A review of the Knops blood group: separating fact from fallacy

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Introduction
It has been more than 10 years since the topic of “high-titer, low-avidity” (HTLA) antibodies was reviewed in *Immunohematology*. We have learned a lot about these antibodies in the past 10 years and that knowledge has helped us to understand some of the unusual characteristics of these antibodies. Furthermore, it has helped us to name and delineate the various associated blood group systems. Although we will begin with a general review of HTLAs, this manuscript will focus on the recent findings in the Knops blood group system. *Immunohematology* 2002;18:1–8.

Key Words: Knops, polymorphism, CR1, blood group

Historical Perspective

HTLAs
To really understand some of the fallacies that have arisen regarding HTLAs, we must delve into their history, i.e., how they were discovered and how they were named. In the late 1960s and early 1970s, blood group serologists from the American Association of Blood Banks (AABB) and the American Red Cross (ARC) reference laboratories would gather at wet workshops and share samples for the classification of antibodies and the formation of blood group systems. During this time a number of weak antihuman globulin-reactive (AHG) antibodies that appeared to have similar characteristics were studied. When the serum was titrated and the reactions examined microscopically, positive reactions could be observed at dilutions of 1:64 or greater. This was very unusual, as the neat reactions would only be m+ to 1+. The agglutinates often were fragile and could be easily “shaken out.” Hence, several reference laboratory technologists began referring to this group as “high-titer, low avidity” antibodies. They never meant this to denote a blood group system, only an identifying characteristic (D. Mallory, personal communication).

Members of this newly formed group included: Chido, Rodgers, Knops, McCoy, Swain-Langley, Cost, York, JMH, Holley, Gregory, and, sometimes, Cartwright. The latter was already known to be an independent blood group system, i.e., International Society of Blood Transfusion (ISBT) 011. Since that time, all have achieved blood group status with the exception of Cost (Cs⁺), which remains a “collection” as defined by the ISBT Working Party on Terminology of Red Cell Surface Antigens. Chido and Rodgers were the first antigens in the group to be located on a membrane protein and given blood group status (ISBT 017). They were found to be antigens carried on the C4d fragment of the C4B and C4A isotypes, respectively. The Knops antigens were identified as polymorphisms of complement receptor type one (CR1) and accordingly Knops became the 22nd blood group system. Serologic and biochemical studies resulted in the assignment of both Holley and Gregory to the Dombrock system (ISBT 014) and, most recently, JMH (CDw108) was named system 026.¹

Knops
Although many of the HTLA antibodies were simultaneously being investigated in the 1960s, one of the first to be reported was anti-Cs⁺. Giles et al.² found that the sera from three patients had the same specificity and they were mutually compatible. Thus, Cs⁺ (Cost) was named after two of the first antibody producers, i.e., Copeland (CO) and Stirling (ST). Although anti-Cs⁺ appeared to have some association with York, it would later be shown not to be part of the Knops blood group system.³,⁴

The Knops system began to take form when anti-Kn¹ was described in a transfused Caucasian female who had a saline-reactive anti-K plus an unidentified antoglobulin-reactive antibody to a high-frequency antigen.⁵ A blood bank technologist (Helgeson) found that her red blood cells (RBCs) were compatible with the Knops serum. The “Helgeson phenotype” would later be identified as the serologically null phenotype for the Knops blood group. York (Yk¹) was the next high-incidence Knops antigen (KN5) to be reported but it was initially believed to be associated with Cost (Cs⁺)
rather than Kn4,5. Several years later, Molthan and Moulds7 described a new antigen, McC6, that seemed to be related to Kn7. Interestingly, a majority of McC6 antibody producers were black, while most of those making anti-Kn4 were Caucasian, thus suggesting that ethnic differences might exist in their respective gene frequencies. Finally, Sl8 and Vil were reported in separate abstracts, with one author using the term McC6 for Sl8,9.

Antigen/Antibody Characteristics

The description of HTLA antibody characteristics and their corresponding antigens has remained fairly unchanged over the past 30 years. Prominent characteristics of the Knops system antibodies and antigens are summarized in Tables 1 and 2. Urine or saliva did not inhibit any of these antibodies; however, it was found that only anti-Chido or -Rodgers could be inhibited with plasma. Because the antibodies were found in multiply transfused individuals, the serum often contained additional antibodies, such as anti-K, -E, and Duffy antibodies. The HTLA specificities were not considered “clinically significant” because they did not cause overt hemolytic transfusion reactions or hemolytic disease of the newborn.

