The Rh blood group system in review: A new face for the next decade

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Rh is the most well-recognized blood group system after ABO, probably because of the dramatic presentation of a fetus suffering hemolytic disease of the newborn (HDN) following maternal alloimmunization to the D antigen. Even individuals not associated with medicine have heard of the “Rh factor” and are aware that it has some importance in pregnancy. The earliest recorded description of the syndrome dates to the 1600s from a French midwife who attended the delivery of a set of twins, one of which was hydropic and the other was jaundiced and died of kernicterus.1 The agent responsible for the wide range of fetal symptoms, from mild jaundice to fetal demise, remained obscure until 1941. Levine and colleagues2 observed that the delivery of a stillborn fetus and the adverse reaction in the mother to a blood transfusion from the father were related and were the result of an immune reaction to a paternal antigen.

Serologists’ relationship with the offending blood group system began when it was confused with a Rhesus monkey red blood cell (RBC) protein, now termed LW, and much argument and debate ensued over who should receive credit for its discovery.3 Becoming aware of the antigen, however, was only the beginning of the story. This blood group system would become notorious for its complexity, with numerous antigens and multiple nomenclatures defining it.

Several seminal events characterized the history of the Rh system. One of the most important was the observation that ABO mismatch between a mother and the fetus had a partial protective effect against immunization to D. This suggested the rationale for the development of Rh immune globulin (RhIG).4 Although immunoglobulin M (IgM) antibodies did not provide protection, immunoglobulin G (IgG) anti-D was effective. By the early 1960s, a mere 20 years after the discovery of Rh incompatibility, an effective treatment was available.

Despite their clinical importance, the extremely hydrophobic nature of the Rh proteins made biochemical studies difficult, and the proteins were not successfully isolated until the late 1980s.5 This led to the cloning of the genes in the 1990s6 and to major advances in our understanding of the Rh system. The molecular bases of most Rh antigens have been determined, and the RH gene structure explains why this system is so polymorphic. Specifically, the conventional Rh antigens are encoded by two genes, RHD and RHCE, but numerous gene conversion events between them create hybrid genes. The resulting novel hybrid proteins containing regions of RhD joined to RhCE, or the converse, generate the myriad of different Rh antigens.

The goal of this review is to highlight the insights gained since the cloning of the genes, describe applications for RH molecular testing to clinical practice, introduce other members of the Rh family of proteins that are present in other tissues, and focus on the next piece of the Rh puzzle, that is, efforts to determine the structure and function of the Rh family of proteins.

TERMINOLOGY

Now that the genes and the proteins have been elucidated, current Rh terminology attempts to distinguish these from the antigens, which are referred to by the letter designations, D, C, c, E, e, etc. Capital letters and italics are used when referring to the RH genes, which include RHD, RHCE, and RHAG, as well as the nonerythroid RHBG and RHCG. The different alleles of the RHCE gene are designated RHce, RHCe, and RHcE, according to which antigens they encode. The proteins are indicated as RhD, RhCE (or according to the specific antigens they carry Rhce, RhCe, or RhcE), and RhAG, RhBG, and RhCG. Rh haplotypes are designated Dce, DcE, DcE, etc., or ce, Ce, cE when referring to a specific CE haplotype.

ABBREVIATIONS: RhIG = Rh immune globulin; SNP = single-nucleotide polymorphism.

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RBC RhCE, RhD, and RhAG

Two genes (RHD, RHCE) in close proximity on chromosome 1 encode the Rh proteins, RhD and RhCE; one carries the D antigen, and the other carries CE antigens in various combinations (ce, Ce, cE, or CE).\(^6\) The genes are 97 percent identical, each has 10 exons and encode proteins that differ by 32 to 35 amino acids. This is in contrast to most blood group antigens that are encoded by single genes with alleles that differ by only one or a few amino acids. The large number of amino acid differences explains why exposure to RhD can result in a potent immune response in an RhD− individual.

