The burden of false RhD phenotyping in partial D (RHD*10 DAU)

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Abstract

The human Rhesus genes *RHD* and *RHCE* are two closely linked genes in a two-locus system encoding for the *D* and the *CcEe* blood group antigens. The Rh blood group is a polymorphic system with significant issues in blood transfusion. This holds especially true for Rhesus D (RhD). Genotypes derived from gene conversion, polymorphism and mutation are responsible for the synthesis of polymorphic proteins such as weak D, partial D, and DEL phenotypes. The identification of D variants is important in cases of transfusion or pregnancy because RhD-negative patients and patients with D variants are in danger of immunization by RhD-positive blood cells. Serologic RhD typing is often uncertain because monoclonal anti-D reagents may or may not react with weak and partial D types. The behavior of reagents with D variants is not consistent even within a certain Rhesus cluster such as *DAU*. We learned that variants are able to react as strong as normal RhD-positive erythrocytes with monoclonal antibodies while others react weak, faint or with no detectable agglutinations. In a pregnant case with later proven RHD*10 (DAU) and categorized as partial D, we were close on false RhD phenotyping because all serologic tests resulted in full strength reaction with several different anti-D reagents in different standard tests. At no time, agglutination signs for a weak or partial D variant have occurred. This case marks a perfect example for dangerous errors in the serology of RhD phenotyping that could have unwanted consequences in the omission of appropriate anti-D prophylaxis, or in general terms, in possible transfusion situations. The *DAU* cluster and other genetic *RHD* variants may be rare in the European population, while certain D variants are more frequent in some ethnicities than in others. The possibility of false results in serologic D antigen typing must give consideration because D variants are prone to be undetected by serology. The correct determination of the RhD status is relied on molecular analysis.

Key words

Blood group – blood group serology – RHD gene – RhD phenotypes – RhD variant – Rh weak D – Rh partial D – Rhesus DAU cluster

Introduction

In the Rh blood group system, the correct identification of RhD-positive and RhD-negative erythrocytes (RBCs) is significant for transfusion and obstetric medicine because the RhD antigen is very immunogenic. It is standard that laboratories have policies for RhD typing in order to detect and interpret either the D-positive or the D-negative status of a patient. Despite serologic advances, discrepancies in Rh typing still occur due to genetic reasons in the variability of D antigen expression leading to many different weak D and/or partial D phenotypes (ISBT 2019). The great variability is seen in RhD testing by use of monoclonal anti-D reagents (JUDD WJ et al. 2005, MOULDS MK 2006). In the case of weak D or partial D

types, patients susceptible to form anti-D antibodies is not safe by serologic testing (SANDLER SG et al. 2015).

Reasons for the present study was the case of a pregnant woman with suspect Rhesus D antigen expression because of different RhD typing results in two laboratories. In one laboratory, the patient's RBCs typed serologically as blood group **0 RhD-positive ccDee** and **Kell negative** without any conspicuous features of the RhD antigen. Another laboratory issued RhD as **RhD variant**, and the patient received a document with these results: **0 RhD-negative ccddee, K negative**. The reverse side quoted relevant Rhesus classifications: as donor Rhesus positive and as recipient Rhesus negative. Since the introduction of molecular techniques for *RHD* typing it is known that serologic procedures are liable to fail in the detection of clinically relevant D variants. Thus, the above case deemed worthy to revisit the different obtained Rhesus D results and to work off classical assays of serology to mark out the D antigen as a regular phenotype or as a D weak or a D partial variant.

It is nontrivial to note that a strict division of RBCs into RhD-positive or RhD-negative phenotypes can be difficult in the presence of quantitative and qualitative alterations of the D antigen. This is a challenge in diagnostics as well as in clinical practice because non-detection of weak D or partial D types involves the risk of anti-D immunization in transfusion situations (not usually in weak D but more often in partial D or in "weak partial D"). Such alloantibodies are capable of hemolytic reactions in blood transfusion and are the cause of hemolytic disease of the fetus and newborn (MAYNE KM et al. 1991, ROBSON SC et al. 1998, MCBAIN RD et al. 2015).

