Immunohematology

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CONTENTS

The Cromer blood group system: a review

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The antigens of the Cromer blood group system reside on decay accelerating factor (DAF), a protein belonging to the regulators of complement activation family. The blood group system consists of eight high-incidence antigens and three low-incidence antigens. The molecular basis for the antigens is known and, with the exception of IFC, each antigen is the product of a single nucleotide polymorphism in the *DAF* gene and has been localized to one of the four short consensus repeat regions on the DAF protein. The red blood cells (RBCs) of people with the Cromer null phenotype, Inab, lack DAF. Antibodies to Cromer antigens are rarely encountered although there is evidence that the antibodies may cause accelerated destruction of transfused RBCs. There is no risk of hemolytic disease of the newborn associated with Cromer system antibodies because the placenta is a rich source of fetally derived DAF, which is thought to adsorb the antibodies. *Immunohematology* 2002;18:95–103.

Introduction

The Cromer blood group antigens are carried on decay accelerating factor (DAF, CD55), a member of a family of proteins known as the regulators of complement activation. In 1965, an antibody in the serum of a Black prenatal patient, Mrs. Cromer, which reacted with all red blood cells (RBCs) except her own and those of two siblings, was reported.[1] RBCs from Mrs. Cromer were Go(a+) and initially her antibody was named anti-Go[b] because it was thought to detect the high-incidence antigen antithetical to Go[a]. In 1975, Stroup and McCreary[2] reported four additional examples of the antibody and renamed it anti-Cr[a] after the proposita. In 1982, Daniels et al.[3] described an antibody in the serum of a Japanese male (Inab). The antibody was reactive with all RBCs, including those of his mother, father, and brother. Inab RBCs were shown to be the null phenotype of the Cromer system.

Subsequently, other examples of antibodies to high-incidence antigens were linked to Cromer by failure of the antibody to react with Inab RBCs, sensitivity of the antigen to α-chymotrypsin treatment of RBCs, and by specific inhibition of the antibody by concentrated plasma or urine from an antigen-positive person. To date, eight high-incidence antigens and three low-incidence antigens have been described in the Cromer system (Table 1). Transfusion data regarding the clinical significance of antibodies to Cromer antigens are mixed; however, no cases of hemolytic disease of the newborn (HDN) have been described. This is discussed later in more detail.

Table 1. Antigens of the Cromer blood group system

ISBT number	Antigen name	Frequency	Examples of antibody*	Ethnic association
021001	Cr[a]	High	Many	Cr(a–) in Blacks; 1 Spanish-American
021002	Tc[a]	High	Many Few	Tc(a–) in Blacks Tc(a–) in Whites
021003	Tc[b]	Low	Few	Tc(b+) in Blacks
021004	Tc[c]	Low	Few	Tc(c+) in Whites
021005	Dr[a]	High	Several	Dr(a–) in Uzbekistani Jews and Japanese
021006	Es[a]	High	Few	No association
021007	IFC	High	Several	3 Japanese 2 Italian American 1 Jewish American
021008	WES[a]	Low	Several	WES(a+) in Finns and Blacks
021009	WES[b]	High	Few	WES(b–) in Finns and Blacks
021010	UMC	High	Few	Japanese
021011	GUTI	High	Few	GUTI– in Chileans

*Few = 1–3; Several = 4–10; Many = > 10

Cromer Blood Group Antigens are Carried on DAF

In 1987, a human cell-surface glycoprotein with an approximate M_r of 70 kDa, found on RBCs, white cells, and platelets, was characterized and shown to carry Cromer antigens.[4] Telen et al.[5] and Parsons et al.[6] showed independently that this glycoprotein was DAF and that people with the Inab phenotype lacked DAF on their RBCs. DAF is linked to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor and is expressed on all hemopoietic cells and the vascular endothelium.[7] It is also widely expressed on epithelium in the gastrointestinal, genitourinary, and

central nervous systems. A soluble form is present in plasma and body fluids.[8]

The gene encoding DAF was mapped to chromosome 1 in 1987.[9] It was cloned and sequenced in the same year: *DAF* encodes a protein of 381 amino acids that includes a 34-residue signal peptide.[10,11] The mature protein is arranged into four short consensus repeat (SCR) units of ~ 60 amino acids, followed by a 70-amino acid region that is rich in serine and threonine residues.[7] In this review, for the numbering of nucleotides and amino acids in DAF, we use the system described by Lublin and Atkinson, with nucleotide A of the initiation methionine codon designated 54 and the first amino acid of the mature protein (without the signal peptide) designated as residue 1 (Fig. 1).[7] These numbers may differ from those in the original reports.
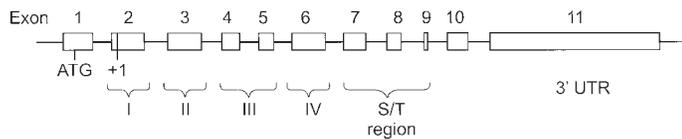
Fig. 1. The organization of the *DAF* gene.[63] ATG in exon 1 is the initiator codon, the A of which is nt-54.[7] The mature protein on the RBC membrane is encoded by *DAF* from within exon 2 (+1). Roman numerals indicate the region of the gene that encodes the short consensus repeat (SCR) regions of the protein. The S/T region refers to the exons that encode a serine/threonine rich region. 3' UTR is the 3' untranslated region.

Before DAF was identified as the carrier protein for Cromer blood group antigens, traditional methods of establishing exclusion of new Cromer antigens from existing blood groups were used. These methods include comprehensive family studies and serologic characterization of the antibodies. Consanguinity has been shown in four families in which the propositus lacks a high-incidence Cromer blood group antigen.[12–15] Now DNA sequencing provides a rapid method of identifying the genetic basis of the antigens. Furthermore, two elegant sets of experiments, one using the monoclonal antibody immobilization of erythrocytes assay (MAIEA)[16] and the other using a series of DAF deletion mutant proteins expressed in Chinese Hamster Ovary (CHO) cell lines, permitted epitope mapping of the antigens to one of the four SCRs on DAF (Fig. 2).[17–19] These experiments are important because, while DNA sequencing can be used to identify a missense mutation in *DAF*, proving that the mutation is responsible for the presence or absence of an antigen is difficult when there are only one or two examples of the phenotype to test. The MAIEA technique confirmed that Cromer antigens are on DAF,

and the use of well-characterized monoclonal anti-DAF in combination with human polyclonal antibodies to different Cromer antigens permitted spatial mapping. Western blotting experiments using CHO cell lines that expressed all or part of the DAF protein were tested with specific antibodies to Cromer antigens and the DAF protein. The results of these assays correlated with the DNA sequence predictions.

Characteristics of the antigens and antibodies

Cr[a]

The first example of anti-Cr[a] was described in 1965 in the serum of a Black female.[1] The antibody reacted with the RBCs of all random donors and with those of more than 4000 Black donors but not with the proposita's own cells or those of two siblings. With the exception of one example found in the serum of a Spanish-American woman,[20] all examples of anti-Cr[a] have been found in Black persons.[21] Although anti-Cr[a] may be stimulated by pregnancy,[22] there have been no cases of HDN attributable to the antibody.

The molecular basis of the Cr(a–) phenotype is a nucleotide mutation, 679G>C in exon 6, which encodes an amino acid substitution of Ala193Pro in the SCR4 of DAF.[19] No low-incidence antigen has been identified as being associated with this change.

Tc[a], Tc[b], Tc[c]

Tc[a], a high-incidence antigen, was first reported in 1980.[23] The antibody was found in the serum of two Black females (GT and DLC). Neither patient had been transfused. Presumably, their antibody had been stimulated by pregnancy, although there was no evidence of HDN. The DLC antibody was distinct from all other antibodies to known high-incidence antigens. Tests with a panel of RBCs that lacked an unidentified high-incidence antigen revealed compatibility with the RBCs of GT. Crosstesting confirmed that both antibodies were mutually compatible.
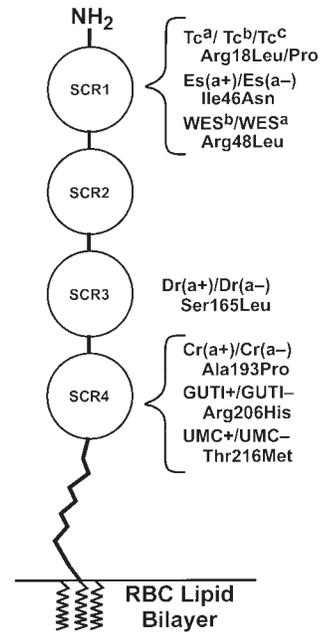
Fig. 2. Schematic representation of *DAF* showing the location of the Cromer blood group antigens. The IFC antigen is not shown since it has not been defined.

Studies on 28 members of DLC's family revealed two additional Tc(a–) people, neither of whom had produced anti-Tc[a].[12] Previously, Stroup and McCreary had found that the RBCs of one of their Cr(a–) patients, AJ, reacted very weakly with the GT serum, and a possible relationship between the two antigens was proposed.[2] The RBCs of Inab were compatible with the "new" antibody; however, the Inab serum was reactive with RBCs from both AJ and DLC. The new antigen defined by the G<u>T</u> and DL<u>C</u> sera was named Tc[a] after the two propositi.

The low-incidence antigen Tc[b] was identified when the Tc(a–) RBCs of WM reacted with one of four sera containing anti-Go[a].[24] After adsorption of the anti-Go[a], the serum reacted with 6 of 103 RBC samples from randomly selected Black individuals. Additionally, the RBCs of 11 Tc(a–) individuals were agglutinated by the adsorbed serum. Thus, it was concluded that the new antibody detected an antigen, Tc[b], which was antithetical to Tc[a]. The incidence of Tc[b] in a random Black population was calculated to be 5 percent.[25] No Tc(b+) Whites have been found.

The Tc(a–) phenotype has been described in Whites.[26] An antibody was detected in the serum of an 18-year-old woman (DWL) who had been transfused with four units of RBCs during her second pregnancy. Her RBCs typed as Tc(a–b–) and the antibody was compatible with Tc(a–b+) RBCs. A family study showed that the proposita's parents and three of four siblings were incompatible, but the RBCs of DWL's sister also typed Tc(a–b–) and were nonreactive with the antibody. The antithetical low-incidence antigen was named Tc[c] and is the low-incidence allele of Tc[a]. At 6 months, DWL's serum was reactive with Tc(a+b–) and Tc(a–b+) RBCs but not with her own RBCs or those of her Tc(a–b–c+) sister. Subsequent testing showed that the antibody was compatible with cells of the Inab phenotype. It was proposed that the antibody was an inseparable anti-Tc[a]Tc[b].[26]

The molecular basis of the Tc[a]/Tc[b] antigens was defined by direct sequence analysis as a single nucleotide polymorphism 155G>T that encoded Arg18Leu in the SCR1 of DAF.[19] Another nucleotide change at the same position (155G>C) was found in a Tc(a–b–c+) sample, which encodes a change of Arg18Pro.[17]

Dr[a]

Another antibody to an apparent Cromer-related high-incidence antigen was reported in 1984.[13] Before surgery, an antibody to a high-incidence antigen was identified in the serum of MD, an Israeli woman of Buhkaran origin. In family studies, MD's serum was compatible with the RBCs of her sister, whose serum also contained an antibody that demonstrated identical specificity. Both women were multiparous and had been transfused. The RBCs of both MD and her sister had weakened Cr[a] and Tc[a] antigens and were only weakly incompatible with the Inab serum and that of Owens (see IFC).[13] The antibody was shown to be distinct from anti-Cr[a] and anti-Tc[a] and was named anti-Dr[a], after MD. Two other examples of anti-Dr[a] in unrelated individuals of Uzbekistani Jewish descent were described.[27,28] Family studies performed on the second reported example revealed three Dr(a–) individuals, two sons and a daughter, none of whom had anti-Dr[a] in their serum. Weakened expression of Cr[a] and Tc[a] antigens (and subsequently of other high-incidence Cromer antigens) was also observed in these individuals. Reid et al.[29] described a 38-year-old Russian woman (KZ), who presented with a chronic intestinal disorder. KZ was originally reported as an example of the Inab phenotype but subsequent studies showed that her Dr(a–) RBCs were weakly reactive with anti-Tc[a] and anti-IFC.[30] Although KZ's siblings were not available for testing, samples from her parents were tested. Both parents were positive for IFC; however, the reactivity was weaker than that of control cells, consistent with heterozygosity.

