A Study on Weak D and the Function of the Rh Complex in Red Blood Cells

Een studie over zwak D en de functie van het Rh complex in rode bloedcellen

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Chapter 1

General introduction

Rhesus (Rh) is the most complex of the blood group systems. In this general introduction several aspects of Rh are described. The immunogenicity is responsible for its discovery and establishes its clinical importance. Subsequently the following characteristics of the Rh complex are described: the molecular basis of the Rh blood group system, the membrane organisation of the Rh proteins, Rh-complex formation, association of the Rh complex to the membrane skeleton, and the development and distribution of the Rh polypeptides. With regard to this thesis, much emphasis is given to the variation in expression of Rh. This variation in expression of Rh is not only between the different phenotypes (qualitative versus quantitative), but also between ethnic groups (prevalence of certain phenotypes). To understand the development of a protein like Rh, processing from DNA to membrane protein is described. And a transfection model, mimicking the RBC, was used to follow this process. Insight in the function of Rh proteins might also be informative for the complexity of the Rh blood group system (and vice versa).

1.1 Immunogenicity

The Rh blood group was discovered in 1939 because of its high immunogenicity. At that time, Levine and Stetson (Levine *et al.*, 1939) investigated a haemolytic reaction resulting from the transfusion of a woman with blood from her husband, after she had given birth to a stillborn baby.

An antibody in the mother's serum agglutinated her husband's red blood cells (RBCs) and those of 80% of ABO-compatible blood donors. Levine and Stetson showed that this new antigen, which they did not name, was independent of the then known blood groups ABO, MN and P. They suggested that the mother had been immunised by a foetal antigen of paternal origin, and that the haemolytic episode was caused by a maternal antibody reacting with that antigen on the transfused husband's RBCs. One year later, Wiener and Peters (Wiener *et al.*, 1940) were able to identify the same antibody in the serum of individuals showing transfusion reactions after receiving ABO-compatible blood transfusions. In 1941, Levine *et al.* reported that the antibody responsible for this reaction had the same specificity as the antibody obtained by Landsteiner and Wiener (Landsteiner *et al.*, 1940) after injecting rabbits and guinea pigs with RBCs of rhesus monkeys. However, in the course of further investigations, it became obvious that the human antibodies responsible for the haemolytic disease of the newborn and the 'anti-rhesus' antibody produced in animals by Landsteiner and Wiener recognise different antigens (Fisk *et al.*, 1942). Therefore, the term anti-Rh was chosen to designate the clinically significant human alloantibody RhD, whereas the hetero-antibody was renamed anti-'LW', in honour of its discoverers Landsteiner and Wiener. The other major antigens of the Rh system (C, c, E and e) were discovered shortly afterwards (Race *et al.*, 1944), (Fisher, 1944).

More than 45 different Rh antigens have been described till now, but very few are of clinical importance. The major Rh antigen is RhD. Depending on the presence or absence of this antigen, human RBCs are divided into RhD-positive or RhD-negative cells. After the ABO blood group system, RhD is the most important RBC antigen (Mollison *et al.*, 1997). Eighty percent of RhD-negative individuals who receive RhD-positive blood will produce anti-D antibodies after their first blood transfusion (Pollack *et al.*, 1971), (Urbaniak *et al.*, 1981). Only 7 to 8 % of the RhD-negative individuals remain complete non-responders (Issitt, 1994). RhD antibodies can cause haemolytic transfusion reactions, autoimmune haemolytic anaemia and, despite the introduction of anti-D prophylaxis in 1969 (in the Netherlands), anti-D is still the major cause of haemolytic disease of the newborn (HDN).

Rhc is clinically the most important Rh antigen after RhD. Between

14 and 21 % of Rhc-positive babies born to women with anti-c required exchange transfusion (Astrup *et al.*, 1977), (Hardy *et al.*, 1981), (Kozlowski *et al.*, 1995). Anti-C, -E, -e and -G can all cause HDN, but the occurrence is rare and the outcome seldom severe (Mollison *et al.*, 1997).

1.1.1 Haemolytic disease of the newborn

In the Netherlands, maternal antibodies against foetal RBCs threaten the infant's health in about 200 cases per year, of which 170 cases are due to RhD antagonism (Klumper *et al.*, 2000). Maternal antibodies may be produced after exposure to an antigen that is not present on maternal RBCs, e.g. by foetal-maternal transfusion, after blood transfusion or after organ transplantation. In the foetus, RBCs covered by maternal antibodies will be degraded, mainly extravascularly by phagocytosis and antibody-dependent cellular cytotoxicity (ADCC), leading to hyperbilirubinaemia and anaemia. Destruction of foetal RBCs is recognised by signs of anaemia (oedema), increased levels of bilirubin in amniotic fluid and extramedulary hematopoiesis (resulting in hepatosplenomegaly). Foetal anaemia may lead to foetal hydrops and death. After birth, hyperbilirubinaemia may lead to kernicterus.

To prevent immunisation, anti-D is given within 48 hours after exposure to RhD-positive RBCs, for example as a consequence of chorion villi biopsy, abortion or ectopic pregnancy and, of course, as a result of birth. The precise mechanism for this antibody-mediated immune suppression is not known, but probably depends on interactions with the IgG Fcγ receptor type IIb on B lymphocytes (Kumpel *et al.*, 2001). Routine postnatal prophylaxis with 1000 IE anti-D has reduced the immunisation from 14.7% to 1.6% of the RhD-negative women with an RhD-positive child (Crowther *et al.*, 2000). If a massive foetal-maternal transfusion (>20 mL RhD-positive blood) is expected, e.g. after caesarean section, manual placenta removal or multiple pregnancy, the dosis of anti-D has to be adjusted according to the volume of foeto-maternal transfusion.

In the Netherlands, antenatal prophylaxis (1000 IE anti-D) is given since July 1998 to all RhD-negative mothers in the 30th week of gestation, which is expected to decrease RhD immunisation from 1.5% to 0.2% (Crowther *et al.*, 2000). Moreover, all pregnant women are screened early in pregnancy for the presence of irregular antibodies against RBCs. This will be repeated

in all RhD-negative pregnant women in the 30th week of gestation, before administration of antenatal RhD prophylaxis. If irregular antibodies are present, their specificity, concentration and haemolytic activity (by ADCC test) will be determined, as well as the blood group of the father. If the ADCC is above the critical value (>50% for anti-D), echoscopic monitoring of foetal anaemia is performed once a week. Echoscopically, foetal anaemia is characterised by increased flow velocity in the fetal arteria cerebri media, vena umbilicalis and aorta decendens, and by cardiomegaly and spleno- or hepatomegaly. When indications for foetal anaemia are present, foetal blood sampling by cordocentesis for haemoglobin determination and by amniocentesis for bilirubin determination has to be performed.

Early treatment of severe foetal anaemia consists of intra-uterine transfusion, performed between the 17th and 35th week of gestation. In the 37-38th week of gestation, labour will be induced, because the intra-uterine disadvantages (continuing haemolysis, severe anaemia) are greater than the advantages, and the diagnostic tools will have less predictive value. Directly after birth, in the neonatal period, jaundice may be expected. Therefore, the foetal blood group, the direct antiglobulin test and the haemoglobin concentration have to be determined from cord blood directly after birth. Also the concentration of bilirubin has to be monitored during the first days after birth.

1.1.2 Transfusion reactions by red blood cell antibodies

Transfusion reactions caused by alloimmunisation can occur when the donor blood group is not compatible with that of the recipient.

Mild transfusion reactions include fever, chills and anaemia due to the shortened lifespan of the transfused RBCs. Breakdown of these RBCs causes increased levels of bilirubin and lactate dehydrogenase.

Major haemolytic transfusion reactions may cause hypotension, shock, disseminated intravascular coagulation, renal failure and death.

Haemolytic transfusion reactions due to incompatibility of Rh occur especially when matching of the Rh blood group is not a routine procedure, for example in China, where 99.5% of the population is RhD-positive (Guo *et al.*, 1994).

However, the most common alloantibody problem is not due to typing neglectance but to an antibody present in such a low titer that it is

not detected by the antibody screen or crossmatch. After transfusion, the patient has a rise in antibody titer, usually in 5 to 10 days, a rapid fall in haemoglobin and a rise in bilirubin and lactate dehydrogenase. This is termed a delayed haemolytic transfusion reaction.

1.1.3 Autoimmune haemolytic anaemia

Autoimmune haemolytic anaemia (AIHA) comprises a diverse group of disorders that have in common the presence of an RBC autoantibody resulting in a shortened RBC lifespan as the major mechanism of the cause of anaemia. Warm autoantibodies with a broad "Rh" specificity are typically found in the serum and can be eluted from the patients' RBCs. They are usually of the IgG class, with or without fixed complement. A relative specificity of the autoantibody for anti-e is sometimes observed, but also anti-c, -E, -D and -C autoantibodies can occur, roughly in that order of prevalence. Cold autoantibodies are usually of the complement-activating IgM class, and autoanti-D has been described (Longster *et al.*, 1988).

Clinical signs and symptoms of AIHA can be insidious. A gradual emergence of symptoms of anaemia is often associated with fever and jaundice. If the onset is sudden, it can present with pain in the abdomen and back, general malaise and manifestations of rapidly increasing anaemia. A history of dark urine is common as the result of the presence of bile pigments or hemoglobinuria. Only 25% of the patients have no enlargement of spleen, liver or lymph nodes. There is a positive direct antiglobulin test and signs of haemolysis, but although anaemia can be severe, platelet count and white blood cell count are usually normal.

The incidence of AIHA in the Netherlands is 1-3 per 100,000 per year, of which 70-84% due to warm autoantibodies. Therapy of AIHA due to IgG and/or IgA warm autoantibodies consists of corticosteroids, splenectomy and intravenous immunoglobulin, whereas AIHA due to IgM cold autoantibodies are more benificially treated with alpha-interferon. The prognosis is unpredictable. A minority of the patients has a complete resolution of their disease, and others have chronic but manageable courses (Petz, 1992).

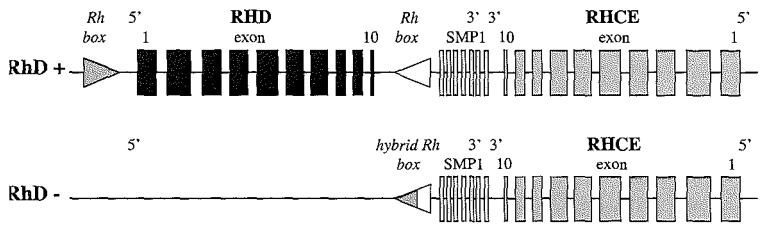


Figure 1.1: Location of *RHD* and *RHCE* on chromosome 1.
Genomic organisation of the *RH* genes in typical RhD-positive (above) and RhD-negative (below) haplotypes, showing *RHD*, the two homologous *Rh boxes* and *SMP1* in 5' to 3' orientation, and *RHCE* in 3' to 5' orientation. In the RhD-negative haplotype, there is a deletion of *RHD* and a part of each *Rh box* (Wagner *et al.*, 2000-II).

1.2 Rh blood group system

1.2.1 Molecular basis

The Rh blood group system is encoded by two highly homologous genes, *RHD* and *RHCE* , located on chromosome 1 (p34.3-p36.1) (Chérif-Zahar *et al.*, 1991), (MacGeoch *et al.*, 1992). Each gene is composed of 10 exons and produces transcripts of 1251 base pairs (bp), resulting in a protein of 417 amino acids (aa) (Chérif-Zahar *et al.*, 1994), (Chérif-Zahar *et al.*, 1997).

The whole *RHD* and *RHCE* genes consist of 57295 and 57831 bp, respectively (exons and introns together) (Okuda *et al.*, 2000) and are separated by approximately 30 kb, in which the coding sequence of *SMP1* (Small Membrane Protein 1) is located (Wagner *et al.*, 2000-II). *RHD* and *RHCE* are located in opposite direction on the chromosome (5'*RHD*3'–3'*RHCE*5'), with *RHD* centromeric of *RHCE* . The *RHD* sequence is flanked by two highly homologous (98.6%) regions, termed the upstream and the downstream *Rh box* (Figure 1.1).

The *RHCE* gene encodes the CcEe polypeptides and the *RHD* gene gives rise to the D polypeptide. Forty nucleotide (nt) differences are found between the *RHD* gene and the *RHCE* gene, resulting in 35 amino acid substitutions (Figure 1.2) (Mouro *et al.*, 1993), (Simsek *et al.*, 1994).

In Caucasians the deletion of both *RHD* genes results in the RhD-negative phenotype, leaving a hybrid Rh box originating from the upstream and downstream box. In non-Caucasians, mutated genes have been found

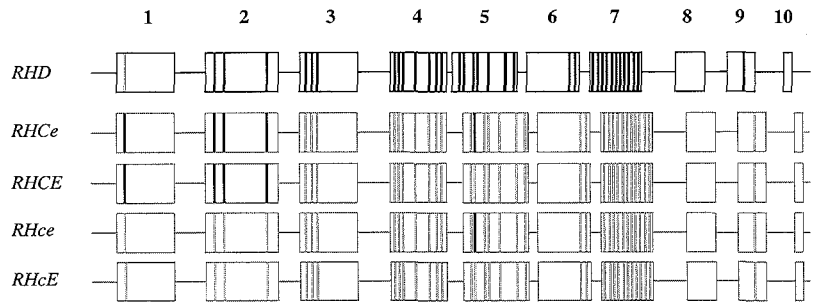


Figure 1.2: *RH* transcripts.
The positions of nucleotide mutations that lead to amino acid substitutions are indicated by bars. Compared to the *RHcE* transcript, which was the first to be described, the *RHD* transcript reveals 35 amino acid substitutions. Exon 1 of the *RHcE*, *RHce* and *RHD* transcripts are identical. All *RH* transcripts share identical exons 8 and 10. Exons 1 and 2 of *RHCE* gene show four amino acid substitutions, which are responsible for the C/c antigenic switch. At position 226 in exon 5 of the *RHCE* gene proline to alanine substitution is responsible for the E/e polymorphism. The Alanine 226 is also present in the *RHD* gene. *RHD* intron 4 is smaller than that of *RHCE*.

to result in the RhD-negative phenotype.

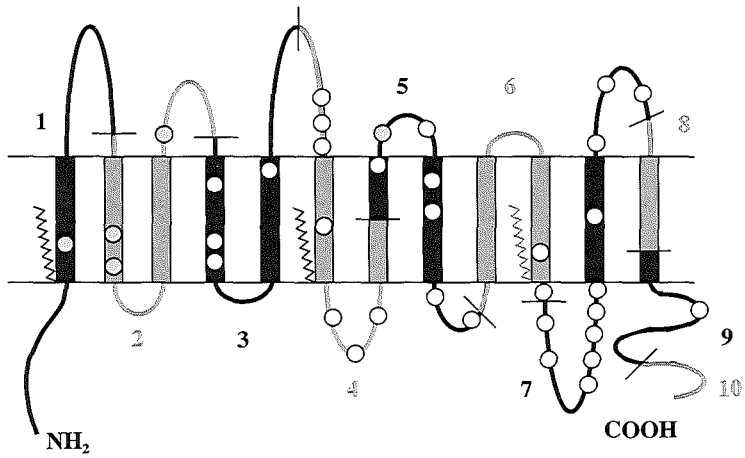
The *RHCE* gene has four alleles: *RHCE*, *RHCe*, *RHcE* and *RHce*. Nucleotide substitutions of the *RHCE* gene in exon 1 (nt48 G→C, aa16 Cys→Trp) and exon 2 (nt150 T→C, silent mutation; nt178 A→T, aa60 Ile→Leu; nt201 G→A & nt203 G→A, aa68 Ser→Asn; nt307 T→C, aa103 Ser→Pro) account for the C/c polymorphism. One nucleotide substitution in exon 5 (nt676 C→G, aa226 Pro→Ala) of the *RHCE* gene is responsible for the E/e antigenic difference (Figure 1.2) (Faas *et al.*, 1995).

Exons 1 of the *RHce* and *RHcE* alleles are identical to the *RHD* gene. Exons 2 of the *RHCe* and *RHCE* alleles are identical to exon 2 of the *RHD* gene.

Knowledge about the molecular background of Rh is of essential importance for genotyping in case RBCs are not available, for example in prenatal diagnostics, after massive transfusions and in severe cases of AIHA.

1.2.2 Membrane organisation

The two Rh polypeptides, RhD and RhCcEe, have an almost identical organization. Both share 12 transmembrane spanning domains, with the NH_2-



Location of Cc or Ee polymorphism.
Location of amino acid residues difference between D and CE.
Probable sites for palmitoylation (Cys-Leu-Pro motifs),
(in grey) palmitoylation site only present on the CcEe polypeptide.
Border between the different exons.

Figure 1.3: Model of the RhD and RhCcEe polypeptides in the red blood cell membrane.

The Rh polypeptides have 12 membrane spanning domains with cytoplasmatic N- and C- termini. The regions encoded by the 10 exons (numbered 1 to 10) are alternately represented in black and grey . The zig-zag lines represent possible sites of palmitoylation. The circles represent sites where the amino acid residues of the D, Cc and Ee may differ.

and the COOH-terminal parts intracellulary (Avent *et al.*, 1990), (Kajii *et al.*, 1993). Three of the cysteine residues in the RhCcEe polypeptide and two in the RhD polypeptide form Cys-Leu-Pro motifs at the point of entry of the polypeptide into the cytoplasmic leaflet of the lipid bilayer and probably represent the major sites for attachment of palmitic acid (De Vetten *et al.*, 1998), (Hartel-Schenk *et al.*,1992). The extracellular parts, against which the immune responses are directed, are very small and comprise no more than 25% of the polypeptide (Figure 1.3).

It is assumed that the RhD protein monomer has a diameter of about 50Å (Liu *et al.*, 1999).

1.2.3 Rh complex

The molecular mass of the Rh complex in non-ionic detergent-solubilized membranes has been estimated to be 170 kDa, after correction for bound detergent. This size is consistent with the complex being a $\alpha_2\beta_2$-type tetramer (Hartel-Schenk *et al.*, 1992). Most probably, therefore, the Rh polypeptides exist in a macromolecular complex, comprising two Rh polypeptides and two RhAg polypeptides (Rh-associated glycoprotein, also known as GP50, Rh50 or CD241) These four peptides interact through their N-terminal domains, as was shown by proteolysis studies. After treatment with *S. aureus* V8 protease (cleaving RhAg specifically at Glu34 and Lys196) a 28.5-kDa RhAg peptide was co-precipitated with RhD/RhCcEe, suggesting that RhAg interacts with RhD/RhCcEe between Glu34 and Lys196 (Eyers *et al.*, 1994), (Avent *et al.*, 1996). Several membrane glycoproteins, such as LW, CD47 and glycophorin B (GPB), are associated with the tetrameric Rh complex (Figure 1.4) (Cartron *et al.*, 1994).

The *RHAG* gene is located on chromosome 6 (q21-qter). RhAg has a homology of about 36% with the Rh polypeptides, but there is no indication of polymorphisms in RhAg (Ridgwell *et al.*, 1992). For that reason RhAg is not immunogenic. Hydropathy analysis suggests that RhAg closely resembles Rh polypeptides, with 12 transmembrane spanning domains and the NH_2- and COOH- terminal parts also located intracellularly (Ridgwell *et al.*, 1992), (Matassi *et al.*, 1998), (Huang *et al.*, 1998). In contrast to the Rh polypeptides, RhAg possesses one extracellular N-glycosylation site (Asn37, on the first extracellular loop) (Eyers *et al.*, 1994) and no sites for palmitoylation.

The involvement in the Rh complex of other proteins (such as LW, GPB, CD47) has been deduced from their simultaneous absence or extreme reduction in the Rh_{null} phenotype, a phenotype completely deficient in RhD and RhCE (see below).

LW antigens have a higher expression in RhD-positive than in RhD-negative RBCs; this convergence has probably led to confounding of Rh with LW in 1940. In addition, monoclonal anti-LW co-precipitates LW glycoprotein and Rh polypeptide (Bloy *et al.*, 1989).

The Fy5 antigen of the Duffy system is also totally absent in Rh_{null} RBCs and weakly expressed in D-- RBCs, suggesting an association in the membrane of this protein with the Rh proteins or with the Rh-associated

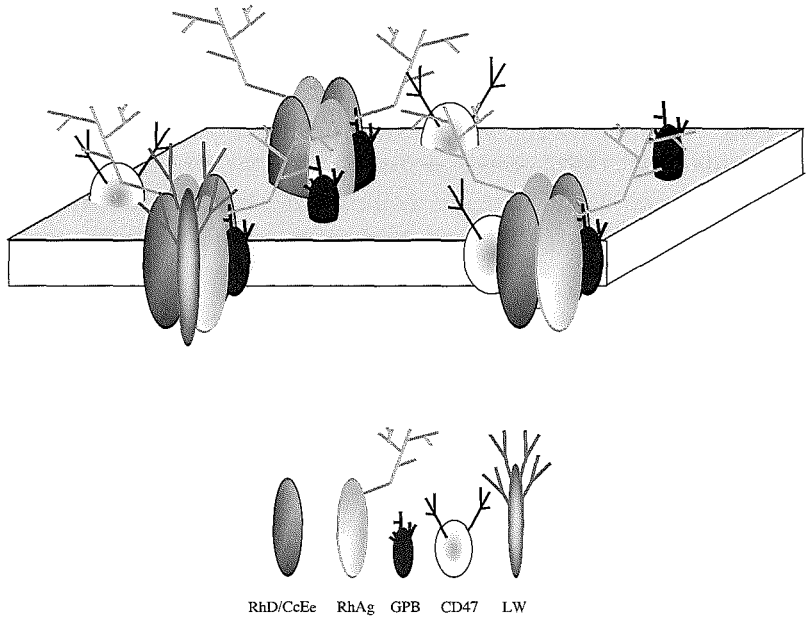


Figure 1.4: The RhD complex.
The Rh complex consists of a tetrameric core of two RhAg proteins and two RhD/RhCcEe proteins, accompanied by GPB, CD47 and LW.

glycoprotein. However, there is no evidence of reduced expression of the Duffy glycoprotein or other Duffy antigens (Daniels, 2002). This implies that the involvement of the Duffy system in the Rh complex is still unclear.

Rh_{null} RBCs (regulator and amorph type) have a 60-70% reduction in GPB (Dahr *et al.*, 1987), reflected as reduced levels of S, s and U antigens. The association of GPB with the Rh complex was confirmed by the co-precipitation of GPB with anti-RhAg MoAb 2D10 (Von dem Borne *et al.*, 1990). RhAg of GPB-deficient RBCs is more heavily glycosylated than in normal RBCs. GPB might facilitate transport of RhAg to the cell surface membrane, which, in the absence of GPB, remains longer in the intracellular compartment, permitting more glycosylation (Ridgwell *et al.*, 1994)

CD47 or integrin-associated protein (IAP) is a widely distributed heavily N-glycosylated glycoprotein in RBC membranes (Avent *et al.*, 1988). It is present in reduced quantity (75% of normal) on Rh_{null} RBCs

(Avent *et al.*, 1988), (Lindberg *et al.*, 1994), (Miller *et al.*, 1987). It may function as a marker of self by binding signal regulatory protein α (SIRPα) on macrophages, generating a negative signal that prevents phagocytosis of RBCs (Oldenborg *et al.*, 2000).

The presence in the Rh complex of the Diego system is not clear. There is no evidence that antigens of the Diego system show altered expression on Rh_{null} cells, but an association of band 3 (the Diego antigen) with Rh has been demonstrated by co-transfection experiments. K562 cells transfected with *RHD* or *RHcE* cDNA express RhD or RhcE antigens, respectively. When those cells are subsequently co-transfected with cDNA encoding band 3, the levels of RhD or RhcE and of endogenously produced RhAg are increased (Beckmann *et al.*, 2001). This suggests that an interaction between band 3 and the Rh-RhAg complex either enhances their translocation to the cell surface or affects their conformation in the plasma membrane.

1.2.4 Association of the Rh complex with the membrane skeleton

The Rh complex represents a major attachment between the lipid bilayer and the erythroid skeleton, as was concluded from the following observations; i) Rh_{null} cells are characterized by abnormalities of the RBC shape, cation transport and phospholipid organization (Cartron, 1999), (Huang *et al.*, 2000); ii) The major members of the Rh complex (RhD, RhCcEe, RhAg and CD47) are partly resistant to membrane solubilization by the non-ionic detergent Triton X100, indicating an association with the membrane skeletal matrix (Gahmberg *et al.*, 1984), (Ridgwell *et al.*, 1984), (Paradis *et al.*, 1986). And iii) fluorescence-imaged microdeformation, a method which quantifies redistribution in situ of fluorescently labelled RBC membrane proteins during mechanically induced membrane deformation following cell aspiration into a micropipette, showed that the behaviour of RhD, RhCcEe and CD47 proteins is intermediate between that of actin (a component of the membrane skeleton) and that of band 3 (a membrane glycoprotein that is firmly attached to the skeleton), whereas the behaviour of RhAg is similar to that of actin (Gimm *et al.*, 2000), (Dahl *et al.*, 2003). These results imply that the Rh complex is firmly linked to the actin-spectrin-based RBC membrane skeleton, presumably via RhD/RhCcEe, RhAg and/or CD47.

From the study of RBCs from hereditary spherocytosis patients, asso-

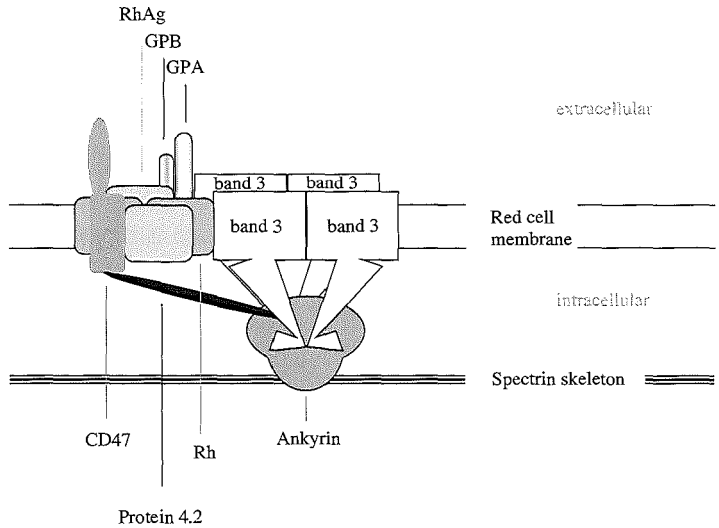


Figure 1.5: Association of the Rh complex with the membrane skeleton. Tetramers of band 3 are attached to the spectrin cytoskeleton via ankyrin. Protein 4.2 binds ankyrin, band 3, and CD47, providing a link between the Rh complex and the band-3 complex.

ciated with the complete lack of protein 4.2, it has been suggested that protein 4.2 through interaction with CD47 is involved in the linkage of the Rh complex to the RBC skeleton and/or its translocation through the cell membrane (Mouro-Chanteloup *et al.*, 2003). Band 3 is also playing a major role in the association of Rh to the membrane skeleton, as was suggested by the reduced expression of the Rh complex in band-3 deficient RBCs and by a similar number of tetrameric Rh complexes and band-3 tetramers. Direct evidence that Rh and band 3 are associated was obtained by co-precipitation of band 3 with the Rh-protein complexes (Bruce *et al.*, 2003). Band 3 in tetrameric form binds ankyrin and protein 4.2 and is the major attachment site of the RBC membrane to cytoskeleton (Rybicki *et al.*, 1996). A proposed model for the band-3 macrocomplex is shown in Figure 1.5.

1.2.5 Development and distribution of Rh antigens

RhD, RhCcEe and RhAg appear to be specific for RBCs. There is no evidence that these antigens, also referred to as erythroid Rh homologues, are expressed on other tissue types. RhAg is detected early in the erythropoiesis on erythroid blast-forming units (BFU-E), after glycophorin C and Kell glycoprotein, but before glycophorin A and band 3. The Rh antigens appear substantially later, after glycophorin A and band 3 (Southcott *et al.*, 1999), (Bony *et al.*, 2000). Their expression increases during erythroid differentiation and erythroid ageing (Rearden *et al.*, 1977). Besides the erythroid Rh homologues, closely related non-erythroid Rh homologues have been described, located in liver, skin, proximal tubules of the kidney (RhBG) (Liu *et al.*, 2000) and the testis, and in the collecting tubules of the kidney (RhCG= RhGK) (Liu *et al.*, 2001).

1.3 Expression of RhD

1.3.1 Qualitative variations

Qualitative variations of RhD were discovered when individuals who where typed RhD-positive had produced alloanti-D after exposure to RhD-positive blood. The RhD antigen harbours a number of epitopes; therefore, individuals who lack one or more epitopes (partial D) may be alloimmunized to the missing epitopes.

Tippett and Sanger divided partial-D antigens into six catagories (I-VI), based on reaction patterns of RBCs with antibodies of RhD-positive individuals who made anti-D. The introduction of monoclonal antibodies enabled the classification of partial-D antigens to different patterns of reactions, considered to represent different epitopes of the RhD antigen. Lomas *et al.* (1989) defined seven reaction patterns by testing 29 monoclonal anti-D, representing most of the partial-D antigens, and two more were added later. However, the more different partial-D RBCs were identified, the more subsplits in the original reaction pattern were created. The latest pattern of reactions between partial D and monoclonal antibodies includes 30 epitopes, with a terminology consisting of the original 9 epitopes, followed by numbers representing subdivisions of these epitopes (Fourth International Workshop on Monoclonal Antibodies to Red Cell Surface Antigens, Paris,

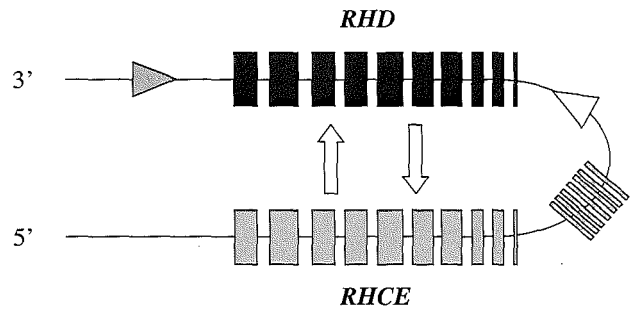


Figure 1.6: Hairpin formation resulting in *RHD-CE-D* or *RHCE-D-CE* hybrids. Probable mechanism for *RH* gene conversion, with pairing between *RHD* and *RHCE*, *in cis*.

France, 2001).

Gene rearrangements and point mutations form the molecular basis for partial-D variants. That many *RHD* and *RHCE* hybrids are formed is probably due to the high homology and opposite orientation of these two genes. Gene conversion, resulting in *RHD-CE-D* or *RHCE-D-CE* hybrids or amino acid substitutions, can easily occur after hairpin formation, pairing *RHD* and *RHCE in cis* (Figure 1.6) (Wagner *et al.*, 2000-II), (Suto *et al.*, 2000).

An overview of all known partial Ds is shown in Table 1.1, together with the molecular basis (amino acid substitutions or hybrid genes), nucleotide changes, protein sequence, exon and membrane localisation, antigen density, similarity index (if known) and whether alloantibodies have been observed in this phenotype.

1.3.2 Quantitative variations

The number of RhD antigens expressed on the RBC membrane has been determined by Scatchard plot analysis with ^{125}I-labelled monoclonal antibodies. A continuum of RhD-antigen expression was found in the following increasing order: R1r, R2r, R1R1, R1R2, R2R2 (Appendix, Table 2), ranging from 15,000 to 33,000 sites per cell (Hughes-Jones *et al.*, 1988), (Van Bockstaele *et al.*, 1986), (Nicholson *et al.*, 1991).

Trivial Name	Molecular basis (allele)	Nucleotide change	Protein sequence	exon	membrane localization	mean antigen density	RhD Similarity Index	allo-anti-D
D II	*RHD* (A354D)	C->G at 1060	Ala to Asp at 354	7	EC	3200		yes
		C->A at 1061	"	7	EC			
D IIIa	*RHD* (N152T, T201R, F223V)	A->C at 455	Asn to Thr at 152	3	TM	12300		yes
		C->G at 602,	Thr to Arg at 201	4	IC			
		T->G at 667,	Phe to Val at 223	5	TM			
D IIIb	*RHD* -CE (exon2) -D	T->C at 90	no change	1	-	?		yes
		A->C at 180	Ile to Leu at 60	2	TM			
		G->A at 201	Ser to Asn at 68,	2	TM			
		T->C at 307	Ser to Pro at 103,	2	EC			
D IIIc	*RHD* -CE (exon3) -D	T->A at 361,	Leu to Met at 121	3	TM	22300	0.69	yes
		T->C at 380,	Val to Ala 127	3	TM			
		A->G at 383,	Asp to Gly at 128	3	TM			
		A->C at 455	Asn to Thr at 152	3	TM			
D III type IV	*RHD* (L62F, A137V, N152T)	G->C/T at 186	Leu to Phe at 62	2	TM	33255	0.45	yes
		C->T at 410	Ala to Val at 137,	3	IC			
		A->C at 455	Asn to Thr at 152	3	TM			
D IVa	*RHD* (L62F, N152T, D350H)	G->C/T at 186	Leu to Phe at 62	2	TM	9300	0.21	yes
		A->C at 455	Asn to Thr at 152	3	TM			
		G->C at 1048	Asp to His at 350	7	EC			
D IVb	*RHD* -CE (exon7, 8 ,9) -D	G->C at 1048	Asp to His at 350	7	EC	4000		yes
		C->T at 1053	no change	7	-			
		G->T at 1057	Gly to Trp at 353	7	EC			
		A->G at 1059	"	7	"			
		G->A at 1060	Ala to Asn at 354	7	EC			
		C->A at 1061	"	7	"			
		T->C at 1170	no change	9	-			
		A->T at 1193	Glu to Val at 398	9	IC			
D IV type III	*RHD* -CE (exon6, 7, 8, 9) -D	G->A at 916,	Val to Ile at 306	6	IC	607	0	?
		A->G at 931	Tyr to Cys at 311	6	IC			
		& all nt of D IVb	& all aa of D IVb	7, 8, 9	EC/IC			
D IV type IV	*RHD* (D350H, G353W, A354N)	G->C at 1048,	Asp to His at 350	7	EC	?		?
		G->T at 1057,	Gly to Trp at 353	7	EC			
		A->G at 1059,	"	7	"			
		G->A at 1060,	Ala to Asn at 354	7	EC			
		C->A at 1061	"	7	"			
D Va	*RHD* -CE (exon5) -D	T->G at 667,	Phe to Val at 223	5	TM	9400	?	yes
		G->C at 676,	Ala to Pro at 226	5	EC			
		G->C at 697,	Glu to Gln at 233	5	EC			
		G->A at 712,	Val to Met at 238	5	TM			
		G->C at 733,	Val to Leu at 245	5	TM			
		G->A at 787,	Gly to Arg at 263	5	IC			
		A->T at 800	Lys to Met at 267	5	IC			
D VI type I	*RHD* -CE (exon4, 5) -D	A->C at 505,	Met to Leu at 169	4	EC	300	0	yes
		T->G at 509,	Met to Arg at 170	4	EC			
		A->T at 514,	Ile to Phe at 172	4	EC			
		T->A at 544,	Ser to Thr at 182	4	TM			
		G->A at 577,	Glu to Lys at 193	4	IC			
		A->T at 594,	Lys to Asn at 198	4	IC			
		C->G at 602,	Thr to Arg at 201	4	IC			
		& all nt of D Va	& all aa of D Va	5	EC/IC/TM			
D VI type II	*RHD* -CE (exon4, 5, 6) -D	all nt of D VI type I,	all aa of D VI type I,	4, 5	EC/IC/TM	1600	0	yes
		G->A at 916,	Val to Ile at 306	6	IC			
		A->G at 931	Tyr to Cys at 311	6	IC			
D VI type III	*RHD* -CE (exon3, 4, 5, 6) -D	T->A at 361,	Leu to Met at 121	3	TM	14502	0	?
		T->C at 380,	Val to Ala 127	3	TM			
		A->G at 383,	Asp to Gly at 128	3	TM			
		A->C at 455	Asn to Thr at 152	3	TM			
		& all nt of D VI type II	& all aa of D VI type II	4, 5, 6	EC/IC/TM			
D VII	*RHD* (L110P)	T->C at 329	Leu to Pro at 110	2	EC	3600	0.48	yes
DAR	*RHD* (T201R, F223V, I342T)	C->G at 602,	Thr to Arg at 201	4	IC	?		yes
		T->G at 667,	Phe to Val at 223	5	TM			
		T->C at 1025	Ile to Thr at 342	7	TM			
DBT type I	*RHD* -CE (exon5, 6, 7) -D	all nt of D Va	all aa of D Va	5	EC/IC/TM	4300	?	yes
		G->A at 916,	Val to Ile at 306	6	IC			
		A->G at 931	Tyr to Cys at 311	6	IC			
		G->C at 1048	Asp to His at 350	7	EC			
		C->T at 1053	no change	7	-			
		G->T at 1057	Gly to Trp at 353	7	EC			
		A->G at 1059	"	7	"			
		G->A at 1060	Ala to Asn at 354	7	EC			
		C->A at 1061	"	7	"			
DBT type II	*RHD* -CE (exon5, 6, 7, 8, 9) -D	all nt of DBT type I	all of DBT type I	5, 6, 7	EC/IC/TM	?	?	?
		T->C at 1170	no change	9	-			
		A->T at 1193	Glu to Val at 398	9	IC			
DFR	*RHD* (M169L, M170R, I172F)	A->C at 505,	Met to Leu at 169	4	EC	5300	?	yes
		T->G at 509,	Met to Arg at 170	4	EC			
		A->T at 514	Ile to Phe at 172	4	EC			
DFW	*RHD* (H166P)	A->C at 497	His to Pro at 166	4	EC	?	?	?
DHAR	*RHCE* -D (exon5) -CE			5		?	?	yes
DHK (DYO)	*RHD* (E233K)	G->A at 697	Glu to Lys at 233	5	EC	?	?	?
DHMI	*RHD* (T283I)	C->T at 848	Thr to Ile at 283	6	TM	2400	0.12	yes
DHO	*RHD* (K235T)	A->C at 704	Lys to Thr at 235	5		1300	?	?
DHR	*RHD* (R229K)	G->A at 686	Arg to Lys at 229	5		3800	?	?
DIM	*RHD* (C285Y)	G->A at 854	Cys to Tyr at 285	6		192	0	?
DMH	*RHD* (L54P)	T->C at 161	Leu to Pro at 54	1		?	?	?
DNB	*RHD* (G355S)	G->A at 1063	Gly to Ser at 355	7		?	?	?
DOL	*RHD* (M170T, F223V)	T->G at 509,	Met to Arg at 170	4	EC	4700	?	yes
		T->G at 667,	Phe to Val at 223	5	TM			

Table 1.1: All partial Ds

RBCs expressing quantitative variations can be divided into those with enhanced expression (e.g. D--) and those with decreased expression (weak D).

