Immunohematology Case Studies 2020 - 4

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Clinical History

Mrs D. 34 years old, pregnant, G2P1, ABO:-1,-2,-3 (O); RH:-1,-2,-3,4,5 (dccee (D negative))

Previous pregnancy marked by an important fetomaternal hemorrhage discovered at delivery.

The Kleihauer-Betke test was found at 230 FRBC (fetal red blood cells) /10 000 ARBC (adult red blood cells), corresponding to a volume of **115 ml of fetal blood**.
Clinical History

The newborn’s phenotype was RH:1,2,-3,4,5 (DCcee) (D positive). The Hb level at birth was 6g/dl. A red blood cell transfusion was required at day 1.

The mother’s antibody screening was negative at delivery.

To prevent anti-D alloimmunization: 7000 IU/ml (1400 µg) of anti-D Immunoglobulin (Ig) was administered to the mother the day after the delivery. 72h after the end of the anti-D Ig IV infusion, the Kleihauer-Betke test control was at 0 FRBC/10,000 ARBC.
Serologic History

Despite the reversion of the Kleihauer-Betke test, a failure of prophylaxis is sometimes observed in these kind of cases, because the fetomaternal hemorrhage often started several days before delivery and anti-D Ig administration.

Hence, a **control of the maternal antibody screening** was done 6 months after delivery.
Conclusion: anti-RH1 (D) and anti-RH2 (C) alloimmunization
Serologic History

**Antibody titration** (to evaluate the risk for a future pregnancy)

- **Anti-RH1 + RH2** titer was 16 (indirect antiglobulin test, tube method, saline medium, with RH:1,2,3,4,5 [DCcEe] red blood cells (RBC)].
- **Anti-RH1** titration with RH:1,-2,-3,4,5 RBC (Dccee) : 8
- **Anti-RH2** titration with RH:-1,2,-3,4,5 RBC (dCcee) : 2

![Image of antibody titration](image1.jpg)

**Anti-RH1 + anti-RH2 quantitation** by continuous flow analysis (CFA) (hemagglutination) on Astoria WHS autoanalyzer with RH:1,2,3,4,5 RBC (DCcEe): 5 IU/ml

![Image of continuous flow analysis](image2.jpg)
Serologic History

Postnatal medical consultation in our center:

- Explanations given about the alloimmunization and its impact in case of a future pregnancy or a future transfusion

- Recommendation for waiting at least 2 years before beginning a new pregnancy, to let the antibody level reach a minimum
Current Sample Presentation Data

Current pregnancy 2 years later:

- Antibody screening at the 10th week of gestation (GW): anti-RH1 + RH2, with the same picture as 2 years ago

- Antibody titration:
  - Anti-RH1 + RH2 titer: 4
  - Anti-RH1 titer: 4
  - Anti-RH2 titer: 2

- Anti-RH1+RH2 concentration (CFA on Astoria WHS autoanalyzer): 2 IU/ml
Fetal *RHD* genotyping

*Same father* as 2 years ago (RH:1).

Fetal *RHD* genotyping realized by automated extraction (EasyMag™) and real time PCR (*ViiA™ 7*), using exons 5,7,10 and maize DNA extraction control (*Free DNA fetal kit RHD ®, J Boy*)

Result at 12 GW: fetus *RHD* negative
Control at 16GW: fetus *RHD* negative

➤ No anti-RH1 fetomaternal incompatibility
➤ No need to follow the anti-RH1 titer during the pregnancy

Because of the anti-RH2 present in the maternal serum and the unknown RH2 phenotype of the father, the lab recommended a new titration of the anti-RH2 at 32 GW.

➤ But this control titration was not done (not prescribed by the clinicians)
Delivery at 39 GW: the newborn was anemic, with a hemoglobin level of 9 g/dl. He also had jaundice: the total bilirubin level was 110 micromol/l at hour 11.

