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CHAPTER 16

Disorders of the red cell membrane

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1. Introduction

The red cell membrane designates, in a strict sense, the plasma membrane of the erythrocyte, the only membrane remaining in the circulating red cell. It consists of a lipid bilayer, a variety of proteins studded therein, and the glycans that stick outward, being linked covalently either to proteins or to lipids. Protein or glycan domains constitute the structural bases of blood groups. In a wider sense, the red cell membrane includes, in addition, an unusually thick, bidimensional protein network that provides the red cell with its mechanical properties of both resistance and flexibility. This protein network is named the red cell skeleton. Most of the genes encoding the membrane proteins are known. Mutations in these genes account for a variety of different conditions, most of which are haemolytic anaemias of various descriptions.

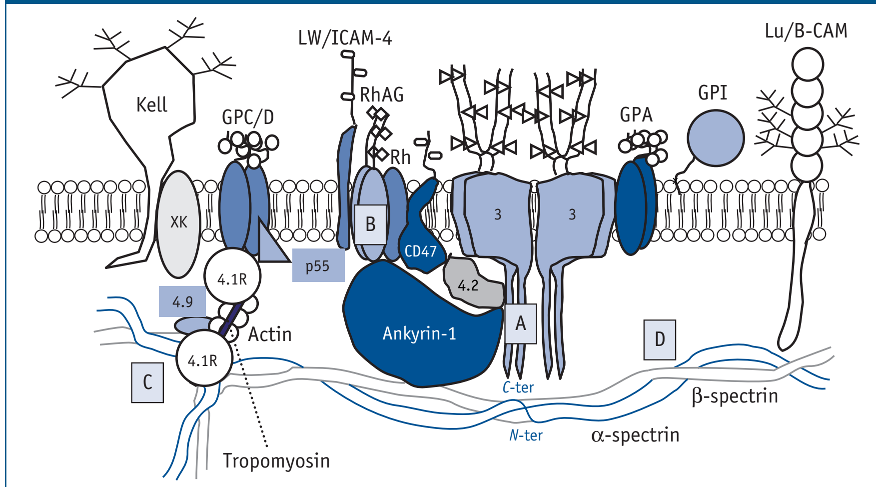
2. The red cell membrane

A schematic picture of the red cell membrane is shown in Figure 1. A classical description of the lipid bilayer was provided in a review by Lux and Palek (1). During the last decade, a major breakthrough has been the discovery of lipid rafts in membranes in general, and in the red cell membrane in particular. Rafts are detergent-resistant plasma membrane microdomains. They are rich in sphingolipids. They are also rich in cholesterol. They exist as islets having a phase different to that of the loosely packed disordered state of the rest of the bilayer. Shingolipids contain long, largely saturated fatty acids allowing them to pack tightly together (2). Lipid rafts show a higher density of some proteins, such as phosphatidyl-inositol linked proteins, stomatin (Table 1), and flotillin-1 and -2. Related to lipid rafts are caveolae, which are invaginated plasma membrane microdomains. Flotillins may act as scaffolding proteins within caveolar membranes. The main properties of membrane proteins and their genes are summarised in Table 1. Their main functions are summarised in Table 2. Briefly, proteins may be divided into several main categories.

2.1 Skeletal proteins

Skeletal proteins (mainly spectrin α- and β-chains, protein 4.1 and actin) are organised as a network, largely in the form of a hexagonal mesh. They provide the circulating red cell with its resilience and elasticity. They are connected with one another at two sites: (i) Two or more spectrin αβ dimers articulate head to head (α-chain N-terminus vs. β-chain C-terminus) at the spectrin self-association site; (ii) The extremities of several tetramers (or oligomers) converge toward a complex where protein 4.1 (through its 10 kDa domain interacting with the spectrin β-chains

Figure 1: **A schematic view of the red cell membrane proteins and skeleton interactions**



Key proteins which interact in the red cell membrane are depicted (not to scale). The lipid bilayer forms the equator of the cross-section with its polar heads (open circles) turned outward and their apolar fatty acid chains facing one another and forming the inner core.

Band 3 complex (Box A)*, centered by a band 3 tetramer, but band 3 may also exist as a dimer. The bulky part of each monomer represents transmembrane segments towered by a long, branched polylactosaminoglycan chain, which stems from asparaginyl residue 642. The stalky part of band 3 monomer accounts for its cytoplasmic domain which serves to anchor proteins, such as ankyrin-1, which also binds to spectrin β-chain (C-ter region of the latter), protein 4.2 and a number of cytoplasmic proteins (deoxyhemoglobin, glyceraldehyde-3-phosphate dehydrogenase, aldolase and others, not shown). Interaction between band 3 and glycophorin A, which exists as a dimer and is decorated by numerous short, sialic-acid-containing glycans, form the* Wr^b *antigen.*

Rh complex (Box B) *includes the Rh polypeptides and the RhAG (Rh-Associated glycoprotein) glycoprotein most likely arranged as a heterotrimer (here as* $RhAG_2Rh$*) which are associated with the accessory chains CD47, the Landsteiner-Wiener glycoprotein (LW, also called ICAM-4) and glycophorin B (not shown). CD47 interacts with protein 4.2, but Rh and RhAG proteins still bind to skeletal proteins in the absence of 4.2-CD47 interaction, as seen in 4.2-deficient individuals. Rh/RhAG proteins interact with ankyrin-1 to link the Rh complex and the membrane skeleton. The Rh complex and the band 3 complex are thought to form a "macrocomplex" involved in gas transport. A glycosylphosphatidylinositol-linked protein (GPI) appear on the upper right corner as a balloon attached to the outer lipid monolayer through a complex oligoglycan chain.*

The junctional complex (Box C)*: protein 4.1R interacts, through its 10 kDa domain, with one extremity of several spectrin tetramers (site located in the β-chain N-terminal part), in a region containing actin short filaments and an array of actin-binding proteins: dematin (protein 4.9), tropomyosin, β-adducin (not shown) and tropomodulin (not shown). Outside this complex, protein 4.1R also interacts through its 30 kDa domain with transmembrane glycophorin C and p55 in a triangular fashion. The Kell and*

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*Xk proteins (*on the left*) are covalently linked by a disulphide bond to form a complex. (Studies of 4.1-deficient* mouse *RBCs have shown that on* mouse *RBCs, the Xk protein as well as Duffy and Rh proteins (not Rhag) also binds to protein 4.1R (not shown)).*
Spectrin (Box D)*. The α2β2 tetramers of spectrin form a dense network lining the inner surface of the lipid bilayer. The α- and β-chains are antiparallel. Two dimers associate side-by-side, a process set off at the nucleation sites on both chains, not far from the C-terminal and N-terminal regions of the α- and β-chains, respectively. Dimers associate head-to-head, N-terminal region of α-chains vs C-terminal region of β-chains, at the self-association site in order to generate tetramers, as shown here, and higher order oligomers. Lu/BCAM also interacts with α-spectrin.*

N-terminal region) brings them together. Short actin filaments and a variety of other proteins (adducin, tropomyosin, tropomodulin) participate in this protein 4.1-based complex (3).

2.2 Transmembrane proteins

Transmembrane proteins represent the bulk of the membrane proteins. Many are involved in the transport of ions and small organic molecules, but various other functions have also been assigned to them (Table 2). A prominent member of this class is band 3, a polytopic membrane protein organised in two distinct functional domains. The N-terminal cytoplasmic domain mediates the anchoring of the skeleton via ankyrin (see below). The C-terminal region mediates chloride-bicarbonate exchange, a function which is critical for the transport of carbon dioxide from the tissues to the lungs. Transmembrane proteins usually carry a variety of outwardly oriented N-and/or O-glycans, which carry some of the blood group antigens, including the ABO antigens. ABO antigens are also carried by carbohydrates attached to glycolipids, as are some other blood group antigens (P and Ii). In many instances, however, blood group antigens are determined by oligopeptide motifs on the external surface of the membrane. Among the most important ones for blood transfusion are the Rh, Kell, Duffy, and Kidd antigens (Table 1). Some of these blood group carrying proteins interact directly or indirectly with the membrane skeleton (see below and Figure 1).