Enhanced expression of RhD

The Rh phenotypes D-- and related complexes, which lack expression of RhE and Rhe and sometimes also of the RhC and Rhc antigens, show elevated expression of the RhD antigen (between 50,000 and 100,000 sites per cell) (Van Bockstaele *et al.*, 1986), (Nicholson G *et al.*, 1991), (Issitt, 1985) Most of the D-- haplotypes consist of a complete, or almost complete, *RHD*, paired with a hybrid gene containing a substantial portion of *RHD* (Figure 1.7). This may provide an explanation for the enhanced RhD expression. The D-- haplotype has a frequency of 0.0005 in Sweden, 0.0047 in Iceland and 0.001 in Japan (Rasmuson *et al.*, 1966), (Olafsdottir *et al.*, 1983), (Okubo *et al.*, 1983).

Weak expression of RhD

In 1946, a phenotype was described in which the RhD antigen could only be detected by some of the anti-RhD reagents available at that time or when an antiglobulin reagent was added (Stratton, 1946), (Stratton *et al.*, 1948). This antigen was first called D^u, later weak D.

A fraction of the weak-D phenotype is caused by the suppressive effects of the Cde haplotypes in trans position. When the haplotype producing the weakly expressed D is partnered by a haplotype expressing neither C nor D (thus cde or cdE) in another family member, the D is expressed normally. The mechanism for this phenomenon is unknown (Ceppellini *et al.*, 1955). This weak-D phenotype, formerly called high-grade D^u, likely possesses the normal *RHD* allele, because the parents and children of individuals with this phenotype often express a normal RhD density. This weak-D phenotype is characterised by a minor reduction of RhD-antigen expression and is nowadays often typed as normal RhD, because of the increased sensitivity of monoclonal anti-D.

The majority of weak-D phenotypes, is caused by variations either at the Rh gene locus itself or in its proximity, because the weak expression is inherited along with the RhD phenotype. Besides the mere quantitative re-

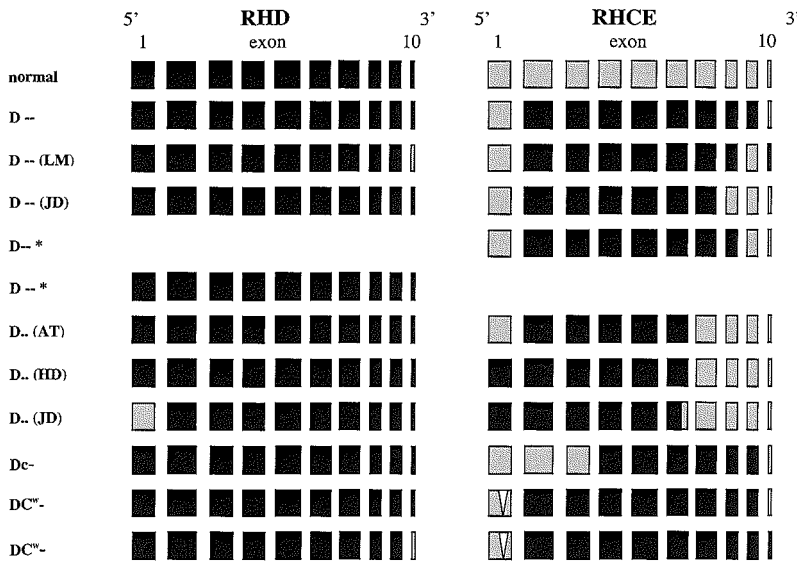


Figure 1.7: Genomic arrangement of D-- and related phenotypes.
Representation of the 10 exons of *RHD* (black) and *RHCcEe* (grey) of D-- and related phenotypes. D-- indicated with a * are the proposed haplotypes in one D-- propositus. White triangle in exon 1 encodes Arg41 characteristic of C^w.

duction, no qualitative differences have been discerned in the RhD antigen of this group. Since 1997 (Avent *et al.*, 1997), (Legler *et al.*, 1998), evidence has been accumulating that the underlying molecular basis of these weak-D phenotypes might be heterogenous, and that some weak-D individuals may carry structurally abnormal *RHD* alleles. Wagner *et al.* (1999, 2000-I) revealed 20 different molecular causes of these weak-D phenotypes. The amino-acid substitutions of the weak-D phenotypes were located in intracellular and transmembrane protein segments and clustered in four regions of the protein (amino acid positions 2 to 13, around 149, 179 to 225, and 267 to 397) (See Table 1.2). The weak-D types display type-specific antigen densities between 70 and 5200 RhD antigens per cell. Each weak-D type expresses a particular epitope density profile, which was established by flowcytometry with 59 IgG anti-D MoAbs. An Rh similarity index, the ratio of the 10th percentile and the 90th percentile of the epitope densities, was devised to indicate qualitative differences of RhD phenotypes. Ideally,

normal RhD antigens would have an index of 1 (all MoAbs recognize the same epitope density, the epitope density profile is a single narrow peak), grossly aberrant partial RhD would have an index of 0.

A very weak form of RhD expression in the Far East is called DEL (originally Del). This phenotype can only be detected reliably by adsorption-elution tests and is characterised by several genotypes. In Taiwan, DEL is associated with a 1013bp deletion of *RHD* extending from intron 8 to intron 9 and encompassing the whole of exon 9 (Chang *et al.*, 1998). Two types of RNA mutations with the potential to disrupt RNA splicing were found. The first is a G to A substitution on the first nucleotide of intron 1, which leads to the loss of exon 1 in most mRNA molecules. The second is a G to A substitution on nt 1227 (exon 9), which might lead to abnormal splicing of exon 9 (Singleton *et al.*, 2001).

1.3.3 Rh-deficiency phenotypes

Rh_{null} RBCs are completely deficient in RhD and RhCE (Avent *et al.*, 1988)

Rh_{null} RBCs of the regulator type have a molecular defect in the *RHAG* gene (Chérif-Zahar *et al.*, 1996) resulting in the lack of expression of the complete Rh complex. Rh_{null} RBCs of the amorph type have a molecular change in the *RHCE* gene in tandem with a deleted *RHD* gene (Chérif-Zahar *et al.*, 1998), (Huang *et al.*, 1998) resulting in the lack of expression of RhD and RhCE; however, RhAg is still expressed in reduced amounts. Some mutations in the *RHAG* gene give rise to low-level expression of Rh antigens, a phenotype called Rh_{mod} (Chown *et al.*, 1972). Molecular analysis of three individuals with the Rh_{mod} phenotype revealed missense mutations in *RHAG* (aa1 Met→Ile, aa79 Ser→Asn and aa399 Asp→Tyr). This indicates that the expression of RhAg is essential for the expression of the Rh polypeptides.

Rh_{null} RBCs are morphologically and functionally abnormal (Schmidt *et al.*, 1967), (Schmidt *et al.*, 1969). Most Rh_{null} and Rh_{mod} individuals have some degree of haemolytic anaemia. Typical signs of Rh-deficiency syndrome are the presence of stomatocytes and some spherocytes, increased red cell osmotic fragility and increased reticulocyte count. Rh_{null} RBCs have an abnormal organisation of their membrane phospholipids (Kuypers *et al.*, 1984) and increased cation permeability, compensated by an increased number of sodium-potassium pumps (Lauf *et al.*, 1976).

Table 1.2: All weak Ds

Trivial Name	Molecular basis (allele)	Nucleotide change	Protein sequence	exon	membrane localization	no.	% of weak RhD	mean antigen density	RhD Similarity index
Weak RhD type 1	*RHD* (V270G)	T->G at 809	Val to Gly at 270	6	TM	95	70.29	1285	0.57
Weak RhD type 2	*RHD* (G385A)	G->C at 1154	Gly to Ala at 385	9	TM	43	18.01	489	0.68
Weak RhD type 3	*RHD* (S3C)	C->G at 8	Ser to Cys at3	1	IC	7	5.19	1932	0.77
Weak RhD type 4	*RHD* (T201R, F223V)	C->G at 602,	Thr to Arg at 201,	4	IC	6	1.30	2288	0.36
		T->G at 667,	Phe to Val at 223	5	TM				
		G->A at 819	no change	6	-				
Weak RhD type 4.1	*RHD* (W16T, T201R, F223V)	G->C at 48,	Trp to Cys at 16	1	TM			3811	0.48
		C->G at 602,	Thr to Arg at 201,	4	IC				
		T->G at 667,	Phe to Val at 223	5	TM				
		G->A at 819	no change	6	-				
weak RhD type 4.2 = DAR	*RHD* (T201R, F223V, I342T)	C->G at 602,	Thr to Arg at 201,	4	IC			1650	0.21
		T->G at 667,	Phe to Val at 223	5	TM				
		T->C at 1025,	Ile to Thr at 342	7	TM				
Weak RhD type 4.2.1	*RHD* (T201R, F223V, I342T)	C->G at 602,	Thr to Arg at 201,	4	IC				
		T->G at 667,	Phe to Val at 223	5	TM				
		G->A at 957	no change	6	-				
		T->C at 1025,	Ile to Thr at 342	7	TM				
Weak RhD type 4.2.2	*RHD* (T201R, F223V, I342T)	C->G at 602,	Thr to Arg at 201,	4	IC				
		T->G at 667,	Phe to Val at 223	5	TM				
		C->T at 744,	no change	5	-				
		G->A at 957	no change	6	-				
		T->C at 1025,	Ile to Thr at 342	7	TM				
Weak RhD type 5	*RHD* (A149D)	C->A at 446	Ala to Asp at 149	3	TM	2	0.84	296	0.39
Weak RhD type 6	*RHD* (R10Q)	G->A at 29	Arg to Gln at 10	1	IC	1	0.74	1053	0.79
Weak RhD type 7	*RHD* (G339E)	G->A at 1016	Gly to Glu at 339	7	TM	1	0.74	2409	0.03
Weak RhD type 8	*RHD* (G307R)	G->A at 919	Gly to Arg at 307	6	IC	1	0.74	972	0.75
Weak RhD type 9	*RHD* (A294P)	G->C at 880	Ala to Pro at 294	6	TM	1	0.42	248	0.43
Weak RhD type 10	*RHD* (W393R)	T->C at 1177	Trp to Arg at 393	9	IC	1	0.42	1186	0.34
Weak RhD type 11	*RHD* (M295I)	G->T at 885	Met to Ile at 295	6	TM	1	0.22	183	0.53
Weak RhD type 12	*RHD* (G277E)	G->A at 830	Gly to Glu at 277	6	TM	0	<2.22	96	nc
Weak RhD type 13	*RHD* (A276P)	G->C at 826	Ala to Pro 276	6	TM	0	<2.22	946	0.33
Weak RhD type 14	*RHD* (S182T, K198N, T201R)	T->A at 544,	Ser to Thr at 182,	4	TM	0	<2.22		
		A->T at 594,	Lys to Ans at 198,	4	IC				
		C->G at 602	Thr to Arg at 201	4	IC				
Weak RhD type 15	*RHD* (G282D)	G->A at 845	Gly to Asp at 282	6	TM	0	<1.26	297	0.21
Weak RhD type 16	*RHD* (W220R)	T->C at 658	Trp to Arg at 220	5	TM	0	<1.26	235	0.44
Weak RhD type 17	*RHD* (R114W)	C->T at 340	Arg to Trp at 114	3	TM			66	nc
Weak RhD type 18	*RHD* (R7W)	C->T at 19	Arg to Trp at 7	1	IC				
Weak RhD type 21	*RHD* (P313L)	C->T at 938	Pro to Leu at 313	6	IC			5200	
Weak RhD type 22	*RHD* (W408C)	G->C at 1224	Trp to Cys at 408	9	IC				
Weak RhD type 23	*RHD* (G212C)	G->T at 634	Gly to Cys at 212	4	TM				
Weak RhD type 24	*RHD* (L338P)	T->C at 1013	Leu to Pro at 338	7	IC				

nc=not calculated, because 90th percentile is less than 200 antigens per cell

1.3.4 Ethnic diversity

The Rh blood group is very polymorphic and frequencies of the Rh haplotypes can differ markedly between different ethnic groups. About 15% of Europeans and North-American Caucasians have the RhD-negative phenotype. Of the black population, less than 5% has the RhD-negative phenotype, whereas in the Asian population the frequency of RhD-negativity can even be lower (Mourant *et al.*, 1976), (Tills *et al.*, 1983). As described before, RhD negativity in Caucasians is mainly caused by homozygosity for a deletion of *RHD*. In blacks, the RhD-negative phenotype can also be caused by the *RHD* pseudo (*RHDΨ*) gene, in which a 37bp sequence duplication of the last 19 nucleotides of intron 3 and the first 18 nucleotides of exon 4 results in a reading-frame shift and introduces a premature translation stop codon. *RHDΨ* has also a nonsense mutation in exon 6 (269Tyr→stop), which ensures that no RhD protein is present on the membrane (Singleton *et al.*, 2000). The r's gene is another gene causing RhD negativity in African blacks, in which the 5' part of exon 3 and exons 4, 5, 6, 7 and 8 have been replaced by the *RHCE* counterparts.

A very weak form of the RhD antigen, called DEL, as described above, is found in the Japanese and Chinese population. DEL accounts for 10% and 30%, respectively, of the RhD-negative population in Japan and China (Mak *et al.*, 1993), (Okubo *et al.*, 1984).

Some qualitative RhD variants (partial D) are mainly seen in certain ethnic populations, for example: $D^{III a}$, $D^{III b}$, $D^{IV a}$ and DOL are frequently found in blacks (Tippett *et al.*, 1996), (Avent *et al.*, 1999). DBT type II (Huang *et al.*, 1999) and DHK (Hyodo *et al.*, 2000) are more often seen in Japanese.

1.4 Protein development

1.4.1 Transcription and translation

The production of all proteins, including transmembrane proteins such as the Rh polypeptides, starts in the nucleus. The envelope around the nucleus consists of an inner and an outer membrane, with passageways through nuclear pores. The outer nuclear membrane continues in the endoplasmatic reticulum (ER) membrane, and the space between it and the inner mem-

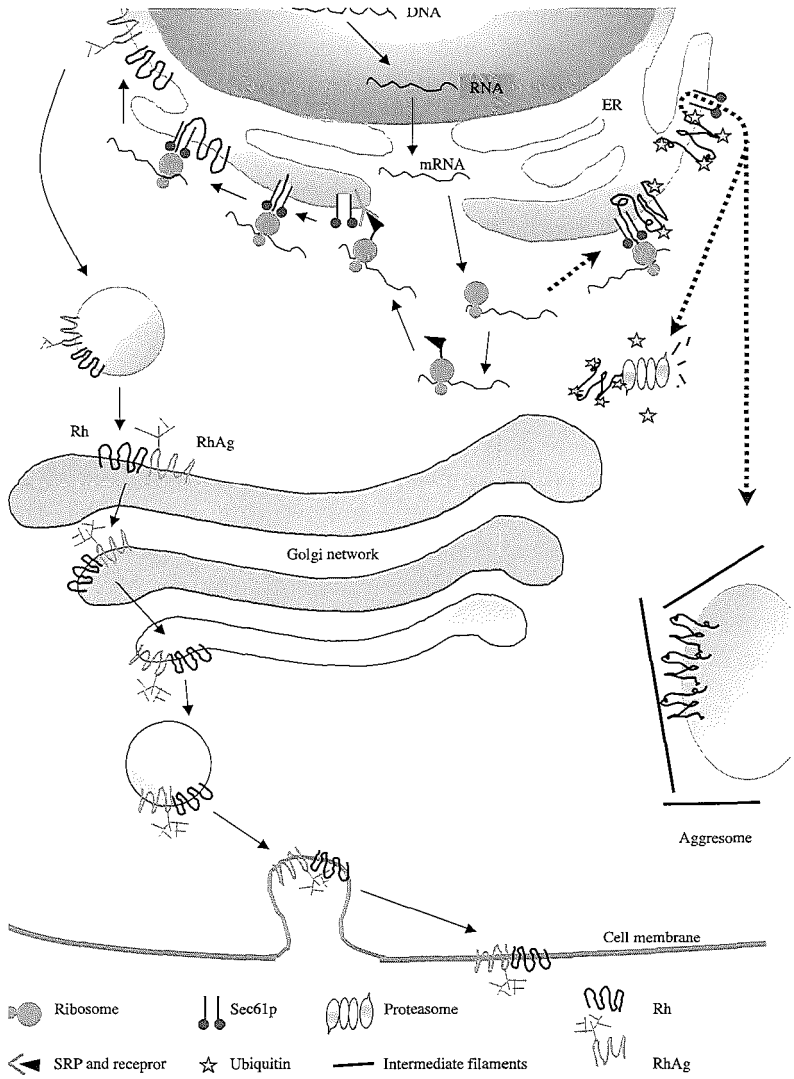


Figure 1.8: Protein development (Pollard *et al.*, 2002).
(Left) The production of a protein starts in the nucleus where DNA is transcribed. In the ER translation, glycosylation and protein oligomerisation takes place. In the Golgi the proteins are processed and directed to their final destination. (Right) When proteins are misfolded, they will be eliminated. In the ER ubiquetin tags will mark the misfolded or unassembled proteins. Degradation takes place in the proteasome. Aggresomes are formed when the degradation capacity of the proteasome is exceeded.

brane continues in the ER lumen. mRNA molecules, which are transcribed from the DNA in the nucleus, and ribosomal subunits, which are assembled there, are exported from the nucleus to the cytosol. Many ribosomes in the cytosol can bind to single mRNA molecules, and the polyribosome that is formed will attach to a docking protein on the rough ER membrane through the signal recognition peptides (SRP) present on the growing polypeptide chains. This binding to the membrane initiates the translocation process that threads a loop of polypeptide across the ER membrane through a hydrophyllic pore in a protein translocator (the Sec61p complex). Transmembrane proteins destined for the cell-plasma membrane are translocated across the ER membrane, but are not released into the lumen. Instead, they remain anchored in the lipid bilayer by one or more membrane-spanning α-helical regions in their polypeptide chain. These hydrophobic portions of the protein can act either as start-transfer or stop-transfer signals during the translocation process. When a polypeptide such as the Rh protein contains multiple alternating start-transfer and stop-transfer signals, it will pass back and forth across the bilayer multiple times. ATP-driven heat-shock proteins, such as BiP, serve as chaperones that bind nascent proteins and assist in their folding.

1.4.2 Glycosylation and protein oligomerisation in the ER

N-glycosylation (oligosaccharide attachment to asparagines) of RhAg starts in the ER by synthesis of high-mannose-oligosaccharide from dolichol-phosphate carriers.

During synthesis of the Rh polypeptides by the ribosomes, these peptides are transferred to the ER as subunits of a hetero-oligomeric protein complex (the Rh complex). Assembly of proteins generally occurs prior to export and also involves chaperones that protect hydrophobic surfaces found in subunit interfaces. Because synthesis of protein subunits occur on distinct ribosomes and may be unbalanced, additional chaperones may promote subunit interactions and prevent premature export or degradation.

1.4.3 Routing and processing of proteins: Golgi complex and beyond

The Golgi complex consists of at least three stacked compartments or cisternae. Golgi stacks are highly polarised with respect to the composition of the individual cisternae. The complexed Rh proteins move from ER through endocytic compartments, carried by lipid-bound transport containers. These vesicles generally enter the cis side of the stack proximal to the nucleus, while processed material exits from cisternae found on the trans side. Each Golgi cisterna contains a different spectrum of processing enzymes. All newly synthesized proteins that pass inspection proceed through the Golgi complex, even if they are not substrates for further processing, such as RhD and RhCE. But most proteins, like RhAg, receive post-translational modifications, such as terminal glycosylation, before being shipped to their destination. Successful passage through the Golgi complex brings the near-final products to the trans-Golgi network (TGN), the major distribution centre, where they are packaged and sent to the cell membrane.

1.4.4 Misfolded proteins

A highly efficient quality control system in the ER guarantees that only properly folded proteins are delivered, whereas misfolded proteins or unassembled subunits of protein complexes are eliminated. This process is called: ER-associated degradation (ERAD) (Sommer *et al.*, 1997). Unregulated proteolysis would be disastrous, therefore tightly regulated compartmentalized intracellular proteolytic activity must be used. Proteasomes are compartments for proteolysis of misfolded membrane proteins. These proteolytic machines, located in the cytoplasm, are assembled from multiple protein subunits that form a small cylindrical compartment with the proteolytically active sites sequestered on the inside. The misfolded membrane proteins located in the ER must be identified. The small polypeptide ubiquitin is central in this process by targeting molecules for degradation by proteasomes (Bonifacino *et al.*, 1998). Ubiquitin binds post-translationally to Lysine residues on protein substrates and is recognised by the cellular machinery that targets them for proteolysis. The transfer of these misfolded membrane proteins, from the ER to the cytoplasmic proteosome, is

mediated by Sec61p, the same translocon that is used for translocation of nascent polypeptides through membranes (Plemper *et al.*, 1999). Dyslocated, ubiquitinated, misfolded proteins can either be rapidly degraded by cytosolic proteasomes or, when the degradation capacity of the proteasomes are exceeded, they aggregate. Misfolded, aggregated proteins are transported to the microtubule organising centre, where they are ensheathed by the intermediate filament vimentin. This structure is called the aggresome (Johnston *et al.*, 1998).

1.5 Transfection model for Rh expression

To study intracellular processes in RBCs a model had to be developed, because erythrocytes do not have a nucleus anymore. In-vitro expression of *RH* genes in eukaryotic cells has been tried by transfection with plasmid expression vectors but initially lacked success (Hermand *et al.*, 1993), (Suyama *et al.*, 1993). In 1996, Smythe *et al.* successfully used a retroviral gene transfer method to express RH-cDNA in K562 cells. K562 cells are erythroleukemic cells that already express RhAg, which is considered essential for the expression of Rh antigens. Although the K562 cell line was derived from an RhD-positive patient, these cells express RhD to such a low extent that after transfection with *RHD* a clear rise in expression can be observed.

1.6 Function of the Rh complex

1.6.1 Similarities with the ammonium transport family

The predicted topology of the Rh proteins in the cell membrane (polytopic, with cytoplasmic N- and C- termini) is characteristic for membrane transporters. RhAg bears an even closer resemblance to RBC membrane transporters, because it has an N-glycan on one of its extracellular loops.

Ammonium transporters of the Mep/Amt family are polytopic membrane proteins, highly conserved in eubacteria, archaebacteria, fungi, plants and invertebrates.

In vertebrates no Mep/Amt (methylammonium permeases /(methyl-)ammonium transporter) protein has yet been found. In the human

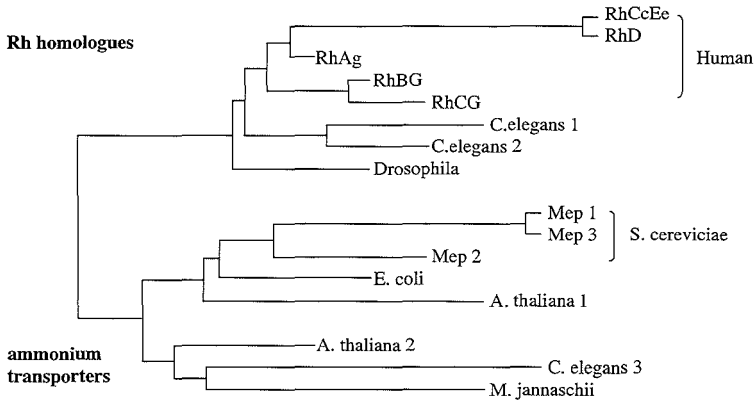


Figure 1.9: The ammonium transport family.
Phylogenetic tree of multiple sequences from human Rh blood group antigens, human Rh glycoproteins, non-human sequences with Rh homology, and ammonium transporters from yeast, bacteria, plants and worms.

genome, the Rh family proteins are the most homologous to Mep/Amt transporters (Liu *et al.*, 2001); a sequence identity of 20-27% exists between them (Marini *et al.*, 2000). The Rh family consists of a primitive group (proteins located in uni-cellular slime molds, multi-cellular protozoans and metazoans), an erythroid group (RhAg and RhD/RhCcEe), and a non-erythroid group (RhBG and RhCG) (Liu *et al.*, 2001) (Figure 1.9). The tissue specificity of RhBG (expressed in kidney convoluted tubules and Henle's loop and in liver and skin) and RhCG (expressed in kidney collecting tubules and in testis seminiferous tubules), along with the sequence similarity to the ammonium transporters raised the possibility that these non-erythroid Rh homologues might be the long sought ammonium transporters in humans (Liu *et al.*, 2001).

1.6.2 RhAg and RhCG transport ammonium bi-directionally in yeast cells

To test whether human RhAg and RhCG are capable of ammonium transport activity, Marini *et al.* performed functional complementation assays in a yeast mutant with deletions in the three endogenous ammonium trans-

porter genes (yeast cells deleted of Mep1, Mep2 and Mep3, further referred to as triple-MepΔ yeast cells). In yeast, the Mep proteins import ammonium as a nitrogen source necessary for growth. The triple-MepΔ yeast cells cannot grow on minimal media that contain less than 5mM ammonium. Triple-MepΔ yeast cells expressing RhAg or RhCG did grow, even at 1mM of ammonium, suggesting ammonium uptake activity. Also an enhanced resistance to toxic concentrations of methylammonium was found in triple-MepΔ yeast cells expressing RhAg, suggesting that RhAg mediates also the export of ammonium (Marini *et al.*, 2000).

1.6.3 RhAg mediates ammonium uptake in Xenopus oocytes

Contrary to yeast cells, RhAg was fully glycosylated when expressed in Xenopus oocytes, suggesting that it was processed appropriately and trafficked correctly to the oocyte plasma membrane.

Oocytes expressing RhAg demonstrated an 8-10 fold increase in uptake of ammonium, measured with the radioactive ammonium analogue ^{14}C-methylammonium. Because the uptake was increased by raising the external pH or lowering the intracellular pH, it was assumed that ammonium uptake might be coupled to proton extrusion. Uptake mediated by methylammonium-H^+ counter transport would therefore be an attractive mechanism to accumulate ammonium without compromising pH (Westhoff *et al.*, 2002).

1.7 Scope of this thesis

The first aim of this study was to define the molecular basis of weak-D phenotype, because that was not yet known at the start of my investigations. However, shortly after the start it was published that amino-acid alterations in *RHD* are present in individuals with the weak-D phenotype. We have investigated whether there is a direct correlation between these amino-acid substitutions and the lower expression of RhD, using an erythroleukemic K562 cell line model (Chapter 4). This eythroleukemic K562 cell line model was furthermore used to investigate the processing of the weak-D antigen in the cell.

Transfusion of RhD-positive blood to individuals with qualitatively altered RhD may result in alloimmunization. Therefore, it is important to know whether the weak-D phenotypes are purely a quantitative variant or whether also qualitative changes are present in the weak-D polypeptide. Because no alloantibodies have been found in individuals with the most common weak-D phenotypes, the weak-D polypeptide was scrutinized by Scatchard plot analysis to detect possible qualitative alterations (Chapter 3). We identified a weak-D phenotype with a high frequency in African blacks, investigated its molecular basis and studied whether it was only a quantitative or also a qualitative variant (Chapter 2). The reason for the heterogenicity of Rh and the differences in haplotypes between different ethnic populations have not been solved yet. Maybe some Rh phenotypes have functional advantages. Already for a long time, a transport function for the Rh protein had been assumed, because of its predicted topology in the cell membrane. After the discovery of the non-erythroid Rh-homologues (RhBG and RhCG), the missing link was identified in the nucleotide sequence similarity between Rh and the ammonium transporter family (Amt). By transfection studies, a transport function of ammonium in yeast cells has been shown for RhAg. The aim of our study was to investigate the involvement of the Rh complex in the transport of ammonium in human red blood cells (Chapter 5).

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Chapter 2

DAR, a new RhD variant involving exon 4, 5 & 7, often in linkage with ceAR, a new Rhce variant; frequently found in African Blacks

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Abstract

The highly polymorphic Rh system is encoded by two homologous genes *RHD* and *RHCE*. Gene rearrangements, deletions, or point mutations may cause partial D and CE antigens.

In this study, a new *RHD* variant, *DAR*, and a new *RHCE* variant, *ceAR*, are described in four Dutch African Blacks. Serologically, DAR showed weaker reactions with a MoAb and a polyclonal antiserum against D. The DAR phenotype was characterized by complete loss of at least 9 of 37 RhD epitopes. RBCs expressing ceAR were all typed as VS^-, V^+.

DNA analysis showed a partial D allele with only three mutations: C602G (exon 4), T667G (exon 5) and T1025C (exon 7). The ceAR allele carried G48C (exon 1), a hybrid exon 5 (A712G, C733G, A787G and T800A) and A916G (exon 6).

To study the frequency of these new variants, a group of 326 South-African Blacks was screened genomically. Of the 326 donors, 16 (4.9%) carried the *DAR* allele, 20 (6.1%) the *ceAR* allele, and 14 (4.3%) both mutated alleles. Five of these donors (1.5%) had the DAR phenotype, indicating that they carried the *DAR* allele homozygously, or next to an RhD-negative allele. Immunogenicity of the RhD antigen for individuals with the DAR phenotype was proven, since one of the four Dutch individuals produced alloantibodies against RhD after multiple transfusions with RhD-positive blood. In a multi-ethnic society, the prevalence of this new RhD phenotype will increase and is therefore relevant in transfusion practice and in prevention of hemolytic disease of the newborn.

2.1 Introduction

The Rh blood group system is clinically important, because antibodies against Rh antigens are involved in haemolytic disease of the newborn, haemolytic transfusion reactions, and autoimmune haemolytic anaemia.

The Rh system is complex; as many as 45 different antigens have been serologically defined (Issitt, 1994), (Daniels, 1995). These antigens are carried by non glycosylated, non phosphorylated polypeptides. The Rh polypeptides are predicted to have 12 transmembrane-spanning domains with intracellular N- and C- termini, resulting in 6 extracellular loops on which the Rh antigens are located (Chérif-Zahar *et al.*, 1990), (Avent *et al.*, 1992). Two highly homologous genes, *RHCE* and *RHD*, encode the Rh antigens. Both genes are localized on chromosome 1p34.3-p36.1 and are inherited together (Chérif-Zahar *et al.*, 1991). *RHCE* gives rise to the C/c and E/e polymorphisms. *RHD* encodes the RhD antigen. Total or partial deletion of the *RHD* gene can result in the RhD-negative phenotype (Colin *et al.*, 1991), (Hyland *et al.*, 1994), (Umenishi *et al.*, 1994), (Faas *et al.*, 1997-I), (Blunt *et al.*, 1994). In non-Caucasians, it has been found that

RhD negativity can appear in individuals carrying the complete *RHD* gene (Daniels *et al.*, 1997), (Okuda *et al.*, 1997).

The most immunogenic Rh antigen is the RhD antigen, comprising at least 30 epitopes (Scott *et al.*, 1996), (Scott, 1996).

Partial-D phenotypes, characterized by loss of epitopes, can arise from replacement of *RHD* exons by their *RHCE* counterparts as has been shown in D^{IIIb}, D^{IIIc}, D^{IVb}, D^{Va}, D^{VI}, DFR and DBT and by point mutations in the *RHD* gene that occur in D^{II}, D^{IVa}, D^{VII}, DHMi, DNU and DHR. Frequencies of D^{VII}, D^{VI}, D^{IV}, D^{V}, D^{II}-like, and DFR are 1:900, 1:6800, 1:10,000, 1:30,000, 1:30,000 and 1:60,000, respectively, as established with serological methods in a Caucasian population (Flegel *et al.*, 1996). Alloantibodies may be produced against missing epitopes in individuals expressing RhD variants, when exposed to the complete antigen by blood transfusion or during pregnancy.

Three types of *RHCE* variants have been described (Faas *et al.*, 1997). Single point mutations are found in VS, V, C^{w}, C^{x} and Rh:26. *RHCE* exon replacements, in which exons of different alleles of *RHCE* are exchanged, were found in $r^{G}r$ and a variant in which exon 1 and intron 1 of the *RHc* allele are replaced by the corresponding part of the *RHC* allele. Finally, replacement of *RHCE* exons by their *RHD* equivalents may occur, as is found in D-- , Dc-, R_{o}^{Har}, R^{N} and partial E. As in *RHD* variants, these exon replacements or mutations not only result in loss of epitopes, but may also account for the formation of new epitopes.

In the present report, we describe a new partial-D antigen, called *DAR*, expressed in four unrelated Dutch women of African Black origin. In these four individuals, also a variant *RHCE* gene, called *ceAR* was found. That more African Blacks are carrier of the mutated *RHD* gene was suggested by the fact that three of these four women were noticed in a routine pregnancy screening. Thereafter, blood was sent for confirmation of the Rh typing to the Central Laboratory for Blood Transfusion (CLB). We also screened 326 African Black donors from the South African Blood Transfusion Service in Johannesburg (South Africa) for *DAR* and *ceAR*.

