GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF DOMBROCK (Do) BLOOD GROUP ANTIGENS IN BLOOD DONORS

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Introduction: The Dombrock blood group system was discovered in 1965 by recognizing antibodies to Do^a in a patient's serum, followed by the discovery of the antithetical antigen Do^b in 1973 (1;2). Later, three additional high incidence antigens Gregory (Gy), Holly (Hy) and Joseph (Jo) were included into the Do blood group system, since these antigens are linked to either Do^a or Do^b alleles (3). By means of expressed sequence tags, the Do gene was identified encoding a glycoprotein identical to the formerly described mono-ADP-ribosyltransferase 4 (ART4) (4;5). This enzyme is attached to the red blood cell (RBC) membrane by a glycosylphosphatidylinositol (GPI) anchor (5). So far, in humans no enzyme activity could be demonstrated. Anti-Do antibodies may cause severe hemolytic transfusion reactions(6) and especially anti-Do^a has been described in mild hemolytic disease of the newborn. Their differentiation is notorously difficult due to a lack of Do phenotyped reagent red cells (RRC). Provision of Do phenotyped blood products is usually impossible due to lack of anti-Do antisera for donor typing. Furthermore, anti-Do antibodies are weakly reactive, deteriorate in vitro and are often present in antibody mixtures. Therefore we established methods to reliably identify Do genotype as well as phenotype using molecular genotyping and flowcytometry circumventing the need of reagent tools. Thus we studied the Do allele frequencies in our donor collective and set up RRC panels for anti-Do identification.

Material and Methods:

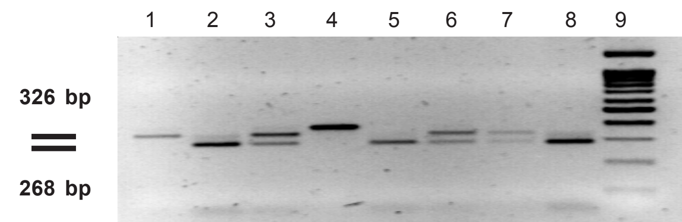
PCR-RFLP assay for determination of DoA/B polymorhism: genomic DNA was prepared from EDTA blood samples of consecutive blood donors (BD) using QIAamp DNA Mini Kit, according to the manufacturer's recommendations. PCR amplification (Taq PCR Master Mix Kit; Qiagen No.201443) was performed as described by J.R. Storry(7), using the primers DoF and DoR, which contained deliberate changes in the nucleotide sequence of the 793 A > G polymorphism in exon 2 of the Do gene, distinguishing DoA and DoB. PCR products were digested by *BseRI* restriction enzyme (Bioconcept No R 0581S), separated on 2% agarose gel and visualized with ethidium bromide.

Surface staining of Dombrock/ART4 protein on red blood cells (RBC): To rule out rare Do(Null) phenotypes due to splice site and premature stop mutations (8), we set up a FACS protocol for generic detection of Do/ART4 protein on RBC surface. Fresh RBC of EDTA anticoagulated blood were washed and incubated (4°C/30 min) with hybridoma supernatants (kindly provided by Prof F. Koch-Nolte, Hamburg) containing the monoclonal rat-anti Do/ART4 (CD297) antibodies NONI-B4, recognizing a N-terminal antigen of Do/ART4, and NONI-B63, recognizing a C-terminal antigen of Do/ART4 protein. After washing, the cells were incubated (4°C/30 min) with phycoerythrin-conjugated goat anti-rat Fab$_2$ fragments (Jackson Immuno Research, USA). The final cell suspension was analyzed with a FACScalibur flowcytometer using Cellquest software (BD Bioscience). The fluorescence signal was compared to the signal of RRC stained with the appropriate control antibodies (rat IgG2a antibody, Clone No R35-95, BD/Pharmingen). In order to assess confomational and proteolytic susceptibility of Do/ART4 protein, the RRC were pre-incubated (37°C, 30 to 45 min) with dithiothreitol (DTT), DTT + papain (ZZAP) and papain.