One of the problems in studying such serum and cell samples was that they did not travel well. The antigens varied greatly in strength and often weakly reactive RBCs were negative by the time they arrived at a secondary consultation laboratory. Although ficin destroyed the Ch, Rg, and JMH antigens, it had no effect on Knops or McCoy. Later it was shown that chemicals that could disrupt disulfide bonds, i.e., dithiothreitol (DTT) and 2-aminoethylisothiouronium bromide (AET), could also destroy Knops, McCoy, and York. This was the dogma, although we had little insight into why these antibodies behaved as they did.

Biochemical Identification of Knops Antigens

In 1991, two groups identified CR1 as the protein carrying the Kn4, McC6, and Sl8 (McC6) blood group antigens.3,10 In addition, Moulds et al.3 identified Yk6 on CR1 and suggested that the Helgeson phenotype was due to low CR1 copy numbers on the RBCs (E-CR1). The CR1 gene also exhibited two other polymorphisms besides the Knops blood group. A structural polymorphism results from four different alleles that encode four different molecular-weight kiloDalton (kD) proteins: 190 kD (CR1*3), 220 kD (CR1*1), 250 kD (CR1*2), and 280 kD (CR1*4). The third commonly recognized polymorphism is based upon quantitative differences in E-CR1. A HindIII restriction fragment length polymorphism (RFLP), identified in Caucasians, is detected by two allelic fragments of 7.4 or 6.9 kilobase (kb) on Southern blots. Homozygotes for the 7.4 kb-fragment (HH) are high CR1 expressors, heterozygotes (HL) express intermediate levels of CR1, and homozygotes for the 6.9-kb fragment (LL) are low expressors of CR1.11 Alternatively, a polymerase chain reaction (PCR)-RFLP can be used, which results in bands of 1.8 kb for H alleles or 1.3 and 0.5 kb for L alleles.12 Although this RFLP correlates with RBC expression in Caucasians and Chinese,13 there is no relationship between this polymorphism and CR1 expression in African Americans14 or West Africans (J.M. Moulds, unpublished data).

Molecular Identification of Knops Antigens

The CR1 gene resides on chromosome 1 (1q32) and comprises 39 exons spread out over approximately 133 kb pairs of DNA.15 These exons encode regions called short consensus repeats (SCRs) of approximately 60 amino acids in the functional CR1 protein. Seven SCRs are organized into larger units called long homologous repeats (LHRs). The most common size protein product, CR1-1, is made up of 4 LHRs (A, B, C, D), a transmembrane region, and a cytoplasmic tail domain (Fig. 1). The binding sites for C3b and C4b have been localized to SCRs 8–9 and 15–16 (LHRs B and C) and to SCRs 1–2 (LHR-A), respectively.
Fig. 1. Schematic drawing of the most common form of CR1. Thirty short consensus repeats (SCRs) are extracellular and are arranged into four long homologous regions (LHRs) followed by a transmembrane region (TMB) and a cytoplasmic tail (CTY).

Using CR1 deletion constructs, Moulds et al. first localized the McCoy and Sl antigens to LHR-D of CR1. By direct DNA sequencing they were then able to identify two separate mutations in SCR 25 that correlated with these two blood group antigens. The McCα/McCβ polymorphism is at bp 4795, where an A encodes proline (McCα) and a G encodes aspartic acid (McCβ). The Sl/Vil mutation is only 33 bp (11 amino acids) away at bp 4828; an A encodes arginine while a G encodes glycine. Accordingly, the ISBT has now assigned these antigens to the Knops system with the following numbers: Sla as KN4, McCb as KN6, and Vil as KN7. Two other mutations have been identified in SCR 25, one of which was found in a Caucasian and is related to Sl. It is unknown at this time if the other DNA mutation correlates with any of the other McCoy antigens named by Dr. Molthan, e.g., McCc, McCd, or if this represents “Kn/McC.”