RBCs express yet another Rh protein termed RhAG, for Rh-associated glycoprotein, which carries one Nglycan chain.\(^10\) RhAG shares 38 percent identity with RhCE and RhD, has the same predicted topology in the membrane, and is encoded by a single gene on chromosome 6. RhAG is not polymorphic, so it does not carry blood group antigens, but it is important for targeting RhCE and RhD to the membrane. Two molecules of RhAG associate with two molecules of RhCE and/or RhD.\(^11\) RhAG mutations are responsible for loss of expression of Rh antigens (Rhnull), because without RhAG, neither RhCE nor RhD reach the RBC membrane.\(^12\) RhAG is expressed early during erythroid differentiation, being detectable on CD34+ progenitors, whereas RhCE appears later, followed by RhD.\(^14\) The timing of expression may reflect the assembly of these proteins in the RBC membrane.

Variations of RhCE

RHCE encodes both C/c and E/e antigens on a single molecule.\(^4\) Rhce probably represents the oldest human locus and encodes the c and e antigens. RHCe arose from RHce via gene conversion with exon 2 from RHD (Fig. 1A). This resulted in three new amino acids in the protein, but only the amino acid change at position 103 is predicted to be extracellular (Fig. 2). The fact that the amino acids encoded by exon 2 of RHCE are identical to those encoded by exon 2 of RHD explains the expression of G antigen on both RhD and Rhc proteins.

The antigens C\(^{09}\) and C\(^{5}\), which were thought to be antithetical to C, are the result of single amino acid changes on the first extracellular loop of RhCE (Fig. 2).\(^15\) The fact that the amino acids encoded by exon 5 of RHCE resulted in RHcE (Fig. 1A). A single point mutation acquired in exon 5 of RHCE resulted in RHcE (Fig. 1A). The requirement for e antigen expression, however, is more complex than the 226AAla polymorphism, and weak or altered expression is frequently encountered in Black persons and people with mixed ancestry.\(^16\) Variant e expression occurs on the RBCs of the more than 30 percent of Black persons who are V+ and VS+. Both antigens are the result of a single Leu245Val substitution located in the predicted eighth transmembrane segment of RhCE (Fig. 2),\(^17\) which causes a local conformation change affecting expression of e. This haplotype encoding 245Val and V and VS antigens is referred to as ce\(^{0}\) (Fig. 1B). The subsequent loss of V expression (the V/VS+ phenotype) results from a Gly336Cys change on this background.\(^18\) Loss of VS expression (the V+VS− phenotype) is associated with additional amino acid changes and is characteristic of the ceAR haplotype (Fig. 1B).\(^19\) Altered e expression is also associated with a Trp16Cys amino acid polymorphism in exon 1 of the RHce gene.\(^20\)

Antibodies. Individuals who are homozygous for RHCE genes that encode variant e antigens type as e+, but they often make alloantibodies with e-like specificities. The antibodies, designated anti-hr\(^5\), -hr\(^6\), -RH18, and -RH34, are difficult to identify serologically and, importantly, they are clinically significant and have caused transfusion fatalities.\(^21\) The prevalence of e variants in Black persons, together with the incidence of sickle cell disease requiring transfusion support often provided by Caucasian donors with conventional Rhce, make the occurrence of alloanti-e in the e variants not uncommon. Some of the RH genetic backgrounds of individuals who make these antibodies have now been defined and include the RHCE haplotypes designated ce\(^{6}\), ceAR, ceMO, ceEK, and ceBI.\(^22\) All encode the Trp16Cys difference in exon 1 and have additional changes, primarily localized to exon 5. Homozygous ce\(^{6}\) RBCs lack the e-related antigen hr\(^6\), and RBCs from ceAR, ceMO, ceEK, and ceBI lack the hr\(^a\) antigen (Fig. 1B and summarized in Reid and Lomas-Francis\(^22\)). Importantly, because of the multiple molecular backgrounds responsible for the hr\(^5\)–hr\(^6\)– phenotypes, some of which are not yet elucidated, the antibodies they produce are not all serologically compatible. This explains why it is difficult to find compatible blood for patients with these antibodies, and often only rare deleted D− RBCs appear compatible. As an additional complication, these variant RhCE can often be inherited with an altered RHD, for example, DAR or D\(^{ii}\) (Fig. 1B), so they can also make anti-D. With the implementation of molecular testing, patients with altered Rhce/RhD can now be identified. The next challenge is to institute molecular screening of minority donors to develop a registry of units that are genotyped for these variants. This is critical to meet the transfusion needs of these alloimmunized and often critically ill patients, especially as rare D− units are in very limited supply and are not always compatible.

