Neonatal alloimmune thrombocytopenia due to anti-HPA-2b (anti-Ko\textsuperscript{a})

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Most severe cases of neonatal alloimmune thrombocytopenia (NAIT) are due to anti-HPA-1a (anti-Pl\textsuperscript{a}) antibodies. We report a case of NAIT due to anti-HPA-2b that resulted in intracranial hemorrhage. A 33-year-old G2P1A0 Caucasian woman had a routine ultrasound at 34 weeks. The fetus appeared to have a left hemispheric hematoma. IVIG, 1g/kg, was started immediately and administered weekly until delivery. One day after receiving the first dose of IVIG, fetal platelet count was 18 × 10\textsuperscript{11}/L and Hb was 116 g/L. Eleven mL of matched platelets compatible by monoclonal antibody immobilization of platelet antigens (MAIPA) assay were transfused in utero, raising the platelet count to 62 × 10\textsuperscript{11}/L. Repeat transfusions were done later that week and 1 week later, with pretransfusion counts of 19 × 10\textsuperscript{11}/L and 16 × 10\textsuperscript{11}/L, respectively. Delivery by C section was done at 35.5 weeks, after the third platelet transfusion. Platelet count at birth was 77 × 10\textsuperscript{11}/L. Drainage of the hematoma was performed after transfusion. Testing with a solid phase ELISA revealed reactivity against GPIb/IX. MAIPA testing after platelet treatment with the protease inhibitor leupentin demonstrated the presence of anti-HPA-2b. On PCR-SSP the mother was HPA-2a homozygous, the father was HPA-2a/2b. Antibodies against the HPA-2b antigen located on the GPIb/IX complex have been reported in rare cases of NAIT. Testing is complicated by proteolytic degradation of the antigen-bearing fragment. Compatible platelets are easily found since approximately 85 percent of donors are HPA-2a/2a. ImmunoHematology 2003;19:43–46.

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Neonatal alloimmune thrombocytopenia (NAIT) is caused by maternal antibodies directed against an antigen on paternal and fetal platelets. NAIT can result in intracranial hemorrhage and death in severe neurologic sequelae.\textsuperscript{1} Unlike its RBC counterpart, erythroblastosis fetalis, NAIT may affect the firstborn infant. In the Caucasian population, most severe cases of NAIT are due to anti-HPA-1a (anti-Pl\textsuperscript{a}). Anti-HPA-5b (anti-Br\textsuperscript{a}) antibodies account for approximately 20 percent of serologically proven NAIT. They usually are associated with milder thrombocytopenia and therefore lead to less severe clinical manifestations. HPA-2 (Ko, Sib) platelet autoantigens are located on GPIbα.\textsuperscript{2} Anti-HPA-2b antibodies have been found in multitransfused patients and in rare cases of NAIT.\textsuperscript{3,5} We report an additional case of severe NAIT due to anti-HPA-2b. The case illustrates the importance of performing sufficient laboratory investigations to detect antibodies other than anti-HPA-1a in a clinical setting suggestive of NAIT, in order to supply appropriate phenotyped platelets for transfusion support.

Case Report

The patient, a 33-year-old Caucasian woman of Greek origin, G2P1A0, had an unremarkable first pregnancy. During her second pregnancy, a routine ultrasound of the fetus at 34 weeks' gestation showed a left hemispheric hematoma. She was taking no medication and had no other medical problems. IVIG (1g/kg/week) was started. Percutaneous umbilical cord blood sampling (PUBS) done 1 day post IVIG administration showed a fetal Hb of 116 g/L and a fetal platelet count of 18 × 10\textsuperscript{11}/L. Microplate ELISA testing demonstrated strong reactivity against GPIb/IX in the

![Fig. 1. Perinatal transfusions and the fetal platelet count are illustrated.](image-url)
maternal serum as well as against HLA Class I antigens. Three intrauterine transfusions of 11 mL each were given using platelets that were crossmatch compatible in the monoclonal antibody immobilization of platelet antigens (MAIPA) assay. Perinatal transfusions and the fetal platelet count are illustrated in Figure 1. After Cesarean delivery, a fourth crossmatch-compatible transfusion was given and the hematoma, reconf irmed on ultrasound, was evacuated. No petechiae or other signs of bleeding were noted at delivery. The baby was discharged home and is developmentally normal to date, although a seizure disorder is present.

