# Hemolytic disease of the newborn: anti-C and anti-D, or anti-G

A 28-year-old woman who is gravida 3 para 1 presents with vaginal bleeding at 7 weeks gestation. She is otherwise healthy and has never been transfused.

Blood bank testing:

| | |
|---|---|
| ABO/Rh | B negative |
| Antibody screen | Positive (cell I and cell II on a 3-cell screen) |
| Direct antiglobulin test | Negative |

Records from 7 years ago at 28 weeks gestation of her first pregnancy:

| | |
|---|---|
| ABO/Rh | B negative |
| Antibody screen | Negative |
| Rh immune globulin | 1 vial issued |

Cord blood testing after delivery at 38 weeks gestation of the first pregnancy:

| | |
|---|---|
| ABO/Rh | O positive |
| Direct antiglobulin test | Negative |
| Rh immune globulin | 1 vial issued |
| Fetomaternal hemorrhage screen | Negative |

Records from 3 years ago obtained from another facility prior to first trimester pregnancy termination:

| | |
|---|---|
| ABO/Rh | B negative |
| Antibody screen | Positive |
| Antibody ID | Anti-D, anti-C |
| Rh immune globulin | 1 vial issued |

Extended panel workup now shows anti-D and anti-C:

| | |
|---|---|
| Anti-D titer | 2048 |
| Anti-C titer | 128 |

### *Anti-G versus anti-C and anti-D*

Allen and Tippett reported the Rh antigen G in 1958. All RBCs that are C positive carry G, and almost all RBCs that are D positive carry G. Almost all RBCs that are negative for C and D are also negative for G, but rare C-negative and D-negative RBCs are G positive ($r^G$). The G antigen is defined by Ser103, which is common to both the D and the CE polypeptide when C is expressed. The G antigen explains the observation that about 30% of D-negative subjects immunized with Dccee RBCs make an antibody (anti-G) that reacts with D-negative, C-positive RBCs. Similarly, a D-negative patient (ccee) who receives D-negative blood that happens to be C positive (Ccee) may develop anti G, although this is rare.[1] In a pretransfusion specimen, the discovery of apparent anti-C and anti-D specificities do not warrant investigation for anti-G, because blood selected for transfusion, which will be C and D negative, will be G negative as well. In a pregnant woman who is D negative and has D-negative partners or has received appropriate Rh immune globulin prophylaxis for previous pregnancies, the discovery of apparent anti-D and anti-C can cause confusion. The key principle is that pregnant women who have anti-G (with or without anti-C) but no anti-D remain candidates for Rh immune globulin prophylaxis.[2] While anti-D can cause severe hemolytic disease of the fetus and newborn, anti-C typically is associated with less severe manifestations, and anti-G may not cause hemolytic disease of the newborn (HDN) at all.

***Objectives***

- Recognize the importance of distinguishing anti-G from anti-D and anti-C in prenatal workups.
- Describe the role of immunohematologic testing in prenatal management.
- List the steps to take to prepare blood for a neonatal exchange transfusion.