The Dr(a–) phenotype has also been described in the Japanese population. Daniels et al.[31] reported anti-Dr[a] in the serum of a female blood donor who had two children but no history of transfusion. The donor's RBCs reacted weakly with anti-Cr[a], anti-Tc[a], anti-WES[b], anti-UMC, and six of nine monoclonal anti-DAFs but not with anti-Dr[a]. Another Japanese Dr(a–) proband, a blood donor, was reported by Uchikawa et al.[32] The donor's RBCs were only weakly agglutinated with five monoclonal anti-DAFs and with the original anti-IFC, but not with anti-Dr[a].

The molecular basis is the same for Israelis and Japanese. A point mutation of 596C>T changes Ser165Leu.[30,31,33] The nucleotide change creates an alternative splice site that is used preferentially but not exclusively. However, the mis-splicing results in a 44-bp deletion in the coding sequence that alters the open reading frame and results in a premature stop codon. The small amount of full-length DAF that is translated carries the Ser165Leu residue change and RBCs type Dr(a–). The Cromer antigens on Dr(a–) RBCs are very weak and may not be detected by hemagglutination.

Dr(a–) RBCs express only 40 percent DAF when compared with normal RBCs in tests with monoclonal antibodies.[33] The Dr[a] antigen is the receptor for fimbriae from 075X-positive *E. coli*, an organism that is associated with urinary tract infection, cystitis, and protracted diarrhea.[34]

Es[a]

The original antibody, found in the serum of a woman of Mexican descent, was detected in routine compatibility testing and was reactive with all random RBCs tested.[14] The RBCs of two of three siblings, as well as RBCs of the Inab phenotype, were compatible with the antibody. The woman's parents were first cousins. The second example was identified in an African American male who was being treated for Alzheimer's disease, diabetes, and anemia. He had no history of transfusion and no siblings were available.[35]

The molecular basis of the Es(a–) phenotype was determined to be a point mutation of 239T>A, which changes Ile46Asn in SCR1 of DAF.[17]

IFC (the Inab phenotype)

The Inab phenotype is the null phenotype of the Cromer blood group system and the RBCs of persons with this rare phenotype are DAF-deficient.[3] To date, this rare phenotype has been identified in six people: three Japanese,[3,31,36] one Jewish American,[37] and an Italian American woman and her brother.[38] It is likely that Owens, an African American boy, was also of the Inab phenotype; however, his RBCs were never tested.[39] The first two probands with the Inab phenotype, a Japanese man and a Jewish American man, were both diagnosed with protein-losing enteropathies and it was unclear whether the "null" status was an acquired phenomenon associated with their disease or an inherited characteristic.[3,37] However, the parents of the propositus were first cousins, suggesting that the Inab phenotype arose from the inheritance of a rare recessive gene. The Japanese patient, Osad-II,[31] was diagnosed with a capillary angioma of the small intestine. Similarly, the African American boy, Owens, had a diagnosis of protein-losing enteropathy. However, no history of intestinal disease was reported in the 86-year-old Italian American, who had been admitted to hospital with a fractured hip.[38] The association of DAF-deficiency and chronic disease of the intestine remains unproved.

The sera of the first four Inab phenotype propositi contained an antibody that was reactive with all random RBCs tested, including RBCs lacking the high-incidence Cromer antigens Cr[a], Tc[a], Dr[a], Es[a], WES[b], and UMC. The antibody was named anti-IFC, and RBCs of the Inab phenotype are IFC-negative.[15]

The molecular basis of the Inab phenotype has been determined in the three Japanese probands. In two people, a point mutation of 261G>A changes Trp53 to a stop codon.[30,31] In the third Japanese Inab person, a point mutation of 263C>A creates a cryptic splice site in exon 2. Use of this splice site results in a 26-bp deletion and alters the open reading frame. As a consequence, Ser54 is changed to a stop codon.[36] In all cases, there is no DAF protein in the membrane.

DAF is important in the prevention of autolysis of RBCs by complement. It was surprising, therefore, to find that IFC-negative and Dr(a–) RBCs that either lack DAF completely or have reduced DAF do not show a marked susceptibility to lysis in vitro.[40,41] The relative stability of the RBCs in these people contrasts sharply with the state of RBCs in persons suffering from paroxysmal nocturnal hemoglobinuria (PNH). PNH III RBCs have a gross deficiency of all GPI-linked glycoproteins, of which DAF is just one.[42]

WES[a], WES[b]

In 1987, a low-incidence antigen, WES, was described in the Finnish population, with an incidence of 0.6 percent.[43] The antibody was first detected during routine compatibility testing using serum from a 77-year-old woman. Subsequently, many more examples of the antibody were identified when WES+ RBCs were used to screen donor plasma samples. Family studies showed WES to be inherited as an autosomal dominant characteristic. Two further examples of WES+ RBCs were found in 392 samples from Black American blood donors, and five WES+ donors were found in a population of 245 Black North London donors. In a study of 3072 American donors drawn in eight different blood centers across the United States, seven of 1460 Black donors and two of 1612 White donors were WES+, giving an incidence of 0.48 percent and 0.12 percent, respectively.[44]

Daniels et al.[45] described an antibody to a high-incidence antigen produced by a Black WES+ prenatal patient (Wash). At delivery, the infant's RBCs had a weakly positive direct antiglobulin test (DAT) result and the antibody could be eluted, but there was no evidence of clinical HDN. The antibody failed to react with a presumed WES+ homozygote of Finnish origin (Hel). Both the proposita's RBCs and those of Hel were positive for Cr[a], Tc[a], Dr[a], and other Cromer-related antigens. The Wash serum reacted with RBCs lacking

high-incidence Cromer-related antigens but showed weak reactivity only with Dr(a–) RBCs and was nonreactive with RBCs of the Inab phenotype. Additionally, the serum failed to react with α-chymotrypsin-treated or pronase-treated RBCs. It was proposed that the antigen recognized by the Wash serum was antithetical to WES. The low-incidence antigen WES was renamed WES[a] and the high-incidence antigen was named WES[b].

Molecular characterization of WES[a] and WES[b] shows that the antigens are encoded by a single nucleotide difference in codon 48. A point mutation 245G>T of the wild type *DAF* results in the loss of the WES[b] antigen and expression of WES[a]. At the protein level, this encodes a change of Arg48Leu on SCR1 of DAF. Tests with anti-WES[b] showed that Es(a–) RBCs react less strongly than Es(a+) RBCs; additionally, WES(a+b–) RBCs react very weakly with anti-Es[a], reactivity only being demonstrable by adsorption/elution tests.[28] Since the amino acid responsible for the Es[a] antigen is Ile46, just two residues away, this finding is not surprising.[17]

UMC

UMC, another high-incidence antigen of the Cromer blood group system, was reported in 1989.[46] The only example of anti-UMC described was found in the serum of a Japanese blood donor. Although untransfused, the proposita had three children. The antibody reacted with all panel RBCs tested, including RBCs lacking known high-incidence Cromer antigens, although the RBCs of one sibling were compatible, as were the RBCs of a patient with PNH and those of the Inab phenotype. Testing of 45,610 Japanese blood donors did not reveal another UMC– individual. The molecular basis of the UMC– phenotype was shown to be another single nucleotide polymorphism: a change of 749C>T, which encodes Thr216Met on SCR4 of DAF.[17]

GUTI

An antibody in the serum of a Canadian blood donor of Chilean descent (anti-GUTI) was strongly reactive with a panel of RBCs of common phenotype. Tests following different protease treatments showed the antibody reactivity was ablated following treatment of test RBCs with α-chymotrypsin or pronase. Treatment with papain, trypsin, or 200 mM dithiothreitol (DTT) did not affect reactivity. Subsequent tests with RBCs known to lack high-incidence antigens in the Cromer system and with PNH III RBCs showed that the plasma contained an antibody to a novel antigen in the Cromer blood group system. Molecular analysis of the donor's blood identified a polymorphism, 719G>A in DAF, that corresponded to an amino acid change of Arg206His in SCR4 of DAF.[47] Immunoblotting analysis using anti-GUTI with DAF deletion mutants confirmed the location of the antigen on SCR4.

The donor's parents and two daughters were shown by PCR-RFLP analysis to be heterozygous for the mutation. One sister was homozygous for normal *DAF*. Another sister was homozygous for the 719A mutation and her RBCs were compatible with anti-GUTI. Screening of more than 1000 North American blood donors did not reveal any additional GUTI-negative RBCs. PCR-RFLP analysis of DNA samples from 114 Native Chileans showed that the polymorphism was present with an incidence of 5.3 percent.[47]

Characteristics of Cromer Antigens

Cromer blood group antigens are destroyed by treatment with α-chymotrypsin or pronase, but they are not affected by treatment with trypsin, papain, or ficin.[28,48] Reducing agents such as 2-aminoethylisothiouronium bromide (AET), 2-mercaptoethanol (2-ME), or DTT weaken the antigens but generally do not disrupt them completely.[28] This is somewhat surprising, as DAF is known to have a number of intrachain disulfide bonds and the protein is detectable by immunoblotting techniques only under nonreducing conditions.[4]

Although DAF is a cell-membrane bound glycoprotein, small amounts are present in the plasma and other body fluids of normal individuals.[5,49] Hemagglutination inhibition studies have shown that antibodies to Cromer antigens may be inhibited by concentrated plasma and urine from an antigen-positive person.[28] In one study by Judd et al,[50] a commercial preparation of human platelet concentrates for immunohematologic use was shown to adsorb anti-Cr[a] from four different sera. They warned of the potential hazards of using such preparations, designed for the removal of HLA antibodies, in the adsorption of potentially clinically significant antibodies.

Clinical Significance of Cromer Antibodies

Since persons lacking the various high-incidence antigens of the Cromer blood group system are rare, there are few suitable donors for patients who are sensitized. In vivo and in vitro survival studies have been used to determine the consequences of transfusing

antigen-positive RBCs to sensitized patients. In vitro studies, such as the mononuclear phagocyte assay (MPA) or the monocyte macrophage assay (MMA), suggested that these antibodies could be clinically significant in vivo.[12,50–54] A number of in vivo ^{51}Cr-labeled red cell survival studies with Cr(a+) RBCs have been reported.[20,37,50,53,55–57] With the exception of one case,[57] increased destruction of incompatible RBCs, albeit variable, was observed. However, Smith et al.[20] reported the uneventful transfusion of a patient with anti-Cr[a] with two units of Cr(a+) RBCs. Based on the results of a ^{51}Cr survival study in one patient, Ross and McCall proposed that incompatible blood could be transfused "without significant untoward effect."[55] In a case described by Whitsell and Oxendine, normal survival of Cr(a+) RBCs was observed in a patient with anti-Cr[a].[57] The antibody was reactive in an MMA and was IgG. Two incompatible units were transfused successfully, as determined by the absence of clinical signs of hemolysis. In one study, four examples of anti-Tc[a] gave significant values in an MPA (M. Schanfield, unpublished observations). Kowalski et al.[58] reported a hemolytic transfusion reaction in a woman who was transfused with three units of crossmatch-compatible RBCs. Seven days posttransfusion, the patient's hemoglobin had dropped to 5.5 g/dL with no overt signs of bleeding. Anti-Tc[a] was identified in the posttransfusion serum and an MMA was strongly reactive. Anderson et al.[56] observed decreased red cell survival of incompatible RBCs in a patient with anti-Tc[a] and there was no increase in antibody titer. RBC survival at 16 days was normal.

