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with the partial D^{VI} antigen from those with either partial D
antigens or weak D antigens
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M.E. REID, G.R. HALVERSON, F. ROUBINET, P.A. APOIL, AND A. BLANCHER

Historically, red blood cells (RBCs) with partial D antigens have been defined serologically by their pattern of reactivity with polyclonal and monoclonal anti-D. Although numerous variants have been described in tests with well-characterized monoclonal anti-D, definition remains difficult to ascertain serologically. RBCs of known partial D type were tested with LOR-15C9 (a monoclonal anti-D) and commercial anti-D by the tube indirect antiglobulin test (IAT), by micro typing system IgG gel cards, and by immunoblotting. By IAT, LOR-15C9 reacted strongly with D^{IIIa}, D^{IIIc}, D^{Va}, D^{VI}, D^{VII}, and DFR RBCs in addition to RBCs with common D antigens; weakly with D^{II}, DNU, and D^{IIIb} RBCs; and not at all with D^{IVa}, D^{IVb}, DBT, or R₀^{Har} RBCs. Reactivity was variable (1+ to 4+), with RBCs classified as weak D (D^U). As expected, the commercial anti-D agglutinated all D variants and weak D RBC samples by the IAT and by using IgG gel cards; however, the reactivity with D^{VI} RBCs was weaker than with LOR-15C9. By immunoblotting, LOR-15C9 detected a band with an apparent molecular mass of approximate M_r 30,000–34,000 in membranes prepared from D-positive, D^{IIIa}, D^{IIIc}, D^{Va}, D^{VI}, D^{VII}, and DFR RBCs and an additional band of M_r 20,000–22,000 in membranes prepared from D^{VI} RBCs. No band(s) was detected in membranes from D^{II}, DNU, D^{IIIb}, D^{IVa}, D^{IVb}, DBT, R₀^{Har}, weak D, or D-negative samples. LOR-15C9 provides a useful tool to identify positively D^{VI} samples and thereby differentiate this partial D from other D variants and from weak D samples. *Immunohematology* 1998;14:89–93.

Key Words: D antigen, D variants, immunoblotting, monoclonal anti-D, partial D variants, Rh blood group system

Partial D phenotypes were first defined in D-positive people who had made alloanti-D.^{1–4} People with a partial D phenotype have red blood cells (RBCs) that possess some but not all D epitopes and thus can make antibodies to the missing epitopes. Even though numerous variants have been detected with well-characterized monoclonal anti-D,^{2,5–7} detection remains serologically difficult. Mutations resulting in the absence of some D epitopes are a consequence of either gene rearrangements between *RHD* and *RHCE*

genes^{8–14} or point mutations in the *RHD* gene.^{15–17} Historically, partial D phenotypes were categorized serologically by the pattern of reactivity of the person's RBCs with anti-D from other D category people. Antibodies to low-incidence Rh antigens and the reactivity of the person's anti-D with other partial D phenotypes helped to confirm classification.^{1,2} RBCs from nonimmunized people giving the expected pattern of reactions could be fitted into a certain category. It is worth noting that anti-D used for this purpose were well characterized and that although mutually compatible, the reactions of anti-D from category members are not necessarily identical. Although the characterization of some partial D phenotypes is simplified by their association with low-incidence antigens, not all low-incidence antigens are restricted to a single partial D phenotype (Table 1). More recently, attempts were