The direct antiglobulin test was strongly positive (IgG 4+, C3d 0 (column-filtration method – DC Screening II, Bio-Rad®))

The newborn’s RBC phenotype was O RH:-1,2,-3,4,5 (dCcee) showing an anti-RH2 fetomaternal incompatibility

An acid elution test was performed and anti-RH2 but also anti-RH1 were found in the eluate (!?)
A titration of the antibodies in the maternal serum was immediately performed:

- anti-RH1 titer (with RH:1,-2,-3,4,5 RBC) : **256**
- anti-RH2 titer (with RH:-1,2,-3,4,5 RBC) **128**
- anti-RH1+ RH2 concentration (CFA analysis with RH:1,2,3,4,5 RBC): **60 IU/ml**

➢ increase of the anti-RH2, but also of the anti-RH1 titer in the maternal serum !?
Challenge with the Current Presentation

How can we explain:

- the presence of an anti-RH1 in the eluate while the newborn’s RH1 phenotype is negative?

- the increase of the anti-RH1 titer in the maternal serum in an apparently RH1 compatible pregnancy?
Hypothesis 1:

- *RHD* fetal genotyping error and blocked-D phenomenon?

Blocked D phenomenon can be observed in the presence of high titer of anti-D: maternal antibodies present in the newborn’s blood can coat and block the D antigens on RBC. This “blocking” phenomenon prevents agglutination of the newborn’s D positive RBC with IgM anti-D typing reagents, giving false negative results. But maternal anti-D antibodies are found in the eluate.
Challenge with the Current Presentation

**Hypothesis 1:**

*RHD* genotyping on the newborn’s blood cells

To test this hypothesis, a rapid in-house *RHD* genotyping was performed directly on the newborn’s cells (*RHD* exons 4, 7, 10 and intron 4) and was found negative, confirming the RH:-1 phenotype of the newborn.

**ASP (allele specific) PCR**

**Primers**

- Exon 10: D4, D5/LO2, P4, P5
- Exon 7: D6, D7
- Exon 4 + 4 *Dpsi*: RHDIN3F, D9
- Intron 4: I2, E5-1

1 to 4 = patients
Newborn of Mrs D = patient 3

- E10 = exon 10
- E7 = exon 7
- IN4 = intron 4
- E4Ψ = exon 4 + exon 4 *Dpsi*

+ = positive control
- = negative control
Ψ = positive *Dpsi* control
b = blank
Challenge with the Current Presentation

Hypothesis 2:

- Presence of anti-G (RH12) antibodies in the maternal serum that are bound to the newborn’s RBC?

The G (RH12) antigen:
Common epitope of the RhD protein and the RhCe protein carrying the C antigen
Red blood cells positive for the D and/or the C antigen are also positive for the G antigen. The newborn is RH:-1,2,-3,4,5 (dCcee) so positive for C and G antigens.
On the antibody screen, an anti-G (RH12) has the same picture as an anti-D (RH1) + anti-C (RH2) association: so it can explain the “anti-D (RH1) + anti-C (RH2)” picture of the eluate.
Interim Antibody Identification
Possible Answers and Next Steps

Anti-G (RH12) research in the mother’s serum

1) Use of rare red blood cells expressing the r”’G (RHD*01N.07) allele (cells provided by the French National Immunohaematology Reference Center (Centre National de Référence pour les Groupes sanguins (CNRGS)) that express G antigen but not D and C antigens due to RHD - RHCE gene conversion.

The basis of reactivity for G antigen is Ser103, which is encoded by RHD gene and by the RHCE*Ce (C allele of the RHCE gene)).

The RHCE*ce (c allele of the RHCE gene) encodes a Pro103).

Molecular mechanism of the rare allele RHD*01N.07

Adapted from Daniels G. Human Blood Groups 3rd edition (courtesy Dr J Babinet, CNRGS, France)
Further Work

Indirect antiglobulin test performed with the patient’s serum and different types of papain-treated RBC (suspension of 0.8% RBC in Cell Stab® solution, gel-microcolumn assay (LISS Coombs IgG+C3d gel card, Bio-Rad®))

Results:

<table>
<thead>
<tr>
<th>Papainized R0r RBC (D+ C- G+)</th>
<th>Papainized r’r RBC (D- C+ G+)</th>
<th>Papainized RBC with the r’’G allele (D- C- G+)</th>
<th>Papainized rr RBC (D- C- G-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactivity of the serum of Mrs D</td>
<td>4+</td>
<td>4+</td>
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</table>

Conclusion: presence of anti-G (RH12) in the plasma of Mrs. D.