2.3 Linker proteins

Linker proteins mediate the attachment of skeletal proteins to transmembrane proteins. The main member of this class is ankyrin, which links spectrin (at a site close to the β-chain C-terminus) to the cytoplasmic domain of band 3 and to the Rh complex (Figure 1). Protein 4.2 plays a role in this interaction. Band 3 (tetramers), ankyrin, and protein 4.2 contribute to the so-called band 3 complex, which also includes glycophorin A (GPA). Protein 4.1 interacts with band 3 as well. The band 3-protein 4.1 interaction has been better deciphered in the zebrafish (4).

Table 1: **Some major membrane (glyco)proteins or complexes present in the red cell membrane**

MEMBRANE	GENE				(GLYCO)PROTEIN			
Component	ISBT[d]	ISGN	Location	Size (kb) /exons	kDa	Amino acids (TM domains)	N-Glycans	Copies/RBC x10³
Spectrin α-chain		*SPTA1*	1q22-q23	80/52	281	2429 (None)	None	242
Spectrin β-chain		*SPTB*	14q23-q24.1	>100/36	246	2137 (None)	None	242
Ankyrin		*ANK1*	8p11.2	>120/42	206	1880 (None)	None	120
Protein 4.1		*EPB41*	1p33-p34.2	>250/>23	66	588 (None)	None	200
Protein 4.2[a]		*EPB42*	15q15-q21	20/13	77	691 (None)	None	200
Protein 4.9[b]		*EPB49*	8p21.3	23/	43	383 (None)	None	ca.140
p55[a,c]		*MPP1*	Xq28	27/12	55	466 (None)	None	/
Stomatin[a]		*EPB72*	9q33.2	31/7	32	287 (1)	None	/
Band 3 (CD233)	DI	*SLC4A1*	17q21	20/20	102	911 (14)	1	1,000
GPA (CD235a)	MN	GYPA	4q31.22	31/7	36	131 (1)	1	1,000
Rh complex[e]								
• Rh (CD240D/CE)	RH	*RHD/RHCE*	1p36-p34	70/10	30-32	417 (12)	None	200
• RhAG (CD241)	*RHAG*		6p21-p12	30/10	50	409 (12)	1	200
Kell-Kx complex								
- Kell (CD238)	*KEL*	*KEL*	7q33	21/19	93	732 (1)	5/4 (K2/K1)	4-17
- Kx	*XK*	*XK*	Xp21	/3	73	444 (10)	None	1
GPC/D (CD236C/D)	*GE*	*GYPC*	2q14-q21	13.5/4	32/23	128(1)/107 (1)	1	100/50
Duffy (CD234)	*FY*	*DARC*	1q22-q23	1.5/2	35-45	338 (7)	1	13-14
Kidd	*JK*	*SLC14A1*	18q11-q12	30/11	50	389 (10)	1	15
Lutheran (CD239) (B-CAM)	*LU*	*LU*	19q13.2	13/15	85 78	597 (1) 557 (1)	5	1.5-4
LW (CD242) (ICAM-4)	*LW*	*ICAM-4*	19p13.3	2.5/3	42	241 (1)	4	3-5
RAPH (CD151)	*MER2*	*CD151*	11p15.5	4.3/8	32	253 (4)	1	0.05-0.5
AQP1	*CO*	*AQP1*	7p14	17/4	50	269 (6)	1	100-200
AQP3	*GIL*	*AQP3*	9p13	7/6	50	292 (6)	1	15

Mostly proteins and the corresponding genes involved in disease are presented. Other important proteins may be mentioned in the text. The oligomeric state (if relevant) has been omitted. Part of data compiled from Lux and Palek (1) and http://genome.ucsc.edu. ***a*** *Main fatty-acylated proteins.* ***b*** *also known as dematin.* ***c*** *Member of the MAGUKs family (membrane-associated guanylate kinase homologs).* ***d*** *Blood group gene nomenclature from the International Society of Blood transfusion (ISBT). DI stands for Diego blood group locus encoding band 3.* ***e*** *Rh and RhAG proteins define the core of the Rh complex, which also includes LW/ICAM4, CD47 and GPB.*

Table 2: **Main function(s) of some red cell membrane proteins listed in Table 1**

PROTEIN	MAIN FUNCTION
Spectrin α-chain	Skeleton mechanics
Spectrin β-chain	Skeleton mechanics
Ankyrin	Skeleton attachment
Band 3 (CD233)[a]	Skeleton attachment Anion exchange Receptor *P. falciparum*
Protein 4.1	Skeleton mechanics
Protein 4.2	Skeleton attachment
Flotillin 1 **Flotillin 2**	Scaffolding proteins within caveolar membranes
Stomatin	
Rh complex* • Rh (CD240D/CE) • RhAG[a] (CD241)	Skeleton attachment Ammonia (and CO_2?) transport
Kell-Kx complex • Kell • Kx	 Zn^{2+}-metalloprotease Membrane transport (?)
GPC/D (CD236C/D)	Skeleton attachment (?)
Duffy	Receptor for chemokines and *P. vivax* and HIV-1 (?)
Kidd	Urea transport
Lutheran (CD239) (B-CAM)	Adhesion molecule Laminin receptor
LW (CD242) (ICAM-4)	Adhesion molecule Ligands for β_2 and α_V-integrins
RAPH (CD151)	Adhesion function (?)
AQP1	Water channel
AQP3	Aquaglyceroporin

Although most proteins are multifunctional, only the most important function(s) is (are) named.
a *Band 3 and RhAG may have a potential ability to transport monovalent cations under normal conditions.*

It is generally accepted, though this has also been questioned (5) (see below), that the main role of protein 4.1 is to tether the spectrin network to glycophorin C/D, through a site in its N-terminal 30 kDa domain. Protein p55 interacts also with both protein 4.1 and glycophorin C/D (GPC/D) (6, 7). Recently, a more complex model has been suggested, in which band 3 (dimers), flanked by a set of Duffy, Rh proteins and proteins of the Kell-XK complex, would participate in the protein 4.1-based complex on *mouse* erythrocytes. (3) (Figure 1). Whether the latter interactions exist on *human* red cells is not yet proven. Recent studies have also shown, that

the Lutheran glycoproteins (α5-laminin receptor) bind directly to α-spectrin (8) and that this interaction regulates the Lu-adhesive function. It has been also shown that GLUT-1, the major glucose transporteur of human erythrocytes (absent from murine red cells), binds to the protein 4.1-based complex ("junctional complex") through interaction with dematin (protein 4.9) and adducin (9).
Glycosyl-phosphatidyl-inositol (GPI) linked proteins, floating off the lipid bilayer, are linked to structures made of short and complex glycans, phosphoethanolamine, and a phosphatidylinositol residue (PI) plugged in the outer monolayer. Some polypeptides carry blood group antigens such as Cromer (or CD55), Cartwright (or acetylcholinesterase), JMH (or CD108), and Dombrock. These antigens are collectively absent from the red cells in patients suffering from paroxysmal nocturnal haemoglobinuria (PNH), an acquired disorder characterised by chronic intravascular haemolysis, variable cytopenia and an increased risk of thrombosis (10). PNH is caused by clonal somatic mutations, within haematopoietic stem cells, in an X-linked gene called PIG-A (11). This enzyme catalyses the first step of synthesis of the glycan moiety by transferring N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to phosphatidyl-inositol (PI) to form GlcNAc-PI, which will be further processed by sequential addition of monosaccharides and phosphoethanolamine. This leads to the complete GPI anchor to which the protein is eventually transferred.
A critical point is that genes encoding red cell membrane proteins are usually also expressed in a wide array of other cell types. They often appear as isoforms resulting from alternative splicing (spliceoforms), alternative initiation of transcription or translation. Other isoforms stem from post-translational modifications. This may be glycosylation, which involves most proteins emerging at the outer surface of the membrane, and fatty-acylation which concerns proteins looking inwards and having only one transmembrane domain (stomatin), or none at all. In the latter case, the protein remains tangentially associated with the lipid bilayer (protein 4.2, p55). Many proteins of wide interest were first discovered in the red cell.
A dramatic example is erythrocyte aquaporin, the firstborn of a universal water channel family (http://nobelprize.org/chemistry/laureates/2003/agre-lecture.pdf). Conversely, some proteins were first identified in non-erythroid tissues and subsequently found in the red cell membrane. An example is flotillin-2, which was initially identified as a cell surface antigen (ESA) in the epiderm.
Another critical point, yet to be elucidated, is that the expression of many red cell membrane genetic conditions are restricted to these cells, even in the case of serious mutations in the homozygous state. This may be explained by functional redundancy, that is, co-expression of proteins with similar function.

