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

J.M. MOULDS

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^a), 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^a. Giles et al.[2] found that the sera from three patients had the same specificity and they were mutually compatible. Thus, Cs^a (Cost) was named after two of the first antibody producers, i.e., Copeland (CO) and Stirling (ST). Although anti-Cs^a 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-Kn^a was described in a transfused Caucasian female who had a saline-reactive anti-K plus an unidentified antiglobulin-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 (Yk^a) was the next high-incidence Knops antigen (KN5) to be reported but it was initially believed to be associated with Cost (Cs^a)

rather than Kn^a.[6] Several years later, Molthan and Moulds[7] described a new antigen, McC^a, that seemed to be related to Kn^a. Interestingly, a majority of McC^a antibody producers were black, while most of those making anti-Kn^a were Caucasian, thus suggesting that ethnic differences might exist in their respective gene frequencies. Finally, Sl^a and Vil were reported in separate abstracts, with one author using the term McC^c for Sl^a.[8,9]

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.

Table 1. Prominent characteristics of Knops system antibodies

- High-titer, low-avidity
- Can demonstrate variable reactions
- Not neutralized by pooled serum or other body fluids
- Difficult to adsorb and elute
- IgG, reacting by AHG* technique
- Do not bind complement
- Reactive with enzyme-treated RBCs†
- Usually not clinically significant

* Antihuman globulin
† Red blood cells

Table 2. Prominent characteristics of the Knops antigens

- Inherited as Mendelian dominant traits
- High-frequency RBC* antigens
- Generally developed on cord RBCs
- Not denatured by proteolytic enzymes
- Not found on platelets
- Not found as soluble antigens
- Denatured by reducing antigens, e.g., DTT, AET

* Red blood cell

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 Kn^a, McC^a, and Sl^a (McC^c) blood group antigens.[3,10] In addition, Moulds et al.[3] identified Yk^a 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 *Hin*d III 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 Americans[14] 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.

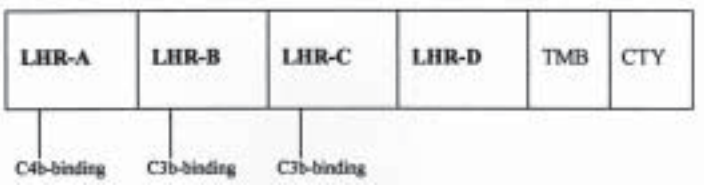

Fig. 1. Schematic drawing of the most common form of CR1. Thirty short consensus repeats (SCRs) are extracellular and are arranged into four long homologous regions (LHRs) followed by a transmembrane region (TMB) and a cytoplasmic tail (CTY).

Using CR1 deletion constructs, Moulds et al.[16] first localized the McCoy and Sl^a 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 McC^a/McC^b polymorphism is at bp 4795, where an A encodes proline (McC^a) and a G encodes aspartic acid (McC^b). The Sl^a/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[1] has now assigned these antigens to the Knops system with the following numbers: Sl^a as KN4, McC^b 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^a.[17] 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., McC^e, McC^f, 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.[18] 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.[19] 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)[20] 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),[21] *Legionella pneumophila* (monocyte-macrophage),[22] *Mycobacterium leprae* (monocyte-macrophage),[23] and *Mycobacterium tuberculosis* (monocyte-macrophage).[24]

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."[25] 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

Table 3. Titer of Knops system antibodies is dependent on the RBC* CR1 copy number (E-CR1)

Anti-	Low E-CR1	Medium E-CR1	High E-CR1
Kn^a	64 (8) †	> 1,024 (34)	> 1,024 (62)
McC^a	32 (5)	1,024 (33)	> 1,024 (45)
Sl^a	8 (3)	64 (19)	> 1,024 (48)

*Red blood cell
† Score is shown in parenthesis

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]

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 McC^a, McC^b, Sl^a, 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 Yk^a antigen on one allele and whose RBCs gave variable results when typed with several examples of anti-Yk^a.[27] To clarify, the total E-CR1 may be in the normal range, i.e., 300 copies, but only one allele would produce Yk^a. Consequently, only 50 percent of the CR1 molecules (~150 copies) would have Yk^a, and this is in the range where variable results would be obtained. Since heterozygosity has been found to affect McCoy, Sl^a, and York typings, it is very likely that a similar situation exists for Kn^a and Kn^b.

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 Sl^a and Vil encoding genes. If each gene was equally expressed the result would be 250 copies for Sl^a and 250 copies for Vil, which is sufficient to detect serologically. However, the cells might still type as Sl(a–) if the Sl^a-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.[30,31] 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.[32] reported that the *In(Lu)* gene often suppressed Kn^a, McC^a, Sl^a, Yk^a, and Cs^a. However, not all Lu(a–b–) families showed suppression and, when present, the suppression was not as dramatic as that for P_1 and Au^a. 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[33] 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,[26,31] others have not found any reduction in antigen strength.[34] 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[35] reported that adsorption performed with buffy coats (white blood cells [WBCs]) was able to remove anti-Kn^a from serum. This led to the speculation that anti-Kn^a 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[36] and plasma.[37] This is believed to be the result of proteolytic cleavage of CR1 from WBCs.[38,39] Serum CR1 is present only in nanogram amounts[39] and, therefore, the levels are insufficient to neutralize Knops antibodies using routine serologic techniques. Hence, Moulds and Rowe[40] developed an inhibition technique using recombinant, soluble CR1 (sCR1). Since their source of sCR1was positive for Kn^a, McC^a, Sl^a, and Yk^a, it would not inhibit anti-Kn^b or -McC^b. 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^b and Vil.[16]

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^c and Sl^b have now been officially named Sl^a and Vil, additional specificities described by Molthan[41] have yet

to be identified at the molecular level. These include McC^e and McC^f, 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^a/McC^a, 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^a may be heterogeneous. Furthermore, two amino acids may be involved, including the arginine at amino acid 1601 (Sl^a) 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^a, 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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A gel microtyping system for diagnosis of paroxysmal nocturnal hemoglobinuria

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Paroxysmal nocturnal hemoglobinuria (PNH), an acquired stem cell defect, is underdiagnosed because of its atypical symptoms in some patients and because available methods, which are time consuming and complicated, are not widely used. The hemolysis of PNH red blood cells (RBCs) is attributed to their enhanced susceptibility to complement lysis caused by a deficiency in glycosylsphosphatidylinositol (GPI)-anchored complement regulatory membrane proteins, especially membrane inhibitor of reactive lysis (MIRL [CD59]). We evaluated the diagnostic value of a simple hemagglutination test using the gel microtyping system by comparing it with lytic tests (the Ham test and the sucrose lysis test) and with flow cytometry (FC) assessment of expression of GPI-anchored proteins (CD59 and CD55). Examining 51 blood samples from 48 patients, we found that the gel test is useful as a screening test for PNH diagnosis and can replace the Ham test and the sucrose lysis test. The threshold of the gel test is about 10 percent of defective RBCs detected by FC. It should, however, be supplemented with FC so as to analyze precisely the defective RBCs and granulocytes in patients with positive gel test results, and, in case of negative results, to detect a small clone of defective cells in atypical cases. Due to the simplicity of the gel test, its wide use can facilitate the diagnosis of PNH. ***Immunohematology* 2002;18:9–12.**