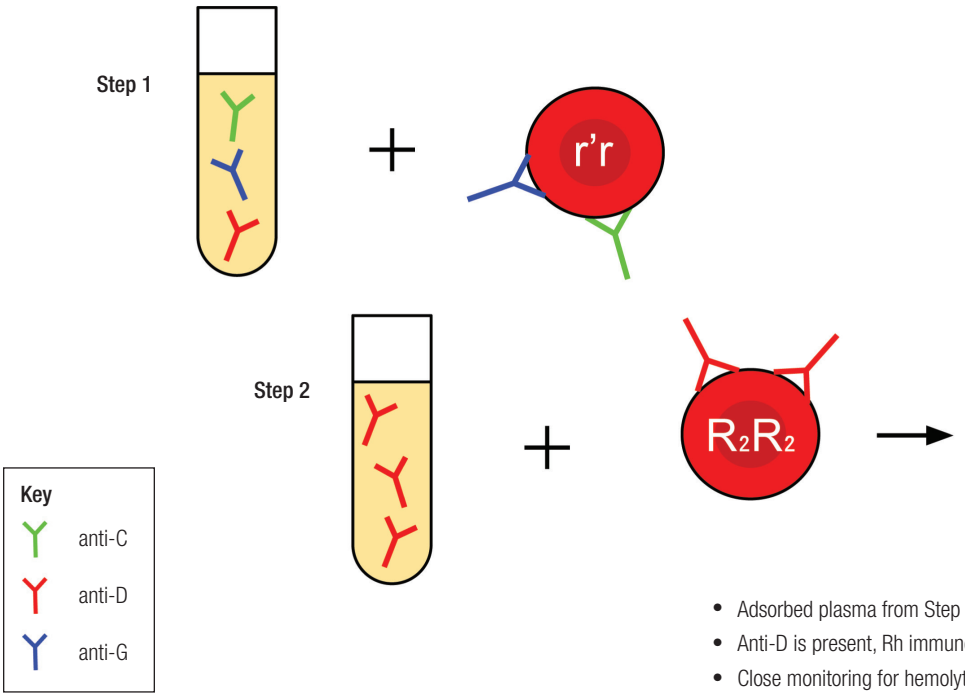
A clue to the possibility of anti-G occurs when titering samples containing apparent anti-D and anti-C, and noting an anti-C titer that is higher than anti-D. Isolating anti-G requires a tedious double adsorption and elution procedure. A simpler procedure has been reported recently that allows most transfusion service laboratories to confirm the presence or absence of anti-D with a single adsorption procedure. In a series of 27 prenatal specimens with apparent anti-D and anti-C specificities that were tested with this simpler method, anti-D was excluded in 8 of 27 (30%) patients, who then received Rh immune globulin prophylaxis; of these, 6 women had anti-C and anti-G, while only 2 had anti-G alone. Of the 19 patients who had anti-D, almost half (9 of 19) had anti-D, anti-C and anti-G.[3] Details of a procedure based on this method are given in Appendix 1-1. Briefly, patient plasma is adsorbed with RBCs that are r′r (D-C+) to adsorb out anti-C and anti-G, then the adsorbed plasma is run against an $R_2R_2$ cell (D+C-). If agglutination is observed, anti-D is present; if there is no agglutination, anti-D is not present and Rh immune globulin is indicated. A schematic representation of this adsorption procedure is shown in Figures 1-1a and 1-1b. To determine whether anti-C is present, a parallel adsorption can be set up with $R_2R_2$ cells to adsorb out anti-D and anti-G, then the adsorbed plasma is run against r′r cells. If agglutination occurs, anti-C is present.

The index patient's plasma was tested using this method and the results shown are the indirect antiglobulin test using untreated and adsorbed plasma at IgG phase:

| | $R_2R_2$ | r′r | Interpretation |
|---|---|---|---|
| Untreated plasma | 3+ | 2+ | |
| Plasma adsorbed with r′r RBC | 2+ | 0 | Anti-D present |
| Plasma adsorbed with $R_2R_2$ | 0 | 1+ | Anti-C present |

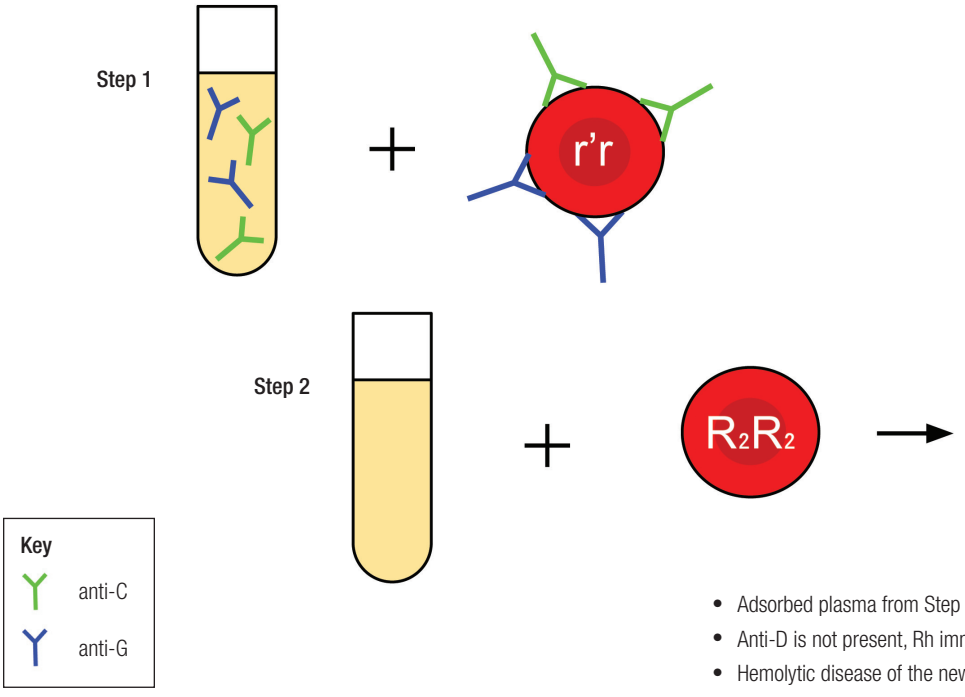
Since anti-D was present without any recent exposure to Rh immune globulin, the patient was diagnosed with Rh(D) sensitization and referred to the high-risk obstetric clinic for close monitoring. Anti-C was present as well, but the concurrent presence of anti-G was not specifically elucidated.