Other modifications of RHCE include the hybrids D\(^{i}\), R\(^{2}\), and I\(^{2}\) and several E/e variants\(^23\)-\(^27\) (Fig. 1B). Some of these can cause discrepancies in serologic typing between monoclonal and polyclonal reagents. For example, D\(^{i}\) is found in people of German ancestry. The RBCs are negative with polyclonal reagents and with some mon-
oclonal anti-D including the weak D test, but type as D+ with monoclonal anti-D containing IgM clone MS201 (Immucor, Norcross, GA) (Table 1). These individuals do not carry RHD, but, instead, exon 5 of RHCE has been replaced by exon 5 from RHD (Fig. 1B). Not surprisingly, they can be stimulated by conventional D antigen and should be treated as D– for transfusion and RhIG prophylaxis. RN RBCs carry a hybrid RHCE in which exon 4 is replaced with RHD, and they may type as e– or weak with polyclonal reagents, but are indistinguishable from “normal” e+ RBCs with monoclonal anti-e.

Variations of RhD

D. D– is much more prevalent in Caucasian persons of European descent (15%-17%), less likely in individuals of African background (3%-5%), and rare in Asian populations (<0.1%). The distribution of D– individuals worldwide attracted the early attention of population biologists, who attempted to make predictions of human origins and migrations based on Rh phenotype. How the D– phenotype reached the high proportions seen in European persons remains a mystery. Genetic selection models dictate that there must have been some advantage to individuals who were heterozygous (balancing selection). What that advantage might have been is obscure, as the only obvious selection pressure is against the heterozygote, reflected in morbidity and mortality of D/d infants born to D– alloimmunized women.

It is now clear that the D– phenotype has arisen numerous times on different genetic backgrounds. In

Fig. 1. (A) Origin of the common RHCE genes. The 10 RHD exons are shown as black boxes, and the RHCE are in gray. RHCE and RHcE have arisen from RHce by gene conversion or point mutation, respectively. See text for details. (B) Examples of some RHD and RHCE gene rearrangements. Shown are those that are more frequent in Black and Caucasian persons. (C) Examples of some rearrangements responsible for elevated expression of D antigen.
Caucasian persons of European descent, it is primarily the result of deletion of the entire RHD gene. This deletion occurred on a Dce (R0) haplotype; that is, the RHCE allele carried with the deletion is ce. In Caucasians rare exceptions exist and include RHD that is not expressed because of a premature stop codon, nucleotide insertions, point mutations, or RHD/CE hybrids. Most of the exceptions arose on the DCe (R1) or DcE (R2) background and result in the less common Ce (r¢) or cE (r≤) haplotypes. D– phenotypes in African and Asian persons are often caused by inactive or silent RHD, rather than a gene deletion. Asian D– phenotypes result from mutations in RHD most often associated with Ce, indicating that they originated on a DCe (R1) haplotype. Many Asian persons, however, who type as D– are D el and actually have low-level expression of D, which is detectable only by adsorption and elution. Del results from deletion of exon 9 or the missense mutation 1227G>A. In D– African Black persons, 66 percent have RHD genes that contain a 37-bp insertion, which results in a premature stop codon, and 15 percent carry a hybrid RHD-CE-D linked to ceS, termed (C) ceS, characterized by expression of weak C and no D antigen.