Table 1. Low-incidence antigens associated with partial D antigens

D category	Associated low-incidence antigen
D ^{II}	None
D ^{III}	None
D ^{IVa} ce	Go ^a
D ^{IVa} (C)-	Go ^a , Rh33, Riv, FPTT
D ^{IVb}	Unusual Evans
D ^{Va}	D ^W
D ^{VI} Ce	BARC (76 of 78)
D ^{VI} cE	None
D ^{VII}	Tar
DFR	FPTT
DBT	Rh32
R ₀ ^{Har}	Rh33, FPTT

made to establish alternative means to accurately categorize partial D phenotypes and monoclonal antibodies (Mabs) that give distinct patterns of reactivity with different partial D antigens have been used for

this purpose.^{6,7,18} However, similar to the classification of partial D antigens with polyclonal anti-D, the use of monoclonal anti-D is hampered by technical limitations, including low D copy number or the weak expression of some epitopes. The serologic pattern of reactions with monoclonal anti-D is, in part, method dependent. Further, the specificity and strength of a reaction depends on the concentration of the antibody as well as on all parameters of the technique used. It was evident during the second and third International Workshops that standardization of the characterization of partial D antigens by using Mab anti-D panels is not yet easy.

Using monoclonal anti-D, the incidence of D^{VI} has been estimated to be between 0.02 percent and 0.04 percent.¹⁹⁻²¹ People with this partial D can make anti-D, possibly because their RBCs lack many of the D epitopes.² In contrast, people with a weak D phenotype (D^u), which can be difficult to distinguish from D^{VI}, have all D epitopes and do not make anti-D. Thus, in the clinical setting, it may be important to determine whether RBCs from prospective mothers express D^{VI} or weak D antigens.

LOR-15C9, a previously reported Mab anti-D and the first to react by immunoblotting,^{22,23} defines a pattern by immunoblotting that is characteristic for D^{VI}.²⁴ We describe here the practical use of this Mab to distinguish D^{VI} from other partial D variants and from weak D antigens.

Materials and Methods

Blood samples with known D phenotypes were either tested fresh or following recovery from liquid nitrogen storage. These samples had been received from local patients and donors and from many colleagues. Of the partial D phenotypes, 17 had been categorized at the Blood Group Unit, MRC, London. RBCs were washed and used as a 5% suspension in phosphate-buffered saline for the indirect antiglobulin test (IAT) or 0.8% in micro typing system (MTS) diluent 2 for the MTS IgG gel card testing (Ortho-Clinical Diagnostics, Raritan, NJ). Monoclonal LOR-15C9 anti-D (IgG1; Eurobio, France) and commercially blended polyclonal/monoclonal anti-D reagents (Bioclone; Ortho-Clinical Diagnostics; and Gamma Biologicals, Houston, TX) were tested by the IAT read in tubes after centrifugation and by the MTS IgG gel card system. Mab anti-D, HAM-A, RUM-1, and MAD-2 (Blood Products Laboratory, Elstree, UK) also were used to test the majority of samples. Immunoblotting, using 50 μ L per

lane of freshly solubilized RBC ghosts, was performed as described.²⁴

Results

Hemagglutination

LOR-15C9 strongly agglutinated (3+ to 4+) D-positive RBC samples with common Rh phenotypes by the tube IAT. It did not agglutinate D-negative RBC samples (rr, r'r, r'r', r''r, r''r'', r^yr^y) and reacted variably (1+ to 4+) with RBCs (n = 11) classified as weak D (D^u). LOR-15C9 agglutinated (2+ to 4+) RBCs with partial D antigens associated with D^{IIIa} (n = 8), D^{IIIc} (3), D^{Va} (7), D^{VI} (21), D^{VII} (5), and DFR (1). LOR-15C9 reacted weakly (1+ to 2+) with D^{II} (1), DNU (1), and D^{IIIb} (2) RBCs; and did not react with D^{IVa} (6), D^{IVb} (3), DBT (2), or R₀^{Har} (7) RBCs. LOR-15C9 agglutinated RBCs of all three types of D^{VI} more strongly than the commercial anti-D reagents tested both by the IAT and by using IgG gel cards. In agreement with others, we found considerable variation of reaction strengths in testing category D^{Va} and D^{VI} RBCs with various anti-D. The D^{II} and DNU RBCs were strongly agglutinated by commercial anti-D and by HAM-A, RUM-1, and MAD-2, whereas the D^{IIIb} RBCs were weakly agglutinated by all of these reagents.