Extensive studies of the genetic background of the Rh system enabled the understanding of RhD antigen expression. Molecular basis of weak D and partial D phenotypes are actions of gene conversion, polymorphism and mutation within the Rhesus gene locus. It was also posed that the pattern of DNA variation in certain gene regions is consistent and determined by balancing gene conversion and strong selection in order to maintain the state of *RHD*/*RHCE* variation (CHÉRIF-ZAHAR B et al. 1991, COLIN Y et al. 1991, LE VAN KIM C et al. 1992a and 1992b, ARCE MA et al. 1993, CARTRON JP 1994, HYLAND CA et al. 1994, HUANG CH et al. 1996, SMYTHE JS et al. 1996, FLEGEL WA et al. 1998, MATASSI G et al. 1999, WAGNER FF et al. 1999, AVENT ND and REID ME 2000, WAGNER FF and FLEGEL WA 2000, WAGNER FF et al. 2000, INNAN H 2003, FLEGEL WA 2007a and 2007b, DANIELS G 2013, WAGNER FF and FLEGEL WA 2014, RHESUSBASE 2018, OMIM 2019).

The vast number of D variants as of yet described, the so-called weak D, partial D, and DEL phenotypes, concern genetically defined modifications of the D antigen (STRATTON F 1946, FLEGEL WA and WAGNER FF 2002, WESTHOFF CM 2005, FLEGEL WA 2007a, FLEGEL WA 2011, RIZZO C et al. 2012, SANDLER SG et al. 2017). Such genetics include ethnic variability as another important aspect to be reconciled (MÜLLER TH et al. 2001, WAGNER FF et al. 2002, CHEN Q and FLEGEL WA 2005, DENOMME GA et al. 2005, SRIVASTAVA K et al. 2016).

The term "serologic weak D phenotype" does not reflect the relevant clinical properties of the D antigen, especially with respect to qualitative differences from a normal RhD antigen. This, however, is important in clinical issues such as transfusion. Changes in D epitopes are the main reason for the induction of transfusion related anti-D antibodies. The best way for clarification of RhD molecular features is *RHD* typing (TIPPETT P et al. 1996, DOMEN RE 2000, WAGNER FF et al. 2000, LURIE S et al. 2001, DENOMME GA et al. 2005, FLEGEL WA 2006, FLEGEL WA et al. 2007, FLEGEL WA et al. 2009, PHAM BN et al. 2011, FLEGEL WA and DENOMME GA 2012, DANIELS G 2013, SANDLER SG et al. 2014, SANDLER SG et al. 2015, VIRK M and SANDLER SG 2015, SANDLER SG and QUEENAN JT 2017, SANDLER SG et al.

2017, LUKACEVIC KRISTIC J et al. 2018). For safe red blood cell transfusion, only molecular typing can secure the correct categorization of Rhesus D.

Material and Methods

The assumption of incorrect D antigen typing in a pregnant woman was imperative to reexamine that blood group. Special needs were to focus on RhD typing. Blood group typing for diagnostic purpose must comply the guidelines for hemotherapy and immunohematology released by the German Medical Association (BUNDESÄRZTEKAMMER 2017).

Claims arising from the guidelines:

- The test systems must comply with national and international standards. The use of certified in vitro diagnostics (reagents and devices) is mandatory
- Compliance with legal regulations for quality assurance in context with the Act of Parliament governing medical products MPG and MPBetreibV (Medizinproduktegesetz, Medizinprodukte-Betreiberverordnung) including the obligatory regulations of the German Medical Association for diagnostic laboratories (BUNDESÄRZTEKAMMER 2014)
- Testing of Rhesus D factor has to be done with at least two test reagents. Monoclonal antibodies of the IgM class are advised, and two different clones should to be used. Anti-D reagents must not record the category DVI
- In discrepant, questionably positive or weakly positive results for the D antigen, the patient is declared at this stage as "recipient RhD-negative". Molecular biological methods should be employed for further differentiation of the RhD type, particularly for girls, women of childbearing potential and patients with chronic transfusion needs

Apart from these main issues, more details and requirements are referenced in the above quoted guidelines (BUNDESÄRZTEKAMMER 2017).

Standard methods in serologic immunohematology are hemagglutination tests for which several assay designs exist:

- Direct agglutinations for slide, tube, spot plate, microplate
- Microtube column agglutination techniques by the principle of agglutination and gel filtration
- Microplate techniques developed for automated work-up, solid-phase technology

Agglutination in test tubes

This is the classic way of detecting hemagglutination as visible clumping of erythrocytes. Agglutination results from specific antigen-antibody binding, precipitation is due to bimolecular reactions. They obey the law of mass actions (HUGHES-JONES NC 1963, HUGHES-JONES NC et al. 1963, CHAK KC and HART H 1980, HART HE and CHAK KC 1980). Antibodies of the IgM type are suitable for direct agglutination without additional aids.