Although the antigens of the Cromer blood group system are well developed on fetal RBCs, there have been no cases of HDN attributed to these antibodies. This might appear somewhat anomalous, as Cromer antibodies are mostly IgG, predominantly of the IgG1 subclass.[43,53,59] Elegant studies on human trophoblast cells by Holmes et al.[60] showed that there was an elevated concentration of DAF on cells at the immediate fetomaternal interface. They proposed that the elevated concentration of DAF in the trophoblast is a protection mechanism of the fetus against complement-mediated maternal attack. Three different reports provide evidence for this theory: three women whose serum contained anti-Cr[a] with titers that exceeded 64 in the first trimester demonstrated loss of reactivity of the antibody as the pregnancy progressed; in one case to undetectable levels at delivery. Another woman, whose serum contained anti-Dr[a] with a titer of 512 at 22 weeks, had no detectable antibody at delivery. The DAT performed on the cord RBCs of all four babies was negative and there was no evidence of HDN.[22,61,62] In two of the cases, the antibody was detected again in the serum of both women postdelivery, suggesting an adsorption mechanism rather than the cessation of antibody production.[61]

Conclusion

The Cromer blood group system is a polymorphic system of antigens on DAF that are characterized serologically by their sensitivity to α-chymotrypsin and pronase. With the exception of IFC, molecular analysis has shown that each antigen is the result of a single nucleotide change in DAF. In general, the different phenotypes demonstrate an ethnic bias that suggests a spontaneous mutation in that population. The recent identification of the GUTI antigen suggests that there might be other rare polymorphisms of DAF that await discovery.

Acknowledgments

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Significance of platelet-reactive antibody screening for patients facing frequent platelet transfusions

T. Tasaki, K. Fujii, K. Gotoh, S. Satoh, J. Takadate, S. Sasaki, M. Tachibana, and K. Yamamoto

It is not clear whether platelet-reactive antibody screening is clinically significant for patients facing frequent platelet transfusions. On the basis of data from 96 patients who had been examined for platelet-reactive antibodies by the mixed passive hemagglutination method for a variety of reasons, we investigated the following three issues retrospectively: (1) the relationship between platelet-reactive antibodies and the occurrence of problems in platelet transfusions, such as refractoriness or nonhemolytic reactions; (2) the influence of a history of transfusion on the production of those antibodies; and (3) the effect of screening for those antibodies on the prompt administration of appropriate platelet components. More than half of the platelet transfusion-related problems were associated with platelet-reactive antibodies. For patients with a history of transfusion, the mean period before a clinical problem occurred with platelet transfusions was 9 days, compared with 66 days for those without such a history. Accordingly, during the period, patients with a history of transfusions received fewer units of platelets and had fewer donor exposures than did patients without such a history. On the other hand, most patients who had been screened in advance for those antibodies received appropriate platelet components without delay, whereas an average of 10 days was needed before those who had not been screened received compatible platelets. The patients who had not been screened were transfused with 68 units of random platelets on average during the period. When frequent platelet transfusions are anticipated, especially for patients with a history of transfusion, screening for platelet-reactive antibodies beforehand would be helpful for prompt administration of appropriate platelets, although problems, such as the cost of those platelets and the burden on donors, remain to be resolved. *Immunohematology* 2002;18:104–108

Platelet transfusion is an important supportive therapy for the treatment of hypoplastic thrombocytopenia following intensive chemotherapy for hematologic and/or malignant diseases. In Japan, the number of platelet components supplied yearly continues to increase. One of the distressing problems in platelet transfusion is the development of refractoriness to platelets, a state in which the number of platelets does not increase despite consecutive transfusions. When platelet-transfusion refractoriness occurs, its cause should be investigated to distinguish between alloimmunization and nonimmune mechanisms such as disseminated intravascular coagulation, splenomegaly, and infection, using a platelet corrected count increment (CCI)[1] and the patient's clinical features. These efforts do not always identify the cause of the refractoriness. When the cause turns out to be platelet-reactive antibodies, usually HLA antibodies, HLA-matched leukocyte-reduced platelets are prepared. This process is somewhat complex and time-consuming; depending on the patient's needs, the process might include characterizing the alloantibodies, selecting HLA-matched donors, crossmatching, and performing plateletpheresis. Therefore, like screening for allo-antibodies to red blood cell (RBC) antigens before RBC transfusion, it seems desirable to periodically screen the sera of patients facing frequent platelet transfusions for platelet-reactive antibodies; and, when screening results are positive, to identify the antibodies. Since 1997, we have been screening for platelet-reactive antibodies using a mixed passive hemagglutination (MPHA)[2] method. The purpose of this report is to evaluate the efficacy of the screening retrospectively. The relationship between those antibodies and transfusion-related problems was also reviewed in terms of the presence or absence of a history of transfusions.

Methods

Patients

From March 1997 to April 2001, a total of 1934 specimens, from 520 patients with various diagnoses, were tested for platelet-reactive antibodies in accordance with physicians' instructions at the division of blood transfusion and testing laboratory of Iwate Medical University Hospital. For 424 of those patients, such testing was performed only once or no platelet transfusion was given. Therefore, data from the remaining 96 patients were available for retrospective assessment. These patients fell into three broad groups, according to the reason why the testing had been ordered: (A) patients with platelet-transfusion refractoriness defined as a less-than-expected increment of platelets after at least two consecutive platelet transfusions; (B) patients with nonhemolytic transfusion reactions (NHTR) such as fever, urticaria, anaphylactic shock, etc., upon receiving platelets; (C) patients facing frequent platelet transfusions at the time they were admitted to our hospital. In other words, tests for groups A and B were carried out to clarify the association between platelet-reactive antibodies and clinical problems in the course of supportive therapy with platelets. In group C, on the other hand, the MPHA test that was performed before the initial platelet transfusion at our hospital was considered screening for those antibodies.

Information such as transfusion history, diagnosis, and clinical course was obtained directly from the patients and/or their medical records. As antibodies can sometimes be detected at 2 to 6 months after the primary immune response, patients were regarded as "having a history of transfusion" if they had received blood components more than a year before admission to our hospital. Details of the blood components that had been used in our hospital came from transfusion records.

MPHA test as a screen for platelet-reactive antibodies

The MPHA test for the detection of platelet-reactive antibodies to human platelet-specific antigens (HPA) was developed by Shibata, et al.[2] Since platelets have HLA class I antigens in addition to HPA, MPHA has been widely used in Japan to screen for HPA and HLA antibodies, which together are called platelet-reactive antibodies. The testing procedure is quite simple[2]: A serum sample is placed in a U-type well where platelets with known antigen specificity (HLA and HPA) have already been fixed. The next step is the addition of

washed sheep RBCs coated with purified antibodies to human IgG, serving as indicator cells. The plate is then placed on a damp towel for 3 hours at room temperature. The interpretation of the result was straightforward, as the reactive patterns were judged by the naked eye. Since a diffuse sedimentation pattern implies simply that a patient has some antibody against HLA and/or HPA, further examination using the standard microlymphocytotoxic (LCT) assay[3] was used to identify specificity. In this hospital, a commercially available MPHA test kit called anti-PLT•Olybio MPHA II (Olympus®, Tokyo, Japan) was used.

LCT

The standard LCT assay using a panel of 15 lymphocytes covering almost 95 percent of the known HLA specificities in Japan was performed to characterize the antibodies. In short, 1 µL of prepared lymphocytes was placed in each well with 2 µL of patient serum. The mixture was left at room temperature for 30 minutes, followed by the addition of 5 µL of rabbit-derived complement. After 60 minutes of incubation, 3 µL of eosin was added and the mixture was left for an additional 3 minutes. Finally, formalin (8 µL) was injected and the panel was evaluated under a phase-contrast microscope for serum reactivity against lymphocytes. When more than 20 percent of the cells in the well were killed, the serum was judged positive for HLA antibody and was further examined for its specificity.

Frequency of testing and assessment of platelet transfusions

Sera from patients with platelet-transfusion refractoriness and/or NHTR were examined for platelet-reactive antibodies, using both the MPHA and LCT methods. For patients without such issues, the MPHA test was carried out as a screening for those antibodies at least twice a week during the period of consecutive platelet transfusions. When the specificity of antibodies was clarified, crossmatch-negative ABO-compatible platelets from a donor without HLA that react to those antibodies (i.e., HLA-matched platelets in a narrow sense) were transfused through a leukocyte reduction filter. Until then, random, single-donor ABO-compatible platelets were used.

The effectiveness of platelet transfusions was judged from the results of CCIs: platelet-transfusion refractoriness due to an immune mechanism was diagnosed when the increase at one hour after transfusion was below 5000/µL. When the increase at 1 hour was normal (> 7000/µL) but that at 20 hours

was low (< 4000/μL), a nonimmune mechanism was suspected.

Statistical analysis

Statistical analysis of the results was based on the unpaired t test; i.e., the F test for equality of variances was carried out first and, depending on the results, either Student's t test or Welch's t test followed. We considered a variance significant when the p value was < 0.05.

Results

Table 1 shows the number of patients distributed into the three groups described in the Methods section. Of the 96 patients, 87 (97%) had diagnoses of hematologic or malignant diseases.

Table 1. Categorization of group A, group B, and group C patients by disease

Disease	Group A*	Group B†	Group C‡	
Leukemia	7	7	10	
Hematologic diseases other than leukemia	11	6	25	
Malignancy other than hematologic diseases	5	7	9	
Other	1	1	7	
Total	24	21	51	96

*Patients with platelet-transfusion refractoriness
†Patients with NHTR with platelet transfusions
‡Patients facing frequent platelet transfusions

Table 2 shows the results of platelet-reactive antibodies evaluated by the MPHA and LCT. Of the 24 patients with platelet-transfusion refractoriness in group A, 13 were positive by both MPHA and LCT, while ten were negative for both tests. However, the results for four of these ten cases simultaneously turned positive at a later date (Table 2). Finally, 17 (71%) out of 24 patients with platelet-transfusion refractoriness produced platelet-reactive (HLA) antibodies. These cases had, without exception, low CCIs at 1 hour after platelet transfusions. For four of the remaining seven cases, a nonimmune mechanism was thought to be the cause of platelet-transfusion refractoriness, taking into account CCIs and clinical features; in three cases, however, the reason for the low level of CCIs at 1 hour was not clarified.

In group B (Table 2), of the 21 patients with some adverse reactions in association with platelet transfusion, nine showed positive results by MPHA. Seven of them were also positive for the LCT; however, one remained negative for the LCT, probably due to antibodies against HPA or antibodies that were

Table 2. Results of MPHA and LCT for groups A, B, and C

Patients with platelet-transfusion refractoriness—Group A

		LCT			
		Pos	Neg	NT*	Total
MPHA	Pos	13	0	0	13
	Neg	0	10(4)†	1	11
					24

Patients with nonhemolytic transfusion reactions with platelet transfusion—Group B

		LCT			
		Pos	Neg	NT*	Total
MPHA	Pos	7	1	1	9
	Neg	4(2)†	4	4	12
					21

Patients facing frequent platelet transfusions—Group C

		LCT			
		Pos	Neg	NT*	Total
MPHA	Pos	17	2(1)†	4	23
	Neg	1(1)	11(9,2)	16	28
					51

*Not tested
†Numbers in parentheses are for cases with subsequent positive results for MPHA and/or LCT. Details are described in the Results section of this paper.

adsorption-positive but cytotoxicity-negative. Of the 12 patients with negative results by MPHA, four were positive for the LCT. Of particular interest was the observation that two of them later became positive by MPHA, which suggested that the LCT was more sensitive than the MPHA. The specificities of HLA antibodies for the four cases were anti-B7, -B67; anti-A31, -A33; anti-A11, -A26; and multispecific, respectively. Of the 21 patients in Group B, 13 (62%) were, in the end, positive for platelet antibodies that might be associated with transfusion-related reactions.