Immunoblotting

By immunoblotting, LOR-15C9 detects a band with an apparent molecular mass of approximately M_r 30,000–34,000 in membranes freshly prepared from D+ RBCs (R₁R₁, R₂R₂, R₁R₂, R₀r, R_zR_z, and D+G-) but not in membranes prepared from D- RBCs (rr, r'r', rr'', and r^yr^y).²⁴

When membranes prepared from RBCs with partial D antigens were tested by immunoblotting with LOR-15C9, the M_r 30,000–34,000 band was detected with D^{IIIa} (n = 7), D^{IIIc} (3), D^{Va} (9), D^{VI}Ce (Type II, 30), D^{VI}cE (Type I, 13) D^{VI}Ce (Type III, 1), D^{VII} (5), DFR (1), and weak D (1); but not with D^{II} (1), DNU (1), D^{IIIb} (2), D^{IVa} (6), D^{IVb} (6), DBT (2), R₀^{Har} (1), or weak D (18) RBCs. An additional band of M_r 20,000–22,000 was obtained with membranes prepared from D^{VI} RBCs (n = 44) of all three types (Fig. 1).

Discussion

It is likely that the propensity of D^{VI} people to develop an anti-D alloimmune response is related to the lack of many D epitopes as a consequence of gene rearrangements. At the genomic level, three variants of D^{VI} have been defined: Type I has an exchange of exons 4 and 5 of the *RHD* gene with exons 4 and 5 of the *RHCE*

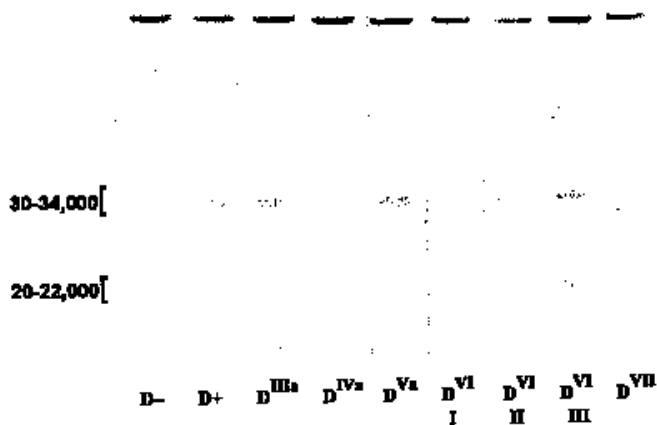


Fig. 1. Immunoblotting with LOR-15C9. Freshly prepared red blood cell membranes were separated on SDS-PAGE and electroblotted onto nitrocellulose paper. On blotting with LOR-15C9, a band with an approximate M_r of 30,000–34,000 is present in D+ and some partial D samples. This band is not present in D^{IVa}, D^{IVb}, DBT, or D- samples. Representative samples are shown. All three types of D^{VI} gave a characteristic banding pattern with the 30,000–34,000 band and an additional band with an approximate M_r of 20,000–22,000.

gene;^{12,13} type II has an exchange of exons 4 to 6;¹⁴ and type III has an exchange of exons 3 to 6 from *RHCE* into *RHD*.^{25,26} The first type is associated with the RHcE haplotype, whereas types II and III are associated with the RHCE haplotype. In one study, 76 of 78 D^{VI}Ce samples tested possessed the low-incidence antigen BARC, whereas all D^{VI}Ce RBCs tested were BARC-negative.² Because not all D^{VI} RBCs possess the BARC antigen, such typing will only aid in the identification of some D^{VI} RBCs. LOR-15C9 belongs to the unusual group of Mabs that agglutinate D^{VI} variants, and with its unique ability to react by immunoblotting, provides a useful tool to identify D^{VI}.