2.2 Materials and Methods

Samples. EDTA anti-coagulated blood samples were obtained from four unrelated African Black women (identification numbers 3308, 3424, 3895 and 4413). Three samples were noticed because of weak-D expression, during routine pregnancy screening and sent to the CLB for confirmation. A blood sample of individual 4413 was referred to our lab because antibodies were detected. Red blood cells (RBCs) of these individuals were Rh phenotyped according to standard protocols with monoclonal antibody (MoAb) MS-201 (CLB, Amsterdam, The Netherlands), recognizing RhD epitope 6/7 (9 epitope model) equal to RhD-epitope 12 (37 epitope model), a polyclonal reagent (anti-D with bromelain as enhancer, CLB) and MoAbs (all obtained from CLB, Amsterdam, Netherlands) recognizing RhC (MS 24), Rhc (MS 32), RhE (MS 260) or Rhe (MS 21, MS 63). Polyclonal reagents 97-501639 (patient serum) and Q-sera were used to phenotype VS and V, respectively. The presence of the low incidence antigen D^W, thus far only found in D^{Va}, was tested with an anti-Rh23 by C. Green and G. Daniels (International Blood Group Reference Laboratory (IBGRL), Bristol, UK). Phenotyping for partial D was performed with a panel of selected MoAbs with known epitope specificity (Third International Workshop on Monoclonal Antibodies against Red Cell and Related Antigens, 1996, Nantes, France). RBCs of donor 3424 were sent to the IBGRL for confirmation.

In the blood sample of individual 4413, RBC antibodies were present with the specificity of anti-D, -C, -E, $-Fy^a$, Jk^a, M and Sl^a. This individual suffered from sickle cell anemia and received multiple transfusions over the years. Adsorption-elution techniques were used to determine whether the anti-D antibodies were alloantibodies or autoantibodies.

Blood samples of 326 South-African Black donors were randomly collected by the South African Blood Transfusion Service in Johannesburg (courtesy of J. Hooydonk, Johannesburg, South Africa). Seven donors were serologically typed as RhD negative, all other donors were RhD positive.

cDNA sequence analysis. White blood cell-reduced RBCs were enriched with reticulocytes as described before (Murphy *et al.*, 1973), (Simsek *et al.*, 1994). RNA was isolated from the reticulocyte-enriched fraction (Chomzynski et al., 1987). cDNA was obtained by RT-PCR (reverse

transcriptase polymerase chain reaction) using Superscript II RNAse H-RT (Gibco BRL, Gaithersburg, MD), and full-length amplification was performed with consensus primers as described in Table 2.1. PCR products were ligated into pGEM-T vector (system I, Promega, Madison, WI) and the vectors were introduced into competent *Escherichia coli* by electroporation (Sambrook *et al.*, 1989). Inserts were cycle-sequenced automatically (ABI-PRISM 377, DNA sequencer, Perkin Elmer, Norwalk, CT) on both strands.

Genomic DNA analysis. Genomic DNA was isolated from peripheral blood leukocytes with a DNA isolation kit (Puregene, Minneapolis, MN).

Sequence analysis. On genomic DNA, exon-specific PCRs were used. All primers are listed in Table 2.1. Exons 4 to 5 (including intron 4) and 7 were amplified with *RHD*-specific primers (R496/Rex5AD$_2$ and R973/R1068, respectively) and cycle-sequenced automatically (ABI-PRISM 377, DNA sequencer). Exon 5 and exon 6 were amplified with consensus primers (Rex5S$_2$/Rex5A and Rex6S/Rex6A, respectively); PCR products were subcloned and sequenced.

PCR assays. Five PCR allele specific primer amplifications (ASPAs) were designed specifically for detection of mutations. Primerset R31/R147 and internal control primer R-15, all three primers located in exon 1, were used to recognize the *RHC*-specific nucleotide at position 48 (Faas *et al.*, 1996). An ASPA specific for *RHCE* nucleotides at position 602 and 667 (primerset R581/R667) was developed to amplify intron 4. We applied an exon 5 ASPA, using an *RHCE*-specific sense primer (R678) and an *RHD*-specific antisense primer (Rex5AD$_2$) to detect a hybrid exon 5. An exon 5 to 6 ASPA was used to amplify intron 5 with the *RHCE*-specific sense primer R678 in exon 5 and the *RHD*-specific antisense primer R933 in exon 6. An exon 6 to 7 ASPA (primerset R973/R1044) with an *RHD*-specific sense primer in exon 6 and an *RHCE*-specific antisense primer in exon 7 was developed to detect the *RHCE*-specific mutation in *RHD*-exon 7.

RHD-specific multiplex PCR. RHD exons 3, 4, 5, 6, 7 and 9 were amplified with *RHD*-sequence-specific primers in a one-reaction mixture assay as described before (Maaskant-van Wijk *et al.*, 1998).

Restriction fragment length polymorphism of RH intron 5. To determine the origin of the intron 5 of the *ceAR* allele, intron 5 was amplified

Primer		sequence	Annealing time and temperature	amplicon	specificity
R-15	sense	5'tatctagagacggacacaggATGAGC3'	1' 60 °C	exon 1	consensus
R31	sense	5'CGCTGCCTGCCCCTCTGC3'		exon 1, *C* specific	*C* specific: nt 48
R147	antisense	5'TTGATAGGATGCCACGAGCCCC3'			consensus
R496	sense	5'CACATGAACATGATGCACA3'	1'55 °C	exon 4 to 5	*D* specific: nt 514
$Rex5AD_2$	antisense	5'cacCTTGCTGATCTTACC3'			*D* specific: nt 787
R581	sense	5'ACGGAGGATAAAGATCAGAG3'	1'55 °C	intron 4, *CE* specific	*CE* specific: nt 602
R667	antisense	5'CTCAGCAGAGCAGAGTTGAC3'			*CE* specific: nt 667
$Rex5S_2$	sense	5'cctctctggccccaggCGCC3'	1'55 °C	exon 5	consensus
Rex5A	antisense	5'cagcgccctgctcac3'			consensus
R678	sense	5'CTGCTGAGAAGTCCAATCC3'	1'55 °C	exon 5, *CE-D* hybrid	*CE* specific: nt 707
$Rex5AD_2$	antisense	5'cacCTTGCTGATCTTACC3'			*D* specific: nt 787
R678	sense	5'CTGCTGAGAAGTCCAATCC3'	1'55 °C	exon 5 to 6, *CE-D* hybrid	*CE* specific: nt 707
R933	antisense	5'GTACTTGGCTCCCCCGAC3'			*D* specific: nt 916
R716	sense	5'TCAACACCTACTATGCTG3'	1'55 °C	intron 5	VS&*D* specific: nt 733
R870	antisense	5'AGAAGGGATCAGGTGACACG3'			consensus
Rex6S	sense	5'gctatttctttgcag3'	30''48 °C	exon 6	consensus
Rex6A	antisense	5'tgtctagtttcttca3'	α taq added		consensus
R973	sense	5'AGCTCCATCATGGGCTACAA3'	1'67 °C	exon 6 to 7, *D-CE* hybrid	*D* specific: nt 992
R1044	antisense	5'CACCAGCAGCACAATGTAGG3'			*CE* specific: nt 1025
R973	sense	5'AGCTCCATCATGGGCTACAA3'	1'55 °C	exon 7	*D* specific: nt 992
R1068	antisense	5'ATTGCCGGCTCCGACGGTATC3'			*D* specific: nt 1068
R-15	sense	5'tatctagagacggacacaggATGAGC3'	1.5'55 °C	Full Length cDNA	consensus
R1339	antisense	5'gcgtttctcacgtacaaatgc3'			consensus

Table 2.1: Nucleotide sequences and positions of primers.
The sequences of the oligonucleotides are given in capital letters when exon sequences are indicated and in small letters when intron sequences are indicated.

with sense primer R716 (specific for nt 733G, present in the *RHCE* allele of VS^+ individuals and in the *RHD* allele) and antisense primer R870 (consensus primer). This product was *RHD*-specifically digested with restriction enzyme *ApaI* (New England Biolabs Inc., Beverly, MA) and analyzed by electrophoresis in a 1% agarose gel.

Southern blot analysis. 10 μg DNA of all donors was digested with the endonuclease *BamHI* and after electrophoresis transferred to a nitrocellulose membrane. Blots were hybridized with a ^{32}P-labelled *RH* full-length cDNA (kindly provided by Dr. D. Anstee, IBGRL). The results were visualized by autoradiography.

PCR conditions. All PCR assays were performed in a Perkin-Elmer Cycler Model 480 (Norwalk, CT) on 200 ng of cDNA or gDNA in a total volume of 50 μL. Reaction mixtures contained 50 ng of each primer, 0.2 mM of each dNTP (Pharmacia) and 2 U of Taq DNA polymerase (Promega) in the appropriate buffer, supplemented with 1.5 mM $MgCl_2$. PCR conditions were 1 cycle of 5 minutes at 95^0C, followed by 35 cycles of 1 minute at 95^0C, with an annealing time and temperature as described in Table 2.1 and, depending on the size of the expected product, the extension time at 72^0C varied between 45 seconds and 2.5 minutes. Extension was completed during 5 minutes at 72^0C.

2.3 Results

Serology. Individuals 3308, 3424, 3895, and 4413 were serologically typed as C^-, c^+, E^- and e^+, VS^- and V^+. RBCs of these four individuals showed weaker reactions with anti-D MoAb MS-201 and polyclonal anti-D antiserum than did normal RhD-positive control cells. Therefore, with restricted screening protocols, these donors might be considered as expressing weak D. However, extensive serological studies of all four individuals showed a new partial-D pattern (see Table 2.2), in which 9 of the 37 epitopes were completely missing and 6 of the 37 epitopes showed different results with several MoAbs. These results were confirmed by Joyce Poole's laboratory of the IBGRL. RBCs of these four donors did not carry the low incidence antigen D^W.

epitope-1-9	model 1-37	Nantes	DAR
1	1	LHM169/81	±
1	2	LHM70/45, LHM174/102	-
2	3	LOR12-E2, LORE	±
2	4	LOR28-7E6	-
3	5	LOR11-2D9, LHM76/55, H41.11B7, H41	+
4	6	LOR17-6C7	+
5	7	CAZ7-4C5	-
		HIRO-6	±
5	10	AUB-2F7/Fiss, MAR-1F8	-
		C205-29, CLAS1-126, BS229, BS231	±
5	11	819	-
6/7	12	175-2, LOR17-8D3, P3AF6, RUM-1, MS201, P3x61	±
6/7	13	D-89/47, 17010C9, LOR28-21D3, H2D5D2F5	±
		F5S	+
6/7	15	D-90/7, D-90/17, NOI, SAL20-12D5, LHM50/2B, LHM169/80, BIRMA-DG3, BIRMA-D56, L87.1G7, D10	±
6/7	17	HeM-92, NaTH53-2A7, P3F17, HIRO-I, HS114	-
		B9A4B2, NaTH28-3C11, BS232	±
6/7	18	LHM59/20, T3D2F7, P3F20	-
		HG/92, LHM50/3.5, LHM59/25, HM10	±
		ID6-H8	+
6/7	21	VOL-3F6	-
		D-90/12, BRAD5	±
		HIRO-2	+
8	22	LHM59/19	-
9	23	P3x21223B10	-
		BIRMA-D6, P3G6, MS26, HIRO-4, HIRO-7, HIRO-8	±
	31	NOU	-
	32	ZIG-189	-
	33	NaTH87-4A5	-
	34	LORA	-
	35	SALSA-12	-
	36	NAU3-2E8, BTSN6, LHM76/59	±
		NAU6-4D5	+
	37	822, HIRO-3	+

Table 2.2: Epitope models (9 and 37) with the reaction pattern of the new partial D, tested with MoAbs described in the Nantes workshop (1996).
The serology was performed as described before (Avent *et al.*, 1997). This pattern was found in all nine individuals expressing *DAR* homozygously or next to a RhD-negative allele. These results were confirmed by Joyce Poole's laboratory of the IBGRL
- (or +) = negative (or positive) MoAb reaction
± = positive MoAb reaction, but weaker than that of normal RhD-positive contol cells

The antibodies present in the serum of patient 4413 were characterized with the adsorption-elution technique. It was shown that antibodies directed against RhD were present, among other antibodies. With agglutination studies it was shown that these antibodies did not react with her own RBCs, nor with the RBCs of other donors with the DAR phenotype, but did react with RBCs from normal RhD-positive donors, indicating that these antibodies are alloanti-D.

cDNA sequence analysis. Sequencing of cDNA from one individual (identification number 3424) showed the presence of three different transcripts (Figure 2.1). At least three clones per different transcript were completely sequenced.

1. A normal *RHce* transcript.
2. An *RHce*-like transcript carrying G48C (Trp16Cys) in exon 1; A712G (Met238Val), C733G (Leu245Val), A787G (Arg263Gly) and T800A (Met267Lys) in exon 5 and A916G (Ile306Val) in exon 6.
3. An *RHD*-like transcript carrying C602G (Thr201Arg) in exon 4, T667G (Phe223Val) in exon 5 and T1025C (Ile342Thr) in exon 7.

Genomic DNA analysis. To confirm the mutations found in cDNA of individual 3424, as well as to show the presence of the mutations in the other three individuals 3308, 3895 and 4413, analysis on genomic DNA was performed. All of these results were in full concordance with the cDNA analysis.

From individual 4413, not only the exons of interest, but all exons were amplified from genomic DNA and subsequently cycle sequenced. No other mutations were found.

The mutations found in the *RHce*-like transcript were confirmed on genomic DNA by an ASPA which recognized the *RHC*-specific nucleotide at position 48, by sequence analysis of *RHce*-exon 5 which showed the mutations A712G, C733G, A787G and T800A; and by sequence analysis of *RHce*-exon 6 which showed A916G. The *RHce* origin of intron 5 was indicated by a remaining uncut part, after *RHD*-specific digestion with *ApaI* of the 1791bp product, obtained after *RHD/VS*-specific amplification of intron 5.

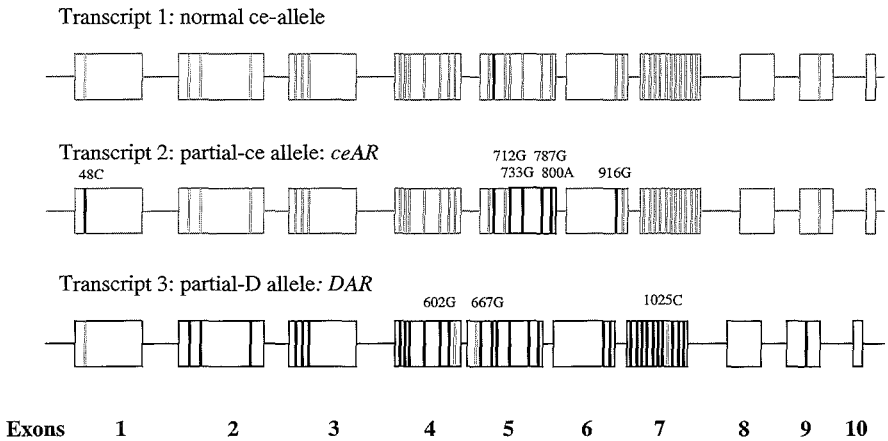

Figure 2.1: Transcripts as found by sequencing cDNA.
Nucleotides derived from the *RHCE*-specific sequence are represented by a grey bar, whereas nucleotides derived from the *RHD*-specific sequence are represented by a black bar. *RHD*-specific exons or exon parts are represented by a black outer line, whereas those of *RHCE* are represented by a grey outer line.

The mutations found in the *RHD*-like transcript were detected by sequence analysis of *RHD*-exon 4 to 5 and exon 7. Beside the mutations C602G and T667G, sequence analysis revealed a normal *RHD*-intron 4 (Avent *et al.*, 1997) and the T1025C mutation in *RHD*-exon 7. To show that only the mutated *RHD* transcript was present, in the absence of a normal *RHD* allele, the *RHD*-specific multiplex was performed. From all four DNA samples the 200bp internal control fragment from the β-actin gene was amplified. Amplification products from exons 3, 6, 7 and 9 were detected; whereas exons 4 and 5 were not amplified.

Southern blot analysis. The *BamHI* digestion pattern of the genomic DNA from the four individuals (identification numbers 3308, 3424, 3895 and 4413) showed no difference in the Rh patterns compared to a normal RhD-positive donor.

Screening of the South-African Black donors. In 56 of the 326 South-African Black donors (17.2%), the *RHCE* intron 4 PCR demonstrated the two *RHCE* specific nucleotides in the exon 4 and 5 of the *RHD* gene, with an *RHD* intron 4 in between. All 56 of these donors were sero-

logically RhD positive. In 16 of these 56 donors, the presence of T1025C was proven by the *D-CE* hybrid exon 6 to 7 PCR, indicating the presence of the *DAR* allele.

Because a linkage between the *DAR* and the *ceAR* allele was assumed, we tested all 326 donors for the presence of the *ceAR* allele. In 20 of the 326 donors (6.1%) the *CE-D* hybrid exon 5 PCR and the *CE-D* hybrid exon 5 to 6 PCR gave positive results, indicating the presence of a *ceAR* allele. Of those 20 donors, 14 were also carrier of the *DAR* allele. Thus, 2 donors carried the *DAR* allele without the *ceAR* allele and 6 donors carried the *ceAR* allele without the *DAR* allele.

Five of the 14 donors carrying the *DAR* and *ceAR* allele (1.5%) had the deviant *RHD* multiplex PCR pattern, missing exon 4 and 5. This indicates that these 5 donors were either homozygous for the *DAR*, or carried *DAR* on one allele and lacked *RHD* on the other. The results found on gDNA of these five donors were serologically confirmed and gave with all five the same 37 epitope pattern as shown in Table 2.2.

In DNA of the 40 donors only carrying the C602G and T667G mutations in exon 4 and 5 of the *RHD* gene and not the T1025C mutation in exon 7, the *CE-D* hybrid exon 5 PCR and the *CE-D* hybrid exon 5 to 6 PCR gave negative results, indicating the absence of the *ceAR* allele. One of these 40 donors showed the deviant *RHD* multiplex PCR pattern, missing exon 4 and 5.

2.4 Discussion

In this study, a newly discovered RhD variant named DAR that occurs frequently in African Blacks, is described. This new RhD variant consists of an *RHD* allele with three point mutations on polymorphic sites, in which *RHD*-specific nucleotides are replaced by *RHCE*-specific ones. These mutations are located in exon 4 (nt602), exon 5 (nt667) and exon 7 (nt1025). The four probands also had a variant *RHce* allele, called *ceAR*. This allele had an *RHC*-specific mutation on nt48 in exon 1, a hybrid exon 5 in which the polymorphic sites between nt712 and nt800 were replaced by *RHD*-specific nucleotides, and an *RHD*-specific point mutation on polymorphic site nt916 in exon 6.

This new RhD variant was previously described by us, as ARRO-I

(Hemker *et al.*, 1998). At that time, sequence analysis was not completed. Further analysis revealed the mutation in exon 7 and the mutant *RHce*-allele in these individuals.

Serologically, this new RhD variant showed weaker reactions with a monoclonal anti-D and with polyclonal antiserum used for routine screening, indicating weak-D expression. The genomic features of the new variant have much resemblance with the recently described weak D type 4, in which also the mutations C602G and T667G are present, but the mutation T1025C is not (Wagner *et al.*, 1999). However, with extensive serological testing, the RhD characteristics of the DAR phenotype gave a different, not previously described pattern of serological reactions with MoAbs. The loss of epitopes, as well as the finding of alloanti-D formation in an individual with the DAR phenotype, indicates that we are dealing with a new qualitative RhD variant with low expression. In one of the 40 South African Black donors in whom PCR-based analysis showed the mutation in exon 4 and 5 but not in exon 7, the expression was not masked by the presence of a normal *RHD* gene. This donor is expected to present the weak D type 4 phenotype. Preliminary serological results of this donor suggest a quantitative instead of a qualitative loss of epitopes, as can be expected from the results of Wagner *et al.* (1999). This is an intriguing observation in perspective of the effect of the mutation in exon 7 for the loss of epitopes. An explanation for this phenomenon can perhaps be found in the fact that the mutations in exon 4 and 5 do not change the polarity of the amino acids in the protein. In contrast, the transmembranal mutation in exon 7 causes the incorporation of a hydrophilic amino acid (Thr) instead of a hydrophobic amino acid (Ile). This might result in a severe change in the conformation of the Rh protein, explaining the described loss of epitopes. In future, transfection studies will be performed to study this phenomenon in detail.

The loss of so many RhD epitopes in DAR is the result of only three mutations in *RHD*, because from the variant Rhce-allele only addition of RhD epitopes can be expected. The mutations at the 3'end of exon 4 and the 5'end of exon 5 of the *RHD* allele are due to point mutations. This is suggested by the presence of a normal *RHD* intron 4, as shown by sequence analysis. However, aberrant nucleotides may occasionally be introduced if incorrect mismatch repair of the heteroduplex DNA takes place during

gene conversion (Huang *et al.*, 1995). So, the *DAR* mutations on the 3'side of exon 4 and the 5'side of exon 5 also could be due to the occurrence of heteroduplex repair, rather than to spontaneous point mutations. The same phenomenon has been described for the glycophorins A and B, which are also encoded by highly homologous genes (Huang *et al.*, 1995).

The variant *RHce*-gene, *ceAR*, is characterized by a mutation in exon 1, a hybrid exon 5, and a mutation in exon 6. The presence of G48C (Trp16Cys), the *RHC*-specific nucleotide in exon 1, without expression of this antigen, frequently occurs in African Blacks (Faas *et al.*, 1999). The *RHD*-specific nucleotide found in exon 6 of the *RHCE* allele is probably caused by a point mutation or might be due to heteroduplex repair (see above), because restriction site analysis suggested a normal *RHce*-intron 5. Besides the *ceAR* allele also a complete *RHce*-allele was found, so it was not possible to test the loss of Rhce-epitope expression. All four individuals were expressing the rare VS^-, V^+ phenotype. Daniels has previously described that the *VS* mutation (G733, in exon 5) surrounded by *RHD*-specific nucleotides in exon 5 and the *V* specific nucleotide 1006G (Gly336), provided the VS^-, V^+ serotype (Daniels *et al.*, 1998). In this study, *RHCE* exon 6 was not sequenced. Therefore, four VS^-, V^+ samples of African Black donors, provided by Dr. G. L. Daniels (IBGRL) were sequenced, and indeed, these samples also showed the mutation in exon 6 as well as the *RHC* mutation (G48C). These results suggest that the most common genetic basis of the VS^-, V^+ phenotype is the ceAR variant. Furthermore, also these four donors carried the *DAR* allele.

The four original probands in whom this new serological reaction pattern was found, all proved to be of African Black origin. No Caucasians carrying this variant have so far been found during routine screening. This suggested that more African Blacks might be carriers of these genes. Therefore, a group of 326 South-African Blacks was screened by genomic PCR. We found that 4.9% of this group are carriers of a *DAR* allele. Five donors (1.5%) had the gene homozygous or in combination with a RhD-negative allele, as was shown by the absence of the amplification products from exons 4 and 5 in the multiplex PCR. These donors also had the same serological partial-D pattern as was shown in the 37 RhD-epitope model. The fact that so many South African Blacks are carriers of this gene suggests that these donors have an evolutionary advantage, as described for Duffy involving

malaria (Gelpi *et al.*, 1976). So far, no correlations between Rh phenotypes and the occurrence of malaria have been found. However, these studies have been performed on immunologically recognized antigens, whereas this new variant is primarily recognized at the DNA level. The frequency of the DAR phenotype (1.5%) in the African Black population is much higher than the frequency of D variants in the Caucasian population (0.1% - 0.001%), and therefore might have an impact on monoclonal reagent design. Testing the African Blacks for the presence of the ceAR revealed that 6.1% were carriers of this allele.

The finding that the first four individuals tested expressed both variant alleles suggested that these two genes were inherited en bloc. By screening the 326 African Blacks, we expected to find only donors, carrying both mutated alleles or both normal alleles. The screening results did not confirm this idea, because besides donors with both variant alleles, also donors with only the *DAR* or *ceAR* allele were found. Nevertheless, the incidence of the combination is much higher than expected to occur by chance, indicating linkage of *DAR* and *ceAR*.

Individuals with the DAR phenotype may form anti-D antibodies when exposed to a complete RhD antigen. Despite the loss of so many epitopes, a complete RhD antigen does not seem to be highly immunogenic for individuals expressing DAR. Otherwise, the highly frequent DAR variant should have been recognized much earlier. A possible explanation for this low-responsiveness may be that the footprints of most anti-D antibodies are related to one another, as was published recently (Chang *et al.*, 1998). It is postulated that, in the alloimmune response against the RhD antigen in different individuals, a similar and restricted "pathway" is used. It is tempting to speculate, that in this variant, the apparent low immunogenicity of the complete RhD antigen is due to the fact that the most common "pathway" could not be used, because these B cells have been clonally deleted or have become anergic to avoid self-reactivity. Probably a less common "pathway" has produced the anti-D made by the multi-transfused donor 4413.

The clinical significance of these anti-D antibodies in this individual is not clear yet. Nevertheless, pregnant women and recipients of blood transfusion expressing the DAR variant should be regarded as RhD negative. As donor, people expressing DAR should be carefully distinguished from RhD-

negative donors by the use of selected reagents, because alloimmunization is likely to occur when administering RBCs expressing the DAR variant to RhD-negative recipients. Therefore, donors expressing DAR should be regarded as RhD positive. Hemolytic disease of the newborn could occur in fetuses expressing DAR when carried by an immunized RhD-negative mother, or in fetuses with the complete RhD antigen carried by mothers with the DAR phenotype. Therefore, in the future, anti-D monoclonals for immunoprophylaxis should be guaranteed to cover this new variant, especially because of the high incidence of this new variant in a multi-ethnic society, in which RhD negativity is found more frequently than in the original African Black population. In addition, the chance of getting a population with an even higher frequency of people expressing DAR is increased, because the expression will not be masked by the presence of a normal *RHD* allele.

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Chapter 3

No altered RhD-protein structure and transcription level for the weak-D phenotype

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Abstract

About 0.2% to 1% of the Caucasian population have RBCs with a reduced expression of the RhD antigen (weak D). Whether the weak-D phenotypes (caused by 22 different *RHD* alleles, with one or more point mutations) are purely quantitative variants or also have qualitative variations, is important for transfusion policy.

The lower expression of RhD in the weak-D phenotype is not due to a change in the amount or stability of *RHD* mRNA, as was shown by RQ-PCR in this study. By flowcytometry, no significant changes in expression of other proteins of the Rh complex were found, contributing to or caused by the weak-D antigen.

A low Rhesus similarity index (RI), which is assumed to indicate qualitative alterations, was measured in partial and weak-D

phenotypes by flowcytometry. Scatchard plot analysis and flowcytometry confirmed a significant lower epitope density on RBCs with the weak-D phenotype compared to the normal RhD-positive phenotype. But Scatchard plot analysis did not confirm possible qualitative alteration of the weak-D antigen. The affinity of each of the 7 MoAbs tested was not significantly different for the weak-D antigen from that found for the normal RhD antigen. The low RI is not a good tool to observe qualitative alterations, because differences in epitope densities can be due to differences in affinity of antibodies, as was shown by Scatchard plot analysis.

3.1 Introduction

The Rh blood group system is clinically important, because antibodies against Rh antigens are involved in haemolytic disease of the newborn, haemolytic transfusion reactions and autoimmune haemolytic anaemia.

Variations in RhD expression are either qualitative or quantitative, or a combination of these two. Red blood cells (RBCs) expressing qualitative variations (partial D) lack or gain epitopes, or some epitopes are changed in such a way that they seem to be present, but are less well recognised by the antibody. Individuals expressing a partial-D phenotype are able to form alloantibodies against the missing epitopes, when they encounter the complete RhD antigen. RBCs expressing quantitative variations can be divided in those with increased expression (e.g. D--) and those with decreased expression (weak D).

About 0.2% to 1% of whites have RBCs with the weak-D phenotype. Because serologically it has been impossible to show qualitative differences between normal RhD and weak-D RBCs, it has become generally accepted that most weak Ds, if not all, possess a normal RhD antigen. Indeed, so far donors with the most common weak-D phenotypes do not form alloantibodies when the immune system gets in contact with the complete RhD antigen.

A purely quantitative variation could be due to altered transcription activity of the *RHD* gene, or to reduced mRNA stability. The amount of *RHD* mRNA in reticulocytes of donors carrying the weak-D phenotype was measured before with a semi-quantitative PCR (Rouillac *et al.*, 1996,

Beckers *et al.*, 1997). Rouillac *et al.* reported reduced steady-state levels of *RHD*-mRNA transcripts in donors with a weak-D phenotype compared to levels of *RHD*-mRNA transcripts in donors with a normal expression of RhD. This is in contrast to Beckers *et al.*, who found no differences in the amount of *RHD*-mRNA transcripts between normal and weak-D donors. To solve these discrepant results, we now determined the amount of *RHD*-mRNA transcripts in reticulocytes of donors with weak-D phenotypes once again, but now more precisely with real-time quantitative PCR (RQ-PCR).

Wagner *et al.* (1999) have shown that the weak-D phenotype is caused by many different *RHD* alleles encoding aberrant RhD proteins. In all 22 different *RHD* alleles described for the weak-D phenotype, there are one or more point mutations located in the transmembrane or intracellular part of the RhD protein (http://www.uni-ulm.de/~wflegel/RH/, Wagner, Blood 1999). However, it is unclear whether these point mutations alter the exofacial structure of the RhD polypeptide, or only affect the formation of the Rh complex or the integration of the RhD polypeptide into the membrane without conformational changes in its extracellular loops. This distinction is important for transfusion policy. So far, no definite proof for qualitative variation has been obtained, since alloantibodies have never been found in individuals carrying the most common weak-D phenotypes. Alloantibodies have been found in two donors with weak-D phenotypes that are not frequently occurring (Wagner *et al.*, 2000). One of these two donors carried the weak-D type 4.2, a genotype we like to refer to as the partial D DAR. The other donor carried the weak-D genotype 15, which might possibly also be a partial D.

But, in a subsequent study, Wagner *et al.* (2000) suggested that these point mutations lead to qualitatively altered RhD antigens. This was based on the observation that in the different weak-D types the reduction of expression, as measured by flowcytometry, is not observed to the same extent for all epitopes, as expressed with the Rh similarity index (RI). The RI is calculated as the ratio of the 10 percentile to the 90 percentile of the epitope densities detected with 59 anti-D MoAbs. As these studies were only done by flowcytometry with a single antibody concentration, it cannot be formally excluded that differences in affinity and/or concentrations of the antibodies are responsible for the differences in the RI. Therefore, in the present study we investigated by means of Scatchard plot analysis if

qualitative changes in the expression of the RhD protein of the two most common weak-D antigens (weak-D type 1 and weak-D type 2) occur.

3.2 Materials and Methods

Samples. Blood samples anticoagulated with EDTA were collected from Caucasian blood donors and characterised. RBCs of donors with the weak-D phenotype (3-59, 3-62, 3-63, 3-66, 3-69, 3-77, 3-78, 3-82) were formerly used by Beckers *et al.* (1997). For Scatchard plot analysis also RBCs from other donors with the weak-D phenotype (40-1415, 40-1416, 40-1507, 40-1511, 40-1514, 3-5166, 3-5328 (RBCs of this last donor were kindly provided by W.A. Flegel, Ulm, Germany) were used. RBCs were used of five donors with the D-- phenotype (3-5062, 3-2070, 3-2288, 3-2499, 3-2677), of one donor carrying the Rh_{null} phenotype (3-980), of two donors with the D^{VI} type III (40-1510, 40-1517), of four donors with the DAR phenotype (3-5089, 3-5093, 3-5064, 3-5126) and of two donors with the DFR phenotype (3-5008, 3-5106). RhD-positive RBCs were used from blood donors with phenotypes R2R2 (n=1), R1R2 (n=6), R1R1 (n=9), R2r (n=2), R1r (n=12), R0r (n=1), and RhD-negative RBCs of blood donors with phenotype rr (n=2) were used.

Genomic DNA. Genomic DNA was isolated from peripheral blood leukocytes with a DNA isolation kit (Puregene, Minneapolis, MN, USA)

Four allele-specific primer amplifications (ASPAs) were designed specifically to detect weak-D types 1 to 4. For weak-D type 1 (nt809 T→G), allele specific-sense primer WeakD1 (5'GCTATTTCTTTGCAGACTTATGG3') and D-specific antisense primer RexAD3 (5'TGTCTAGTTTCTTACCGGCAAGT3') were used. For weak-D type 2 (nt1154 G→C), allele specific sense primer WeakD2 (5'TATGCATTTAAACAGC3') and antisense primer Rex9a (5'GGTGAAAAATCTTAC3') were used. For weak-D type 3 (nt8 C→G), allele specific sense primer WeakD3 (5'AGACGGACACAGGATGAGCTG3') and antisense primer R127 (5'CCTTTTGATCCTCTAAGGAAGC3') were used. And for weak-D type 4 (nt602 C→G, nt667 T→G and nt819 G→A), CE-specific sense primer R581 (5'ACGGAGGATAAAGATCAGAG3') and CE-specific antisense primer R667 (5'CTCAGCAGAGCAGAGTTGAC3') were used, discriminating between *RHD* and

RHCE by their difference in length of intron 4. Primers specific for β actin (sense primer βACTs: 5'CCTTCCTGGGCATGGAGTCCTG3' and antisense primer βACTas: 5'GGAGCAATGATCTTGATCTTC3') were added as internal control. All ASPAs were performed in 50-μL mixtures and contained 200 ng of genomic DNA, 1.5 mM $MgCl_2$, 200 μM of each dNTP (Pharmacia, Uppsala, Sweden), 300 nM of each primer (Gibco BRL, Gaithersburg, MD) and 2 U of Taq polymerase (Promega, Madison, WI), pre-incubated with TaqStart antibody (Clonetech Laboratories Inc, Palo Alto, CA, USA) The amplification was performed in a thermal cycler (Perkin-Elmer Applied Biosystems, model 9700, Shelton, CT) and consisted of 5 minute 95^0C, followed by 35 cycles of 1 minute at 95^0C, 1 minute of 50^0C to 64^0C (64^0C for weak-D type 1 and 3, 50^0C for weak-D type 2 and 59^0C for weak-D type 4), 1 minute at 72^0C and 5 minutes of 72^0C.

Sequence analysis. White blood cell-reduced RBCs were enriched for reticulocytes as described before (Murphy, 1973), (Simsek *et al.*, 1994). mRNA was isolated from the reticulocyte-enriched fraction by standard procedures (Chomzynski *et al.*, 1987). Each time mRNA was isolated from reticulocytes of weak-D donors, mRNA from reticulocytes of normal RhD-positive donors was isolated at the same time. cDNA was obtained by reverse transcriptase. 1 μg of RNA in a total reaction volume of 20 μL (containing 1x First strand buffer, 20 mM DTT, 1 mM dNTP's, 1 μg random primers, 20 U RNAse OUT and 100 U MMLV-enzyme (Promega) was incubated for 60 minutes at 42^0C. After inactivation for 3 minutes at 99^0C, 30 μL of H_2O was added to make a total volume of 50 μL.

Full-length amplification on cDNA was performed with consensus primers as described before (Hemker *et al.*, 1999]. PCR products were ligated into pGEM-T vector (Promega) and the vectors were introduced into competent *Escherichia coli* by electroporation (Sambrook *et al.*, 1989). Inserts were cycle sequenced automatically (ABI-PRISM 377, DNA sequencer; Perkin-Elmer, Norwalk, CT) on both strands.

Quantification of cDNA by real time quantitative PCR (RQ-PCR). All primers and probes used in the RQ-PCR were designed with the Primer Express version 1.0 software (PE Biosysytems, Foster City, CT, USA) and the Oligo 6.1 software (Dr W. Rychlik, Molecular Biol-

ogy, Cascade, CO) according to the manufacturer's guidelines. cDNA specific primers and probes were developed for *RHD*, located in exon 6 and 7 (sense primer Taq 915F: 5'CGTCGGGGGAGCCAAGTA3', antisense primer Taq 1064R: 5'CCGGCTCCGACGGTATC3' and probe Taq 986T: 5'CCCACAGCTCCATCATGGGCTACAA3') To correct for differences in the total amount of RNA input and for RT efficiency, the quantity of *RHD* transcript in RBCs was "normalized" to the amount of *KELL* (sense primer Kell 1142F: 5'TGGAATATTTGAAAAACATGTCACAA3', antisense primer Kell 1380R: 5'CTCGAAGAACGTGCCTGTCTC3', and reverse probe Tr-Kell 1260: 5'CTTTCTGCGTGCCTCCTGGAATTGA3'), and in K562 cells to the amount of Cyclophilin (Control gene plate, Applied Biosystems).

