A review: modification of the red blood ce ll membrane and its application in blood group serology

J. R. STORRY

Key Words: red cell antigens, enzymes, modification of red cell membrane, antibody specificity

Introduction

Blood group antigens are carried on proteins and carbohydrates located on the extracellular surface of the red blood cell (RBC) membrane. The RBC membrane is composed of a lipid bilayer, through which a number of proteins pass or are anchored. Proteins bearing blood group antigens vary widely in their structure and function. Some proteins (e.g., glycophorin C, band 3) are anchored to the intracellular membrane skeleton and play a role in maintaining red cell shape and integrity; others are transport proteins for specific molecules (e.g., urea transporter, channel-forming integral protein), and other proteins are receptors (e.g., the LW, Lutheran and Fy glycoproteins) and play a role in cell–cell interaction.[1–3]

Blood group antigens may be simple linear sequences of amino acids (with or without any associated sugar molecules), or may be formed by the secondary and/or tertiary conformation of the protein. Antigens such as A, B, H, I, and P_1 are entirely carbohydrate, but the oligosaccharides on which they are carried are attached to proteins and lipids of the RBC membrane.

Proteins may be modified by the action of certain enzymes and chemicals. The action of different modifiers on proteins that carry blood group antigens may be used selectively in antibody identification and may eliminate the need to test rare RBCs.

Enzymes

Proteases have been used in blood group serology since 1947, when Morton and Pickles showed that enzyme solutions obtained from culture extracts of *Vibrio cholerae* (sialidase) or from hog stomach (trypsin) could be used to enhance agglutination reactions. Other sources of proteases were shown to be suitable for use in the blood bank. These included plant-derived proteases: namely, papain extracted from papaya, ficin extracted from figs, and bromelin extracted from pineapples: and pronase which was isolated from the culture supernate of *Streptomyces griseus* (reviewed in Issitt and Anstee[4] and Judd[5]).

Proteases have been traditionally used to enhance detection of Rh and Kidd system antibodies, enhancement that results primarily from cleavage of sialic acid-bearing glycophorin A and glycophorin B. However, the expansion of knowledge regarding the proteins that bear the different human blood groups has made the action of proteases more valuable in routine serologic tests. Proteases cleave proteins at defined sites along a linear peptide sequence, and as such may be used to modify the RBC antigen expression. Blood group antigens that are above the enzyme cleavage site on a protein will be removed following treatment of the RBCs (Fig. 1).

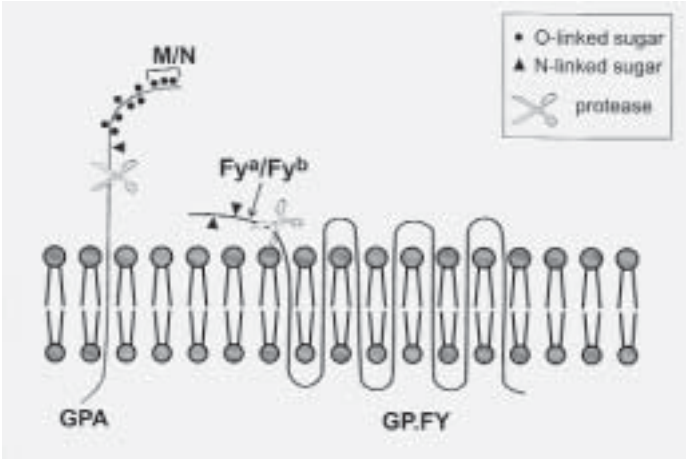

Fig. 1. Effect of proteases: Antigens located above an enzyme-cleavage site will be removed when RBCs are treated with enzymes. Much of the sialic acid on the RBC also will be removed by protease treatment as these oligosaccharides are attached to the *N*-terminal portions of the glycophorins A, B, and C that are cleaved.

Although proteases are more commonly used in blood group serology, glycosidases (enzymes that cleave sugars) also may help determine an antigen's characteristics. Table 1 lists the most commonly used enzymes and their substrates.