3. General and specific features of genetic disorders of the red cell membrane

Nearly all genetic conditions of the red cell membrane are characterised by a more or less compensated increase in red cell breakdown, though there is a wide spectrum of severity. The clinical features are those of haemolytic anaemia (pallor, icterus, anaemia, splenomegaly, hyperbilirubinaemia, hypohaptoglobinaemia) and its complications (gallstones, iron overload). The salient haematological features are the red cell indices, including the percentage of hyperdense cells, the cell shape alterations visible on smears, and the deformability and/or hydration of the erythrocyte (osmotic gradient ektacytometry is the most straightforward way to assess the latter parameters). SDS-PAGE of membrane proteins is also a key method for diagnosis. The temperature dependence of the monovalent cation leak, a specialised test, is an important step toward the characterisation of stomatocytoses.

Some conditions are distributed worldwide, whereas others are more or less clearly restricted to specific ethnic groups. In the first case, there are a wide variety of mutations, each being virtually confined to one family or to a group of unrelated families. In the second case, one finds some recurrent mutations, hinting at the possibility that a selective advantage played in favor of the heterozygotes over the ages.

Treatment is mainly symptomatic: transfusions, splenectomy, and/or the use of erythropoietin. One of the potential complications of splenectomy in these conditions is the possible generation of a hypercoagulable state. Indeed, splenectomy is strongly contra-indicated in the stomatocytoses because it inevitably leads to thrombo-embolic events, as will be discussed below (12).

4. Genetic disorders affecting the mechanical properties of the red cell

The main genetic disorders affecting the mechanical properties of the red cell are hereditary spherocytosis (HS) and hereditary elliptocytosis (HE) (13, 14).

4.1 Hereditary spherocytosis

Spherocytes, the defining feature of hereditary spherocytosis, are mechanically fragile because of the reduction in the normal "excess" of membrane surface area (140 μm^2) with respect to volume (85 μm^3). Whether the diminished surface occurs during erythropoiesis (suggested by the fact that reticulocytes are smaller than normal) or after the red cell is launched into the circulation by way of microvesicle loss, or both, is uncertain. Osmotic gradient ektacytometry shows that spherocytes are osmotically fragile and dehydrated, in keeping with the increased percentage of hyperdense cells. Parvovirus infections produce a dramatic, yet reversible, drop of the red cell count, haemolysis enhancing the effect of the acute erythroid aplasia

while this lasts. In infants, the “sluggish” phase of erythropoiesis induces a temporary aggravation of HS, during which erythropoietin treatment may be considered (rather than transfusion).
The mutated genes that cause HS are shown in Table 3. Generally speaking, the mutations lead to reduction in, or the absence of one protein. They have, or approach the status of a null mutation, that is a mutation leading to failure of production of the protein. Whether absent or low protein levels result from reduced synthesis, from impaired addressing (transfer to its physiological destination) or, ultimately, from a lack of binding to its partner protein(s), is generally unknown unless one finds a peremptory mutation (i.e. a mutation that does not allow any protein to be produced, such as a splice site mutation or premature stop codon). HS thus stems from a deficient covering of the inner surface of the lipid bilayer by the skeleton, more specifically by spectrin, whether the primarily missing protein is a chain of spectrin itself, or a protein contributing to tether spectrin to linker proteins (protein 4.1 however seems not to be involved).

Table 3: **Genes whose mutations are responsible for hereditary spherocytosis, hereditary elliptocytosis and Southeast Asian ovalocytosis, and some genetic disorders affecting the leak of monovalent cations**

GENES	INHERITANCE PATTERN
Hereditary spherocytosis	
ANK1	Dominant[a]
SLC4A1	Dominant
SPTB	Dominant[a]
SPTA1	Recessive
EPB42	Recessive
Hereditary elliptocytosis	
SPTA1	Dominant, with modulation[b]
SPTB	Dominant
EPB41	Dominant
Southeast Asian ovalocytosis	
SLC4A1[c]	Dominant
Cryohydrocytosis	
SLC4A1	Dominant
Overhydrated hereditary stomatocytosis	
RhAG	Dominant

For each condition, the genes are tentatively presented according to decreasing mutation frequencies.
***a** De novo mutation are rather common. **b** Through allele α^{LELY}. **c** 27 nucleotide deletion resulting in the absence amino acids 400 to 408 at the junction of the cytoplasmic and membrane domains of band 3.*

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The decrease in band 3 may also cause a variety of distal renal tubular acidosis (DRTA) because the SLC4A1 gene is also expressed, though as a distinct isoform (alternative initiation of transcription), in the basolateral membrane of the distal tubules α-intercalated cells. In the heterozygous state, SLC4A1 mutations usually produce either HS or DRTA (both dominantly inherited) rather than both together, but understanding why one or other is produced is not as obvious as it seems.

Severe cases of HS stem from homozygosity or compound heterozygosity. The primary absence of either α- or β-spectrin chain, or ankyrin have never been observed in humans, nor have they been obtained in the mouse by targeted disruption of the corresponding genes. These deficiencies must therefore be lethal. Missing protein 4.2 generates a moderately severe form of HS in humans and in mice. (i) The absence of band 3 (or its existence as traces) due to mutations affecting the two SLC4A1 genes, or (ii) a marked reduction in spectrin (-40%), stemming from mutations in both SPTA1 genes, lead to a similarly severe clinical picture with transfusion-dependence, especially during the 'sluggish' phase of erythropoiesis in infancy. Early partial splenectomy must be performed, the splenic stump being removed some years later. Mouse Slc4a1 gene targeted disruption produces a phenotype in keeping with the phenotype caused by missing band 3 in man (see below). The wan/wan mouse is a severely anaemic mouse (C3H/heJ strain) devoid of band 3, due to a spontaneous mutation in the Slc4a1 gene (15). The phenotype is even more severe than that of targeted band-3 null mouse strains. When the wan gene was transferred to mice with different genetic backgrounds through appropriate crosses, mutation wan itself, in the homozygous state, showed a decreased severity. This led to the suspicion of a quantitative trait locus (QTL) in the original strain. This QTL mapped to the region of the Spnb1 gene, encoding spectrin β-chain. It is currently assumed that some yet to be identified abnormality of the Spnb1 gene accounts for a further disruption of the membrane skeleton and the aggravated phenotype.

An interesting case concerns a polymorphism of the SPTA1 gene (ca. 5% of alleles among Caucasians), referred to as allele αLEPRA (16). The wild type allele of the SPTA1 gene produces over a fivefold excess of α-chains so that allele αLEPRA (triggering a very subtle splicing abnormality) is symptomless in the heterozygous state. Should it lie in *trans* of a null allele of the SPTA1 gene, the output of spectrin would then drop below a critical threshold and result in very fragile spherocytes and severe HS.