**Weak D.** An estimated 0.2 to 1 percent of Caucasian persons and a greater number of African-American persons have reduced expression of the D antigen but the number of samples classified as weak D depends on the characteristics of the typing reagent. The molecular basis of weak D expression is primarily the result of point mutations that cause amino acid changes predicted to be intracellular or in the transmembrane regions of RhD and not

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**TABLE 1. Reactivity of some Partial D and variant CE with current FDA licensed anti-D**

<table>
<thead>
<tr>
<th>Partial D</th>
<th>Reactivity</th>
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<tr>
<td>D--</td>
<td>Positive</td>
<td>IS Tube*</td>
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<tr>
<td>D--</td>
<td>Negative</td>
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<td>D--</td>
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<td>IAT†</td>
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<td>D--</td>
<td>Positive</td>
<td>IS Tube (Gamma, Immucor)</td>
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<td>D--</td>
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<tr>
<td>D--</td>
<td>Positive</td>
<td>IAT (Ortho)</td>
</tr>
<tr>
<td>Variant CE</td>
<td>Negative</td>
<td>IS Tube and IAT (Ortho)</td>
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<tr>
<td>Crawford (Rh43)§</td>
<td>Negative</td>
<td>IS Tube (Gamma, Dominion, RUM1)</td>
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* IS = immediate spin.  † IAT = indirect antiglobulin test.  § predominantly found in Blacks.  ** most frequent partial D in Blacks.  # most frequent partial D in Caucasians.
Most partial D RBCs result from the inheritance of a hybrid gene with portions of \(RHDI\) replaced by the corresponding portions of \(RHCE\) (Fig. 1B) (reviewed in Huang\textsuperscript{23} and Avent and Reid\textsuperscript{41}). Some of the replacements involve single or multiple exons, whereas others involve short regions encompassing several codons. Different rearrangements can also cause the same partial D category. For example, \(D^\text{VI}\), which is the most common form of partial D in Caucasian persons,\textsuperscript{42} can result from the replacement of two, three, or four \(RHDI\) exons with the corresponding regions of \(RHCE\) (Fig. 1B).\textsuperscript{43} These rearrangements are designated as \(D^\text{VI}\) Types 1, 2, and 3 to distinguish them. Additionally, the novel sequences of the hybrid protein resulting from regions of RhD joined to RhCE can generate new antigens, and \(D^\text{VI}\) RBCs carry the BARC antigen. Because individuals with partial D can make anti-D, they should receive D– donor blood. In practice, many will type as D+ by direct tests and will only be recognized after they have made anti-D.

**Elevated D.** Several phenotypes, including D–, Dc–, and DCw–, have an enhanced expression of D antigen and no, weak, or variant CE antigens, respectively.\textsuperscript{39} They are caused by replacement of portions of \(RHCE\) by \(RHDI\), the converse of the partial D rearrangements described above (Fig. 1C and other examples in Reid and Lomas-Francis\textsuperscript{22}). The additional RHD sequences in \(RHCE\), along with a normal \(RHDI\), explain the enhanced D and account for the reduced or missing CE antigens. Immunized people with these CE-depleted phenotypes make anti-Rh17.

