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Biochemistry of the Erythrocyte Rh Polypeptides: A Review

PETER AGRE, M.D., BARBARA L. SMITH, B.S.,
AND SABINE HARTEL-SCHENK, Ph.D.

Department of Medicine and Department of Cell Biology/Anatomy, Johns Hopkins University School of Medicine, Baltimore, Maryland

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The clinically important Rh blood group system is complex, consisting of multiple distinct antigens. Despite clinical recognition for over 50 years, the Rh blood group antigens have remained poorly understood on a molecular level until the recent identification and characterization of the "Rh polypeptides," the core structural proteins of the Rh antigens. This group of erythrocyte membrane proteins of molecular weight 30,000–35,000 daltons was first recognized by employing Rh-specific antibodies to immunoprecipitate radiolabeled components of erythrocyte membranes. By using antibodies specific for the Rh D, c, and E antigens, a series of highly related non-identical proteins were immunoprecipitated, indicating that the Rh antigens are composed of multiple related proteins. The Rh polypeptides have been purified and characterized, and they were found to have several unusual biochemical characteristics. The Rh polypeptides penetrate the membrane bilayer; they are linked to the underlying membrane skeleton; they are covalently fatty acid acylated with palmitate. While the Rh antigenic reactivity is unique to human erythrocytes, the Rh polypeptides have been isolated from erythrocytes of diverse species and are thought to be fundamental components of all mammalian erythrocyte membranes. The functional role of the Rh polypeptides remains undefined, but a role in the organization of membrane phospholipid is suspected.

CLINICAL RECOGNITION OF Rh

The Rh antigens are one of the most important blood group systems in clinical and transfusion medicine. The Rh antigen was first described in 1939 by Levine and Stetson, who identified a young mother recently delivered of a stillborn infant. The baby had succumbed to intrauterine hemolysis, then a disease of unknown cause [1]. The mother was herself anemic and received a transfusion with blood from her husband, who was ABO-compatible. She had an immediate transfusion reaction, and the investigators postulated that she had developed antibodies to a substance on the surface of her infant's erythrocytes, which was inherited from the father. Presumably the antibodies had crossed the placenta, causing the infant's death.

The presence of a new and pathological antibody was confirmed; the patient's serum agglutinated the father's erythrocytes and cells from 80 of 104 random donors. At the same time, Landsteiner and Wiener were independently seeking evidence of blood group antigenic variations in several non-human species and had raised rabbit antibodies to erythrocytes from rhesus monkeys [2]. These anti-rhesus antisera were found to react with human erythrocytes with the same specificity as the antiserum from Levine and Stetson's patient. The membrane antigen was therefore named "Rhesus factor" or "Rh." Ironically, the similarity in cell specificity was subsequently found to result from a different antigen named, in honor of Landsteiner and Wiener, "LW."

TABLE 1
Abbreviated Nomenclature of Rh Antigens[a]

Rosenfield	Fisher Race (CDE)	Weiner (Rh-Hr)
Rh 1	D	Rh_o
2	C	rh′
3	E	rh″
4	c	hr′
5	e	hr″

[a]Derived from [35]

Rh serology is exceedingly complex, and two conflicting genetic theories, which are still not resolved, were put forth by early investigators. This problem has led to the development of different nomenclatures, which are often confusing (refer to Table 1). The theory proposed by Wiener postulates that different Rh antigens reside upon a single molecule, which is the product of a complex gene [3]. The theory proposed by Fisher and Race postulates that multiple distinct antigens exist (referred to as D, C, c, E, and e), which are the products of multiple closely linked genes [4]. Approximately 85 percent of the general population have the major Rh antigen (D) and are referred to as "Rh-positive" (genetically *DD* or *Dd*), while 15 percent of the population are "Rh-negative" (*dd*); the latter are, of course, completely normal. While the d phenotype is recognized by the lack of D, no d antigen has actually been detected serologically. The C and c antigens and the E and e antigens are found on all normal Rh-positive and -negative erythrocytes.