But finally, does Mrs. D also have anti-D and/or anti-C alloimmunization? ➤ Adsorption - elution test
2) Adsorption/elution test performed in the CNRHP lab

Example of a sample with an association of anti-D, anti-C and anti-G

A) Serum adsorption with papain-treated $R_0r$ (D+C-) RBC for exhaustion of anti-D and anti-G: the adsorbed serum contains anti-C (adsorbate number I). Elution of the adsorbed antibodies = eluate number 1 (containing anti-D + anti-G)

Adsorption of the eluate 1 with papain-treated $r'r$ (D-C+) RBC for exhaustion of anti-G: the adsorbate (adsorbate number III) contains only anti-D
B) Serum adsorption with papain-treated r’r (D-C+) RBC for exhaustion of anti-C and anti-G: the adsorbed serum contains anti-D (adsorbate number II). Elution of the adsorbed antibodies = eluate number 2 (containing anti-C + anti-G)

Adsorption of the eluate 2 with papain-treated R₀r (D+C-) RBC for exhaustion of anti-G: the adsorbate (adsorbate number IV) contains only anti-C
C) Titration by indirect antiglobulin test of the eluates 1 and 2 and the adsorbates II, III and IV with papain-treated R_{0r}, r’r and rr RBC (column filtration method).

<table>
<thead>
<tr>
<th></th>
<th>Eluate 1</th>
<th>Eluate 2</th>
<th>Adsorbate II</th>
<th>Adsorbate III</th>
<th>Adsorbate IV</th>
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<td>r’r (D-C+)</td>
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<td>r’r (D-C+)</td>
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<td>Adsorbate IV</td>
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<td>r’r (D-C+)</td>
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<td>rr (D-C-)</td>
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</table>
# Results of the adsorption/elution test for Mrs D

## Table

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<tr>
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<th>RBC</th>
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<th>1/2</th>
<th>1/4</th>
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<td>R₀r (D+C-)</td>
<td>Anti-D+G</td>
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<td>4+</td>
<td>4+</td>
<td>4+</td>
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<td>3+</td>
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<td>Anti-G</td>
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<td>rr (D-C-)</td>
<td>Neg Ctl</td>
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<tr>
<td><strong>Eluate 2</strong></td>
<td>R₀r (D+C-)</td>
<td>Anti-G</td>
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<td>4+</td>
<td>3+</td>
<td>2.5+</td>
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<td>r'r (D-C+)</td>
<td>Anti-G+C</td>
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<td>rr (D-C-)</td>
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<tr>
<td><strong>Adsorbate II</strong></td>
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<td>Anti-D</td>
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<td>r'r (D-C+)</td>
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<td>rr (D-C-)</td>
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<td><strong>Adsorbate III</strong></td>
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<td>Anti-D</td>
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<td><strong>Adsorbate IV</strong></td>
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**Conclusion:** Presence of anti-D, anti-G and anti-C

Neg ctl = negative control / Ads ctrl = adsorption control
Interpretations

Mrs. D had anti-D (RH1), anti-C (RH2) and anti-G (RH12) alloimmunization.

Anti-RH2 (C) and anti-G (RH12) concentration and titers have increased in the mother’s sera in the presence of RH:1,2,3,4,5 cells (DCcEe), RH:-1,2,-3,4,5 (dCcee) but also RH:1,-2,-3,4,5 (Dccee) cells, which are all RH:12 (G positive)). It had given the illusion of an increase of the anti-RH1 titer.

In fact, antibodies titers in the maternal serum at delivery were
anti-RH1 + anti-RH12 titer (with RH:1,-2,-3,4,5 RBC) : 256
anti-RH2 + anti-RH12 titer (with RH:-1,2,-3,4,5 RBC) 128
anti-RH1+ anti-RH2 + anti-RH12 concentration (CFA analysis with RH:1,2,3,4,5 RBC) 60 IU/ml
(in bold = increasing antibodies because of fetomaternal incompatibility)

The father’s RH phenotype was also determined after delivery : he was found RH:1,2,-3,-4,5 with a predicted combination of Dce/dCe haplotypes (R₁r’).
Updated Clinical Information

Because of the severity of the hemolytic disease of the newborn (HDN) at day 1, the newborn was transferred to another bigger hospital with a neonatal department and more adapted technical support.