Systematic searching for HS-causing mutations is no longer being performed. The involved genes are too large. Besides, the identification of novel mutations has limited bearing on the treatment, is only exceptionally requested for genetic counselling

and seldom brings now any dramatic new insights into gene regulation or protein function. One must then decide carefully in which cases to engage in a costly mutation search. For example, the HS mutation nearest to the 3'-end on the SPTA1 gene was recently elucidated in severely anaemic siblings carrying allele αLEPRA as the other SPTA1 allele (17).

4.2 Hereditary elliptocytosis and poikilocytosis

HE is the paradigm of a genetic condition affecting the mechanical behaviour of the red cells. The proteins involved and their genes are summarised in Table 3. Unlike HS mutations, which tend to be quantitative (weak or null) mutations, most HE mutations are qualitative. Of course, a protein, basically protein 4.1, may be missing in HE due to a null mutation. This will result in an aggravated form of elliptocytosis known as poikilocytosis in the homozygous state (and not severe HS). Many qualitative mutations affecting protein 4.1 (10 kDa domain), as well as the N-terminus end of spectrin α-chain or the C-terminus end of spectrin β-chain, have revealed a great deal about the binding of spectrin β chain to protein 4.1 and the spectrin self-association process, respectively.

Like allele αLEPRA in HS, allele αLELY is the SPTA1 gene weak allele in HE. However, it is not as weak as allele αLEPRA (output reduced by -50%). It is much more frequent (20-30% of the SPTA1 alleles), affecting all the ethnic groups investigated worldwide with a comparable incidence. Although it also acts through a subtle splicing abnormality (18), leading to an impaired nucleation of spectrin αβ dimers (at a site opposite the self-association site), its mechanism of action is very different to that of allele αLEPRA. It aggravates elliptocytosis, often producing poikilocytosis, when it lies in trans of a STPA1 allele carrying a mutation in its 5' region, corresponding to the α-chain self-association site. αLELY provides a classical example of genetic modulation. There is no known low expression allele modulating the expression of SPTB gene HE mutations.

4.3 Southeast Asian ovalocytosis

This symptomless condition in the heterozygous state stems from a unique mutation (Table 3) and is widespread in Southeast Asia, providing an advantage with respect to malaria. In all likelihood, the homozygous state is lethal.

5. Genetic disorders affecting the leak of monovalent cations

These disorders, dominated by stomatocytoses (19), are usually dominantly transmitted. De novo mutations are common in overhydrated hereditary stomatocytosis.

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5.1 Familial pseudohyperkalemia (FP)

We will begin with a peculiar, asymptomatic trait: FP, first recognised by Stewart et al. (20). It consists of a massive outward leak of K^+, not in the bloodstream itself, but in drawn blood allowed to stand for a few hours at room temperature. This feature is more and more unlikely to be detected nowadays because serum tests are carried out without delay in separation of serum. Nevertheless, it appears that FP must be extremely rare.

In a large Scottish family, FP was found to map to 16q23-q24 (21). Another case of FP, detected in a large Flemish family, mapped to 2q35-36 (22). It is odd that such a rare condition as FP maps to more than one locus. It might be that a heterodimeric transporter is involved.

Another interesting point is that pseudohyperkalemia may be part of a pleiotropic syndrome as will be discussed in the following section (5.2) about DHS, a condition showing genetic heterogeneity with one locus known at 16q23-q24 precisely. The idea thus arises that FP is a borderline manifestation of DHS. In addition, patients with either “FP Chiswick” or “FP Falkirk” showed macrocytosis, a hint that FP may be indeed a borderline haematological disorder (23).

5.2 Dehydrated hereditary stomatocytosis (DHS)

DHS is a fully-fledged haemolytic anaemia, though it can be mild or even nearly symptomless. It shows a macrocytosis and a high reticulocyte count, attesting to a hyperregenerative bone marrow. Haemolysis is usually well compensated. Stomatocytes are often ill-formed and rarely numerous. A typical ektacytometric curve confirms the diagnosis. The leak-temperature curves are of various shapes, with the “shallow slope” shape being most common. Although tranfusions are unnecessary, DHS is a heavily iron loading disorder – the main point of follow-up and treatment. We recently observed a case of haemosiderosis that was only found after 10 years to be related an extremely mild form of DHS (unpublished data).

DHS is thus prone to be ill-diagnosed, being commonly mistaken for HS. Its incidence is not negligible, being 20 times lower than that of HS. It is essential, though, that DHS be distinguished from HS. Splenectomy is strictly contra-indicated in DHS, for it causes thrombo-embolic accidents (12), some of which are severe or extremely severe (24). The mechanism of the hypercoagulable state is unknown.

DHS is part of a recently individualised pleiotropic syndrome combining DHS itself, pseudohyperkalemia (as has already been pointed out), and perinatal oedema (25). Perinatal oedema may be severe (26) or remain subclinical (27). Ascites (which is chylous in some instances) is the central feature of the fluid effusions, but fluid may accumulate in other tissues in severe cases. Strikingly enough, effusions dry out spontaneously within weeks or a few months following birth never to reappear

– in stark contrast to life-long hyperhaemolysis. So far, there have been no cases of isolated foetal oedema reported, in which foetal oedema on its own would appear as the only manifestation of the pleiotropic syndrome. This issue remains open, however. Incidentally, a case of DHS has been reported in association with neonatal hepatitis (28), but it is not sure how hepatitis is related to oedema.
The mutated gene in some DHS families maps to 16q23-q24 (29), as in one type of FP. It is not known whether the responsible gene in other DHS kindreds would map to chromosome 2, as is the case in FP. In other words, it remains unknown whether DHS and FP, as facets of the pleiotropic syndrome, split into paired subgroups, one of them mapping to chromosome 16, the other to chromosome 2.

5.3 Hereditary cryohydrocytosis with normal stomatin

Hereditary cryohydrocytosis (CHC) is a variety of stomatocytosis whose salient feature is the dramatic resumption of the leak, upon in vitro tests, when the temperature draws close to 0°C. CHC was first described by Miller et al. (30). It later gained its present name based on the study of four families from the United Kingdom (31, 32). Anaemia was well compensated. The increase in the reticulocyte count was moderate to high. The temperature dependence of the leak showed a minimum at about 20°C and increased again at lower temperatures: values at 0°C were higher than at 37°C. CHC has been associated with mutations in the *SLC4A1* gene (33), a somehow paradoxical situation since the encoded protein, band 3, is an anion exchanger.

5.4 Overhydrated hereditary stomatocytosis (OHS)

This is an exceptional variety of stomatocytosis, commonly arising from de novo mutations. Hyperhaemolysis is pronounced and anaemia may be marked. Stomatocytes are often numerous and well-formed. The ektacytometer curve is highly typical. Here again, there is a high risk of iron overload and splenectomy is absolutely contra-indicated. A salient biochemical feature is the near disappearance of protein 7.2b, or stomatin. Nevertheless, no mutations have been found in the corresponding gene, the EPB72 gene. Stomatin interacts with the major glucose transporteur, GLUT-1, in the red cell membrane. Notably though, this interaction results in the switching of D-glucose to L-dehydroascorbic acid as a main transported substance in man, who is unable to synthesise vitamin C. In OHSt, missing stomatin tends to partly revert the preferred transported substrate to D-glucose (34). The gene whose mutations cause OHSt is RHAG (35). It encodes the Rh associated glycoprotein, a member of the ammonium transporter family (See below). In 6 out of 7 cases, the mutation was Phe65Ser, and in one case, Ile61Arg. All mutations lie in the second transmembrane domain.