No method of directly assessing the Rh genotype of an individual has been developed, but the frequencies of the different Rh antigens have been established in different racial and ethnic groups (Table 2). Such information permits estimation of a "most likely" genotype, and this genotype can be further established by studying other family members. For example, an individual whose blood reacted with antibodies to C, c, D, and e may be genetically *CDe/ce* (R^1/r) or *CDe/cDe* (R^1/R^0). The former would be more likely among whites and Asians; the latter would be more likely among blacks.

The genes encoding the D, C, c, E, and e antigens are inherited *en bloc* with no crossing over during meiosis. Thus an individual with the haplotype *CDe/cDE* (R^1/R^2) could pass on *CDe* (R^1) or *cDE* (R^2) to his progeny but could not pass on CDE (R^z) or *cDe* (R^0).

BIOCHEMICAL BASIS OF THE Rh ANTIGEN

Despite large clinical importance, the membrane structural components of the Rh antigen remain incompletely defined. A series of investigations, beginning in the late 1960s, identified a relationship between Rh and lipid [5]. Several peculiarities of Rh made it resistant to traditional biochemical analysis. Rh immune globulin fails to give a specific reaction with membrane components on immunoblots or with detergent-solubilized membranes. Membrane proteins from Rh-positive and -negative erythrocytes appear identical when studied with traditional electrophoresis methods. Moreover, Rh serological reactivity survives extensive proteolytic digestion of intact erythrocytes.

TABLE 2
Selected Rh Gene Frequencies[a]

Symbol	CDE	Frequency		
		Whites	Blacks	Asians
R^1	*CDe*	0.41	0.10	0.55
r	*cde*	0.39	0.15	0.10
R^2	*cDE*	0.14	0.10	0.35
R^0	*cDe*	0.03	0.60	<0.01
R^Z	*CDE*	<0.01	<0.01	<0.01

[a]Derived from [35]

THE Rh POLYPEPTIDES

A major advance in the molecular understanding of the Rh antigens was provided by two European groups who independently discovered the Rh polypeptides in 1982 [6,7]. As will be explained, the "Rh polypeptides" and "Rh antigens" are not synonyms, since the Rh polypeptides are core components of the antigenic complex, but by themselves are devoid of the immunoreactivity which defines the Rh antigens.

The Rh polypeptides were first identified by labeling intact erythrocytes with ^{125}I. A membrane protein of approximately 30,000 daltons was avidly labeled on the surface of Rh-positive erythrocytes and more weakly on Rh-negative erythrocytes. Rh immune globulin was added to the membranes, and the immune complex was solubilized in detergent and isolated. Unlike core components of other blood group antigens, the Rh polypeptide contains no detectable carbohydrate [8]. The Rh polypeptide also was found to be entirely linked to the spectrin-actin membrane skeleton [9,10]. This latter observation explains the long-recognized finding that Rh-specific IgG fails to fix complement, and the Rh antigen-antibody complexes are unable to diffuse in the plane of the lipid bilayer. These phenomena presumably result from physical constraints upon the Rh antigens at the cell surface by the intracellular spectrin-actin membrane skeleton. IgG can only activate complement when antigens are clustered at a critical distance from one another. Linkage of the Rh antigen to the underlying membrane skeleton prevents this process. Moreover, while the Rh polypeptide was found to be entirely restricted to the membrane bilayer, it survived proteolytic digestions at either the inner or outer face of the bilayer [11]. Taken together, these observations are strong evidence that the Rh polypeptides are core components of the Rh antigens.

Rh_{null} PHENOTYPE

A rare phenotype known as Rh_{null} has been recognized since the early 1970s [12]. Rh_{null} patients suffer chronic mild hemolytic anemia. Rh_{null} erythrocytes contain no detectable Rh antigens (no D, C, c, E, or e), and, when transfused with Rh-negative blood, the patients develop antibodies to c or e. The Rh_{null} phenotype is heterogeneous; some patients appear to lack Rh-specific genes, whereas others carry Rh genes but fail to express Rh antigens.