Key Words: paroxysmal nocturnal hemoglobinuria, gel test, Ham test, sucrose lysis test, flow cytometry

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired stem cell disorder of a clonal nature, resulting in intravascular hemolysis, cytopenia of variable degrees, and recurrent thrombotic events. PNH has been described in patients already affected by bone marrow aplasia and, conversely, in PNH patients who later develop aplasia and, rarely, leukemia.[1-4] Moreover, PNH clones have been observed in patients with other diseases.[5]

Hemolysis of red blood cells (RBCs) is attributed to the enhanced susceptibility of affected RBCs to lysis by complement. The molecular mechanism of this increased susceptibility is a deficiency in complement regulatory membrane proteins, such as the decay-accelerating factor (DAF) and the membrane inhibitor of reactive lysis (MIRL), which are covalently attached to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor.[6-10] In patients with PNH, a somatic mutation in an X-linked gene, *PIG-A,* leads to impaired synthesis of the GPI anchor. A proportion of RBCs is therefore deficient in GPI-linked proteins, including MIRL (CD59) and DAF (CD55).

Despite a great deal of work on its molecular basis in recent years, PNH remains not fully understood. For instance, it is not clear how the nonhemolytic sequelae are related to the deficiency of GPI-anchored proteins and what other defects associated with clinical events seen in PNH (e.g., thrombosis) may be acquired by other than RBCs. One reason why PNH is not fully understood is that it is underdiagnosed, partly due to its heterogeneity. Also, PNH is underdiagnosed partly because many patients have atypical symptoms that do not resemble classic cases of PNH. Moreover, available laboratory methods of diagnosing PNH, some of which are time-consuming and complicated, are not widely used. The use of the hemagglutinating gel test for the diagnosis of PNH seems to open new possibilities.[11-13] This test can detect CD59- and CD55-deficient RBCs. It is based on the antiglobulin test using murine monoclonal antibodies against CD59 and CD55 proteins. Defective cells are not agglutinated by these antibodies.

The aim of this study was to evaluate the usefulness of the gel test for the diagnosis of PNH by comparing the gel test with lytic tests and with flow cytometry (FC) assessment of the expression of CD59 and CD55 on RBCs.

Materials and Methods

Fifty-one blood samples from 48 patients with anemia (suspected for PNH, aplastic anemia, or other hemolytic anemias) and 30 samples from blood donors (control group) were evaluated for this study. Blood samples were examined by the gel test, the Ham test, sucrose lysis, and FC on the same day as they were

drawn. Informed consent was obtained from all patients studied.

Gel test

The gel test (DiaMed-ID Micro Typing System PNH test, DiaMed AG, Cressier, Switzerland) was performed according to the manufacturer's instructions. In brief: 50 μL of an 0.8% RBC suspension in LISS diluent and 50 μL of murine monoclonal antibody (anti-CD59 or anti-CD55) were pipetted into a microtube that contained an antiglobulin reagent, incubated at 37°C for 15 minutes, and then centrifuged and read. Defective cells, i.e., those deficient in CD59, CD55, or both, were found at the bottom of the microtube (positive result), while normal cells were agglutinated and found on the top of the gel (negative result). In each card, a microtube with the patient's RBCs and without monoclonal antibodies was included as a negative control. In patients with RBC-bound immunoglobulins, a crossreaction may occur between them and the antiglobulin reagent directed against mouse immunoglobulins. In these cases the manufacturer suggests repeating the test after autoantibody elution.

Lytic tests

The acid lysis test (the Ham test) and the sucrose lysis test were done as part of the routine workup; results are evaluated by spectrophotometry and expressed as the percentage of lysis. Zero lysis was regarded as a negative Ham test, and lysis below 5 percent as a negative sucrose test.[14]

Flow cytometry

FC was performed with RBCs by the indirect immunofluorescence technique, using murine monoclonals anti-CD59 (BRIC 229, IBGRL, Bristol, UK), anti-CD55 (a mixture of BRIC 216, 110, 230, and 220, IBGRL, Bristol, UK), and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (Ortho-Clinical Diagnostics, Raritan, NJ, or DAKO, Denmark).[15,16] FC was performed with granulocytes (separated by dextran sedimentation and ficolgradient centrifugation with subsequent hemolysis of RBCs), using FITC-conjugated anti-CD59 (BRIC 229, IBGRL, Bristol, UK) and anti-CD67 (CLB-B 13.9, Ortho-Clinical Diagnostics).[17]

Results

In all 30 samples from blood donors the gel test was negative with both anti-CD59 and anti-CD55. The Ham test and the sucrose lysis test were also negative. The FC analysis in each case consistently showed less than 1 percent CD59-negative RBCs and less than 6 percent CD55-negative RBCs. The percentage of negatively staining granulocytes was always below 2 percent with anti-CD59 and 5 percent with anti-CD67.

In 23 patients (25 blood samples), the gel test showed GPI-deficient RBCs with both anti-CD59 and -CD55 (Table 1). A parallel examination of 22 of the 25 samples by lytic tests demonstrated a positive sucrose lysis test in all 22 and a positive Ham test in all but one sample (Table 1, No. 15). The FC analysis, together with the above results, indicated a diagnosis of PNH (suspected from clinical symptoms in most of the patients), even in the one patient with a negative Ham test. In addition, we found defective granulocytes in all 22 patients (not shown in Table 1). The percentage of defective granulocytes was usually higher than that of defective RBCs.

Table 1. Patients with positive results in the gel test; comparison with lytic tests and FC analysis

No.	Tests % of lysis		Flow cytometry analysis % of red cells		
	Ham test	sucrose lysis	CD59*	CD59†	CD55*
1	8	27	71		52
2	3	8	33		14
3	10	28	47	32	59
4	3	13	31		23
4a	4	24	52	16	56
5	6	33	55	22	51
6	2	14	17		15
7	3	34	35	25	61
8	7	43	67	7	60
9	8	39	83		89
9a	2	15	22		24
10	14	35	52	36	62
11	2	10	19		21
12	6	33	46	28	66
13	2	24	54		30
14	11	83	89		83
15	0	8	39	3	28
16	2	27	66	6	49
17	7	26	43	4	36
18	6	21	63		65
19	15	49	53		53
20	nt‡	nt	22		25
21	nt	nt	8		14
22	nt	nt	16	3	16
23	3	8	23		27

*CD59 and CD55: PNH type III, completely deficient cells
†CD59: PNH type II, partially deficient cells
‡Not tested
Control values: The Ham test = zero lysis; sucrose lysis test = < 5% lysis; FC = < 1% CD59 defective red cells and < 6% CD55 defective red cells.

In 25 of the remaining 26 samples from patients, the results of the PNH gel test were negative. These cases were divided into two groups: (1) six patients (Nos. 1 to 6) with a PNH clone found by FC (Table 2) and (2) 20 patients without such a clone.