Material and Methods

GTI PAK™ 12 assay

In the ELISA assay, patient's serum was added to the microwells of the GTI PAK™ 12 platelet antibody screening kit (GTI, Brokfield, WI) coated with platelet glycoproteins and HLA class I antigens, allowing for antibody to bind when present. After washing away unbound immunoglobulins, an alkaline phosphatase-labeled anti-human IgG was added. Unbound anti-IgG was washed away and the enzyme substrate P-nitrophenyl phosphate was added. After incubation, the reaction was stopped by a sodium hydroxide solution and the optical density was measured in a spectrophotometer at 405 nm.

MAIPA

The MAIPA assay was slightly modified from the method described by Kiefel et al.1 Platelets (20 × 10^6) isolated from EDTA-anticoagulated blood from known donors were washed, suspended in 50 μL of PBS-BSA 2%, and incubated with 50 μL of human serum at 37°C for 30 minutes. Platelets were then washed in PBS-BSA 2% and incubated with 0.2 μg of the glycoprotein-specific monoclonal antibody (anti-GP1b/IX, Immunotech, Marseille, France) at 37°C for another 30 minutes. Platelets were washed in 100 μL of isotonic saline and centrifuged × 3 at 12,000–14,000 g for 2 minutes. After the last centrifugation, the platelets were suspended in 100 μL solubilization buffer (containing 1% leupeptin) and allowed to lyse at 4°C for 30 minutes. The lysates were then centrifuged at 12,000–14,000 g for 30 minutes at 4°C and 50 μL of the supernatant was diluted in 200 μL of Tris buffer saline (TBS). One hundred μL of the dilution was distributed into wells of a microtiter plate coated with goat anti-mouse IgG (1 in 500). After overnight incubation at 4°C the microplate was washed × 5 with TBS and incubated 120 minutes at 4°C with 100 μL of goat anti-human IgG labeled with peroxidase (1 in 500). The wells were then washed × 5 and filled with 100 μL of orthophenylenediamine (OPD) substrate solution. After a 15-minute incubation at room temperature in the dark, the enzyme reaction was measured in a spectrophotometer at 490 nm.

Allele-specific PCR (PCR-SSP)

Amplification was run using 25 μM each of the specific primers, as described by Skogen et al.7 The primers are designed as follows:

- HPA-2a specific primer: 5'-CCCAGGCCTCGTGCAG-3'
- HPA-2b specific primer: 5'-CCCCAGGGCTCTGTAT-3'
- HPA-2c common primer: 5'-GGCAGGCAGCGACAAATA-3'

The cycling protocol used includes one cycle of 94°C for 5 minutes; 30 cycles of 94°C for 1 minute, 65°C for 2 minutes, and 72°C for 1 minute; and one cycle of 72°C for 10 minutes. The mixture is made using 1.5 mM MgCl2, 10 X reaction buffer, 25 μM dNTPs mix, 5 U/μL Taq polymerase (2.5 U/amplification), and the human growth hormone gene as an internal control.

| Table 1. MAIPA testing of maternal serum: optical density |
|-----------------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Platelet Genotypes         | HPA-2ab     | HPA-2ab     | HPA-2ab     | HPA-2ab     | HPA-2aa     | HPA-2aa     |
| Control serum               | 0.121       | 0.009       | 0.026       | 0.009       | 0.028       | 0.009       |
| Maternal serum              | 0.365       | 0.269       | 0.298       | 0.319       | 0.029       | 0.019       |

Results

On GTI PAK™ 12 testing, strong reactivity was demonstrated against GPIb/IX in the maternal serum (OD = 0.919 vs. OD = 0.036 for negative control), as well as against HLA Class I (OD = 1.741 vs. OD = 0.056 for negative control).

MAIPA testing using leupeptin-treated platelets and monoclonal antibody anti-GPIb/IX demonstrated reactivity against paternal and known HPA-2b(+) platelets, as demonstrated in Table 1.

In PCR-SSP, the mother was found to be HPA-2a/2a, while the father was HPA-2a/2b.