Results:

BseR I digestion of PCR product of exon 2 of the Do gene allows to distinguish unambigously between the three genotypes at the DoA/B polymorphic site (Figure 1)

Figure 1: PCR-RFLP of exon 2 of Do gene using *BseRI* endonuclease



Lane: 1: DoAA, 2: DoBB, 3: DoAB, 4: undigested, 5: DoAA, 6: DoAB, 7: DoAB, 8: DoBB, 9: Marker

Using PCR-RFLP we genotyped 221 consecutive Swiss BD. The results are shown in table 1 and 2. The frequencies of Do genotypes and the respective alleles are in line with earlier publications for the caucasian population (9,5)

Table 1: Frequency of Do genotypes in caucasion population

Genotype	Swiss BD	% Swiss BD	% Caucasion (9)
DoAA	38	17.2	18
DoAB	107	48.4	49
DoBB	76	34.4	33
Total	221	100	100

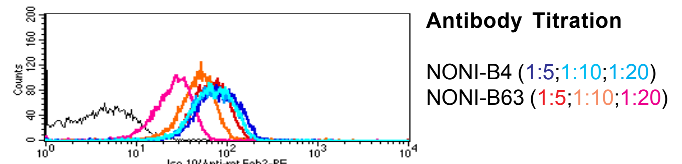
Table 2: Frequency of Do alleles in the caucasion population

Allele	Swiss BD	% Swiss BD	% Caucasion (5)
DoA	183	41.4	42.5
DoB	259	58.6	57.5
Total	442	100	100

To rule out rare Do(Null) phenotypes, we established a FACS based method to assess the presence of generic Do/ART4 protein on the red blood cell membrane. This is undispensable for accurate Do phenotyping of RRC.

Literature: (1) Swanson J et al (abstract), Nature 1965;206:313; (2) Molthan L et al, Vox Sang 1973;24:382-4; (3) Banks JA et al, Vox Sang 1995;68:177-82; (4) Gubin AN et al, Blood 2000;96:2621-7; (5) Parusel I et al, Cell Immunol 2005;236:59-65; (6) Baumgarten R. et al, Transfusion 2006;46:244-9; (7) Storry JR et al, Immunhematol 2003;19:73-6; (8) Rios M. et al, BJH 2002;117:765-7; (9) Reid M, Transfusion 2005;45:92S-99S

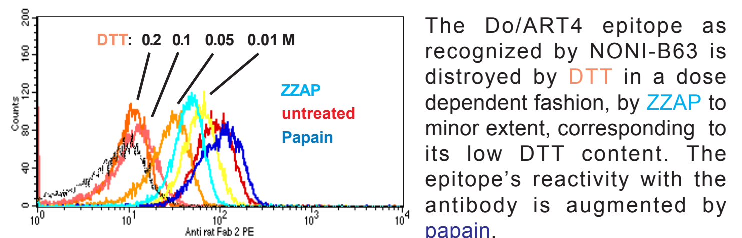
Figure 2: Do/ART4 (CD297) examination of RBC membrane by flowcytometry



NONI-B4 and NONI-B63 show different binding avidity with saturation kinetics.

NONI-B4 probably recognizes an epitope in the N-terminal half of Do/ART4 and NONI-B63 recognizes an epitope in the C-terminal half of the protein (5). Both epitopes are different from the clinically relevant DoA/B polymorphic site (D219N) of Do/ART4, which lies close to the GPI-attachment site at the C-terminal end of the protein on the external leaflet of RRC membrane. Since the GPI-anchored membrane proteins are stabilized by disulfid bounds, we assessed the conformational susceptibility of Do/ART4 protein upon incubation with DTT and ZZAP. For assessment of proteolytic susceptibility of Do/ART4, we pre-incubated the RRC with papain (Figure 3).

Figure 3: Chemical susceptibility of Do/ART4 epitopes



The Do/ART4 epitope as recognized by NONI-B63 is distroyed by DTT in a dose dependent fashion, by ZZAP to minor extent, corresponding to its low DTT content. The epitope's reactivity with the antibody is augmented by papain.

Conclusions:

1. Molecular genotyping by PCR-RFLP as provided allows to accurately assess the genotype/phenotype status of the DoA/B polymorphism and therefore allows to provide compatible BP in cases carrying anti-Do antibodies.

2. For phenotyping of RRC, we recommend to exclude rare Do(Null) phenotypes by generic FACS assessment of the Do/ART4 protein in order to prevent erronous prediction of DoA/B phenotype based on molecular genotyping.

3. Do/ART4 epitopes are conformation depended (DTT susceptible) but are not cleaved off by peptidases such as papain. Based on our results, we predict a decreased reactivity of DaA/B epitopes upon incubation with DTT and unchanged or increased reactivity upon incubation with papain.

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