The following anti-D reagents are used for D antigen typing:

- Immucor Anti D (IgM + IgG): anti D monoclonal IgM (TH28) and anti-D monoclonal IgG (MS26)
- Immucor Anti D (Anti-D fast IgM): anti D monoclonal IgM (D175-2)
- Immucor Anti CDE (IgM + IgG): anti C monoclonal IgM (MS24), anti D monoclonal IgM (MS201), anti E monoclonal IgM (MS80) and anti D monoclonal IgG (MS26)

Column agglutination

The detection principle is a combination of agglutination reaction and centrifugation both carried out in microtubules filled with gel or glass beads (LAPIERRE Y et al. 1990, RUMSEY DH and CIESIELSKI DJ 2000). Technical approaches and products exist for agglutination purposes.

The microtyping systems from Bio-Rad Laboratories and Ortho Clinical Diagnostics are used:

– Ortho BioVue System
  ID-Cassette 707119 containing A, B, AB, D (D^{VI-}), D (D^{VI-}), ctrl
  anti D monoclonal IgM (D7B8)
  anti D monoclonal IgM (RUM1)

– Bio-Rad ID-Micro Typing System
  ID-Card 001344 containing A, B, D (D^{VI-}), D (D^{VI-}), ctl, DAT
  anti D monoclonal IgM (LHM50/3 [LDM1], TH-28, RUM-1)
  anti D monoclonal IgM (LHM59/20 [LDM3], 175-2)

The ID-Micro Typing System (Bio-Rad) served for the determination of Rhesus subgroups and the K antigen with monoclonal antibodies:

– ID-Card 002124 containing C, c, E, e, K, ctl (details described in Bio-Rad catalogue)

– ID-Card 002224 containing C, c, E, e, K, ctl (details described in Bio-Rad catalogue)

Autoanalyzer for microplate testing

The test relies on the principles of agglutination in microplates with pattern recognition and an automated work-up of all working steps such as transport of microtiter plates, distribution of liquid reagents and blood samples for incubation, washing, centrifugation and recording by a CCD camera (Beckman Coulter, Olympus PK 7300). Analyzers read the settling patterns of the red blood cells in each well of the plate. Readings follow the threshold settings chosen for each reagent. Absence of agglutination indicates a negative reaction of red blood cells for the antigen tested. Patient samples are processed using two instruments with different reagents.

Anti-D reagents adapted to the autoanalyzer:

– Immucor Series 4 anti D (Blend IgG und IgM): anti D monoclonal IgM (MS201) und anti D monoclonal IgG (MS26)

– Immucor Series 5 anti D (Blend IgG und IgM): anti D monoclonal IgM (TH28) und anti D monoclonal IgG (MS26)

– Sifin anti D (monoclonal): anti D monoclonal IgM (BS225)

Other serologic assays

The indirect antiglobulin test (IAT, COOMBS RR et al. 1945) in LISS-Coombs milieu at 37 ºC (LISS/Coombs, Card 004014, Bio-Rad) with a panel of test erythrocytes (ID-DiaCell I-II-II, Bio-Rad) served for alloantibody screening. The direct antiglobulin test (DAT, (LISS/Coombs Card 004014, Bio-Rad) was used to detect sensitization of the patient's erythrocytes.

Approaches to test for weak D and partial D phenotyping include the indirect antiglobulin assay (MUIRHEAD EE and JENNINGS ER 1964) in all cases with negative direct agglutination of erythrocytes when using Anti D duo (IgM + IgG) and Anti CDE (IgM + IgG) reagents.

Controls

Quality controls are according to legal regulations (BUNDESÄRZTEKAMMER 2014 and 2017).

RHD analysis

Genotyping is realised by the Blood Transfusion Service of the German Red Cross in Bad Kreuznach using the SSP-PCR technique.

