Immunohematology Case Studies 2020 - 4

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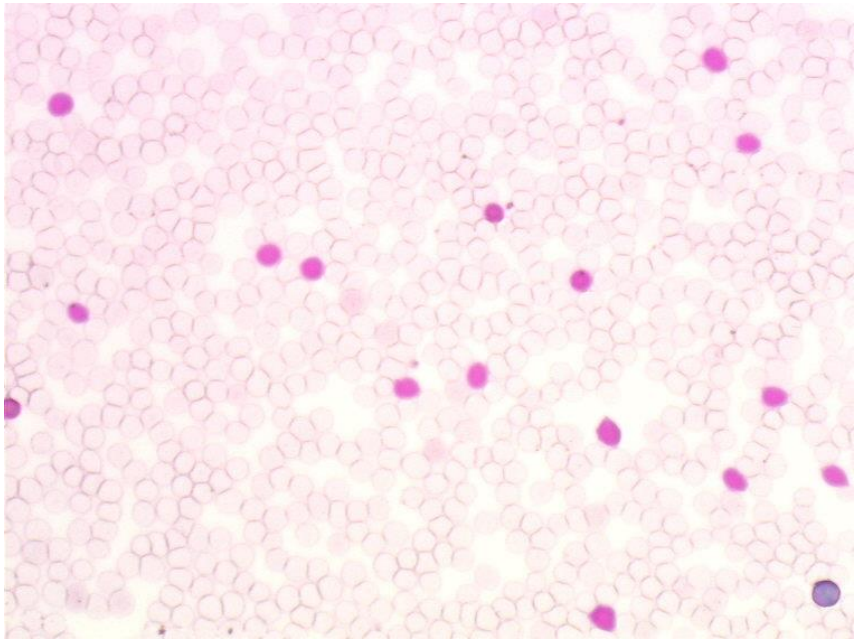
Clinical History

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.

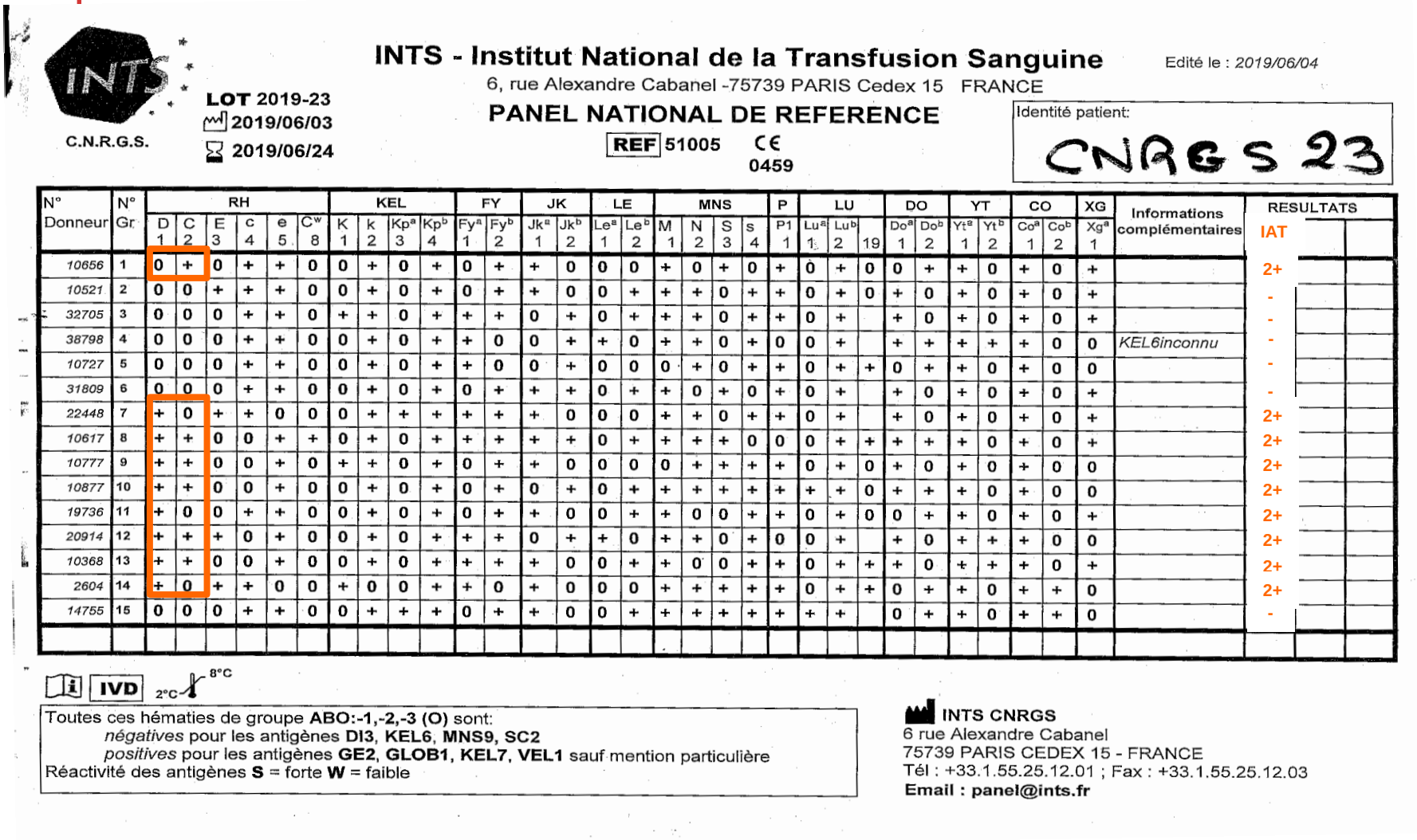
To prevent anti-D alloimmunization : 7000 IU/ml (1400 µg) of anti-D Immunoglobulin (Ig) was administered to the mother the day after the delivery. 72h after the end of the anti-D Ig IV infusion, the **Kleihauer-Betke test control was at 0** FRBC/ 10,000 ARBC.