Rh_{null} erythrocytes show several phenotypic abnormalities, including abnormal stomatocytic morphology (cup-shaped rather than disc-shaped erythrocytes), abnormal cation transport [13,14], and abnormal membrane phospholipid organization with

increased aminophospholipid in the outer leaflet of the bilayer [15]. Employing an antibody raised in rabbits to the denatured Rh protein, immunoblots of erythrocyte membranes from common Rh phenotypes were found to give a specific reaction with the Rh polypeptides, whereas Rh_{null} membranes failed to react [16]. Also, Rh serological reactivity is known to be destroyed by sulfhydryl oxidants. Employing a sulfhydryl probe, two membrane proteins of 34,000 and 32,000 daltons (presumably Rh polypeptides) were labeled in all erythrocyte membranes except those from Rh_{null} individuals [17]. While the Rh_{null} membranes invariably lack the Rh polypeptide, other polypeptides are also deficient in these membranes [18]. It is very likely that a complex of several mutually dependent membrane proteins are expressed together with the Rh polypeptide, a hypothesis termed the "Rh cluster" [19].

PURIFIED Rh POLYPEPTIDE

Methods for purifying the Rh polypeptide were developed, employing either immunological or non-immunological methods. Large-scale immunoprecipitations followed by preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [20] or large-scale separations employing hydroxylapatite chromatography, gel filtration, and preparative SDS-PAGE [11] resulted in identical, pure proteins of approximately 30,000 daltons. The serological reactivity which defines the Rh antigens was totally lost during the purifications, however, presumably due to alteration of conformation or loss of additional components of the Rh antigen.

Determination of N-terminal amino acid sequence from immunoprecipitated and from chromatographically isolated Rh polypeptides yielded the same unique sequence [21–23]. Moreover, the N-terminal amino acid sequences of the Rh polypeptides isolated from Rh-positive and Rh-negative erythrocytes were identical [23]. Nevertheless, two-dimensional iodopeptide analysis of the proteins after digestion in chymotrypsin demonstrated a polymorphism which was specific for the Rh polypeptide isolated from Rh-positive membranes, confirming that the Rh polypeptide is linked to the expression of Rh antigenic phenotype. Calculating back from recovery, it was estimated that 60,000 Rh polypeptides exist per cell. It is likely that the Rh polypeptides exist naturally as a complex of multiple subunits [11,19]; nevertheless, this number is compatible with the known number of Rh D antigens per cell, approximately 20,000 [24].

COMPLEXITIES

The Rh polypeptides are now recognized to be more than a single molecular species. Employing human antisera specific for Rh D, a protein of approximately 30,000 daltons was immunoprecipitated, whereas antisera against c or E consistently brought down a slightly larger protein [25]. When Rh D, c, and E polypeptides were compared by two-dimensional iodopeptide analyses, the polypeptides were found to be strikingly similar. The c and E preparations were identical except for a single iodopeptide, whereas the D preparation was approximately 60 percent identical to c and E [26]. These differences confirmed that the Rh polypeptides associated with the different Rh phenotypes were similar but non-identical; these differences may result from expression of Rh polypeptides from different D, c, and E genes. Alternatively, these variations may represent variant splicing of mRNA from a single Rh gene. Nevertheless, the issue is as yet unresolved. While three distinct Rh polypeptides have been identified so

far, there may be several others, such as polypeptides corresponding to the Rh C and e antigens. The existence of a Rh d polypeptide can neither be confirmed nor refuted.

The Rh antigen may be a complex of Rh polypeptides and additional components. In addition to the 30,000 dalton Rh polypeptide, monoclonal antibodies to D immunoprecipitated a hazy protein of molecular weight ranging from 45,000–100,000 daltons. This larger protein bears polylactosaminoglycan-type carbohydrate and was initially thought to represent glycosylated Rh polypeptides [25]. Determination of N-terminal amino acid sequence of the glycosylated Rh protein demonstrated a unique sequence, which was approximately 50 percent homologous with the N-terminal Rh polypeptide sequence [18], suggesting duplication and divergent evolution of an ancestral Rh gene. Thus a family of Rh proteins exists.