Table 2, for group C, shows the results of platelet-reactive antibody screening tests for 51 patients faced with a high likelihood of frequent platelet transfusions; the tests were conducted upon their admission to our hospital. Of these 51 patients, 23 were already MPHA-positive, of which 17 were positive by both the MPHA and LCT. Of two cases that were initially MPHA-positive and LCT-negative, one had an LCT-positive result thereafter. The specificity of the HLA antibody was anti-B67. Twenty-eight of the 51 patients were negative for the initial MPHA test; however, one was already LCT-positive (anti-A11, -B35, -B39) and was found to be MPHA-positive by subsequent testing. Eleven patients were negative by both MPHA and LCT at first, but nine of them turned positive for both tests, and the remaining two ultimately tested positive only by the

Table 3. Transfusion history and platelet-reactive antibody production

History of previous transfusions	Red cell transfusion		Platelet transfusion		Period (days)*
	Units[†]	Donors[‡]	Units[†]	Donors[‡]	
Yes (n=21)	7.1±6.9	3.9±3.8	67.7±34.9	5.3±3.4	9.3±3.3
No (n=16)	13.7±6.2	7.1±3.3	129.4±75.3	8.8±5.8	66.2±32.1
p values	0.005	0.014	0.002	0.027	< 0.001

All values are shown as mean ±SD (range)

*Period (days) before a patient experienced platelet-transfusion refractoriness and/or encountered adverse reactions (groups A, B)

†Number of units (red blood cells or platelet components) transfused to one patient during the period

‡Number of donors to whom one patient was exposed during the period

LCT (Table 2). Specificities of the HLA antibodies for those two cases, respectively, were anti-B51 and -B52; and anti-A26, -A33, -B51, and -B52. Since the results of the subsequent MPHA test for 16 patients remained negative, the LCT was not carried out.

Table 3 shows the effect of transfusion history on the production of platelet-reactive antibodies based on data from a total of 37 cases in groups A, B, and C. The subjects chosen for this investigation consisted of 13 cases from group A, 13 from group B, and 11 from group C. We considered that the 26 patients from groups A and B had experienced platelet-transfusion refractoriness and/or had some reactions due to platelet-reactive antibodies provoked by consecutive platelet transfusions in our hospital. Data from 11 patients in group C whose sera turned positive for platelet-reactive antibodies by MPHA, LCT, or both were also added to this investigation. The 37 cases were divided into two groups, depending on the history of transfusion and the number of units of blood components transfused, or the number of days before antibodies were produced. The mean number of days before a patient had a positive result for the tests was 9.3 for patients with a history of transfusion and 66.2 for those without such a history. The overall average was 33.9 days. For the patients with a history of transfusion, the number of units of blood components transfused and the number of donor exposures in this hospital were significantly smaller than those for the patients without a history of transfusion.

For 24 patients (13 group A, 11 group B) with HLA antibodies of identified specificity, the mean length of time before the transfusion of compatible platelets was 10.7 days and the amount of random but single donor-derived apheresis platelets transfused was 68.0 units. However, seven of 11 patients (group C) who became seropositive for platelet-reactive antibodies by the screening test received no random platelets thereafter, and the other four patients received the following: 20 units (1 donor, 1 day before receiving compatible platelets), 20 (1,1), 40 (2,2), and 40 (2,3), respectively.

Discussion

Once a patient becomes refractory to platelet transfusion, treatment with random platelet transfusions occasionally becomes ineffective.[4] In such cases, HLA-matched platelets are required. However, this process is time-consuming. Therefore, along the same lines as screening for RBC antibodies before RBC transfusion, a platelet-reactive screening test followed by characterization of the antibodies in advance would be meaningful for prompt preparation of appropriate blood components for patients with a greater likelihood of receiving a platelet transfusion.

The relationship between transfusion history and rapid production of alloantibody induced by secondary stimulation of antigens is well known as an anamnestic immune response. The amount of platelet-reactive antibodies might initially be too low to detect and/or too low to bring about clinical symptoms. We assumed that the day when patients in groups A and B experienced platelet-transfusion refractoriness and/or encountered adverse reactions during the course of treatment was the day when platelet-reactive antibodies had risen significantly due to stimulation by platelet transfusion.

Concerning the sensitivity of the MPHA method for detecting platelet-reactive antibodies, some reports have found that this test's ability is by no means inferior to that of the LCT.[2] In this study, most of the results of the MPHA and LCT paralleled each other; however, some cases in groups B and C initially were MPHA-negative and LCT-positive, results that later changed to MPHA-positive, and vice versa. Although the principles underlying these methods are different, other reasons for changes in results could be differences in the spectrum of antigens tested and/or characteristics of the antibodies.[5]

The MPHA test is quite simple in technique and is able to detect HLA as well as HPA antibodies.[2] This method would be acceptable as a screening test for the speedy detection of those antibodies, if such problems as cost and labor involved with transfusion of expensive HLA-matched platelet concentrates (about 20% more expensive than random platelets) or frequent or massive transfusion of random platelets could be resolved. In one report, a platelet apheresis program for the supply of HLA-matched platelets was

less expensive than that of random platelets.[6] These results should be compared with those in cases of patients receiving leukocyte-reduced or just crossmatch-negative platelets.[7,8] The selection of platelets according to the patient's HLA antibody specificity has been reported to be as effective as HLA matching or crossmatching.[9]

It cannot be denied that unnecessary HLA-matched platelet transfusions were performed during the study period, and we recognize that one disadvantage to this study is that we did not identify the number of women who had ever been pregnant. Therefore, more prospective controlled studies must be conducted before we can know the real advantage of a screening test for platelet-reactive antibodies.

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Clinically significant autoimmune hemolytic anemia with a negative direct antiglobulin test by routine tube test and positive by column agglutination method

M. LAI, C. RUMI, G. D'ONOFRIO, M.T. VOSO, AND G. LEONE

In two to five percent of cases of autoimmune hemolytic anemia (AIHA), the patient's RBCs are negative in the direct antiglobulin test (DAT). We describe a patient with AIHA with a negative DAT when tested by the traditional tube test but with a strongly positive one when tested by column agglutination. When the DAT was repeated by tube test using 4°C saline washes, it became positive. This phenomenon has been observed when low-affinity antibodies are involved in AIHA. The patient's history and serologic findings are detailed in this report. *Immunohematology* 2002;18:109–113.

Key Words: direct antiglobulin test, column agglutination method, autoimmune hemolytic anemia, low-affinity antibodies

In some studies, autoimmune hemolytic anemia (AIHA) with a negative direct antiglobulin test (DAT) had a frequency between 2 percent and 5 percent.[1–3] In other reports, authors have suggested autoantibody characteristics and laboratory errors as possible causes for AIHA with a negative DAT.[1,3–11] In some cases, an AIHA with a negative DAT may have life-threatening pathology.[7] In this study we describe a case of AIHA with a negative DAT when performed by a traditional tube test (TT), but a strongly positive one when it is performed by the column agglutination test (CAT). The DAT by TT became positive when the wash steps were performed with saline cooled to 4°C, the "cold-wash" method.[2,3,6,12] This phenomenon has previously been associated with AIHA due to "low-affinity" antibodies.[3] Affinity maturation is a process that leads to increased affinity of the antibody for its antigenic determinant.[13] In a study of a murine model of AIHA,[14] it was suggested that the affinity maturation process of an antibody could not play a critical role in the production of autoantibodies with pathologic consequences.[14–17] In fact, in spite of their misleading name, the "low-affinity" antibodies are characterized by a high pathogenic potential and are capable of causing severe hemolysis in humans.[14] The "low-affinity" antibodies associated with AIHA are more often of the warm type; however, the cold type may be involved. A patient's history and serologic findings are detailed in this report, and an analysis of the possible cause of a negative DAT when the test is performed by TT is presented.

Case Report

The patient is a 44-year-old female admitted to the hematology department with a 10-day history of malaise and jaundice, with no previous history of anemia or transfusion. A physical examination revealed pallor and jaundice accompanied by splenomegaly.

The hemoglobin level at diagnosis was 4.6 g/dL, reticulocyte count 119×10^9/L, bilirubin level 3.9 mg/dL (direct 0.3 mg/dL), LDH 1167 UI/L, and the urine was positive for bilirubin and hemoglobin. Abdominal ultrasonography revealed an enlarged spleen, but without lesions. The liver was within normal limits, and no lymphadenopathies were detected in the abdominal region. In the acute phase and in the convalescent phase, serologic studies were negative for cytomegalovirus (CMV), Epstein-Barr (EB) virus, Mycoplasma, HCV, HBV, HAV, and HIV 1 and 2. Additional negative laboratory tests included those for nuclear antibodies, rheumatoid factor, and the Donath-Landsteiner (D-L) antibody. The DAT performed by the

TT was negative both macroscopically and microscopically. The indirect antiglobulin test (IAT) performed by the CAT was positive with a low titer. The tests for cold agglutinins and cryoglobulins were both negative. After admission to our hematology department the patient was started on a regimen with methylprednisolone 100 mg/day. The steroid treatment gave positive results with stable hemoglobin values at 11–12 g/dL after 4–6 weeks. During the first 2 weeks after diagnosis, four RBC units were transfused.

Materials and Methods

All of the patient's samples were tested within 2 hours in both the acute and convalescent phases. The original TT DAT was performed as follows: a 3 percent suspension of patient RBCs was dispensed into a tube and washed × 3 with saline at room temperature. The final wash was completely decanted; immediately, a polyspecific anti-human globulin (AHG) reagent (Immucor Inc., Norcross, GA) was added to the tube and the contents mixed. The tube was centrifuged according to the manufacturer's directions for 15 seconds at 900 × g. At diagnosis, the DAT was carried out in two tubes with the following changes: before the polyspecific AHG reagent was added, the washing steps were performed in the first tube using saline warmed to 37°C and in the second tube using saline cooled to 4°C. After centrifugation, the tubes were observed for agglutination macroscopically and microscopically and graded and scored according to Marsh.[18] For the positive control, RBCs coated with IgG were used (Ortho-Clinical Diagnostics®, Raritan, NJ). As negative controls, RBCs from normal donors were washed at 4°C before a DAT. The TT DAT was further tested using monoclonal anti-IgG, -IgA, -IgM, -C3c, and -C3d (Biotest Diagnostics Corp., Denville, NJ).

The CAT DAT was performed using Ortho-Clinical Diagnostics® Coombs ID-Cards, following the manufacturer's instructions and without any wash step. ID-Diamed Cards (DiaMed AG, Cressier, Switzerland) equipped with microcolumns with anti-IgG, -IgA, -IgM, -C3c, -C3d, and a negative control were used to determine the immunoglobulin class on the RBC surface. Agglutination strength was graded according to manufacturer's directions.

Cold agglutinin test

The cold agglutinin test was carried out with a 3 percent saline suspension of adult group O RBCs, cord RBCs, and adult group O enzyme-treated RBCs. Three drops of each serial dilution of the serum that had been separated at 37°C were added to one drop of each RBC sample. After incubation for 1 hour at 4°C, each tube was centrifuged and macroscopically examined for agglutination. The tubes were also examined after incubation at room temperature and centrifugation.