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.

Serologic History



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Edité le : *2019/06/04*

C.N.R.G.S.

LOT 2019-23
2019/06/03
2019/06/24

PANEL NATIONAL DE REFERENCE

REF 51005 CE 0459

Identité patient: CNRGS 23

N° Donneur	N° Gr	RH						KEL				FY		JK		LE		MNS				P	LU			DO		YT		CO		XG	Informations complémentaires	RESULTATS
		D 1	C 2	E 3	c 4	e 5	C^w 8	K 1	k 2	Kp^a 3	Kp^b 4	Fy^a 1	Fy^b 2	Jk^a 1	Jk^b 2	Le^a 1	Le^b 2	M 1	N 2	S 3	s 4	P1 1	Lu^a 1	Lu^b 2	19	Do^a 1	Do^b 2	Yt^a 1	Yt^b 2	Co^a 1	Co^b 2	Xg^a 1		IAT
10656	1	0	+	0	+	+	0	0	+	0	+	0	+	+	0	0	0	+	0	+	0	+	0	+	0	0	+	+	0	+	0	+		2+
10521	2	0	0	+	+	+	0	0	+	0	+	0	+	+	0	0	+	+	+	0	+	+	0	+	0	+	0	+	0	+	0	+		-
32705	3	0	0	0	+	+	0	+	+	0	+	+	+	0	+	0	+	+	+	0	+	+	0	+		+	0	+	0	+	0	+		-
38798	4	0	0	0	+	+	0	0	+	0	+	+	0	0	+	+	0	+	+	0	+	0	0	+		+	+	+	+	+	0	0	KEL6inconnu	-
10727	5	0	0	0	+	+	0	0	+	0	+	+	0	0	+	0	0	0	+	0	+	+	0	+	+	0	+	+	0	+	0	0		-
31809	6	0	0	0	+	+	0	0	+	0	+	0	+	+	+	0	+	+	0	+	0	+	0	+		+	0	+	0	+	0	+		-
22448	7	+	0	+	+	0	0	0	+	+	+	+	+	+	0	0	0	+	+	0	+	+	0	+		+	0	+	0	+	0	+		2+
10617	8	+	+	0	0	+	+	0	+	0	+	+	+	+	+	0	+	+	+	+	0	0	0	+	+	+	+	+	0	+	0	+		2+
10777	9	+	+	0	0	+	0	+	+	0	+	0	+	+	0	0	0	0	+	+	+	+	0	+	0	+	0	+	0	+	0	0		2+
10877	10	+	+	0	0	+	0	0	+	0	+	0	+	0	+	0	+	+	+	+	+	+	+	+	0	+	+	+	0	+	0	0		2+
19736	11	+	0	0	+	+	0	0	+	0	+	0	+	+	0	0	+	+	0	0	+	+	0	+	0	0	+	+	0	+	0	+		2+
20914	12	+	+	+	0	+	0	0	+	0	+	+	+	0	+	+	0	+	+	0	+	0	0	+		+	0	+	+	+	0	0		2+
10368	13	+	+	0	0	+	0	0	+	0	+	+	+	+	0	0	+	+	0	0	+	+	0	+	+	+	0	+	+	+	0	+		2+
2604	14	+	0	+	+	0	0	+	0	0	+	+	0	+	0	0	0	+	+	+	+	+	0	+	+	0	+	+	0	+	+	0		2+
14755	15	0	0	0	+	+	0	0	+	+	+	0	+	+	0	0	+	+	+	+	+	+	+	+		0	+	+	0	+	+	0		-