Functions of CR1 and the Knops Antigens

CR1 is a membrane-bound glycoprotein and is found on most human peripheral RBCs. Depending upon the methods used, RBCs display approximately 300–800 CR1 molecules per cell while leukocytes display ~10,000–30,000 molecules per cell. Because RBCs are present in the peripheral circulation at concentrations 10^3-fold higher than the peripheral blood mononuclear cells (PBMCs), they account for greater than 85 percent of CR1 in the blood. RBC CR1 binds immune complexes (ICs), which are shuttled to the liver or spleen for transfer to and ingestion by macrophages, leading to their elimination. IC-free RBCs return to the circulation, where they can continue participating in IC clearance.

In 1997, Rowe et al. identified CR1 as a ligand for the rosetting of Plasmodium falciparum-infected RBCs among uninfected cells. The ability of RBCs infected with P. falciparum to form rosettes is a property shown by only some parasite isolates, but is of importance because it has been associated with severe malaria. They showed that CR1 on uninfected RBCs was required for the formation of rosettes in some laboratory-adapted parasite strains, by demonstrating that CR1-deficient erythrocytes (Helgeson phenotype) had reduced rosetting and soluble recombinant CR1 could inhibit rosetting. RBCs having the Sl(a–) phenotype (found more frequently among African-derived persons) showed reduced binding to the parasite rosetting ligand P. falciparum erythrocyte membrane protein 1 (PfEMP1). Thus, the authors hypothesized that this polymorphism may have been selected for in malarious regions by providing protection against severe malaria.

CR1, as well as other complement receptors, has been identified as a receptor facilitating cell entry for a variety of pathogenic organisms. Pathogens utilizing CR1 include Babesia rodhaini (erythrocyte), Leishmania major (monocyte-macrophage), Legionella pneumophila (monocyte-macrophage), Mycobacterium leprae (monocyte-macrophage), and Mycobacterium tuberculosis (monocyte-macrophage).

Separating Fact From Fallacy

Titer and avidity

Clearly we have learned a lot in the past 10 years regarding the Knops blood group system. We can now use that knowledge to explain some of the earlier observations and clarify some of the misconceptions that have arisen over the years. Let’s start with the name “high titer, low avidity (HTLA).” According to the AABB technical manual (W.V. Miller, ed., 1974) in use at the time HTLAs were first described, “titer” was defined as “the reciprocal of the highest dilution at which macroscopic agglutination is observed.” Most of the Knops antibodies give w+ to 1+ reactions, even using RBCs having moderate expression of CR1, and would not appear to be of high titer using the technical manual definition. However, the term “high titer” was applied because weak reactions were observed microscopically that could give positive results past a dilution of 1:64 and sometimes into the thousands. But even determining an antibody titer for any Knops antibody can be problematic, because the CR1 expression polymorphism complicates the choice of RBCs, which ideally should be homozygous for the corresponding antigen. As shown in Table 3, antibody titer is very dependent on the indicator RBC that is chosen. RBCs with low expression may give only microscopically positive reactions, resulting in low scores, while RBCs with abundant CR1 give both high
titers and high scores. Thus, using titration as the sole means for classifying these antibodies is not recommended.

The term “low avidity” may be more accurate in its description of these antibodies, as avidity refers to the speed and intensity of an antigen-antibody reaction. It is this author’s experience that the Knops system antibodies are not very avid and give the strongest results when using a 60-minute incubation in saline at 37°C. This recommendation was also made by some early investigators but was subsequently lost in the rush to speed up serologic testing by using low-ionic-strength conditions or additives. Low-ionic-strength saline (LISS), polyethylene glycol (PEG), and even albumin do little to enhance the strength of the reactions for Knops system antibodies.26

### Table 3

<table>
<thead>
<tr>
<th>Anti-</th>
<th>Low E-CR1</th>
<th>Medium E-CR1</th>
<th>High E-CR1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kna</td>
<td>64 (8)†</td>
<td>&gt; 1,024 (34)</td>
<td>&gt; 1,024 (62)</td>
</tr>
<tr>
<td>McCa</td>
<td>52 (5)</td>
<td>1,024 (33)</td>
<td>&gt; 1,024 (45)</td>
</tr>
<tr>
<td>Sla</td>
<td>8 (3)</td>
<td>64 (19)</td>
<td>&gt; 1,024 (48)</td>
</tr>
</tbody>
</table>

*Red blood cell
† Score is shown in parenthesis

### Variable reactivity

Early investigations of the Knops blood group system were hampered by the inability to duplicate reactivity between laboratories, especially when the RBCs had to travel long distances. This led to arguments between serologists and misidentification of many serum and cell samples. Although the genetic differences in RBC expression of CR1 contribute to this variability,27 we now know that it is even more complex than first believed. The first variable is the method used for antibody detection. As mentioned previously, Knops antibodies prefer longer incubations, with 1 hour being optimal. In fact, when either LISS or PEG is used for testing, antibody strength may be weakened.26 This author has also observed reduced strength of reactions when saline containing azide is used; this may actually be an advantage when doing compatibility testing! Finally, increasing the serum-to-cell ratio when weak reactions are observed may be counterproductive and result in prozoning if the RBCs have below average E-CR1.

