Effect of pronase on high-incidence blood group antigens and the prevalence of antibodies to pronase-treated erythrocytes

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Pronase is a useful and relatively nonspecific protease that cleaves many red blood cell (RBC) membrane proteins that carry blood group antigens. Unexpected findings in tests using pronase-treated RBCs during the investigation of a patient's blood sample led us to test which high-incidence blood group antigens were sensitive and which were resistant to pronase treatment, and to determine the prevalence of antipronase in the serum of blood donors. Our results show that antigens in the Cromer and Lutheran blood group systems and the JMH antigen were sensitive to pronase treatment of RBCs. Antigens in the Dombrock blood group system and Sc1 were either sensitive to or markedly weakened by pronase treatment of RBCs. The following high-incidence antigens were resistant to treatment of RBCs with pronase: AnWj, At^a, Co^a, Co3, Di^b, En^{a}FR, Er^a, Fy3, Jk3, Jr^a, k, Kp^b, Js^b, K14, Lan, Ok^a, Rh17, U, Vel, and Wr^b. Over half of the serum samples from normal blood donors contained antibodies to pronase-treated RBCs. When testing human serum against pronase-treated RBCs, it is essential either to use an autocontrol or to perform the testing with an eluate. ***Immunohematology*** 1996;12:139–142.

In 1992, Daniels[1] compiled, tested, and tabulated the effect of enzymes on high-incidence red blood cell (RBC) antigens. Daniels pointed out several pitfalls in the interpretation of his serologic results when using enzyme-treated RBCs: (1) different antibodies with apparently the same specificity may behave in different ways with enzyme-treated RBCs, and while weakly reactive antibodies may not react with treated RBCs, stronger examples may react; (2) often only one example of an antibody specificity could be tested; (3) since many sera agglutinate pronase-treated RBCs "nonspecifically" and negative controls were not always tested, care should be taken in interpreting results. Nevertheless, it is common practice to rely on the data as presented by Daniels in his table.[1]

Antibodies to enzyme-treated RBCs, which are known to be present in adult sera, react with all RBCs (including autogeneic) that have been treated with the specific enzyme. Antibodies to RBCs treated with papain, trypsin, or bromelin are present in 0.1 percent, 1 percent, and 2 percent of adult sera, respectively.[2] We could not find documentation of the prevalence of antibodies to RBCs treated with pronase.

While investigating a patient's sample whose serum contained anti-Dr^a, we consulted the table prepared by Daniels[1] and were surprised that the Dr^a antigen in our tests was sensitive to pronase treatment. After reading the possible pitfall of autoanti-pronase described by Daniels, we decided to determine the prevalence of antibodies to RBCs treated with pronase and to test the effect of pronase on high-incidence blood group antigens.

Materials and Methods

Plasma samples from 60 random donors were obtained from segments containing whole blood or, in the case of 60 known immunized donors, from plasma retention samples. In order to prevent false-positive reactions due to antipronase in tests versus pronase-treated RBCs, either monoclonal antibodies (MAbs) were used or acid eluates (Elu-KitII, Gamma Biologicals, Inc., Houston, TX) were prepared from antigen-positive RBCs following incubation with serum containing the antibody of interest. The immune sera had been obtained from patients or from colleagues through the Serum, Cell, and Rare Fluid (SCARF) Exchange Program. When possible, more than one source of each antibody specificity and more than one specificity within a blood group system was used. MAbs were also obtained from many generous colleagues. Antibodies to Cromer-related antigens were anti-Cr^a (6 examples), anti-Dr^a (2), anti-Tc^a (2), anti-WES^b (1), and anti-DAF (MAb BRIC 110, BRIC 216, BRIC 230, and PMC6/S1). Lutheran antibodies were anti-Lu^b (1), anti-Lu3 (1), anti-Lu4 (1), anti-Lu5 (1), anti-Lu6 (1), anti-Lu7 (1), anti-Lu12 (1), anti-Lu13 (1), and anti-Lu17 (1). Kell system antibodies were anti-k (MAbF71E7), anti-Kp^b (1), anti-Js^b (1), and anti-K14

(MAb 610/B11). Antibodies to antigens in the Dombrock blood group system were anti-Do^a (1), anti-Gy^a (3), anti-Hy (1), and anti-Jo^a (1). Other antibodies were anti-AnWj (1 and MAb H86), anti-At^a (1), anti-Co^a (1), anti-Co3 (1), anti-Di^b (2 and MAb OK), anti-En^{a}FR(1), anti-Er^a (2), anti-Fy3 (MAb 512-1), anti-Jk3 (1), anti-JMH (3 and MAb H8), anti-Jr^a (MAb HMRO921), anti-Lan (1), anti-Ok^a (MAb TRA 1.85), anti-Rh17 (MAb CYN ISI405), anti-Sc1 (1), anti-U (2), anti-Vel (2), and anti Wr^b (MAb R7).

