Case Report

Posttransplant maternal anti-D: a case study and review

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Plasma from a 35-year-old, D– woman was found to have anti-D, -C, and -G at 5 weeks' gestation and again at 8 weeks' gestation, when she presented with a nonviable intrauterine pregnancy. The anti-D titer increased with a pattern that suggested it was stimulated by the 8-week pregnancy. Six years before this admission, the patient's blood type changed from group O, D+ to group O, D– after a bone marrow transplant for aplastic anemia. Three years after transplant, the antibody screen was negative. After the patient was admitted for the nonviable pregnancy, the products of con-ception were found to be D+ by DNA testing for *RHD*. There were no documented transfusions or pregnancies during the interval in which anti-D appeared. The timing of the alloimmunization was unusual. In a subsequent pregnancy, fetal D typing was performed by molecular methods. ***Immunohematology* 2012;28:55–9.**

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Case Report

A 30-year-old, D+ woman with aplastic anemia underwent allogeneic bone marrow transplant (BMT) from her HLA-identical, D– brother in December 2003. The pretransplant antibody screen (by indirect antiglobulin test [IAT]) was negative both in donor and in host. The patient received red blood cell (RBC) and platelet transfusions from D+ donors before the BMT. RBC transfusions were restricted to D– components from the time of conditioning. The patient received three units of group O, D+ RBCs on Day –70 and five units of group O, D– RBCs between Day –30 and Day +12 (Table 1). She also received 30 units of single-donor platelets (SDP) between Day –70 and Day +12; these included SDPs from D+ and D– donors. The conditioning regimen consisted of cyclophosphamide and antithymocyte globulin (ATG). The patient initially received cyclosporine and methotrexate for graft-versus-host (GVH) prophylaxis. On Day +20, she still typed as D+ with a negative antibody screen. She required no further blood or platelet transfusions after posttransplant Day 12, and she was tapered off cyclosporine immunosuppression 9 months after transplant. The patient delivered a healthy, D– baby in 2006. During that pregnancy and at delivery, the patient typed as D– with a negative antibody screen by solid-phase technology. The cord blood typed as group O, D–, weak D–, and the father typed as group O, D–.

In January 2009, the patient presented with a pregnancy at 5 weeks' gestation and was found to have anti-D, -C, and -G with a titer of 128 against a D+, C+, G+ RBC sample. At 8 weeks' gestation, the patient presented with a nonviable intrauterine pregnancy, and the anti-D and -C titers were 1024 and 64, respectively. The patient's RBCs were negative by the direct antiglobulin test (DAT). As the G antigen is expressed in the presence of D or C or both, it is highly unlikely that the patient and thus the products of conception would express G because the D+G– and C+G– phenotypes are extremely rare and the D+C+G– phenotype has not been reported. After the patient was admitted for the nonviable pregnancy, the products of conception were predicted to be D+ by DNA testing for *RHD*. Polymerase chain reaction (PCR)–based assays predicted the following phenotypes: mother D+ C+ E– c+ e+ by buccal swab and D– C– E– c+ e+ by peripheral blood white blood cells; father D– C– E– c+ e+; and conceptus D+ C+ E– c+ e+.

In October 2009, the patient became pregnant for the third time. Fetal *RHD* typing, performed at 18 weeks' gestation from amniotic fluid, predicted a fetal phenotype of D– C– E– c+ e+. An analysis of variable number of tandem repeats was used to confirm that the results were not affected by maternal cell contamination of the amniocyte sample. At that time, the anti-D and -C titers were 2048 and 64, respectively.

