Case Report

Severe hemolytic disease of the fetus and newborn due to anti-C+G

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Anti-G is commonly present with anti-D and/or anti-C and can confuse serological investigations. In general, anti-G is not considered a likely cause of severe hemolytic disease of the fetus and newborn (HDFN), but it is important to differentiate it from anti-D in women who should be administered anti-D immunoglobulin prophylaxis. We report one woman with three pregnancies severely affected by anti-C+G requiring intrauterine treatment and a review of the literature. In our case, the identification of the correct antibody was delayed because the differentiation of anti-C+G and anti-D+C was not considered important during pregnancy since the father was D–. In addition, anti-C+G and anti-G titer levels were not found to be as reliable as is generally considered in Rh immunization. Severe HDFN occurred at a maternal anti-C+G antibody titer of 8 and anti-G titer of 1 in comparison with the critical titer level of 16 or more in our laboratory. Close collaboration between the immunohematology laboratory and the obstetric unit is essential. In previously affected families, early assessment for fetal anemia is required even when titers are low. ***Immunohematology* 2015;31:123–127.**

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Anti-D is the most common antibody responsible for severe hemolytic disease of the fetus and newborn (HDFN), but antibodies against other antigens belonging to the Rh blood group system can also cause HDFN. G, first described by Allen and Tippett,[1] is part of the Rh blood group system and is dependent on expression of both the *RHCE*Ce* and *RHD* alleles. There are some exceptions, but most individuals who do not carry an *RHD* or *RHCE*Ce* allele would be expected to be negative for G. Anti-G often presents with anti-D and/or anti-C, but seldom alone. Anti-G should be suspected in cases where the anti-C titer is higher than the anti-D titer and confirmed by additional tests.[2,3] Anti-G is present in approximately 30 percent of cases with apparent anti-D+C.[4]

Anti-G has been associated with HDFN. A few cases have been reported where anti-G was the probable cause of severe HDFN, but usually the disease is mild and does not require therapeutic intervention in the offspring.[3,4–9] A case initially considered anti-D+C may turn out to be anti-C+G instead, and in these cases, anti-D prophylaxis should be administered to prevent anti-D alloimmunization and manifestation of HDFN in subsequent pregnancies.

In this case report, we describe the outcomes of three pregnancies of a Caucasian woman and her African husband, where anti-C+G caused severe HDFN requiring intrauterine (IU) transfusions.

Case Report

The patient had a history of two miscarriages and one extrauterine pregnancy. Subsequently, she had a normal pregnancy, where antenatal antibody screening was negative. In the following pregnancies, alloimmunization was identified, resulting in three cases of severe HDFN. In this case report, we present these affected pregnancies, designated as the first, second, and third (Table 1). According to the national protocol for antenatal antibody screening, maternal sampling began at 8–12 weeks of gestation in all pregnancies. The postnatal blood samples were drawn on the day of delivery.

In her first affected pregnancy (in 2009), the antibody screening was positive for what were at the time assumed to be anti-D and anti-C. Followed monthly, the antibody titers remained at moderate levels (4–8) until 34 weeks of gestation. Because the titer level was below the critical level of 16, ultrasound examinations to detect fetal anemia were not considered necessary. At 37 weeks of gestation, an emergency caesarean section was performed because of reduced fetal movements and a sinusoidal heart rate pattern in cardiotocography. A D– boy was delivered with a hemoglobin (Hb) of 3.1 g/dL and a positive direct antiglobulin test (DAT). Unexpectedly, the anti-D+C titer on a sample taken 5 days prior to the delivery and available postnatally was 128. Immediately postnatally, the anti-D+C titer was 64. The anti-C titer was higher than the anti-D titer, as had been the case already during the pregnancy, thus prompting suspicion of anti-G instead of anti-D. The infant recovered well after successful treatment with red blood cell (RBC) transfusion and treatment with intravenous immunoglobulin (IVIG).

At the beginning of her second affected pregnancy (in 2012), anti-D–like antibody, anti-C, and anti-Jk^a were

Table 1. The courses and outcomes of three pregnancies (in the years 2009, 2012, and 2013) of an alloimmunized D−C−c+E−e+Jk(a−b+) mother