Figure 1-1a. Adsorption of patient plasma with r′r RBCs in a patient who has anti-C, anti-D, and anti-G




- Adsorbed plasma from Step 1 agglutinates $R_2R_2$ RBCs
- Anti-D is present, Rh immune globulin is not indicated
- Close monitoring for hemolytic disease of the newborn is needed

Figure 1-1b. Adsorption of patient plasma with r′r RBCs in a patient who has anti-C and anti-G




- Adsorbed plasma from Step 1 does not agglutinate $R_2R_2$ RBCs
- Anti-D is not present, Rh immune globulin is indicated
- Hemolytic disease of the newborn from anti-C is typically milder

### *Immunohematologic testing in the prenatal patient*

Prenatal specimens are typically submitted during the first prenatal visit, for first-trimester vaginal bleeding (representing threatened or actual spontaneous abortions), or prior to pregnancy terminations. A second sample for blood bank testing is sent at least on Rh(D)-negative women at 28 weeks gestation. The majority (80%) of transfusion service laboratories in the US do not perform routine weak D testing on specimens that test negative with anti-D by direct agglutination.[4] Occasionally, Rh(D) discrepancies may arise; for instance, a woman who tests D negative during pregnancy or hospitalization may have been told she is Rh positive, particularly if she has been a blood donor, because blood donor testing includes a test for weak D.

Unless the patient's partner is known to be D negative as well, all D-negative women in the US receive Rh immune globulin prophylaxis at 28 weeks. Rh immune globulin prophylaxis at 28 weeks and again after the delivery of a D-positive infant reduces the risk of sensitization from 12% to 0.1%. Some countries in Europe, such as Denmark, use automated DNA typing to determine fetal D type from cell-free fetal DNA in maternal blood at 25 weeks gestation, avoiding the unnecessary use of prophylactic Rh immune globulin in 37% (862 of 2312) of D-negative women.[5] Circulating cell-free fetal DNA for fetal *RHD* genotyping is commercially available and increasingly used. Fetal DNA in maternal plasma is derived from apoptotic syncytiotrophoblasts. After extraction, fetal DNA is evaluated by real-time quantitative polymerase chain reaction (QT-PCR). Most protocols amplify 3 exons or more, including *RHD* exons 4-7 and 10. Exon 4 contains the target pseudogene sequence that accounts for the majority of D-negative mutations in individuals of African descent. Confirmation of nonmaternal markers is required—typically the Y chromosome in male fetuses and/or housekeeping genes. A recent metaanalysis of noninvasive *RHD* genotyping from 3000 maternal plasma samples reported an accuracy of 95%.[6-8]

It has recently been proposed that weak D testing for prenatal specimens should be reintroduced; specimens that are weak D positive (or react at 2+ or weaker with anti-D reagent) should be referred for *RHD* genotyping. The rationale is that 3 common missense mutations (weak D types 1, 2, and 3) account for >90% of weak D phenotypes in white individuals, and these mutations do not confer any risk of anti-D development (see case 7 for a discussion of Rh genetics). Such women can safely avoid Rh immune globulin.[9]