Table 2. Patients with negative results in the gel test; comparison with lytic tests and FC analysis (remaining 20 patients with a negative gel test are described in the text)

	% of lysis		Flow cytometry analysis, % of defective			
		sucrose	red cells		granulocytes	
No.*	Ham test	lysis test	CD59	CD55	CD59	CD67
1	0	4	4	6	12	16
2	0	4	12	10	54	56
3	0	1	2	9	15	14
4	0	3	4	5	9	9
5	0	2	8	6	7	8
6	nt	nt	8	7	86	91

*__Clinical comments:__ Patients No. 1–5 had bone marrow aplasia/hypoplasia with pancytopenia; Patient No. 4 also had severe aplastic anemia and severe thrombocytopenia; Patient No. 6 was diagnosed previously as PNH (see Table 1, Patient No. 9).
__Control values:__ Ham test = zero lysis; sucrose lysis test = < 5% lysis; FC analysis = < 1% CD59 defective red cells and < 6% CD55 defective red cells; < 2% defective granulocytes with anti-CD59 and < 5% defective granulocytes with anti-CD67.

In all six cases with a PNH clone detected by FC and with a negative PNH gel test (Table 2), the results of lytic tests were also negative. The percentage of CD55-deficient RBCs by FC for some of the patients was within the range observed in the blood donor samples. However, the percentage of CD59-deficient RBCs and deficient granulocytes was above the percentage found in blood donor samples. Thus, all of these patients had an identifiable PNH clone, although in four cases it was small. Review of clinical data revealed that five patients had bone marrow hypoplasia/aplasia, usually with pancytopenia. One patient (see Table 1, No. 9) had been previously diagnosed with PNH.

In 19 of 20 patients (not included in Table 2) with negative gel tests and negative lytic tests, defective RBCs detected by FC were within the range found in donor samples. In one patient, however, the gel test was only positive with anti-CD55. The Ham test was weakly positive, but low (1%), and FC analysis identified 18 percent of CD55-defective RBCs. This patient had anemia with myelofibrosis. The other 19 patients had various kinds of anemia (e.g., due to different deficiencies, other types of hemolysis, accompanying myeloproliferative diseases, and bone marrow aplasia).

Discussion

Our data show that the gel test is useful as a screening test for the diagnosis of PNH and can replace the lytic tests. When we compared the PNH gel test results with FC results, we found that the gel test can detect 10 percent of RBCs deficient in CD59 and CD55. This is consistent with previous observations.[13] However, others[11,12] reported 2 to 5 percent of defective RBCs detectable by the gel test. However, they used an earlier version of the gel test that required the user to pipette into the gel the mixture of the antiglobulin reagent with RBCs that had previously been incubated with monoclonal antibodies.

Our method also allowed us to define which protein(s) (CD59 and/or CD55) was/were lacking from the patient's defective cells, and this was not possible by lytic tests. In one case, for instance, CD55-deficient RBCs only were detected; this information may be useful since DAF-defective RBCs alone are less important from a clinical point of view.[18] The inclusion of both anti-CD55 and -CD59 monoclonal antibodies in the gel system is advantageous because the person who reads the test can be surer about the result. The main advantage of the gel test, compared to the lytic tests, is that it is simple to use, and the results are ready within ½ hour.

Our comparison of a relatively large group of patients with both positive and negative results in the gel test showed that the positive results correlated well with the presence of abnormal cells. In all patients suspected of having PNH, a preliminary diagnosis by a positive gel test was possible. The FC analysis was, however, more sensitive, which was always useful to evaluate how numerous the defective cells were, and whether type III and type II RBCs were present. This was not possible using the gel test. Similar observations have been published but they were based on single cases.[12]

Negative gel test results were less reliable than positive results. In 6 of 26 (23 percent) samples with a negative gel test, CD59-deficient RBCs, as well as granulocytes deficient in both CD59 and CD67, were detected by FC. Most of these patients had bone marrow hypoplasia/aplasia, conditions known to be associated with the presence of PNH (or PNH-like) clones.[1,2] It should be stressed that the detection of a defective clone, in spite of a negative gel test, is not inconsistent with our opinion that this test can replace the lytic tests, since the Ham and sucrose lysis tests were also negative in this group of patients. It is, however, worth noting that 77 percent of negative gel test results (20 of 26 samples) predicted properly the lack of defective cells confirmed by FC in the population studied.

In summary, the gel test appears to be useful as a screening test for PNH and can replace lytic tests. It cannot, however, replace the FC, since only the latter can diagnose a small clone of defective RBCs and can detect PNH defects on granulocytes in transfused patients. Because of the simplicity of the gel test, its

wide use can result in an increased ability to diagnose PNH. The use of this test would be especially easy in laboratories that already use the microtyping gel system for antibody detection and blood grouping and have an appropriate centrifuge and persons experienced in interpreting the results.

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Antibody screening in 37°C saline. Is it safe to omit it using the indirect antiglobulin (gel) test?

J. Duran and M. Figueiredo

Pretransfusion tests must detect antibodies that can shorten the life of red blood cells (RBCs). Some studies have demonstrated the existence of clinically significant antibodies detected at 37°C in saline that are not detected by the indirect antiglobulin test (IAT) when the conventional tube test is used. Our aim was to determine whether these antibodies, detected with a 37°C saline tube test, are also detected when a sensitive column gel agglutination method is used. The 2373 pretransfusion samples were tested as they were received (from May 1998 to December 1999), in 37°C saline and by IAT using the DiaMed gel system. The screening tests were performed using 50 μL of 0.8% low-ionic-strength saline suspended RBCs and 50 μL of plasma. The tests were examined for agglutination and hemolysis. Two hundred and thirty three samples (9.81%) were reactive by IAT and 88 (3.70%) by 37°C saline. All 88 samples reactive by 37°C saline also reacted by IAT. These data indicate that 37°C saline is not an essential pretransfusion procedure when the DiaMed gel test is used. ***Immunohematology* 2002;18:13–15.**

Key Words: antibody screening, pretransfusion tests, gel test

Pretransfusion tests must detect antibodies that can cause hemolysis of red blood cells (RBCs). For many years, routine pretransfusion RBC antibody detection has been performed using the conventional tube method. The tube tests use saline-suspended RBCs, with or without low-ionic-strength saline solution (LISS), that are mixed with the sera to be tested. A 37°C incubation phase is performed before centrifugation and reading. After this step an indirect antiglobulin test (IAT) is performed. Several authors have demonstrated that some clinically significant antibodies were missed when the 37°C reading phase was eliminated.[1,2] These antibodies likely were predominantly IgM, representing a primary immune response.[3,4] Nevertheless, about 20 percent of these antibodies could be considered clinically significant (Rh, K, or Jk^a specificity).[1] Over the years, new methods, such as column agglutination and solid phase, have been introduced in an effort to improve the sensitivity, safety, and ease of pretransfusion tests.[5] Our aim was to evaluate whether clinically significant antibodies would be missed if the 37°C saline test was omitted when a sensitive IAT column gel agglutination method developed by Lapierre et al.[6] (the DiaMed gel system) was used.[7-9]