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5.5 Cryohydrocytosis with reduced stomatin

This is a subset of OHS and there are only two (unrelated) cases described (36). The temperature dependence of the leak showed a minimum and then increased back again, the leak becoming "torrential" near 0°C. The interesting thing is that neurological manifestations were present in these cases: mental retardation, seizures and, in addition, cataract. Such observations should be substantiated by additional cases, however the possibility exists of a new haemato-neurological syndrome. This assumption was strengthened by the fact that no mutations were found in the *RHAG* gene (35).

6. Association of stomatocytosis and macrothrombocytopenia

This syndrome associating stomatocytosis and macrothrombocytopenia was first identified by Ducrou and Kimber (37) in Australian residents of Mediterranean origin, and repeatedly found thereafter. It is not categorised in the disorders affecting the leak monovalent cation since there is no such leak. In addition, out of keeping with this group, the inheritance pattern is recessive.

This syndrome was recently related to phytosterolaemia, a condition in which aborption of sterols is unselective, allowing the intake of normally rejected sterols such as phytosterols (38). Phytosterolaemia stems from mutations in the ABCG5 and ABCG8 genes (39), adjacent genes at 2p21 encoding sterolin-1 and sterolin-2, respectively. In 5 families with the stomatocytosis/macrothrombocytopenia syndrome, mutations were found in the ABCG5 or the ABCG8 genes (40). How the haematological symptoms result from hypersterolaemia has yet to be explained.

7. Genetic disorders affecting blood group components

The molecular basis of the 29 currently known blood group systems has been determined through extensive biochemical and molecular genetic investigations which have revealed that blood group gene products encode a wide structural and functional diversity of molecules (41-43). Only some representative groups are listed in Table 1. Many blood group antigens are present in non-erythroid tissues (a typical example is the carbohydrate-specified antigen ABO, which is predominantly expressed on endothelial cells and epithelial cells along the gastro-intestinal tract). Currently, only a few molecules such as glycophorins A and B (carriers of MN and Ss antigens, respectively), Rh, LW/ICAM-4 and ERMAP (carriers of Sc and Rd antigens) appear erythroid-specific. Other antigens have a restricted tissue distribution; for instance, a band 3 isoform (see above) and the Duffy protein are expressed respectively in the distal tubules of the kidney and on endothelial cells of post-capillary venules in many organs. Still other protein

antigens, such as AQP1 (water channel, carrier of CO antigens) or Lutheran proteins, have a much broader tissue distribution.
Insights into the structure and function of blood group antigens have been gained from investigations performed with rare "null phenotype" variants that are defective for blood group antigens. Several of these phenotypes are associated with mild-to-moderate haemolysis, indicating a role for blood group molecules in red cell membrane integrity or function. Additionally, since some blood groups exhibit a broad tissue distribution, clinical or subclinical manifestations may extend to non-erythroid tissues. The management of null phenotypes is crucial since in all instances they confer a high risk of immunisation by transfusion or pregnancy. With the exception of band 3 defect described in the previous section, three phenotypes associated with the defect of Rh-RhAG complex, Kx-Kell complex and glycophorins C and D (carriers of Gerbich antigens) have been extensively investigated.

7.1 Rh-deficiency syndrome

Rh blood group antigens are defined by a complex association of membrane polypeptides that includes the non-glycosylated Rh proteins (carriers of RhD and RhCcEe blood group antigens), and RhAG (Rh-Associated Glycoprotein), a glycoprotein strictly required for cell surface expression of Rh antigens (44, 45) which carries the blood group antigens "Ol^{a}" and "Duclos" (46). In red cells, the core of the Rh complex is presumably a trimer composed of Rh and RhAG subunits, to which accessory chains (CD47, LW/ICAM-4, GPB) are associated by non covalent bonds (Figure 1). The Rh complex represents a major site of interaction between the membrane lipid bilayer and the spectrin-based skeleton and may thus be involved in the regulation of the shape, deformability and mechanical properties of red cells. Indeed, recent studies of the erythroid ankyrin deficient normoblastosis (Ank1nb) mice (by analysis using the yeast two-hybrid system) have shown that ankyrin may interact directly with the C-terminal cytoplasmic domains of Rh and RhAG (47). In addition, primary deficiencies in protein 4.2 (48, 49) or band 3 (50) (particularly band 3) were found to be associated with a decrease in CD47 and various members of the Rh complex. Homologues of the RhAG glycoprotein, RhBG and RhCG, have been recently identified in non-erythroid tissues, thus defining a new Rh superfamily (51). Functional studies have also shown that RhAG in red cells, and the kidney RhBG and RhCG in heterologous expression systems, facilitate NH_3 transport across cell membranes (52, 53). This is fully consistent with features deduced from the crystallographic structure (with or without ammonia) of the bacterial ammonia transporter AmtB of *E. coli*, a member of the Rh protein superfamily (54, 55), notably with the presence of conserved histidine residues in the hydrophobic channel in Rh glycoproteins (RhAG, RhBG and RhCG), but not in Rh proteins (RhD or RhCE). More

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recently, the crystal structure of the Rh protein homolog of *Nitrosomonas europaea* (called NeRh50) has been resolved (56, 57) which revealed structural similarities and differences potentially useful to clarify the mechanisms of substrate permeation. One characteristic feature was the lack of the NH_4^+ binding site present in AmtB proteins which should favor the conduction of NH_3 by a pH-dependent mechanism. *In silico* homology models of Rh glycoproteins with using NeRh50 as template (43; and I. Callebault, personnal communication) indicate similarities of the pore structure, suggesting that NeRh50 may be a better model to study structure/function relationship of Rh proteins, until the human Rh glycoproteins themselves are purified and crystallised.

Other studies, however, suggest that the Rh glycoproteins, perhaps as a band 3-based macrocomplex (50), might rather function as CO_2 gas channels (58), which recently received experimental support by showing that Rh_{null} (see below) and $AQP1_{null}$ red cells each exhibit a 50% decrease of membrane permeability to CO_2, as measured by the exchange of ^{18}O between CO_2, HCO_3^- and water in a red cell suspension, using a mass spectrometry technique (59). These proposed functions are not necessarily mutually exclusive.

Rh-deficiency is a rare autosomal recessive condition, which results from the lack (Rh_{null}) or severe reduction (Rh_{mod}) of the red cell Rh and LW antigens, and, to a variable extent, of the other accessory chains (CD47, GPB) of the Rh complex. Rh-deficient phenotypes are caused by several different mutations that occur in either the *RHAG* or *RH* loci on chromosome 6p12-p21 and 1p34-p36, respectively (60, 61). Interestingly, among the RHAG mutations, one mutation is able to abolish the interaction between the Rh complex and ankyrin (47). The Rh complex is missing or severely reduced in Rh-deficient red cells, but there is no alteration of the genes encoding the accessory chains. Because of a variable expressivity, some mutations of the *RHAG* gene result in the total lack of RhAG (and Rh) protein, defining the Rhnull of the "regulator type", but others result in weak RhAG (and Rh) protein levels, defining the Rhmod phenotype. Mutations of the *RH* gene resulting in the total lack of Rh and only a reduced expression of RhAG (20% of normal) define Rhnull of the "amorph type". Accordingly, primary defects of either RhAG or Rh result in defective cell surface expression and/or transport of the whole Rh complex.