Results

The patient's RBC typed as blood group **0 RhD positive, Rh subgroups ccee** and **K antigen negative** with several serologic techniques. The Transfusion Service of the Red Cross obtained comparable results by serologic typing without identification of conspicuous features of some RhD variant. Due to the known and above described discrepancy, the specimen was genetically analysed with the result that the serologic phenotype proved to be misleading. The molecular genetic analysis revealed the **Rhesus D variant RHD*10 (DAU)**. This D variant applies to partial D in accordance with the International Society of Blood and Transfusion (ISBT 2019) and The Human Rhesus Base (RHESUSBASE 2018). Because D variants in general may cause alloimmunization, the patient has to be classified Rhesus D positive as blood donor and Rhesus D negative as blood recipient in transfusion situations.

Table 1 shows the results of the patient's D antigen typing. Serologic reactions in immediate spin with agglutination strengths from 3+ to 4+ reflect the typical feature of a normal D protein with the evaluation as RhD-positive. From D variants caused by mutations in *RHD* one expects weak or negative agglutination reactions. However, the strong agglutination of the patient's erythrocytes in the actual tests do not give indications for the presence of weak D or partial D.

Results are identical with either of the employed methods and irrespective of the assay type. In this connection, the agglutination behavior is straight for a normal RhD phenotype.

Tabelle 1. Case No. 71465841, results of RhD antigen tests with different anti-D reagents

Anti-D reagent source and clones	**Tube agglutination**	**Bio-Rad gel matrix**	**Ortho BioVue glass beads**	**Microplate analyzer**
Immucor (Duo IgM + IgG) monoclonal IgM and monoclonal IgG TH28, MS26	+++ to ++++ *	n/a *	n/a	positive **
monoclonal IgM D175-2	++++	n/a	n/a	n/a
anti CDE monoclonal IgM and monoclonal IgG MS201, MS26	++++	n/a	n/a	n/a
Bio-Rad ID-System monoclonal IgM LHM50/3, TH-28, RUM-1	n/a	+++	n/a	n/a
monoclonal IgM LHM59/20, 175-2	n/a	++++	n/a	n/a
Ortho BioVue System monoclonal IgM D7B8	n/a	n/a	++++	n/a
monoclonal IgM RUM1	n/a	n/a	++++	n/a
Microplate analyzer Immucor Series 4 and 5 monoclonal IgG, IgM	n/a	n/a	n/a	positive

a) MS26, MS201 b) MS26, TH28				
Sifin monoclonal IgM BS225	n/a	n/a	n/a	positive

* Strength of agglutination reaction (f. e. 3+ and 4+)
n/a (not applicable), reagent or method not used in the assay
** Results (positive or negative), agglutination result reported according to the instrument settings

Further results: control reactions are regular. In DAT, no sign of autoagglutination and sensitization of the patient's erythrocytes occurred. Tests for alloantibodies are negative.

Discussion

The blood sample of the index-patient reacted in all tests of the participating laboratories in the same manner. Due to the strong reactivity of the different anti-D reagents, the patient's RhD feature was straightforward conclusive RhD-positive. This because no serologic discrepancies were obvious. No doubts were left about the patient's blood group being 0 RhD-positive. To clarify the diagnostic results, case history and all technical results were discussed with the Blood Transfusion Service of the German Red Cross (Bad Kreuznach) to clear up diagnostics. Finally, a molecular analysis of the *RHD* gene was phased.

Molecular analyses of the *RHD* gene revealed RHD*10 (DAU), a *DAU* variant categorized as partial D (RHESUSBASE 2018). This result was at first surprising from all the findings of standard serology. D variants are quite unusual in cases of clear-cut D serology, but they may occur. Several studies with monoclonal antibodies have shown that serologic detection of weak D or partial D types is not easy at all, and that extensive variability in serologic testing can be expected (JONES J et al. 1995a and 1995b, JUDD WJ et al. 2005, DENOMME GA et al. 2008, LAI M et al. 2009, SANDLER SG et al. 2017).

Usually, *DAU* variants show variable strength of reaction with anti-D reagents (WAGNER FF et al. 2002, DUNCAN JA et al. 2017). Yet, surprisingly, in the present case of RHD*10 (DAU) full strength reactions occurred with monoclonal anti-D antibodies. RHD variants derived from the *DAU* allele cluster (a cluster of closely linked homologous genes) occur frequently in Africa, while in Europe they are very rare. The present case highlights the limitations of standard serologic procedures. Generally, possible anti-D immunizations as in other weak D phenotype or partial D variants must be considered in transfusion or pregnancy (WAGNER FF et al. 2000, WAGNER FF et al. 2002, RIZZO C et al. 2012, MCBAIN RD et al. 2015, SRIVASTAVA K et al. 2016). The incidence of anti-D immunization, however, is difficult to predict. For patients with *DAU* alleles, the following Rhesus D classification for medical issues such as blood transfusion or anti-D prophylaxis in pregnancy is advised:

– Rhesus D classification as a donor: Rhesus positive
– Rhesus D classification as a recipient: Rhesus negative.