Moulds et al.27 first reported that the weak and variable reactions obtained with Knops antibodies were due to variable expression of CR1. However, the complexity of this observation was not fully appreciated at the time. Black Africans were found to have higher E-CR1 levels than Caucasians,20 but Moulds et al.16 found that heterozygosity could result in a falsely negative phenotype even when E-CR1 was adequate. Combined phenotyping of fresh RBCs along with genotyping of the same donor for McCa, McCb, Sla, and Vil showed that there may be as much as 20 percent discordance between the methods. This had been previously reported in a Caucasian donor who only expressed the Yka antigen on one allele and whose RBCs gave variable results when typed with several examples of anti-Yka.27 To clarify, the total E-CR1 may be in the normal range, i.e., 300 copies, but only one allele would produce Yka. Consequently, only 50 percent of the CR1 molecules (~150 copies) would have Yka, and this is in the range where variable results would be obtained. Since heterozygosity has been found to affect McCoy, Sla, and York typings, it is very likely that a similar situation exists for Kna and Knb.

The above scenario assumes equal expression of both CR1 alleles; however, we now know that not all alleles are equally expressed. This variation in expression can be easily visualized using immunoblotting techniques for the CR1 protein. Immunoblotting, in combination with genotyping, has shown that falsely negative serologic typings can be obtained even with high E-CR1 if a low-expressing allele is present in combination with heterozygosity for a particular gene.16,20 For example, a person with 500 copies of CR1 (high copy number) is shown by genotyping to have both Sla and Vil encoding genes. If each gene was equally expressed the result would be 250 copies for Sla and 250 copies for Vil, which is sufficient to detect serologically. However, the cells might still type as Sl(a–) if the Sla-encoding gene had low expression, e.g., only 100 copies.

Clearly E-CR1 is important in determining the strength of the reaction, but there are many other related factors that could also impact the final results. If test RBCs being used are several weeks old, e.g., commercial reagent RBCs near the expiration date, reactions may be weaker than if RBCs from a donor unit just recently drawn are used. CR1 is lost from the RBC membrane during storage through vesiculation and budding.28,29 Thus, RBCs that may have given weak reactions at the time they were drawn due to “borderline E-CR1” may give negative reactions after prolonged storage. Consequently, if one is trying to perform Knops phenotyping, the RBCs should be as fresh as possible to obtain accurate results.
To summarize, many factors can affect the final Knops phenotype result, including: antibody titer, detection method, total E-CR1 copy number, heterozygosity, low-expressing or nonexpressing alleles, and prolonged storage of the RBCs. Is it any wonder that early investigators were unable to duplicate each other’s work and were frustrated with the Knops system?

Reactivity with enzyme-treated cells

Again, some confusion exists in the early reports of Knops system antibodies and can be attributed to misleading statements such as “reactive with enzyme-treated cells.” This, of course, depends not only on the enzyme but also on the length of time used for premodification of the RBC membrane. There are no examples of these antibodies that are enhanced by enzyme treatment of RBCs, and most are still reactive (sometimes more weakly) with either ficin- or papain-treated cells. However, all Knops system antibodies identified to date are nonreactive with trypsinized RBCs. It is known that a trypsin cleavage site exists in SCR 28 of the CR1 protein. Since the blood group antigens identified to date have been found in SCR 25, they are lost upon trypsin treatment of the RBCs. This can be a useful tool not only in antibody identification, but also for adsorption, to remove other antibodies such as anti-A or -B from a serum sample.