All Rh-deficient phenotypes result in the same clinical syndrome, which is characterised by a chronic haemolytic anaemia of varying severity and a persistent moderate reticulocytosis (3 to 20%) (60, 62-64). Only few patients have been examined in detail. The blood smear typically shows stomatocytosis and spherocytosis. The red cell osmotic fragility without incubation is often slightly abnormal but becomes markedly increased after 24 hours incubation at 37°C. In the few cases investigated, red cells have an abnormal deformability profile in the ektacytometer

indicating an increased osmotic fragility, a reduced surface area and dehydration, suggesting membrane instability *in vivo* (65). Some patients may present signs of accelerated red cell destruction *in vivo* and the half-life of autotransfused cells was found to be between 7 to 17 days (normal 24-28 days by the ^{51}Cr method). In severe cases, clinical improvement has been observed after splenectomy with a normalisation of autologous life-span. Rh-deficient erythrocytes also have increased rates of passive and active cation transport (K^+, Na^+) and increased Na^+-K^+ ATPase activity, which results in cell dehydration (66). These cells also exhibit an abnormal membrane organisation of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [increased exchangeability of PC, increased access of PE to phospholipases], indicating a change in the phospholipid asymmetry of the bilayer and an enhanced passive transmembrane flip-flop (67).
How the lack or severe reduction of the Rh complex may cause pleiotropic or specific effects on membrane ion transports and lipid organisation is still not resolved. Similarly, the physiological consequence of reduction in NH_3 transport in Rh-deficient erythrocytes remains mysterious. Of note, it has not been possible to evaluate the role of erythroid CD47 in the Rh complex in man because of the lack of variants targeting the CD47 locus. However, CD47-deficient mice express normal levels of red cell Rh and Rhag, do not become anaemic and do not exhibit red cell abnormalities. CD47 might function as a marker of self on red blood cells (68) , at least in mice, and it has been suggested that the severe decrease in CD47 (80-90%) in Rh-deficient red cells may represent a pathway for the control of haemolytic anaemia. However, a similar decrease of CD47 expression in human variants with a rearranged RHCE gene (D- -, D.., R^N phenotypes) is not associated with a haemolytic syndrome (49). Finally, the haematological, biochemical and biological features of Rh-deficiency reinforced pioneer observations pointing to the similarity between the Rh-deficient phenotypes and hereditary spherocytosis. We currently know that the latter disease is notoriously heterogeneous at the genetic, clinical and molecular levels, and Rh-deficiency could now be considered as a further subgroup of this red cell disorder.

7.2 McLeod syndrome

McLeod is a rare phenotype initially described as an X-linked trait in which red cells lack the common Kx antigen and have a marked decrease in all blood group Kell antigens. In the red cell membrane, the Kx and Kell proteins (carriers of Kx and Kell antigens, respectively, Table 1) are covalently linked by a single disulphide bond presumably forming a functional complex (69). The Kx protein has a membrane topology typical of a transporter, but its function is still unknown (70). The Kell protein is a member of the neprilysin (M13) family of zinc-metalloproteases (71)

that cleaves big endothelins (preventially ET-3) into biologically active peptides. The KX and KEL transcripts are present in erythroid as well as non-erythroid tissues, notably brain, skeletal muscle and heart. The Kell protein has been detected in skeletal muscle, testis (Sertoli cells) and lymphoid organs (follicular dendritic cells), but the presence of the Kx protein is difficult to establish (due to lack of potent and specific antibody) (72, 73). Recent immunohistochemistry studies in skeletal muscle, however, indicate that Kx protein is restricted to sarcoplasmic reticulum and Kell protein to sarcoplasmic membranes, suggesting that the transport of these proteins is differently regulated in red cells and muscles.

Long after the McLeod phenotype was described, it was realised that McLeod individuals suffer from a chronic compensated haemolytic anaemia with modest reticulocytosis and that their red cells have an acanthocytic morphology (about 25-30% of the cells) and reduced in vivo survival (reviewed in 74 and 75). The ATP content of red cells was normal. The deformability profile in the ektacytometer indicated a reduced surface area and dehydration but a normal osmotic fragility. A decrease in water permeability of about 30% has been reported. There is a normal phospholipid composition and distribution, but an enhanced transbilayer mobility of phosphatidylcholine. Acanthocytosis occurs with a normal serum lipoprotein level, and can be corrected in vitro by substances (chlorpromazine, phosphatidylserine) accumulating in the inner bilayer leaflet of the membrane, suggestive of some lipid bilayer imbalance, but how this is directly or indirectly related to the Kx protein defect is unknown.

Early observations indicated that McLeod patients also suffer from a slowly progressive disease with a late-onset of neurological and muscular anomalies, and so the serological, haematological and neuromuscular defects were collectively called “McLeod syndrome” (74). Of note, Kellnull (K0) individuals who lack Kell but express Kx on their red cells are apparently healthy and do not suffer from the clinical manifestations associated with the McLeod syndrome. The McLeod syndrome sometimes accompanies large deletions of the X chromosome, including XK and neighbouring genes such as CGD (chronic granulomatous disease), DMD (Duchenne muscular dystrophy) and RP (retinitis pigmentosa). However, “pure” XK gene defects affecting either splicing of the transcripts or creating a frameshift that results in premature termination of translation have been described and were instrumental to unambiguously demonstrate the critical role of the XK gene in the McLeod phenotype and associated syndrome (76). Interestingly, one missense mutation (E327K) causing the serological McLeod phenotype (no Kx and Kell antigens reduced on red cells) was not associated with systemic manifestations; there were no haematologic abnormalities and no neuromuscular or cerebral involvement (no acanthocytes, no compensated anaemia, normal serum creatine kinase). An impaired

complex formation or cell surface transport of Kx was suspected (as observed in a R222G mutant, which unfortunately was not examined for haematological and clinical manifestations). However, another McLeod phenotype caused by a different missense mutation (C249R) was associated with neurological signs.

All patients with the McLeod syndrome are males and exhibit elevated serum creatine kinase suggestive of muscular damage. Females carriers are symptomless but show Kell blood group mosaicism and erythrocyte morphological mosaicism with a dual population of acanthocytes (varying from 2 to 95%) suggesting X chromosomal inactivation by the Lyon effect (74). A single case of McLeod phenotype in a female with severe clinical manifestations has been reported. The propositus was heterozygous for a frameshift mutation in XK (caused by a single nt deletion - del90), and the probable explanation was inactivation of the X chromosome carrying the normal XK locus.

The late-onset neurological and muscular anomalies (4th-5th decades) in McLeod patients are variable and include central nervous involvement (dystonic or choreiform movements, seizures, neuropsychiatric manifestations such as depression, anxiety and cognitive impairment) and neuromuscular manifestations (areflexia, generally mild myopathy sometimes with cardiomyopathy, but with normal dystrophin) (74). Cerebral imaging by positron emission tomography and magnetic resonance volumetry revealed atrophy of the basal ganglia (neural loss and astrocytic gliosis), notably of the caudate nucleus and putamen. Altogether, these findings suggest that the McLeod syndrome could be a variant form of neuroacanthocytosis, a group of neurological diseases associated with chorea and acanthocytosis (76, 77). Cerebral involvement in these diseases shows a strong resemblance to the striatal dysfunction and caudate atrophy found in Huntington's disease.

Although these findings implicate the XK locus in a multi-system disorder involving red cell, muscle and neurological abnormalities, the role of the Kell (or a Kell-like) protein and the pathophysiological relationship with the clinical manifestations of the McLeod syndrome remain unknown. However, it is intriguing that Kell, an endothelin-converting enzyme (see above) and endothelin-3 (ET-3) are present in brain, as endothelins might serve as basal ganglia neurotransmitters (78).