Intensive phototherapy was introduced. The hyperbilirubinemia reached a peak of 250 µmol/l at H36, but no exchange transfusion was needed.

As the hemoglobin level reached a nadir of 7 g/dl at Day 5, a top-up transfusion was required.
No further transfusion was needed.
The baby left the hospital at Day 13.
Conclusions

- We present here a case of a **severe hemolytic disease of the newborn (HDN)** due to anti-C (RH2) and anti-G (RH12) maternal alloimmunizations.

- The **levels of the antibodies** had not been correctly **followed up** during pregnancy, **because the patient was first considered having an anti-D (RH1) + anti-C (RH2) alloimmunization**, and the negative results of the **RHD fetal genotyping** were reassuring.

- **Thus, the HDN has not been anticipated at birth**, and its diagnosis has been delayed, inducing a **non-optimal management** of the hemolytic disease in the newborn’s first hours of life.
Summary of Case Challenges

- The presence of a positive direct antiglobulin test and an **anti-RH1 (D) + anti-RH2 (C)** picture in the newborn’s eluate, whereas the newborn’s D phenotype is negative, could be due to an **anti-G(RH12)** maternal antibody.

- In case of an **anti-RH1 (D) + anti-RH2 (C)** alloimmunization, **when the anti-RH1 (D) titer increases** in the maternal serum whereas the **fetal RHD genotyping is negative**, you have to think of an **anti-RH12 (G)** antibody.
Lessons Learned by the Case

1) Huge fetomaternal hemorrhage discovered at birth often leads to the mother’s immunization even if an anti-RH1 prophylaxis has been correctly given.

2) Anti-RH2 (C) and anti-G (RH12) antibodies can cause severe postnatal HDN, even if they are often less severe than those induced by anti-RH1 (D).

3) This case highlights the need for continuous monitoring of pregnancies complicated by anti-RH1 (D) + anti-RH2 (C) immunization, even if the fetus is found RHD negative (at least a titration test during the third trimester, to anticipate a potential HDN due to anti-RH2 (C) and/or anti-G (RH12) maternal antibodies).

4) Knowing the paternal phenotype is important. In this case, the presence of a paternal RH2 homozygosity should have drawn clinicians attention not to forget the titration at the third trimester.
Lessons Learned by the Case

In case of confirmed negative fetal RHD genotyping, if an anti-RH1 alloimmunization is (at least) present, our center recommends to continue to perform an antibody screening (with antibody quantification) every 6 weeks.

This allows:

- To detect early the appearance of new antibodies, as new immunizations are quite often observed during pregnancies of women who have already developed an antibody.

- Not to forget to quantitate the non anti-D antibodies that could be associated.
G (RH12) antigen:
The amino acid basis of reactivity for G antigen is Ser103, which is encoded by \(RHD\) gene and by the \(RHCE^*Ce\) (\(C\) allele of the \(RHCE\) gene).

Anti-G (anti-RH12):
Differentiating this antibody from an anti-D + anti-C (anti-RH1+ anti-RH2) association has no real impact for transfusion practice, as RH:-1,-2 (D-C-) RBC will be chosen and serologically crossmatched. It will allow detection if a rare RH:-1,-2,12 unit has been selected for transfusion.

But its characterization is important in obstetrics: this antibody can cause HDN, mild in general, but some cases of severe HDFN have already been described with high level of antibodies.

Moreover, in case of anti-G or anti-G + anti-C immunization without associated anti-D, prophylactic anti-D Immunoglobulins must be administered to the pregnant woman if the fetus is \(RHD\) positive or has an unknown \(RHD\) status.
References

- Allen FH, Tippet PA: A new Rh blood type which reveals the Rh antigen G, *Vox Sanguinis* 1958;3;321-30

- Hadley AG, Poole GD, Poole J et al Hemolytic disease of the newborn due to anti-G, *Vox Sanguinis* 1996;71:108-12


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Dr Paul MAURICE
Dr Loriane FRANCHINARD
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