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Detection of weak A antigen associated with *Proteus vulgaris* infection

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ABSTRACT

Background: A person with weak A antigen expression is presented.

Objectives: The study aimed to reveal weak A antigen by different methods applied in laboratories.

Materials and methods: The reaction of adsorption, agglutination, and inhibition of agglutination were performed at 4 °C, 10 °C, 13 °C, 20 °C, and 37 °C for 1-13 hrs and one-day incubation. The complement of the guinea pig was added to the heated serum to reveal hemolysis.

Results: A person's red blood cells reacted with commercial anti-B sera. Whereas anti-A and anti-B (reactive at 37 °C) antibodies were found in the serum. The person demonstrated anti-A antibody adsorbing ability at 4 °C. The plasma of the person inhibited agglutination of A and B RBCs by polyclonal sera. The blood culture was positive for *Proteus vulgaris* infection.

Conclusion: The presence of weak A antigen was detected the best by the adsorption method at low temperatures with polyclonal sera from the O blood group.

Introduction

Weak A subgroups (A_3, A_x, A_{end}, A_m, A_y, and A_{el}) are usually detected as O groups, which may lead to transfusion complications. The cases of weak A antigen are presented by researchers. The patient's RBCs were not agglutinated by anti-B antibodies and showed mixed field agglutination of agglutination with anti-A and 1^+ with anti-A_1 antibodies, the serum showed the presence of anti-B antibodies. The presence of a weak A subgroup with Type II discrepancy was stated.[1]

Weak A antigen variants may be revealed by various methods.[2,3] Special techniques (adsorption-elution, glycosyltransferase estimation, and genotyping) are performed to confirm these blood groups.[4] ABO discrepancy was detected when in forward typing O blood type was revealed and reverse typing showed the presence of anti-B antibodies. No agglutination of the RBCs with anti-A and anti-AB antibodies was obtained, A_m, A_y or A_{el} phenotype was suggested. The RBCs were investigated in adsorption-elution methods with polyclonal anti-A antibody and eluate agglutinated A RBCs pointing to the presence of A antigen on the red cells. The eluate showed agglutination with two different A RBCs, did not agglutinate O RBCs and the final wash solution did

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not agglutinate A, B, AB, and O RBCs. Blood groups of the husband and son appeared to be of O type. The person was a secretor and showed A and H substances in saliva. A_y is considered to be similar to Am and adsorption-elution tests often fail to differentiate these variants. Since the eluted anti-A antibody agglutinated weakly A RBCs (1^+ by tube technique) A_y phenotype was reported. In the case of Am phenotype, the agglutination strength is much stronger; thus, a person of weak A subgroup was typed.[4]

A blood group person showing weak B antigen associated with *Proteus vulgaris* bacteremia and anti-B antibody in the serum has been described. The antigen was associated with *Proteus vulgaris* bacteremia. The acquired B antigen reacted with a fraction of the anti-B antibody missing in the person's anti-B serum.[2] The relevance of antigen acquisition through bacteria has been widely discussed. Microorganism's effects on RBCs are various: causes of autoimmune diseases, effect *in vivo* and *in vitro* on RBCs, blood group specificity, and stimulation of anti-A and anti -B antibody production.[3]

Acquired B antigen is well described in the literature.[5-8] Marsh demonstrated an association *E. coli* with acquired B antigen. Springer showed 137 gram-negative with a blood group activity (20% of them showed high *in vitro* activity).[9] *E. coli* O88 had the most B activity, as well as *Proteus vulgaris*. The mechanisms of acquired B antigen are the adherence of bacterial antigen to the erythrocyte membrane and enzymatic alteration of the erythrocyte membrane.[9] The bacterial antigen may be adsorbed on the RBCs membrane and be the basis of bacterial hemagglutination tests. The lipopolysaccharide from *E. coli* O86 was found to be similar to the B blood group antigen and eluted back off the RBCs by washing them at 58 °C.[10]