The Donath-Landsteiner test

The D-L test used patient serum from a freshly collected blood sample maintained at 37°C, group AB fresh serum, and a 50 percent suspension of group O RBCs tested for the presence of the P antigen. The mixture was incubated for 30 minutes at 0°C (melting ice), observed for the presence of hemolysis, and then incubated for 1 hour at 37°C; this was followed by mixing, centrifugation, and examination for hemolysis.

Indirect antiglobulin test

IAT screens were performed using the CAT cards and RBC panels for IAT (BioVue Screen™, Ortho-Clinical Diagnostics®), following the manufacturer's instructions. Antibody serum identification was performed using the RBC panel Resolve C® (Ortho-Clinical Diagnostics) and the IgG-CAT (Ortho-Clinical Diagnostics), following the manufacturer's instructions. For serum antibody titration we used the CAT IgG cards and a group O RBC reagent, Selectogen® (Ortho-Clinical Diagnostics), with a serial twofold dilution of the serum, following manufacturer's instructions.

IAT screens were also performed versus ficin-treated RBCs (Ortho-Clinical Diagnostics BioVue Screen™ FICIN) to rule out the presence of glycophorin autoantibodies, which are nonreactive versus enzyme-treated RBCs.

Eluate

An eluate was made using the Elukit II™ for rapid acid elution (Gamma Biologicals, Inc., Houston TX), following the manufacturer's instructions except for the washing solution that was replaced with saline cooled to 4°C. To eliminate precipitate and cellular debris, the eluate was recentrifuged at 5000 rpm, and then the eluate was transferred to a clean tube for examination. Specificity and titer for the eluate antibody were performed as described for the serum antibody.

Results

The RBCs of the patient were group O, C+D+E–c+e+, K–k+. The DAT performed with the TT at diagnosis (Table 1) was repeatedly negative when the RBCs were washed at room temperature and at 37°C. The DAT was positive with agglutination strength of 3+ when the RBCs were washed with saline

Table 1. Results of direct antiglobulin tests (DATs)

DAT	Room Temp.*	37°C*	4°C*	CAT†
At diagnosis	neg	neg	3+	4+
Week 1	neg	ND‡	ND	4+
Week 2	neg	ND	ND	4+
Week 3	neg	ND	ND	3+
Week 4	neg	ND	ND	2+
At 6 mos	neg	ND	ND	1+

*Tube tests
†Column agglutination test
‡Not done

Table 2. Results of indirect antiglobulin tests (IATs) and eluates by CAT

	IAT	IAT titer	Enz*	Eluate (4°)	Eluate titer
At diagnosis	4+	8	4+	2+	4
Week 1	4+	8	4+	ND†	ND
Week 2	4+	4	4+	ND	ND
Week 3	2+	4	3+	ND	ND
Week 4	2+	4	2+	ND	ND
At 6 mos	0	0	0	ND	ND

*Versus ficin-treated RBCs
†Not done

Table 3. Results of direct antiglobulin tests (DATs) using the column agglutination test and monospecific antisera

	IgG	IgA	IgM	C3c	C3d
At diagnosis	3+	Neg	Neg	Neg	.5+
Week 1	3+	Neg	Neg	Neg	.5+
Week 2	3+	Neg	Neg	Neg	.5+
Week 3	2+	Neg	Neg	Neg	.5+
Week 4	1+	Neg	Neg	Neg	.5+
At 6 mos	1+	Neg	Neg	Neg	Neg

cooled to 4°C. As a control for autoagglutination, the patient's RBCs, washed at 4°C, were suspended in albumin, with a negative result. In addition, normal donor RBCs, washed at 4°C, were DAT negative. The D-L test was also negative.

The DAT performed at diagnosis by a CAT was 4+ positive. The DAT CAT remained 4+ at week 1 and week 2 (Table 1). The agglutination strength was reduced to 3+ at week 3 and to 2+ at week 4. After 6 months the DAT with the CAT was still 1+ positive.

The eluate made at diagnosis by washing the patient's RBCs with saline at room temperature gave a negative result (Table 2). The same test performed by washing the RBCs with saline cooled to 4°C was 2+ positive for IgG by the CAT. A panagglutinin was eluted from the RBCs.

The DAT performed at diagnosis (Table 3), using the CAT cards equipped with monospecific antisera (IgG, IgM, IgA, C3c, and C3d), was positive for IgG (3+) and C3d (0.5+); the same result was obtained at week

1 and week 2. After week 3 the DAT was positive for IgG (2+) and C3d (0.5+). At week 4, it was positive only for IgG (1+); 6 months after the diagnosis the CAT DAT was still positive for IgG (1+).

The IAT at diagnosis (Table 2) was 4+ positive both by CAT and versus ficin-treated RBCs. The titer was 8. The IAT was still positive after 4 weeks with an agglutination strength of 2+ and a titer of 4. After 6 months the IAT was completely negative. The IAT antibody specificity performed at diagnosis with a panel of 11 untreated RBCs and AHG ID cards yielded a panagglutin with an agglutination strength of 4+ for each RBC tested.

Discussion

In this report we describe a case of severe AIHA in which the DAT performed with the routine TT was negative, but positive when performed with the "no-wash" CAT. The patient was a 44-year-old woman with severe AIHA sensitive to steroid therapy. The patient's hemoglobin level in the acute phase dropped to 3.9 g/dL, and urine was positive for bilirubin and hemoglobin. In patients with AIHA a negative DAT may result from many causes, such as the amount of immunoglobulins on the RBC surface,[5,16,17,19] involvement of immunoglobulins other than IgG, selective antibody activity,[4–11] depression of antigenic determinants on the RBC surface,[8–11] or the involvement of multiple immunoglobulins. Nevertheless, in our case, none of these explanations is in agreement with the strong agglutination strength obtained with the CAT and the positive DAT obtained with the TT "cold-wash" method.

The most plausible explanation for the negative DAT with the routine TT in our case was the involvement of a warm "low-affinity" antibody.[12] In this condition most of the antibody is lost when the RBCs are washed with saline at room temperature. The use of cold saline (4°C) for the wash step permits stabilization of the bond of the antibody to the RBCs. In our study, the DATs performed by washing the RBCs with saline cooled to 4°C were positive, with an agglutination strength of 4+. The TT DAT was positive for IgG only when a cold wash was used and for IgG and C3d when the CAT was performed with monospecific antisera.

Our observation is in agreement with a previous report in which the authors had shown that, in a patient with warm AIHA with "low-affinity IgG" antibodies on the RBCs, the DAT performed with the CAT was strongly positive. Unfortunately, in this

previous work, the DAT with the CAT was performed only in one of the two patients with AIHA and "low-affinity" antibodies. Since the CAT does not include washing steps, the elution of "low-affinity" auto-antibody may be avoided but the eluate may yield a negative result. In our case, the CAT eluate was positive only by washing the RBCs with saline cooled to 4°C. This means that the involvement of a "low-affinity" antibody must be kept in mind in the presence of a positive DAT with the CAT plus a negative DAT with the TT and a negative eluate.

It is most important to improve methods that are capable of detecting "low-affinity" antibodies on the RBC surface. This kind of antibody, in spite of its low incidence, has high pathogenic potential.[14] The CAT, because it is a no-wash method, could be the system indicated for initial testing when AIHA is suspected. This technology would permit a prompt diagnosis when the "low-affinity" antibodies are the cause of the hemolysis. It is especially true for centers where a cold-wash method is not used. The results detailed in this report and in previous studies encourage further investigations to establish whether or not the CAT is effective in detecting "low-affinity" antibodies in AIHA when the DAT is negative with the TT.

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Enzyme and DTT treatment of adherent RBCs for antibody identification by a solid phase immunoassay system

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Treatment of RBCs with protease enzymes or dithiothreitol (DTT) causes denaturation of several RBC antigens and is regularly used in antibody identification. In this study, we have standardized enzyme and DTT treatment of adherent RBCs in the magnetic-mixed passive hemagglutination assay (M-MPHA) for antibody identification. We have also tried drying these treated RBCs. The optimal enzyme and DTT treatment conditions for intact adherent RBCs were determined, in addition to the optimal condition for drying RBCs. All tested agents with the exception of chymotrypsin could be applied to RBC drying, but the optimal condition for drying was different from that for intact RBCs. Enzyme or DTT treatment of adherent RBCs and their application in M-MPHA provide a simple and convenient method for antibody identification. Furthermore, the technique of drying treated RBCs provides an even stronger antibody identification tool, since dried RBCs can be stored for a long period. *Immunohematology* 2002;18:114–119.

Key Words: RBC treatment, solid-phase, M-MPHA, irregular antibodies, antibody identification, enzyme, DTT, RBC drying

Treatment of RBCs with certain enzymes or dithiothreitol (DTT) is useful for antibody identification, but may cause denaturation of certain RBC antigens.[1] Variable results have been reported, i.e., some reports demonstrate antigen denaturation, others report that antigens are unchanged.[2] We believe this variability occurs because RBCs are treated using nonstandard methods.

Several new techniques, namely microcolumn agglutination[3] and solid phase red cell adherence (SPRCA),[4] have been used widely as alternatives to conventional tube tests. These methods contribute to simplification and automation of pretransfusion testing. We have developed a variation of the solid phase method, which we call the magnetic-mixed passive hemagglutination assay (M-MPHA), which is a modification of the mixed-passive hemagglutination assay (MPHA).[5,6]

M-MPHA has high sensitivity and only a few falsely positive reactions are seen. A more advanced technique, M-MPHA-Dry,[7] which uses dried RBCs instead of intact RBCs, has an advantage over the commonly used hemagglutination methods because microplates containing dried RBCs can be stored for long periods.

In the tube test, RBC treatment with enzymes or DTT can be a complicated procedure. However, treatment of RBCs adhering to microplate wells is easy and convenient, since centrifugal washing is not needed and the ratio of RBCs in the treatment agent solution is always kept the same.

In this study, we standardized the treatment of conventional or dried RBCs adhering to microplates with various enzymes and DTT, which are commonly used in serology, for the purpose of applying this information to antibody identification by M-MPHA.

Materials and Methods

Enzymes and DTT

Trypsin and chymotrypsin were obtained from Sigma Chemical Co. (St Louis, Missouri). The enzyme activity listed on the label was used for calculations in this study. Enzyme concentrations were expressed in units (U) per 50 µL. Papain (ID-Papain) and bromelin (ID-Diluent 1) were obtained from DiaMed AG (Morat, Switzerland), and the activity of these enzymes was assayed by the casein method of Hagihara et al.[8] Enzyme activities of ID-Papain (papain) and ID-Diluent 1 (bromelin) were determined to be 163 U and 86.5 U per 50 µL, respectively. DTT was obtained from Wako Junyaku (Osaka, Japan).

Reagent RBCs

Group O reagent RBCs were obtained from Ortho-Clinical Diagnostics (Raritan, NJ).

Antibodies

Human polyclonal reagent antibodies (anti-D, -E, -C, -e, -c, -Fy^a, -Fy^b, -S, -s, -Xg^a, -Js^b, -Kp^a, -Kp^b, -K, -k, -Jk^a, -Jk^b, -Lu^a, -Lu^b, -Di^a, -Wr^a, and anti-HLA B7) were obtained from Ortho-Clinical Diagnostics, Bio Test AG (Dreieich, Germany), or Gamma Biologicals, Inc. (Houston, Texas). Anti-M, -Le^a, -Jr^a, -Rg, bromelin one-stage falsely positive samples, and antibody-negative sera were obtained from donors at the Tokyo Metropolitan Blood Center. These samples were stored at –20°C until used.