**Testing for weak D in transfusion practice.** Weak D red cells have stimulated the production of anti-D in D– individuals.\textsuperscript{16,39} Testing for weak D in the donor setting is straightforward; that is, donor center typing procedures must detect and label all weak D RBCs and components as D+. In contrast, transfusion service policies vary, and the test for weak D is optional.\textsuperscript{44} Historically, the weak D test was performed on transfusion recipients and on prenatal samples to conserve supplies of D– components and to prevent unnecessary RhIG administration, respectively. Several developments in the past decade, however, have appropriately caused many to reevaluate their policy of weak D testing in the transfusion and prenatal setting. For example, the current generation of monoclonal anti-D reagents contains an IgM component that causes direct agglutination of many RBCs with reduced D antigen. Samples that would previously have been classified as weak D type as D+ and patients receive Rh+ donor units, eliminating concerns regarding the unnecessary use of Rh– supplies. In addition, some partial D and, importantly, category DVI, the most common partial D in Caucasian persons,\textsuperscript{42} react only in the IAT and not by direct testing (Table 1). Because individuals with partial D RBCs make anti-D, elimination of the IAT classifies them as Rh– for transfusion and for RhIG administration and avoids the potential for the production of anti-D. Furthermore, the
distinctions between weak D and partial D have blurred with the finding that some people with weak D RBCs have produced anti-D12,38, suggesting that they would also be better served by elimination of the IAT. These considerations, along with the cost savings realized by elimination of this extended testing step and the prospect of avoiding the potentially serious error of misinterpreting the D type when RBCs are coated with immunoglobulin (positive direct antiglobulin test [DAT]), often result in a decision to eliminate the IAT phase of D testing for transfusion recipients and prenatal screening.

Ideally, tests to determine an individual’s D status would distinguish those with RBCs that lack, or have altered, D epitopes and are at risk of immunization to conventional D from those who carry mutations that reduce expression levels of D and are not at risk of producing anti-D. Unfortunately, serologic reagents cannot discriminate between these RBCs, and reactivity patterns often reflect the characteristics of the reagent rather than the characteristics of RhD expressed on RBCs. These limitations suggest that molecular testing to determine D status may be commonplace in the future.

**Applications of RH molecular genotyping for transfusion medicine practice**

Molecular assays for blood group genes currently rely on polymerase chain reaction (PCR) amplification to generate sufficient material for analysis. One of the most common approaches uses oligonucleotide primers that are sequence-specific, with amplification indicating the presence of an allele and lack of amplification denoting the absence of the allele. Another common method is PCR-restriction fragment length polymorphism (RFLP) with primers designed to amplify the region containing an allele single-nucleotide polymorphism (SNP) followed by digestion with a restriction enzyme to detect the SNP. The size of the products, as determined by electrophoresis, indicates the presence or absence of the SNP. Automated platforms with fluorescence-based detection and microchip technologies are being actively explored.

**RH** molecular testing can aid resolution of D typing discrepancies. These often are the result of differences in manufacturers’ reagents, but in the donor setting they can be FDA reportable. Molecular genotyping can be used to determine the phenotype of RBCs in patients who have been recently transfused or whose RBCs are coated with IgG. RH testing by molecular methods has applications in the prenatal setting to determine paternal RHD zygosity, to predict fetal D status to prevent invasive procedures, and to determine D– status in the presence of a strongly positive DAT.

**Molecular genotyping in the prenatal setting**

**RHD zygosity determination.** To determine paternal RHD zygosity, assays are designed to probe the region, termed Rhesus boxes, generated by deletion of **RHD**.41 A PCR-RFLP assay can be discordant when testing individuals from mixed ethnic groups40 and a PCR assay that directly detects the hybrid region is often included.47 These tests, combined with a direct screen for the 37-bp insert **RHD** pseudogene and for the **RHD-CE-D** hybrid that causes D– in individuals of mixed ancestry are reliable for determination of **RHD** zygosity.

**Prediction of fetal risk for HDN.** DNA analysis to determine the D status of the fetus is important to determine when an infant is D– and is not at risk for HDN, thereby preventing invasive and costly monitoring of the mother during pregnancy. These assays rely on detecting the presence or absence of portions of **RHD**. Fetal DNA can be obtained by amniocentesis or villus sampling, but fetal-derived DNA extracted from maternal plasma is attractive as a noninvasive sample source.48 For the prediction of HDN, samples from the mother, and the father if known, are analyzed serologically and molecularly to identify D– genotypes in the parents. This is important for accurate interpretation in situations where families carry inactive, rather than deleted, **RHD**.