Serologic reactivity of LOR-15C9 appears to be anti-epD3 and is dependent on amino acids 350 to 354 (in the 6th external loop), which are encoded by exon 7 of *RHD*.²⁴ The alteration or replacement of exon 7 of *RHD* in D^{IV} and DBT,^{10,11} respectively, explains why LOR-15C9 does not react with RBCs of either of these partial D types. It has been reported that most MAb anti-D that react with D^{VI} RBCs do not react with D^{IV} or DBT RBCs.² R₀^{Har} RBCs that result from exon 5 of *RHD* replacing exon 5 of *RHCE*²⁷ are also expectedly nonreactive with LOR-15C9.

The weak reactivity of LOR-15C9 and other anti-D with the D^{IIIb} and weak D samples implies a reduced amount of D antigen in these RBCs. It is likely that the negative immunoblotting results with membranes from these RBCs is due to a quantitative effect and limits of

sensitivity of the test. It should be noted that the two examples of D^{IIIb} RBCs were those reported by Rouillac et al.⁸ These Caucasian donors were found during a population study with different examples of Mab anti-D.²¹ Neither had anti-D in their serum but were G-. In Tippett's original classification, the partial D category D^{IIIb} is associated with a G-,ce haplotype found in black African donors who had made anti-D;²⁸ therefore, the possibility exists that these two Caucasian D^{IIIb} donors are G-, weak D (D^u).

In contrast, the weak hemagglutination results with D^{II} and DNU RBC samples are likely to be due to a qualitative effect. Although LOR-15C9 reacted weakly with these RBCs, other Mab anti-D (HAM-A, RUM-1, MAD-2) were strongly reactive. D^{II} is associated with a change of Ala³⁵⁴ → Asp, and DNU is associated with Gly³⁵³ → Arg.¹⁷ Because LOR-15C9 recognizes an epitope that is dependent on amino acid residues 350 to 354 of RhD, it is quite possible that a change in residue 353 or 354 would reduce its affinity. It is interesting to note that although there is an enzyme cleavage site in the sixth external loop of the RhD protein between amino acid residues 353 and 354,¹⁷ D epitopes epD3, 4, and 9 are insensitive to treatment of RBCs by papain or bromelain. Avent et al.¹⁷ suggest that such cleavage is unable to destroy the D epitopes because of an interaction of the RhD polypeptide with other components of the Rh complex. Thus, we conclude that the affinity of LOR-15C9 is diminished in D^{II} and DNU variants but is not affected by treatment of D-positive RBCs with either papain or bromelain,²² whose cleavage site is between amino acid residues 353 and 354.

Although Mab anti-Ds provide valuable tools to characterize RBCs with partial D antigens, there are many pitfalls with using this approach. Limitations include variation of D antigen strength on RBCs with different phenotypes, the possible presence of a weak D (D^u) encoded by the partner chromosome, condition of RBCs due to conditions of storage (e.g., some D epitopes deteriorate on storage, particularly in a saline suspension),² variation in technique, deterioration of anti-D during storage, testing with mischaracterized anti-D or D category RBCs, and concentration of Mab.

Although it would be possible to use a molecular biology approach to identify D^{VI} RBCs, it is practical to study the D polypeptides by immunoblotting with LOR-15C9.²⁴ We have shown here in tests with membranes made from RBCs with partial D antigens or weak expression of the D antigen that category D^{VI} is the only type to exhibit the M_r 22,000 band by

immunoblotting with LOR-15C9. This provides a practical test to positively identify D^{VI} RBCs of the three reported molecular types. In such testing, blood samples should be as fresh as possible and RBC membranes should not be stored in solubilizing buffer.