Chemical Modification

Sulfhydryl reagents

Chemicals such as dithiothreitol (DTT) and 2-aminoethylisothiouronium bromide disrupt the secondary structure of polypeptides by reducing the double bonds between cysteine residues. These bonds form under oxidizing conditions between the sulfhydryl groups of cysteine residues in close spatial proximity within a protein and give the protein its secondary structure.[6] Many antibodies to blood group antigens recognize conformational epitopes; thus, disruption of the secondary structure of blood group proteins on the RBC surface will result in the failure of the specific antibody to react (Fig. 2). Blood group-bearing proteins that are sensitive to reducing agents include Kell, Dombrock, Knops, Lutheran, Indian, Cromer, JMH, AnWj, Yt, LW, and Scianna. Antigens on these proteins may show variable susceptibility to DTT treatment. Within the Kell blood group system, Js^a and Js^b are exquisitely sensitive to reducing agents and are denatured by as little as 1 to 2 mM DTT. Other Kell antigens require a concentration of 200mM for their inactivation.[7]

ZZAP reagent (marketed as W.A.R.M.; Immucor, Inc., Norcross, GA) is a mixture of papain and DTT, and incorporates the modification properties of both reagents.[7,8] ZZAP reagent is commonly used for removing bound IgG from DAT-positive RBCs prior to autoadsorption. However, it also can be used as a one-step treatment of RBCs as an aid in antibody identification.

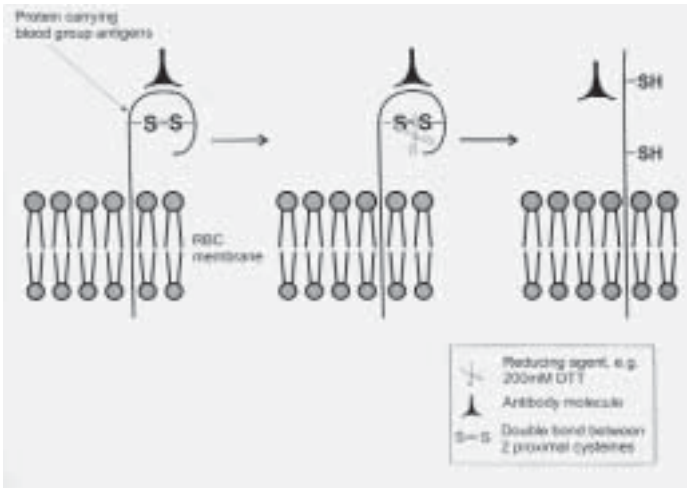

Fig. 2. Antibodies that recognize conformation-dependent antigens will be nonreactive following treatment of the RBCs by a reducing reagent, which perturbs the secondary structure of the protein; e.g., 200mM DTT.

Chloroquine diphosphate (CDP)

The mechanism by which CDP modifies RBC membrane proteins is not understood. However, chloroquine is known to cleave or inhibit noncovalent antigen-antibody binding and is used in that context to remove IgG bound in vivo from the RBCs of patients with autoimmune hemolytic anemia.[9] CDP is known to inactivate Bg antigens on RBCs.[10] Bg antigens were shown to be HLA antigens and were originally thought to have been adsorbed from the plasma. Although most RBCs do not express HLA antigens, Giles et al.[11] determined that Bg antigens are residual HLA class I antigens that are normally lost upon maturation to the erythrocyte. CDP specifically removes β-2-microglobulin, part of the heterodimer that composes class I HLA antigens, and thus interferes with the conformation of the HLA molecule.[11]

Table 1: Enzymes commonly used in blood group serology[4,20]

Enzyme	Source	Classification	Cleavage site
Papain	Papaya	Thiol endoprotease	arginine, lysine, glutamine, tyrosine, glycine, histidine, (next but one to) phenylalanine
Ficin	Fig	Thiol endoprotease	arginine, lysine, glutamine, tyrosine, glycine, asparagine, leucine, valine
Bromelin	Pineapple	Thiol endoprotease	arginine, lysine, tyrosine, glycine, serine, phenylalanine
Trypsin	Pancreas (bovine or porcine)	Serine endoprotease	arginine, lysine
α-chymotrypsin	Bovine pancreas	Serine endoprotease	phenylalanine, tryptophan, tyrosine, leucine
Pronase	*Streptomyces griseus*	Metalloendoprotease	tyrosine, glycine, leucine, valine, alanine, isoleucine, tryptophan, phenylalanine
Neuraminidase (Sialidase)	*Vibrio cholerae*	Exoglycosidase	$\alpha 2\rightarrow 3$ and $\alpha 2\rightarrow 6$ linkages between neuraminic acid and galactose

Note: Proteases hydrolyze the C-terminal bonds of amino acids.

