A Combination of the Effects of Rare Genotypes at the XK and KEL Blood Group Loci Results in Absence of Kell System Antigens From the Red Blood Cells

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The 22 antigens of the Kell blood group system are located on a red blood cell (RBC) membrane glycoprotein that shows sequence homology with a family of metalloendoproteases. Expression of the Kell system antigens is partially governed by XK, an X-linked gene that encodes the Kx protein; absence of Kx results in reduced Kell antigen expression. Almost total absence of Kell antigens from the RBCs of a German man with no symptoms of neuroacanthocytosis could not be due to the Kell-null phenotype, Kx0, because his RBCs had very weak expression of Kx antigen and his three children were Kp(a+b+). Kell antigens were normal on the RBCs of his son but weak on those of his two daughters.

The surface of the human red blood cell (RBC) has been analyzed in tremendous detail by immunological means. In recent years, the unravelling of the molecular nature of many RBC surface antigens has provided valuable information on the variety of genetic mechanisms available for varying the structure of cell surface proteins and glycoproteins.1

Kell is a complex blood group system composed of 22 RBC antigens1 located on a glycoprotein of an apparent molecular weight of 93 kD, with a large highly folded C-terminal extracellular domain, a single membrane spanning domain, and a short N-terminal cytoplasmic domain.2,3 The function of the Kell glycoprotein is unknown, but its amino acid sequence shows homology with a family of metalloendopeptidases, in particular, the human common acute lymphoblastic leukemia antigen.3 Therefore, the Kell glycoprotein may serve to catalyze the activation or inactivation of biologically important peptides in the peripheral blood. Kell glycoprotein appears to be restricted to erythroid cells.4 Kell mRNA transcripts have been detected in human bone marrow and fetal liver but not in the brain, kidney, lung, or adult liver.5

The Kell gene spans 21.5 kb organized into 19 exons of coding sequence6 and is located on chromosome 7q33-q35.5,7,8 The molecular backgrounds of five antigenic polymorphisms (K and k; Kp, Kpb, and Kp); Js and Js; Ul; K1 and K17) have been determined; all result from single base changes.9,11

The rare Kell-null phenotype (Kx0), in which none of the Kell system antigens is present on the RBCs, arises from homozygosity for an inactive Kell gene.1 A number of phenotypes exists in which all high-frequency Kell system antigens, and K when encoded, are expressed only weakly.1 One of these, the McLeod phenotype, results either from a deletion of an X-linked gene (XX)1,2 or from a mutant allele at that locus,10 resulting in the absence of Kx, an RBC surface protein, the product of the common allele at the XK locus.10,11McLeod RBC phenotype is generally associated with acanthocytosis and a variety of muscular and neurological defects, collectively known as McLeod syndrome.1,2 The reasons for the variety of diverse characteristics associated with McLeod syndrome are unknown.

When the McLeod phenotype is caused by a deletion at the XK locus, it is also usually associated with X-linked chronic granulomatous disease (CGD), the result of the deletion also encompassing CYBB, the gene for a large subunit of cytochrome b555.12-15 Two unrelated men with McLeod syndrome, but with no CGD, had intact XK genes, but each contained a different single base mutation within the second intron, one within the 5′ donor splice consensus sequence and the other within the 3′ splice consensus sequence.16 Each mutation would be expected to disrupt RNA splicing, resulting in the production of little or no normal Kx protein.

Depression of Kell system antigens sometimes occurs in the rare Kp(a+b−) phenotype.1 The amino acid substitution within the Kell glycoprotein responsible for expression of the Kpα antigen appears to cause reduced expression of all other Kell system antigens present on that molecule. The reason for this is not clear, but it is possible that the Arg→Trp substitution responsible for the Kpα antigen causes conformational changes throughout the glycoprotein. The weakening of Kell system antigens because of a Kpα allele is not easily detected in Kpα/Kpβ homozygotes but is obvious in serological tests with RBCs of individuals heterozygous for Kpα and for a null (K0) allele (Kp/K0), as shown by family studies.16,19 The k antigen is often weak in K+k+ Kp(a+b+) individuals, in whom one allele at the KEL locus produces k and Kp, and the other produces K and Kpα.