Materials and Methods

The study was performed at Centro Hospitalar de Vila Nova de Gaia (CHVNG), a general hospital with 600 beds, and at Centro Regional de Sangue do Porto (CRSP), the Oporto Regional Blood Center, from May 1998 to December 1999. The samples tested at CHVNG (1528) were from patients with orders for blood transfusions. Many of the 845 samples tested at CRSP were sent for antibody identification from small hospitals where a positive antibody screen had been detected. The 2373 samples were tested when received, in 37°C saline and by IAT using the DiaMed gel system (DiaMed-ID Micro Typing System, Cressier, Switzerland). The 37°C saline tests were performed using "NaCl/enzyme" cards (neutral gel) and the IAT using "LISS/Coombs" cards (anti-IgG + C3d). Fifty μL of 0.8% LISS-suspended R_1R_1, R_2R_2, and rr RBCs (ID-DiaCell I+II+III) and 50 μL of plasma were used. When the screening was positive, either in 37°C saline or in IAT, antibody identification was performed both in 37°C saline and in IAT again, using 0.8% LISS RBC panels (ID-DiaMed Panel) and 50 μL of the patient's plasma. The RBCs and the plasmas were not warmed before being mixed in the cards. The cards were incubated at 37°C for 15 minutes, then centrifuged at 900 rpm for 10 minutes, according to the manufacturer's instructions (DiaMed's ID-DiaCent centrifuge). The tests were then examined for agglutination and hemolysis.

Results

The results of antibody screens were analyzed retrospectively. All positive samples were from different

patients. When multiple antibodies were present, each specificity active at 37°C was verified. Among the 2373 samples, 233 (9.81%) were reactive by IAT and 88 (3.70%) by 37°C saline. All 88 samples reactive by 37°C saline did, in fact, also react by IAT (Table 1). Clinically

Table 1. Samples with positive antibody screening tests

Results	IAT*	37°C saline
Clinically significant antibodies	176 (7.41%)	57 (2.40%)
Clinically insignificant antibodies	35 (1.47%)	20 (0.84%)
Undetermined antibodies	22 (0.92%)	11 (0.46%)
Total	233 (9.81%)	88 (3.70%)

*Indirect antiglobulin test

significant antibodies (anti-D, anti-C, anti-c, anti-E, anti-e, anti-K, anti-Fy, anti-Jk, anti-S, and anti-s) were detected in 176 samples (7.41%) by IAT and 57 samples (2.40%) by 37°C saline (Table 2). Antibodies considered clinically

Table 2. Clinically significant antibodies detected by IAT and by 37°C saline using the DiaMed gel system

Antibodies	No. reactive by IAT*	No. reactive by 37°C saline†
Anti-D	74	28
Anti-D+C+K	11	0
Anti-D+C+E+Fy^a	1	1
Anti-D+Fy^a	2	1
Anti-D+K	2	1
Anti-D+C	14	7
Anti-D+C+E	3	1
Anti-D+C+Le^a	2	2
Anti-D+Jk^a	3	2
Anti-D+S	1	0
Anti-C	2	2
Anti-C+e	3	1
Anti-c	3	0
Anti-c+E	6	3
Anti-c+E+Fy^a	1	0
Anti-c+Fy^a+Jk^a+M	1	1
Anti-E	15	3
Anti-E+K	1	0
Anti-E+K+Fy^a	1	1
Anti-E+Jk^a+M	1	1
Anti-e	1	0
Anti-K	12	0
Anti-K+Fy^a	1	0
Anti-Fy^a	1	0
Anti-Fy^b	2	0
Anti-Jk^a	7	0
Anti-S	3	0
Anti-s	1	1
Anti-S+P_1	1	1
Total	**176**	**Total 57**

*Indirect antiglobulin test
†All antibodies reacting in saline at 37°C also reacted by IAT.

insignificant (anti-Le^a, anti-Lu^a, anti-M, and anti-P_1) were detected in 35 samples (1.47%) by IAT and in 20 samples (0.84%) by 37°C saline (Table 3). Antibodies of undetermined specificity were detected in 22 samples (0.92%) by IAT and in 11 samples (0.46%) by 37°C saline (Table 1).

Table 3. Clinically insignificant antibodies detected by IAT and 37°C saline

Antibodies	No. reactive by IAT*	No. reactive by 37°C saline†
Anti-Le^a	14	7
Anti-Le^b	1	
Anti-Le^a+Lu^a	1	1
Anti-Lu^a	1	1
Anti-M	12	8
Anti-P_1	6	3
Total	**35**	**20**

*Indirect antiglobulin test
†All antibodies reacting in saline at 37°C also reacted by IAT.

Discussion

The objective of antibody screening is to detect antibodies that can cause accelerated destruction of RBCs by transfusion or pregnancy. These clinically significant antibodies can be screened by several methods. The IAT and 37°C saline tube tests still remain the tests of choice for many. Some earlier studies demonstrated that, using the tube test, some clinically significant antibodies could remain undetected if the 37°C saline reading phase were eliminated.[1,2] More recently, Judd et al.,[10,11] discussing the risk of omitting 37°C saline readings, reported that no increase in transfusion reactions or cases of immune hemolysis have been reported since they decided to eliminate this test in 1996.[10,11]

The gel test is an RBC-affinity column method introduced in 1988 that has several advantages: small sample size, no cell washing step, and easy-to-read and stable results.[12,13] It is a sensitive and specific method, avoiding most falsely positive rouleaux-induced results. When the tube test is used, the reading after 37°C saline incubation is a simple and fast step before performing IAT. It consumes very little time and does not significantly increase the costs or work time. When using the gel test, the 37°C saline screening doubles the work and costs and delays patient care. We decided to evaluate whether we could eliminate it from our routine work without risk of missing clinically significant antibodies. Our data show a high percentage of positive antibody screening results (9.81%). This is because many of the samples that were tested at CRSP had been referred from small hospitals where unexpected antibodies had already been detected. All 88 samples positive in saline at 37°C also reacted by the IAT gel test, indicating that a 37°C saline test is not an essential pretransfusion procedure.

Judd et al.[11] found that the risk of eliminating 37°C saline testing was lower than or similar to that of eliminating other procedures, such as the direct

antiglobulin test and antihuman globulin crossmatch, and that no increase in reported transfusion reactions or cases of immune hemolysis has occurred since they eliminated this test in 1996. Based on these data and our own, we have decided to omit testing at 37°C in saline from our routine pretransfusion tests.