Acknowledgments

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The Lutheran blood group system: a review

M.L. BECK

At first sight, the Lutheran system would seem to have little interest to blood bankers. Lutheran antibodies are rarely, if ever, clinically relevant, although their identification has the potential to be enormously time-consuming. However, other complexities of this system are proving to be extraordinarily intricate. Genetically, the system appears to be composed of at least three closely linked loci, each with two alleles and, so far, 20 antigens, which have laid claim to at least an honorary affiliation. Two antigens, Lu10 and Lu15, have become obsolete. Three different interpretations of the genetic events leading to the Lu null phenotypes have been needed to accommodate the hereditary observations. The biochemistry of the cell membrane structures on which Lutheran antigenic activity resides is starting to be unravelled. Information about Lutheran has been accumulating for 50 years. This review will summarize what is known to date.

Granulocyte transfusions: a review

D.F. STRONCEK AND S.F. LEITMAN

Granulocyte transfusions have been used for over 25 years to treat neutropenic patients with severe bacterial or fungal infections that are unresponsive to antimicrobial therapy. Methods to mobilize and collect granulocytes have changed, but the use of granulocyte transfusions has not increased. The availability of hematopoietic growth factors that markedly increase donor granulocyte counts, collection yields, and possibly clinical efficacy has resulted in renewed interest in these transfusions. Clinical trials of growth factor-mobilized granulocytes are being planned or are under way. As the results of these studies become known, use of these components is likely to increase.

Paroxysmal cold hemoglobinuria and the elusive Donath-Landsteiner antibody

R.J. SOKOL, D.J. BOOKER, AND R. STAMPS

some of the difficulties in making the diagnosis. A 46-year-old male presented with anemia, a weakly positive direct antiglobulin test (DAT) with anti-IgG, a haptoglobin < 0.1 g/L, and a cold autoagglutinin showing anti-P specificity. A 9-year-old female had a 4-day coryzal illness, a 20 g/L fall in hemoglobin over 24 hours, and a haptoglobin < 0.1 g/L; the DAT was positive with anti-C3d. A 3-year-old female was referred following a rapid drop in hemoglobin of 30 g/L; the DAT was positive with anti-C3d. A 17-month-old female, unwell for 2 weeks, had a hemoglobin of 41 g/L; the DAT was strongly positive with anti-C3d and weakly positive with anti-IgG and -C3c. In all patients, PCH was confirmed by positive indirect Donath-Landsteiner tests, and the autoantibodies demonstrated P specificity. In two patients, the test was strongly positive; in the third patient, it was only positive using papainized red cells; and in the fourth patient, a two-stage papainized procedure was needed before a positive result was obtained. PCH must always be considered in a child with a rapid drop in hemoglobin, even if initial tests are negative. *Immunohematology* 1998;14:109-112.

Use of the MAIEA assay to demonstrate that Fy3 is on the same glycoprotein as Fy6, Fy^a, and Fy^b

J. TZENG, R. DODD, AND D. MALLORY

The monoclonal-antibody immobilization of erythrocyte antigens (MAIEA) assay is a technique that detects trimolecular complexes formed by a human antibody and a mouse monoclonal antibody with specific red cell epitopes. This enzyme-linked immunoadsorbent assay test gives a positive reaction when two different epitopes on the same membrane protein are separately recognized by human and mouse antibodies. In this study, the MAIEA test was used to determine if the Duffy system antigen Fy3 is on the same membrane protein as Fy6. The well-established location and relationship of Duffy blood group antigens Fy^a, Fy^b, and Fy6 were again confirmed by the MAIEA assay, and those facts were used to standardize a variation of the assay to establish the relationship between Fy3 and Fy6 using red cells with various Fy phenotypes. The MAIEA assay generated high absorbance values when Fy6 and Fy^a (or Fy^b) antigens were evaluated. Similarly, high absorbance values were seen when Fy3 and Fy6 antigens were tested using Fy(a+b+), Fy(a+b-), and Fy(a-b+) red cells. The MAIEA assay is a valid technique for detecting Duffy system antigens. By this technique, it was shown that Fy3 and Fy6 antigens are carried on the Duffy glycoprotein D and by extension that Fy3 is also located on the Duffy glycoprotein D. *Immunohematology* 1998;14:113-116.