**Determination of D type when DAT-positive.** Infants born to D– mothers sometimes have a positive DAT, often due to ABO mismatch, which can result in an invalid test for weak D. If the infant cannot be shown to be weak D–, the mother is a candidate for RhIG.44 Because RhIG is not completely without risk (HCV and unknown variant Creutzfeldt-Jakob disease), is costly, and is derived from human source material that is in short supply, it is prudent policy to avoid unnecessary use. Molecular testing to assess the D– status of the infant can prevent needless administration of RhIG.

In conclusion, **RH** molecular testing is an important tool that, when combined with serologic methods, can resolve discrepancies and ambiguous typing results, aid the management of HDN, and improve transfusion safety and outcomes for patients.

**Function of the Rh/RhAG proteins**

Clues to a possible function for the Rh/RhAG proteins initially came from structural predictions which suggested they were transport proteins. Database searches for proteins with similarities to RBC erythrocyte Rh/RhAG, however, were not successful until the genomic sequencing of *Caenorhabditis elegans* revealed two Rh-like proteins. These proteins were similar to ammonium transporters from bacteria and yeast, thus linking the human proteins to a family of transporters with ammonia or ammonium as the substrate. Rh/RhAG protein analysis is a good example of the power of comparative genomics, in which the sequencing of the genomes of other organisms, along with computer analysis, allows a testable hypothesis for protein function.
Difficulty in expressing the Rh/RhAG proteins in nonerythroid cells has hampered functional studies. _Xenopus_ oocytes are used for expression because of their well-documented ability to reveal the function of ion channels and transporters. Single-cell oocytes are micro-injected with cRNA, and expression can be monitored by Western blot. It was found that RhAG was readily expressed in oocyte membranes and mediated transport of the ammonium analog, [14C]methylammonium, a radioactive substrate often used to study ammonium transport. Transport of the analog was readily competed by ammonium, demonstrating specificity. Functional characterization revealed that uptake was independent of the membrane potential and the Na\(^+\)/H\(^+\) gradient, but was dramatically stimulated by raising extracellular pH or lowering intracellular pH. This suggested that uptake was coupled to an outwardly directed \(\Delta \mu \text{H}^+\) gradient and led us to hypothesize that RhAG might function by \(\text{NH}^+/\text{H}^+\) countertransport.

Additional evidence for RhAG-mediated transport of ammonium comes from complementation experiments in _Saccharomyces cerevisiae_ yeast cells that are deficient in ammonium transport. Studies reported by Marini and coworkers and data from our laboratory showed that expression of RhAG could rescue yeast cells lacking the ammonium transport proteins (MEPS) when the cells were grown on low ammonium. This is important because the ability of a mammalian protein to functionally substitute for the yeast protein is one of the most powerful indications that the proteins have a related function. Several hundred mammalian proteins have now been shown to function in yeast and to genetically complement mutants in _S. cerevisiae_.

Complementation in yeast only occurs when the pH value of the medium was raised to 6.1 to 6.5, which may explain the failure of others (C.H. Huang, personal communication) to see complementation, which has led to criticism of the earlier report. But the most striking evidence for RhAG-mediated transport of ammonium or methylammonium was seen when RhAG was expressed in wild-type yeast cells. RhAG dramatically rescued wild-type cells from the toxic effects of high concentrations of methylamine (Fig. 4). Rescue was associated with efflux of methylammonium and was also pH-dependent, but in contrast to complementation, was enhanced at acid pH values. These data revealed that in yeast RhAG mediates both uptake and efflux of substrate, and that the direction of transport depends on the pH value of the medium.

It has also been speculated that Rh/RhAG proteins may mediate movement of CO\(_2\), based on the observation that expression of _RH1_, one of two Rh-like proteins in the blue green algae, _Chlamydomonas reinhardtii_, increased when these cells were grown in 3 percent CO\(_2\) and decreased when the cells were shifted to air. More than 2000 genes, however, are induced in _C. reinhardtii_ when shifted to growth in CO\(_2\). _RH2_ did not show this effect, and no direct evidence for _RH1_-mediated movement of CO\(_2\) has been demonstrated. This hypothesis, if substantiated with direct transport data, could be reconciled with the ammonium data if Rh/RhAG are found to mediate nonspecific movement of uncharged molecules, like CO\(_2\) and NH\(_3\).