Materials and Methods

At the hospital laboratory in 2006, the maternal antibody screens were performed on an automated analyzer (ABS 2000, Immucor Inc., Norcross, GA) using a four-cell screen by solid phase technology (Capture-R Ready Screen IV, Immucor Inc.). Cord blood typing on the newborn was performed by manual tube testing, including weak D testing. In 2009, the maternal antibody screen was performed on a newer analyzer (Galileo, Immucor Inc.) using a solid phase two-cell screen (Capture R Ready Screen II, Immucor Inc.). At the New York Blood Center immunohematology laboratory, the anti-D, -C, and -G specificities were confirmed using manual tube

Table 1. Condensed case description including transplant Day 0 (December 2003), transplant Day +20 (December 2003), full-term pregnancy (2006), pregnancy at 5 weeks' gestation (January 2009), nonviable pregnancy at 8 weeks' gestation (January 2009), and subsequent pregnancy (2010)

	Date					
Case description	2003	2003	2006	2009	2009	2010
Patient Rh phenotype	D+C+E–c+e+	D+	D–	D–	D–C–E–c+e+	D–
Antibody screen	Negative	Negative	Negative	Positive	Positive	Positive
Anti-D titer	—	—	—	128	1024	2048
Anti-C titer	—	—	—	—	64	64
RBC transfusion	3 units, group O D+, Day –70	5 units, group O D–, Day–30 to Day +12	—	—	—	—
SDP transfusion	30 units (Day–70 to Day +12)	—	—	—	—	—
Fetal gestational age	—	—	40 weeks	5 weeks	8 weeks (nonviable)	18 weeks (viable)
Fetal source material	—	—	Cord blood	—	Products of conception	Amniotic fluid
Fetal Rh phenotype	—	—	D–	—	D+C+E–c+e+	D–C–E–c+e+

RBC = red blood cell, SDP = single-donor platelet.

methods including a sequential adsorption/elution procedure. PCR-based assays for *RHD, RHCE*C/c,* and *RHCE*E/e* were performed on the products of conception, maternal and paternal buccal epithelial cells, and maternal blood sample. At a reference laboratory during the third pregnancy (early 2010), amniotic fluid was used for genomic DNA testing by allele-specific gene amplification.

Results

Table 1 contains pertinent features of the case in a timeline beginning with the BMT and associated transfusions, followed by a total of three pregnancies. In summary, Table 1 depicts the patient's change in type from D+ to D– after the BMT, the negative maternal antibody screen at the time of the healthy full-term delivery, the rising anti-D titers during the nonviable pregnancy, and the molecular test results from the second and third pregnancies. Details of the case history have been described earlier in this report.

Table 2 summarizes the results of chimerism testing by fluorescence in situ hybridization (FISH) during the year after the BMT. The patient's chimerism results indicated a tiny percentage of host-type lymphocytes in the peripheral blood a year after transplant. The posttransplant specimens did not show mixed-field reactivity in the patient's RBC typing.

Table 2. Chimerism results by fluorescence in situ hybridization (donor XY, host XX) to show percent engraftment of the bone marrow transplantation in the patient (host)

Time	Source	Cells analyzed	Interpretation
Day +30	Marrow	497 XY, 3 XX	99.4% donor, 0.6% host
Day +100	Marrow	495 XY, 5 XX	99% donor, 1% host
Day +365	Blood, unsorted	497 XY, 3 XX	99.4% donor, 0.6% host
Day +365	Blood, T cells	96 XY, 4 XX	96% donor, 4% host

Discussion

The timing of this patient's alloimmunization to D was unusual. If it was caused by the nonviable pregnancy, the alloimmunization occurred unusually early in gestation. If it was caused by peritransplant exposure to D, it is unusual that the antibody did not appear soon after immunosuppression was tapered. Posttransplant formations of anti-D are uncommon because the donor-related primary immune response usually starts about 6 months after the BMT; after this period, if recipient bone marrow is 100 percent of donor origin, residual D+ recipient RBCs are no longer detectable.[1] Anti-D was identified in one of seven D+ recipients of a D– graft, who was also exposed to D+ blood components before and after the nonmyeloablative transplant.[2] In one case, anti-D appeared only when immunosuppression was discontinued, 2 years after the BMT.[3] Our patient's plasma contained no anti-D 3 years after the BMT, yet anti-D was present 6 years after transplant. In one study of D– hosts with D– bone marrow donors, the alloimmunization rate by D+ blood components was less than 5 percent,[4] but some authors suggest prophylaxis with intravenous anti-D immunoglobulin if D+ platelets must be used.[1] Our patient received SDPs from both D+ and D– donors, and only D– RBCs were given from the start of conditioning for the BMT. There were no

documented transfusions or pregnancies during the interval in which anti-D appeared (between late 2006 and early 2009).