Additionally, CDP has been shown to weaken other blood group antigens, including Fy^b, Lu^b, Yt^a, JMH, Knops, Dombrock, and Rh blood group antigens.[12,13]

Glycine/EDTA

Glycine/EDTA is used primarily to elute IgG bound to RBCs. Dissociation is affected by the low pH of the glycine solution.[14] It is useful in testing samples from patients with strongly positive direct antiglobulin tests as IgG is quickly and efficiently removed, permitting phenotyping tests with sera that require the secondary addition of an antiglobulin reagent.[15] However, it has been shown to weaken or eliminate the expression of Kell blood group antigens, Er^a, and Bg antigens.[14,16,17]

4,4'-di-isothiocyanatostilbene-2,2'-disulfonic acid(DIDS)

DIDS is one of a group of stilbene disulfonates that are used widely to inhibit anion transport in vitro. It binds readily to band 3, the RBC anion transporter (reviewed in Tanner[18]). Rouleaux is a common cause of falsely positive reactions in serologic tests that result from the ordered aggregation of RBCs in the presence of high concentrations of macromolecules such as albumin or dextran. DIDS has been shown to inhibit rouleaux in vitro by binding to band 3. Norris et al.[19] obtained complete inhibition of rouleaux formation by the pretreatment of reagent RBCs using 150μL of a 0.05mg/mL solution of DIDS in 0.15M NaCl for 10 minutes at 37°C.

Practical Applications of Enzymes and Chemicals

Although it is useful to know of the existence of other RBC protein modifiers, perhaps the two most practical (and therefore, most widely used) solutions in blood group serology are 1% solutions of papain or ficin and 200mM DTT. Antibody identification of difficult cases, such as antibody mixtures or antibodies to high-incidence antigens, can be greatly simplified by testing parallel sets of reagent RBCs: unmodified, enzyme-treated, and DTT-treated. The pattern obtained can help in quickly determining the blood group specificity (if not the antigen specificity). For example, an antibody to a high-incidence antigen that is nonreactive with papain-treated RBCs but reactive with DTT-treated RBCs immediately limits the specificity to anti-Ch, anti-Rg, anti-Ge2, or anti-Ge4. Conversely, the specificity of an antibody that is reactive with both papain-treated RBCs and DTT-treated RBCs is excluded from the MNS, Kell, Yt, LW, Chido/Rodgers, Scianna, Knops, and Indian blood group systems. There are always exceptions to any rule, of course, but knowledge of an antigen's sensitivity or resistance to or enhancement by different modifying reagents will reduce the number of rare RBCs and sera required to determine the specificity (see Table 2).[20]

Table 2. Red cell antigens sensitive or resistant to, or enhanced by different modifying reagents

Blood group systems[4]	Protease	Sulfhydryl AET/DTT	CDP[3]	Glycine EDTA Acid
ABO	+ ↑↑	+	+	+
AnWj[4]	+	$+^w$	+	+
Chido/Rodgers	o	+	+	+
Colton	+	+	+	+
Cost	+	+	+	+
Cromer	+	$+^w$	+	+
Diego	+	+	+	+
Dombrock	$+^1$	+/o [2]	+	+
Duffy	o [1]	+	+	+
Er	+	+	+	o
Gerbich	o	+	+	+
Globoside	+ ↑↑	+	+	+
Hh	+ ↑↑	+	+	+
Ii	+ ↑↑	+	+	+
Indian	o	o	+	+
JMH[4]	o	o	+	+
Kell	+	o	+	o
Kidd	+ ↑↑	+	+	+
Knops	o	+/o [2]	+	+
Kx[4]	+	+	Unknown	Unknown
Lan[4]	+	+	+	+
Lewis	+ ↑↑	+	+	+
Lutheran	$+^1$	+	+	Unknown
Lw[4]	+	o	+	+
MNS	o	+	+ (presumed)	+
P	+ ↑↑	+	+	+
Rh	+ ↑↑	+	+	+
Scianna	+	o	+ (presumed)	+ (presumed)
Vel[4]	+ ↑↑	+	+	+
Yt	$+^1$	+/o [2]	+	+
Xg[4]	o	+	+	Unknown

1—Except trypsin; 2—Some antigens are resistant and some weakened; 3—Effect at RT; at 37°C, Fy^b, Lu^b, Yt^a, JMH, Kn, Do, and Rh may have a weakened expression; 4—Blood group systems except as noted
o Negative
+ Positive
↑↑ Enhanced