**RhAG**

Ascribing a physiologic role for an ammonia or ammonium transporter in RBCs is rather speculative at present, but it may be more complex than simply allowing the RBC to survive transit through regions of high ammonium in the kidney. Because total blood ammonia consists of NH\(_4^+\) and NH\(_3\) in equilibrium (NH\(_4^+\) \(\rightarrow\) NH\(_3\) + H\(^+\), pK\(_a\) 9.25), at blood pH 7.4 most of the total blood ammonia exists as charged NH\(_4^+\) and about 1 percent as uncharged NH\(_3\). The charged molecule NH\(_4^+\) is very toxic to brain cells and mitochondria and any increase in blood ammonium (NH\(_4^+\)) levels will also increase the amount of the toxic, uncharged NH\(_3\) molecule. Our hypothesis is that RhAG serves as an ammonia or ammonium scavenger, keeping the total blood ammonia (NH\(_4^+\) + NH\(_3\)) level low by trapping ammonium (NH\(_4^+\)) inside the RBC. In support, total ammonia levels in blood plasma are low (0.1-0.2 mmol/L), but total RBC ammonia levels are, on average, three times greater. The large total RBC mass in the blood would enable these cells to carry away a significant amount of ammonia or ammonium, and the bidirectional nature of RhAG transport would allow ammonium to be

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**Fig. 4.** _S. cerevisiae_ cells expressing RhAG (Clones 5 and 9) or with the empty vector grown on medium with or without methylamine. A concentration of 50 mmol per L methylamine is toxic to the cells, but expression of RhAG confers resistance.
exchanged in the liver and kidney, where other Rh/RhAG homologs are found.

**RhBG and RhCG**

N erythroid Rh homologs, designated RhCG and RhBG, have now been found in the kidney, liver, brain, and skin\(^{56,57}\) and these proteins have the same predicted 12-transmembrane structure as erythroid RhAG/Rh.

When expressed in oocytes, kidney RhBG and RhCG also mediate transport of ammonium.\(^{58}\) Significantly, in the kidney, ammonium ions act as expendable cations that facilitate excretion of acids, and renal ammonium metabolism and transport are critical for acid-base balance.\(^{256,59}\) In the collecting segment and collecting duct, where large amounts of ammonium are excreted, RhBG and RhCG are found on the basolateral and apical membranes, respectively, of the intercalated cells.\(^{58}\) These localization studies suggest that RhBG and RhCG are ideally situated to mediate transepithelial movement of ammonium from the interstitium to the lumen of the collecting duct. In support, mouse collecting duct (mIMCD-3) cells, which show polarized expression of these proteins, demonstrate transporter-mediated movement of ammonium, and the transport characteristics are consistent with electroneutral NH\(_4^+\)/H\(^+\) exchange activity.\(^{62}\)

The liver is a critical organ for ammonium metabolism. Localization studies in the liver reveal that RhBG is found on the basolateral membrane in perivenous hepatocytes, where it may function in ammonium uptake. RhCG is present in bile duct epithelial cells, where it is ideally positioned to contribute to ammonium secretion into the bile fluid.\(^{58}\)

In conclusion, RhCG and RhBG have been localized to regions where ammonium production and elimination are critical in mammalian tissues, strongly suggesting a role for these proteins in ammonium homeostasis. Future functional studies of the kidney, liver, and brain Rh homologs, along with the RBC RhAG/Rh proteins, promises to lead to development of a unifying hypothesis of ammonium transport in mammals by the Rh family of proteins.