We have identified an individual whose RBCs express...
almost no Kell system antigens, yet his three children had inherited a KP<sup>α</sup> allele from him. DNA analysis showed a splice consensus sequence mutation within intron 2 of XK, suggesting the McLeod phenotype, plus homozygosity for a KP<sup>α</sup> allele at the KEL locus. It appears that a combination of these two rare genotypes, both of which are known to cause weakening of Kell system antigens, has resulted in these antigens being virtually undetectable.

**MATERIALS AND METHODS**

Ascertainment and case history of the propositus. M.D., the propositus, is a 53-year-old German man who presented with a benign prostate tumor. Initial testing of his RBCs showed them to be O DCE/dce K–k–. Further typing showed a K<sub>α</sub>-like phenotype.

M.D. has no acanthocytosis and no signs of muscular or neurological defects. Raised serum creatinine kinase levels are a symptom of muscle damage and, consequently, usually a symptom of McLeod syndrome. The serum creatinine kinase level of M.D. of 21 U/L is within the normal range of 10 to 190 U/L.

**RBC serology.** Standard blood grouping techniques were used throughout. Indirect agglutination tests were performed with antihuman IgG (BPL, Elstree, UK) or antihuman IgG (Dako, Glostrup, Denmark), and were read after either centrifugation or in a capillary after 10, 20, and 30 minutes. Titrations were performed by testing RBC samples with series of doubling dilutions of serum or supernatant. Titration scores are the sum of scores for each dilution according to the following scoring system: 4+, 12; 3+, 10; 2+, 8; 1+, 5; 0, 3; weak, 2; negative, 0. Eluates were prepared by an acid-elution technique with a commercial kit (Eli-Kit; Gamma Biologicals, Houston, TX). The antibody described as anti-K<sub>x</sub> is serum from a McLeod phenotype boy with CGD. This serum behaved serologically as anti-K<sub>x</sub>; therefore, no attempt was made to separate potential anti-K<sub>x</sub> and anti-K<sub>m</sub> specificities.

**DNA analysis.** Whole blood was collected into EDTA, and 5 mL was added to 5 mL of low-salt buffer containing 10 mmol/L Tris-HCl (pH 7.6), 10 mmol/L KCl, 10 mmol/L MgCl<sub>2</sub>, and 2 mmol/L EDTA. Cells were lysed by the addition of 125 μL Nonidet P-40 (Sigma, Poole, Dorset, UK). After centrifugation, the supernatant was then resuspended in high-salt buffer containing 300 mmol/L NaCl, and the suspension was centrifuged. The supernatant was then removed and the nuclear pellet was washed in 5 mL low-salt buffer. The pellet was then resuspended in high-salt buffer containing 10 mmol/L Tris-HCl (pH 7.6), 10 mmol/L KCl, 10 mmol/L MgCl<sub>2</sub>, 0.4 mol/L NaCl, and 2 mmol/L EDTA. After the addition of 50 μL 10% sodium dodecyl sulfate, the suspension was mixed thoroughly and then incubated at 55°C for 10 minutes. A total of 300 μL 6 mol/L NaCl was then added, and the suspension was mixed well and then centrifuged. Two volumes of 100% ethanol were added to the supernatant, and the precipitated DNA was removed into ice-cold 70% ethanol and centrifuged. The DNA pellet was then dried and reconstituted in 0.5 mL 10 mmol/L Tris-HCl, 1 mmol/L EDTA at pH 8.0 and incubated at 65°C for 15 minutes.

Exons 7-8 of KEL were amplified by the following oligonucleotide primers: 5’CCCATGAGACGTCGAGAGT3’ and 5’GGCCCTTCGATCGTGGAGTA3’. The following polymerase chain reaction (PCR) conditions were used: 35 cycles of 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds. PCR product (10 μL) was incubated with 10 U of Mlu II (New England Biolabs, Beverly, MA) in the buffer provided for 1 hour at 37°C. DNA fragments were visualized by ethidium bromide staining after electrophoresis on a 1% agarose gel. The PCR products derived from DNA of the propositus were excised from a 1% agarose gel, purified with a QIAEX II kit (QIAGEN, Dorking, UK), and sequenced on an Applied Biosystems 373A DNA sequencer (Applied Biosystems, Warrington, Lancs, UK).