For RQ-PCR analysis the Taqman 1000 Reactions Gold with buffer A kit (PE Biosystems) was used. Reaction mixtures of 50 μL contained the Taqman buffer A with the ROX dye as a passive reference, 5 mM $MgCl_2$, 200 μM of each dNTP's, 300 nM primers, 100 nM probe, 1.25 U AmpliTaq Gold (PE Biosystems) and cDNA made from 10 ng of mRNA of reticulocytes of weak-D donors and normal RhD-positive and RhD-negative donors or K562 cells.

To quantify, a standard curve was made in each run from a ten-fold dilution of one of the samples, ranging from cDNA made of 100 ng to 0.00001 ng of RNA.

Serologic Analysis. Innitially, the weak-D samples were typed negative with polyclonal anti-RhD in direct testing, but all were positive in the indirect antiglobulin test (IAT). All weak-D samples gave a weak reaction with MoAb MS-201(CLB)

The expression of different epitopes was tested in the indirect agglutination assay following standard procedures (as described before (Avent *et al.*, 1997)) with 70 selected monoclonal antibodies (MoAbs) with known epitope specificity (Third International Workshop on Monoclonal Antibodies against Red Cell and Related Antigens, 1996, Nantes, France)

Flowcytometry. Expression of RhD and parts of the Rh complex on RBCs was tested by FACS analysis. Murine monoclonal antibodies (MoAbs) BRIC 69 (anti-Rh29, kindly donated by Neil Avent, Bristol, UK),

5W2 (anti-Rh29, SBTS, Glasgow, Scotland), BS46 (19W3), BS56 (19W4) (anti-LW^{ab}, Biotest AG, Dreieich, Germany), LA18.18, 2D10 (anti-RhAg, CLB, Amsterdam), BRIC 126 (anti-CD47, F Spring, UK) FuJ30 (anti-Kell, Hisami Ikeda, Japan) and the human MoAb FLOS2/2 (anti-RhD, CLB, Amsterdam) were used. For each MoAb the optimal concentration was determined by means of serial dilution. Antibody binding was demonstrated by Fluoresceine IsoThio Cyanate (FITC)-labelled goat-anti-human-Ig Fab fragments (Jackson ImmunoResearch), FITC-labelled goat-anti-mouse-Ig or PhycoErythrin (PE)-labelled goat-anti-mouse-Ig (DAKO A/S, Glostrup, Denmark).

150,000 RBCs (concentration of $3*10^6$/mL) or 100,000 K562 cells were incubated with one of the MoAbs for 30 minutes, followed by incubation with either FITC-labelled goat-anti-human-Ig, FITC-labelled or PE-labelled goat-anti-murine-IgG for another 30 minutes. All incubations were performed at room temperature. Cells were washed with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA). Flow-cytometric analysis of at least 10,000 cells was performed with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

To compare epitope densities measured with different MoAbs in one experiment on different RBCs, flowcytometry was also performed with six of the MoAbs also used in the Scatchard plot experiments (HIRO 2 (III-1-122), HIRO 55 (123), L87 1G7 (108), P3G6 (101), P3AF6 (102) and 170 10C9 (136)) and a selection of MoAbs (6D10, HIMA 35, 415 1E4, OSK3-3, BRAD 8, 5C8, HIRO95) used in the experiments of Wagner *et al.* (2000). All these MoAbs were obtained from the Third and Fourth International Workshops on Monoclonal Antibodies Against Red Blood cell and Related Antigens. The calculation of epitope densities was based on the median fluorescence detected with 'test RBC' relative to the 'standard RBC' (with the R1R2 phenotype, known epitope density 27,500 antigens/cell). These calculations were performed with the spreadsheet version 1.1 <http:/www.uni-ulm.de/~wflegel/RH/Paris2001/>. Epitope densities, as calculated from flowcytometry experiments, performed by Flegel *et al.* were personally obtained.

Scatchard plot analysis. Seven IgG anti-RhD MoAbs were used: HIRO-2 (epitope specificity (e.s.) 6.7 in the new epitope model, according to the Fourth International Workshop, Paris (= e.s. 6.7-IV)), HIRO-55 (=D6D02 e.s. 2.1-IV) (both kindly provided by Dr. Uchikawa, Japanese Red Cross Central Blood Centre, Tokyo, Japan) H41-11B7 (e.s. 3.1-IV), L87 1G7 (e.s. 6.3-IV) (both kindly provided by Dr. A. Rapaille, BTS, Liège, Belgique), P3G6 (e.s. 9.1-IV), P3AF6 (e.s. 6.1-IV) (both kindly provided by Dr. A. Martin, CRTS de Rennes, France) and 170 10C9 (e.s. 6.2-IV) (kindly provided by W. M. Eberlie, Dominion Biologicals Limited, Dartmouth, Nova Scotia, Canada). All MoAbs used for this assay were purified by passage through a protein A bead column, and labelled with the Ionogen method. Specific activity of the labelled antibody was determined by means of trichloric acid precipitation before and after dialysis.

A two-fold serial dilution, starting at a concentration of 250 nM to 0.5 nM, of the radioactive labelled antibody was made. An equal volume with $7x10^6$ RBCs were added and incubated at 37^0C till equilibrium had been reached (120 minutes for all MoAbs, as determined with an association experiment for each MoAb). To prevent precipitation of RBCs during incubation, reaction tubes were mixed every 15 min. Unbound ^{125}I-MoAb was removed by spinning the RBC suspension through a layer of 20% (w/v) sucrose / 0.1% (w/v) bovine serum albumin (BSA) in PBS. The amount of ^{125}I-MoAb bound to the RBCs was measured for 1 minute in the RBC pellet on a multichannel gamma counter (Packard Instrument Company, Meriden, CT, USA). For each MoAb, the experiment was repeated three times.

To calculate the number of epitopes on RBCs (Bmax) and the reciprocal of the affinity (Kd) the following assumptions were made: (i) for all MoAbs tested, non-specific binding was determined as the binding to RhD-negative cells, (ii) the molecular mass of all MoAbs is 160 kDa, (iii) only one MoAb can bind to a single epitope and (iv) binding of the MoAb to the RBCs follows the law of mass action and is reversible. To analyse the data, GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com), non-linear regression technique 'one-site binding (hyperbola) was used.

Statistics. All results were expressed as the mean of at least three experiments ± standard deviation (SD). Significance levels were determined with the two-sided Student's t-test. The data of Scatchard plot analysis did not have a normal distribution and were therefore compared with the Kruskall-Wallis test and Mann-Whitney test by the statistical computer program SPSS (SPSS Inc., Chicago, IL., www.spss.com).

3.3 Results

Molecular typing of weak-D samples. All 8 weak-D donors used by Beckers *et al.* (1997) carried one of the weak-D alleles described by Wagner *et al.* (1999) as shown by sequencing cDNA. Donors 3-62, 3-66, 3-69, 3-77, 3-78, 3-82 all carried the weak-D type 1 allele (nt809 T→G), donor 3-63 carried the weak-D type 2 allele (nt1154 G→C) and donor 3-59 carried the weak-D type 3 allele (nt8 C→G). These mutations had been missed in our previous analysis (Beckers *et al.*, 1997). Mutations were confirmed by ASPA-PCR on gDNA of these donors.

The donors 40-1514 (weak-D type 1), 40-1415, 40-1416, 40-1507, 40-1511 (weak-D type 2), 3-5328 (weak-D type 3) and 3-5166 (weak-D type 4, nt602 C→G, nt667 T→G and nt819 G→A) were identified by ASPA-PCR on gDNA.

Quantification of *RHD* mRNA by RQ-PCR. To compare the amount of *RHD*-mRNA transcripts of weak-D phenotypes with the RhD-positive phenotype, RQ-PCR was performed.

Reticulocytes of donors with weak-D phenotypes (n=9) had comparable amounts of *RHD* mRNA independent of type, corrected for input, as reticulocytes of donors with a normal RhD-positive phenotype (R1r n=3, R1R1 n=2). This RQ-PCR assay was not accurate enough to measure a twofold difference. The mean amount of *RHD* mRNA corrected for input (ratio *RHD* over Kell mRNA) of the weak-D phenotype was 1.4 ±0.6 and of the normal RhD phenotype 1.6 ±0.7 (p=0.68), as shown in Figure 3.1. No *RHD* mRNA was amplified from reticulocytes of donors carrying the RhD-negative or Rh_{null} phenotype, as expected.

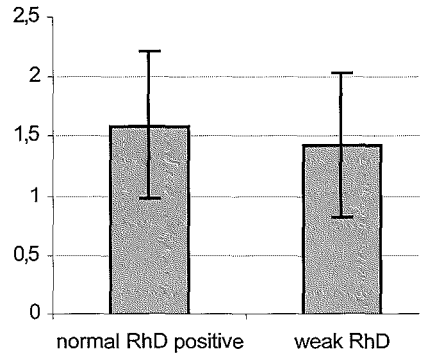

Figure 3.1: The amount of *RHD* mRNA corrected for input.
The amount of *RHD* mRNA corrected for input in reticulocytes from donors expressing normal RhD phenotypes (1.6 ±0.7) and different weak-D phenotypes (1.4 ±0.6). Values in ratios of *RHD* over Kell mRNA.

Agglutination tests with a panel of RhD-specific MoAbs. RBCs from one donor of each weak-D phenotype (weak-D type 1, weak-D type 2, weak-D type 3 and weak-D type 4), three donors with a DAR phenotype and two donors with a normal RhD-positive phenotype (both R1r) were tested with 70 MoAbs of the Third International Workshop on Monoclonal Antibodies against Red Cell and Related Antigens (1996, Nantes, France). Results are shown in Table 3.1.

More positive reactions (with MoAb III-1-82 (LHM169/81), 48 (LOR28-7E6), 46 (LOR17-8D3), 77 (LHM77/64), 53 (NAU3-2E8)) were detected in the weak-D phenotypes, compared to the partial-D phenotype DAR (Hemker *et al.*, 1999), also described as weak-D type 4.2 by Wagner *et al.* (1999). Some epitopes seem to be absent in the weak-D phenotypes, but the MoAbs against those epitopes (III-1-79 (LHM70/45), 48 (LOR28-7E6), 54 (NAU6-1G6), 57 (NOU), 38 (NaTH87-4A5) also gave a weak reaction (2 instead of 4) for the RhD-positive phenotype. It might be that these epitopes cannot be detected by agglutination, because of low affinty of the antibody. The serologic results, data not shown, mainly indicate that it is hard to demonstrate qualitative variations by serologic analysis.

Weak-D type 2 RBCs show a weaker reaction pattern than weak-D type 1 RBCs.

pattern			antibodies		RhD positive	partial RhD	weak D type			
1-9	1-37	epitope model WS IV	IgG	IgM	R1r	DAR	1	2	3	4
					18-862	*3-5089 3-5093 '3-5126*	*3-69*	*3-63*	*3-59*	*3-5166*
1	1	1.1	82		3	-	2	2	3	2
1	2	1.2	79		2	-	-	-	+	-
			83		2	-	+	(+)	2	(+)
2	3	2.1	44		3	2	+	+	2	3
			51		3	-	ND	ND	ND	ND
2	4	3.1	48		2	-	-	-	-	-
3	5		75		3	2	+	2	3	3
			106		3	2	2	+	2	3
			179		3	3	ND	ND	ND	ND
4	6	4.1	45		3	2	-	-	+	(+)
5	7	5.1		116	3	+	-	-	+	-
				42	4	-	ND	ND	ND	ND
5	10	5.4	88,89		3	2	2/+	+	2	3/+
			41		3	1	ND	ND	ND	ND
5	11		52		4	-	-	-	+	-
6/7	12	5.5	69		3	-	-	-	-	-
		6.1	102		3	3	2	+	2	3
			46		4	-	2	+	2	2
				113	4	2	-	-	(+)	+
				35	4	-	+/-	-	+	+
6/7	13	6.2	29,36,47,90,93,105		3	2	2	2	3	3
6/7	15/16	6.3	71,81,97		3	2/-	2	2	2/3	3
			32,58,108,114		3					
			56		4	3	ND	ND	ND	ND
			31,95		3					
			59		3	3	2	+	2	3
			80		3	2	2	2	2	2
6/7	17	6.4		134	3	-	-	-	(+)	-
				115	3	-	-	-	(+)	-
				99	3	-	-	-	(+)	-
				98	3	ND	-	-	(+)	-
				28,34	4	(+)/-	+/-	-	+/2	+
				37	3	(+)	-	-	+	(+)
				39	4	-	ND	ND	ND	ND
6/7	18	6.5	30		4	3	3	2	3	3
			119		3	-	-	-	-	-
				84,85	4	+	+	-	+/2	+
				86,87	4	-	-	-	+	(+)/-
				100	3	-	-	-	-	-
6/7	20/21	6.7	33		3	+	2	+	2	3
			94		4	-	+	2	3	3
			122		4	4	2	2	3	3
			61		3	-	ND	ND	ND	ND
8	22	8.1	74,78		3	-	ND	ND	ND	ND
9	23	9.1	112		3	2	2	+	3	3
			101 118		3	2	2	2	2/3	2/3
			120 121		3	3/-	+	+	2	3
			96		4	3	2	+	2	3
			77		3	-	3	3	3	3
N/A	31	10.1		54,57	2	-	-	-	-	-
N/A	32	11.1	62		3	-	ND	ND	ND	ND
N/A	33	12.1		38	2	-	-	ND	ND	-
N/A	34	13.1	50		2	-	ND	ND	ND	ND
N/A	35	14.1	60		3	-	ND	ND	ND	ND
N/A	36	15.1	72,76		3	2	2	2	3	3
			55		3	2	(+)	+	2	2
			53		3	-	(+)	(+)	+	+
N/A	37	16.1	68,73,117,124		3	2/3	2	2/+	3/2	3/2

Table 3.1: Epitope models (9 and 37) with the reaction pattern of the weak Ds tested with MoAbs described in the Nantes Workshop 1996
More positive reactions were detected with RBCs expressing weak RhD phenotypes than with RBCs expressing the weak partial-D phenotype DAR. Positive reactions were found in the range of "4" till "+" in declining agglutination strengths, negative agglutination reactions were described as "-"

Expression of proteins of the Rh complex on weak-D RBCs. To determine whether the expression of other parts of the Rh complex were altered, contributing to or caused by the weak-D antigen, the numbers of total Rh (Rh29), RhD, RhAg, LW and CD47 sites on RBCs were determined on RhD-positive RBCs (R1r, R2r, R1R2, R2R2 (n=2)), RhD-negative RBCs, weak-D phenotypes (type 1, 2 and 3), D-- and Rh_{null} RBCs. Results are depicted in Figure 3.2. No differences were observed between the different weak-D types; therefore, these results are pooled as weak D in this figure.

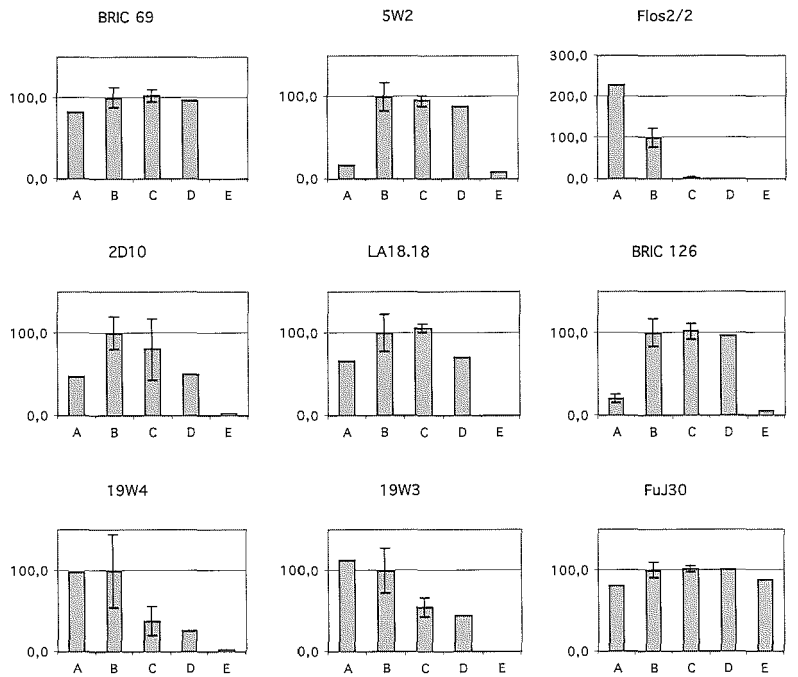

Figure 3.2: The expression of different proteins of the Rh complex and control antigen (Kell).
RBCs were tested for total Rh (BRIC69, 5W2), RhD (Flos2/2), RhAg (2D10, LA18.18), CD47 (BRIC126), LW (19W3, 19W4) and Kell (FuJ30). The expression is related to RhD-positive RBCs (B), which is normalized at 100. A= D-- RBCs; B= RhD-positive RBCs; C= weak RhD RBCs; D= RhD negative RBCs; E= Rh_{null} RBCs.

The expression of total Rh as measured both with MoAb BRIC69 and 5W2 showed no significant difference between the weak-D phenotype (weak-D type 1 (n=4) weak-D type 2 (n=1) and weak-D type 3 (n=1)), RhD-

positive (n=4) and RhD-negative phenotype (rr, n=2). The D-- phenotype showed a lower expression of Rh29 with MoAb 5W2, whereas the expression measured with MoAb BRIC69 was normal.

RBCs with the weak-D phenotype showed significant lower expression of RhD, measured with FLOS 2/2 than RBCs with the normal RhD phenotype. (Mean epitope density of weak-D RBCs was 910 ±450, and the mean epitope density of the normal RhD-positive RBCs was 27,500 ±8,000).

The expression of RhAg measured with MoAb 2D10 and LA18.18, was not different among RhD-positive, weak-D or RhD-negative RBCs.

The number of LW antigens, measured with MoAbs 19W3 and 19W4, were significantly lower on RBCs carrying the weak-D phenotype than on RBCs carrying the normal RhD phenotype (resp. p=0.005 and p=0.1), as described before by Beckers *et al.* (1997).

The number of CD47 antigen, measured with BRIC 126, is equal on RBCs carrying the weak-D phenotype and RBCs with the normal RhD phenotype, but a significant lower ($p < 0.001$) number is expressed on RBCs carrying the D-- phenotype (n=5)

The expression of Kell, as an internal control, measured with FuJ30, was similar for all RBCs tested.

By flowcytometry, the epitope densities of six weak-D type 1 and two normal RhD-positive (R1R2) phenotypes were determined with six of the MoAbs that were also used for Scatchard plot analysis and a selection of 7 MoAbs used by Wagner *et al.* (2000). These 7 MoAbs were selected because of their relatively high (6D10, HIMA35, 415 1E4 and OSK3-3) or low (BRAD8, 5C8, HIRO95) expression levels on the weak-D type 1 in the flowcytometry experiments (data kindly obtained from Dr. W. A. Flegel, Ulm, Germany). In Table 3.2, the expression of the weak-D phenotypes is related to the expression of the R1R2 phenotype. The expression of the weak-D type 1 phenotype in our experiment was comparable to theirs for the selection of 7 MoAbs of Wagner *et al.* (2000). The 6 MoAbs used in the Scatchard plot analysis, could be ranked among the other MoAbs for those that gave relatively high epitope densities on weak-D type 1 (1200 ±350; P3AF6; 4.9% of the number of epitopes on normal R1R2 cells), intermediate epitope densities (550 ±250; HIRO2; 170 10C9; HIRO55; 2.4%) and those with relatively low epitope densities (250 ±80; L87 1G7; 1.4%).

relatively high expression of weak RhD type 1

	6D10		HIMA35		415 1E4		OSK3-3	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
wD1-A	3,7	5,1	3,1	7,6	2,7	3,2	3,3	7,7
wD1-B	4,3	5,3	3,8	8,0	3,3	3,7	4,0	7,9
wD1-C		5,1		4,5		7,1		6,5
wD1-D		3,5		2,8		4,4		4,7
wD1-E		2,3		1,9		3,4		3,5
wD1-F		2,4		2,6		3,9		4,5
mean wD1	4,0	4,0	3,4	4,6	3,0	4,3	3,6	5,8

relatively low expression of weak RhD type 1

	BRAD8		5C8		HIRO95	
	A	*B*	*A*	*B*	*A*	*B*
wD1-A	0,9	2,6	0,5	1,3	1,1	1,0
wD1-B	1,3	1,1	0,6	1,1	1,2	0,9
wD1-C		1,3		2,1		1,3
wD1-D		0,6		1,0		1,7
wD1-E		0,1		0,2		1,0
wD1-F		0,5		0,5		1,4
mean wD1	1,1	1,0	0,6	1,0	1,2	1,2

Scatchard Plot MoAbs

	P3AF6	P3G6	HIRO55	L87 1G7	HIRO 2	170 10C9
	B	*B*	*B*	*B*	*B*	*B*
wD1-A	5,2	4,7	5,6	2,5	2,0	3,7
wD1-B	6,0	1,4	3,1	1,7	1,6	2,8
wD1-C	6,4	3,0	2,7	1,4	4,0	4,1
wD1-D	4,3	1,8	1,7	1,2	2,0	2,9
wD1-E	3,2	1,1	0,5	0,6	1,1	2,1
wD1-F	4,0	1,4	0,7	1,0	1,4	2,2
mean wD1	4,9	2,2	2,4	1,4	2,0	3,0
	relatively high			relatively low		

Table 3.2: RhD expression of weak-D type 1 RBCs as a percentage of RhD expression of R1R2 RBCs, measured by flowcytometry.
Anti-D MoAbs are classified in relatively high expression (6D10, HIMA35, 415 1E4, OSK3-3) and relatively low expression (BRAD8, 5C8, HIRO95) on weak-D type 1 RBCs. MoAb P3AF6 could be ranked among the MoAbs with relatively high expression, MoAb L87 1G7 could be ranked among MoAbs with relatively low expression on weak-D type 1, compared to RBCs with the R1R2 phenotype. (A) Data obtained from Dr. W. A. Flegel (Ulm, Germany); (B) current study.

Scatchard plot analysis. To determine the affinity of a MoAb for the different RhD phenotypes and to compare the affinities between the different MoAbs, Scatchard plot analysis was performed. RBCs of the following phenotypes were used: weak-D type 1 (n=2), weak-D type 2 (n=4), D^{VI} type III (n=2), DAR (n=2), DFR (n=2) and as normal control RhD positive (n=5) and RhD negative (n=2).

The data in Table 3.3 and 3.4 are presented as the mean of three experiments, with the 95% confidence interval. The data of the number of epitopes (Bmax) are presented in Table 3.3, the data of the Kd (reciprocal of the affinity) are presented in Table 3.4.

With the different RhD MoAbs about the same amounts of RhD antigens were measured per RBC. Homozygous RhD-positive (DD) RBCs expressed 31,915 ±2,275, and heterozygous RhD-positive (Dd) RBCs R2r: 27,140 ±2,526 and R1r: 12,970 ±4,782. However, from Table 3.3 it is clear that only minor differences were observed in the quantification of RhD epitopes with the same MoAb on the phenotypically same RBCs of different donors, but that slightly larger differences were observed in the quantification of different RhD epitopes (measured with different MoAbs) of one donor. These differences are probably due to differences in specific activity of the labelled antibodies. This latter effect does not hamper the comparison of Kd values of one antibody for the different RBC types.

With each MoAb a significantly lower amount ($p<0.0001$) of epitopes was found on the weak-D RBCs (930 ±450) compared to RhD-positive RBCs (21,000 ±9,700). The expression of RhD antigens was significantly lower ($p<0.001$) on weak-D type 2 RBCs (630 ±200) than on weak-D type 1 RBCs (1,450 ±400), for all MoAbs tested. The RhD epitopes tested with the different MoAbs on partial-D RBCs varied between no expression at all (D^{VI} type III phenotype with MoAb HIRO55 (epitope 2.1-IV) and L87 1G7 (epitope 6.3-IV) and DFR phenotype with MoAb 170 10C9 (epitope 6.2-IV) and HIRO2 (epitope 6.7-IV)) and weak expression compared to the normal RhD phenotypes, as expected (Fourth International Workshop on Monoclonal Antibodies against Red Blood Cell and Related Antigens, 2001, Paris, France) These results support the validity of our method.

Most importantly, no significant differences in the Kd (reciprocal of the affinity) of the different MoAbs between weak-D RBCs and the normal RhD-positive RBCs were found, as shown in Table 3.4. If there would have

been qualitative differences between the normal RhD antigen and the weak-D antigen an increase in Kd had been observed. Only for MoAb HIRO55 a lower Kd (higher affinity) was observed in weak-D type 2 RBCs. Indeed, in partial-D phenotypes reduced affinities were observed for some of the MoAbs. Significant reduction in affinity was found in 170 10C9 for DAR, P3AF6 for DFR and H41 11B7 for D^{VI}. No affinity was found in 170 10C9 and HIRO2 for DFR and HIRO55 and L787 1G7 for D^{VI}. All expected, according to the Fourth International Workshop on Monoclonal Antibodies against Red Blood Cell and Related Antigens.

To investigate whether the difference in epitope density, as measured by flowcytometry, between the different MoAbs could be due to differences in affinity of the MoAbs, we closely examined the Kds of these MoAbs and their epitope densities measured by flowcytometry. In Figure 3.3, all seven MoAbs tested by flowcytometry and Scatchard plot are compared with each other (42 comparisons), the coordinates of a dot is the ratio of the % epitopes measured with MoAb A to MoAb B on a weak-D phenotype (x-axis) and the ratio of the Kd of MoAb A to MoAb B (y-axis).

We noticed, that if two MoAbs have comparable affinities (Kd MoAb A: Kd MoAb B = 1), also the epitope density detected by flowcytometry is comparable (% expression measured with FACS with MoAb A: % expression measured with FACS with MoAb B = 1), depicted in Figure 3.3 as black diamonds. And when the affinity of one MoAb is significantly higher than the other (Kd MoAb A : Kd MoAb B < 1 or >1), this MoAb detects a higher epitope density than the other (% expression measured with FACS with MoAbA: % expression measured with FACS with MoAbB > 1 or <1), depicted as grey squares. This indicates that the epitope density measured by flowcytometry is greatly influenced by the affinity of the antibody. The combinations of MoAbs that had significant ($p< 0.05$) differences in Kd were : HIRO55 & P3AF6, H41 11B7 & P3AF6, H41 11B7 & 170 10C9, H41 11B7 & L87 1G7, H41 11B7 & P3G6, P3AF6 & 170 10C9, P3AF6 & L87 1G7, P3AF6 & HIRO2, P3AF6 & P3G6, 170 10C9 & HIRO2, L87 1G7 & HIRO2 and HIRO2 & P3G6.

antibody	HIRO 55	H41 11B7	P3 AF6	170 10C9	L87 1G7	HIRO 2	P3G6
epitope (9-37)	2-?	3-5	6/7-12	6/7-13	6/7-15/16	6/7-20/21	9-23
Controls							
R1R1	34790	34030	ND	ND	31270	ND	ND
	(33330-36250)	(32220-35840)	ND	ND	(28850-33680)	ND	ND
R1R2	32410	30110	ND	ND	28880	ND	ND
	(30690-34120)	(28200-32020)	ND	ND	(30890-34490)	ND	ND
R2r	30050	25850	ND	ND	25520	ND	ND
	(27600-32490)	(24340-27350)	ND	ND	(24260-26770)	ND	ND
R1r	ND	ND	10900	8169	ND	20770	7570
	ND	ND	(10440-11350)	(7816-8521)	ND	(19860-21670)	(7260-7879)
R1r	19100	14080	9659	9654	15960	17950	8863
	(17770-20430)	(12800-15370)	(9322-9996)	(9333-9975)	(15490-16420)	(17200-18710)	(8400-9327)
Weak D							
Type 1	ND	ND	1793	1511	ND	2052	1266
R0r	ND	ND	(1616-1971)	(1476-1545)	ND	(1937-2168)	(1205-1327)
Type 1	1002	1510	1801	866	1649	1661	891
R1r	(824-1180)	(1342-1678)	(1685-1917)	(834-899)	(1578-1720)	(1499-1822)	(833-949)
Type 2	371	555	726.7	392	697	1052	398
R2r	(271-471)	(451-658)	(679-774)	(359-424)	(601-792)	(967-1138)	(344-352)
Type 2	746	731	523	572	948	912	587
R2r	(650-841)	(657-806)	(464-582)	(541-603)	(905-992)	(837-987)	(524-650)
Type 2	380	611	ND	ND	624	ND	ND
Rzr	(291-489)	(551-672)	ND	ND	(591-657)	ND	ND
Type 2	384	878	ND	ND	609	ND	ND
Rzr	(334-433)	(683-1073)	ND	ND	(572-648)	ND	ND
Partial D							
DAR	ND	ND	867	331	ND	1580	632
R0r	ND	ND	(728-1006)	(265-396)	ND	(1458-1703)	(562-703)
DAR	ND	ND	1636	775	ND	2882	1327
R0r	ND	ND	(1528-1744)	(692-858)	ND	(2694-3070)	(1265-1388)
DFR	ND	ND	1241	0	ND	0	5762
?	ND	ND	(804-1680)		ND		(5090-6434)
DFR	ND	ND	950	0	ND	0	1436
R1r	ND	ND	(364-1537)		ND		(1343-1529)
D VI	0	4874	ND	ND	0	ND	ND
R1r		(4279-5470)	ND	ND		ND	ND
D VI	0	9088	ND	ND	0	ND	ND
R1r		(8519-9658)	ND	ND		ND	ND

Table 3.3: RhD Epitope densities (Bmax) as determined by Scatchard plot analysis using 7 different MoAbs.
Between brackets is the 95% confidence interval of three independent experiments. The epitope density of normal RhD-positive RBCs (controls) is significantly higher than the epitope density of weak-D RBCs (weak D). The epitope density on RBCs expressing weak-D type 1 is significantly higher than the epitope density on weak-D type 2 RBCs. As expected, epitope loss was demonstrated on RBCs expressing partial D in concordance with the variant analysed. ND= not done.

antibody	HIRO 55	H41 11B7	P3 AF6	170 10C9	L87 1G7	HIRO 2	P3G6
epitope (9-37)	2-?	3-5	6/7-12	6/7-13	6/7-15/16	6/7-20/21	9-23
Controls							
R1R1	11.2	10.9	ND	ND	5.6	ND	ND
	(9.7-12.7)	(9.0-12.9)	ND	ND	(3.9-7.3)	ND	ND
R2r	11.8	9.2	ND	ND	4.1	ND	ND
	(8.7-15.0)	(7.4-11.0)	ND	ND	(3.2-4.9)	ND	ND
R1R2	10.3	9.2	ND	ND	3.8	ND	ND
	(8.6-12.0)	(7.1-11.4)	ND	ND	(1.9-5.8)	ND	ND
R1r	ND	ND	1.9	2.6	ND	9.5	2.1
	ND	ND	(1.5-2.3)	(2.2-3.1)	ND	(7.9-11.1)	(1.7-2.6)
R1r	8.3	5.8	1.7	3.0	3.0	7.4	2.7
	(6.3-10.3)	(3.8-7.7)	(1.4-2.0)	(2.6-3.4)	(2.6-3.4)	(6.2-8.7)	(2.1-3.3)
Weak D							
Type 1	ND	ND	2.3	4.6	ND	11.7	2.9
R0r	ND	ND	(1.3-3.4)	(4.3-5.0)	ND	(9.3-14.1)	(2.3-3.5)
Type 1	5.2	12.4	2.1	4.2	7.2	8.3	5.6
R1r	(1.6-8.7)	(8.5-16.3)	(1.5-2.8)	(3.6-4.8)	(6.0-8.4)	(5.3-11.3)	(4.2-7.0)
Type 2	1.8	4.9	1.4	2.4	4.2	7.4	4.0
R2r	(0.4-4.0)	(1.4-8.4)	(0.9-1.8)	(1.6-3.2)	(1.9-6.6)	(5.0-9.8)	(1.7-6.3)
Type 2	3.8	3.8	1.8	3.2	2.9	7.7	2.3
R2r	(2.0-5.6)	(2.2-5.4)	(0.8-2.8)	(2.5-3.8)	(2.3-3.5)	(5.2-10.2)	(1.2-3.5)
Type 2	2.2	8.1	ND	ND	3.4	ND	ND
Rzr	(0-4.4)	(5.6-10.7)	ND	ND	(2.6-4.2)	ND	ND
Type 2	3.5	13	ND	ND	3.1	ND	ND
Rzr	(1.8-5.1)	(4.9-21.1)	ND	ND	(2.2-3.9)	ND	ND
Partial D							
DAR	ND	ND	2.8	16.2	ND	11.1	2.9
R0r	ND	ND	(0.7-4.8)	(7.5-24.9)	ND	(7.9-14.2)	(1.6-4.2)
DAR	ND	ND	2.3	18	ND	14.9	4.4
R0r	ND	ND	(1.6-3.1)	(12.6-23.3)	ND	(11.6-18.3)	(3.5-5.3)
DFR	ND	ND	202	0	ND	0	4.6
?	ND	ND	(96.0-308)		ND		(2.4-6.9)
DFR	ND	ND	233	0	ND	0	1.8
R1r	ND	ND	(28.7-437)		ND		(1.2-2.4)
D VI	0	23.8	ND	ND	0	ND	ND
R1r		(15.4-32.2)	ND	ND		ND	ND
D VI	0	16.9	ND	ND	0	ND	ND
R1r		(13.6-20.2)	ND	ND		ND	ND

Table 3.4: The Kd (M^{-1}) as the reciprocal of the affinity of 7 anti-D MoAbs by Scatchard plot analysis.
Between brackets is the 95% confidence interval of three independent experiments. The Kd of each MoAb did not show significant difference between the weak-D phenotype and normal D-positive phenotype. Between several MoAbs, the Kd of both normal D-positive and weak-D RBCs, did show significant differences. The Kd for the partial-D phenotype ranged from normal to extremely increased.

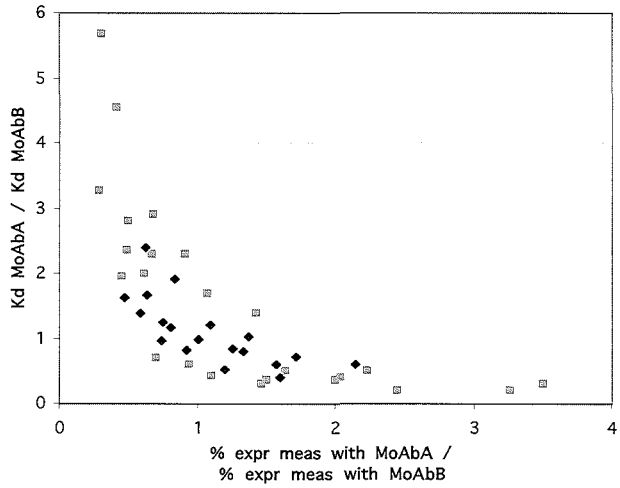

Figure 3.3: Kds measured with Scatchard plot analysis (y-axis) vs expression measured with flowcytometry (x-axis).
Comparison of two MoAbs with Kds in the same range are depicted as black diamonds. Comparison of two MoAbs with significantly different Kds are depicted as grey squares. When the Kds of two MoAbs are comparable, they also detect the same amount of epitopes by flowcytometry. If the Kd of a MoAb is significantly higher (affinity is lower), less epitopes are detected and if the Kd of a MoAb is significantly lower, more epitopes are detected by that MoAb with flowcytometry.

3.4 Discussion

Serologically, the weak-D phenotype is characterized by weak or negative agglutination reactions with polyclonal anti-RhD in an immediate-spin test. This agglutination can be enhanced in the indirect antiglobulin test (Race *et al.*, 1948), (Stratton *et al.*, 1948). *RHD* transcripts from people whose RBCs express a weak form of the RhD antigen were found to have missense mutation(s) within the predicted transmembrane or cytoplasmic domains of the RhD (Wagner *et al.*, 1999).