7.3 Leach phenotype

The Leach phenotype is characterised by the lack of GPC and GPD (carriers of Gerbich antigens) on red cells, a mild form of elliptocytosis (79) and an increased osmotic fragility. Leach erythrocytes are also mechanically unstable (80). GPC and GPD are two single pass transmembrane proteins produced from a single gene (GYPC) by translation of the same mRNA at two in-phase AUGs by a leaky translation mechanism. The loss of GPC and GPD in Leach individuals results from a partial gene

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deletion (exons 3 and 4) or a frameshift mutation in GYPC (81). Leach erythrocytes also lack the p55 protein, and protein 4.1 is reduced by 20-25%, which presumably accounts for the variable elliptocytosis of these cells (6). Of note, p55 is absent and GPC/D is reduced by about 70% in homozygous 4.1 deficiency, causing hereditary elliptocytosis (see above). Although the GPC-protein 4.1-spectrin/actin linkage occurs physiologically, it was shown recently that experimental conditions that disrupt the GPC-protein 4.1 bridge apparently have no impact on the mechanical properties of red cells (5). Although a complete dissociation of the GPC-protein 4.1 bridge has not been observed, these studies raise some doubts about the contribution of this link to membrane stability. However, they remain consistent with protein 4.1 reduction as a critical factor of membrane instability in Leach erythrocytes. The ternary complex between GPC, protein 4.1 and p55, however, may play an important role during invasion and growth of *P. falciparum* parasites. Both 4.1-deficient and Leach erythrocytes resist invasion by malaria while intracellular parasite developement is severely reduced in 4.1-deficient but not Leach red cells (82). There is also some serological evidence that the Kell protein and GPC/D interact with each other, because there is a weak expression of Kell antigens on Leach erythrocytes which lack GPC/D and on other Gerbich-negative red cells which carry altered GPC/D proteins (no intact GPC/D proteins present).

7.4 Miscellaneous disorders associated with blood group defects

Blood group abnormalities have been described in dyserythropoietic anaemia. In CDA type II (formerly called "HEMPAS" for Hereditary Erythroblastic Multinuclearity with a Positive Acidified Serum test), the red cells can be lysed (in acidic conditions) by a naturally occurring, complement binding IgM antibody present in some sera. In these cells, there is an incomplete synthesis of complex N-glycans on band 3 (and GLUT1-glucose transporter) associated with the accumulation of polylactosaminoglycans on membrane lipids, but the biochemical nature of the HEMPAS antigen detected by the IgM antibody is unknown. A new form of dyserythropoietic anaemia has also been described in a patient presenting a persistence of embryonic and foetal haemoglobins. The patient has an erythroid-restricted defect characterised by the lack of CD44 (carrier of Indian antigens), and of the water channel AQP-1 (carrier of Colton antigens). This patient, therefore, has the extremely rare phenotype In(a-b-), Co(a-b-), but its molecular basis is unknown (83).

Recently, CD151 (carrier of MER2 antigens; *RAPH* blood group system) has been identified on human red cells. CD151 is a member of the tetraspanin superfamily of proteins which facilitates the interaction of membrane and intercellular signalling molecules by formation of specific microdomains (84). Three patients of Indian Jewish origin with end-stage kidney disease were found to be homozygous for a single-

nucleotide insertion (G383) in exon 5 of the *CD151* gene, causing a frameshift and premature stop signal in codon 140 (85). The CD151 defect nephropathy is associated with pretibial epidermolysis bullosa and deafness, suggesting that CD151 may be essential for the correct assembly of basement membranes in the human kidney and may have functional significance in the skin and the inner ear. The patients have severe anaemia attributable, at least in part, to the co-existence of β-thalassaemia minor, but there is an impaired marrow response to erythropoietin. CD151 null mice generated on a C57/Bl6 background are grossly normal and healthy (86), but *CD151* gene inactivation on a mixed FVB/N x129 background results in a severe glomerular disease (87). Most interestingly, however, CD151 null mice on C57/Bl6 background develop a severe glomerular disease associated with proteinuria after backcross with FVB/N background (88) and further studies suggested that CD151 could be involved in assembly and maturation of the glomerular basement membrane in collaboration with integrins α3β1 (88).
Yet another example of rare mild red cell disorders is the severe lack of red cell antigen Lutheran (Lu). The defect occurs with a red cell restricted suppression of antigens such as CD44, CD151, AnWj (erythroid *Haemophilus influenza* receptor) and the P1 glycolipid. It is caused by a gene "inhibitor of Lutheran" unlinked to the *LU* locus, called *In(Lu)* which is dominantly inherited. The molecular basis of the In(Lu) phenotype found in 21 of 24 individuals is related to various mutations, at the heterozygous state, in the promotor or coding sequence of the EKLF/KLF1 protein, a transcription factor involved in erythroid differentiation (89).
In some individuals, the In(Lu) phenotype is characterised by an abnormal red cell morphology (mild poikylocytosis, acanthocytosis), but there is no anaemia (90), thus indicating that a single functional *EKLF* gene is required for normal erythropoiesis. The osmotic fragility is normal, but during incubation (24h at 37°C) the cells lose K^+ and become osmotically resistant. Of note, Lu_{null} phenotypes caused either by homozygosity for a very rare recessive *lu* gene at the *LU* locus (chromomose 19), or by hemizygosity for an uncharacterised X-linked suppressor gene, exhibit a normal red cell morphology and no abnormal electrolyte transport. Various mutations (nonsens, deletion) of the *lu* gene causing the recessive type of Lu(a-b-) phenotype have been identified (91), but the molecular basis of the X-linked phenotype is still unknown.
A functional role of some blood groups may be revealed under pathological conditions. For instance, sickle red cell adhesion to vascular endothelium is increased via an erythroid pathway implicating the PKA-dependent phosphorylation of LU and ICAM-4 (LW) glycoproteins and binding to endothelial ligands (αVβ3 integrin for ICAM-4), which in turn may contribute to vaso-occlusive episodes in sickle cell disease

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(92) and such adhesive effects may be modulated upon treatment (93). Another example is the up-regulation of the Fy/DARC protein (a promiscuous receptor for CXC and CC chemokines, also known as DARC-Duffy/Antigen receptor for chemokines) on endothelial cells during renal inflammation (HIV nephropathy, haemolytic uremic syndrome, renal transplant rejection, crescentic glomerulonephritis), which suggests that DARC may facilitate chemokine migration across the endothelium and promote neutrophil transmigration (94, 95). Interestingly, overexpression of DARC may regulate growth and metastatic potential of tumours (prostate, breast, lung, melanoma) in murine models, by clearing angiogenic CXC chemokines (that carry an ELR motif) from the tumor and inhibiting neovascularisation. It was recently reported (96) that tumor cells disseminating from a primary tumor that expresses tetraspanin CD82 (synonym KAI1), a previously identified suppressor of metastasis, may interact with endothelial DARC and that this interaction ultimately leads to inhibition of cancer cell proliferation at distant sites and to the senescence of tumour cells. DARC is also known as a red cell receptor for *P. vivax* (97) and recent studies have shown that HIV-1 particles attached to red cells via DARC and might promote viral dissemination and transfer to target cells (98). Moreover, the previously described disease-accelarating effect of a CCL5 chemokine polymorphism (asociated with an increased expression of CCL5) is present in DARC-expressing individuals only, suggesting that DARC (which binds CCL5) may influence HIV/AIDS susceptibility.

7.5 Clinically silent phenotypes

It is surprising that the defect of some major membrane proteins such as GPA, which is as abundant as band 3 (10^6 copies/RBC), or GPB, either alone or together (as in the homozygous MkMk condition), is not associated with any membrane defect or reduced red cell survival. However, although the function of these proteins is obscure, their high sialic acid content contributes most of the net negative charge of the erythrocytes, a critical factor in minimising interaction with other cells in the circulation. Moreover, it has been reported that GPA may facilitate the transit of band 3 to the cell surface (99). GPA-deficient cells (En(a-), MkMk), but not GPB-deficient cells (S-s-U-), exhibit a 60% decrease of band 3-mediated sulfate and iodide transport, wich is presumably related to an increased flexibility of the membrane domain of band 3 in the absence of GPA. GPA may also contribute upon ligand binding to membrane rigidity by association of its C-terminal domain with the membrane skeleton (100).

It is much more surprising that the defect of membrane proteins with well defined functions such as AQP1, UT-B1 (Kidd protein, urea transporter), Lu, LW/ICAM4 or the Fy protein (Table 2) is clinically silent. Either the function of these proteins is not vital function or there may be functional redundancy. Alternatively, the

phenotype might only be revealed under certain stress or pathological conditions. For instance, under water deprivation, AQP-1 deficient individuals exhibit a defective urinary concentrating ability and a decreased pulmonary vascular permeability (101).