Other antigen characteristics

The inherited expression, polymorphism, and instability of the antigen upon storage have caused misinterpretation of many test results. In 1986, Daniels et al. reported that the In(Lu) gene often suppressed Kna, McCa, Sla, Yka, and Csa. However, not all Lu(a–b–) families showed suppression and, when present, the suppression was not as dramatic as that for P1 and Au. The questionable variability in the results was recently readdressed in light of the CR1 expression polymorphism. Using samples less than 72 hours old, Moulds and Shah found there was no suppression of the high-frequency Knops antigens. They suggested that the previous results may have been due to prolonged storage of the RBCs either before or after frozen storage in glycerol.

Although RBCs from cord blood samples have been reported to have weakened Knops antigens, others have not found any reduction in antigen strength. In our studies of black African children, we have typed infants less than 1 year old and found no difference in strength of reactions as compared to older children and adults (J.M. Moulds, unpublished observation).

Other antibody characteristics

Two other characteristics attributed to Knops antibodies were they were not neutralized with plasma, saliva, or urine and they were difficult to adsorb and elute. The latter most likely reflects the low density of the CR1 protein on the RBC membrane. However, Race and Sanger reported that adsorption performed withuffy coats (white blood cells [WBCs]) was able to remove anti-Kn from serum. This led to the speculation that anti-Kn and related specificities were WBC antibodies (see next section).

Although CR1 has not been found in saliva, low levels have been found in both urine and plasma. This is believed to be the result of proteolytic cleavage of CR1 from WBCs. Serum CR1 is present only in nanogram amounts and, therefore, the levels are insufficient to neutralize Knops antibodies using routine serologic techniques. Hence, Moulds and Rowe developed an inhibition technique using recombinant, soluble CR1 (sCR1). Since their source of sCR1 was positive for Kn, McC, Sl, and Yk, it would not inhibit anti-Kn or -McC. It must be remembered that the Knops phenotype of the sCR1 will be dependent upon the gene chosen for its production. More recently, these investigators have used mutated CR1 constructs to produce peptides capable of inhibiting anti-McC and Vil.

The name game

Because antibodies in the Knops system were often found in sera containing HLA antibodies and because they could be adsorbed on WBCs, some investigators initially believed that they were antibodies to WBCs. This concept was eventually proved to be incorrect and consequently the term “HTLA antibodies” came into vogue. But even then there were some serologists who were not comfortable with this terminology and pointed out that many examples of these antibodies did not have a high titer. With the assignment of most of these specificities to blood group systems, the term HTLA should be discarded. It now becomes part of our history, along with terms like “non-specific cold agglutinins” (anti-I) and “non-A, non-B hepatitis” (hepatitis C).

But the confusion over terminology is bound to remain with us for at least a few more years. Although McC and Sl have now been officially named Sl and Vil, additional specificities described by Molthan have yet...
to be identified at the molecular level. These include \(McC^e\) and \(McC^o\), along with "Kn/McC," which have been used by many laboratories to denote Knops system antibodies that are nonreactive with the Helgeson RBCs. Finally, the compound specificities, such as anti-Kn*/
McC*, may actually represent conformational epitopes similar to Rg2.

Indeed, Moulds et al.\(^{17}\) have reported evidence for the existence of even more complex Knops specificities. They have shown that sera containing \(Sl^b\) may be heterogeneous. Furthermore, two amino acids may be involved, including the arginine at amino acid 1601 (\(Sl^P\)) and a new mutation at amino acid 1610, resulting in a total of five \(Sl\) epitopes. If the Knops, McCoy, and York antisera prove to be as diverse as \(Sl^b\), we can expect a rapid increase in the numbered antigens for this system similar to what has recently occurred with the Diego system!

**Summary**

Although this author has tried to clarify some of the misunderstandings and confusion regarding the Knops blood group system, interested readers are urged to obtain the original publications to better appreciate the complexities of this system. Some of the early investigators were criticized for their work and their viewpoints; yet we now know that they were correct (at least in some of their interpretations). In light of our current knowledge of the Knops system, I would like to end this review with a quote from Dr. Lyndall Molthan, who in 1983 predicted what has now been scientifically proved regarding CR1 expression and the Knops antigens. She stated "Other difficulties attributed to (Knops) antigens are their variations in strength, partly due to zygosity, or unrelated to zygosity but genetically determined, partly due to race, or on the basis of presence or absence of related antigens. All of these factors account for unexpected negative typings in working with patients’ samples, donors’ RBCs, and commercial panel cells."\(^{26}\)

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