IVD 2°C 8°C

Toutes ces hématies de groupe **ABO:-1,-2,-3 (O)** sont:
négatives pour les antigènes **DI3, KEL6, MNS9, SC2**
positives pour les antigènes **GE2, GLOB1, KEL7, VEL1** sauf mention particulière
Réactivité des antigènes **S** = forte **W** = faible

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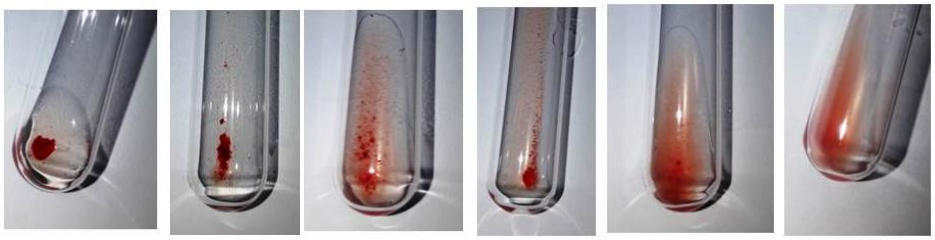
Conclusion: **anti-RH1 (D) and anti-RH2 (C) alloimmunization**

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 t**itration with RH:-1,2,-3,4,5 RBC (dCcee) : **2**



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**

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

<u>Current pregnancy</u> 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**

Current Sample Presentation Data

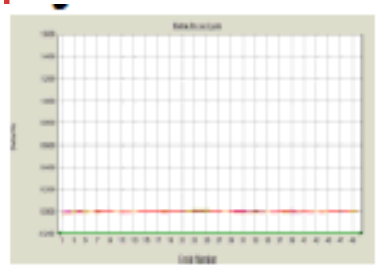
Fetal *RHD* genotyping

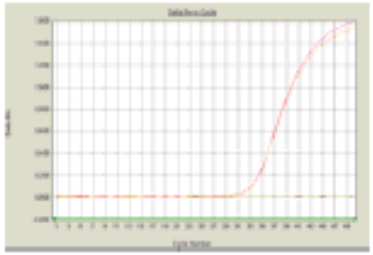
Same father as 2 years ago (RH:1).

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**





	Exon10	Exon7	Exon5	Maize
Well1	-	-	-	33.2
Well2	-	-	-	33.2

Ct amplification

➢ **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)

Current Sample Presentation Data

<u>Delivery at 39 GW</u>: 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 **<u>anti-RH2 but also anti-RH1</u> were found in the eluate (?!)**

Current Sample Presentation Data

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?**

Challenge with the Current Presentation

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:

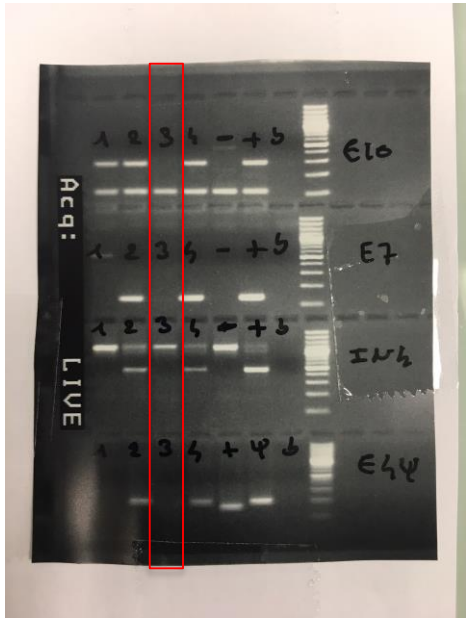
RHD genotyping on the newborn's blood cells

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

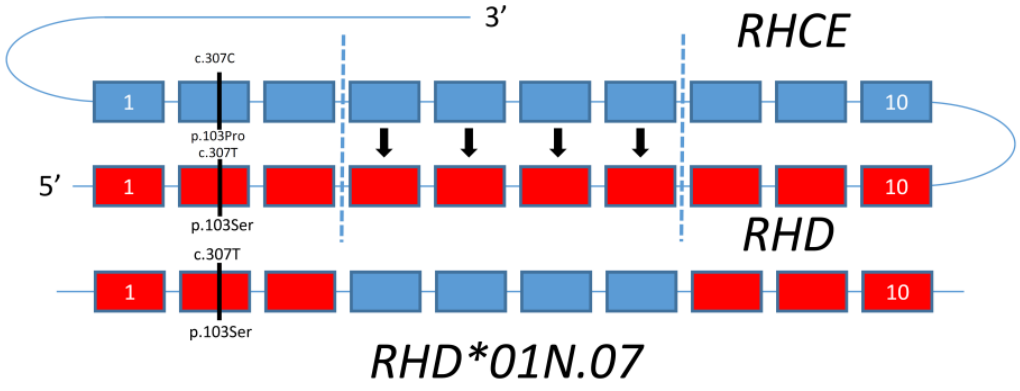
Anti-G (RH12) research in the mother's serum

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:

	Papainized R0r RBC (D+ C- G+)	Papainized r'r RBC (D- C+ G+)	Papainized RBC with the *r''G* allele (D- C- G+)	Papainized rr RBC (D- C- G-)
Reactivity of the serum of Mrs D	4+	4+	4+	-

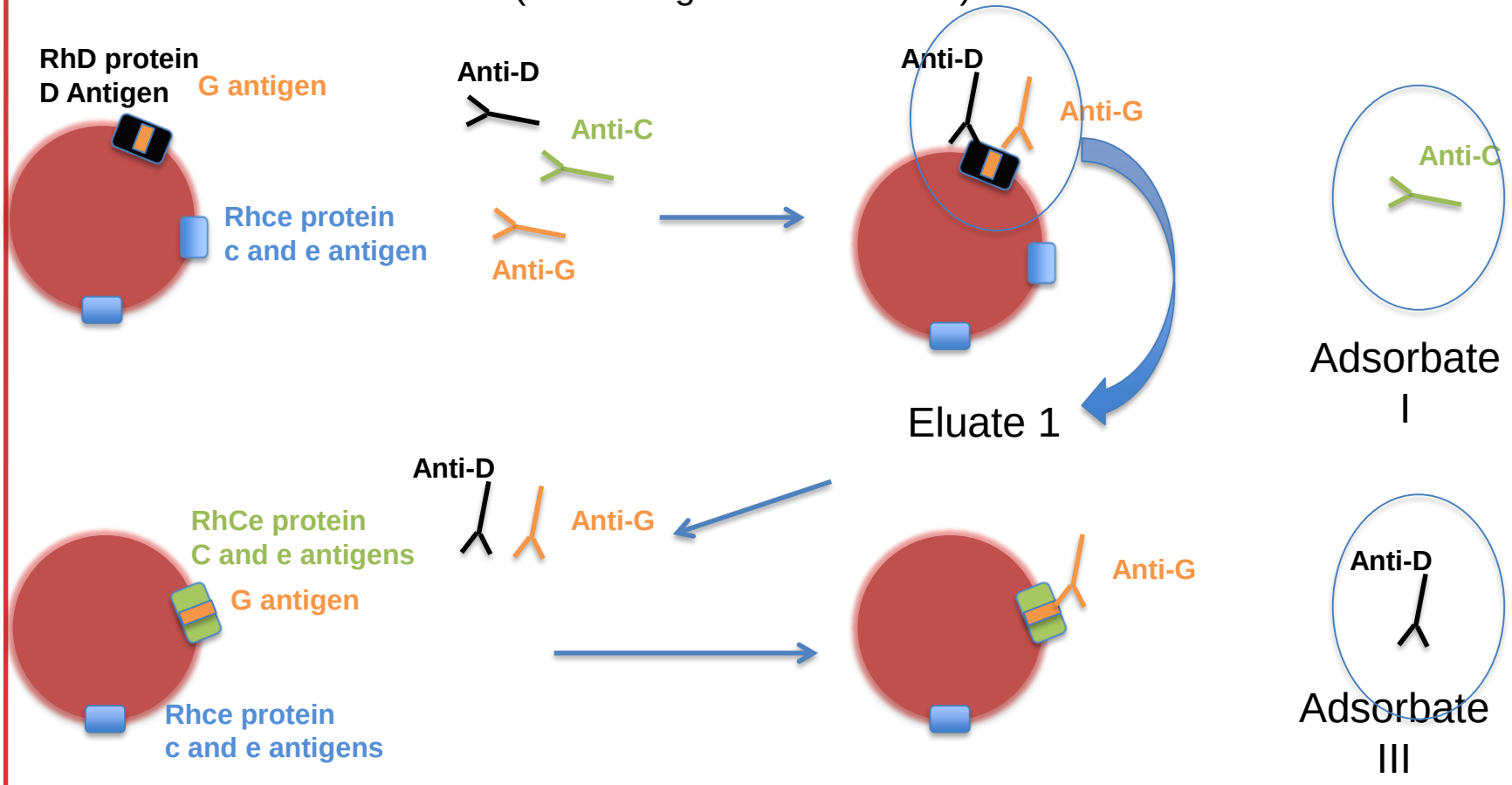
Conclusion : **presence of anti-G (RH12) in the plasma of Mrs. D.**