In the present study, we definitely prove that the lower expression of RhD in the most frequently occurring weak-D phenotypes is not due to a reduced amount or stability of *RHD* mRNA. By RQ-PCR, we showed in this study that equal amounts of *RHD* mRNA were isolated from the reticulocytes of donors carrying the weak-D phenotype types 1 & 2 and of donors carrying a normal D antigen. Preceding studies of Rouillac *et*

al. (1996) and Beckers *et al.* (1997) have studied the amount of mRNA before, with opposite results. Both studies used semi-quantitative PCR and therefore can be considered less accurate than RQ-PCR.

By flowcytometry, we examined whether the expression of other components of the Rh complex (RhAg, CD47 and LW) was altered, contributing to, or caused by, the weak-D antigen. The lower expression of LW, also described by Beckers *et al.* (1997), was confirmed. Most probably the lower expression of LW is caused by the weak expression of RhD, since the mutations found in the weak-D phenotype have already been shown to be responsible for the lower expression of RhD (Chapter 4). No significant changes in expression of CD47 were observed. We also made two noteworthy side observations by means of flowcytometry. The first observation was that the MoAb 5W2 (anti-Rh29) is probably directed against CE and not against total Rh, because 5W2 did not show expression in D-- and Rh_{null} RBCs. The second observation was a significant decrease in expression of CD47 (with MoAb BRIC126), not only on Rh_{null} RBCs, but also on D-- RBCs of all five donors, as described before (Gardner *et al.*, 1991). This reduced expression of CD47 in the absence of RhCcEe suggests a correlation between RhCcEe and CD47. Recent studies have revealed more about the attachment of the Rh complex to the cytosketon (see Figure 1.5). The spectrin cytoskeleton is attached to band 3 via ankyrin. Band 3 is linked to CD47 through interaction with protein 4.2 (Rybicki *et al.*, 1996), (Mouro-Chanteloup *et al.*, 2003), (Bruce *et al.*, 2003). And CD47 is associated with the Rh complex (Dahl *et al.*, 2003) probably as concluded from this study via RhCcEe.

The observation that the weak-D phenotype has normal levels of mutated *RHD* mRNA leads to two possible hypotheses. The mutation found in the weak-D phenotype leads to a qualitatively altered weak-D antigen, like for example the partial-D variant DAR. Or the mutation found in the weak-D phenotype leads to misfolding of a part of the RhD proteins, as has been observed for the Cystic Fybrosis Transmembrane Conductance Regulator protein (CFTR) (Cheng *et al.*, 1990). Thirteen of the 24 amino acid substitutions in the 22 different weak-D types are located in the preserved regions of the Rh homologues (regions with identical or similar amino acids in RhD, RHCcEe, RhBG and RhCG). These well-preserved areas might be essential for the processing/routing of the protein to the membrane. If RhD proteins

are misfolded, they will be unable to form a complex with the accessory proteins, therefore cannot be properly transported to the RBC membrane and will be degraded intracellularly (by proteasomes or lysosomes). If so, the small amount of properly folded RhD protein expressed on the RBC membrane is like the normal D antigen, and therefore the weak-D phenotype would purely be a quantitative variant. The difference between the two hypotheses is very important for the transfusion policy. The first hypothesis implicates that alloantibodies can be formed, when an individual with the weak-D phenotype is transfused with RhD-positive RBCs. In consequence, individuals with the weak-D phenotype should always be transfused with RhD-negative RBCs or RBCs with the same weak-D phenotype. The second hypothesis implicates that alloantibodies will never be formed and individuals can be considered as RhD positive. Till now, alloantibodies have never been found in persons carrying the weak-D phenotype, even though weak-D recipients receive RhD-positive RBCs, but this does not exclude that the weak-D antigen is not a qualitative variant. The normal RhD phenotype is not very immunogenic for some qualitative variants, like the DAR variant, which was frequently found in a South African population (phenotype frequency 1.5%). Only two persons expressing the DAR phenotype, described in the literature, have been identified with alloanti-D (Hemker *et al.*, 1999). The location of the mutations found in the different weak-D types (transmembrane or intracellular) is not a sufficient argument for this lack of alloantibody response. Although most partial-D antigens, and other immunogenic polymorphic bloodgroup antigens, have amino acid substitutions on the extracellular side of the protein, some partial-D antigens ($D^{III a}$, $D^{III c}$, D^{IV} type III, DAR and the VS epitope) have amino acid subsitutions only on the transmembrane or intracellular side of the protein (Avent *et al.*, 2000). Also individuals with these variants are known to produce alloantibodies. This indicates that not only extracellular mutations lead to conformational changes (qualitative alterations) in the exofacial loops.

One way to demonstrate possible qualitative changes of the weak-D antigen is to look at the expression level of the different epitopes. If there is a change among the expression levels of the different epitopes, a qualitative variation is likely. A tool to observe qualitative changes in expression of the different epitopes is the Rh similarity index (RI). The RI is calculated as

the ratio of the 10^{th} percentile to the 90^{th} percentile of the epitope densities detected with 59 anti-D MoAbs by flowcytometry. The RI may range from 1 in normal RhD to 0 in partial D which are lacking many epitopes. Wagner *et al.* (2000) suggested that most weak-D types carry altered RhD antigens, because of their low RI.

A disadvantage of the RI is that the affinities of the MoAbs are not taken into consideration. By flowcytometry, antibodies with high affinity will still demonstrate high numbers of epitopes on weak-D antigens, while MoAbs with low affinity will fail to demonstrate all epitopes on weak-D antigens because of early dissociation of the antibody with the antigen (Van Bockstaele *et al.*, 1986). Indeed, in the present study we demonstrated, by Scatchard plot analysis, that the differences in epitope densities measured by the different MoAbs could be due to the affinity of the MoAbs. Moabs with equal affinities for the RhD antigens demonstrate equal epitope densities on weak-D samples in flowcytometry, whereas MoAbs with large differences in affinity demonstrated similarly large differences in epitope densities. Therefore, it cannot be concluded that the wider variance in epitope densities as reflected in the RI, is due to qualitative changes of the RhD antigen, as the lower RI might also be caused by differences in affinity of the antibodies. Moreover, with each MoAb, no significant difference in affinity was found between weak-D and normal-D antigens, again suggesting that the conformation of these antigens was comparable. As expected, the affinity for some epitopes on partial D antigens was significantly lower ($p< 0.0001$). Besides neglecting the MoAb properties, in another way the RI does not only represent the qualitative differences of RhD. Small differences in fluorescence (for example of technical origin, therefore independent of cell type) in RBCs with high expression will be negligible (i.e., epitope density 10^{th} percentile=27,350, 90^{th} percentile=27,650, RI=0.99), while the same small difference in fluorescence in RBCs with low expression has an enormous impact on the RI (i.e. epitope density 10^{th} percentile=500, 90^{th} percentile=800, RI=0.6). These two remarks do not withstand that flowcytometry is very robust and reproducible, as has been tested in 12 laboratories (Flegel *et al.*, 2002).

Scatchard plot analysis could be used to obtain the affinity (=1/Kd) of MoAbs to the antigenic site (as described above), but also to obtain the epitope densities (=Bmax). The epitope densities on the weak-D RBCs were

significantly lower than on normal RhD-positive RBCs, as expected, and were comparable to the flowcytometry values of Wagner *et al.* (2000). Also a significantly lower number of RhD epitopes was observed on cells with the weak-D type 2 phenotype compared to the weak-D type 1 phenotype, conform the flowcytometry values and agglutination strength difference between these phenotypes.

As encountered before, the number of epitopes measured was dependent on the MoAb used in the Scatchard plot. This problem was encountered before (Moreau *et al.*, 1987), and three hypotheses were formed. The Rh polypeptides could be present at varying depths within the lipid bilayer (Gorrick *et al.*, 1988); this was suggested by the finding that the addition of cholesterol into the RBC membrane increases the number of available RhD-antigen sites (Shinitky *et al.*, 1979). Also the estimation of the number of epitopes is ultimately dependent on determination of specific activity of the ^{125}I-labelled MoAbs. This measurement is extremely sensitive to the presence of non-protein contaminants in the MoAb preparations. Thus the determination of the specific activity may have been incorrect, leading to an incorrect estimate of epitope number (Gorrick *et al.*, 1993). The last hypothesis suggested that some MoAbs measure a reduced number of epitopes because they are unable to bind to the epitope due to partial steric hindrance of neighbouring molecules (Gorrick *et al.*, 1993).

Concluding, with Scatchard plot analysis and flowcytometry a significant lower epitope density was found in RBCs with the weak-D phenotype than with the normal D-positive phenotype. This is not due to a difference in amount or stability of RhD transcripts, because similar amounts of *RHD* mRNA were found in normal RhD and weak-D RBCs. Scatchard plot analysis did not confirm possible qualitative alteration of the weak-D antigen, because the affinity for the weak-D antigen and the normal RhD antigen for each MoAb did not differ significantly. We also proved that the differences in epitope density on the weak-D RBCs measured with the different MoAbs (rendered by a low RI), are caused by properties of the MoAbs and do not have to be due to a qualitative change of the weak-D antigen. Besides, also technical aspects can cause a low RI when measuring low epitope densities. Therefore, there are still no solid arguments to consider weak D as not only a quantitive, but also a qualitative variant.

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Chapter 4

A K562 erythroleukemic cell line transfection model for the weak D type 1 and 3

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Abstract

About 0.2% to 1% of Caucasian population has red blood cells (RBCs) with a reduced expression of the RhD antigen (weak D). Different mutations in the *RHD* gene have been found in donors with the weak-D phenotype. The lower expression of RhD in the weak-D phenotype is not due to a change in the amount or stability of mutated *RHD* mRNA, as was shown by RQ-PCR in RBCs. The expression of other components of the Rh complex was measured, by flowcytometry. Only the expression of LW showed significant lower expression, probably caused by the weak-D antigen. To formally prove that the mutations are responsible for the reduced expression of RhD, a K562 transfection model was used expressing the weak-D type 1 (transmembrane nt809 T→G) or 3 (intracellular nt8 C→G). In these K562 cells, a decreased expression of RhD without affecting the transcription level in the K562 model was shown. We postulated that the translocation

of the protein to the cell membrane might be interrupted by the weak-D mutations. To investigate this possibility, RhD was fused to green fluorescent protein (GFP) to localise the weak-D protein in the K562 cell. Degradation of intracellular misfolded proteins was prevented by proteasome inhibition. However, the genesis of the weak-D phenotype remains unsolved, because the inhibition of proteasomes did not lead to an accumulation of mutant proteins in our K562 model. This might be due to the experimental set-up, because K562 cells lack some essential components to yield complete Rh complexes, and GFP might possibly lead to a change in conformation or processing of the fusion protein.

4.1 Introduction

About 0.2 - 1% of Caucasians has red blood cells (RBCs) with reduced expression of the RhD antigen (weak D). Serologically, the weak-D phenotype is characterized by weak or negative agglutination reactions with polyclonal anti-RhD in an immediate-spin test. This agglutination is enhanced in the indirect antiglobulin test (Race *et al.*, 1948), (Stratton *et al.*, 1948).

The lower expression of RhD in weak D is not due to a change in the amount or stability of *RHD* mRNA, as was shown before by RQ-PCR (Chapter 3). By flowcytometry, no significant changes were found in the expression of other components of the Rh complex (such as RhAg and LW) that might contribute to or be caused by the weak-D antigen (Chapter 3).

In *RHD* transcripts from weak-D individuals, missense mutation(s) have been found within the predicted transmembrane or cytoplasmic domains of RhD (Wagner *et al.*, 1999). Subsequently, the Rhesus similarity index (RI) was introduced, a way to express the variation between the expression densities of 59 epitopes measured by flowcytometry, and qualitative alteration of the weak-D protein was assumed. In a subsequent study, we showed by Scatchard plot analysis that the RI not merely reflects qualitative changes of the RhD antigen, because the RI is strongly influenced by the affinity of the antibodies used to detect RhD.

Rh antigens are defined by a complex association of membrane polypeptides, assembled by non-covalent bonds. Rh and RhAg form the core of the Rh complex and are accompanied by CD47, LW and Glycophorin B (GPB).

Protein 4.2 provides a link between band 3 and the Rh complex via CD47 (Figure 1.5). A current model suggests that if one subunit of the major components (Rh, RhAg) is missing, the Rh complex is not assembled or transported to the cell surface. As seen in the Rh_{null} phenotype, mutations in *RHAG* not only affect the transcription of the *RHAG* gene but can also affect the processing of the RhAg protein in several ways, which in turn may alter the formation of the Rh complex. Since it was shown that mutations causing weak D do not affect transcription of *RHD*, the processing of the RhD protein to the cell membrane might be affected, resulting in weak expression of RhD. Also mutations in Rh-associated proteins may result in decreased expression of Rh antigens. For example, a lower expression of RhD was found on Southeast Asian Ovalocytosis red cells, which are heterozygous for a mutant band 3 containing a nine-amino-acid deletion (Tanner, 1997), (Tanner, 2002), and on band-3 Coimbra red cells, which are homozygous for a V488M mutation in band 3 (Ribeiro *et al.*, 2000).

The *RH* gene expression has been investigated in K562 cells, which appeared to be a good in vitro model to mimick red blood cells (Smythe *et al.*, 1996). Retroviral gene transfer was used to transduce K562 cells with cDNAs, because transfection with plasmid expression vectors had met with very little success (Hermand *et al.*, 1993), (Suyama *et al.*, 1993). K562 cells are originally chronic myeloid erythroleukaemia cells that already express RhAg, which is considered to be essential for the expression of Rh antigens. Although the K562 cell line was derived from an RhD-positive patient, these cells express RhD to such a low extent that after transfection with *RHD* a clear rise in RhD expression can be observed.

The present study was performed to examine whether and in which way the point mutations found in weak-D alleles lead to weak expression of the RhD protein. For this purpose, K562 cells were transfected with *RHD*, weak-D type 1 (wD1) or weak-D type 3 (wD3), or with *RHD*-GFP, weak-D type 1-GFP (wD1-GFP) or weak-D type 3-GFP (wD3-GFP) (GFP=green fluorescent protein).

4.2 Materials and Methods

Retroviral transduction. The construction of K562 cells expressing normal RhD (*RHD*-K562), the weak-D type-1 phenotype (wD1-K562)

labelled, PE-labelled or Cy5-labelled goat-anti-human-IgG for another 30 minutes. All incubations were performed at room temperature. The cells were washed with phosphate-buffered saline (PBS) containing 0.2% (v/v) bovine serum albumin (BSA). Flowcytometric analysis of at least 10,000 cells was performed with a FACScan flowcytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Confocal microscope imaging. Cells were prepared as for flowcytrometric analysis. The following additional antibodies and fluorochromes were used: C-20 (polyclonal IgG goat-anti-Vimentin, Santa Cruz Biotechnology, Santa Cruz, CA), Hoechst 33258 (to visualise the nucleus, Molecular Probes, Eugene, OR) TRITC-labelled goat-anti-human-IgG (Jackson ImmunoResearch) and Alexa568-labelled rabbit-anti-goat-IgG (Molecular Probes). K562 cells were analysed as cytospins or on poly-L-lysine coated slides. Vectashield (Vector laboratories, Burlingane, CA) was added between the cells and coverslip, to enhance fluorescence. Images were recorded with a ZEISS LSM510 confocal microscope with appropriate filter settings. Cross-talk between the green and red channel was avoided by use of sequential scanning.

Immunoblotting. For immunodetection, 10^6 cells were boiled in 50 μL of sodium dodecyl sulfate sample buffer (125 mM Tris, pH 6.8; 20% [wt/vol] sodium dodecyl sulfate; and 12.5% [vol/vol] β-mercaptoethanol) and were loaded on a 12.5% polyacrylamide gel, according to Laemmli, in a gel apparatus (Mini-Protean II, BioRad, Hercules, CA). Western blotting was performed (Mini Trans-Blot cell, BioRad) according to the manufacturer's recommendations. Detection of proteins was performed as described previously, with JL-8 (anti-GFP, BD Biosciences Clonetech). In the dot blot assay, an antibody directed against GPDH was used as an internal control.

Proteasome inhibition. Proteasomes were inhibited with the proteasome inhibitor Lactacystin (Calbiochem, La Jolla, CA). Cells were cultured overnight in IMDM complete containing 10 μM Lactacystin and compared to cells that were cultured in IMDM complete without additives. Proteasome inhibition was checked by reduction of cell growth. Colouring

Vimentin (with MoAb C-20) to observe typical ring-like structures in the cells was used to check for the formation of aggresomes.

4.3 Results

Mutant *RHD* transfected K562 cells. To examine the influence of the mutations found in weak-D genotypes, a model was constructed for the weak-D type-1 phenotype and the weak-D type-3 phenotype in the erythroleukemic cell line K562.

After transduction, K562 single cell colonies were selected with higher expression of total Rh (measured with MoAb BRIC 69) than the wild-type K562 cells (WT-K562 cells). By making single cell colonies, it is possible to compare the variation in Rh expression among different colonies and to correct for differences in transfection efficiency. Twelve colonies of the wD1-K562 cells, wD3-K562 cells and *RHD*-K562 cells were used for further flowcytometric analysis. WT-K562 cells, pB-K562 cells (K562 cells transfected with an empty pBabe-puro vector) and *RHcE*-K562 (K562 cells transfected with *RHcE*) (Faas *et al.*, 2001) were used as control samples.

As shown in Figure 4.1, the expression of total Rh (Rh29) measured with MoAb BRIC69, was significantly lower (p=0.002) in wD3-K562 cells than in *RHD*-K562 cells or wD1-K562 cells. WT-K562 cells and pB-K562 cells showed a very low expression of Rh29. The expression of RhD, measured with MoAb FLOS2/2, was significantly lower in wD1-K562 cells and in wD3-K562 cells than in *RHD*-K562 cells ($p < 0.04$ and p=0.001, respectively). The expression of RhAg, measured with MoAb 2D10, was comparable for wD1-K562 cells, wD3-K562 cells, *RHD*-K562 cells, *RHcE*-K562 cells, WT-K562 cells and pB-K562 cells. The expression of LW antigens, measured with MoAbs 19W3 and 19W4, was significantly lower ($p < 0.001$) in wD3-K562 cells than in *RHD*-K562 cells or wD1-K562 cells. The expression of the CD47 antigen, measured with BRIC 126 did not differ among wD1-K562 cells, wD3-K562 cells and *RHD*-K562 cells, but was significantly higher (p=0.02) on *RHcE*-K562 cells.

To exclude that the lower expression of RhD caused by the weak D point mutations in the K562 model is due to a lower transcription level or lower stability of mRNA, the amount of *RHD*-mRNA was quantified by RQ-PCR. All 12 colonies of each cell type were tested. The results are shown in

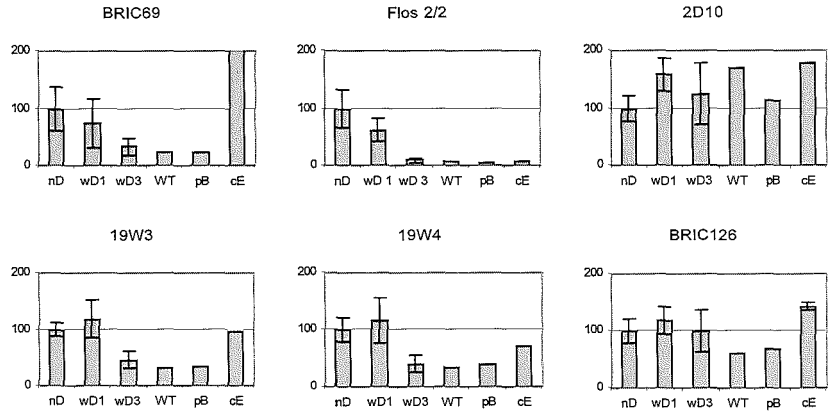

Figure 4.1: The expression of different proteins of the Rh complex on K562 cells, as measured by flowcytometry.
The expression of total Rh (BRIC69), RhD (Flos2/2), RhAg (2D10), CD47 (BRIC126) and LW (19W3, 19W4) was measured on K562 cells expressing normal RhD (nD), weak D type 1 (wD1), weak D type 3 (wD3), wild type (WT), empty pBabe puro vector (pB) and RhcE (cE). Expression is related to K562 cells expressing normal RhD.

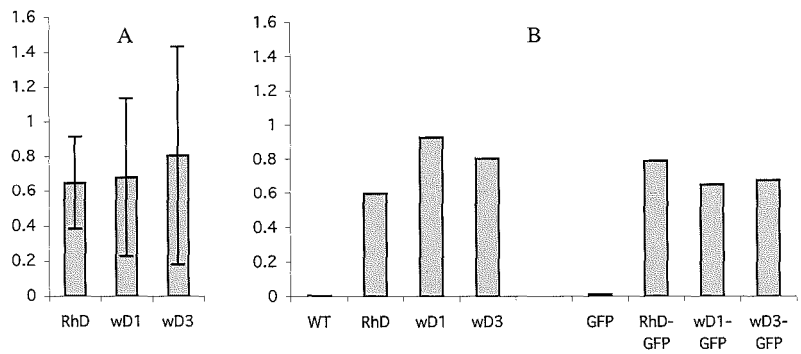

Figure 4.2: Amount of *RHD* mRNA corrected for input.
The amount of *RHD*-mRNA corrected for input in K562 cells expressing (A) normal RhD (RhD) (n=12), weak D type 1 (wD1) (n=12), and weak D type 3 (wD3) (n=12) and (B) RhD (n=1), wD1 (n=1), wD3 (n=1), green fluorescent protein (GFP) (n=1), *RHD*-GFP fusion protein (*RHD*-GFP) (=1), wD1-GFP fusion protein (wD1-GFP) (=1), wD3-GFP fusion protein (wD3-GFP) (=1). No differences could be found in the amount of *RHD*-mRNA among the different K562 cell types.

Figure 4.2A. The amount of *RHD*-mRNA corrected for input of wD1-K562 cells (ratio *RHD*-mRNA to cyclophillin-mRNA ± SD = 0.68 ±0.45) and wD3-K562 cells (0.81 ±0.62) was comparable to the amount of *RHD*-mRNA in *RHD*-K562 cells (0.65 ±0.26) (n=12). A small amount of *RHD*-mRNA (0.014 ±0.007) was found in WT-K562 cells (As was previously described by Suyama *et al.*, 1994), pB-K562 cells and *RHcE*-K562 cells. Also in the K562 cells transfected with GFP-fusion proteins, the same amounts of *RHD*-mRNA were found with RQ-PCR for *RHD*-GFP, wD1-GFP and wD3-GFP (mean ratio in all GFP-fusion K562 cells ± SD=0.7 ±0.4; Figure 4.2B).

Based on these results, it can be concluded that the amino-acid alterations found in individuals with weak-D phenotypes lead to decreased expression of RhD without affecting the transcription level in the K562 model.

RhD and GFP expression in GFP-fusion K562 cells. The GFP-fusion proteins were developed to visualize the localisation of the RhD protein or weak-D protein in or at the surface of K562 cells and to be able to use an antibody against GFP for immunodetection.

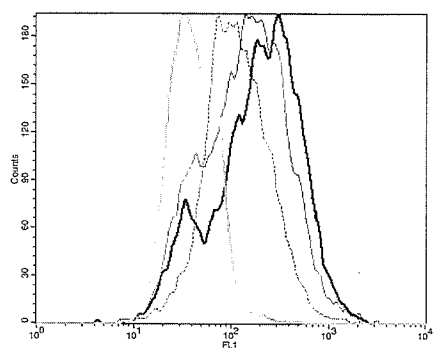

Figure 4.3: Green fluorescent protein (GFP) expression.
GFP expression measured on K562 cells expressing GFP fusion proteins. The GFP expression in the GFP-fusion K562 cells was highest for *RHD*-GFP K562 cells (black line), followed by wD3-GFP (dark-grey line) and lowest for wD1-GFP K562 cells (dashed dark-grey line. GFP expression of all GFP-transfected K562 cells was higher than the autofluorescence of WT-K562 cells (light-grey line).

The GFP expression in the GFP-fusion K562 cells was highest for *RHD*-

GFP K562 cells, followed by wD3-GFP and lowest for wD1-GFP K562 cells (Figure 4.3).

Localisation of the GFP-fusion protein. Confocal Laser Scanning Microscopy (CLSM) was performed to study the localisation of the GFP-fusion proteins in K562 cells.

A distinct GFP expression on the membrane of the cell was observed in *RHD*-GFP, wD1-GFP and wD3-GFP K562 cells. GFP expression in the GFP-K562 cells was diffusely distributed through the whole cell, including the nucleus. No difference in expression of GFP was observed between *RHD*-GFP, wD1-GFP and wD3-GFP K562 cells. There was no relevant autofluorescence in the GFP-channel in WT-K562 cells.

RhD expression, measured with MoAb Flos2/2, was co-localised with GFP on the cell membrane in the *RHD*-GFP-fusion K562 cells. No intracellular staining was seen in these cells. No difference in RhD expression was observed among *RHD*, wD1, wD3, *RHD*-GFP, wD1-GFP and wD3-GFP K562 cells (Figure 4.4).

Immunodetection of the GFP-fusion protein. As a control for the amount of free GFP, immunoblotting was performed with an antibody against GFP (JL-8).

In the GFP-K562 cells a product of 27kD, representing free GFP, was detected in a major amount. Also in the GFP-fusion K562 cells a product of 27kD was detected, but only in a minor amount. In the *RHD*-GFP K562 cells, wD1-GFP K562 cells and wD3-GFP K562 cells no *RHD*-GFP fusion product (of 57kD) was detected, which might be due to the multi-spanning organisation of RhD and therefore to complexes that are too large to run into the gel (Figure 4.5).

Proteasome inhibition. To investigate whether proteasome inhibition results in accumulation of mutant RhD proteins, and therefore enhances the intracellular expression of RhD and/or GFP, K562 cells were overnight incubated with proteasome inhibitor Lactacystin. Proteasome inhibition was confirmed by the observation of growth reduction. No other control could be used since MHC class I, downregulated after inhibition of proteasomes, is not present in K562 cells.

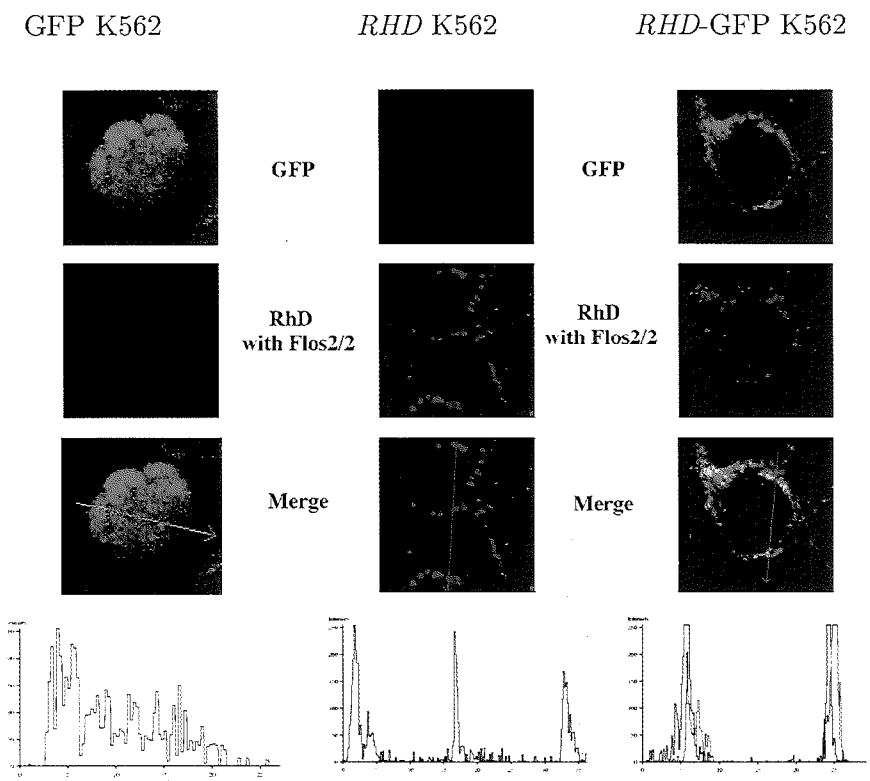

Figure 4.4: Confocal image of K562 cells.
Confocal image of K562 cells expressing green fluorescent protein (GFP), RhD and *RHD*-GFP fusion proteins (*RHD*-GFP). The upper images only show GFP (green), the middle images only show RhD (red), the lowest images merge GFP and RhD detection (co-localisation=yellow). The profile shows the expression of GFP (green) and RhD (red) of a cross-section of the K562 cell.

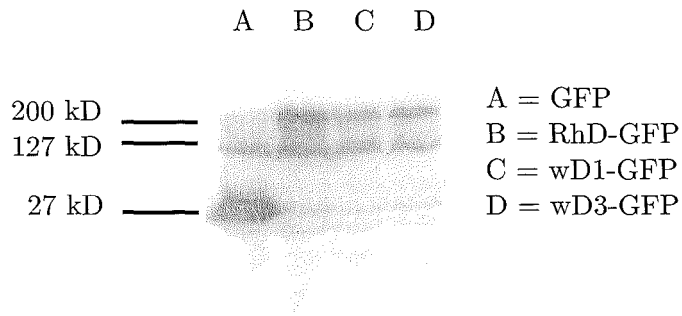

Figure 4.5: Western blot of GFP-fusion proteins.
In the GFP-K562 cells a product of 27kD, representing free GFP, was detected in a major amount. Also in the GFP-fusion K562 cells a product of 27kD was detected, but only in a minor amount. In the *RHD*-GFP K562 cells, wD1-GFP K562 cells and wD3-GFP K562 cells no *RHD*-GFP fusion product (of 57kD) was detected.

By flowcytometry, no increase in expression of RhD, measured with the conformation-independent MoAb LOR15C9, was observed in any of the permeabilised non-GFP K562 cell lines (RhD, wD1, wD3) after incubation with Lactacystin (Figure 4.6). Also no increase in expression of GFP was observed in any of the GFP-fusion K562 cell lines after incubation with the proteasome inhibitor Lactacystin (Figure 4.7).

By CLSM no increase in intracellular expression of GFP was seen in the GFP-fusion K562 cells after incubation with lactacystin (Figure 4.8).

Aggresomes, typical ring-like Vimentin structures (Johnston *et al.*, 1998), were not observed by CLSM in permeabilised cells after proteasome inhibition.

Immunoblotting was performed to quantify the amount of GFP in the GFP-fusion K562 cells before and after proteasome inhibition. Dot-blot analysis was performed, because immunodetection of the GFP-fusion proteins by Western blot with SDS-PAGE was not possible. A large amount of GFP was present in *RHD*-GFP K562 cells, wD3-GFP K562 cells and wD1-GFP, representing free GFP and GFP fused to RhD. A small amount of GFP was detected in the GFP K562 cells, representing only free GFP. For all GFP-fusion K562 cells no increase in amount of GFP was observed after incubation with Lactacystin. Equal amounts of all cells were used, because

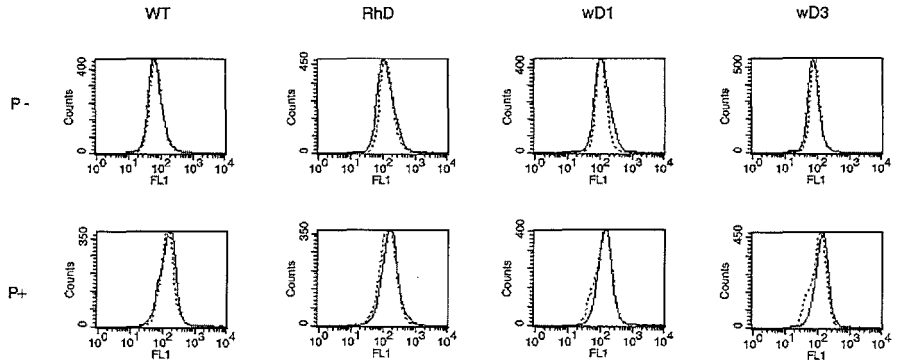

Figure 4.6: Expression of RhD

Expression of RhD before (continuous line) and after (dashed line) proteasome inhibition of wild type K562 cells (WT), K562 cells expressing RhD (RhD), weak D type 1 (wD1) and weak D type 3 (wD3), by flowcytometry with anti-D MoAb LOR15C9. Cells were permeabilised (P+) or not permeabilised (P-). No increase in RhD expression was observed in any of the K562 cell lines, with or without permeabilisation.

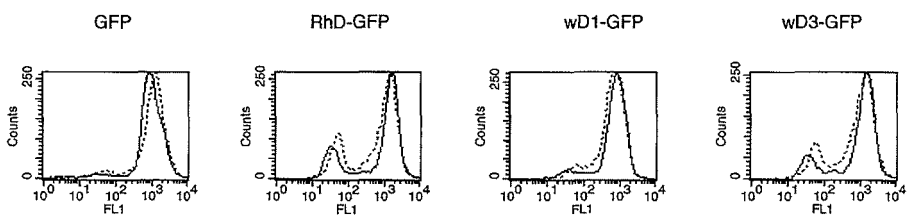

Figure 4.7: Expression of GFP.

Expression of green fluorescent protein before (continuous line) and after (dashed line) proteasome inhibition of K562 cells expressing GFP (GFP), K562 cells expressing *RHD*-GFP fusion proteins (*RHD*-GFP), weak D type 1-GFP fusion proteins (wD1-GFP) and weak D type 3-GFP fusion proteins (wD3-GFP). No increase in GFP expression was observed in any of the GFP fusion K562 cell lines.

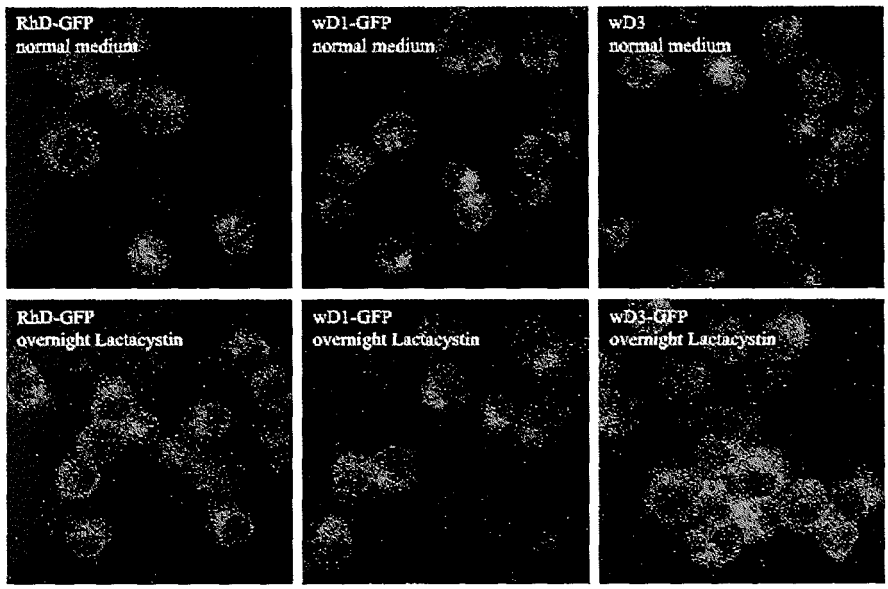

Figure 4.8: Confocal image of K562 cells.
Confocal image of K562 cells expressing green fluorescent protein (GFP) fusion proteins (*RHD*-GFP. wD1-GFP, wD3-GFP) before (upper) and after (lower) proteasome inhibition. No increase in intracellular expression of GFP was seen in the GFP-fusion K562 cell types after incubation with lactacystin

no difference in the amount of the control protein GPDH was detected (Figure 4.9).

4.4 Discussion

In *RHD* transcripts from weak-D individuals missense mutation(s) have been found within the predicted transmembrane or cytoplasmic domains of RhD (Wagner *et al.*, 1999). In RBCs, these point mutations do not affect the amount or stability of the *RHD*-mRNA and neither do they lead to qualitative alterations of the weak-D antigen, as was determined by RQ-PCR and Scatchard plot analysis (Chapter 3).