The patient's chimerism results by FISH showed that her engraftment status was almost completely donor, and her RBCs typed as donor without mixed-field reaction. RBC chimerism was not excluded by more sensitive methods. For example, flow cytometry was not performed to look for D+ cells.

Based on the analogous scenario of minor ABO mismatched transplants, we would expect this patient to have a low risk of alloimmunization for the following reasons: the regimen was myeloablative; the source was marrow, not blood stem cells; and GVH prophylaxis included both cyclosporine and methotrexate. On the other hand, passenger lymphocyte–mediated hemolysis has been reported to be more common in recipients of related donor versus unrelated donor transplants.[5] The conditioning regimen for aplastic anemia is somewhat less aggressive than myeloablative regimens for other clinical indications and may be considered submyeloablative. Low-intensity conditioning regimens may leave the host's antigen-presenting machinery relatively intact, possibly resulting in more potent stimulation of donor memory B cells.[6]

The maternal anti-D titer increased from the 5-week gestation sample to the 8-week gestation sample, suggesting the antibody was stimulated by very early pregnancy. Few D– women form anti-D that is IgG during their first uncomplicated pregnancy. The average rate reported for this is 0.9 percent, range 0.3 to 1.9 percent. The risk of D immunization in a D– woman is considered to be 1 to 2 percent during a pregnancy with a D+ (ABO compatible) fetus, increasing to 14 to 17 percent during delivery.[7] During normal pregnancy, transplacental hemorrhage (TPH) can occur as early as at 4 weeks after fertilization, or 6 weeks after last menstrual period (LMP). This is the time when fetal and maternal circulations in the placenta have been formed and when the vascularization of the villi and the pumping action of the fetal heart begin. At this stage of pregnancy, TPH of 0.004 mL has been detected.[7]

Bergström et al.[8] found Rh antigens on the RBCs of a 10-mm fetus, obtained approximately 38 days after conception or 52 days after LMP. The embryo was found in a 47-year-old woman, with systemic lupus erythematosus (SLE), who was undergoing hysterectomy because of a cervical myoma. The embryo had a yolk sac and was obtained within intact membranes. Microscopic examination of the suspension showed almost exclusively nucleated megaloblasts of fetal type. The fetal material was found to be D+. Theoretically, early abortion could induce anti-D immunization in the D– woman. However, there is no evidence in the literature to show that spontaneous abortion occurring in the first trimester can cause anti-D immunization.[7,9] In this patient, the high-titer, IgG maternal response is unexpected given the 8-week gestation without trauma.

The occurrence of anti-D detected in D– individuals who have never been exposed to D+ RBCs through either pregnancy or tranfusion is rare. Six cases have been reported in the literature. These include a never-pregnant 26-year-old woman, three women who delivered D– babies, and two male patients.[9] The authors excluded alloimmunization after intravenous drug use with shared needles and administration of intravenous immunoglobulin. They suggest other possible mechanisms: occult pregnancy followed by undetected abortion; the transfer of D+ RBCs from mother to D– fetus resulting in alloimmunization (the grandmother theory); anamnestic response induced by infection or malignancy; or exposure to an unidentified antigen similar to D.