It is estimated that up to 0.4% of pregnant women have antibodies to clinically significant Rh (non-D) and other minor RBC antigens. Common maternal non-D alloantibodies are anti-E, anti-K, and anti-c. Some jurisdictions provide K-negative blood for transfusion to women of childbearing potential to prevent the development of anti-K, which can cause severe HDN, but the cost-effectiveness of this approach has not been rigorously studied. In countries where this is a national standard, routine K phenotyping is performed on all blood donors, and the information is displayed on the blood bag label.[10]

If a woman is discovered to be alloimmunized, the titer of the antibody should be determined using a standardized method.[11] Titers above a predefined threshold (such as 16 for anti-D) should be reported as a critical alert result to the ordering provider. Anti-D titers should be tested monthly until 28 weeks gestation and every 2 weeks thereafter. Testing should ideally be performed in parallel with a frozen antecedent sample to reduce subjectivity in titer results. A difference of two dilutions or more is considered a significant change. Once a predefined critical titer is reached (8-32 for anti-D in the US), dictating more intensive fetal monitoring such as ultrasonography or amniocentesis, further antibody titration is not necessary.[6] A caveat to note is the poor correlation between anti-K titers and HDN severity: anti-K is known to cause severe fetal anemia by suppressing erythroid precursors.[12] Lewis antibodies are common during pregnancy but do not cause HDN. Other antibody specificities that have been associated with moderate or severe HDN include C, Cw, e, k, Kp(a), Kp(b), Js(a), Js(b), Jk(a), Fy(a), M, U, $PP_1P^k$, Di(b), Ge, Lan, LW, Wr(a), and Jr(a).[1]

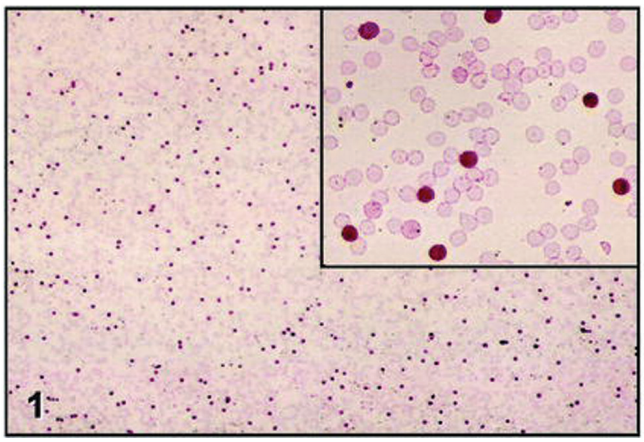
For sensitized pregnancies, traditionally amniocentesis was performed to determine the amount of bile pigment in amniotic fluid by measuring the OD450, the difference in optical density at 450 nm between the observed density and an extrapolated baseline. Liley charts plot OD450 values against gestational age to generate different risk zones; zone 3 indicates a high probability of hydrops. In recent years, noninvasive monitoring by fetal Doppler middle cerebral artery blood flow velocity as a surrogate of anemia has greatly reduced the number of amniocentesis procedures performed. Blood flow velocity values are reported as *multiples of the median,* or *MOM,* for gestational age, and correlate with moderate or severe fetal anemia when they exceed 1.5 MOM.[6] Measurements can be initiated as early as at 18 weeks of gestation and repeated at 1- to 2-week intervals until 35 weeks gestation, after which false-positive results become more common. Percutaneous umbilical vein sampling is performed in cases identified to be at high risk of hydrops by either amniocentesis or blood flow velocity. Cordocentesis hemoglobin result determines if intrauterine transfusion is needed. Intrauterine transfusions are performed at highly specialized antenatal centers; blood should be less than 72 hours old, O negative, K negative, antigen-matched to the mother as much as possible, crossmatched with maternal serum, irradiated, leukoreduced, and centrifuged to have a hematocrit of around 80. Intrauterine transfusion carries a risk of pregnancy loss estimated to be 1.6% per procedure and also risks sensitizing the mother to additional RBC antigens.[6]