To formally prove that the single nucleotide mutations found in donors with weak-D phenotypes really cause decreased expression of RhD, K562 cells were transfected with a construct containing normal *RHD* (*RHD*-

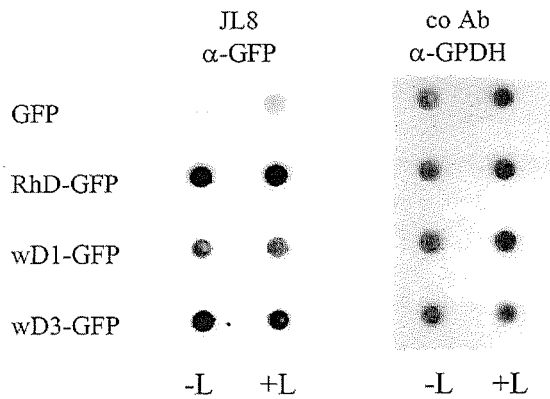

Figure 4.9: Dot blot.
Dot blot detecting GFP (with JL8) and a control for input (co Ab GPDH), before (-L) and after (+L) proteasome inhibition of K562 cells expressing GFP (GFP), K562 cells expressing *RHD*-GFP fusion proteins (*RHD*-GFP), weak-D type 1-GFP fusion proteins (wD1-GFP) and weak-D type 3-GFP fusion proteins (wD3-GFP). A large amount of GFP was present in *RHD*-GFP K562 cells, wD3-GFP K562 cells and wD1-GFP K562 cells, representing free GFP and GFP fused to RhD. A small amount of GFP was detected in the GFP K562 cells, representing only free GFP. In none of the GFP fusion K562 cell types an increase in amount of GFP was observed after proteasome inhibition. The amount of the control protein GPDH was equal in all cell types, indicating that equal amounts of all cell types were used.

K562) or with constructs containing the weak-D type 1 mutation (wD1-K562) (T→G at nt809, transmembrane, exon 6) or the weak-D type 3 mutation (wD3-K562) (C→G at nt8, intracellular, exon 1) both obtained by site-directed mutagenesis. As shown by flowcytometry, both wD1-K562 cells and wD3-K562 cells expressed RhD at reduced levels compared to *RHD*-K562 cells. Equal *RHD* mRNA levels were detected in wD1-K562 cells, wD3-K562 cells and *RHD*-K562 cells by RQ-PCR. This confirms that, as seen in RBCs, the weak-*RHD* point mutations introduced in the *RHD*-K562 cells did not have an effect on the transcription level of *RHD*.

Although the reduction of RhD expression in wD1-K562 cells and wD3-K562 cells compared to *RHD*-K562 is significant (40% of *RHD*-K562), it is not as low as in the weak-D RBCs (3% of normal RhD-positive RBC). The fact that the point mutations found in RBCs with the weak-D phenotypes did not cause the same strong reduction of RhD expression in K562 cells (even more pronounced for the wD1-K562 cells than for the wD3-K562

cells), might be due to the artificial expression system used in transfected K562 cells. The expression system with the SV40 promoter could lead to an excess of *RHD* transcripts, resulting in a relatively high expression of mutant RhD protein on the membrane. The relatively low expression of RhD in *RHD*-K562 cells might be due to limiting amounts of other proteins of the Rh complex, such as RhAg (Mouro-Chanteloup *et al.*, 2002). The mutation of the weak-D type 3 phenotype is located intracellularly near the N-terminus of the protein. It has been suggested that the tetrameric core of the Rh complex is formed by binding of the N-termini of two Rh30-proteins (RhD or RhCE) and two RhAg proteins (Eyers *et al.*, 1994). A mutation in the N-terminus might interfere with the Rh complex formation with the RhAg proteins. Mutations in RhAg in Rh_{null} cells of the regulator type have taught us that complex formation is necessary for transport to the membrane (Cartron, 2000). Furthermore, K562 cells lack the expression of band 3. Beckmann *et al.* have co-transfected band 3 with *RHD* in K562 cells and observed an enhanced expression of RhD. They concluded that band 3 might play a role in the translocation (and so enhances the movement of Rh components to the cell membrane) of the Rh complex or affects its conformation in the plasma membrane (Beckmann *et al.*, 2001). Bruce *et al.* have shown that band 3 is involved in the association of the Rh complex to the membrane skeleton and proposed a band-3 macrocomplex in which the Rh complex and band 3 are linked via protein 4.2 and CD47 (Bruce *et al.*, 2003). The stronger expression of RhcE in *RHcE*-K562 transfected cells compared to RhD expression in *RHD*-K562 transfected cells after band-3 co-transfection may be explained by an interaction that is stronger between band 3 and the Rh complex containing the CcEe polypeptide than between band 3 and the Rh complex containing the RhD polypeptide. This notion and the proposed band-3 macrocomplex is in line with our observation that CD47 might have a stronger interaction with the RhCcEe polypeptide, as was shown by an decreased expression of CD47 in D-- RBCs (Chapter 3) and an increased expression of CD47 in RhcE-transfected K562 cells.

The observation that the weak-D phenotype has normal levels of mutated *RHD* mRNA leads to two hypotheses. The mutations found in the weak-D phenotypes lead to misfolding of all RhD proteins, of which most will not form a complex and are degraded, but some are expressed on the membrane and therefore are qualitatively altered and weakly expressed,

like for example the partial-D variant DAR. Alternatively, the mutation found in the weak-D phenotype leads to misfolding of most of the RhD-proteins, but some are folded correctly, similar to the situation observed for the Cystic Fibrosis Transmembrane conductance regulator protein (CFTR) (Cheng *et al.*, 1990). Thirteen of the 24 amino-acid substitutions in the 24 weak-D phenotypes are located in the preserved regions of the Rh homologues (regions with identical or similar amino acids in RhD, RhCcEe, RhBG and RhCG), and most weak-D amino-acid substitutions are not located on RhD/RhCcEe polymorphic sites. It might be that these well preserved areas or non-polymorphic sites are essential for the translocation of the protein to the cell membrane. If RhD proteins are misfolded, they will be unable to form a complex with the accessory proteins; therefore, they cannot be properly transported to the RBC membrane and will be degraded intracellularly (by proteasomes and lysosomes). Then the small amount of properly folded RhD protein expressed on the RBC membrane has the normal RhD-antigen conformation, and therefore the weak-D phenotype would purely be a quantitative variant. The difference between the two hypotheses is very important for transfusion policy. The first hypothesis implicates that alloantibodies may be formed when an individual with the weak-D phenotype is transfused with normal RhD-positive RBCs. In that case, individuals with the weak-D phenotype should always be transfused with RhD-negative RBCs or RBCs with the same weak-D type. The second hypothesis implicates that alloantibodies will never be formed, and weak-D individuals can be considered as RhD positive.

The GFP-fusion proteins were developed to determine the localisation of the RhD protein in K562 cells and to quantify the amount of protein independently of the conformation. The GFP-fusion proteins wD1-GFP and wD3-GFP had a reduced expression of GFP compared to *RHD*-GFP, without a reduction in the amount of *RHD* mRNA. Confocal microscopy was used to localise the GFP-fusion protein, which was recognised by co-localisation of GFP and RhD. *RHD*-GFP, wD1-GFP and wD3-GFP were almost exclusively present on the extracellular membrane. As expected, GFP-K562 cells only expressed GFP, diffusely distributed throughout the whole cell, and *RHD*-K562 cells only expressed RhD, almost exclusively localised on the extracellular membrane. Not much free GFP was present in the GFP-fusion K562 cells, as was shown by immunodetection on Western

blot.

As the non-GFP and the GFP-fusion K562 cells both seem to be a good model for the weak-D phenotype, they were used to test the hypothesis about the genesis of the weak-D phenotype. To remove intracellular, misfolded or uncomplexed proteins, the intracellular ubiquitination pathway will be enhanced and proteasomes, which are more prone to remove transmembrane proteins than lysosomes, will be upregulated. By inhibition of these proteasomes with Lactacystin, an intracellular accumulation of misfolded or uncomplexed proteins might be expected. Aggregated misfolded or uncomplexed proteins may also segregate as aggresomes, ensheathed by the intermediate filament Vimentin (Johnston *et al.*, 1998). In our K562 model, proteasome inhibition should result in an increase in intracellular RhD in non-GFP K562 cells, an increase in intracellular GFP in GFP-fusion proteins and the presence of aggresomes. However, after proteasome inhibition, no increase in expression of RhD was observed in non-GFP K562 cells by flowcytometry, and no increase in GFP expression was observed in GFP-fusion proteins by flowcytometry, CLSM or immunoblotting. And aggresomes, typical ring-like Vimentin structures, were not detected by CLSM.

The proteasome inhibition experiments presented in this study did not confirm the hypothesis on disturbance in the translocation of the weak-D proteins. This might be due to the fact that another mechanism is underlying the genesis of the weak-D phenotype, but arguments can also be found to criticise the approach we used to confirm the hypothesis. To follow the processing of the weak-D protein, this model might have some disadvantages, as indicated below. The fact that the non-GFP proteins do not show an increased expression of intracellular RhD after inhibition of the proteasomes might be due to the MoAb LOR15C9. Although LOR15C9 is the only antibody recognizing a linear RhD epitope, it might be that the affinity or specificity is too low to recognise the misfolded or uncomplexed RhD protein. This may be the reason that equal expression levels of RhD were measured before and after incubation with Lactacystin. GFP-fusion proteins were developed to detect the RhD antigen without the use of an antibody. But the GFP-fusion proteins do have several other disadvantages. The first disadvantage concerns the effect of GFP on RhD expression. Since GFP has almost the same size and weight as RhD (27kD vs 30kD), this

might possibly lead to a change in conformation or processing of the fusion protein. Indeed, we observed a much lower expression of RhD as measured by flowcytometry in the GFP-fusion transfectants than in the non-GFP transfectants. The second disadvantage concerns the stability of the GFP-fusion protein. It is unknown whether the misfolded GFP-fusion proteins are completely degraded by the proteasomes or whether only the misfolded RhD part is degraded, leaving a free GFP protein intact. And it might also be that just after translation, part of the GFP-fusion proteins are spliced into separate GFP and RhD proteins. The presence of free GFP was demonstrated by immunodetection in Western blot analysis. In both cases, proteasome inhibition increased the expression of the GFP-fusion protein, but not of free GFP. If there is more free GFP in the cells than GFP-fusion protein, the increase after proteasome inhibition cannot be noticed by flowcytometry or CLSM. However, these options are not very likely, because free GFP is present in the cells, but only in limited amounts. The third disadvantage concerns the use of an artificial promotor in the K562 transfection model. The use of the artificial promotor can lead to an excess of (weak) RhD(-GFP) proteins, as described before. The production of the other Rh-complex proteins, such as RhAg, are regulated by the natural promotor of the K562 cell, and may therefore not be produced in sufficient amounts to form complete Rh complexes with all the (weak) D(-GFP) proteins. From Rh_{null} cells of the regulator type, we know that complex formation with RhAg is necessary for transport of the RhD(-GFP) to the membrane (Mouro-Chanteloup *et al.*, 2002). When the proteasome activity is insufficient to degrade the excess of RhD (-GFP) proteins, no difference will be observed before and after proteasome inhibition.

In conclusion, the mutations found in the weak-D phenotype directly lead to a decreased expression of RhD without affecting the transcription level in the K562 model. The genesis of the weak-D phenotype remains unsolved, since the inhibition of proteasomes did not lead to an accumulation of mutant proteins in our K562 model. This might be due to the approach we used. Fusion of RhD with another, smaller and better immunodetectable tag (such as HA) might bypass some of the disadvantages encountered with the GFP fusion. Also co-expression of other parts of the Rh complex, which are not sufficiently produced to yield complete Rh complexes (e.g. RhAg) or to properly translocate or present the Rh com-

plex on the cell membrane (e.g. band 3), might be an approach to further investigate the genesis of the weak-D phenotype.

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Chapter 5

The Rh complex exports ammonium from human red blood cells

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Abstract

The Rh blood group system represents a major immunodominant protein complex on red blood cells (RBCs). Recently, the Rh homologues RhAG and RhCG were shown to promote ammonium ion transport in yeast. In this study, we show that also on RBCs the human Rh complex functions as an exporter of ammonium ions.

We measured the ammonium import during the incubation of the RBCs in a solution containing a radiolabelled analogue of NH_4Cl (^{14}C-methyl-NH_3Cl). Rh_{null} cells of the regulator type (expressing no Rh-complex proteins) accumulated at room temperature radiolabelled methyl-ammonium ions to significantly higher levels (p=0.05) than normal RBCs. Rh_{null} cells of the amorph type (expressing limited amounts of Rh-complex proteins) accumulated an intermediate amount of methyl-ammonium ions. To

show that a decreased export contributes to its accumulation, the release of intracellular methyl-ammonium from the cells was measured over time. In 30 seconds, normal RBCs released 87% of the intracellular methyl-ammonium ions, whereas Rh_{null} cells of the regulator type released only 46%.

We conclude that the Rh complex is involved in the export of ammonium from RBCs.

5.1 Introduction

The Rh blood group system, expressed on red blood cells (RBCs), is of clinical importance, because antibodies against Rh antigens are involved in haemolytic disease of the newborn, haemolytic transfusion reactions and autoimmune haemolytic anaemia. Rh antigens are expressed on polypeptides encoded by the two highly homologous genes *RHD* and *RHCE* (reviewed in Avent *et al.*, 2000). Several RBC membrane glycoproteins are associated with these Rh polypeptides, such as RhAG (GP50), LW, CD47 and glycophorin B. (Cartron *et al.*, 1998) The Rh complex has an estimated molecular mass of 170 kD (Hartel-Schenk *et al.*, 1992), and consists of a tetramer with two RhAG molecules and two RhCcEe or RhD polypeptides (Eyers *et al.*, 1994). The Rh_{null} phenotype, in which no Rh antigens can be detected on RBC, is a rare autosomal recessive disorder characterized by a varying degree of haemolytic anaemia and spherostomatocytosis (Huang *et al.*, 1999).

DNA sequencing revealed that the erythroid Rh homologues RhD/RhCE and especially RhAG are related to the family of ammonium transporters, although nucleotide homology was not high (Marini *et al.*, 1997). Recently, the non-erythroid Rh homologues RhBG and RhCG have been described, which seem to be the missing link in the ammonium transporter phylogenetic tree (Liu *et al.*, 2000 & 2001). For these closely related non-erythroid Rh homologues, located in liver, skin, proximal tubules of the kidney (RhBG) and the testis, and in the collecting tubules of the kidney (RhCG), an ammonium transport function has been proposed. Marini *et al.* (2000) have shown that RhAG and RhCG (=RhGK) transfected into yeast promote ammonium ion (NH_4^+) transport. However, it is not known whether RhAG and/or RhD/CE also function as an ammonium transporter

in RBCs.

It has been known for a long time that RBCs can be selectively lysed by a buffer containing NH_4Cl and $KHCO_3$ (Figure 5.1) (Roos *et al.*, 1970). We postulated that a possible role for the Rh complex in NH_4^+ transport might be revealed in an ammonium lysis assay. The enzyme carbonic anhydrase, present in RBCs, plays a central role in this selective lysis, because lysis is restricted to RBC and is strongly inhibited by inhibitors of this enzyme. Ammonia (NH_3), formed by dissociation from NH_4^+ in the lysis buffer, diffuses into the RBC. Intracellularly, this NH_3 is bound to H^+ derived from H_2CO_3, formed by carbonic anhydrase from H_2O and CO_2. The remaining HCO_3^- inside the cell is exchanged for Cl^- from the outside of the RBC by the anion exchanger band 3 (Tanner *et al.*, 1988). Accumulation of NH_4^+ and Cl^- in the RBC results in osmotic swelling and successive lysis of the RBCs. In initial experiments we found that Rh_{null} cells, which express no (regulator type) or limited amounts (amorph type) of the Rh proteins, were more sensitive to NH_4Cl-induced lysis than normal RBCs ($p<0.0001$). Moreover, Rh_{null} cells of the regulator type were most sensitive to lysis. Although these results indicate that the Rh complex is involved in the export of NH_4^+ from the RBC, we cannot exclude that this decreased resistance is due to the known decreased osmotic resistance of Rh_{null} cells.

Therefore, we studied the role of the Rh complex in NH_4^+ transport directly by analysis of the transport of radiolabelled methyl-ammonium in normal and Rh_{null} RBCs.

5.2 Materials and Methods

Samples. Cryopreserved (in 38% glycerol) RBC samples (anti-coagulated in citrate) were obtained from 21 normal donors with common Rh phenotypes (R1r: 18-880, 18-882, 18-908; R2R2: 18-919, 18-911, 18-923; R2r: 18-756, 18-877, 18-930; R1R1: 13-7818, 13-7920, 13-7922; R0r: 13-7772, 13-8701, 13-8498; rr: 18-919, 18-911, 18-923, 18-903, 18-830, 18-921) and from 6 donors with the Rh_{null} phenotype (3-510, 3-980, 3-2301, 3-371, 3-617, 3-5134). The Rh_{null} cells were completely deficient in RhD and RhCE as determined by both FACS analysis with BRIC 69 (a monoclonal antibody (MoAb) against RhD/CE) (Avent *et al.*, 1988) and agglutination with a panel of human antisera. Four of the six Rh_{null} donors were of the regulator

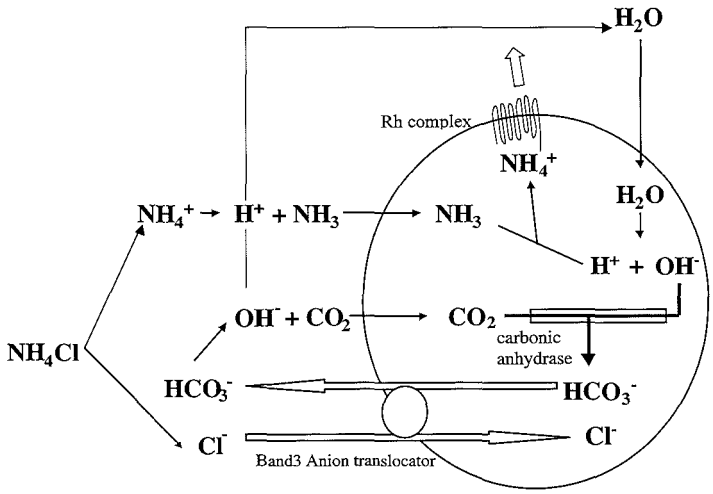

Figure 5.1: Scheme of RBCs incubated in a solution containing NH_4Cl and $KHCO_3$.

NH_3 diffuses into the RBC. The enzyme carbonic anhydrase in the RBC converts CO_2 and H_2O into H^+ and HCO_3^-. HCO_3^- inside the cell is exchanged for Cl^- from the outside. NH_3 is protonated to NH_4^+. Accumulation of NH_4^+ and Cl^- in the RBC results in osmotic swelling and successive lysis. The Rh complex promotes the export of the intracellularly formed NH_4^+, thereby preventing the attraction of H_2O and lysis.

type, which is due to a molecular defect in the *RHAG* gene (Chérif-Zahar *et al.*, 1996), resulting in the lack of expression of the complete Rh complex. The absence of these proteins was determined by FACS analysis with the MoAbs 2D10 (anti-RhAG) and LA18.18 (anti-RhAG) (Von dem Borne *et al.*, 1990; Mallinson *et al.*, 1990). The other two Rh_{null} donors were of the amorph type because their RBCs lacked expression of RhD and RhCE, but RhAG was still expressed. Rh_{null} of the amorph type is the result of a molecular change in *RHCE* in tandem with a deleted *RHD* (Avent *et al.*, 1998; Chérif-Zahar *et al.*, 1998 ;Huang et al, 1998). All RBCs were incubated in sorbitol for 5 minutes directly after thawing from liquid nitrogen storage, suspended in erythrocyte preservation medium (CLB, Amsterdam, The Netherlands) and used within 4 days after thawing. Before use, the RBCs were washed three times with phosphate-buffered saline (PBS).

The presence of ATP in all cryopreserved RBCs used, was demonstrated

by means of bioluminescent determination with luciferase/luciferin (Sigma Chemical Company, St.Louis, MO, USA); comparable amounts of ATP were found in all RBC suspensions.

Scatchard plot analysis. The expression of RhAG was determined by Scatchard plot analysis with the ^{125}I-labelled anti-RhAG MoAb 2D10 (^{125}I-2D10). The MoAb was labelled with the iodogen method. Two-fold serial dilutions from 250 nM to 0.5 nM of ^{125}I-2D10 were made in a volume of 35 μL. RBCs were added (7 x10^6 RBCs in 35 μL) and incubated at 37^{0}C until equilibrium was reached (120 minutes), as determined with a 2D10 association experiment. To separate free ^{125}I-2D10 from ^{125}I-2D10 bound to the RBCs, 50 μL of the reaction volume was spun through a layer of 20% (w/v) sucrose / 0.1% (w/v) bovine serum albumin (BSA) in PBS. Radioactive counts were measured in the RBC pellet for 1 minute on a Wallac multi-channel gamma-counter (Packard Instrument Company, Meriden, CT, USA). Non-specific binding was determined as the binding of ^{125}I-2D10 to Rh_{null} cells of the regulator type. To analyse the data, GraphPad Prism version 3.00 for Windows was used (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com/welcome.htm) with the non-linear regression technique 'one-site' binding (hyperbola).

^{14}C-methyl-ammonium loading. Because the radioactive isotope of nitrogen, ^{13}N, has a short half life (only 10 minutes), the radiolabelled ammonium analogue ^{14}C-methyl-ammonium was used in our studies of ammonium transport (Hackette *et al.*, 1970). The ability of the Mep/AMT/Rh family (including RhAG) to transport ^{14}C-methyl ammonium has been demonstrated before (Marini *et al.*, 2000; Hackette *et al.*, 1970). Indeed, in RBCs, similar kinetics of lysis were seen with methyl-NH_3Cl as with NH_4Cl (data not shown). At RT and at 0^{0}C, the intracellular concentration of ^{14}C-methyl-ammonium was measured at different times after adding this ammonium chloride analogue to the cells. In this experiment, we used RBCs of four normal phenotyped donors, two donors with Rh_{null} of the regulator type and two donors with Rh_{null} of the amorph type. The experiment was repeated three times. RBCs (1.6 x10^8 suspended in 160 μL of PBS) were incubated with an equal volume of an isomolar PBS solution, containing 31 mM methyl-NH_3Cl, of which 2‰ was ^{14}C-methyl-NH_3Cl

(143.4 Bq/μL), 10 mM $KHCO_3$ and 0.1 mM EDTA. RBCs were separated from the solution by centrifugation through a Ficoll layer (1.077 g/cm^3, Amersham Pharmacia AB, Uppsala, Sweden). The minimal time needed for the procedure of sample taking, centrifugation and removing the Ficoll gradient was 2 minutes. The pellet was then resuspended in 116 μL of PBS, and the proteins were precipitated by the addition of 6 μL of perchloric acid (PCA). After 15 minutes of incubation on ice, the samples were centrifuged. One hundred μL of clear supernatant was mixed with 10mL of the scintillation fluid Ultima Gold (Packard Instrument Company), and the radioactivity was measured in a 1900TR liquid scintillation analyser (Packard Instrument Company). The Transformed Spectral Index of the External Standard Spectrum (tSIE) was used to monitor and compensate for the quench level of each sample. The quench index was calculated from the Compton spectrum, induced by an external standard source (^{133}Ba), and the measurements are, therefore, described as dpm instead of cpm. Radioactive counts are given as the average of 5 independent measurements. By measuring the optical density of the supernatant at 405 nm it was shown that in 31 mM methyl-NH_3Cl no lysis of RBC occurred.

^{14}C-methyl-ammonium release. Release of ^{14}C-methyl-ammonium/ammonia in the supernatant and the amount of ^{14}C-methyl-ammonium/ammonia left in the pellet was measured after different release periods at RT in normal RBCs (n=4), Rh_{null} cells of the amorph type (n=1) and Rh_{null} cells of the regulator type (n=2). The experiment was repeated three times.

RBCs (2 x10^8) suspended in 200 μL of PBS were incubated on ice for 30 minutes with an equal volume of an isomolar PBS solution, containing 31 mM methyl-NH_3Cl, of which 2‰ was ^{14}C-methyl-NH_3Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA. The RBC suspension was divided into two parts, and the RBCs in both were separated from the solution by centrifugation through a Ficoll layer. The RBC pellet of one part was resuspended in 116 μL of PBS. The counts measured by means of scintillation in the clear supernatant obtained after the PCA precipitation procedure of these RBCs represent the total uptake of the ^{14}C-methyl-NH_3^+. The RBC pellet of the second part was resuspended in 300 μL of ice-cold PBS or in 300 μL of 31 mM unlabelled isomolar methyl-NH_3Cl solution. Samples of 100 μL

were taken at RT, and the RBCs were spun down after 30 seconds, 90 seconds and 180 seconds. The minimal time needed for separating the RBCs from the supernatant with the released counts is 30 seconds. The counts measured by means of scintillation in the supernatant of these samples represent the release of both ^{14}C-methyl-NH_3^+ and ^{14}C-methyl-NH_2. The RBC pellets of these samples were resuspended in 116 μL of PBS. Counts measured by means of scintillation in the clear supernatant obtained after PCA precipitation of these RBCs represent the remaining ^{14}C-methyl-NH_3^+ in the RBC pellet after release.

^{14}C-benzylamine and ^{14}C-urea loading and release. Loading and release experiments with ^{14}C-benzylamine and ^{14}C-urea were similarly performed as with ^{14}C-methyl-ammonium. We used RBCs of four donors with the normal phenotype and of three donors with the Rh_{null} phenotype (regulator (n=2) or amorph type (n=1)). The RBCs were incubated in an isomolar solution of PBS solution containing 31 mM benzylamine, of which 2‰ was ^{14}C- benzylamine (138.8 Bq/μL), 10 mM of $KHCO_3^-$ and 0.1 mM EDTA or 200 μM urea, of which 10% (37 Bq/μL) was ^{14}C-urea. In the urea loading and release experiments also RBCs were tested from two donors (13-9172, 13-7995) with the $Kidd_{null}$, Jk(a^-b^-), phenotype, missing the urea transporter (Sands *et al.*, 1997).

Correction for residual water. To measure the adherence of residual water to the different RBC types, we used (3000 MW) FITC-dextran (Molecular probes, Eugene, Oregon, USA). FITC-dextran is a fluorescent molecule that does not enter the RBC, but can be spun through the Ficoll layer in the watermantle of the RBC. We incubated the RBCs in an isomolar solution containing FITC-dextran (diluted 1:50). RBCs were precipitated with PCA as described above. The pH of the supernatant was restored with K_2CO_3, precipitated salts were removed by spinning the samples for 15 minutes at 4^0C. The fluorescence of the supernatant was measured in a flat-bottomed white polystyrene plate on the HT Soft multi-well reader (Perkin-Elmer, Norwalk, CT, USA)

Statistics. The data of the different donors with the same phenotype were comparable and were, therefore, considered as one group. All results

were expressed as the mean of at least three experiments ± standard deviation (SD). Significance levels were determined with the two-sided Student's t-test.

5.3 Results

RhAG expression on RBCs. The amount of RhAG expressed on the two Rh_{null} cells of the amorph type in comparison with normal RBCs of eight different donors, representing four different phenotypes, was determined by Scatchard plot analysis. The number of binding sites for MoAb 2D10 on the RBCs of the two Rh_{null} RhAG-positive donors was 33% ±4% and 38% ±7%, respectively, of the binding sites on normal cells (n=8) as determined in three independent experiments. No expression of RhAG was seen on Rh_{null} cells of the regulator type. No clear differences in the amount of RhAG expressed on RBCs of the various Rh phenotypes were found, in particular no clear gene dosage effect was seen for RhD-positive and RhD-negative RBCs (data not shown).

^{14}C-methyl-ammonium loading. The RBCs were incubated at room temperature (RT) or at 0^0C with a solution containing ^{14}C-methyl-ammonium chloride in the presence of an excess of unlabelled methyl-ammonium chloride (31 mM) (see Material & Methods). Under these conditions, methyl-NH_2 diffuses freely into the RBCs and is quickly protonated to methyl-NH_3^+. After different time points, the amount of intracellular ^{14}C-methyl-NH_3^+/NH_2 in the RBCs was measured.

At RT in normal RBCs, the plateau of a low concentration of intracellular methyl-NH_3^+/NH_2 was immediately reached, possibly because all methyl-NH_2 converted to methyl-NH_3^+ was pumped out of the RBC. In Rh_{null} cells, the intracellular methyl-NH_3^+/NH_2 concentration increased during the incubation period (Figure 5.2A). After 32 minutes, Rh_{null} cells of the regulator type and the amorph type contained about 2 times more ^{14}C-methyl-ammonium than the normal RBCs did ($p<0.008$). At 0^0C in normal RBCs, an increase in methyl-ammonium content was observed with time; with similar kinetics, the same plateau was reached as was observed for the two types of Rh_{null} cells (Figure 5.2B).

^{14}C-methyl-ammonium release. Loading of RBCs with ^{14}C-methyl-NH_3^+/NH_2 was performed for 30 minutes at 0^0C, because all RBCs accumulated the same amount of ^{14}C-methyl-NH_3^+/NH_2 under these conditions. After loading, the cells were incubated at RT. The release of ^{14}C-methyl- NH_3^+/NH_2 was expressed as a percentage of the total intracellular amount after loading with ^{14}C-methyl-NH_3^+/NH_2 (Figure 5.3). Normal RBCs had released almost all intracellularly loaded ^{14}C-methyl-NH_3^+/NH_2 within the first 30 seconds. The Rh_{null} cells of the regulator type (without RhAG) did not release all loaded methyl-ammonium within 3 minutes ($p<0.006$). In Rh_{null} cells of the amorph type (with RhAG) the release of methyl-ammonium was slower than in normal RBCs. It took significantly more time (90 seconds at RT, $p=0.013$) to release the radiolabelled methyl-ammonium than in normal RBCs. No clear differences were seen between the release of ^{14}C-methyl-NH_3^+/NH_2 when the loaded RBCs were resuspended in PBS or when the cells were resuspended in an isomolar solution containing 31 mM methyl-NH_3Cl concentrations (data not shown). In all samples, the sum of the radioactivity in the supernatant and in the pellet constituted the radioactivity of the input.

^{14}C-benzylamine and ^{14}C-urea loading and release. To determine whether the observed differences between normal RBCs and Rh_{null} cells were specific for ammonium transport, the transport of radiolabelled benzylamine and urea was measured as well. Loading the RBCs at RT with ^{14}C-benzylamine, a plateau of low concentration of intracellular benzylamine was immediately reached in Rh_{null} and normal RBCs. The percentages of released ^{14}C-benzylamine after 30 seconds, 90 seconds and 180 seconds, measured in three separate experiments after loading the RBCs with equal amounts of ^{14}C-benzylamine, were similar in Rh_{null} cells and in normal RBCs. In 30 seconds, normal RBCs released 82.9% $\pm$6.5%, Rh_{null} amorph type 83% $\pm$10.2% and Rh_{null} regulator type 84% $\pm$8.5%. After 180 seconds, normal RBCs released 85.9% $\pm$5%, Rh_{null} amorph type 88.2% $\pm$3.5% and Rh_{null} regulator type 88.4% $\pm$4.2% (n=3).

Also by loading with ^{14}C-urea, a plateau of low concentration of intracellular urea was immediately reached in normal and Rh_{null} RBCs, but in $Kidd_{null}$ cells the intracellular urea concentration increased during the incubation period (Figure 5.4). After 15 minutes $Kidd_{null}$ RBCs contained

about 7 times more ^{14}C-urea than did normal and Rh_{null} RBCs ($p \leq 0.006$).

To measure the release of intracellular urea, all RBCs were loaded with equal amounts of ^{14}C-urea and then incubated in an isomolar phosphate-buffered solution (Figure 5.5). Both normal and Rh_{null} (regulator and amorph type) RBCs released 87% of the intracellular accumulated urea within 30 seconds, while the $Kidd_{null}$ RBCs released only 42% ($p << 0.0001$). In 180 seconds, $Kidd_{null}$ RBCs released 82% of the intracellular urea, which was still significantly less than the normal and Rh_{null} RBCs ($p < 0.02$).

Correction for residual water. To exclude a difference in the amount of residual water, we measured the amount of FITC-dextran adhered to the different cell types. No differences were measured in fluorescence between the different RBC types (n=3). The proportion of contamination of external medium in the RBC pellet in Rh_{null} cells was 0.21% $\pm$0.02% and in normal RBCs 0.22% $\pm$0.11%. This indicates that the proportion of contamination of external medium in the RBC pellet between the different RBC types is comparable.

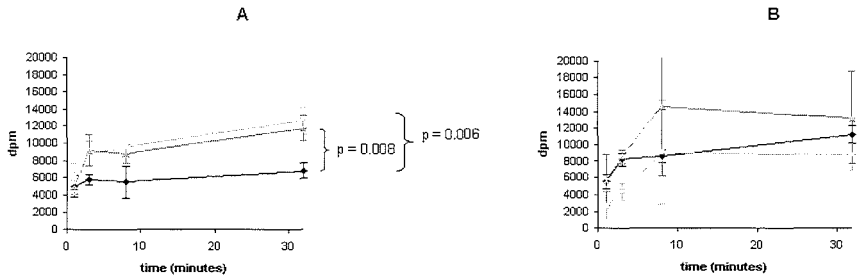

Figure 5.2: ^{14}C-methyl-ammonium loading at RT (A) and 0^0C(B).

Radioactivity of ^{14}C-methyl-ammonium measured in pellets of normal RBCs (n=4) (black diamond) and Rh_{null} cells of the amorph type (n=2) (grey triangle) and the regulator type (n=2) (grey square) after different incubation times in a 31 mM methyl-NH_3Cl (2‰ ^{14}C-methyl-NH_3Cl) solution. Before counting the radioactivity, the proteins were precipitated as described in "Materials and Methods". The data are depicted as the mean ± SD of three experiments. Data of the different donors are pooled. The radioactive counts are given as the average of five independent measurements, corrected with an external control (dpm). Significantly more ^{14}C-methyl-ammonium/ammonia was loaded in Rh_{null} cells than in normal RBCs at RT (A). In normal RBCs significantly more radioactivity was loaded at 0^0C than at RT (B).

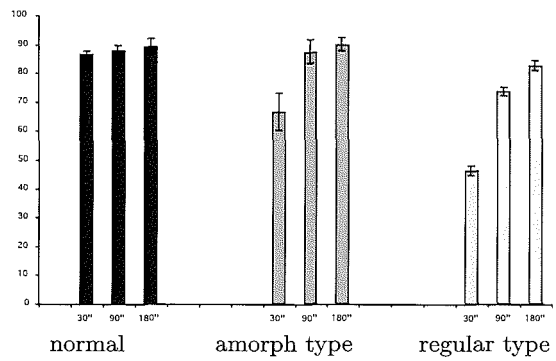

Figure 5.3: ^{14}C-methyl-ammonium release.

RBCs were loaded with ^{14}C-methyl-ammonium/ammonia by incubation on ice for 30 minutes in an isomolar buffer containing 15.5 mM methyl-NH_3Cl of which 2‰ was ^{14}C-methyl-NH_3Cl. Loaded RBCs were incubated in PBS at RT. Release of ^{14}C-methyl-ammonium/ammonia in the supernatant and the amount of ^{14}C-methyl-ammonium/ammonia in the pellet was measured after different release periods at RT in normal RBCs (n=4), Rh_{null} cells of the amorph type (n=1) and Rh_{null} cells of the regulator type (n=2). The results are expressed as percentage of total intracellular amount after loading with ^{14}C-methyl-ammonium. The sum of radioactivity in the pellet and in the supernatant was always 100%. The data are depicted as the percentage of radioactivity released by the cells after 30, 90 and 180 sec, mean ± SD of three independent experiments. Data of the different donors are pooled.

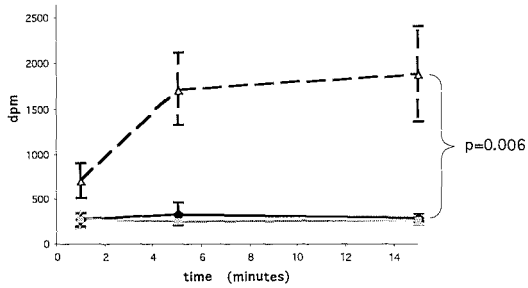

Figure 5.4: ^{14}C-urea loading at RT.