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Autoanti-D in a patient after cladribine treatment for lymphoplasmocytic lymphoma

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We report the case of a 62-year-old woman who developed an autoanti-D after cladribine treatment. In May 2000, the patient underwent splenectomy for a stage IV-B lymphoplasmocytic lymphoma. She was transfused with ABO- and Rh(D)-matched blood. A month later, she received chemotherapy with cladribine. In February 2001, blood grouping showed her to be AB, D+ and the direct antiglobulin test was positive for IgG. An autoanti-D was identified in the eluate. Genotypic analysis confirmed the Rh phenotype of the patient as ccDEe. No hemolysis was evident, as judged by the absence of anemia, a bilirubin of 15.7 μmol/L, and lactic dehydrogenase of 412 IU/L. When an anti-D is identified in a D+ blood recipient, a passive transfer of anti-D, and an alloimmunization in a recipient with a weak D phenotype, should be ruled out. Finally, as in our case, an autoantibody is an additional possibility. ***Immunohematology*** **2002;18:16–18.**

Key Words: autoantibody, direct antiglobulin test, cladribine, anti-D in a D+ recipient

The occurrence of anti-D in a D+ blood recipient is unusual and may arise by several potential mechanisms.[1] The receipt of intravenous immune globulin or plasma-containing components from an immunized D– donor may be the source of passively transferred anti-D. The production of an alloantibody in a D+ subject with weak D expression is also a possibility. Also, in a D+ allograft recipient, the production of an alloantibody by the persistent presence of donor immunocytes, i.e., microchimerism when the donor is D–, is also possible.[2] Finally, an autoantibody is an additional, though rare, possibility.[3]

The introduction of the purine analogs has dramatically changed the treatment of lymphoproliferative diseases, particularly chronic lymphocytic leukemia (CLL) and certain low-grade non-Hodgkin's lymphomas. The use of the purine analogs is associated, however, with a spectrum of toxicities different from those seen in patients treated with alkylating agents.[4] Among such toxicities, severe autoimmune hemolysis has been reported, even with fatal outcome.[5] In contrast, autoimmune hemolytic anemia (AIHA) occurring in the setting of lymphoplasmocytic lymphoma is much rarer and usually involves cold agglutinating IgM antibodies produced by the malignant clone.[6] Prior reports documenting cladribine-induced autoimmune hemolysis in patients with non-Hodgkin's lymphoma are rare. Tetreault and Saven[7] reported four patients who developed AIHA after cladribine treatment for Waldenström's macroglobulinemia (WM).

We report a case of autoanti-D in a D+ blood recipient after cladribine treatment for lymphoplasmocytic lymphoma without evidence of hemolysis.

Case Report

In May 2000, a 62-year-old woman underwent splenectomy. She was transfused with ten units of AB, D+ packed red blood cells (RBCs) because of bleeding. No antibodies were found in her serum at that time. No other cellular or plasma-containing components nor Rh immune globulin were transfused. The diagnosis of lymphoplasmocytic lymphoma was established by pathology study. One month later, she received two cycles of cladribine. This drug was administered as a 2-hour intravenous infusion for 5 successive days at a dose of 0.12 mg/kg/day, repeated every 28 days.

Materials and Methods

Routine serologic testing, elution, and antibody identification were performed by standard methods.[1] Briefly, ABO and D antigens were detected by tube technique following the manufacturers' instructions (Diagast, France, Gamma Biologicals, Houston, TX, respectively).

The antibody screen test was performed by low-ionic-strength saline (LISS) indirect antiglobulin test (Ortho-Clinical Diagnostics, Raritan, NJ) and an Rh panel of 11 reagent RBCs was used to identify any antibody specificity, using the gel test (Ortho-Clinical Diagnostics).

The direct antiglobulin test (DAT) was performed by tube test, using polyvalent antihuman globulin (Gamma Biologicals), monospecific anti-IgG (Ortho-Clinical Diagnostics), and anti-complement (Diagast).

Eluates prepared from the patient's RBCs using the Elu-kit™ II (Gamma Biologicals) according to the manufacturer's instructions were tested with panels of 11 reagent RBCs, to detect any autoantibody specificity and to rule out alloantibodies, using the gel test.

Genotypic analysis by polymerase chain reaction using 12 sequence-specific primers (PCR-SSP), according to the technique described by Gassner et al.,[8] was performed, to confirm the Rh phenotype, in the immunohematology laboratory of our center.

Results

The DAT and antibody screen were negative before cladribine treatment and were also negative at 1, 3, and 6 months after cladribine treatment. Laboratory tests performed 9 months after cladribine treatment yielded the following results. Blood grouping showed the patient to be AB, D+. The antibody screen test was positive and an anti-D was identified. The DAT was positive (3+) with anti-IgG and an autoanti-D was identified in the eluate. Genotypic analysis confirmed the Rh phenotype as ccDEe. No hemolysis was detected, as evidenced by a hemoglobin of 143 g/L (normal range: 120 to 140), a bilirubin of 15.7 μmol/L (normal range: 1.7 to 17.1) and lactic dehydrogenase of 412 IU/L (normal range: 210 to 460). Reticulocyte count and haptoglobin level were not determined at that time nor was an adsorption with D– RBCs done to rule in or out a mimicking autoanti-D.[9]

Discussion

We report a case of autoanti-D in a D+ woman with a hematologic malignancy who developed autoanti-D following cladribine treatment.

In our case, a passive transfer of anti-D is excluded because the patient only received ten units of AB, D+ packed RBCs. In addition, an antibody acquired by passive transfer would not be detected nine months after the last transfusion, as the half-life of human anti-D is approximately 21 days.[9] Alloimmunization in a weak D recipient is also excluded in our case because a genotypic analysis using the PCR-SSP technique confirmed the normal D phenotype. Thus, the presence of an autoanti-D is the most likely explanation for our case.

An autoantibody could produce a positive DAT and no signs of hemolysis, as in our case. Warm type IgG autoantibodies can attach to the patient's RBCs, optimally at 37°C, and may result in splenic sequestration and extravascular hemolysis.[10] Although most of these autoantibodies appear to be "nonspecific," many have specificity to an Rh antigen, notably to e. Autoanti-D have been described previously, often accompanying alloimmunization,[11] but are uncommon by themselves.[12] Other authors have published an incidence < 1 percent in a 5-year period.[13]

Long before the introduction of treatment using purine analogs, lymphoproliferative diseases were known to be associated with AIHA. Fludarabine, a purine analog, may induce severe autoimmune hemolysis, even with fatal outcome,[14] particularly in patients with CLL.[5] Cladribine (2-chloro-2′-deoxy-β-D-adenosine, Leustatin, Ortho Biotech, Raritan, NJ) is a synthetic purine analog used to evaluate any effect on autoimmune diseases. Interestingly, the first CLL patient treated with cladribine was included due to AIHA.[15,16] More recently, one study shows some evidence that cladribine may suppress the autoimmune hemolytic process in some patients with CLL.[17] Cladribine therapy has been beneficial in patients with a variety of chronic lymphoproliferative disorders and immediate toxicities generally are mild and consist chiefly of myelosuppression and infections.[18] Few data are available on the long-term toxicities of cladribine. In this setting, Kong et al.[19] reported Sjögren's syndrome in one patient and second malignancies in three patients. Recently, cladribine has also been associated with AIHA in single CLL patients[17,20,21] and Tetreault and Saven[7] reported four cases of delayed onset of AIHA after cladribine treatment for WM. On the one hand, it seems that hemolysis could be considered an early complication, occurring generally within 6 weeks, in CLL patients treated with fludarabine.[22] On the other hand, Tetreault and Saven[7] reported a median interval of 40 months (range, 24 to 60 months) from the administration of cladribine to the onset of hemolysis. In our case, autoantibody was detected at 9 months after cladribine treatment.