The lipopolysaccharide of *Proteus vulgaris* OX-19 was found to have receptors for anti-B antibodies and antisera to *Proteus* OX-19 agglutinated B RBCs.[11] Platelet-activating ureases produced by *Proteus mirabilis* were described. The cytotoxic and pro-inflammatory activities of enzymes in *Proteus* were detected.[11] The mechanisms involving endogenous or extraneous enzymes that transform A-specific antigen into B antigen were discussed. Marsh showed, the B antigen may be acquired through an enzymatic reaction, since the B-producing mechanism was destroyed by heat, and the B substance was absent in the filtrate (the filtrate did not inhibit the reaction of anti-B serum with normal B RBCs).[9] A fraction of anti-B antibody against acquired B antigen, neutralized by the bacterial polysaccharide was demonstrated. The patient's cells were not agglutinated by antisera against *Proteus*. A factor, enzymatic in nature, associated with polyagglutinable cells in rabbits with enteritis, was to have an ability to persist in the circulation and transfer from cell to cell. The interesting feature of the acquired B is the presence of anti-B antibody reacting with B RBCs except with own cells with anti-B antibody. The researchers showed, when anti-B serum is adsorbed with the patient's cells all activity for the acquired B antigen is removed, however, a strong agglutinin for usual B RBCs is present.[9] The importance of anti-A and anti-B antibodies reacting with acquired A and B antigen show a defensive mechanism and a person lacking this anti-B antibody may develop an infection with bacteria (*Proteus*), that has a B-like antigen on the membrane. Hyperimmune anti-B sera are able to kill *E.coli* 086 in the presence of complement.[1]

Thus, microorganisms cause immune responses within the ABO blood group system, that may influence blood group specificity. Platelet-activating ureases produced by *Proteus mirabilis* were described. The cytotoxic and pro-inflammatory activities of enzymes in *Proteus* were detected.[4] Weak A subgroups are reported to require advanced methods such as adsorption and elution. Molecular tests are required for confirmation and typing of the subgroups. Hemagglutination-based methods are used to detect weak subgroups. However, variation in reagents and techniques leads to the missing of weak phenotypes, often mistyped as the O group. Meanwhile, weak subgroups of A may cause transfusion reactions, if the donor mistyped as O is transfused to O blood type recipient, causing intravascular hemolysis. Therefore, it is necessary to reveal weak subgroups since the accurate determination of ABO type would help in better managing transfusion practice.

The objective of this study was to reveal weak A antigen by different special methods.

Material and methods

The person with B blood group and anti-A antibody adsorbing ability was examined at Sytenko Institute of Spine and Joint Pathology because of skin infection. The blood was drawn into an EDTA tube. On common blood analysis: white cells: 7.8×10^9, lymphocytes: 18.6%, monocytes: 5.7%, granulocytes: 75.7%, platelets: 210×10^9, MCV 96 fL, RBCs: 4.94×10^{12}/L, Hb: 161 gm/L, Hematocrit 0.46. The patient's RBCs reacted with the anti-B antibody, the indirect antiglobulin test of the patient's cells, and the anti-A antibody was negative when tested with a broad-spectrum antiglobulin reagent. The patient's cells reacted with commercial anti-B sera, and 10 group A donor sera. The results obtained with the group A donors varied from weak microscopic agglutination to strong macroscopic positives. The serum contained an antibody, that reacted with B cells at 37 °C at a low titer 1:2 at 37 °C. No family was available for genetic studies. The person's RBCs could adsorb anti-A antibodies at 4°C.

The RBCs and sera were taken from 25 healthy volunteers aged 62.4±2.20 years, among those were 11 men and 14 women. Washed RBCs of O, A, B, AB, and O blood groups were used for the study.

The RBCs' diameter and their quantity were fixed by microscope with a digital camera Sigeta DCM-900, 9.1 Mp, recorded on the hard disk, quantity and diameter of each erythrocyte were measured (in µM). Antibody titration was performed by diluting 100 µL of the serum with normal saline in 1:2-1:32 titer. Titrations were carried out by making doubling dilutions of the test sera in saline. Two drops of serum or diluted serum were mixed with one drop of a suspension of cells in saline and

incubated. The RBCs were washed three times with saline and centrifugated at 1,000 g for 10 min. The reaction of adsorption, agglutination, and inhibition of agglutination was performed at 4 °C, 10 °C, 13 °C, 20 °C, 37 °C for 1-13 hrs and one-day incubation. The guinea pig complement was added to the heated serum to reveal hemolysis. To obtain IgG antibodies the sera were heated for 30 mins at 60 °C. Fifty µL of RBCs were added to 100 µL of the serum in different dilutions and incubated at 20 °C. One hundred µL of the guinea pig complement was added to the reaction for revealing immune antibodies. Agglutination reactions were performed by standard tube methods and checked microscopically. Antiglobulin test was performed with a broad-spectrum antiglobulin reagent following incubation at 37 °C for 30 min.