Discussion

The HPA-2 (Ko) alloantigen system was first described by van der Weerdt et al. in 1961.8 The gene frequency of HPA-2b is 0.09 in Caucasians in the United
States, 0.18 in African Americans, and 0.13 in Koreans. The GPIb/IX/V complex is the platelet receptor for von Willebrand Factor (vWF). The HPA-2 alloantigens are located in the N-terminal globular portion of the molecule, as is the vWF binding site. In vitro, anti-HPA-2b antibodies have been shown to inhibit vWF binding. It is therefore possible that the antibody may affect platelet function as well as platelet survival.

The N-terminal globular portion of the GPIb molecule is sensitive to proteases. Therefore, it is preferable to use a protease inhibitor, such as leupeptin, during MAIPA testing to avoid degradation of the antigen. In our protocols, 1% leupeptin is added to platelet suspensions before testing, and also at the step of solubilization in the lysing buffer.

The GTI PAK™ 12 microplate testing system permits relatively rapid identification of reactivity against a given platelet glycoprotein. However, we and others have found that alloantibodies may be undetectable in PAK™ 12 testing or may give nonspecific reactions. Therefore, although the GTI kit is useful as a screening test, it must be complemented by other laboratory investigations. The MAIPA assay, first described by Kiefel et al., is used in many platelet serology laboratories. At the 10th ISBT International Platelet Genotyping and Serology Workshop, in 2000, the MAIPA assay was reported to be used alone, or in combination with other methods, by 28 of 38 laboratories. Although this assay may be more sensitive and specific than the GTI kit, it also requires a minimum of 10 hours of technical time and an overnight incubation period. In addition, a monoclonal antibody against the glycoprotein complex of interest must be added. MAIPA testing using monoclonal antibodies solely against GPIIb/IIIa and GPIa/IIa in the expectation of finding an anti-HPA-1a or anti-HPA-5b antibody, respectively, would have given a negative result in this case. In addition, the use of a frozen platelet panel or of platelets that have been stored without protection from proteases may lead to antigen degradation and a negative result. Finally, allele-specific PCR confirms the alloantigen incompatibility between the two parents. However, it does not prove that an alloantibody is actually present. Because alloantisera are extremely rare, genotyping permits the laboratory to develop a panel of platelets of known HPA-2 specificities and to establish a bank of HPA-2a/2a donors.

There have been several other reported cases of NAIT due to anti-HPA-2b. Kroll et al. reported two cases in Germany. In the first case, the third child of healthy parents was found to have a postpartum platelet count of $53 \times 10^9/L$ at delivery, which decreased to $11 \times 10^9/L$ on day 4. Three maternal platelet transfusions were necessary to prevent bleeding. In the second case, a second pregnancy was complicated by erythroblastosis fetales due to anti-CD treated by intrauterine transfusion. Fetal death occurred, and a platelet count of $3 \times 10^9/L$ was found. Because of the small numbers of cases reported and the laboratory diagnostic difficulties discussed, it is difficult to know if these cases represent the true range of severity of NAIT due to anti-HPA-2b.

The frequency of NAIT due to anti-HPA-2b is difficult to determine. In a large prospective study by de Moerloose et al., of 8388 newborns in Switzerland, 40 newborns had NAIT; anti-HPA-1a and anti-HPA-5b antibodies were detected in three and two mothers, respectively, and no anti-HPA-2 antibodies were present. In a study by Hohlfeld et al., of 5194 neonates in France undergoing fetal blood sampling for cytogenetic analysis and various infections and hematologic disorders, anti-HPA-1a and anti-HPA-5b were detected in 23 and 2 mothers, respectively, but no anti-HPA-2 antibodies were present. In a series of 975 cases of NAIT reported by McFarland et al. spanning 11 years of testing by a tertiary referral center, only three cases of anti-HPA-2b were detected. In all three cases, anti-HPA-2b was found in the presence of other alloantibodies (anti-HPA-3a in one, anti-HPA-3b in another, and anti-HPA-1a and anti-HPA-3a in the third).

Although anti-HPA-2b antibodies are a rare cause of NAIT, they should be considered in a clinical setting that is suggestive of NAIT, when more commonly seen antibodies have not been found. The detection of these antibodies in the MAIPA assay requires fresh platelets, treatment with leupeptin, and the use of an anti-GPIb/IX monoclonal antibody. The GTI PAK™ 12 assay may be useful in screening for these antibodies.

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References


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