Radioactive counts of ^{14}C-urea measured in pellets of normal RBCs (n=4) (black diamonds) and Rh_{null} cells of the regulator type and amorph type (n=3) (grey squares) and the $Kidd_{null}$ RBCs (n=1) (open triangles) after different incubation times in a 100 μM urea (10% ^{14}C-urea) solution. Before counting the radioactivity, the proteins were precipitated as described in "Materials and Methods". The data are depicted as the mean $\pm$ SD of three experiments. Data of the different donors were pooled; no differences were seen between the two Rh_{null} types. The radioactive counts are given as the average of five independent measurements, corrected with an external control (dpm). Significantly more ^{14}C-urea was loaded in $Kidd_{null}$ cells than in normal RBCs and Rh_{null} cells.

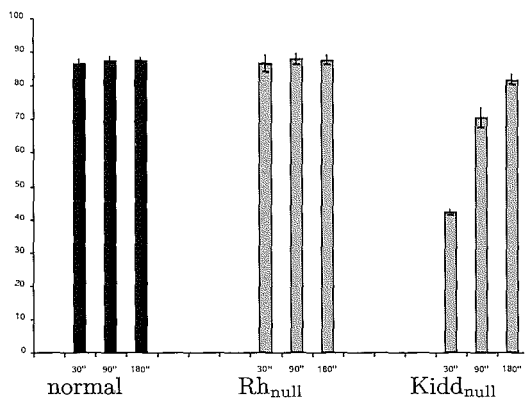

Figure 5.5: ^{14}C-urea release.

RBCs were loaded with ^{14}C-urea by incubation on ice for 30 minutes in an isomolar buffer containing 100 μM urea of which 10% was ^{14}C-urea. Loaded RBCs were incubated in PBS at RT. Release of ^{14}C-urea in the supernatant and the amount of ^{14}C-urea in the pellet was measured after different release periods at RT in normal RBCs (n=4), Rh_{null} cells (n=3) and $Kidd_{null}$ cells (n=2) and expressed as percentage of total intracellular amount after loading with ^{14}C-methyl-ammonium. The sum of radioactivity in the pellet and in the supernatant was always 100%. The data are depicted as the percentage of radioactivity released by the cells after 30, 90 and 180 sec, mean $\pm$ SD of three independent experiments. Data of the different donors were pooled.

5.4 Discussion

Previously, it has been shown that RhAG transfected into yeast promotes NH_4^+ transport, both import and export (Marini *et al.*, 2000). In the present study, we show that also in RBCs the Rh complex functions as an ammonium/ammonia exporter. Based on experiments with Rh_{null} cells, we postulate that upon incubation with an aqueous solution of NH_4^+, in which ammonium ions are in equilibrium with ammonia, NH_3 can diffuse over the RBC membrane, independent of the presence of the Rh complex. Within the red cell, NH_3 binds H^+ derived from H_2CO_3, formed by carbonic anhydrase from H_2O and CO_2 (Figure 5.1). The Rh complex promotes the export of this intracellularly formed NH_4^+. Initially, we noticed that upon incubation in a lysis solution, containing 77.5 mM NH_4Cl and $KHCO_3$, Rh_{null} cells lyse significantly faster than normal RBCs (data not shown). Because it is known that Rh_{null} cells are osmotically more fragile, this observation might be independent of any transport function of the Rh complex. But it already argues against a major role for the Rh complex in the import of ammonium. It has previously been shown that the RBC membrane is permeable for NH_3, whereas no NH_4^+ influx was seen (Labotka *et al.*, 1995). Whether the import of ammonium takes place by free diffusion of the gaseous ammonia through the lipid bilayer or whether another carrier protein in the RBC membrane is needed, as suggested by Ludewig *et al.* (2001), has to be investigated.

To investigate whether increased accumulation of ammonium ions takes place upon incubation in a solution containing NH_4Cl and $KHCO_3$, the concentration of radioactive intracellular methyl-ammonium was measured over time after incubation in a non-lysing isomolar solution with 15.5 mM methyl-NH_3Cl/2‰ ^{14}C-methyl-NH_3Cl. Indeed, the amount of ^{14}C-methyl-NH_3^+/NH_2 in Rh_{null} cells increased over time and was two times higher in Rh_{null} cells than in normal RBCs after 30 minutes, which again demonstrates that the presence of the Rh complex is not a prerequisite for ammonium influx. Since this increased accumulation might be due to a missing exporter for ^{14}C-methyl-ammonium in Rh_{null} cells, the release of radioactivity from ^{14}C-methyl- NH_3^+/NH_2-loaded RBCs was measured. Rh_{null} cells of the regulator type showed a low release of ^{14}C-methyl-NH_3^+/NH_2 in 30 seconds compared to an almost complete release in normal RBCs. The low release measured in Rh_{null} cells of the regulator type is probably the

gaseous $^{14}CH_3$-NH_2 that can freely diffuse out of the RBCs. An alternative explanation might be the presence of an additional ammonia transporter in RBCs, which is present in normal RBC as well as in Rh_{null} cells (Ludewig *et al.*, 2001). The release of ^{14}C-methyl-NH_3^+/NH_2 by Rh_{null} cells of the amorph type was intermediate after 30 seconds, but increased in 3 minutes to an almost complete release. This is most probably due to a decreased export capacity by the limited amount of RhAG proteins and/or due to the lack of RhD/CE proteins in the complex.

The differences in counts measured in the loading and release experiments are not due to differences in residual water between the cell types, because the proportion of contamination of external medium in the RBC pellet between the different RBC types is comparably low.

To show that the Rh_{null} cells, except for (methyl-) ammonium transport, behave normally in transport processes, the export of other radioactive molecules was also measured. Neither ^{14}C-benzylamine nor ^{14}C-urea showed increased uptake in Rh_{null} cells compared with normal RBCs. Also the release of these radioactive substances from preloaded cells was comparable. These results indicate that Rh_{null} cells do not differ in cell volume and surface area from normal RBCs. The $Kidd_{null}$ RBCs (missing the urea transporter Jk (Sidoux-Walter *et al.*, 1999)), used as a control for urea transport, did show an increased accumulation of ^{14}C-urea and a decreased release, compared with normal RBCs and also Rh_{null} cells. This implies that our methods are capable of measuring impaired transport.

It has been described that the Kidd protein, after transfection into the Xenopus oocyte is involved in import of urea (Sands *et al.*, 1997; Sidoux-Walter *et al.*, 1999). Also, RhAG transfected into the Xenopus oocyte or yeast cell functions as an importer of ammonium (Marini *et al.*, 2000; Westhoff *et al.*, 2002). Our experiments with null cells demonstrate that both the Kidd protein and the Rh complex in RBCs are involved in the export and are not essential for the import of urea and ammonium, respectively. However, possible differences in kinetics of import between normal and null cells might have been missed by our technical approach, because this process is too fast.

In RBCs lacking the Rh proteins, completely as in Rh_{null} cells of the regulator type or partially as in Rh_{null} cells of the amorph type, the transport of NH_4^+ is diminished. The Rh_{null} cells of the amorph type express

33-38% of RhAG antigens compared to RhD-positive and RhD-negative phenotypes. Because the accumulation in the Rh_{null} cells of the amorph type shows intermediate results in all tests, there may be a dose-dependent effect proportional to the amount of RhAG proteins. Whether the polymorphic RhD and RhCE polypeptides are similarly involved in NH_4^+ export cannot be concluded from our experiments. The Rh_{null} cells of the amorph type show that the polymorphic Rh polypeptides are not a prerequisite for ammonium transport, but since so far RhD or RhCE polypeptides have never been expressed in a complex with a non-functional mutant of the Rh-family (RhAG, RhBG or RhCG), this question remains unanswered. We did not find functional differences between RhD-positive and RhD-negative RBCs (data not shown).

The ammonium transport function of the Rh proteins could serve as a protection for the RBC in an environment with high ammonium levels, as may be found in the kidney during the excretion of acids. If Rh proteins are absent, haemolysis might then occur, as noticed in Rh_{null} donors.

5.5 References

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Chapter 6

General discussion

The expression of Rh can vary. This thesis mainly involves the RhD proteins with weak expression. A central question in this thesis is whether these RhD proteins also have qualitative variations, because then alloantibodies can be formed by weak-D individuals against the normal D antigen or against the weak-D antigen by normal D individuals. This would have implications for transfusion policy. To investigate qualitative variations, knowledge about the tertiary structure of RhD is indispensible. Also insights in the processing of the Rh proteins might give us a clue about the genesis and nature of the weak D proteins. The variations of Rh phenotypes between ethnic populations is already known, but only speculations can be made for the reason of this variation; it might be related to the function of Rh. Involvement of the Rh complex in ammonium transport has been shown, but the complete process has not been fully elucidated.

6.1 Variation in expression of Rh antigens

The RhD-antigen expression varies considerably between individuals, from the greatly enhanced expression of the D-- phenotype, via the normal phenotypes (in decreasing order: R2R2, R1R2, R1R1, R2r, R1r) to the weak-D phenotypes. Besides quantitative variations also qualitative variations occur. In qualitative variants (partial Ds), one or more epitopes of RhD are missing and the remainder is expressed normally or weakly, whereas in purely quantitative variants, all epitopes are considered to be expressed.

However, the division between qualitatively and purely quantitatively altered antigens is often not distinct and they are difficult to differentiate, especially in qualitatively altered antigens that are weakly expressed. The ability to generate alloantibodies against RhD in partial-D phenotypes, but not in weak-D phenotypes, is probably the most suitable distinction.

An example of a variant with weak expression of a qualitatively altered D antigen is the DAR phenotype (Chapter 2). This partial D is genotypically characterised by *RHCE*-specific nucleotides in exons 4, 5 and 7 and is frequently found in African Blacks. Because of its weak expression, this RhD variant is also known as weak-D type 4.2.0 (Wagner *et al.*, 2000), but the presence of alloantibodies in individuals with the DAR phenotype, after transfusion with RhD-positive blood, is the definite proof that DAR is also a qualitatively altered antigen. Other proven qualitatively altered RhD antigens with a very weak expression (less than 4000 sites/cell) are D^{II}, D^{IVb}, D^{IV} type III, D^{VI} type 1 and 2, D^{VII}, DMHI, DHO, DHR and DIM (Table 1.1, General Introduction). The distribution of the amino acid (aa) alterations over the RhD protein (intracellular or transmembrane vs extracellular regions, RhD-specific aa's replaced by RhCE-specific aa's vs novel aa's, specifically located in the preserved region of the Rh super family or not) is not different from partial-D antigens with normal expression. The complete RhD antigen can be very immunogenic for these partial-D phenotypes with weak expression (e.g. D^{VI} type 1 and 2) (Lacey *et al.*, 1983)(Jones *et al.*, 1995).

Most partial-D antigens are characterised by amino-acid alterations in the extracellular domain of the RhD protein. However, in some partial D's (D^{IIIa}, D^{IIIc}, D^{III} type VI and DAR) amino-acid substitutions are only present in the transmembrane or intracellular part of the protein. Because individuals carrying these variants are known to produce alloantibodies, it can be concluded that epitope loss can be caused by amino-acid substitutions in every part of the protein. The mutations found in the different weak-D types are all located in regions of the *RHD* gene encoding transmembrane or intracellular parts of the protein. These mutations might cause conformational changes or regions of local unfolding by affecting charged residues in transmembrane domains, which break the hydrophobicity of the helices (Bonifacino *et al.*, 1991)

To elucidate whether weak-D phenotypes are purely quantitative vari-

ants, or whether, besides weak D type 4.2.0 (DAR), also other weak D types are due to qualitative variations, was one of the aims of this thesis. Besides alloantibody formation, other indications for qualitative variations have not been helpful. The Rhesus similarity index (RI), a way to express variation among the expression of 59 epitopes measured by flowcytometry, was thought to give an indication on qualitative variation. Because in most weak D's not all epitopes are expressed in equal amounts, represented by a low RI, qualitative alteration of the weak-D protein have been assumed. (Wagner *et al.*, 2000). However, our Scatchard plot analysis did not confirm qualitative alteration of the weak-D antigen, because the affinities of several antibodies tested (directed against different epitopes) for the weak-D antigen were not significantly different from those for the normal RhD antigen (Chapter 3). The same study revealed that the wider variance in epitope expression, reflected by a lower RI, is due to differences in affinity of the antibodies. This was concluded from the observation that MoAbs with equal affinities for the RhD antigen demonstrated equal epitope expression, whereas MoAbs with large differences in affinity demonstrated similarly large differences in epitope expression. Therefore, the RI does not merely reflect qualitative changes of the RhD antigen.

By expressing weak-D mRNA in K562 cells, we clearly found that the mutations in individuals with the weak-D phenotypes are indeed responsible for the weak expression of RhD (Chapter 4). The query then was how these mutations lead to a reduced expression of RhD. Weak-D mutations do not involve the amount or stability of the *RHD* mRNA, as we have shown by RQ-PCR in both RBCs and K562 cells. Weak-D mutations also do not lead to obvious qualitative alterations of the weak-D antigen, as we have shown by Scatchard plot analysis on RBCs (Chapter 3). Subsequently, we investigated whether the weak-D point mutations might affect the formation of the Rh complex or the integration of the RhD polypeptide into the membrane without conformational changes in its extracellular loops.

Weak-D proteins that are not properly processed for integration into the membrane will be degraded by proteasomes. Inhibition of these proteasomes with Lactacystin would then lead to intracellular accumulation of RhD proteins. However, in our K562 model no increase in intracellular RhD, weak-D type-1 or weak-D type-3 protein was observed by flowcytometry after incubation with the proteasome inhibitor Lactacystin (Chapter

4). To circumvent the possibility that the MoAb LOR15C9 is unable to recognise unprocessed weak D, a K562 model was created expressing GFP-fusion proteins. The *RHD*-GFP, the weak-D type-1-GFP and the weak-D type-3-GFP fusion proteins were recognized by membrane localization of GFP and by co-localization of RhD with GFP. But again, no increase in intracellular expression of GFP was observed by flowcytometry and confocal microscopy after inhibition of the proteasome activity. Nevertheless, the misfolded or uncomplexed protein hypothesis should not immediately be rejected, because the approach to prove the hypothesis might not be right. The GFP-tag has the same size as the Rh protein, possibly leading to a change in conformation or processing of the fusion protein. Also the use of an artificial promotor and K562 cells that do not resemble RBCs in all characteristics could be a point of concern in this approach.

The existence of phenotypes with qualitatively altered RhD antigens with low expression raises some questions concerning their genesis. When the partial D antigen is due to more than one amino-acid change (e.g. D^{IVb}, D^{IV} type III, D^{VI} type 1 and 2, DAR), then one amino-acid change may cause the conformational change, while another one may affect the processing of the protein to the membrane. But in case only one amino-acid change causes the partial D (D^{VII}, DHMI, DHO, DHR, DIM), maybe the amino-acid changes in these partial-D antigens do not only influence the conformation of the proteins, but also influence the stability of the mRNA transcripts or the processing of the proteins that lead to their stable integration in the membrane. The latter is more plausible because reduced stability of *RHD* mRNA has never been observed. It is remarkable that the mutations in the partial-D antigens with weak expression due to one point mutation are never located on polymorphic sites, and are thus not replaced by *RHCE*-specific nucleotides, and that they are always located extracellularly (except for DHMI). In contrast, partial-D antigens with normal expression always have their mutations on polymorphic sites, and weak-D antigens always have intracellular or transmembrane mutations.

Alloantibody formation in individuals with a variant-RhD phenotype. Until now, no alloantibodies have been found in individuals with the most common weak-D phenotypes. This implicates that it is not likely that these antigens are qualitatively altered. When individuals with partial

D phenotypes are transfused with normal RhD-positive blood, they may produce alloantibodies. For carriers of partial-D variants, complete RhD antigen might be relatively immunogenic (as with D^{VI}) or less immunogenic (as with DAR). New insight in the epitope model provides an explanation for the low immunogenicity of complete RhD in individuals with some variant-RhD antigens. The conventional idea was that epitopes overlap but are spatially different. Chang *et al.* (1998) proposed a completely new model in which epitopes do not spatially differ, but differ only in number and arrangements of contact residues present (Figure 6.1). In other words, the footprints of most, if not all, anti-D antibodies are essentially identical. They came to this model by analysing anti-RhD antibodies that were obtained by a Fab/phage display-based method, using a library made from peripheral blood. Detailed analysis of randomly selected clones revealed that, whereas the clones had a specificity for at least half of the major RhD epitopes, in all clones only four closely related heavy-chain germline genes were used (VH3-33 superspecies) and nearly all V_k light chains were derived from one germline gene (DPK9). This new model suggests that in the alloimmune response against RhD, similar and restricted pathways are used in different individuals. It is tempting to speculate that, for example, in the partial-D variant DAR, the apparently low immunogenicity for the complete antigen is due to the fact that the most common pathway could not be used, because these B cells have been clonally deleted or have become anergic to avoid self-reactivity. Probably, in those individuals with the DAR phenotype who did make alloantibodies, a less common pathway has been used to produce the anti-D (Chapter 2).

The mechanism for low immunogenicity, as described above for the DAR phenotype, might also account for the weak-D phenotype, and for that reason we cannot exclude that weak D might be a qualitatively altered antigen.

To elucidate whether specific parts of the RhD allele are provoking an alloimmune respons, Stott *et al.* (2000) investigated the helper response that drives the production of alloanti-D. They studied the proliferation of peripheral blood mononuclear cells (PBMCs) from RhD-negative individuals who developed alloanti-D upon stimulation with a panel of 68 overlapping synthetic 15-mer peptides, spanning the complete sequence of the RhD protein. The number of peptides that induced a proliferative re-

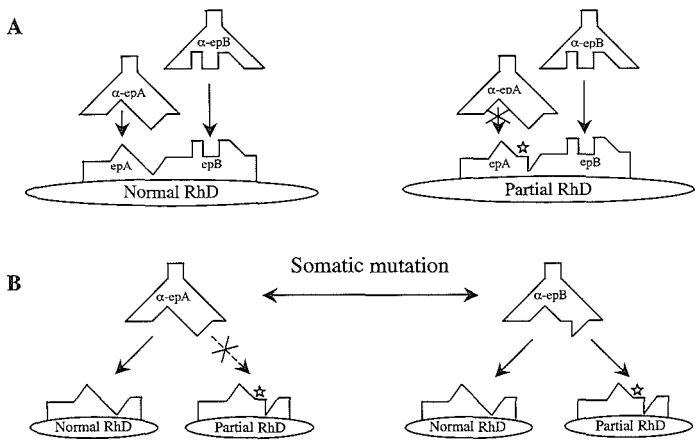


Figure 6.1: : Models for RhD antigen/antibody binding.
Conventional model (A) and model proposed by Chang *et al.* (1998) (B).The conventional model shows spatially different epitopes, while the proposed model only differs in number and arrangement of contact residues presented. In the proposed model of Chang *et al.* a genetic relationship between the antibodies can be expected.

sponse was unrelated to either the frequency of, or the time since, exposure to RhD-positive RBCs, but seemed to be strongly correlated to the level of alloanti-D antibodies. The identification of alloreactive T-cell epitopes on the Rh protein showed that not only extracellularly located RhD sequences stimulate T-cells from alloimmune donors. Most peptides contained RhD-specific polymorphisms, but also responses to peptides that are identical in RhD and RhCE were observed. This could be explained by a difference in processing between RhD and RhCcEe proteins by the antigen-presenting cells (APCs). If so, highly stimulatory peptides, although not containing RhD-specific polymorphisms, are only generated from the RhD protein.

From this study, it can be concluded that also the T-cell epitopes (15-mer peptides) in which the amino-acid substitutions of the weak-D antigens are located, can be very alloreactive to PBMCs. Thus, this cannot be the reason that no alloantibodies have been found in individuals with the weak-D phenotype.

Tertiary structure of the RhD protein. To investigate the tertiary structure of the RhD protein, the patterns of RhD-epitope expression have been related to the regions of RhD amino-acid alterations in the partial-D phenotypes (Scott *et al.*, 1996), (Cartron *et al.*, 1996). Approximately five to eight amino acids are usually critically involved in determining a protein epitope, although a further ten amino acids may interact with the antigen-binding site of an antibody (Crumpton, 1974), (Amit *et al.*, 1986), (Van Regenmortel *et al.*, 1988). Epitopes are formed on a sequential run of amino-acid residues along a protein chain, or composed of amino acids drawn from different parts of the polypeptide chain that are brought together by the conformational folds in the tertiary structure of the protein (Atassi *et al.*, 1978). The use of partial-D antigens to assess information on the tertiary structure of RhD can never be absolute, because the majority of partial-D phenotypes are knock-outs, in which loss of RhD epitopes is observed. Additional information will be obtained with a knock-in model, because most partial-D phenotypes are a combination of mutations in different loops. Also, only a limited panel of partial-D phenotypes is provided by nature. It cannot be assumed that the molecular changes associated with the epitope deficiency are directly responsible for their expression. Changes in one region of the RhD protein may affect other domains, and the substitution of amino acids for others with different size or charge may significantly alter RhD presentation.

To study the role of various amino acids in RhD-epitope presentation, a method in which RhD-specific amino acids were introduced by site-directed mutagenesis into an RhcE protein (knock-in phenotypes) has been described by Avent *et al.* (2000) The 3rd, 4th and 6th extracellular loops were introduced, because only these loops comprise RhD-specific sites. With the results, a model was proposed for the arrangement of the RhD protein in the RBC membrane (Figure 6.2). This model takes into account that the possible physical size of an anti-RhD paratope is not more than 20 Å in diameter. It was assumed that loops 3 and 4, as well as 4 and 6, must be in close proximity, whereas loop 3 and 6 are non-adjacent, because no RhD epitopes are dependent on this structure. The model proposed the localisation of six different RhD epitope clusters. Epitope 3 and 9 require RhD-specific sequences on exofacial loop 6 (cluster A), and possibly epitope 4 also requires loop 6 (cluster E). The expression of epitopes 5 and 6/7 is

dependent on a combination of loop 3 and 4 (cluster F). The expression of epitope 5 is dependent on loop 4 and 6 (cluster B). The expression of epitope 2 (partly), 5 and 6/7 is dependent on loop 3, 4 and 6 (cluster C). Expression of epitope 1, part of 2 and the complete epitope 8 is lacking in this model. This was due to the c-critical residue Pro-103, because K562 cell lines expressing an RhD-CE(loop 2,3,4,6)-D hybrid containing Ser 103 did express epitope 8. This model is entirely consistent with the known partial-D variants and will be useful until high-resolution three-dimensional structures of RhD-protein crystals are available.

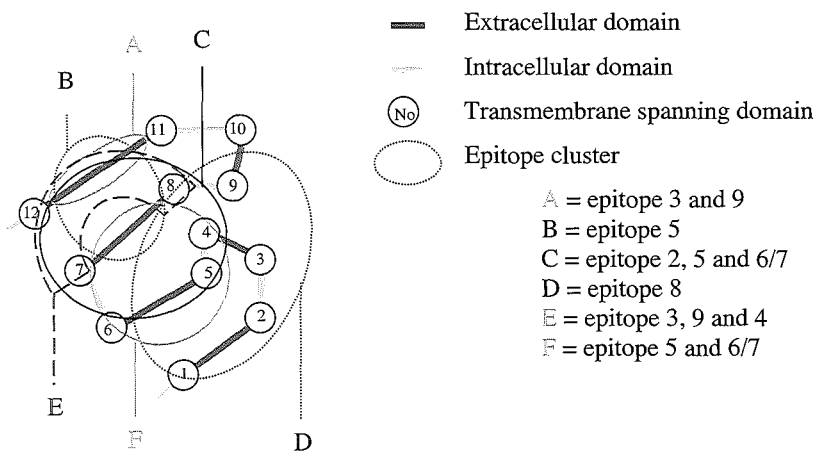


Figure 6.2: Diagrammic model of the RhD protein viewed from outside the membrane.
Circles (numbered 1-12) represent membrane-spanning domains, black lines indicate the extracellular loops and grey lines represent the cytoplasmatic loops. The proposed localisation of the six different epitope clusters are represented by dashed circles.

The above-presented study shows that the expression of one RhD epitope is dependent on a combination of multiple RhD-specific sequences in different parts of the RhD polypeptide. This phenomenon is also observed for the epitopes VS (RH20) and V (RH10). The VS and V epitopes, variants of Rhce, are almost exclusively found in the black African population. Anti-VS and anti-V have been found frequently, often in sera containing other antibodies, but they have never been reported to be clinically important. The $VS^{+}V^{+}$ phenotype is caused by a point mutation in exon 5,

nt733 C→G (aa245 Leu →Val), of the *RHce* allele. An additional mutation in exon 7, nt1006 G →T (aa336 Gly→Cys) leads to the loss of the V epitope (VS^+V^-) (Figure 6.3) (Daniels *et al.*, 1998). But the *ceAR* allele leads to the VS^-V^{+w} phenotype. This allele is characterised by an *RHCE-D-CE* gene, with nt733 C→G and additional mutations in exon 5 and 6 (nt712 A→G, nt787 A→G, nt800 T→A and nt916 A→G) (Chapter 2). Probably, the amino-acid change aa245 Leu→Val, in the surrounding of *RHD* polymorphic sites, does not give rise to the VS epitope, although it still contributes to the expression of the V epitope.

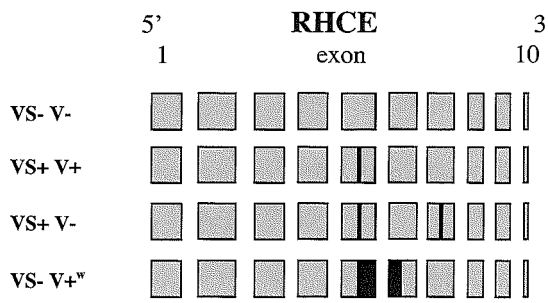


Figure 6.3: Representation of the 10 *RHCE* exons of several VS/V phenotypes.
Codon 245Val (exon 5) gives rise to the VS^+V^+ phenotype. Codons 245Val and 336Cys (exon 7) give rise to the VS^+V^- phenotype. Codon 245Val surrounded by codons 238Val, 263Gly and 267Lys (exons 5 and 6) gives rise to the VS^-V^{+w} phenotype.

Different roles for RhAg and RhD/RhCcEe in the processing of the Rh complex. RhD/RhCcEe and RhAg appear to be both necessary for the surface expression of the Rh complex, but they differ notably in the factors that determine their cell-surface expression. Whereas RhD and RhCcEe have never been found in the absence of RhAg, RhD/RhCcEe might be partially dispensable for the routing of RhAg to the membrane. This can be concluded from four observations: i) a significant level of RhAg is still expressed on the RBC surface in Rh_{null} RBCs of the amorph type (Chérif-Zahar *et al.*, 1996), ii) a significant level of RhAg is still expressed on K562 cells that display no or very low amounts of RhD/RhCcEe (Anstee *et al.*,1992), (Suyama *et al.*, 1994), (Chérif-Zahar *et al.*,1998), iii) RhAg

is expressed before RhD and RhCcEe during differentiation of erythroid progenitors *in vitro* (Bony *et al.*, 1999), (Southcott *et al.*, 1999) and iv) evolutionary, *RHD* and *RHCE* are probably derived from *RHAG* (Carrit *et al.*, 1997).

RhD and RhCcEe might be involved in the stability of RhAg once expressed at the cell membrane, rather than in the dynamic translocation process of RhAg towards the membrane. This can be concluded from the fact that the membrane instability of RhAg in the absence of RhD/RhCcEe is not observed in nucleated cells, presumably because of the constant neosynthesis of the RhAg polypeptide in these cells (Mouro-Chanteloup *et al.*, 2002). However, the underglycosylation of RhAg in Rh_{null} cells suggests that the transit time of RhAg in the Golgi might also be regulated by RhD/RhCcEe (Ridgwell *et al.*, 1994).

In contrast to RhD and RhCcEe, RhAg is not known to be polymorphic. That is, no alloantibodies have been found against polymorphic RhAg epitopes. The only mutations of the *RHAG* gene that have been found result in defective membrane expression of RhAg in Rh_{null} individuals (Cartron, 1999), (Huang *et al.*, 2000), (Huang, 1998), (Cartron, 2000), (Huang *et al.*, 1997). This is due to one of the following mechanisms: i) the mutations in RhAg result in the absence or instability of the mRNA transcript, due to aberrant splicing, and the RhAg protein cannot be formed. ii) the mutations cause a premature stopcodon, most often leading to diminished mRNA levels, but if translated, the predicted RhAg mutant is a truncated polypeptide, rapidly degraded in the ER. Or iii) the mutations alter the conformation required for the assembly and/or intracellular transport of the protein to the membrane. This last possibility has been deduced from 6 patients with missense mutations (M1I; S79N; V270I & G280R; G279E; G380V and D399Y) in RhAg, in whom both RhAg and RhD/RhCE are expected to be produced. Examples of this third hypothesis have been observed for other cell surface molecules, such as Cystic Fibrosis transmembrane regulator protein (CFTR) and Aquaporin-2 (AQP-2) (Cheng *et al.*, 1990), (Deen *et al.*, 1995).

Clinical implications for blood-transfusion policy. If the weak-D phenotype is solely characterized by quantitative variations, individuals carrying this phenotype can be considered as RhD positive. Our results do

not indisputably prove that there are not qualitative alterations of the weak-D antigen, but show that there are no hard indications for their existence. With Scatchard plot analysis we demonstrated that the Rhesus similarity Index (RI) is not the right tool for determining qualitative differences, especially not for phenotypes with a low number of antigenic sites.

Therefore, a transfusion policy should be recommended in which not only *donors* but also *recipients* with the weak-D phenotype should be considered as RhD-positive. Also, antenatal immune prophylaxis should not be recommended for women with the weak-D phenotype. In this regard, it is important to mention that, with sensitive techniques and monoclonal antibodies, weak-D phenotypes are nowadays often typed as normal RhD positive. We observed, by serological screening for weak-D donors among the donors of the Sanquin Bloodbank Region Southwest that the distribution of the different weak-D types was not as expected from the genomic population study of Wagner *et al.* (1999) in Germany. This led us to conclude that only the weaker weak-D types are still recognized by serological screening. In our group, the weak-D type 2 (~500 sites/cell) is more frequently observed than weak-D type 1 (~1300 sites/cell) (61% vs 30%), although weak-D type 1 was far more common in the German study (18% weak-D type 2 vs 70% weak-D type 1). Thus, although the most common weak-D type 1 is mostly typed as RhD positive, this has not led to alloimmunisation in individuals with this phenotype. This indicates that the immunogenicity of normal RhD antigen in these donors is extremely low, otherwise immunisations would have been observed. By typing the partial-D phenotype R_o^{Har} as normal RhD-positive, alloimmunisations did occur.

Partial-D types with weak expression (such as DAR = weak-D type 4.2.0) need to be distinguished from the normal RhD-positive phenotypes, and transfusion policy or antenatal prophylaxis should be adjusted accordingly, because alloimmunisation may occur.

Variations between various ethnic populations. Many RhD variants are more frequently found in certain ethnic populations. The DAR phenotype (Chapter 2) is frequently occurring in African Blacks (1.6%), but this phenotype has not yet been identified in other populations. About 0.2-1% of the Caucasians express the weak-D phenotype. The allele frequency of

the weak-D phenotypes is about 0.0025-0.0125. Probably, weak-D alleles do not occur with this frequency or even at all in African Blacks or Asians. However, it cannot be excluded that weak-D phenotypes are disguised by the presence of an RhD-positive allele, abundantly found in these populations.

It has been suggested that distinct differences between the various populations and the great diversity of RhD variants in the African population are due to major population events. Diversity is influenced by several factors, such as natural selection, population admixture, recombinant and gene conversion, population size and demographic structure. Starting from the idea that the world population originated in Africa some 200,000 to 100,000 years ago, small fractions of this African population dispersed to other parts of the world about 50,000 years ago. (Wright *et al.*, 1999). These small fractions of founders are the starting points of new populations. Because these populations were very small, they determine most of the haplotypes that exist today. A second important population event for Northern Europe is the time period of the last ice age (30,000 till 15,000 years ago), with subsequent recolonysation by small numbers of founders. Again a bottleneck, explaining the high incidence of certain RhD phenotypes (such as RhD negativity) in this population (Reich *et al.*, 2001).

But, of course, it might also be that a certain RhD phenotype has resulted in an evolutionary benefit. An example of selective advantage of a blood group is found in individuals with the Duffy phenotype Fy(a-b-), often Blacks who are resistent to tertian malaria, because the *Plasmodium vivax* parasite is unable to invade the RBC with this phenotype. (Miller *et al.*, 1976) Another example of balanced genetic polymorphism is sickle cell haemoglobin (HbS), characterised by an amino-acid substitution in the beta-globin molecule (β_6 Glu→Val). Many epidemiologic studies have shown that HbS is common in areas of Africa with a high incidence of *Plasmodium falciparum*. Malaria is seldom found in carriers of the sickle cell trait, which suggests that this genetic determinant imparts a selective advantage to people living in areas where the parasite is common. Furthermore, *in vitro* studies have shown that at oxygen tensions similar to those in tissue, the parasite grows poorly in RBCs with HbS.

Now that the ammonium-transport function of Rh has been demonstrated, we can postulate that trivial changes in the Rh complex caused by

mutations in the *RH* genes might result in an altered ammonium concentration within the cells, which might affect the suitability of the cells as a host for certain pathogens. In this way, an evolutionary balance between the pathogen and its host could be responsible for the high level of Rh polymorphism.

6.2 Function of the Rh complex

For a long time, the function of Rh has remained a mistery. The predicted topology of the Rh proteins in the cell membrane (polytopic, with cytoplasmic N- and C-termini and N-glycan on one of its extracellular loops [for RhAg]) is characteristic for membrane transporters. Rapid progression in understanding the fuction of Rh was made when the non-erythroid Rh homologues RhBG and RhCG were identified and ammonium transport by RhAg and RhCG was observed in yeast cells.

We have shown that the Rh complex is involved in the export of ammonium from human RBCs (Chapter 5). This was concluded from differences between RBCs with various phenotypes in the uptake and release of a radiolabelled analogue of ammonium. Rh_{null} cells of the regulator type (expressing no Rh-complex proteins) accumulated significantly higher levels of radiolabelled methyl-ammonium ions than did normal RBC. This accumulation was due to a decreased export, as was shown by a significantly reduced release of intracellular radiolabelled methyl-ammonium ions by Rh_{null} cells of the regulator type. However, the more problems have been solved, the more questions can be raised about the function of Rh, for instance i) What is the impact of mutations in the preserved regions of the Rh homologues on their function? ii) How is the energy provided for the transport function? iii) Is RhAg or RhD/RhCcEe responsible for the ammonium-transport function? And iv) might other ions than ammonium ions be the target molecules for Rh?

i) Similarities of Rh with the ammonium-transport family. As described in the general introduction, DNA sequencing has revealed that the erythroid Rh homologues and non-erythroid homologues are related to the family of ammonium transporters (Marini *et al.*, 1997). The expansion of the Rh family during mammalian evolution implies two possible outcomes

with regard to their specification. Either they serve to transport the same or similar ligands, but differ in kinetics and regulatory modes, or they may each perform a completely different function (Liu *et al.*, 2001). The latter is less likely, considering the remarkable evolutionary conservation of the entire Rh family (Figure 6.4).