**Function of RhCE and RhD**

If RBC RhAG functions as an ammonium transporter, what is the function of the more recently evolved Rh proteins RhCE/D? Because RhCE and RhD have arisen from RhAG, it might be assumed then that they also function as transporters, but assays for a transport function are hindered by their dependence on RhAG for membrane expression. To determine whether RhCE could enhance, modulate, or influence RhAG-mediated transport, transport was measured in oocytes injected with equimolar RhAG and RhCE cRNAs. Coexpression of RhCE/RhAG did not influence the rate or total substrate accumulated in oocytes compared to that seen with expression of RhAG alone (unpublished observations). Although additional studies are necessary to determine whether RhCE/D are involved in membrane transport, our hypothesis is that RhCE/D may have lost transport function and are evolving to have a structural role in the RBC membrane. Evidence in support of an alternative role for RhCE/D in the RBC comes from DIVERGE analysis (personal observations) and evidence from others\(^{64,65}\) that indicate the RhCE/D proteins are rapidly evolving, suggesting that their function may be changing.

A structural role for the RBC Rh proteins is suggested from the RBC shape defects, fragility, and the resulting anemia seen in patients with Rhnull disease.\(^{66,67}\) Rh/RhAG are part of a membrane protein complex that includes CD47, also known as integrin-associated protein, glycoporphin B, and LW (ICAM-4) (reviewed in Avent and Reid\(^{41}\) and Huang et al.\(^{49}\)). Band 3 may also be associated with the complex.\(^{69}\) The Rhnull defect suggests that there is an important membrane-cytoskeletal attachment site in RBC that may be mediated by Rh, RhAG, or a member of the Rh complex. Recent studies reveal that the Rh complex is linked to the membrane skeleton through CD47–protein 4.2 interactions\(^{70}\) and through a novel Rh/RhAG-ankyrin connection.\(^{71}\)

**SIGNIFICANCE AND SUMMARY**

The genetic basis of the Rh blood group proteins has been intensely investigated in the past decade, and the origin of most of the antigens has now been determined. At present, routine RH testing by molecular methods is hampered because of the large number of Rh polymorphisms. More than 77 RH and 22 RHCE gene variants have been reported (summarized in Reid and Lomas-Francis\(^{22}\), and additional variants are still being discovered. Molecular testing, however, as an adjunct to serologic methods, improves transfusion safety and outcomes, and the challenge for the next decade is to develop automated platforms that sample several regions of the genes for unequivocal interpretation.

The discovery that the Rh family of proteins is involved in ammonia or ammonium transport and is ideally positioned in key tissues essential for ammonium elimination is a significant finding because it was long assumed that the high membrane permeability of ammonia (NH\(_3\)) would obviate the need for specific transport pathways in mammalian cells. This is reminiscent of the discovery of the function of the Colton blood group protein as the first member of a family of water transporters (aquaporins) and of the Kidd blood group protein as the first member of a family of urea transporters. The movement of water and urea were also originally thought to
occurs only by passive movement through the lipid bilayer. Like the aquaporins and the urea transporters, homologs of the Rh proteins have been found in other tissues and in many organisms including the sponge, the slime mold, the fruit fly, fish, and the frog (summarized in Huang et al.68), indicating that they have an essential and conserved function throughout evolution.

RBC membrane protein–cytoskeleton and protein–protein interactions are an active area of investigation. Although the major attachment sites between the RBC cytoskeleton and the lipid bilayer are understood to be through glycophorin C and band 3, new evidence for an additional attachment site, mediated by the Rh complex, is indeed exciting. Future studies are needed to determine the protein–protein interactions and the dynamics of the assembly of the Rh–membrane complex to understand the Rhnull defect. Importantly, the findings above highlight the major contributions that the study of the function of blood group proteins continues to make to biology and physiology and emphasize the opportunities for research within the profession of transfusion medicine. The recent revelations about the role of the Rh/ RhAG proteins in RBC membrane structural integrity and their transport function have taken the field of Rh better beyond consideration as simply a family of blood group antigens. Blood groups are not just for blood bankers anymore.

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