The presented results from D antigen typing is an unexpected example for risks of false conclusions from serologic RhD agglutination tests. The key for correct results of the RhD status relies on molecular biological methods. According to the valid hemotherapy guideline, molecular biological methods are allowed to clarify the event of ambiguity within the scope of AB0 determination, RhD determination or other blood group characteristics. However, what about cases with D variants that do not show any serologic abnormalities (see the above described case) and where it only turns out later that a RHD variant is present? There is no statement in official guidelines for this.

Serologic reagents. Generally, discrepancies are inherent in serologic methods. They are by no means to exclude, even not by changing the composition of reagents (LAI M et al. 2009). Anti-D reagents contain various monoclonal antibodies, various additives, and among other things, proteins in different concentrations to act as agglutination enhancers (MOULDS MK 2006). Summing up, this can result into grading of reactions which do not usually corrupt agglutination tests because of weaker reactions with D variants compared with the strong reactions of fully expressed RhD antigens.

D weak or D partial. RBC of weak D or D partial phenotype ususally react in serologic tests by weak agglutination (e.g. 1+ or 2+) when compared to erythrocytes of normally expressed D antigen. (JONES J et al. 1995a and 1995b, DENOMME GA et al. 2005, JENKINS CM et al. 2005, WESTHOFF CM, 2005, LAI M et al. 2009, FLEGEL WA 2011, SANDLER SG et al. 2015). The paradox situation, however, is seen in the present study case which is quite comparable to patients with the variant RHD*DAU5 (DUNCAN JA et al. 2017). It remains to conclude that serologic RhD findings of our index-patient are not suitable to yield the desired diagnostic result.

The DAU cluster. Rhesus DAU is a cluster of at least 18 alleles with a *cDe* haplotype for which one or more mutations of the *RHD* gene are characteristic (WAGNER FF et al. 2002, WAGNER FF and FLEGEL WA 2014, RHESUSBASE 2018, OMIM 2019). Most probably, the D variant of the patient belongs to RHD10.00 (DAU-0) with a single missense mutation at 1136C>T (T379M) whereas all other alleles listed in The Rhesus Base (RHESUS BASE 2018) have multiple missense mutations. The phenotype of DAU-0 is designated to be D-positive (apparently normal), partial D. This will explain the patient's reactivity in serologic D antigen testing as RhD-positive without signs of partial D.

From all the above it is sure that the detection of patients with weak D or partial D types who are susceptible to anti-D immunization is sometimes difficult to realize by the classical way of serologic blood group typing. Mismatching and pitfalls in blood group serology occur possibly more often than thought. Unfortunately, the detection of errors is clearly restricted because of lack of serologic specificity in reading epitope changes in the D antigen. Molecular techniques prove the limits of serology, thus, molecular analyses are required to define partial D from weak D types and normal D from negative D.

Genetic and ethnic factors and other reasons for gene conversion (*RHD, RHCE*) or mutations highlight the limits of serology. This is a challenge for guideline-compliant diagnostics. Studies of *RHD* alleles in D-negative and D-positive Europeans gave evidence that the variety of *RHD* alleles is probably larger as anticipated (WAGNER FF et al. 2001, CHEN Q and FLEGEL WA 2005). Apart from certain genetic-ethnic factors, the overall diversity of *RHD* genes will have an impact on diagnostic strategy. Information about geographical and ethnic origin of patients can be helpful in the diagnostic design (FLEGEL WA 2006, FLEGEL WA 2007a and 2007b, FLEGEL WA 2011, FLEGEL WA et al. 2014).

Acknowledgments

I thank Dr. Brillat (BwZKrhs Koblenz Abt. XXII, Transfusion Medicine) for serologic controls with the Olympus PK 7300 microplate autoanalyzer using monoclonal anti-D reagents, and for serologic controls with the Bio-Rad ID-Micro Typing System (monoclonal and human anti-D antibodies). I thank Dr. Opitz (German Red Cross, Blood Donor Service Bad Kreuznach) for advice and helpful discussions.

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 23.10.2019