M-MPHA

In the first step of M-MPHA, an intact RBC monolayer is immobilized onto a microplate well.[9] Adherent conventional RBCs are incubated with serum antibodies. After washing, either anti-human IgG-sensitized[9] or anti-human IgM-sensitized[10] indicator magnetic particles are added to each microplate well to detect IgG or IgM antibodies on the RBCs. The microplate is then placed on a magnet plate to read the results pattern. In a positive reaction, the indicator magnetic particles are spread over the well; in a negative reaction, they precipitate in the center of the well. Anti-IgG-sensitized particles and anti-IgM-sensitized particles were used for both M-MPHA and M-MPHA-Dry methods. All IgG antibodies, which were detected by either a conventional tube enzyme test or an indirect antiglobulin test, were detected by M-MPHA without using enzyme-treated RBCs.[11]

Treatment of adherent intact RBCs with DTT

RBCs were fixed to microplate wells according to the method of Ohgama et al.[9] Subsequently, 100 µL of 0.0015 M to 0.2 M DTT solutions, which were prepared as serial master dilutions in phosphate buffered saline (PBS) at pH 6, 7, and 8, respectively, were added to the wells. After 30- to 60-minute incubations at room temperature (RT) and at 37°C, plates were washed × 6 with saline. After washing, antisera and negative control sera were tested using anti-IgG-sensitized magnetic particles.[9]

Treatment of adherent intact RBCs with enzymes.

Various concentrations of trypsin (0 to 2560 U), bromelin (0 to 17 U), papain (0 to 30 U), and chymotrypsin (0 to 66 U) per 50 µL were prepared by serial master dilutions with PBS at pH 6, 7, and 8. Chymotrypsin solution was also prepared with a LISS plus glycine solution (Glycine-LISS) at pH 8. Fifty µL of

each enzyme solution was added to the microplate wells with intact RBCs. The solutions were incubated for 10 to 30 minutes at RT or at 37°C, and then plates were washed × 6 with saline. After washing, antisera, including various negative sera, were tested using anti-IgG-sensitized magnetic particles.[9]

Enzyme-treated falsely positive samples

Nine bromelin-treated falsely positive samples found through routine screening of healthy donors by the bromelin one-stage technique were tested with bromelin-treated RBCs by M-MPHA, at the optimal condition of 2 U per 50 µL and pH 6, at RT for 10 minutes. The reactivity of the falsely positive samples was tested with both anti-IgG[9] and anti-IgM[10] indicators. Bromelin-treated falsely positive sera are those that react with reagent RBCs, including autocontrols, but are nonreactive versus papain-treated RBCs.

Drying of enzyme- or DTT-treated RBCs

Optimal conditions for RBC drying were evaluated to develop an enzyme or DTT-treated M-MPHA-Dry. After enzyme or DTT treatment, RBCs were lysed by Triton X-100 and then dried in a vacuum according to the method of Tamai et al.[7] Reactivity of dried RBCs treated under various enzyme and DTT conditions was tested with the various antisera.

Results

Optimal DTT treatment conditions for M-MPHA

The reactivity of anti-K, -k, -Kp^b, and -Js^b was not reduced (compared to untreated RBCs) when the RBCs were treated with DTT in PBS at pH 6, 7, or 8 for 30 minutes at RT. When RBCs were treated with DTT at 37°C for 30 minutes, reactions with all these antibodies were eliminated at concentrations greater than 0.006 M only with PBS at pH 8. A few of the negative sera showed a falsely positive reaction at concentrations greater than 0.1 M DTT. Consequently, the optimal condition was determined to be 0.0125 M DTT, at pH 8, at 37°C for 30 minutes. Under these conditions, the reactions of DTT-treated RBCs with anti-K, -k, -Kp^a, -Kp^b, and -Js^b were negative without falsely positive reactions. Reactivity with anti-Lu^a and -Lu^b, however, did not change (Table 1).

Optimal trypsin treatment conditions for M-MPHA

When RBCs were treated with trypsin in PBS at pH 8 for 10 minutes at RT, reactivity with anti-Lu^a, -Lu^b and -M was eliminated at 1280 U, 320 U, and 1280 U per 50 µL, respectively. When RBCs were treated in PBS at pH

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Table 1. Reactivity of papain-, trypsin-, chymotrypsin-, and DTT-treated RBCs with various antibodies

Antibodies	No. of samples	No. of samples detected versus treated RBCs			
		DTT 0.0125 M pH=8 37°C 30 min.	Trypsin 320 U pH=8 37°C 30 min.	Papain 16 U pH=6 RT 10 min.	Chymotrypsin 4 U Gly-LISS pH=8 37°C 30 min.
D	10	10	10↑	10↑	10↑
C	10	10	10↑	10↑	10↑
E	10	10	10↑	10↑	10↑
e	10	10	10↑	10↑	10↑
c	10	10	10↑	10↑	10↑
S	4	4	4	0	0
s	4	4	4	0	0
M	2	2	0	0	1↓
Xg^a	3	3	0	0	0
Fy^a	9	9	9	0	0
Fy^b	5	5	5	0	0
K	13	0	13	13↑	7↓
k	6	0	6	6↑	4↓
Kp^a	5	0	5	5↑	3↓
Kp^b	9	0	9	9↑	8↓
Js^b	4	0	4	4	4
Jk^a	12	12	12↑	12↑	12↑
Jk^b	9	9	9↑	9↑	9↑
Le^a	2	2	2↑	2↑	2↑
Lu^a	4	4	0	4	0
Lu^b	5	5	0	5	0
Di^a	6	6	6	6	6
Jr^a	8	8	8↑	8↑	8↑
Rg	3	3	1↓	1↓	1↓
Wr^a	1	1	1↑	1↑	1↑
HLA related	5	5	5↑	5↑	5
Negative serum	20	0	0	0	0

↑Antibody reactivity was increased
↓Antibody reactivity was decreased

8 for 30 minutes at 37°C, reactivity with anti-Lu^a, -Lu^b, and -M was eliminated at 40 U, 40 U, and 320 U per 50 µL, respectively. Falsely positive reactions did not occur until 2560 U. Consequently, the optimal conditions were determined to be 320 U, at pH 8, at 37°C for 30 minutes. Under these conditions, trypsin-treated RBCs were nonreactive not only with anti-Lu^a, -Lu^b, and -M, but also with anti-Xg^a. The reactivities of anti-Fy^a, -Fy^b, -S, and -s did not change. In contrast, reactivities with anti-D, -C, -E, -c, -e, -Jk^a, -Jk^b, -Jr^a, -Wr^a, and HLA-related antibodies increased (Table 1).

Optimal chymotrypsin treatment conditions for M-MPHA

The reactivity of chymotrypsin-treated RBCs with anti-Fy^a, -Fy^b, -M, -S, -s, and -Kp^b was not eliminated despite treatment at 66 U per 50 µL in PBS at pH 8 for 30 minutes at RT. Following treatment at 37°C for 30 minutes, however, chymotrypsin-treated RBCs were nonreactive with anti-Fy^a and -S at 8 U and 16 U, respectively, but reactivity with anti-M, -s and -Fy^b was not eliminated until 66 U, and falsely positive reactions occurred at more than 66 U. After treatment at 37°C for 60 minutes, reactivity with anti-Fy^a, -Fy^b, and -S was eliminated at 4U, 33U, and 8U per 50 µL, respectively, but reactivity with anti-M and -s was not eliminated. RBCs that were treated with chymotrypsin in Glycine-LISS at pH 8 for 30 minutes at 37°C caused a falsely positive reaction at more than 8 U per 50 µL. Reactivity with anti-Fy^a, -Fy^b, -S, and -s disappeared at 1 U, but reactivity with anti-M was not eliminated until 8 U. Consequently, from the reactivity with anti-Fy^a, -Fy^b, -S, and -s, the optimal conditions for chymotrypsin treatment were determined to be 4 U in Glycine-LISS, pH 8, at 37°C for 30 minutes. Under these conditions, treated RBCs were nonreactive not only with anti-Fy^a, -Fy^b, -S, and -s but also with anti-Xg^a, -Lu^a, and -Lu^b. In addition, reactivity with anti-M, -K, -k, -Kp^a, and -Kp^b was reduced (Table 1).

Optimal bromelin treatment conditions for M-MPHA

When RBCs were treated with bromelin at RT for 10 minutes with PBS at pH 6, the reactivity with anti-S and -s did not disappear, but the reactivity with anti-Fy^a, -Fy^b, and -M completely disappeared at 0.13 U, 0.13 U, and 0.27 U per 50 µL, respectively. Falsely positive reactions occurred at more than 8.6 U. After RBC treatment, either in PBS at pH 7 or pH 8, reactivity with anti-S disappeared at 4.4 U, but anti-s was still reactive. Based on the reactivity with anti-Fy^a, -Fy^b, and -M and the falsely positive reaction, the optimal conditions were determined to be 2 U at pH 6, at RT for 10 minutes. Eight of nine one-stage bromelin-treated falsely positive samples tested against bromelin-treated RBCs by M-MPHA were detected with anti-IgM-sensitized particles alone, and one was detected with anti-IgG sensitized particles alone.

Optimal papain treatment conditions for M-MPHA

When RBCs were treated with papain in PBS at pH 6, 7, and 8 for 10 minutes at RT, reactivity with anti-Fy^a, -Fy^b, -M, -S, and -s was weakest at pH 6. (Table 2). In more detail, when RBCs were treated at pH 8, their reactivity with anti-Fy^a and -Fy^b disappeared at 0.23 U per 50 µL, but reactivity with anti-M, -S, and -s did not disappear until 30 U. On the other hand, when RBCs were treated at pH 6, the reactivity with anti-Fy^a, -Fy^b, -M, -S, and -s disappeared at 0.03, 0.03, 1.87, 0.94, and 7.5 U per 50 µL, respectively. When RBCs were treated at pH 6.5, reactivity with anti-s did not disappear regardless of treatment and incubation at 37°C for 30

Table 2. Papain concentration (U per 50 μL) at which antibody reactivity disappeared*

pH	Anti-Fy^a	Anti-Fy^b	Anti-M	Anti-S	Anti-s
6	0.03	0.03	1.87	0.94	7.5
7	0.06	0.06	†	0.94	†
8	0.23	0.23	†	†	†

*Treatment was done at RT for10 minutes
†Did not disappear until 30 U

minutes. Falsely positive reactions did not occur until 30 U at pH 6, 7, and 8 (Table 2). Consequently, based on reactivity with anti-Fy^a, -Fy^b, -M, -S, and -s, the optimal conditions were determined to be 16 U per 50 μL at pH 6, at RT for 10 minutes. Under these conditions, treated RBCs were nonreactive with anti-Fy^a, -Fy^b, -M, -S, -s, and -Xg^a (Table 1). On the other hand, reactivity with anti-D, -C, -E, -c, -e, -Jk^a, -Jk^b, -Jr^a, -K, -k, -Kp^a, -Kp^b, -Le^a, and HLA-related antibodies increased (Table 1).

Reactivity of dried treated RBCs

RBCs were treated with papain, trypsin, chymotrypsin, and DTT under various conditions and vacuum dried.[7] The reaction of these dried, treated RBCs with various antibodies was evaluated. Optimal conditions of trypsin and DTT for drying were the same as for conventional RBCs, and the dried RBC reactivity was the same as that of conventional treated RBCs (Table 3).