For these reasons, it is recommended that a DAT be performed at regular intervals during treatment with purine analogs for CLL,[15] and a specific caution is warranted in patients who develop a positive DAT during such treatment.[23] Less caution is required in the treatment of other disorders, such as follicle lymphoma.[15] In our case, cladribine was not given again.

The last follow-up in July 2001 did not show evidence of hemolytic anemia. Repeat DAT and antibody screens were not done.

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Warm autoimmune hemolytic anemia with mimicking anti-c and -E specificities

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An 18-month-old male was admitted to a hospital with a hemoglobin of 4.1 g/dL and a reticulocyte count of 53 percent. There was no history of prior transfusion. Serologic evaluation revealed the presence of both a positive direct antiglobulin test (DAT) and an indirect antiglobulin test (IAT). The patient's red blood cells (RBCs) typed as group A, C–D–E–c+e+ (cde/cde). Evaluation of the IAT revealed the presence of anti-c and anti-E. All other major antibodies were ruled out. Upon adsorption of the patient's serum with ficin-treated Cde/Cde RBCs, both antibody specificities were adsorbed; however, the antibodies were not adsorbed with native (untreated) Cde/Cde RBCs. Furthermore, the autoantibody was not adsorbed by Rh_{null} cells, thereby suggesting Rh specificity. The serum was incompatible with cde/cde RBCs and compatible with Cde/Cde RBCs. The patient was successfully transfused with Cde/Cde RBCs followed by resolution of his anemia, as evidenced by an increased and stable hemoglobin. It was concluded that the autoantibody had mimicking anti-c and -E specificities. This is a report of an unusual case of autoimmune hemolytic anemia because the Rh autoantibody appeared to have dual mimicking specificities, and the patient's RBCs were antigen negative for one of the antibody specificities, i.e., anti-E. ***Immunohematology* 2002;18:19–22.**

Key Words: autoimmune hemolytic anemia, Rh mimicking antibodies

Patients with warm autoimmune hemolytic anemia often require transfusion. These transfusions may be complicated by hemolytic reactions secondary to undetected alloantibodies or the autoantibody proper.[1,2] For these reasons it is critical to perform appropriate tests to obtain compatible or most compatible units for transfusion.

Autoantibodies with Rh specificity commonly cause autoimmune hemolytic anemia.[3] Rh antibodies usually do not fix complement,[4,5] however, one such case describing a complement-binding anti-D in a D^u variant woman was reported.[6] Some of these antibodies are detected by routine serologic methods while others are detected by polybrene or polyethylene glycol techniques.[7,8] Autoantibodies mimicking alloantibodies have been described by others.[9–11] Some of these have Rh specificity[12,13] and several examples of patients with multiple Rh autoantibodies have been reported.[14,15] IgA and IgM autoantibodies with Rh (anti-e) specificity have also been described.[16,17]

In this report we describe a previously untransfused infant with autoimmune hemolytic anemia after a viral illness. The serum antibody had a unique specificity reacting with c and E antigens. Although the serum failed to react with native (untreated) Cde/Cde red blood cells (RBCs), both specificities could be adsorbed with ficin-treated Cde/Cde RBCs. These findings suggest a more complex Rh autoantibody exhibiting mimicking anti-c and -E specificities.

Case Report

An 18-month-old nontransfused male was admitted to a hospital with symptoms of fatigue and fever. The patient had been exposed to several family members with upper respiratory symptoms. Pertinent physical findings included pallor, elevated temperature, and jaundice. A blood count was obtained and revealed a hemoglobin of 4.1 g/dL (normal range 10.5 to 14.0 g/dL), a hematocrit of 13.4 percent (normal range 32 to 42%), a reticulocyte count of 53 percent (normal range 1.0 to 3.0%), a white blood cell count of 27.1×10^9/L (normal range 6 to 17.5×10^9/L), and a platelet count of 331×10^9/L (normal range 150 to 400×10^9/L). The total bilirubin was elevated at 5.3 mg/dL (normal range 0.1 to 1.5 mg/dL) with a direct of 0.3 mg/dL (normal range 0.0 to 0.3 mg/dL). Both the direct antiglobulin test (DAT) and the indirect antiglobulin test (IAT) were 3+. The patient's RBCs typed as group A, C–D–E–c+e+ (cde/cde) with anti-E and -c reactivity detected both in serum and in eluate.

The patient was treated with intravenous prednisolone and then transfused with the least incompatible RBCs available at that time (group A, cde/cde). There was an improvement in the hemoglobin

concentration. Compatible Cde/Cde RBCs were obtained within 8 hours of admission. The transfusion of both cde/cde and Cde/Cde packed RBCs occurred within 30 hours of the hospitalization. A 1.0g/dL increment in hemoglobin was obtained with 4̄2 mL of the packed cde/cde RBCs; however, a similar increment in hemoglobin concentration was obtained with every 36 mL of Cde/Cde RBCs transfused. The patient experienced no complications, was converted to oral prednisone, and was discharged thereafter with a hemoglobin of 10.2 g/dL. Follow-up 3 weeks later revealed complete resolution of his anemia with a hemoglobin of 13.9 g/dL. An additional serum specimen was not available for further serologic studies.

Materials and Methods

The standard immunohematology techniques used are described in the American Association of Blood Banks (AABB) *Technical Manual.*[18] RBC panels were obtained from Immucor, Inc. (Norcross, GA). Phenotyping antisera were acquired from either Immucor or Gamma Biologicals (Houston, TX). Antibody screens and panels were performed in low-ionic-strength saline (LISS). Anti-C (Gamma) and anti-c, -E, and -e (Immucor) were monoclonal reagents requiring incubation at 37°C for 5 to 15 minutes. Positive and negative controls were run with each monoclonal Rh typing reagent. Antibody elution was performed using the Gamma Elu-Kit II. Polyspecific and monospecific anti-C3d and anti-IgG were obtained from Gamma, as were ficin and GammaZyme-S. Adsorptions were performed at 37°C for 30 minutes with ficin-treated or untreated Cde/Cde RBCs. Tests with Rh_{null} cells were done by the Gamma Biologicals Reference Laboratory, Houston, Texas. All specimens used for serologic studies were obtained from the patient within the first days of hospitalization. All other routine patient laboratory data were obtained by standard laboratory procedures using reagents and instruments according to the manufacturers' instructions.