After delivery, cord blood specimens are submitted to the transfusion service laboratory. Routine testing of all cord blood specimens is not necessary. Cord blood testing for ABO/Rh (including weak D testing) and a direct antiglobulin test (DAT) is needed if the mother is D negative, group O,

or sensitized. Any neonate who has undergone intrauterine transfusion will likely type as O negative. A positive DAT on a cord blood specimen should be communicated promptly to the ordering provider. The correlation between the strength of DAT positivity and HDN occurrence or severity is relatively modest. In Rh(D) HDN, infants may have a strongly positive DAT without clinical signs of disease, whereas in ABO HDN, infants may be clinically affected with a negative or weakly positive DAT.[1] In two series of infants whose RBCs were ABO incompatible with maternal serum, 14% to 33% had a positive DAT, but clinically significant hyperbilirubinemia and/or anemia occurred in only 4% of ABO-incompatible infants.[13,14] ABO incompatibility reduces the likelihood of primary Rh(D) sensitization but confers no protection against the secondary immune response or HDN severity once sensitization has occurred. ABO incompatibility is the most common cause of HDN, particularly in group A infants born to group O mothers. Disease course is mild, characterized by hyperbilirubinemia that may require phototherapy. Hemolytic anemia requiring exchange transfusion is rare.

Mothers who are D negative and deliver a D-positive infant require a screening test for fetomaternal hemorrhage (FMH), such as the rosette test. With a negative FMH screen, 1 vial of Rh immune globulin (300 mcg) should be given within 72 hours postpartum. A positive FMH screening result requires quantitation by acid-elution (the Kleihauer-Betke test) or flow cytometry to determine the additional doses of Rh immune globulin needed. The standard vial size of Rh immune globulin covers 30 mL (whole blood) or 15 mL (RBCs) of FMH, and calculations follow accordingly.[15] Massive fetomaternal hemorrhage is not always clinically suspected, as illustrated in Figure 1-2 by the strongly positive acid-elution test from a case reported in the literature.[16]

Figure 1-2




Postpartum peripheral blood smear from another case with unsuspected significant fetomaternal hemorrhage, stained using the Kleihauer-Betke technique. Low-power (original magnification X30) and high-power (inset, original magnification X250) views show numerous darkly staining fetal cells containing hemoglobin resistant to acid elution. From: Pourbabak S et al.[16] Reproduced with permission from *Archives of Pathology & Laboratory Medicine.* © 2004 College of American Pathologists.

### *Exchange transfusion for the postnatal treatment of hemolytic disease of the newborn*

Phototherapy and intravenous IgG are the first-line measures for hyperbilirubinemia in HDN. The indications for "early" exchange transfusion performed within the first 12 hours of life include a cord hemoglobin less than 11 g/dL, cord bilirubin exceeding 5.5 mg/dL, and a rate of rise of total serum bilirubin that exceeds 0.5 mg/dL per hour despite phototherapy.[6] "Late" exchange transfusions are performed when the serum bilirubin threatens to exceed 20 mg/dL to 22 mg/dL in term infants, with the thresholds individualized for postnatal age and other factors. Exchange transfusion achieves the dual purpose of removing antigen-positive RBCs and bilirubin. A double-volume exchange replaces approximately 85% of the infant's blood volume with antigen-negative RBCs. However, the amount of bilirubin or maternal alloantibody removed is significantly less, reflecting redistribution from the extravascular tissue-bound pool. In practice, 170 mL/kg for a 3-kg neonate is 500 mL, which approximates the volume of a unit of reconstituted whole blood. Fresh whole blood less than 7 days old would be ideal for exchange transfusion but is rarely available in the US. To reconstitute whole blood to achieve a final hematocrit of 50, leukoreduced, irradiated, O-negative red blood cells less than 7 days old and screened for hemoglobin S and the offending RBC antigen(s), compatible with maternal serum, are centrifuged and resuspended with an equal volume of group AB plasma. Infants with severe HDN may require more than 1 exchange transfusion. The most common complications after exchange transfusion are thrombocytopenia and hypocalcemia. Infants who received intrauterine transfusions typically do not require exchange transfusion but may require transfusion support for several months after birth while endogenous erythropoiesis recovers.