Table 3. Number of antibodies detected by enzyme- or DTT-treated dried RBCs

Antibodies	No. tested	No. of detected antibodies			
		Intact RBCs	DTT-treated dried RBCs	Papain-treated dried RBCs	Trypsin-treated dried RBCs
D	1	1	1	1	1
C	1	1	1	1	1
E	1	1	1	1	1
e	1	1	1	1	1
c	1	1	1	1	1
S	4	4	4	0	4
s	4	4	4	4	4
M	1	1	1	0	0
Xg^a	1	1	1	0	0
Fy^a	5	5	5	0	5
Fy^b	8	8	8	0	8
K	5	5	0	5	5
k	6	6	0	6	6
Kp^a	5	5	0	5	5
Kp^b	5	5	0	5	5
Js^b	4	4	0	4	4
Jk^a	4	4	4	4	4
Jk^b	6	6	6	6	6
Lu^a	4	4	4	4	0
Lu^b	3	3	3	3	0
Negative serum	20	0	0	0	0

A clear negative pattern could not be obtained with the papain-treated dried RBCs that were treated at more than 3.75 U. Consequently, for drying, the optimal papain treatment condition was determined to be 0.93 U per 50 μL. At this concentration, the reaction of papain-treated dried RBCs with anti-Fy^a, -Fy^b, -M, -S, and -Xg^a was negative but reactivity with anti-s was not completely eliminated. The optimal condition of chymotrypsin treatment for drying could not be determined, since a clear negative pattern could not be obtained under conditions in which anti-Fy^a, -Fy^b, -S, -s, -M, and -Xg^a were negative.

Discussion

K, k, Kp^a, Kp^b, Js^b, Lu^a, and Lu^b antigens are denatured by DTT because DTT disrupts the secondary structure of proteins by reducing disulfide bonds.[12,13] Both pH and temperature are important for DTT treatment. The optimal condition in this study was determined to be 0.0125 M DTT at pH 8, at 37°C for 30 minutes. Under these conditions, RBC reactivity with anti-K, -k, -Kp^a, -Kp^b, and -Js^b was eliminated, confirming that the antigens were denatured. On the other hand, reactivity with anti-Lu^a and -Lu^b showed the same maximum titer when tested with 0.2 M DTT-treated RBCs and untreated RBCs. The Lu^a and Lu^b antigenicity of these treated RBCs did not appear to change, contrary to some reports.[14,15]

Conditions of enzyme treatment are important for RBC antigenicity. When RBCs were treated with increasing concentrations of papain, Fy^a and Fy^b antigens were denatured first, S and M antigens were denatured next, and the s antigen was denatured last. Thus, certain antigens can be denatured selectively by using different papain concentrations. Of course, not only the enzyme concentration but also the pH was important; e.g., at pH 6, M and S antigens disappeared at 1.87 and 0.94 U per 50 μL papain treatment, but at pH 6.5, they did not disappear even at 30 U (data not shown). It has been reported that the S antigen is more easily denatured than the s antigen by chymotrypsin treatment,[16] and the s antigen is more easily denatured than the S antigen by ficin or papain treatment.[17,18] Contrary to these reports, the S antigen in our study seemed to be more easily denatured than the s antigen by chymotrypsin, papain, and bromelin treatment.

Conditions for enzyme treatment of RBCs are also important because overtreated RBCs can cause falsely positive reactions. Comparing papain with bromelin, falsely positive reactions seemed to occur less often with papain. The concentrations of papain and

bromelin needed to denature Fy^a and Fy^b antigens were the same as in the report of Mazda et al.,[19] but papain did not cause a falsely positive reaction even though papain was used at fifteen times the concentration at which bromelin caused a falsely positive reaction. Because of this result, papain was used for drying RBCs for M-MPHA-Dry.

The optimal enzyme treatment concentration was different for testing dried RBCs. When drying RBCs after chymotrypsin and papain treatment, a clear negative pattern could not be obtained at the optimal concentration for conventional RBCs. Enzyme treatment causes reduction of a RBC's charge and density and modification of the membrane,[20] the latter caused by membrane-associated protein cleaving. It is not difficult to presume that modification of RBC membranes is different when comparing enzyme-treated conventional RBCs and enzyme-treated, lysed, and/or dried RBCs.

Falsely positive reactions with enzyme-treated RBCs also sometimes occur with optimally treated RBCs. These types of falsely positive reactions may be caused by IgM agglutinins, because the falsely positive samples, seen with optimally treated RBCs by the tube test, were also detected only by anti-IgM-sensitized particles in this study. To look at it another way, most falsely positive reactions with enzyme-treated RBCs are not detected by M-MPHA, if anti-IgG sensitized particles are used for irregular antibody screening. On the other hand, falsely positive reactions with over-treated RBCs were detected by anti-IgG-sensitized particles. So the cause of these two types of enzyme falsely positive reactions seems to be different. We presume one is caused by nonspecific binding of IgG in the serum and the other by specific IgM binding.

Other RBC treatment agents, such as EDTA/acid[21] or chloroquine,[22] are also routinely used for RBC treatment. These agents could not be used for M-MPHA and M-MPHA Dry, since a clear negative pattern could not be obtained. (Data not shown).

In conclusion, enzyme or DTT treatment of adherent RBCs assessed by M-MPHA provides a simple antibody identification test with minimized falsely positive reactions. Furthermore, the drying technique of treated RBCs provides a more convenient and simple antibody identification tool since dried RBCs can be stored for a long period.

Acknowledgment

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Nondetection of the S antigen due to the presence of sodium hypochlorite

A. Long, L. Tremblay, L. Richard, R. Lemieux, and M. Goldman

Low concentrations of sodium hypochlorite (chlorine bleach) are known to destroy S antigen on intact fresh red blood cells (RBCs). Sodium hypochlorite is commonly used as a disinfectant. We report nondetection of the S antigen in tube and microplate saline indirect antiglobulin testing (SIAT) with a lot of commercial saline utilized in our donor screening and reference laboratories. Known S+s+ RBCs were found to be nonreactive with anti-S by SIAT in our reference laboratory. Our investigation demonstrated the presence of chlorine in the commercial saline. The saline lot was used for several days of donor screening and recall of FFP and platelet concentrates was initiated. Two lots of saline were recalled from blood banks across North America. *Immunohematology* 18;3: 120–122.

Key Words: commercial saline, nondetection of S antigen, tube and microplate saline indirect antiglobulin tests

S and s antigens are carried on glycophorin B and differ from one another in a single amino acid substitution. At position 29 there is a methionine in S and a threonine in s.[1] The intact Ss glycoprotein on intact red blood cells (RBCs) is not affected by trypsin treatment but is inactivated by pronase and by high concentrations of chymotrypsin, bromelin, ficin, and papain.[2] Dahr reported that carboxymethylation or treatment with performic acid, hydrogen peroxide, or cyanogen bromide destroys S antigen without affecting s.[3,4] Rygiel et al.[5] reported that sodium hypochlorite causes destruction of S antigen on intact RBCs.

We report nondetection of the S antigen in tube and microplate SIAT with a production lot of commercial saline contaminated with sodium hypochlorite.

Materials and Methods

Known S+s+ RBCs were found to be nonreactive with anti-S by SIAT tube testing on the first day of use of a lot of commercial 0.9% sodium chloride (saline) in the reference laboratory. Parallel testing of RBCs heterozygous for S was performed using the implicated saline lot (saline A) and saline from a different manufacturer (saline B). Two different S+s+ cell samples were tested with a known donor sample containing anti-S and a commercial anti-S (Lorne, UK). Different automated cell washers and manual washing were used.

Parallel phenotyping of known cells with a single dose "heterozygous" for D, K, k, Kp^a, s, Fy^a, Fy^b, Jk^a, and Jk^b antigens was conducted using commercial antisera and saline A and B. S+s+ and Jk(a+b+) RBCs were also tested after incubation and washing with combinations of saline A, saline B, and saline B with the addition of sodium hypochlorite. Preincubation studies were done to determine whether saline led to destruction of the S antigen or simply interfered with antigen-antibody binding during the performance of the SIAT. Total chlorine levels were measured in saline A using a commercial kit (CHEMetrics).

Results

The S antigen was undetectable with commercial (Lorne) and donor anti-S using saline A and two different cell washers or manual washing (Table 1). Detection of D, K, k, Kp^a, s, Fy^a, Fy^b, Jk^a, and Jk^b was not affected by the different saline solutions.

Table 1. S– and s+ RBCs versus two anti-S (Lorne and donor) using two cell washers and manual washing

| RBCs | | SIAT cell washer* | | | | SIAT manual washing | | | |
| | | Saline A† | | Saline B‡ | | Saline A | | Saline B | |
		Lorne	Donor	Lorne	Donor	Lorne	Donor	Lorne	Donor
S–	s+	–	–	–	–	–	–	–	–
S–	s+	–	–	–	–	–	–	–	–
S+	s+	–	–	++	+++	–	–	+	+++
S+	s+	–	–	++	+++	–	–	+	+++

*Identical results obtained using 2 different cell washers
†Implicated saline lot
‡Saline of a different manufacturer

Table 2. Preincubation studies: effect on S+ and Jka+ phenotypes

Temperature	Pre-incubation time	Pre-incubation saline	Washing saline	Antigen detection S	Jk^a
20°C	45 minutes	A	A	–	+
20°C	45 minutes	A	B	+	+
20°C	3.5 hours	A	B	–	+
20°C	45 minutes	B	B	+	+
20°C	3.5 hours	B	B	+	+
37°C	30 minutes	A	B	+	+
37°C	30 minutes	B	B	+	+

Preincubation of S+ RBCs with saline A for 3.5 hours at 20°C resulted in nondetection of the S antigen. However, after preincubation for 45 minutes at 20°C, or 30 minutes at 37°C, the antigen remained detectable (Table 2). Addition of sodium hypochlorite to saline B resulted in nondetection of S to a concentration of 1/100,000 (0.00005%) (Table 3). The detection of other antigens was not affected. Saline A, the implicated saline, was found to contain 0.1–0.2 mg/L of chlorine, corresponding to a dilution of 1/500,000 to 1/250,000 of sodium hypochlorite using the CHEMetrics kit.

Table 3. Phenotyping results of S+s+ RBCs with addition of sodium hypochlorite to saline B

Concentration of sodium hypochlorite in saline B Dilution*	mg/L†	%	S antigen detection
1/10,000	5 mg/L	.0005%	–
1/100,000	0.5 mg/L	.00005%	–
1/1,000,000	0.05 mg/L	.000005%	+
1/10,000,000	0.005 mg/L	.0000005%	+

Note: No preincubation time prior to adding antiserum to RBC suspension
*Sodium hypochlorite dilution
†Chlorine present

Discussion

Oxidation of methionine is the proposed mechanism of destruction of the S antigen by sodium hypochlorite as suggested by Riegel et al.[5] Sodium hypochlorite, an oxidizing agent, oxidizes methionine, most likely to methionine sulfoxide (Fig. 1). Quality control of saline performed by producers and transfusion laboratories does not routinely include measurement of sodium hypochlorite or detection of anti-S. At our transfusion center, the saline lot was used for several days of donor screening before we detected the problem. Health Canada instructed us to recall FFP and platelet concentrates due to possible nondetection of clinically significant anti-S. The saline manufacturer had recently modified its manufacturing process to reduce the possibility of environmental contamination. After Héma-Québec notified the manufacturer, two lots of saline produced after modification were tested, found to have chlorine present, and recalled from blood banks across North America. Saline manufacturers should add measurement of chlorine levels to their lot quality assurance testing in case the saline may have come into contact with bleach during the manufacturing process. Immunohematology laboratories may want to add anti-S as a positive control after they have cleaned equipment or when they receive a new lot of saline. Since sodium hypochlorite is a disinfectant commonly used for equipment such as cell washers, immunohematology laboratories must be vigilant in removing all traces of it to avoid the problem of the disappearing S.
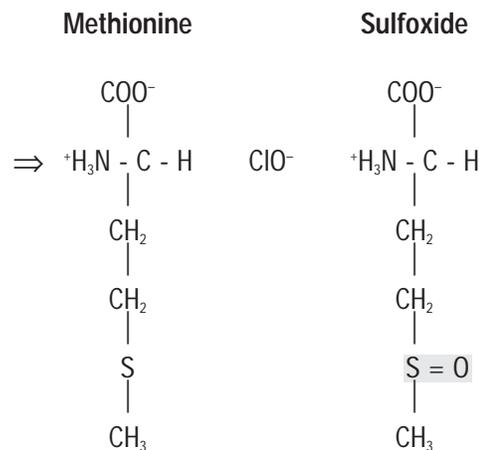
Figure 1. Oxidation of methionine

Acknowledgments

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BOOK REVIEW

Human Blood Groups. 2nd ed. Geoff Daniels, PhD. Malden, MA: Blackwell Publishers, Inc., 2002. ISBN: 0632056460. List price: $195. To order: make checks payable in US funds to Blackwell Publishing, PO Box 30, Williston, VT 05495-0030.