Exons 5-6 of KEL were amplified by the method described by Lee et al. Detection of a K allele was performed with an allele-specific primer as described by Avent and Martin.

Exons of XK were amplified as described by Ho et al. in a 100-μL vol from 50 ng of genomic DNA using one biotinylated and one nonbiotinylated primer. The DNA strands of the resulting products were separated by electrophoresis using Streptavidin-conjugated Dynabeads (Dynal UK, Wirral, Cheshire, UK) and were resuspended in 21 μL distilled water. Sequencing was performed on 7 μL of the separated strands with the USB Sequenase kit, incorporating 32P-autoradiography (US Biochemicals, Amersham, Bucks, UK).

The presence or absence of BsoAl restriction site in the PCR product from exon 2 was assessed by digestion of 10 μL of the PCR product with 10 U of BsoAl in the buffer provided (New England Biolabs) for 2 hours at 37°C. After electrophoresis on a 1.5% agarose gel, DNA segments were visualized by ethidium bromide staining.

**Immunoblotting.** After sodium dodecyl sulfate polyacrylamide gel electrophoresis, RBC membranes prepared under reducing conditions were immunoblotted as described previously. The monoclonal antibody (MoAb; NaM18-1F6) used to immunostain the blot had been raised against immunopurified Kell glycoprotein by Jaber et al. and was kindly provided by Dr D. Blanchard (Etablissement de Transfusion Sanguine Loire-Atlantique/Vendée, Nantes, France).

**RESULTS**

**Serology.** By conventional blood grouping techniques, the propositus has the following Kell phenotype: K–k– Kp(a–b–c–) Js(a–b–) Jk(a–) K–11, –17 Ul(a–) Ku+ K–12, –13, –14, –18, –19, –22, –25. His RBCs did not react with five MoAbs to epitopes on the Kell glycoprotein. However, extremely weak anti-K<sub>x</sub> activity was detected in an eluate prepared from RBCs of M.D. incubated with anti-K<sub>x</sub> serum. No antibody could be detected in an eluate from M.D. RBCs incubated with one of the MoAbs (BRIC107). Unlike most RBCs lacking Kell system antigens, the cells of M.D. have very weak expression of K<sub>x</sub> antigen, as determined by the serum of a transfused boy with McLeod syndrome and CGD. The RBCs of M.D. were also Ge:2,3,4 and no abnormal expression of RBC antigens outside the Kell system was detected. No atypical antibody was detected in the serum of M.D.

The family of M.D. is shown in Fig 1. The wife of M.D. (1-3) has the common Kell phenotype K–k+ Kp(a–b–c–) Js(a–b–) K:11, –17 Ul(a–) Ku+ K:12, 13, 14, 18, 19, 22. His son (1-1) has the common Kell phenotype, apart from being Kp(a–b–c–), and has a normal K<sub>x</sub> antigen. RBCs of the two daughters (11-2 and 11-3) of the propositus are Kp(a–b–c–) but have weak expression of Kp<sub>α</sub> and Kp<sub>β</sub>; they also have weak or undetectable Kell system high-frequency antigens. Expression of Kell system antigens was weaker on the RBCs of II-3 than on those of II-2; II-3 has a very weak or no K<sub>x</sub> antigen, whereas II-2 has a K<sub>x</sub> antigen only slightly weaker than that of normal controls. Some results of titrations of Kell antibodies and RBCs of the family members are shown in Table 1. RBCs of the sister (1-1) of M.D. were less extensively tested but were found to be Kp(a–b–c–), with some weakening of other high-frequency Kell system antigens.

**DNA analysis—KEL gene.** PCR amplification of exon 7, intron 7, and exon 8 provided a product of 353 bp in all members of the family (excluding 1-1 who was not tested) and in control samples. The single base change present in exon 8 of the Kp<sup>α</sup> allele creates an additional Nla III recogni-
Figure 1. M.D. (1-2) and his family showing Kp phenotype and genotype, Xs expression, and XK genotype. XK* represents XK allele with mutation in intron 2.