Most recipients of allogeneic hematopoietic stem cell transplantation (HSCT) suffer from secondary infertility owing to gonadal damage from myeloablative conditioning. However, the use of nontotal body irradiation and nonablative protocols means that more patients may preserve their fertility. Hence, the number of pregnancies involving HSCT recipients is increasing.[10] Furthermore, in the case of aplastic anemia, the post-HSCT outlook for fertility is more favorable than for other conditions that require more aggressive regimens. Indeed, a 1998 study found that at least half of the female patients transplanted for aplastic anemia preserved or regained the ability to become pregnant.[11] For hemolytic disease of the fetus and newborn (HDFN), previous pre-HSCT pregnancy history cannot predict the risk. This is because both the RBC and immune system of the pregnant HSCT recipient have switched to those of donor origin. Fortunately, the incidence of post-HSCT anti-D is low, and clinical hemolysis is rare.[1] Au and Leung[10] state that it is uncertain whether the anti-D titer may increase as a result of anamnestic exposure during post-HSCT; therefore, frequent monitoring may be prudent.

When the patient in our study became pregnant again in October 2009, early fetal genotyping from maternal plasma was desired but was limited by the absence of a reliable internal control, given that the bone marrow donor was male. Fetal D typing from maternal plasma has been performed for several years[12,13] and is now available in the United States[14] using real-time quantitative PCR. Fetal DNA represents between 3 and 6 percent of total free DNA in maternal plasma,[15] and separating fetal DNA from maternal DNA is problematic.[16] *SRY* is a Y chromosome–specific sequence that can be amplified to confirm that fetal DNA has been successfully detected in the case of a male fetus. If the *SRY* gene is used as the control,

cell-free fetal D typing is inconclusive in 14 percent of cases, namely those in which there is a D– female fetus detected. In these cases, the absence of the *RHD* gene in the fetus cannot be verified because there is no internal control to confirm the presence of free fetal DNA in the specimen. Thus, amplification of *SRY* is only effective as a control when the fetus is male.[14] But in this patient, maternal plasma would likely contain traces of male DNA from her bone marrow donor, so *SRY* could not be used as a control. Indeed, Minon et al.[17] reported an unusual false-positive fetal *RHD* result using DNA derived from maternal plasma from a solid-organ recipient. Tests on DNA isolated from the plasma of a D– pregnant woman predicted a D+ male fetus, whereas DNA isolated from amniocytes gave a D– result. The woman, who had received a kidney transplant from a D+ male, delivered a D– girl.

In the current case, cell-free fetal D testing was not performed because of the absence of a reliable internal control, given that the bone marrow donor was male. Instead, fetal *RH* genotyping was performed on DNA isolated from amniocytes. A commercial system in development in the United States, which involves mass spectrometry, will include other genes to be used as controls.[18–20] Tests using other fetal identifiers are also in development outside the United States.[21]

Fetal RBC phenotype may appear to exclude maternal contribution in scenarios such as donor egg or maternal host of HSCT (Table 3). Finally, this case raised the possibility that maternal anti-D might impact early gestational outcomes, although no such relationship exists in the literature. In this patient, two pregnancies with D– fetuses progressed without complication, whereas the pregnancy with a D+ fetus was nonviable.

Table 3. Scenarios in which fetal red blood cell predicted phenotype appears to exclude maternal contribution

Scenario	Explanation
Mother group O, father group A, offspring group B	Donor egg
	Maternal stem cell transplant
Mother group O, father group AB, offspring group AB	Donor egg
	Maternal stem cell transplant
	Cis AB

Conclusions

In this case, maternal anti-D alloimmunization was detected 6 years after BMT, at the time of an early nonviable pregnancy. The 8-week pregnancy may represent a secondary alloimmunization event after a primary event of graft immune cells responding to residual host RBCs, but the timing is unusual. Fetal D typing from maternal plasma is now available in the United States using real-time quantitative PCR. Genes other than *SRY* must be used as fetal identifiers to provide a reliable internal control for female D– fetuses. In a mother who has had a transplant, DNA from the bone marrow donor can be amplified. This is particularly problematic when the mother's type has changed from D+ to D–.

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