Pronase (Boehringer-Mannheim, Germany) was used as described by Daniels.[1] Four volumes of a solution of 2.5 mg/mL pronase in 0.067M phosphate buffer at pH 7.7 were incubated with one volume of washed, packed RBCs at 37˚C for 30 minutes. The RBCs were then washed and used within 48 hours of treatment. Pronase-treated RBCs were incubated with plasma at 37˚C for 30 minutes, centrifuged, and observed for agglutination. The test was then converted to the indirect antiglobulin test (IAT).

Some pronase-treated RBCs were tested with *Arachis hypogea* to demonstrate that they were not T-active. Forty sera containing alloantibodies that had been stored at –20˚C for at least 5 years were also tested versus pronase-treated RBCs.

Results

Prevalence of antibodies to RBCs treated with pronase

Of the 60 plasma samples obtained from segments from blood donations, 59 agglutinated pronase-treated RBCs after incubation at 37˚C for 30 minutes and 38 of these agglutinated the enzyme-treated RBCs in the IAT. Pronase-treated RBCs from the 59 reactive segments were agglutinated when tested with the autogeneic plasma. Sixty plasma samples containing anti-D, anti-C+D, anti-E, anti-K, or anti-Jk^a were tested against pronase-treated, antigen-negative RBCs. Of these 60 samples, 59 agglutinated the enzyme-treated RBCs after a 30-minute incubation at 37˚C and 26 agglutinated the RBCs in the IAT. Collectively, these findings indicated that antibodies to pronase-treated RBCs were present in 98 percent of "fresh" donor plasma samples (118 of 120) at saline 37˚C and in 53 percent of the samples (64 of 120) at the IAT (see Table 1). Approximately 15 percent of the 40 stored sera had antibodies to the pronase-treated RBCs by the IAT (data not shown). From these results it appears that the prevalence of antibodies to pronase-treated RBCs is higher in fresh plasma than in stored plasma. Pronase-treated RBCs were nonreactive versus *Arachis hypogea*.

Table 1. Results of testing "fresh" donor plasma against pronase-treated red blood cells (RBCs)

		Number positive versus pronase-treated RBCs (percent)	
Source of plasma	Number tested	Saline 37˚C	IAT*
Donor plasma	60	59 (98)	38 (63)
Immunized donor plasma	60	59 (98)	26 (43)
Total	120	118 (98)	64 (53)

*Indirect antiglobulin test

Effect of pronase on high-incidence blood group antigens

The following antigens were sensitive to pronase treatment of antigen-positive RBCs: antigens in the Cromer blood group system (Cr^a, Tc^a, Dr^a, and WES^b, and the epitopes on decay-accelerating factor [DAF] recognized by the monoclonal antibodies used), antigens in the Lutheran blood group system (Lu^b, Lu3, Lu4, Lu5, Lu6, Lu7, Lu12, Lu13, and Lu17), and the JMH antigen.

Antigens in the Dombrock blood group system (Do^a, Gy^a, Hy, and Jo^a) and the Sc1 antigen showed reduced reactivity with their corresponding antibodies contained in eluates (see Table 2).

Table 2. Results of eluates containing antibodies in Dombrock and Scianna blood group systems versus untreated and pronase-treated red blood cells Red blood cells

Eluate containing	Untreated	Pronase-treated
Anti-Do^a	1+	weak
Anti-Gy^a (3 samples)	2+	weak
	3+	negative
	4+	±
Anti-Hy	1+	negative
Anti-Jo^a	1+	±
Anti-Sc1	2+	±

Pronase had no apparent effect on Kell blood group system antigens (k, Kp^b, Js^b, and K14) nor on the high-incidence antigens At^a, Er^a, and Jr^a.

Antibody and antigen reactions that were enhanced by pronase treatment of RBCs were Diego blood group system antigens (Di^b and Wr^b), MNS blood group system antigens (En^{a}FR and U), and several high-incidence antigens (AnWj, Co^a, Co3, Fy3, Jk3, Lan, Ok^a, Rh17, and Vel).

Our results and those reported by Daniels[1] are summarized in Table 3. Antigens already determined to be pronase-sensitive by Daniels[1] were not repeated in our study.

Table 3. Effect of pronase on high-incidence blood group antigens

Sensitive	Sensitive/Weakened	Resistant	Resistant/Enhanced
Cr^a,Tc^a,Dr^a,WES^b; Lu^b,Lu3,Lu4,Lu5, Lu6,Lu7,Lu8*,Lu12, Lu13,Lu17;En^aTS*, En^aFS*;LW^{a}*,LW^{ab}*; Ch*,Rg*;Ge2*,Ge3*, Ge4*;Fy6*;In^{b}*;Yt^{a}*; JMH	Do^a,Gy^a,Hy,Jo^a; Sc1	At^a;k,Kp^b, Js^b,K14	En^aFR,U;Di^b,Wr^b; Rh17; Fy3;Vel;Er^a; AnWj;Co^a,Co3; Jk3; Jr^a;Lan;Ok^a

*Results from Daniels[1]; not repeated in our study.