PCR amplification of exons 5-6 showed that all members of the M.D. family also had this region of the KEL gene, and the use of allele-specific primers showed that none had a K allele.

Genomic DNA analysis — XK gene. Sequencing of the XK gene of M.D. showed a substitution of G to A at nucleotide 5 of intron 2, giving the following sequence at the 5' end of exon 2 and the 3' end of intron 2: TGTTGGAGAAAggtacagtattttttatt. This substitution destroys a BsaAI restriction site. In normal controls a 625-bp PCR product spanning the 5' end of intron 2 is cut by BsaAI into 523-bp and 102-bp fragments (Fig 3). The PCR product from M.D. remained uncleaved by BsaAI, suggesting that he is hemizygous for the mutated XK gene; that from I-3 was cut, suggesting that she is homozygous for the normal allele; that of II-1 and II-2, and II-3, Kp/ Kp; II-1, II-2, and II-3, Kp/ Kp; II-1, II-2, and II-3, Kp/ Kp; II-1, II-2, and II-3, Kp/ Kp (Fig 1). Sequencing of the PCR product confirmed that the propositus had the Kp' sequence.

Immunoblotting. Immunoblotting with an MoAb to reduced Kell glycoprotein showed a band representing a structure of an apparent molecular weight of 93 kD with control...
Fig 2. Determination of Kp genotype by Nla III digestion of a 353-bp segment of KEL amplified by PCR. The Kp<sup>+</sup> allele contains two Nla III restriction sites in the 355-bp product, resulting in three fragments of 185 bp, 83 bp, and 87 bp (although the 83-bp and 87-bp fragments are not resolved in this gel); the Kp<sup>−</sup> allele contains one Nla III restriction site in the 355-bp product, resulting in two fragments of 272 bp and 83 bp. Products from Kp<sup>+</sup>/Kp<sup>−</sup> heterozygotes have both 185-bp and 272-bp fragments, in addition to the 83/87-bp fragments. Lanes 1 and 10 show uncut products: lane 1, 1-2 (M.D.); lane 10, Kp(a+b+). Lanes 2 through 9 show NlaIII-digested products: lane 2, 1-2 (M.D.); lane 3, II-1; lane 4, II-2; lane 5, II-3; lane 6, I-3; lane 7, Kp(a+b−) control; lane 8, Kp(a−b+) control; and lane 9, Kp(a+b+) control.

RBC membranes and with membranes from II-1, II-2, and II-3, but no band was apparent with membranes from M.D. (I-2). The staining of the band from II-1 membranes was equal in intensity to that of control membranes, but the bands from the membranes of II-2 and II-3 was of a slightly lower staining intensity (Fig 4).

**DISCUSSION**

After the observation that a German man with a benign prostate tumor had the rare Kell blood group phenotype K<sup>−</sup>k<sup>−</sup>, further serological studies showed that his RBCs lacked all Kell system antigens, a phenotype resembling the Kell-null phenotype, K<sub>0</sub>. However, his abnormal Kell phenotype differed from K<sub>0</sub> in three ways. First, an extremely weak k antigen was detected by adsorption/elution techniques. Second, his three children have Kp(a+b+) RBCs and must have inherited a Kp<sup>+</sup> allele from their father because their mother's RBCs are Kp(a−b+). Third, his RBCs had very weak expression of Kx, an antigen produced by the X-linked gene XK, that is elevated in expression on K<sub>0</sub> cells. Therefore, the RBCs of M.D. resemble the McLeod phenotype in their Kx antigen expression but have substantially weaker Kell system antigens than those generally associated with McLeod.