Results

The patient typed as C–D–E–c+e+ (cde/cde) with Rh monoclonal typing sera. The positive and negative controls run with each monoclonal Rh reagent reacted as expected. Those results excluded interference with the patient's Rh typing by the positive DAT. The initial specimen had a 3+ DAT with monospecific anti-IgG and was negative with anti-C3d. The IAT was 3+ with cDE/cDE RBCs and 2+ with cde/cde screening RBCs using a polyspecific antiglobulin reagent. The CDe/CDe screening cell was nonreactive. Evaluation of the serum with a polyspecific antiglobulin reagent and an eluate from the patient's RBCs with a monospecific anti-IgG revealed anti-c and -E reactivities (Table 1). Three antigen negative and three antigen positive RBCs were used to confirm each reactivity. In both the serum and the eluate, anti-E reactivity was weak and anti-c strong in the IAT phase using monospecific anti-IgG. Antibodies to other antigens were ruled out. Since the serum and eluate had identical specificities, the patient's serum was first adsorbed with untreated Cde/Cde RBCs (37°C for 30 minutes). An eluate was prepared from the Cde/Cde RBCs used for adsorption. No antibody was detected in the eluate (Table 2), illustrating that the autoantibody was not adsorbed by untreated RBCs lacking the corresponding antigens. Another aliquot of serum was adsorbed × 4̄ with ficin-treated Cde/Cde RBCs and reactivity of the adsorbed serum determined (Table 3). The weaker anti-E specificity was readily adsorbed. However, anti-c reactivity remained after three adsorptions, albeit

Table 1. Evaluation of the serum and eluate in LISS at IS and 37°C and by the indirect antiglobulin test (IAT)

Panel Cell #	Rh-Hr Phenotype								Agglutination Results LISS		IAT		
	D	C	c	E	e	f	V	C^w	IS	37°C	Serum	Eluate	CC
1	+	+	0	+	+	0	0	0	0	0	w±	w±	
2	+	+	0	+	+	0	0	0	0	0	w±	+	
3	+	+	0	+	+	0	0	0	0	0	w±	w±	
4̄	+	0	+	+	0	0	0	0	0	0	+++	+++	
5	+	0	+	0	+	+	0	0	0	0	++	++	
6	0	+	+	0	+	+	0	0	0	0	w±	+	
7	0	0	+	+	+	+	0	0	0	0	+++	+++	
8	0	0	+	0	+	+	0	0	0	0	++	++	
9	0	0	+	0	+	+	0	0	0	0	++	++	
10	+	+	0	0	+	0	0	+	0	0	0	0	++
11	+	+	0	0	+	0	0	0	0	0	0	0	++
12	+	+	0	0	+	0	0	0	0	0	0	0	++

Note: Immediate spin (IS) and 37°C reactions were performed in LISS. Cells were washed and polyspecific antihuman globulin was added to the serum and monospecific anti-IgG was added to the eluate. All negative reactions were confirmed using check cells (CC).

Table 2. Results of testing an eluate prepared from Cde/Cde RBCs used to adsorb patient's serum

Cell #	Rh-Hr Phenotype								Agglutination Results IAT*	
	D	C	c	E	e	f	V	C^w	IgG	CC†
1	+	+	0	+	+	0	0	0	0	++
2	+	0	+	0	+	+	0	0	0	++

All adsorptions were performed at 37°C for 30 minutes with native (untreated) Cde/Cde RBCs. An eluate was prepared from the cells and tested for reaction with selected panel cells in LISS at 37°C for 30 minutes and by the indirect antiglobulin test* using monospecific anti-IgG. All negative reactions were confirmed using check cells.†

Table 3. Results obtained with the indirect antiglobulin test (IAT) after the serum was adsorbed × 4 with ficin-treated Cde/Cde red blood cells (RBCs).

Cell #	Rh-Hr Phenotype								IAT Results Adsorption #				
	D	C	c	E	e	f	V	C^w	#1	#2	#3	#4	CC*
1	+	+	0	+	+	0	0	0	w±	0	0	0	+
2	+	0	+	0	+	+	0	0	++	+	w±	0	+

All serum adsorptions with ficin-treated Cde/Cde RBCs were performed at 37°C for 30 minutes. Adsorbed sera were incubated in LISS with a selected panel of RBCs for 15 minutes, then washed, and monospecific IgG antiglobulin reagent was added. All negative reactions were confirmed with check cells.* Reactivity of w± to ++ denotes residual antibody in serum after the respective serial absorption.

markedly decreased, and was removed by the fourth. Furthermore, the unadsorbed serum did not react with Rh_{null} cells nor was reactivity reduced by adsorption with Rh_{null} cells. All crossmatches with Cde/Cde RBCs were compatible.

Discussion

The anti-c and -E activities could be a single autoantibody (anti-cE) reacting with both antigens or with a common determinant expressed on the protein carrying c and/or E antigen. A less likely explanation would be two separate autoantibodies produced by distinct populations of IgG-secreting plasma cells—one producing anti-c and the other producing anti-E. Because additional serum specimens were not available, it is difficult to differentiate between the two possibilities. The data clearly imply that whatever the apparent specificity was, the antibody reacted with an epitope or epitopes exposed on ficin-treated antigen-negative Cde/Cde RBCs. This makes the true nature of the antibody difficult to determine. It is perhaps best described as an autoantibody with mimicking anti-c and -E specificity.

The clinical significance of this autoantibody is not debatable as it was associated with profound hemolytic anemia. Whether the mimicking specificities proper were clinically significant may be questioned. Since the transfusions occurred within a short period of time and immediately after initiation of intravenous prednisolone therapy, the effect of prednisolone on antibody titer and transfused RBC clearance is questionable. However, since the Cde/Cde RBCs resulted in only a 117 percent better increment than the cde/cde RBCs in hemoglobin concentration, the clinical significance of the mimicking specificities is uncertain.

Rh autoantibodies are common, and some that react with more than one Rh antigen have been described. Schonitzer and Kilga-Nolger reported an autoanti-DE in a 16-year-old girl with a cystic ovarian teratoma.[19] Fudenberg et al. were the first to report autoantibodies of apparently the wrong specificity, i.e., to antigens absent on the subject's erythrocytes.[20] Others have also reported autoantibodies to Rh epitopes absent on the patient's RBCs. Such was the case described by Issitt et al. in a Cde/Cde patient with myelofibrosis who developed an autoanti-E.[21] This may not be an uncommon phenomenon, as others have described similar individuals with RBCs that have a positive DAT and yield an eluate that reacts with antigens they phenotypically lack. In this report, an autoanti-E was eluted from the patient's cde/cde cells. This antibody would not react with RBCs with a homozygous expression for the e antigen; however, the autoantibody reacted with RBCs with a homozygous expression for the C antigen and a heterozygous expression for the E antigen. Unfortunately, reagent RBCs with homozygous expression for C and E antigens (CDE/CDE) were not available to further characterize the autoantibody, but one would expect the anti-E component of the autoantibody to react more strongly with RBCs with a homozygous expression for the E antigen. Thus, this autoantibody had dual mimicking specificity with one component of the autoantibody directed to an antigen absent on the patient's RBCs. This unusual case of autoimmune hemolytic anemia exemplifies the complexity of the Rh system.