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

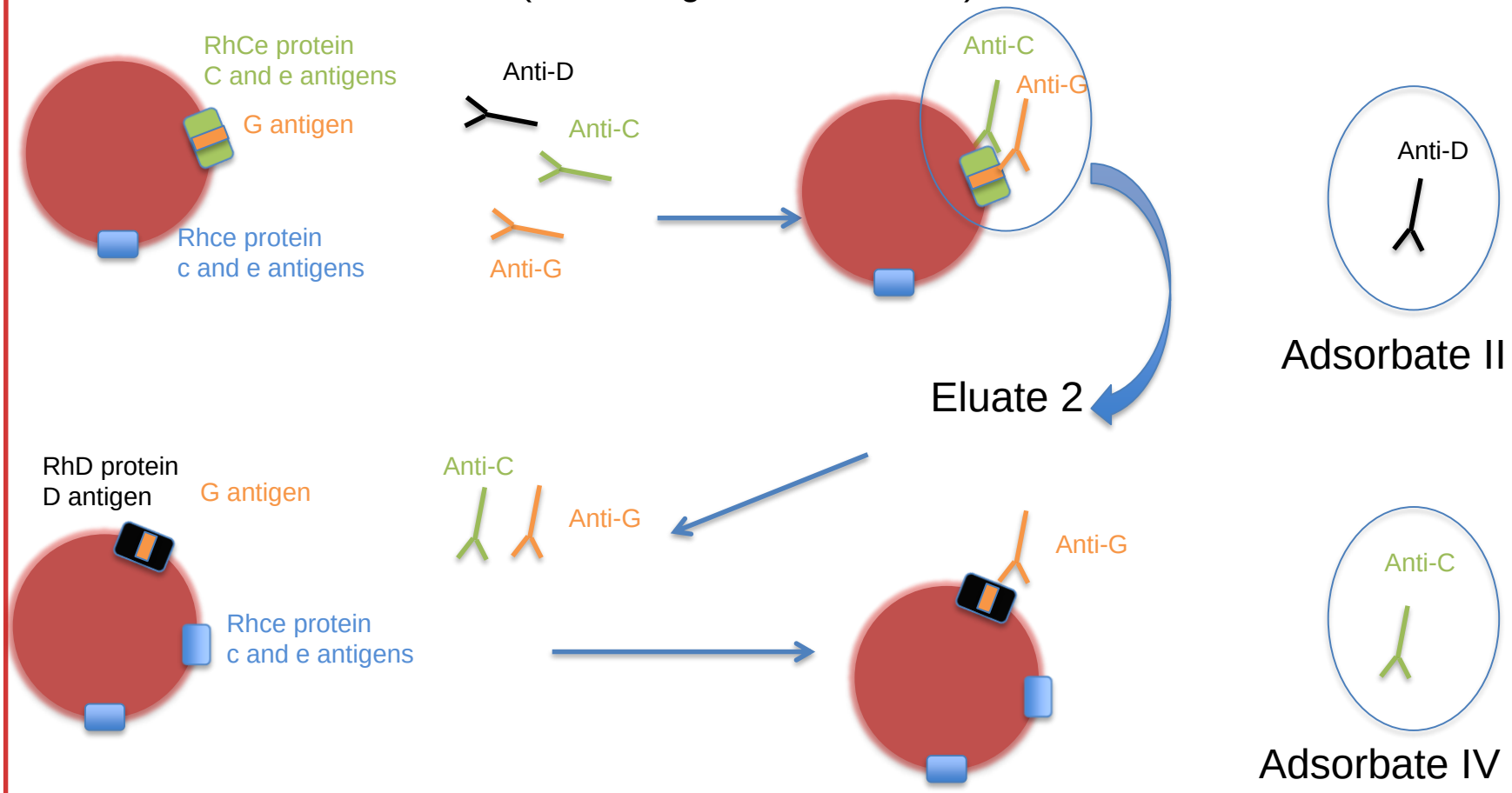
Example of a sample with an association of anti-D, anti-C and anti-G