### *Case follow-up*

The patient admitted to intravenous drug use, which has been associated with Rh alloimmunization, particularly if the woman shares needles with her D-positive sexual partner.[17] Anti-D titer rose to 8192 at 31 weeks gestation, while anti-C titer stayed constant at 128. Serial ultrasounds for peak systolic velocity were reassuring until 36 weeks, when she was admitted urgently for induction but underwent emergent C-section for fetal decelerations. A male infant was delivered and intubated for poor Apgar scores, and noted to have diffuse petechiae and purpura. Cord blood testing was O positive, C positive, DAT positive. An eluate showed anti-D and anti-C. Hematocrit was 23%, platelet count 22,000/μL, bilirubin 9.4 mg/dL. Triple phototherapy was initiated, and 20 mL/kg of packed red blood cells (O negative, C negative) and 10 mL/kg platelets were transfused emergently. Bilirubin

level was 15 mg/dL at 4 hours of age when the first exchange transfusion was performed. Despite continuation of phototherapy and administration of IVIG, bilirubin continued to rise, prompting 3 subsequent exchange transfusions within the first 3 days of life. Thrombocytopenia at birth occurs in up to 25% of neonates with HDN, although severe thrombocytopenia (platelet count <50,000/μL) is less common.[6] Erythropoiesis may be suppressed for many months after exchange transfusion. This infant required 4 additional small-volume RBC transfusions, the last at 7 weeks of age. On clinic follow-up 1 month later, hemoglobin was 9 g/dL.

### *References*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### *Appendix 1-1*

**Sample procedure to demonstrate presence of anti-D +/- anti-C, anti-G in patients showing apparent anti-D and anti-C specificities**

I. Adsorption

1. Perform two adsorptions in parallel (one for anti-C/G using r′r reagent red cells and one for anti-D/G using $R_2R_2$ reagent red cells).
2. Label 10-mL red tops with preprinted accession labels. Number of tubes depends on amount of specimen and number of adsorptions needed. Label one set of tubes as follows: untreated plasma (r′r), r′r red cell aliquot #1, r′r red cell aliquot #2, etc (as many as needed until plasma is nonreactive with r′r red cells).
3. Label a second set of tubes as follows: untreated plasma ($R_2R_2$), $R_2R_2$ red cell aliquot #1, $R_2R_2$ red cell aliquot #2, etc (as many as needed until plasma is nonreactive with $R_2R_2$ red cells).
4. Spin patient specimen for 3 minutes, and pipette half the plasma into the untreated plasma (r′r) tube and half into the untreated plasma ($R_2R_2$) tube.
5. Aliquot 1 mL r′r reagent red cells into each of the r′r red cell tubes.
6. Aliquot 1 mL $R_2R_2$ reagent red cells into each of the $R_2R_2$ red cell tubes.
7. To each of the two red cell #1 tubes (r′r and $R_2R_2$), add 1 mL of polyethylene glycol (PEG) and 1 mL of plasma. Mix well.
8. Incubate for 15 minutes at 37°C water bath. Mix tubes frequently during incubation.
9. Centrifuge the red cell-PEG-plasma mixtures for 5 minutes or until cells are well packed. Harvest the adsorbed plasma.
10. Test the plasma adsorbed with r′r cells against r′r cell. If still reactive, perform second adsorption.
11. Test the plasma absorbed with $R_2R_2$ cells against $R_2R_2$ cell. If still reactive, perform second adsorption.
12. If second adsorptions are needed, add the harvested adsorbed plasma to the next red cell aliquot and repeat steps 7 through 9 without adding PEG.

II. Testing

1. Label a tube for each of the previously adsorbed plasmas to be tested: r′r adsorbed plasma and $R_2R_2$ adsorbed plasma.
2. For the r′r adsorbed plasma, test with $R_2R_2$ reagent red cells. A positive reaction means the patient's plasma contained anti-D (anti-C and/or anti-G adsorbed out).
3. For the $R_2R_2$ adsorbed plasma, test with r′r reagent red cells. A positive reaction means the patient's plasma contained anti-C (anti-D and/or anti-G adsorbed out).
4. Add 4 drops r′r adsorbed plasma to 1 drop $R_2R_2$ cells, and 4 drops $R_2R_2$ adsorbed plasma to 1 drop r′r cells. No enhancement is needed.
5. Incubate all tubes at 37°C for 10 minutes.
6. Wash three times with saline; decant to a dry button after last wash.
7. Add 2 drops of anti-IgG to each tube.
8. Mix well and centrifuge for time specified for antihuman globulin phase testing.
9. Resuspend the cell button, grade reaction, and record results on patient worksheet.
10. Confirm all negative results with 1 drop of Coombs control cells. Tests are invalid if the reaction at the Coombs phase is less than 2+.