	2009			2012			2013		
	Antibody	Titer	Titer cells	Antibody	Titer	Titer cells	Antibody	Titer	Titer cells
Prenatal work-up	Anti-D+C	4–8	R_1r	Anti-D+C	16	R_1r	Anti-C+G	8–16	R_1r
					8–16	r′r	Anti-G	2	R_2r
					1–2	R_2r	Anti-Jk[a]	1	rr
				Anti-Jk[a]	1	rr			
Follow-up	Monthly antibody levels			Monthly/fortnightly antibody levels Weekly/fortnightly MCA PSV flow, MoM			Monthly/fortnightly antibody levels Weekly/fortnightly MCA PSV flow, MoM		
Intervention	Emergency CS at 37 weeks (reduced fetal movements, sinusoid cardiotocography)			IU × 5, Hb (between 24 and 34 weeks), induction of labor at 34 weeks			IU × 3, Hb (between 31 and 35 weeks), induction of labor at 36 weeks		
Delivery	D− boy, DAT+ Hb 3.1 g/dL			D− girl, DAT− Hb 14.1 g/dL			D− boy, DAT+ Hb 14 g/dL		
Postnatal work-up	Anti-D+C*	128	R_1r	Anti-C+G‡	8	R_1r	Anti-C+G	4	R_1r
		64–128	r′r	Anti-G	1	R_2r	Anti-G	2	R_2r
	Anti-D+C†	64	r′r	Anti-Jk[a]	1	rr	Anti-Jk[a]	1	rr
		128	R_1r	Anti-C+G§	2	R_1r			
	Anti-C+G suspected				4	r′r			
	Anti-Jk[a] detected				1	R_2r			
Treatment	IVIG, red cell transfusion			IVIG, phototherapy, red cell transfusion ×2			IVIG ×3, phototherapy		

*Sample taken 5 days prior to the delivery; result available 1 day postpartum.

†Sample taken on the day of delivery.

‡Anti-C+G confirmed postnatally.

§Sample taken from umbilical cord.

MCA = middle cerebral artery; PSV = peak systolic velocity, cm/sec; MoM = multiples of median; CS = caesarean section; IU = intrauterine; Hb = hemoglobin; DAT = direct antiglobulin test; IVIG = intravenous immunoglobulin.

identified. The anti-D+C titer was 16 and the anti-Jk[a] titer was 1. The antibody levels were followed monthly/fortnightly, and they remained unchanged during the pregnancy. Because the father was D−C+, at 16+2 weeks of gestation the fetus was genotyped from amniotic fluid sampling and found positive for a *RHD-CE-D* hybrid gene that is predicted to express no D and instead expresses an altered C. The fetus was then carefully monitored noninvasively with ultrasound examinations. No evidence of fetal anemia was apparent until 23 weeks of gestation, when the peak systolic velocity (PSV) in the middle cerebral artery (MCA) Doppler examinations exceeded 1.5 multiples of median (MoM). In cordocentesis at 24+1 weeks, the fetal Hb was 7.4 g/dL. A total of five IU transfusions were performed successfully between 24+1 and 32+6 weeks of gestation. Vaginal delivery was induced at 34 weeks and a slightly premature but otherwise healthy girl was born with a Hb of 14.1 g/dL and a negative DAT. Neonatal treatment included IVIG, phototherapy, and two top-up transfusions. Anti-C+G was confirmed postnatally.

At the beginning of her third affected pregnancy, in 2013, anti-C, anti-G, and anti-Jk[a] were identified. During the pregnancy, the titers remained stable: 8–16 for anti-C+G, 2 for anti-G, and 1 for anti-Jk[a]. The fetus was genotyped from amniotic cells as a *RHD-CE-D* hybrid at 21+1 weeks of gestation. Weekly ultrasound examinations were begun at 22+6 weeks. Middle cerebral artery PSV stayed under 1.29 MoM until 30+0 weeks, when it reached 1.4 MoM. Given the mother's history, the last finding gave reason to suspect anemia, and the first IU transfusion was then scheduled. The Hb at the first transfusion was 8.8 g/dL. A total of three IU transfusions were performed between 30+5 and 34+6 weeks of gestation. Labor was induced at 36+0 weeks, and a healthy

boy was born with a Hb of 14 g/dL and a positive DAT. The child received IVIG treatment (× 3) and phototherapy, with the latter continued at home after discharge from the hospital.

Materials and Methods

All blood samples and amniotic fluid samples were analyzed in the Finnish Red Cross Blood Service (in Helsinki), which is a national reference laboratory to which the antenatal screening for RBC antibodies is centralized. In the second affected pregnancy, in 2012, a blood sample was also sent to the International Blood Group Reference Laboratory (IBGRL) in Bristol, UK, where absorption studies were performed to differentiate anti-G from anti-D and anti-C.

ABO and D typing of RBC samples from the mother were performed with an analyzer (PK7300, Olympus Corporation, Tokyo, Japan) on microtiter plates.