A relatively high extent of homology is found between the Rh homologues, with 184 (38%) of the 480 amino acids well preserved. These preserved amino acids are mainly located in the transmembrane areas (109 amino acids of the 184). And although RhCcEe has more resemblence to RhAg than RhD, RhD has less amino-acid alterations in the preserved regions of RhAg, RhBG and RhCG than has RhCcEe. Of the 35 amino-acid differences between RhCcEe and RhD, 11 amino acids of RhCcEe have a change of property in the preserved regions (11 of the 35 = 31%). Change of property means a change in the amino-acid properties: hydrophilic, acidic, amido, aromatic, basic, hydroxyl containing, proline and sulphur containing. In contrast, only 2 of the amino acids of RhD have a change of property in the preserved regions (2 of the 35 = 6%). The 24 amino-acid differences that have been found in the 22 different weak-D geno/phenotypes are mainly located in the preserved regions of the Rh homologues (13 of the 24 = 54%). Mutations in the preserved areas might have more effect on the processing, conformation or function of the protein. Thus, RhD might have a higher resemblance to an ammonium transporter than does RhCcEe.

ii) Energy supply of the transport process. The Rh complex is able to export ammonium ions from human RBCs against a concentration gradient, because no clear difference was observed between the release of ^{14}C-methyl-NH_3^+ from loaded cells, whether NH_4Cl was present extracellularly or not (Chapter 5). This thermodynamically uphill transport requires energy. We do not know whether the Rh complex uses energy directly derived from ATP hydrolysis. The Rh proteins do not belong to the family of ATP-binding-cassette transporters and, so far, there are no indications that it can function as an ATPase.

Many active transport processes are not directly driven by the hydrolysis of ATP. Instead, the uphill transport of an ion or molecule is often coupled to the downhill flow of another ion. So, in RBCs, energy to ex-

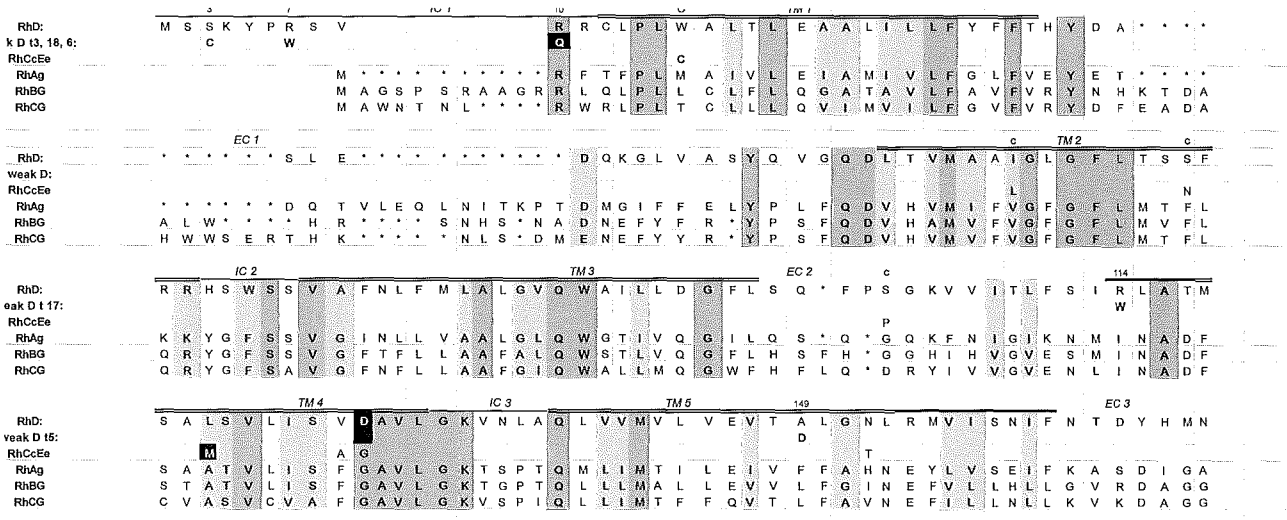
port ammonium might be derived from coupled ion transport such as Na^+-antiport.

iii) Difference in function for RhAg and RhD? RhAg bears a greater resemblance to both primitive and non-erythroid Rh homologues and therefore bears a greater resemblance with the Mep/Amt (methylammonium permeases / (methyl-) ammonium transport) family than do RhD/RhCcEe. RhD and RhCcEe most likely originated from an RhAg-like ancestor. Comparison of the orthologues in various species shows less sequence conservation in genes encoding RhD/RhCcEe than in genes encoding RhAg, indicating that the latter may provide a more basal physiological function (Matassi *et al.*, 1999).

RhAg on its own is able to export ammonium ions, as we showed with RBCs of the Rh_{null} amorph phenotype. Whether RhD and RhCcEe also function as an ammonium transporter in RBCs cannot be concluded from our study, because RBCs with expression of RhD and/or RhCcEe without RhAg do not exist. Because no difference was observed in the transport of ammonium between RBCs with or without expression of RhD (RhD positive vs RhD negative), or with or without the expression of RhCcEe (RhD positive and RhD negative vs D-- , unpublished observations), it can be excluded that RhD and/or RhCcEe play a major role in the transport of ammonium ions. Maybe RhD and/or RhCcEe are involved in transport of other N-containing substances. Transport experiments we performed with other N-containing substances, such as benzylamine (C_7H_9N-HCl), aniline (C_6H_7N-HCl) and dapsone (4,4-diaminodifenylsulfon, a drug used for *M. leprae*, *P. carinii*, chloroquine-resistant malaria, Dermatitis herpetiformis and Polycytemia vera) did not demonstrate involvement of the Rh complex (unpublished observations).

As suggested before, the polymorphic character of RhD and RhCcEe and the great phenotypic differences between ethnic populations, suggest a transport function of ammonium or another molecule that needs adaptability.

Figure 6.4: Alignment of the Rh homologues.
The amino-acid sequences of erythroid Rh homologues: RhD (with the different weak-D types), RhCcEe, and RhAg and of the non-erythroid Rh homologues RhBG and RhCG are aligned. The localisation of the amino acids is indicated above the RhD sequence by a line (intracellular), a double line (transmembrane) or no line (extracellular). Preserved regions of the Rh homologues are marked in dark grey (identical amino acids) or in light grey (amino acids with the same property) A change in (property of) amino acids in the preserved regions of the Rh homologues is marked in black.



TM 6
182
IC 4
198
201
212
TM 7
RhD:
RhCcEe
RhAg
RhBG
RhCG
220
223
6
EC 4
TM 8
IC 5
ak D t16, 4:
RhCcEe
RhAg
RhBG
RhCG
270
TM 9
276
277
282
EC 5
294
295
TM10
307
313
3, 12, 15, 9, 11, 8, 21:
RhCcEe
RhAg
RhBG
RhCG
IC6
338
339
TM 11
ak D t24, 7:
RhCcEe
RhAg
RhBG
RhCG
EC 6
TM 12
385
393
ak D t2, 10:
RhCcEe
RhAg
RhBG
RhCG
IC 7
408
RhD/RhCcEe:
eak D t22:
RhCcEe
RhAg
RhBG
RhCG

iv) Transport of CO_2 by Rh. It has been hypothesised that the molecule to be transported by Rh proteins is CO_2 rather than NH_3, given the distribution of Rh proteins among organisms and the organ and tissue distribution among animals (Soupene *et al.*, 2002). This speculation was tested by Soupene *et al.* with the green alga *Chlamydomonas reinhardtii*, a photosynthetic microbe containing endogenous *RH* genes. The *RH* genes Rh1 and Rh2 of *C. reinhardtii* have a sequence identity with RhAG of 32.3 and 31.8% respectively. The expression of the RH1 gene seemed to be upregulated in air supplemented with 3% CO_2 and downregulated in air supplemented with 0.035% CO_2. The amount of carbonic anhydrase varied in the opposite direction. From this observation, the authors concluded that Rh proteins might be biological gas channels for CO_2. However, by measuring the expression levels of certain proteins in response to a stimulus, an indirect effect may be observed. In the ammonium transport model (Chapter 5), the extracellular CO_2 concentration may also have implications for the expression of Rh, but via NH_4^+; Extracellular CO_2 may, via passive diffusion, increase the concentration of intracellular CO_2, which becomes HCO_3^- with OH^- derived from H_2O. The H^+ from H_2O will form NH_4^+ with intracellular NH_3 and shall be exported by the Rh complex. The increased export may lead to increased expression of Rh proteins. This indirect correlation was confirmed by the observation that upregulation of Rh only occurred when NH_4Cl was used as a nitrogen source (Soupene *et al.*,2002). The downregulation of carbonic anhydrase I expression under hight levels of CO_2 seems contradictory, but no information was given about the expression of the iso-enzyme carbonic anhydrase II, widely distributed and responsible for the highest turnover of CO_2 hydration (Sanyal *et al.*, 1981). Soupene's study also included ammonium transport experiments, in which the authors did not observe intracellular accumulation of ^{14}C-methylammonium when the algae were grown under conditions with NH_4Cl and high CO_2. This can easily be explained by the increased Rh expression under these conditions: the increased export capacity is sufficient to avoid accumulation of ^{14}C-methylammonium. This study of Soupene *et al.* does not convince us that the target molecule for transport by the Rh complex is CO_2 rather that NH_4^+/NH_3.

Bruce *et al.* (2003) also comply to the supposition that Rh is involved in CO_2 transport. They state that the presence of all components within the

band-3 macrocomplex render it likely that the individual components have linked functional or regulatory roles. They state that, since bicarbonate is transported through band 3 to leave the cell in exchange for chloride, this efflux acidifies the cell and causes release of O_2, and thus link is provided between coordinated CO_2 uptake and O_2 release. But this argument that the structural macrocomplex is formed because of its functional roles can also be used for ammonium transport. Band 3 also plays a role in the export of ammonium, since band 3 exports bicarbonate and therefore indirectly stimulates that NH_3 is protonated to NH_4^+.

In conclusion, as long as the function of RhD in the RBC is unknown, it will be difficult to answer the question whether there has been an evolutionary benefit of the RhD-negative phenotype, let alone of the weak-D phenotype. The increased prevalence of RhD negativity in Caucasians, compared to Blacks and Asians might suggest a functional role. Although a founder effect in Caucasians cannot be excluded, the (supposed) increased prevalence of weak D in Caucasians might argue against this.

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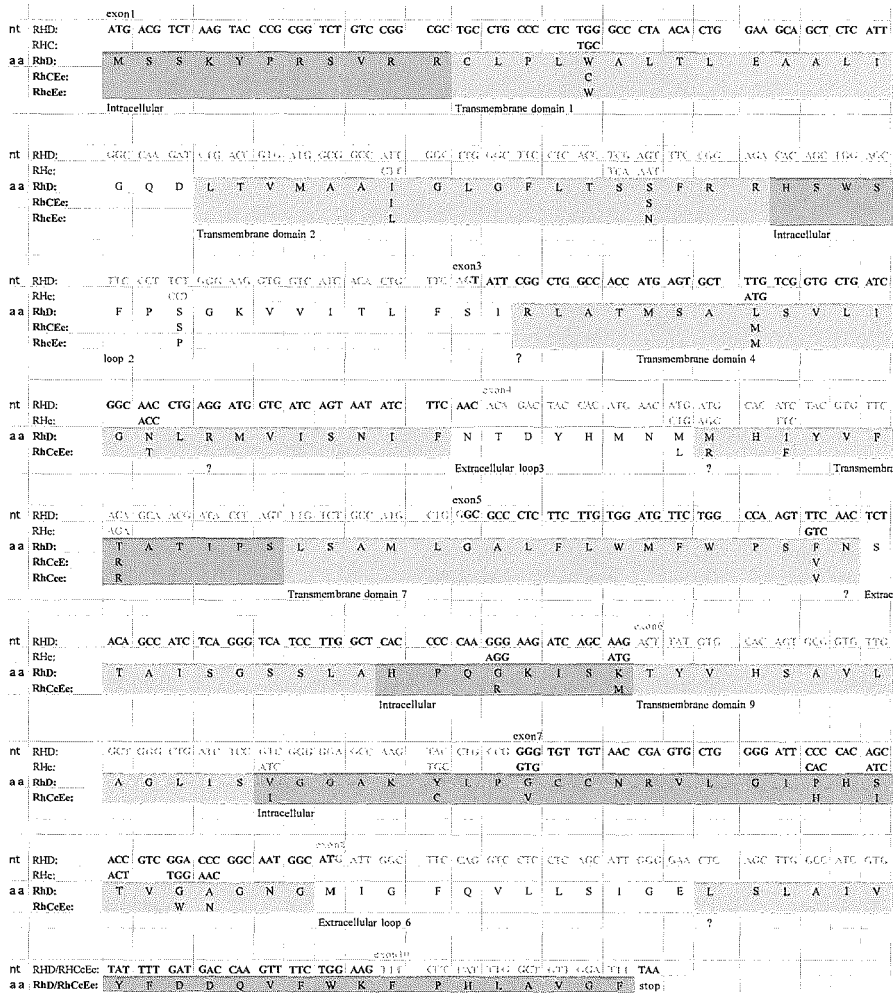
Appendix

Amino acid	1-letter code	3-letter code	codons	property
Alanine	A	Ala	GCU GCC GCA GCG	hydrophobic
Arginine	R	Arg	CGU CGC CGA CGG AGA AGG	basic
Asparagine	N	Asn	AAU AAC	amido
Aspartaat	D	Asp	GAU GAC	acidic
Cysteine	C	Cys	UGU UGC	sulphur containing
Glutamaat	E	Glu	GAA GAG	acidic
Glutamine	Q	Gln	CAA CAG	amido
Glycine	G	Gly	GGU GGC GGA GGG	hydrophobic
Histidine	H	His	CAU CAC	basic
Isoleucine	I	Ile	AUU AUC AUA	hydrophobic
Leucine	L	Leu	UUA UUG CUU CUC CUA CUG	hydrophobic
Lysine	K	Lys	AAA AAG	basic
Methionine	M	Met	AUG	sulphur containing
Phenylalanine	F	Phe	UUU UUC	aromatic
Proline	P	Pro	CCU CCC CCA CCG	proline
Serine	S	Ser	UCU UCC UCA UCG AGU AGC	hydroxyl containing
Treonine	T	Thr	ACU ACC ACA ACG	hydroxyl containing
Tryptophan	W	Trp	UGG	aromatic
Tyrosine	Y	Tyr	UAU UAC	aromatic
Valine	V	Val	GUU GUC GUA GUG	hydrophobic
		stop	UAA UAG UGA	
		start	AUG	

Table 1: Amino acids


exon1
nt RHD: ATG ACG TCT AAG TAC CCG CGG TCT GTC CGG CGC TGC CTG CCC CTC TGG GCC CTA ACA CTG GAA GCA GCT CTC ATT
RHC: TGC
aa RhD: M S S K Y P R S V R R C L P L W A L T L E A A L I
RhCEe: C
RhcEe: W
Intracellular
Transmembrane domain 1
nt RHD: GGC CAA GAT CTG ACT GTG ATG GGG GCC ATC GGC TTG GCC TTC CTC ACT TCG AGT TTC CGC AGA CAC AGC TGG AGC
RHc: CTT TCA AAT
aa RhD: G Q D L T V M A A I G L G F L T S S F R R H S W S
RhCEe: I S
RhcEe: L N
Transmembrane domain 2
Intracellular
exon3
nt RHD: TTC CCT TCT GGG AAG GTG GTC ATC ACA CTG TTC AGT ATT CGG CTG GCC ACC ATG AGT GCT TTG TCG GTG CTG ATC
RHc: CCG ATG
aa RhD: F P S G K V V I T L F S I R L A T M S A L S V L I
RhCEe: S M
RhcEe: P M
loop 2
?
Transmembrane domain 4
exon4
nt RHD: GGC AAC CTG AGG ATG GTC ATC AGT AAT ATC TTC AAC ACA GAC TAC CAC ATC AAC ATG ATG CAC ATC TAC GTG TTC
RHc: ACC CTG AGC TTC
aa RhD: G N L R M V I S N I F N T D Y H M N M M H I Y V F
RhCcEe: T L R F
?
Extracellular loop3
?
Transmembr
exon5
nt RHD: ACA GCA ACG ATA CTC AGT TTG TCT GCC ATC CTG GGC GCC CTC TTC TTG TGG ATG TTC TGG CCA AGT TTC AAC TCT
RHc: AGA GTC
aa RhD: T A T I P S L S A M L G A L F L W M F W P S F N S
RhCcEe: R V
RhCee: R V
Transmembrane domain 7
? Extrac
exon6
nt RHD: ACA GCC ATC TCA GGG TCA TCC TTG GCT CAC CCC CAA GGG AAG ATC AGC AAG ACT TAT GTG CAC AGT GCC GTG TTG
RHc: AGG ATG
aa RhD: T A I S G S S L A H P Q G K I S K T Y V H S A V L
RhCcEe: R M
Intracellular
Transmembrane domain 9
exon7
nt RHD: GCT GGG CTG ATC TCT GTG GGG TCA GCC AAG TAC CTG CTG GGG TGT TGT AAC CGA GTG CTG GGG ATT CCC CAC AGC
RHc: ATC TGC GTG CAC ATC
aa RhD: A G L I S V G G A K Y L P G C C N R V L G I P H S
RhCcEe: I C V H I
Intracellular
exon8
nt RHD: ACC GTC GGA CCC GGC AAT GGC ATG ATT GGC TTC CAG GTC CTC CTC AGC ATT GGG GAA CTC AGC TTG GCC ATC GTG
RHc: ACT TGG AAC
aa RhD: T V G A G N G M I G F Q V L L S I G E L S L A I V
RhCcEe: W N
Extracellular loop 6
?
exon10
nt RHD/RHCeEe: TAT TTT GAT GAC CAA GTT TTC TGG AAG TTC CTC TAT TTG GCT GTG GGA TTT TAA
aa RhD/RhCcEe: Y F D D Q V F W K F P H L A V G F stop

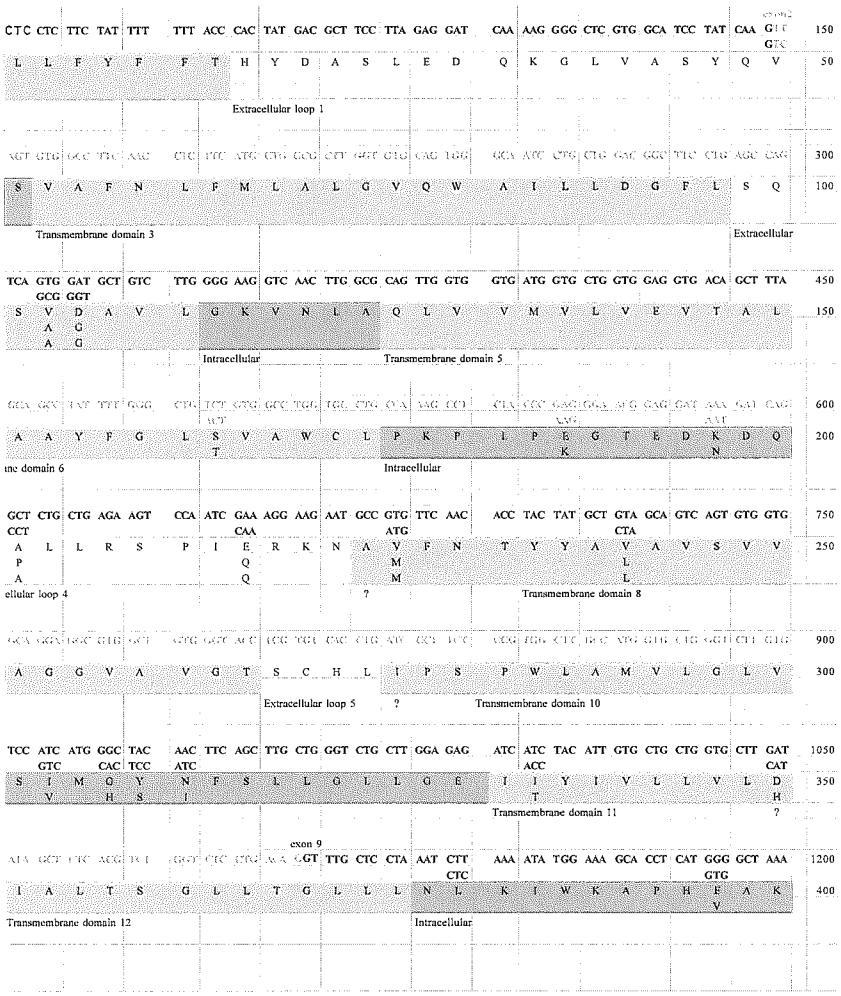


Figure 1: Rh sequence.

The RHD allele sequence (exon borders indicated, alternating in black and grey) and amino-acid sequence (localisation in the membrane indicated; where the precise topology of the protein in the membrane is not known, indicated by a question mark). Differences in the sequence for RHCE allele are shown below the RHD sequence.

	American	**English**	**Numerical**	
RhD positive	R_0	cDe	*RH*	1,2,-3,-4,5
	R_1	CDe	*RH*	1,2,-3,-4,5
	R_2	cDE	*RH*	1,-2,-3,-4,5
	R_z	CDE	*RH*	1,-2,-3,-4,5
RhD negative	r	cDe	*RH*	-1,-2,-3,4,5
	r'	CDe	*RH*	-1,2,-3,-4,5
	r''	cDE	*RH*	-1,-2,-3,4,-5
	r_y	CDE	*RH*	-1,2,3,-4,5
No.		**CDE**	**Rh-Hr**	
RH1	004001	D	Rh_0	
RH2	004002	C	r'	
RH3	004003	E	rh''	
RH4	004004	c	hr'	
RH5	004005	e	hr''	

Table 2: Rh nomenclature

List of publications

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Summary

The Rh blood group system is invloved in haemolytic disease of the newborn, transfusion reactions and autoimmune haemolytic anaemia. The Rh system comprises the RhD and RhCcEe polypeptides. RhD is of most clinical importance, because of its high immunogenicity. The Rh polypeptides are encoded by two highly homologous genes: *RHD* and *RHCE*. The RhD-positive phenotype is determined by the presence of one or two *RHD* gene(s). The Rh polypeptides have twelve transmembrane spanning domains, with the -NH_2 and -COOH termini oriented to the cytoplasm. The Rh complex consists of a tetramer with two RhD/RhCcEe molecules and two RhAg (Rh-associated glycoprotein) molecules. Several membrane glycoproteins such as LW, CD47 and GPB are associated with the Rh complex. The Rh complex is attached to the RBC cytoskeleton via CD47, protein 4.2 and band 3. The Rh-deficiency phenotype (Rh_{null} of the regulator type) lacks the expression of the complete Rh complex. The predicted membrane organisation of the Rh polypeptides and the genetic similarities of *RH* with ammonium transporters suggest a transport function.

Variations in RhD expression are either qualitative or quantitative, or a combination of these two. RBCs with qualitatively altered RhD antigens (partial D) lack or gain RhD epitopes. Individuals with a partial-D phenotype are able to form alloantibodies when they encounter the complete RhD antigen. RBCs with quantitatively altered RhD antigens have an increased or decreased (weak D) expression of RhD. It is supposed that the conformation of the weak-D antigen is not altered, since alloantibodies have never been found in individuals with the weak-D phenotype after transfusion with normal RhD-positive RBCs. In 1999, several mutations (intracellular and transmembrane) have been found in individuals with the weak-D

phenotype. Major differences were observed in the expression level of the various RhD epitopes, measured with 59 anti-D MoAbs by flowcytometry, in RBCs with the weak-D phenotype (indicated with a low Rh similarity index (RI)). Because of the low RI, it was assumed that RBCs with the weak-D phenotype express an RhD protein with an altered conformation.

In this thesis, possible qualitative alterations of the weak-D antigen were examined by Scatchard plot analysis. A direct correlation between the mutations found in individuals with the weak-D phenotype and the lower expression was made, using a transfection model with K562 cells. The K562 cell line model was also used to investigate the processing of the weak-D antigens in the cell. Furthermore, the role of the Rh complex in the transport of ammonium was investigated.

In Chapter 1, a general introduction is given with respect to the biochemical, molecular and functional aspects of the Rh system, and insights in protein development of the cell.

In Chapter 2, DAR, a new RhD phenotype with weak expression of RhD is described. This new RhD variant is encoded by an *RHD* gene with mutations in exon 4, 5 and 7. Besides the weak expression, the DAR antigen is also qualitatively altered. Two individuals with the DAR phenotype produced alloantibodies after contact with the complete RhD antigen. The *DAR* genotype is often in linkage with ceAR, a new *RHce* variant with mutations in exon 5 and 6. Both variants have only been described in African blacks. The frequancy of the DAR phenotype and genotype is high among South African blacks (1.5% and 4.9%).

In Chapter 3, the weak RhD antigens were closely examined for qualitative differences, by Scatchard plot analysis. The affinitiy of all used anti-D antibodies was comparable between normal RhD and weak-D RBCs. This indicates that the epitopes tested have not been qualitatively altered. Affinity differences have been found among the different anti-D antibodies. These differences in affinity can explain the differences in the expression level of the RhD epitopes measured by flowcytometry (represented by a low RI). This suggests that the RI is not only determined by properties of the RhD protein but also by properties of the anti-D antibodies.

In Chapter 4, the weak-D mutations type 1 and 3 were shown to be responsible for the lower expression of RhD, in K562 cells transfected with DNA encoding normal RhD, weak D type 1 and 3. The processing of the

weak-D antigens in the cell was also examined with the K562 transfection model. We hypothesized that the weak-D proteins, due to the intracellular or transmembrane mutations, were misfolded or unable to form a complex. Then, the misfolded or uncomplexed proteins are degraded intracellularly by proteasomes. The amount of RhD (and GFP) was measured before and after proteasome inhibition, in K562 cells expressing RhD, weak-D type 1 and 3 and K562 cells expressing GFP-fusion proteins with RhD, weak-D type 1 and 3. Unfortunately, no differences in expression were observed after proteasome inhibition. Thus, our hypothesis could not be confirmed, but that could be due to the techniques we used.

In Chapter 5, the involvement of the Rh complex in the export of ammonium/ammonia was shown. The uptake and release of ^{14}C-methylammonium (radioactive analogue of ammonium) in RBCs with the Rh complex (RhD positive and RhD negative) was compared to RBCs without the Rh complex (Rh_{null} cells of the regulator type). Rh_{null} cells accumulate significantly more ^{14}C-methylammonium than do RBCs with the Rh complex. This accumulation is due to a decreased release of ^{14}C-methylammonium. The transport of radioactive urea in Rh_{null} cells was comparable to normal RBCs, indicating that there is no general transport dysfunction of the Rh_{null} cells. Thus, the Rh complex is specifically involved in the transport of ammonium/ammonia.

In Chapter 6, the General Discussion, the findings described in this thesis are considered in perspective of the current knowledge of Rh.

Samenvatting

Het Rh bloedgroepsysteem is betrokken bij de hemolytische ziekte van de pasgeborene, bij transfusiereacties en bij autoimmuun hemolytische anemie. Het Rh-systeem bestaat uit het RhD-eiwit en het RhCcEe-eiwit. Van deze twee eiwitten is vooral RhD van klinisch belang, aangezien dit eiwit zeer immunogeen is. De Rh-eiwitten worden gecodeerd door twee zeer homologe genen: *RHD* en *RHCE*. Het RhD-positieve fenotype wordt (meestal) bepaald door de aanwezigheid van één of twee *RHD* genen. De Rh-eiwitten hebben 12 transmembraan domeinen, en zowel het NH_2- als het COOH uiteinde zitten intracellulair. Rh wordt op de rode-bloedcelmembraan gepresenteerd als een complex, bestaande uit een tetrameer van twee RhD/RhCcEe eiwitten en twee Rh-geassocieerde glycoproteinen (RhAg). Ook de eiwitten LW, GPB en CD47 zijn bij dit complex betrokken. Het Rh-complex is via CD47 en proteine 4.2 verbonden aan band 3, een eiwit dat weer via ankyrine stevig aan het rode-bloedcel spectrineskelet vast zit. Bij het Rh-deficientie fenotype (Rh_{null} van het regulator-type) komt het gehele Rh-complex niet tot expressie. De manier waarop Rh in de rode-bloedcelmembraan zit en de genetische overeenkomsten van Rh met ammoniumtransporters doen een transportfunctie vermoeden.

De expressie van RhD kan varieren in kwaliteit en/of in kwantiteit. Bij kwalitatieve variaties zijn, door één of meerdere mutaties in het *RHD* gen, epitopen verloren gegaan of nieuwe epitopen ontstaan. Door de conformatieverandering van het RhD-eiwit kunnen alloantistoffen gevormd worden na contact met het normale RhD-eiwit. Bij kwantitatieve variaties is er alleen sprake van een verlaagde of verhoogde expressie van het RhD-eiwit. Er wordt van uitgegaan dat de conformatie van het RhD-eiwit niet is veranderd; dus alloantistoffen zullen niet gevormd worden na contact

met het normale RhD-eiwit. In 1999 werden verschillende mutaties (alleen intracellulair of transmembraan) beschreven die leiden tot het zwakke-D fenotype. Het expressieniveau van verschillende RhD-epitopen (gemeten met verscheidende anti-RhD antistoffen door middel van flowcytometrie) liet grote verschillen zien op rode bloedcellen met het zwakke-D fenotype (uitgedrukt in een lage Rh-similarity-index). Hierdoor werd gesuggereerd dat rode bloedcellen met het zwakke-D fenotype toch een RhD-eiwit tot expressie brengen, dat een conformatieverandering heeft ondergaan.

In dit proefschrift wordt onderzoek beschreven naar het effect van de mutaties gevonden bij het zwakke RhD op de expressie van dit eiwit. Hiervoor werd een transfectiemodel van K562 cellen gebruikt. Tevens werd onderzocht of deze mutaties naast een effect op de kwantiteit ook kwalitatieve veranderingen teweegbrengen. Ook werd getracht uit te zoeken op welke manier de zwakke-D mutaties leiden tot zwakke expressie. Naast dit al wordt de betrokkenheid van het Rh-complex in het transport van ammonium beschreven.

In hoofdstuk 1 wordt een algemene introductie gegeven over de biochemische, moleculaire en fuctionele aspecten van Rh. Tevens wordt een overzicht gegeven van de posttranslationele veranderingen van een eiwit in een cel.

In hoofdstuk 2 wordt een nieuw RhD fenotype met zwakke expressie beschreven: DAR. Deze nieuwe RhD-variant wordt gecodeerd door een *RHD* gen met mutaties in exon 4, 5 en 7 . DAR heeft niet alleen een verlaagde expressie, maar mist ook enkele epitopen. Bij twee individuen met het DAR fenotype zijn alloantistoffen gevormd na contact met het complete RhD-eiwit. Het *DAR* genotype komt veel voor samen met het *ceAR* gen, een nieuwe Rhce-variant met mutaties in exon 5 en 6. Beide varianten zijn tot dusver alleen beschreven bij Afrikaanse negroïden. De frequentie van het DAR fenotype en genotype is hoog in de Zuidafrikaanse negroïde bevolking (resp. 1,5% en 4,9%).

Voor hoofdstuk 3 werden zwakke-D type 1 en 2 nauwkeurig onderzocht op kwalitatieve veranderingen door middel van Scatchard plot analyse. In dit hoofdstuk wordt beschreven dat de affiniteit van verschillende anti-D antistoffen voor het normale RhD-eiwit en de zwakke-D eiwitten vergelijkbaar is. Hieruit blijkt dat de epitopen waar deze antistoffen tegen gericht zijn niet veranderd zijn bij zwakke RhD expressie. Er bestaan wel grote ver-

schillen tussen de affiniteit van de anti-D-antistoffen onderling. De grote verschillen in het expressieniveau van de verschillende RhD-epitopen die gemeten zijn met flowcytometrie bij het zwakke-D fenotype (uitgedrukt in een lage Rh-similarity-index) worden dus mede veroorzaakt door deze verschillen in antistofaffiniteit.

In hoofdstuk 4 is door middel van het transfecteren van K562 cellen met DNA coderend voor normaal RhD, zwakke-D type 1 of 3 bewezen dat de mutaties die gevonden zijn bij individuen met het zwakke-D fenotype ook daadwerkelijk leiden tot een zwakke expressie. Tevens is een poging ondernomen om de ontstaanswijze van de zwakke-D typen 1 en 3 op moleculair niveau te doorgronden. We formuleerden een hypothese waarbij de zwakke-D eiwitten door de intracellulaire of transmembraanmutatie, fout gevouwen worden of geen complex meer kunnen vormen en daardoor intracellulair worden afgebroken. De RhD-eiwitten die wel goed gevouwen zijn, of een complex vormen, worden op de rode-bloedcelmembraan tot expressie gebracht. Dit zijn er minder dan normaal en daardoor is er een zwakke expressie. De hypothese werd getoetst door middel van transfectie met een fusie van het zwakke-D gen met een gen coderend voor een groen fluorescerend eiwit (GFP). Remming van de proteasomen, die foutgevouwen eiwitten afbreken, zou volgens de hypothese tot een verhoging van intracellulair GFP moeten leiden. Helaas kon dit niet worden waargenomen, hetgeen ook te wijten kan zijn aan de toegepaste technieken.

In hoofdstuk 5 wordt de betrokkenheid van het Rh-complex bij de export van ammonium/ammonia over de rode-bloedcelmembraan aangetoond. Dit werd gedaan door de opname en uitstroom van ^{14}C-methylammonium (een radioactieve analoog van ammonium) in rode bloedcellen mét (RhD-positief en RhD-negatief) en zonder het Rh-complex (Rh_{null}) te meten. Rh_{null}-cellen namen meer ^{14}C-methylammonium op dan rode bloedcellen waarbij het Rh-complex wel tot expressie kwam. Deze ophoping werd mede veroorzaakt door een lagere uitstroom van de ^{14}C-methylammonium. Aangezien het transport van radioactief ureum in Rh_{null}-cellen niet verschilde van 'normale' rode bloedcellen, kan worden geconcludeerd dat het hier niet een algemene transportdisfunctie van de Rh_{null}-cel betreft, maar dat het Rh-complex specifiek betrokken is bij de export van ammonium/ammonia.

In hoofdstuk 6, de Algemene Discussie, worden de bevindingen uit dit

proefschrift in een breder perspectief geplaatst, in het licht van de huidige kennis van het Rh systeem.

Dankwoord

You have brains in your head.
You have feet in your shoes.
You can steer yourself any direction you choose.
You're on your own. And know what you know.
And you are the one who'll decide where to go...
Uit: Oh the places you go (Dr Seuss).

Maar als AIO sta je zeker niet alleen, heb je alle kennis nodig die er om je heen is en bepaal je gezamelijk een lijn in het onderzoek. De mensen hieronder zijn van essentieel belang geweest voor de tot standkoming van dit boekje.

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Bij dag en nacht,
in zon en regen,
kom je leuke dingen tegen.
(Stip naar Seuss)

Curriculum Vitae

Mirte Brigit Hemker werd geboren op 20 juli 1971 te Amsterdam. Ze groeide op in Amsterdam, alwaar ze in 1990 het VWO diploma behaalde aan het Hervormd Lyceum Zuid. In datzelfde jaar werd begonnen met de studie geneeskunde aan de Universiteit van Amsterdam. De doctoraalfase van deze studie voltooide zij in 1994. Haar wetenschappelijke stage over het onderwerp 'Oscillaties in gist', werd gelopen op het E.C. Slater Instituut onder leiding van prof.dr H.V. Westerhoff en prof.dr K. van Dam. Tijdens haar studie was zij in 1992–1993 lid van de faculteitsraad in het Academisch Medisch Centrum (AMC) van de Universiteit van Amsterdam. Voor zij aan haar co-schappen begon heeft zij in het kader van een Erasmus project een wetenschappelijke stage gelopen bij prof.dr. K. Thermos op de afdeling Behavioural Pharmacology van de Universiteit van Heraklion (Kreta, Griekenland). In 1997 werd het artsexamen behaald. Direct hierna begon zij met het onderzoek naar het zwakke Rhesus D fenotype op de afdeling Experimentele Immunohematologie van het Centraal Laboratorium voor de Bloedtransfusiedienst (CLB) te Amsterdam, onder leiding van dr P.A. Maaskant-van Wijk en dr C.E. van der Schoot. Dit onderzoek werd gecontinueerd als promotie onderzoek in samenwerking met het CLB en de Bloedbank ZWN Rotterdam (tegenwoordig: Sanquin Research at CLB en Sanquin Bloedbank, regio Zuidwest) met als promotor prof.dr D.J. van Rhenen. In deze periode kreeg zij twee kinderen: een dochter, Gaya (2001), en een zoon, Tycho (2003).

Tegenwoordig werkt zij als consultatiebureau-arts bij de Jeugdgezondheidszorg van de GG&GD te Amsterdam. In maart 2004 zal zij starten met de opleiding tot huisarts aan de Vrije Universiteit te Amsterdam.