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)



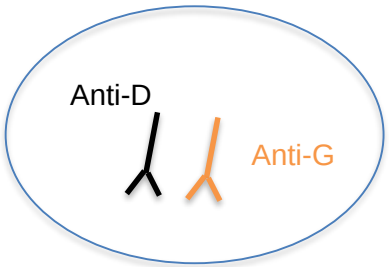
Adsorption of the eluate 1 with papain-treated r'r (D-C+) RBC for exhaustion of anti-G: the adsorbate (adsorbate number III) contains only anti-D

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)

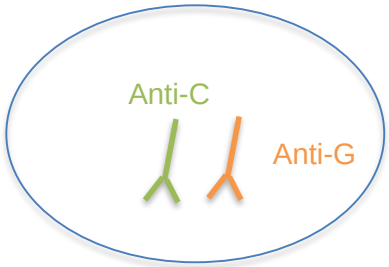


Adsorption of the eluate 2 with papain-treated R_0r (D+C-) RBC for exhaustion of anti-G: the adsorbate (adsorbate number IV) contains only anti-C

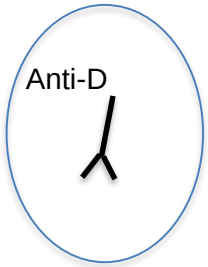
C) Titration by indirect antiglobulin test of the eluates 1 and 2 and the adsorbates II, III and IV with papain-treated R_0r, r'r and rr RBC (column filtration method).