Antibody screening, identification, and DAT studies were carried out with a gel-based analyzer (Diamed ID GelStation, DiaMed, Cressier, Switzerland). Antibody screening and identification were performed using a gel method (LISS/Coombs cards, Bio-Rad, Cressier, Switzerland) for untreated RBCs. Antibody identification with enzyme (papain)-treated RBCs in a gel method (NaCl card, DiaMed) was also used. A DAT was performed on the infant's cord blood sample using polyspecific antihuman globulin (AHG) (anti-IgG and anti-C3D, Bio-Rad) in a gel method (LISS/Coombs cards). Serologic phenotyping was carried out with specific reagent gel cards (Bio-Rad).

Titrations were performed using the tube method for indirect antiglobulin test and R_1r reagent RBCs for anti-D, anti-D+C, and anti-C; r′r for anti-C; and R_2r for anti-G (because reagent cells with only C or G antigen were not available for routine use). During the first affected pregnancy, only R_1r cells were used in titration of anti-D+C, but after delivery of the severely anemic D– child, titration was also performed with r′r cells. In the second affected pregnancy, cells used for titrating anti-D+C, anti-C, and anti-G were R_1r, r′r, and R_2r, respectively, throughout the pregnancy. In the third affected pregnancy, the titer cells used were R_1r for anti-C+G and R_2r for anti-G throughout the pregnancy. Titration for anti-Jk^a was performed with rr Jk(a+b+) reagent RBCs.

Genotyping of the fetal *RHD* and *RHCE* genes was carried out from amniotic cells using polymerase chain reaction with sequence specific primers (PCR-SSP) (PCR-SSP, Inno-Train, Kronberg, Germany) according to the manufacturer's instructions.

Results

The Caucasian mother was phenotyped as D–C–c+E–e+; Jk(a–b+) and the African father as D–C+c+E–e+; Jk(a+b–). The father carried the *RHD-CE-D* hybrid, which encodes an altered C that reacts more weakly than a conventional C when using serologic methods. The father was the same in all pregnancies.

In the first affected pregnancy, the anti-D+C titer remained 4–8 using D+C+ (R_1r) cells followed monthly. The titer was 128 using D–C+ (r′r) cells on a sample taken 5 days prior to the delivery and 64 immediately postnatally.

In the second affected pregnancy, the anti-D+C titer was 16 using D+C+ (R_1r) and D–C+ (r′r) cells, and 2 with D+C– (R_2r) cells. A maternal blood sample had also been sent to the IBGRL in Bristol, UK, where anti-C+G and anti-Jk^a were confirmed and anti-D ruled out. The postpartum maternal anti-C+G titer was 8, and anti-G and anti-Jk^a titers were both 1; the anti-C+G titer from cord blood was 4 and anti-G titer was 1; anti-Jk^a was not detected.

During the third affected pregnancy, the antibody titers remained stable: 8–16 for anti-C+G using D+C+ (R_1r) cells, 2 for anti-G using D+C– (R_2r) cells, and 1 for anti-Jk^a. The postpartum maternal anti-C+G and anti-G titers were 4 and 2, respectively; anti-Jk^a titer was 1 and not obtained for analysis from the cord blood sample.

Discussion

In our patient, anti-C was the dominant antibody in all affected pregnancies, including the first, as could be confirmed retrospectively. The finding of a stronger anti-C compared with anti-D led to the suspicion of anti-G. In the first affected pregnancy, antibody levels remained low until a rapid increase in the final weeks before term, resulting in signs of fetal distress and an emergency caesarean section of a severely anemic child. The last titer level 3 weeks earlier was 8 but had risen to 128 by the time of delivery. In the following pregnancies, despite the fact that the antibody titers remained at moderate levels (2–16), IU transfusions were required.

Though there are several case reports in the literature of HDFN caused by anti-G/anti-C+G, to our knowledge, only one of them required IU transfusion: a mother with a history of several affected pregnancies presented with severe HDFN caused by anti-C+G in a twin pregnancy. Maternal IVIG and plasmapheresis were required before IU RBC transfusions could be initiated, and despite intensive monitoring, one twin

was lost and the other needed prolonged treatment including phototherapy, RBC transfusions, and erythropoietin injections.[9] In a case report by Hadley et al., anti-G was found to be the cause of severe HDFN; cordocentesis was planned but found impossible to perform, and after delivery, the infant required several exchange transfusions.[4] The third reported severe case of HDFN with anti-C+G was described by Jakobowicz and Simmons.[8] Thus, anti-G can be clinically significant in pregnancy and may contribute to the development of moderate or severe HDFN, although most reported cases have been mild. For example, Muller et al. reported a typical case where anti-C+G was mistaken for anti-D+C in a primigravida who had received RBC transfusions prior to her pregnancy. After delivery of a D– baby, anti-C+G was identified in maternal blood by adsorption and elution, but no anti-D was detected.[3] Lenkiewicz and Zupanska reported a pregnancy where anti-C+G antibodies were responsible for moderate hemolytic disease of the newborn, and, based on titration results, anti-G levels were much higher than anti-C.[10]