Discussion

Pronase is a broad-spectrum protease obtained from *Streptomyces griseus*, which hydrolyses almost all peptide linkages in proteins.[3] Although it is not regularly used by blood bankers, its nonspecific properties render it a useful enzyme for screening monoclonal antibodies and antibodies to high-incidence antigens. Prager and colleagues[3] showed that S, s, Kp^b, Jk^a, Fy^a, and Lu^a antigens were sensitive to 1 mg/mL of pronase in 5mM phosphate-buffered saline at pH 7.0. Judson and Anstee[4] reported similar findings in that 0.2 mg/mL of pronase denatured M, N, S, s, Lu^a, and Fy^a, but not K or D antigens.

Cromer blood group antigens are located on DAF.[5–10] The epitopes recognized by anti-DAF MAbs BRIC 110 and BRIC 128 are sensitive to pronase treatment.[11] Our results agree with this finding, and show that all four short consensus repeat (SCR) domains of DAF are cleaved by pronase and that all Cromer blood group antigens are sensitive to this enzyme. Our finding that all Lutheran antigens tested are sensitive to pronase treatment differs from Daniels' data but agrees with others[3,4] who reported that the Lu^a antigen is sensitive to pronase.

Reactions were markedly reduced or abolished when pronase-treated RBCs were tested with antisera in the Dombrock blood group system. This is in agreement with two recent reports.[12,13] Banks et al.[13] found that enzyme-treated and chemically modified RBCs tested with anti-Do^a, -Do^b, -Gy^a, -Hy, or -Jo^a gave the same pattern of reactivity, in that they were enhanced by papain and ficin treatment of the RBCs, were reduced by α-chymotrypsin, and were sensitive to trypsin and pronase. Treatment of RBCs with 6% 2-aminoethylisothiouronium bromide (AET) markedly reduced reactions with antibodies in the Dombrock system and treatment of RBCs with 200 mM dithiothreitol (DTT) destroyed all reactivity. Similarly, Sc1 and Sc2 antigens have been shown by Spring et al.[14,15] to be sensitive to pronase but resistant to papain, trypsin, and α-chymotrypsin.

Our finding that the Fy3 antigen was not denatured by pronase is in agreement with findings reported by Mallinson et al.[16] High-incidence antigens in the Diego blood group system (Di^b and Wr^b) are polymorphisms on the fourth and last extracellular loops of the human erythrocyte anion transporter (AE1, band 3).[17,18] The reactivity of antibodies to these antigens was enhanced after pronase treatment of RBCs. Interestingly, the epitopes (which are dependent on the integrity of the third extracellular loop of AE1) recognized by several murine and rat monoclonal antibodies are sensitive to pronase treatment of RBCs.[19] These data remind us that antigens in various domains of a protein may behave differently to enzyme treatment. Predictions as to the susceptibility of a given antigen can be made if its location on a protein is known; if its location is not known, then specific enzyme testing would need to be performed.

Pronase has proved a useful tool in differentiating anti-LW from anti-D since it cleaves the LW antigen but not the D antigen.[20] However, the very high incidence of nonspecific reactions with pronase-treated RBCs found in our study makes its usefulness questionable. We suggest the use of a sulphydryl reducing reagent (e.g., DTT, 2-mercaptoethanol, or AET), which, like pronase, inactivates the LW antigen but not the D antigen. These reagents have been used for many years in blood group serology and do not appear to cause nonspecificity problems encountered when testing normal human sera with pronase-treated RBCs.

In summary, the high-incidence antigens of the Cromer and Lutheran blood group systems and JMH were sensitive to pronase treatment of intact RBCs. Antigens in the Dombrock blood group system and Sc1 were either sensitive to this treatment or were markedly weakened. The following high-incidence antigens were resistant to (or enhanced by) treatment of intact RBCs with pronase: AnWj, At^a, Co^a, Co3, Di^b, En^aFR, Er^a, Fy3, Jk3, Jr^a, k, Kp^b, Js^a, K14, Lan, Ok^a, Rh17, U, Vel, and Wr^b. Since over half of the plasma samples from normal donors had antibodies to pronase-treated RBCs in the IAT, it is most likely that the discrepancies between our results and those of Daniels[1] were due to antibodies to pronase-treated RBCs in the sera used. When testing human polyclonal antibodies against pronase-treated RBCs, it is essential to use an autocontrol (or other known antigen-negative RBCs) before interpreting the results or to test an eluate containing the antibody of interest.

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