7.6 Targeted disruption of blood group related genes

Gene targeting in mice provides another approach for exploring the functions of blood group proteins in detail. Currently, a small number of genes encoding blood group related proteins have been disrupted by this technology. A severe and identical red cell phenotype was observed in mice targeted for erythroid band 3 (102) or for both the erythroid and kidney isoforms (103). A spontaneous mutation has also been reported in the mouse (wan/wan) that resulted in a band 3 null phenotype (see above). The animals were severely anaemic and there was a high mortality at birth and growth retardation. Accelerated erythropoiesis reflected by an increased reticulocytosis and a marked hepatomegaly/splenomegaly was noted. Red cells were strikingly spherocytic and ektacytometry revealed a pronounced loss of surface area, as seen in human hereditary spherocytosis (see above). Although the ankyrin level is reduced (50%) in band 3 null erythrocytes, the spectrin content is nearly normal and the cells assemble an architecturally nearly normal membrane skeleton. Interestingly, membrane protein analysis also revealed an absence of protein 4.2 and GPA in these cells, supporting the critical role of band 3 for protein 4.2 attachment and as a chaperone for the transport of GPA to the cell surface (99, 104). Despite a normal skeleton with nearly normal spectrin content, band 3 null erythrocytes lose a large amount of membrane by vesiculation, strongly suggesting that horizontal interactions between membrane lipids and integral membrane proteins are critical for membrane bilayer stabilisation ("lipid anchoring" hypothesis). However, bilayer stabilisation by vertical interactions between lipids and skeletal proteins ("skeleton anchoring" hypothesis) may also exist. Targeted disruption of the *GYPA* gene revealed that GPA null mice were not anaemic but that their red cells exhibited a slight increase in osmotic fragility after 24h at 37°C (105). Western blot analysis further confirmed the lack of GPA and the absence of TER antigen, a membrane component present in the erythroid lineage, identified by the monoclonal antibody TER-119 (106), but band 3 migrates normally and was expressed at a normal level.

In contrast to band 3, targeting of blood group related genes in other published examples (CD44, CD47, CD147, AQP1, AQP3, UT-B1 or Fy/DARC) did not compromise the red cell survival or function (but specific functions such as water or urea permeabilities for instance were severely reduced in some instances), although some dysfunctions in other tissues, sometimes severe, were occasionally observed (107). Obviously, "knockout" animals should prove to be useful to address a number of

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remaining questions regarding the functional significance of blood group structures and to correlate the biochemical findings with the pathogenesis of the disorders, particularly when biological and physiological studies cannot be easily performed in humans.

Mice with targeted disruption of the *Lu/BCAM* gene are viable, fertile and develop normally (108). Thus, *Lu/BCAM*-knockout did not reproduce the severe defects seen in mice lacking laminin α5 (which die during late embryogenesis). However, Lu/BCAM-*null* animals exhibited structural alterations of basement membranes expressing laminin-511/521 in kidney and intestine, two organs known to highly express Lu/BCAM. The loss of Lu/BCAM was associated with a thickened basement membrane perturbing the organisation of intestinal smooth muscle layers and of the glomerular basement membrane without any apparent functional defect in *basal* conditions (108). Of note, like knockout mice, rare individuals that lack all Lutheran blood group antigens (natural “Lu-null” of the recessive type, see above) exhibit no phenotype and no clinical syndrome under physiological conditions, but for obvious reasons, this could not be explored further.

Preliminary analysis of mice invalidated for the *Rh* and *Rhag* genes suggest that RBCs from *Rhag*$^{-/-}$ animals lack Rh, Rhag and ICAM-4 proteins and those from *Rh*$^{-/-}$ animals lack Rh and ICAM-4, but Rhag is only reduced by 30% (109). Both types of mice, however, have normal levels of CD47. These findings underline differences between the human and murine models of Rh deficiency (see above) and the structure of the Rh complex in the two species. Of note, mice deficient for the non-erythroid homologs Rhbg and Rhcg have been generated recently (110, 111). Both Rhbg and Rhcg proteins belong to the Amt/Mep family of ammonium transporters and are expressed in tissues involved in ammonium metabolism (kidney, liver, etc). Gene targeting of *Rhbg* in mice does not alter renal acid handling or hepatic ammonium metabolism, and therefore the biological function of this protein remains unclear (110). Mice lacking *Rhcg* have abnormal urinary acidification due to impaired ammonium excretion on acid loading, a feature of distal renal tubular acidosis (111). Moreover, the Rhcg protein is expressed in epididymal epithelial cells and is required for normal fertility. Other putative functions which might implicate these proteins as ammonium-sensors or in carcinogenesis (hepatocarcinomas) are currently explored.

Mice with targeted disruption of the *ICAM-4* gene are viable and fertile, but exhibited a defect in the formation of “erythroblastic islands” (bone marrow niche composed of a central macrophage surrounded by developing erythroblasts) both *in vivo* and in reconstitution assays *in vitro*, although haematocrit, haemoglobin and red cell indices were normal (112). Thus, in *basal* conditions, ICAM-*null* mice have

no anaemia or any red cell phenotype, but stress haematopoiesis was not investigated. In humans, individuals with the rare LW-*null* phenotype (ICAM-4 deficiency) are apparently healthy and their RBCs express the Rh proteins normally.

8. Future directions

The role of the lipid rafts and their involvement in disease will be addressed. The protein three-dimensional structure will be further characterised, helping to decipher the ever-increasing complexity of the membrane supramolecular organisation. Clarification of the functions of blood group antigens, in various tissues and over a wide range of species, will lead to better understanding of the physiological role of these molecules. Some important genes, in particular those whose mutations cause the hereditary stomatocytoses, remain to be identified. The regulation of gene expression and the diversification of their products represents an almost unlimited field of investigation. The red cell, owing to its relative simplicity, will continue to serve as a springboard to the understanding of more complex cells.

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Multiple Choice Questionnaire

To find the correct answer, go to http://www.esh.org/iron-handbook2009answers.htm

1. **Which one of the following statements concerning RhAG is true?**
   a) RhAG is membrane glycoprotein carrying Rh blood group antigens .... ☐

b) RhAG is a red cell specific glycoprotein involved in gas transport (NH_3, CO_2) ☐
c) RhAG is a glycoprotein of erythrocyte and kidney cells mediating ammonium transport ☐
d) Glycoproteins homologous to RhAG are not functional ☐

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2. Which one of the following statements concerning the McLeod syndrome is true?

a) The McLeod syndrome occurs when the Kell glycoprotein is absent on red cells ☐
b) The McLeod syndrome is an X-linked disorder and only males can be affected ☐
c) The McLeod syndrome is a disorder caused by a single gene with pleiotropic effects on red cells, muscle and neurological tissues ☐
d) The McLeod syndrome always occurs by deletions on the X and gene loci such as CGD (Chronic Granulomatous Disease) or DMD (Duchenne muscular dystrophy) may be affected ☐

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3. Hereditary elliptocytosis stems from mutations in which of the following genes?

a) The gene encoding stomatin ☐
b) The gene encoding 4.1R ☐
c) The gene encoding the anion exchanger-1 (band 3) ☐
d) The gene encoding ankyrin-1 ☐

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4. The anion exchanger-1 (band 3) interacts with which one of the following proteins:

a) Glycophorin C ☐
b) Protein 4.2 ☐
c) Actin ☐
d) Protein 4.9 ☐

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5. Which of the following statements concerning dehydrated hereditary stomatocytosis is true?

a) May be associated with a perinatal oedema ☐
b) Is associated with a microcytosis ☐
c) Stems from mutations in the SPTA1 gene ☐
d) Is associated with the absence of stomatin ☐

NOTES