The author of any book on human blood groups is to be admired for their courage in undertaking such a thankless task of keeping up to date on such a fast-moving and constantly evolving field. Geoff Daniels has accepted the challenge, and the second edition of his *Human Blood Groups* is a sleek, modern-looking testimony to his success. In the foreword, Dr. Daniels states that he has achieved that by placing more emphasis on the molecular basis of the genes encoding blood group antigens and omitting some of the history that was present in his last edition. The format of the slightly wider pages has been changed from one to two columns, although the type size remains the same.

Since the publication of the first edition in 1995, the molecular basis of the antigens of the Lutheran, Kidd, Diego, Dombrock, Knops, JMH, and Ok blood groups have been identified. Many of the variants within the ABO, Rh, and MNS blood group systems have been elucidated. The amino acid changes involved in the expression of Kell antigens, as well as the molecular basis for many of the null phenotypes in Kell, Colton, Kidd, and Dombrock, have been identified. The emphasis has changed from asking, "What are these proteins?" to "What do they do?" This has changed the way we look for information about blood groups, and this book reflects those changes.

The book is organized as before, with an introductory chapter on human blood groups, terminology, and arrangement of the blood groups into functional categories. Subsequent chapters tackle the blood group systems one at a time, introducing the blood group by way of a brief history, the gene(s) and carrier protein(s), then a description of each antigen, followed by the antibodies and their clinical significance. The function of the protein(s), as well as any disease association, ends each chapter. It was nice to see the natural inclusion of the Globoside collection in the chapter on the P blood group system, and the Cost antigens included in the Knops blood group chapter. Information on the Ii antigens and cold agglutination was followed by a chapter describing the low-frequency and high-frequency antigens. The penultimate chapters of the book describe the Sid antigen, HLA antigens on red cells, and poly-agglutination. Finally, the book ends with a chapter on blood group gene mapping, bringing us full circle to the role that blood groups have played in the field of human genetics as human gene markers.

Dr. Daniels is to be commended on his comprehensive and current reference lists at the end of each chapter. Although the figures are somewhat stick-like, they are functional and an improvement on those in the first edition. The tables are great and I found information collected together that saved hours of hunting in multiple places of reference.

This is an excellent reference book for those of us who work in the world of blood groups, whether in research or in reference laboratories. As with the first edition (which is constantly consulted in our laboratory), the second edition of *Human Blood Groups* provides readily accessible information on all aspects of blood group antigens and their relevance in transfusion medicine.

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BOOK REVIEW

Transfusion Reactions. 2nd ed. Mark A. Popovsky, MD, ed. Bethesda, MD: American Association of Blood Banks (AABB) Press, 2001, 468 pp. List price: $109; member/student $89. ISBN: 1-56395-140-1. To order: call (866) 222-2498 or fax (301) 951-7150.

The number of new publications that are available every year makes the task of selecting books that could be useful resources for blood bank and clinical staff more than overwhelming. One longs for a comprehensive and thorough discussion of the topic of interest that is succinct enough to provide the answers quickly.

The second edition of *Transfusion Reactions,* edited by Mark Popovsky, was designed to meet this need, and the knowledge and expertise of the authors is impressive. Moreover, the lengthy list of current and historic references at the end of each chapter will allow the reader who needs more information to find it readily. This second edition contains new chapters on reactions associated with progenitor cells, plasma derivatives, and hypotensive reactions, as well as a 28-page chapter on Nursing Management of Transfusion Reactions. This should prove useful for those readers who work with the nursing services to improve the care provided to transfused patients. Each chapter has tables and figures that enhance the text and add to the overall clarity of the discussion.

This book deliberately omits material on the viral infectious complications of transfusions, and considering the length of the text this was an expedient decision. Indeed, as the editor, mindful of the typical patient's concern, notes, "this text is about the 'other' complications of transfusion." This is an important point, as ". . . a patient is 100 times more likely to receive the 'wrong unit' of blood than to be exposed to HIV or hepatitis."

Each chapter is organized in one of three ways: by type of transfusion reaction, including clinical presentation, differential diagnosis, mechanism, treatment, and prevention; by association with a particular type of transfusion product, i.e., progenitor cells; or by patient type, i.e., therapeutic apheresis. If the reader is unsure of what they are looking for, they may struggle to find their topic by chapter. The nursing chapter does contain a useful flow chart of initial signs and symptoms of transfusion reactions.

Of special interest to readers of *Immunohematology* will be the differential diagnosis between hemolytic reactions and other reactions. Blood bankers are well aware that the presence of an antibody in a patient suspected to have had a transfusion reaction can be a source of confusion as a false positive.

The chapter on autologous transfusion complications provides a balanced summary of the latest studies on the advantages and disadvantages of this common practice. Patients and their families often are unaware that there are problems associated with autologous donation and need this information.

In my own experience, working with transfusion committees and nursing continuing education, I found the chapter on Transfusion Related Acute Lung Injury (TRALI) particularly helpful in preparing training material for helping to raise awareness of this clinical problem.

As in any text of such complex material, there are a few confusing typographic errors and inconsistencies. However, considering the breadth of information and depth of detail, most readers looking for a useful text on transfusion reactions, even those who already have the first edition, should find this book a practical and helpful guide to transfusion reactions.

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BOOK REVIEW

Collected Questions and Answers. 7th ed. Mark Brecher, MD, ed. American Association of Blood Banks (AABB) Press 2001. Stock # 012106; softcover, 86 pages; list price: $50.00; member price: $30.00. To order: call (866) 222-2498 or fax (301) 951-7150.

This slim volume is the seventh collection of the popular Questions and Answers (Q & A) that are published in the AABB News. The selection is eclectic but organized into seven sections, namely Blood Donation; Transmissible Disease; Serology; Components; Administration of Blood; Transfusion Practice; and Quality Assurance, Regulatory, and Administrative Issues. While I am a fan of the Q & A column in AABB News, this was the first volume of *Collected Questions and Answers* I had read. I was pleasantly surprised by the variety of questions within the different sections, which covered such topics as why a pelican is associated with blood donation, the clinical implications and side effects of various herbal medicines (from Echinacea to Valerian), how much blood is donated in the world, to the more earnest questions such as how often do we need to repeat an adsorption in a patient with a warm autoantibody, the rationale for using cryosupernatant over FFP for

thrombotic thrombocytopenic purpura, and whether Rh immune globulin is effective if administered 72 hours postdelivery. In the Regulatory section, there is a very nice explanation of the various stages of implementing new drugs and tests, with clear definitions of IND, 510(k), PMA, and the like, and an answer to the definition of QALY. All answers were clear, and background knowledge was provided where necessary. Easily accessible references were given where appropriate, and the regulatory agencies had been consulted in one instance where the *Technical Manual* and the AABB Standards were in conflict.

The size of this book made an easy helping of information, which probably is the easiest way to use the book. I doubt that I would consult it specifically for an answer, but having read it through, I would know where to find the information. Some of the more esoteric questions were sufficiently intriguing to tempt me to read the other volumes, and these books would make a great addition to any blood bank library.

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COMMUNICATIONS

Letter From the Editors

To Contributors to the 2002 issues

There is no doubt that the journal depends on the readers, authors, editorial board, reviewers, and staff who put it together. As much as we would like to thank each of you by name, doing so is not practical. So, some of you we thank individually and others as members of an honored group.

We begin with a thank you to our authors. Our authors came from Italy, Japan, Canada, Australia, Greece, Ireland, Poland, Portugal, Spain, the United Kingdom, and the United States. They have written reviews, scientific studies, case reports, book reviews, and letters to the editors. One of the authors wrote a letter sharing his institution's experience on September 11, 2001.

Our editorial board is a prestigious one with exciting ideas for the journal. We list them by name in each issue. We thank them for their constant support and for, when called upon, also being peer reviewers. Special thanks go to S. Gerald Sandler, MD, our Medical Editor, who reviews every article for medical content, and to Christine Lomas-Francis, MSc, our Technical Editor, who reads every article for technical content. Both editors also do some style editing. Ms. Lomas-Francis did all that and changed jobs this year. Congratulations!

Peer reviewers are thanked each year in this, the December issue. They do a wonderful job and we know that often they do it for more than one journal. If at all possible, we try to keep the number of articles to be reviewed to two per year so that the burden will be minimal. We appreciate their expertise in selecting the most appropriate articles for our readers. We list them here with thanks.

Patricia Arndt, MT(ASCP)SBB
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Finally, the success of *Immunohematology* is directly related to the quality of the papers. For that, we hope the authors, editors, reviewers, and staff will accept our sincere gratitude and admiration.

Delores Mallory
Editor-in-Chief

Mary McGinniss
Managing Editor

ANNOUNCEMENT

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-Rh17 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

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7. Tables—see 6 under Preparation
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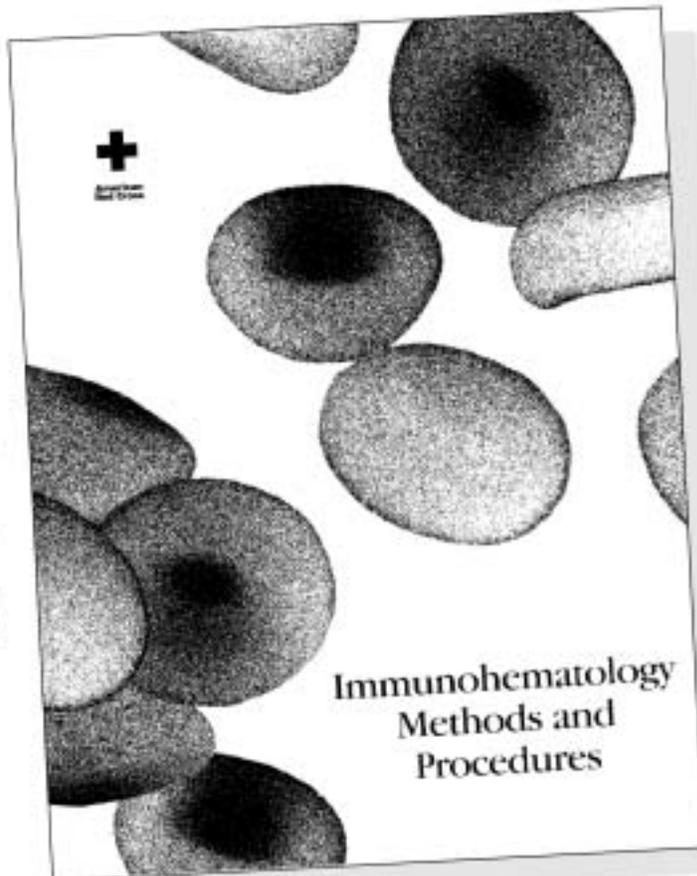
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