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COMMUNICATIONS

Letters to the Editors

Anti-cE (Rh27), a rarely occurring antibody

A serum sample from a 73-year-old female was found to be positive in a pretranfusion antihuman globulin crossmatch. Transfusion was required because of anemia following a gastrectomy. Past history included three pregnancies, and 9 months prior to the gastrectomy she had received a red blood cell (RBC) transfusion. At that time, antibody screening and crossmatch were negative.

Preliminary serologic investigation of the current sample suggested an anti-cE. The serum was further tested vs. group O RBCs of known Rh type using an IgG antihuman globulin test and an enzyme test (bromelin) at room temperature. The serum only agglutinated DcE and dcE (Rh27) RBCs and did not agglutinate DCE, dCE, or dce (Rh27) RBCs, which suggested the presence of anti-cE as a single antibody. The Rh phenotype of the patient was D+C+E–c–e+ (DCe).

Anti-cE was first described in 1961 by Gold et al.[1] in a serum containing eight antibodies, and only a few other samples have been described since then.[2] One example was of a naturally occurring anti-cE (Rh27) that bound complement, described by Kline et al.[3] in 1982.

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September 11

The staff of the American Red Cross Southern Region Reference Laboratory would like to thank our friends at Immucor, Inc., for their support during the events of September 11. The unprecedented donor response posed a dilemma for our reference laboratory: so many potential new rare donors to be found but so few free hands to do the work to find them!

Tama Copeland and her staff at Immucor came to the rescue. They volunteered their time, expertise, facilities, and even their rare antisera to help us with this mass screening. One Vel–, one Lan–, one Js^b–, two U–, one Tj^a–, and numerous multiple-antigen-negative donors were identified and are now enrolled in our American Rare Donor Program. Many of these donors have already given a second donation.

Thanks, Immucor. You were there when we needed you.

Debi Long, MT(ASCP)SBB
American Red Cross Blood Services
Southern Region
Reference Laboratory
Atlanta, Georgia

Letter from the Editors

Thoughts on September 11

The events of September 11, 2001, had a terrible effect on everyone. But, out of the darkness of this event, there was much goodness. People helped each other and we were kind to each other. I am sure that many of you have blood-bank-related stories that you would like to share with the readers of *Immunohematology.* We would like to consider them for publication.

In this issue, you will find a letter to the editors from Debi Long of the Reference Laboratory at the Southern Region of the American Red Cross in Atlanta, Georgia, that relates one of these special kindnesses. If you have a special story and would like to share it, please fax it to Mary McGinniss at (301) 299-7443 or e-mail it to mmanigly@usa.redcross.org.

Feelings of sadness, anger, patriotism, and kindness will be with us forever. Let's build on our patriotism and kindness.

Delores Mallory
Editor-in-Chief

Mary McGinniss
Managing Editor

ANNOUNCEMENTS

Monoclonal antibodies available. The New York Blood Center has developed murine monoclonal antibodies that are useful for donor screening and for typing red cells with a positive direct antiglobulin test. Anti-Rh:17 is a direct agglutinating monoclonal antibody. Anti-Fy^a, anti-K, anti-Js^b, and anti-Kp^a are indirect agglutinating antibodies that require anti-mouse IgG for detection. These antibodies are available in limited quantities at no charge to anyone who requests them. **Contact:** Marion Reid, New York Blood Center, 310 E. 67th Street, New York, NY 10021; e-mail: mreid@nybc.org

Manuscripts: The editorial staff of *Immunohematology* welcomes manuscripts pertaining to blood group serology and education for consideration for publication. We are especially interested in case reports, papers on platelet and white cell serology, scientific articles covering original investigations, and papers on the use of computers in the blood bank. Deadlines for receipt of manuscripts for the March, June, September, and December issues are the first weeks in November, February, May, and August, respectively. Instructions for scientific articles and case reports can be obtained by phoning or faxing a request to Mary H. McGinnniss, Managing Editor, *Immunohematology,* at (301) 299–7443, or see "Instructions for Authors" in every issue of *Immunohematology* or on the Web. Include fax and phone numbers and e-mail address with your manuscript.

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Immunohematology

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Instructions for Authors

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Before submitting a manuscript, consult current issues of *Immunohematology* for style. Type the manuscript on white bond paper (8.5" × 11") and double-space throughout. Number the pages consecutively in the upper right-hand corner, beginning with the title page. Each component of the manuscript must start on a new page in the following order:

1. Title page
2. Abstract
3. Text
4. Acknowledgments
5. References
6. Author information
7. Tables—see 6 under Preparation
8. Figures—see 7 under Preparation

Preparation

1. Title page
   - A. Full title of manuscript with only first letter of first word capitalized
   - B. Initials and last name of each author (no degrees; all CAPS), e.g., M.T. JONES
   - C. Running title of ≤ 40 characters, including spaces
   - D. 3 to 10 key words
2. Abstract
   - A. 1 paragraph, no longer than 200 words
   - B. Purpose, methods, findings, and conclusions of study
   - C. Abstracts not required for reviews
3. Text (serial pages)
   Most manuscripts can usually, but not necessarily, be divided into sections (as described below). Results of surveys and review papers are examples that may need individualized sections.
   - A. Introduction
     Purpose and rationale for study, including pertinent background references.
   - B. Case Report (if study calls for one)
     Clinical and/or hematologic data and background serology.
   - C. Materials and Methods
     Selection and number of subjects, samples, items, etc. studied and description of appropriate controls, procedures, methods, equipment, reagents, etc. Equipment and reagents should be identified in parentheses by model or lot and manufacturer's name, city, and state. Do not use patients' names or hospital numbers.
   - D. Results
     Presentation of concise and sequential results, referring to pertinent tables and/or figures, if applicable.
   - E. Discussion
     Implications and limitations of the study, links to other studies; if appropriate, link conclusions to purpose of study as stated in introduction.
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   - A. In text, use superscript, arabic numbers.
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6. Tables
   - A. Number consecutively, head each with a brief title, capitalize first letter of first word (e.g., Table 1. Results of ...), and use no punctuation at the end.
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7. Figures
   - A. Figures can be submitted either drawn or photographed (5" × 7" glossy).
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   - C. When plotting points on a figure, use the following symbols when possible: ○ ● △ ▲ □ ■.
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   - A. List first name, middle initial, last name, highest academic degree, position held, institution and department, and **complete** address (including zip code) for **all** authors. List country when applicable.

SCIENTIFIC ARTICLES AND CASE REPORTS SUBMITTED AS LETTERS TO THE EDITOR

Preparation

1. Heading—To the Editor:
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4. Author(s)—type flush right; for first author: name, degree, institution, address (including city, state, and zip code); for other authors: name, degree, institution, city, and state.
5. References—limited to ten.
6. One table and/or figure allowed.

Send all submissions (original and two copies) to:
Mary H. McGinniss, Managing Editor, *Immunohematology*, 10262 Arizona Circle, Bethesda, MD 20817.

Include your manuscript on a 3 1/2" disk, in Microsoft Word 97/6.0 or Word Perfect 6.0/5.1 or e-mail your manuscript to mmanigly@usa.redcross.org

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