Two unrelated men with McLeod syndrome, reported by Ho et al., had different single base mutations within consensus splice sequences of the second intron of XK, the gene encoding the Kx RBC antigen. Both men had only very mild symptoms of neuroacanthocytosis and had readily detect-
able, although weakened, Kell system antigens.\textsuperscript{25,26} Similarly, M.D. has a substitution of a nucleotide that is 82\% conserved in the donor splice consensus sequence. This mutation would be expected to cause some degree of abnormal splicing and a gross reduction in the quantity of Kx antigen produced. However, some degree of normal splicing would be expected, which could explain the weakly positive reactions with anti-Kx and the absence of any symptoms of neuroacanthocytosis or muscular defects, including elevated serum creatine kinase levels. Unfortunately, suitable material for isolation of mRNA was not available and no anti-Kx suitable for immunoblotting exists; therefore, it was not possible to show whether there was reduced production of XK transcript or deficiency of Kx protein in the cell membrane. Thus, it has not been possible to prove that the very low level of Kx antigen expression is a direct consequence of the XK point mutation.

As expected, both daughters of M.D. are heterozygous for the XK mutation. Both have weakened Kell antigens and weaker-than-normal Kx. XK is subject to Lyonization; therefore, some RBCs should have normal and others abnormal Kell and Kx antigens.\textsuperscript{27} Mixed-field agglutination was not apparent, but such expected mixed-field reactions are often difficult to detect.\textsuperscript{26} One daughter had substantially weaker Kell and Kx antigens than the other, which might result from nonrandom X-chromosome inactivation. The son of M.D. does not have the XK mutation and has normal Kell and Kx antigen expression.

The extreme weakness of Kell system antigens on the RBCs of M.D. probably derives from his rare Kell genotype, homozygosity for the Kp\(^a\) allele or, possibly, Kp\(^a/Ko\). Kp\(^a\) results from an Arg \textarrow{} Trp substitution\textsuperscript{10} that appears to cause weakness of other Kell system antigens on the same molecule. Therefore, a combination of the effects of McLeod and Kp\(^a\) results in virtually undetectable Kell system antigens. This family shows how the cumulative effects of two very different mutations at independent loci can result in the absence of a number of antigens on a RBC surface glycoprotein. The mechanism by which absence of Kx from the RBCs causes weakness of Kell system antigens is unknown. The Kell glycoprotein and the Kx protein are associated in the RBC membrane: Khamlichi et al\textsuperscript{12} showed that a disulphide-bonded complex containing both structures could be immunoprecipitated from the RBC membrane by anti-K. The Kell glycoprotein could be unstable in the membrane in the absence of Kx protein, or the Kell glycoprotein may be dependent on Kx for transport to the membrane. Alternatively, conformational changes to Kell glycoprotein resulting from the absence of Kx might result in the loss of expression of Kell system antigens. The cause of the Kp\(^a\) effect is also unknown but may be caused by conformational changes of the highly folded glycoprotein occurring as a result of the Arg \textarrow{} Trp substitution. The failure to detect any Kell glycoprotein from reduced membranes of the propositus on immunoblots with an antibody to reduced Kell glycoprotein suggests that there is a very low level of Kell glycoprotein in these RBCs and that conformational changes are of little significance in the absence of Kell antigens in this individual.

The function of the Kell glycoprotein is unknown. It is apparently restricted to erythroid cells and, structurally, is closely related to neutral endopeptidases that process a variety of peptide hormones such as enkephalins, oxytocin, bradykinin, angiotensins, and neurotensin.\textsuperscript{23} However, there is no evidence that the Kell glycoprotein is enzymically active.

Unlike the Kx protein, the absence of which results in neuroacanthocytosis in addition to weakened expression of Kell system antigens, the absence of Kell glycoprotein from the RBCs has no obvious pathological effects. Therefore, if the Kell glycoprotein does serve an important function, it is probable that this function is also performed, at least to some extent, by some other structure.

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REFERENCES

8. Murphy MT, Morrison N, Miles JS, Fraser RH, Spurr NK, Boyd E: Regional chromosomal assignment of the Kell blood group locus (KEL) to chromosome 7q33-34 by fluorescence in situ hybridization: Evidence for the polypeptide nature of antigenic variation. Hum Genet 91:585, 1993
11. Lee S, Wu X, Reid M, Redman C: Molecular basis of the K6,\textendash7 (Js(a+b-)) phenotype in the Kell blood group system. Transfusion 35:822, 1995
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