIg therapy administered the mother prenatally. This finding is in contrast to the apparent success of this approach in case no. 2. Details of the preparation, doses, and schedule of IVIg treatment were not available for case no. 6.

Eight other families with Pt "-positive mothers were also studied (Table 3). In this group, there was again a 100% correlation between Pt " phenotype determined by the two methods. Overall, including typings in the family studies as well as 14 controls, 53 individuals were typed using both allele-specific oligonucleotide hybridization analysis and standard serologic evaluation with complete agreement in results. In seven of the family studies, fresh and/or cultured amniocytes served as a source of fetal genomic DNA.

DISCUSSION

The management of fetuses at risk for NATP is increasingly aggressive due to recognition of a significant rate of in utero intracranial hemorrhage. Accurate prediction of affected fetuses is crucial when the risks and costs of prenatal interventions, including fetal blood sampling, high-dose IVIg administered to the mother, and in utero platelet transfusions, are considered. Until recently, the estimate of risk of NATP in a fetus relied on having made the diagnosis in a previous infant in the same family and on additional gene frequency, serologic, and platelet count data. Using gene frequencies of the Pt " system, one can predict that 87% of subsequent pregnancies will be affected. With such a high probability of affected second pregnancies, without additional information all subsequent pregnancies will be managed as if the fetus is thrombocytopenic. This strategy will result in 13% of fetuses receiving such management unnecessarily. Detection of prenatal platelet-specific antibody in maternal serum has been shown to correlate with thrombocytopenia in the fetus. However, depending on the sensitivity of the antibody assay used, misleading positive (antibody detected, fetus unaffected) or negative (antibody absent, fetus affected) results may be seen. Percutaneous umbilical vein sampling allows clinicians to assess the fetal platelet count early in pregnancy. At as early as 18 weeks gestation, fetal blood sampling can be performed on a pregnancy at risk to obtain fetal platelets for counting or for platelet antigen typing. If early fetal blood sampling indicates that the fetus is affected, subsequent sampling can be performed to monitor prenatal therapy in the mother. We and others have explored methods of elevating the fetal platelet count, including administering IVIg and/or steroid therapy to mothers who have delivered severely affected infants previously and whose fetuses in the current pregnancies are shown to be thrombocytopenic. Although direct fetal blood sampling determines which fetuses are thrombocytopenic at the time of sampling, because the kinetics of fetal platelet destruction in NATP are variable, a fetus with a normal count early in gestation may become thrombocytopenic later during the pregnancy. Therefore, a platelet count determined at a single early date in gestation is potentially misleading if normal. The procedure is associated with a rate of fetal demise on the order of 1%. Alternatively, fetal scalp vein sampling may be used to assess the fetal platelet count immediately before delivery. Scalp vein sampling does not address the problem of in utero intracranial hemorrhage reported to occur as early as 34 weeks gestation. In addition, this procedure is only possible after the fetal head is engaged in the pelvis and early labor is underway. Theoretically, uterine contractions will already have begun and the risk of perinatal hemorrhage is increased. Fetal scalp vein sampling has been associated with a high rate of falsely low platelet counts that result in unnecessary cesarean sections.

Additional techniques are available to aid in the prediction of an affected fetus. Complete serologic platelet phenotyping is performed in a limited number of platelet serology laboratories. Paternal platelets are typed for both

Table 3. Clinical Data From Cases at Risk for NATP Due To Non-Anti-Pt " and Normal Families

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Pt Ag* Inc</th>
<th>Previous Infant Affected</th>
<th>Serologic Typing†</th>
<th>ASO Typing‡</th>
<th>Source of Fetal Tissue§</th>
</tr>
</thead>
<tbody>
<tr>
<td>15. (DC) Normal</td>
<td>No</td>
<td>A1/A1 A1/A1 A1/A1</td>
<td>A1/A1 A1/A1</td>
<td>A</td>
<td></td>
</tr>
</tbody>
</table>

*Platelet antigen incompatibility if any identified.
†Platelet type determined serologically on mother (Mo), father (Fa), and fetus (Fe).
‡Platelet type determined by ASO testing.
§Source of fetal tissue for DNA typing: Fe blood, fetal blood from umbilical vein sample; A, fresh amniocytes; CA, cultured amniocytes; ( ), week in gestation sample obtained.

| Family platelet antigen typing was requested after the first pregnancy was terminated due to massive in utero intracranial hemorrhage in the fetus at 24 weeks gestation. |
alleles of the implicated diallelic antigen system. For instance, in a case of NATP due to anti-\(P^{A}\), the father’s platelets are typed for \(P^{A}\) and \(P^{A}\). If the paternal type is homozygous \(P^{A}\), the father almost certainly will pass the gene for the incompatible antigen to a fetus. Determination of the father’s platelet phenotype (for \(P^{A}\)) allows unequivocal prediction of \(P^{A}\) incompatibility in approximately 74% of subsequent fetuses, as this is the frequency of homozygous \(P^{A}\)-positive fathers of previously affected infants. The remaining 26% of fetuses (paternal heterozygote frequency) will have a 50% probability of being affected. Serologic typing of fetal platelets may be attempted, but on the small volumes of blood available from percutaneous umbilical vein blood samples, reliable platelet typing may not be possible, particularly if the sample is thrombocytopenic.

The current study has shown the utility of DNA-based platelet phenotyping for \(P^{A}\) antigens in the identification of fetuses at risk for developing NATP. Use of fetal leukocytes (in the case of fetal blood samples) or fetal amniocytes (in the case of amniocentesis) for this testing circumvents the problems of serologic typing of platelets isolated from small samples of fetal blood. Indeed, fetal blood is not required for genomic DNA analysis if other fetal tissue is available. The result from allele-specific oligonucleotide hybridization analysis of amniocytes is potentially available at 16 to 17 weeks gestation. Theoretically, the method should also be applicable to chorionic villus samples that may be obtained even earlier in pregnancy, routinely during the first trimester.

Identification of affected fetuses with heterozygous fathers is probably the most important application of this technology at present. Currently, serologic typing of fathers to detect homozygotes for \(P^{A}\), and therefore fetuses with 100% probability of being affected, combined with DNA-based platelet phenotyping of fetal tissue in those pregnancies in which the father is heterozygous, is a reasonable strategy for identifying all subsequent affected offspring in utero. We have recently developed a similar allelespecific oligonucleotide based test for the Bak\(^{+}\)/Bak\(^{-}\} alloantigen system, as a single nucleotide base substitution is also associated with antigenic differences in this system. We anticipate that this technology will be applied to genotypic analysis of the remaining platelet alloantigen systems, including Br\(^{+}\), Pen\(^{+}\), Ko\(^{+}\), and others, as the bases of their underlying polymorphisms are solved at the molecular genetic level.

ACKNOWLEDGMENT

We are indebted to Katherine Krygiel for assistance in performing the oligonucleotide probe typing and to Joyce Truby at The Blood Center of Southeastern Wisconsin for preparation of the manuscript.

REFERENCES

alloantigens PIA1 and PIAZ, are associated with a leucine/proline amino acid polymorphism in membrane glycoprotein IIIa, and are distinguishable by DNA typing. J Clin Invest 83:1778, 1989
Prenatal diagnosis of neonatal alloimmune thrombocytopenia using allele-specific oligonucleotide probes

JG McFarland, RH Aster, JB Bussel, JG Gianopulos, RS Derbes and PJ Newman