Anti-G is only rarely the single antibody responsible for HDFN, but is more often expressed with anti-D, anti-C, or both. Palfi and Gunnarsson analyzed the D/C/G antibody combinations in 27 pregnancies and found that anti-G was present in 24 cases, and in 4 of the 27 cases, anti-C+G was identified without anti-D; anti-G was not found alone in any of these cases.[6] Interestingly, Huber and coworkers reported a patient who did have anti-G as the sole cause of moderate hemolytic disease of the newborn—anti-D and anti-C were excluded.[11]

In our case, the affected fetuses had inherited the hybrid allele *RHD-CE-D* from their father. The allele codes a D– and weak C+ phenotype. G is intact. Thus, both anti-C and anti-G could find the target antigens on the RBCs of the fetuses.

Furthermore, our case demonstrates that anti-C or anti-G titers are poor predictors of the outcome in an immunized pregnancy. In our facility, monitoring of pregnancies at high risk for HDFN is planned in collaboration with the immunohematology laboratory and the obstetric unit. Antibody screening, identification, and follow-up of antibody levels are carried out monthly or every 2 weeks. Usually, if critical antibody titer levels of 16 are reached, fetal ultrasound examinations begin at 18 weeks of gestation and continue weekly/fortnightly throughout the pregnancy. Fetal well-being evaluated with serial ultrasound for signs of hydrops and Doppler measurement of the PSV of the MCA form the basis for follow-up in immunized pregnancies. In anemic fetuses, lower blood viscosity and increased cardiac output result in a higher PSV; a threshold value of 1.5 MoM is predictive of moderate to severe anemia, whereas levels lower than 1.29 are considered normal.[12,13] This noninvasive method has replaced the need for assessment of amniotic bilirubin levels. Moreover, fetal *RHD* and *RHCE* genotyping from maternal serum is now feasible, although blood groups other than Rh still need to be analyzed from amniocytes.[13]

Appropriate titer cells are important in the estimation of the role of anti-G. A significant number of cases where apparent anti-D+C are identified may actually contain only anti-C+G and lack anti-D.[14] Anti-G should be considered if anti-C titer levels exceed or reach the levels of anti-D titers.[3] In our case, cells used in titration were R_1r for anti-D+C, R_2r for anti-D and anti-G, and r′r for anti-C. The titer levels obtained with R_1r cells were consistently higher than with R_2r in all three pregnancies, indicating anti-C as the stronger antibody. In general, in our laboratory, when anti-D+C are identified, only R_1r cells are used in titration. Anti-G should be suspected prior to titration if the antibody identification panel shows stronger reactions for anti-C than anti-D. Then, if titrations performed with R_1r, R_2r, and/or r′r cells also indicate the possibility of anti-G, additional tests should be carried out. In our laboratory, anti-G is demonstrated and anti-D ruled out with absorption studies performed concurrently with D–C+ and D+C– reagent RBCs. In many laboratories, differential absorption and elution is the method of choice, and is recommended for the differentiation of anti-G from anti-D.[2–4,6,10,11] Furthermore, rare r^Gr cells may also be used in titration.[6,7,11] National and international blood group reference laboratories may be of use in cases where antibody identification by in-house methods is challenging.

Identifying anti-G in serum that initially seems to contain anti-D+C is important because D– women shown to have anti-C+G but not anti-D should receive anti-D immunoglobulin prophylaxis when carrying or having delivered a D+ child. Failure to recognize these cases endangers future pregnancies, since alloimmunization may develop. Furthermore, if the father is D–, the identification of anti-C+G should be discussed, because an incorrect report of anti-D+C may lead to unnecessary distress, paternity testing, and social consequences. The number of reported anti-G cases is still low. Nevertheless, anti-G is more common than has traditionally been thought, and may contribute to the development of severe HDFN.

In conclusion, if a pregnant woman seems to have anti-D and anti-C, with anti-C being the stronger antibody, it is important to test for anti-G. Primarily, if anti-D can be excluded, the woman requires anti-D prophylaxis. In addition, anti-G can, especially in combination with anti-C, cause severe HDFN, even with low titer levels.

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