Rh AND LIPID

Early studies demonstrated a relationship between membrane phospholipid and Rh serological reactivity, although the specificity was unclear [5]. Rh serological reactivity could be extracted from membranes with alcohol, but it was restored by addition of exogenous phospholipids. Other investigators noted that Rh serological reactivity was destroyed by phospholipases [27]. Moreover, the non-immune purifications were made especially difficult by the unusually hydrophobic nature of the Rh polypeptide. Recent investigation employing sequential digestions with phospholipase A_2 followed by trypsin resulted in degradation of D but not c or E Rh polypeptides [28]. No proteolytic degradation of any Rh polypeptides was seen without the phospholipase digestion.

When mature erythrocytes are incubated with radiolabeled palmitic acid (a saturated 16 carbon fatty acid), there occurs a dramatic labeling of approximately six erythrocyte membrane proteins of undetermined identities. Of these, the most abundantly labeled is a 30,000 dalton membrane protein, which can be immunoprecipitated with anti-Rh(D). Fatty acid acylation of the Rh polypeptide appears to be the result of covalent thioesterification of the fatty acid on to free sulfhydryl groups within the Rh polypeptide. This fatty acid acylation is post-translational, since mature mammalian erythrocytes contain neither a nucleus nor endoplasmic reticulum and have negligible protein synthesis [29]. While these studies confirmed a relationship between Rh polypeptides and lipid, it remains uncertain whether covalently attached or adjacent lipid is necessary for expression of Rh antigenic reactivity or whether the fatty acids are simply supporting structures.

NON-HUMAN Rh POLYPEPTIDES

While the Rh antigen was mistakely named for the rhesus monkey, Rh antigenic reactivity is restricted to human erythrocyte membranes. Development of the non-immunological method for purification of Rh polypeptides permitted the isolation of putative analogs to the Rh polypeptides from the erythrocytes of diverse species. Erythrocytes from monkey, cow, cat, and rat were each found to contain polypeptides that were biochemically very similar to human Rh polypeptides [30]. When studied by two-dimensional iodopeptide analysis, it was determined that the non-human Rh analogs appeared between 30 and 60 percent identical to the mixture of human Rh D + c + E polypeptides. Curiously, two iodopeptides were conserved among all species, consistent with highly conserved functionally important domain(s); however, the iodopeptide which could be labeled at the surface of the erythrocyte membrane was found only in the human preparations. Thus, while it is likely that the Rh polypeptide is

of fundamental physiological importance to membrane integrity, the surface determinants needed for Rh antigenic reactivity are probably unrelated to the physiological role.

FUNCTIONAL ROLE OF Rh POLYPEPTIDES

The roles of the Rh antigens and Rh polypeptides in normal membrane physiology remain unresolved. The Rh_{null} phenotype indicates that total lack of Rh antigens and polypeptides results in a distinct but clinically mild disorder. The constellation of abnormal cell shape, abnormal cation transport activity, and abnormal phospholipid distribution suggests that the Rh polypeptide may play a structural role in the organization of membrane phospholipids. This effect may result from participation of the Rh polypeptide in active membrane phospholipid translocation [31,32], or it may result from a passive contribution to membrane phospholipid organization by providing a structural restraint to membrane phospholipid mobility.

A structural role for Rh has also been suggested by the linkage of abnormal erythrocyte morphology and Rh phenotype in a subset of hereditary elliptocytosis families. This linkage is now recognized to result from the close chromosomal location of genes encoding structural protein 4.1 and Rh [33,34].

The Rh gene(s) have not yet been isolated and sequenced. With the existing amino acid sequence and antibody probes, however, it is very likely that this work will soon be accomplished. Once cloned, the primary amino acid sequence may reveal homology with a previously characterized polypeptide, and plausible biological roles for Rh may be uncovered. Alternatively, the isolated Rh cDNAs may be expressed after *in vitro* mutagenesis to remove sites of fatty acid acylation or after other modifications. Also, the existance of Rh polypeptide in non-erythroid tissues may be systematically evaluated. In view of these anticipated events, activity in this area of biology is likely to be of significant clinical and basic scientific interest.

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