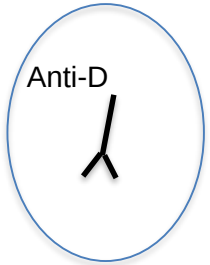
Eluate 1



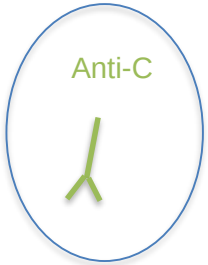
Eluate 2



Adsorbate II



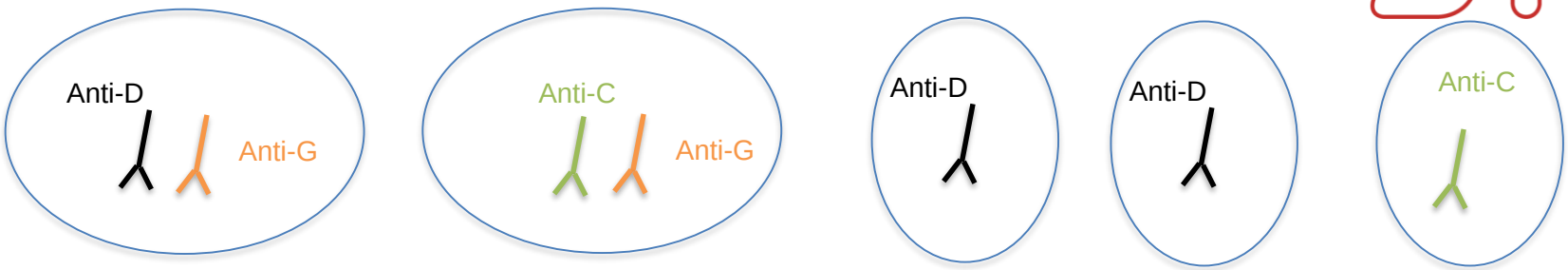
Adsorbate III



Adsorbate IV

	RBC	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
Eluate 1	R_0r (D+C-)												
	r'r (D-C+)												
	rr (D-C-)												
Eluate 2	R_0r (D+C-)												
	r'r (D-C+)												
	rr (D-C-)												
Adsorbate II	R_0r (D+C-)												
	r'r (D-C+)												
	rr (D-C-)												
Adsorbate III	R_0r (D+C-)												
	r'r (D-C+)												
	rr (D-C-)												
Adsorbate IV	R_0r (D+C-)												
	r'r (D-C+)												
	rr (D-C-)												

Results of the adsorption/elution test for Mrs D



	RBC	Antibody(ies) present	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
Eluate 1	R_0r (D+C-)	**Anti-D+G**	4+	4+	4+	4+	4+	4+	3+	2+	1+	(+)	-	-
	r'r (D-C+)	**Anti-G**	4+	3+	3+	2,5+	2+	1+	-	-	-	-	-	-
	rr (D-C-)	**Neg Ctl**	-	-	-	-	-	-	-	-	-	-	-	-
Eluate 2	R_0r (D+C-)	**Anti-G**	4+	4+	3+	2,5+	2+	1+	-	-	-	-	-	-
	r'r (D-C+)	**Anti-G+C**	4+	4+	4+	3+	3+	3+	2,5+	2+	1+	-	-	-
	rr (D-C-)	**Neg Ctl**	-	-	-	-	-	-	-	-	-	-	-	-
Adsorbate II	R_0r (D+C-)	**Anti-D**	4+	4+	4+	4+	4+	4+	3+	3+	3+	2+	1+	-
	r'r (D-C+)	**Ads Ctl**	4+	4+	3+	3+	2+	1+	-	-	-	-	-	-
	rr (D-C-)	**Neg Ctl**	-	-	-	-	-	-	-	-	-	-	-	-
Adsorbate III	R_0r (D+C-)	**Anti-D**	4+	4+	4+	4+	3+	2,5+	2,5+	1,5+	(+)	-	-	-
	r'r (D-C+)	**Ads Ctl**	2+	1+	-	-	-	-	-	-	-	-	-	-
	rr (D-C-)	**Nec Ctl**	-	-	-	-	-	-	-	-	-	-	-	-
Adsorbate IV	R_0r (D+C-)	**Ads Ctl**	2,5+	1,5+	-	-	-	-	-	-	-	-	-	-
	r'r (D-C+)	**Anti-C**	4+	4+	4+	3+	2,5+	2,5+	1,5+	(+)	-	-	-	-
	rr (D-C-)	**Neg Ctl**	-	-	-	-	-	-	-	-	-	-	-	-

Conclusion: Presence of anti-D, anti-G and anti-C

Neg ctl = negative control / Ads ctl = 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 (R_1r')

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) ev**ery 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.

Brief Review of the Blood Group System or Antibody

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

- Cash K Brown T Strupp A UehlingerJ. Anti-G in a pregnant patient. *Transfusion* 1999;39:531-3

- Jianhua Chen and Feng Liu, A case of mild HDFN caused by anti-C, anti-D and anti-G : Diagnostic strategy and clinical significance of distinguishing anti-G from anti-D and anti-C *Transfus Apher Sci*. 2019 Jul 9:102602.

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