As with any test, careful quality control of the process is required. This is achieved very simply by testing treated and untreated RBCs with an antibody to a treatment-sensitive antigen present on the RBCs. For example, following papain treatment, the treated and untreated M+N+ test RBCs can be tested with an example of anti-M. Additionally, it is important to treat the patient's autologous RBCs in parallel, wherever possible. "Enzyme-only" autoantibodies are common and it is easy to be misled by an apparent enhancement of antibody reactivity when, in fact, the patient's serum contains one of these autoantibodies.[21]

References

1. Telen MJ. Erythrocyte blood group antigens: not so simple after all. Blood 1995;85:299–306.
2. Daniels G. Functional aspects of red cell antigens. Blood Rev 1999;13:14–35.
3. Cartron JP, Bailly P, Le Van Kim C, et al. Insights into the structure and function of membrane polypeptides carrying blood group antigens. Vox Sang 1998;74 (Suppl 2):29–64.
4. Issitt PD, Anstee DJ. Applied blood group serology. 4th ed. Durham, NC: Montgomery Scientific Publications, 1998.
5. Judd WJ. Methods in immunohematology. 2nd ed. Durham, NC: Montgomery Scientific Publications, 1994.
6. Alberts B, Bray D, Lewis J, et al. Molecular biology of the cell. 3rd ed. New York: Garland Publishing Co., 1994.
7. Branch DR, Muensch HA, Sy Siok Hian AL, Petz LD. Disulfide bonds are a requirement for Kell and Cartwright (Yt^a) blood group antigen integrity. Br J Haematol 1983;54:573-8.
8. Branch DR, Petz LD. A new reagent (ZZAP) having multiple applications in immunohematology. Am J Clin Pathol 1982;78:161–7.
9. Mantel W, Holtz G. Characterisation of autoantibodies to erythrocytes in autoimmune haemolytic anaemia by chloroquine. Vox Sang 1976;30:453–63.
10. Swanson JL, Sastamoinen R. Chloroquine stripping of HLA A,B antigens from red cells. Transfusion 1985;25:439–40.
11. Giles CM, Darke C, Rowe GP, Botto M. HLA class I (Bg) antigens on red cells of SLE patients: a serological study with polyclonal and monoclonal antibodies. Vox Sang 1989;56:254–61.
12. Sassetti R, Nichols D. Decreased antigenic reactivity caused by chloroquine (letter). Transfusion 1982;22:537–8.
13. Mallory D, Reid M. Misleading effects of chloroquine (letter). Transfusion 1984;24:412.
14. Louie JE, Jiang AF, Zaroulis CG. Preparation of intact antibody-free red blood cells in autoimmune hemolytic anemia (abstract). Transfusion 1986; 26:550.
15. Des Roziers NB, Squalli S. Removing IgG antibodies from intact red cells: Comparison of acid and EDTA, heat, and chloroquine elution methods. Transfusion 1997;37:497–501.
16. Liew YW, Uchikawa M. Loss of Er^a antigen in very low pH buffers (letter). Transfusion 1987;27:442–3.
17. Champagne K, Spruell P, Chen J, et al. EDTA/glycine-acid vs. chloroquine diphosphate treatment for stripping Bg antigens from red blood cells (abstract). Transfusion 1996;36 (Suppl):21S.
18. Tanner MJA. The structure and function of band 3 (AE1): recent developments. Mol Membr Biol 1997;14:155–65.
19. Norris SS, Allen DD, Neff TP, Wilkinson SL. Evaluation of 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid in the inhibition of rouleaux formation. Transfusion 1996;36:109–12.
20. Reid ME, Lomas-Francis C. The blood group antigen factsbook. San Diego: Academic Press, 1996.
21. Reid ME, Green CA, Hoffer J, Øyen R. Effect of pronase on high incidence blood group antigens and the prevalence of antibodies to pronase-treated erythrocytes. Immunohematology 1996;12:139–42.

Jill R. Storry, PhD, FIBMS, Imunohematology Laboratory, New York Blood Center, 310 East 67th Street, New York, NY 10021.

ERRATUM

Vol. 16, No. 2, 2000

Improving transfusion safety by electronic identification of patients, blood samples, and blood units.

The authors have informed the editors of *Imunohematology* that there is an error on page 82, first paragraph, fourth sentence. The sentence should read "In the United States, the incidence of fatalities from transfusion of wrong red cells or whole blood is estimated to be one per 600,000 transfusions." We regret the error.