Results

The RBCs of the studied person in cell grouping were agglutinated by anti-B commercial sera and 10 polyclonal anti-B sera. Anti-A serum agglutinated washed RBCs after 20 hrs of incubation at 13 °C contrary to the incubation with unwashed RBCs (decreased quantity) and contact at 20°C. Two samples adsorbed by B RBCs anti-A,B serum contacted with the studied RBCs at 37 °C for 1 hr did not cause agglutination. The contact of anti-A serum with unwashed RBCs for one day led to the agglutination (RBCs diameter 5.96±0.43 µM), more expressed than after the contact with washed RBCs (diameter 5.83±0.86 µM) (Table 1). However, anti-A serum for one day incubation at 37°C with the studied RBCs did not cause agglutination (diameter 5.65±0.45 µM).

Table 1. The studied RBCs s diameter after contact with anti-A heated serum and complement.

Dilution	without complement			with complement		
	Agglutination	Diameter (µM)	N	Agglutination	Diameter (µM)	N
1:2	3^+	6.08±0.2	29	2^+	4.52±0.94	18
1:4	2^+	4.93±0.77	15	1^+	4.46±0.66	10
1:32	1^+	5.64±0.94	8	w^+	4.02±0.06	8
1:2	3^+	5.83±0.5	24	2^+	4.74±0.38	22
1:4	2^+	6.55±0.39	21	1^+	6.42±0.49	17
1:8	1^+	6.33±0.43	10	w^+	5.85±0.62	10

Note: w: weak agglutination.

One of the two samples adsorbed by B RBCs anti-A,B serum after one-hour incubation at 20 °C showed the tendency to agglutination of the studied RBCs, as well as after the use of antiglobulin serum (AGS) with unwashed incubated RBCs. Adsorbed by B RBCs anti-A,B serum after one-hour incubation at 37 °C did not cause agglutination of the studied RBCs, use of AGS decreased the quantity of RBCs with the tendency to agglutination. Adsorbed by A and B RBCs anti-A,B serum increased the diameter of the studied RBCs at 37 °C. At 37 °C, hemolysis of the studied RBCs in the presence of anti-A serum combined with complement was noted.

The RBCs of the studied person demonstrated an irregular shape with a diameter 4.9±0.89 µM. The contact of the studied RBCs with anti-A serum led to the increased diameter, decreased quantity, and appearance of RBCs shadows. Shadows of objects were detected after the same reaction in a centrifuge tube. However, anti-A serum did not modify the studied RBCs being loaded with serum from an AB person, although decreased their quantity.

The complement use did not induce agglutination of the studied RBCs, however, increased their diameter from 4.0±0.1 µM to 4.38±0.16 µM, from 3.98±0.17 µM to 4.2±0.4 µM, from 4.14±0.38 µM to 4.43±0.28 µM (4.2±0.24 µM initially), as well as diameter of A RBCs s (4.16±0.4 µM to 4.6±0.21 µM, from 4.0±0.2 µM to 4.27±0.1 µM, initially 4.44±0.31 µM) and O RBCs (from 4.03±0.08 µM to 4.24±0.38 µM, from 3.9±0.17 µM to 4.22±0.32 µM, initially 4.6±0.4 µM). The quantity and diameter of the studied RBCs after the contact with anti-A,B serum were modified after the use of Lincomycin (from 3.87±0.9 µM to 3.9±0.18 µM), nystatin (4.16±0.06 µM), Fenbendazole (increased RBCs diameter: 4.13±0.43 µM, decreased titer). Interestingly, the incubation of the studied RBCs with fenbendazole increased the diameter of the studied RBCs to 6.0±0.1 µM contrary to the incubation with Nistatin (5.06±0.21 µM). Incubation of the studied RBCs with normal saline at 37 °C for 13 hrs led to the increased movement of irregularly shaped objects contrary to the incubation of normal B RBCs without anti-A antibody adsorbing ability. The use of the Lanta apparatus led to the agglutination of the moving objects. The diameter of the studied RBCs incubated with Fenbendazole was increased to 6.0±0.34 µM, with Nystatin: 5.06±0.21 µM, with Reosorbilact: 4.7±0.83 µM. The contact of anti-A serum with unwashed studied RBCs for 23 hours at 4 °C led to the rouleaux formation and RBCs diameter 5.0±0.5 µM, with serum from AB blood group: rouleaux formation with diameter 5.37±0.94 µM.

Anti-A heated serum did not agglutinate the studied RBCs at 13 C for 2.5 hrs contact, although increased the diameter of the studied washed RBCs from 4.18±0.38 µM in 1:16 titer to 6.08±0.2 µM in 1:2 titer on the contrary to anti-A heated serum combined with complement (4.02±0.06 µM in 1:32 titer to 4.52±0.94 µM in 1:2 titer, decreased quantity) (Table 2). For the comparison: anti-A,B

Table 2. Incubation of the studied RBCs with various sera.

Contact of the serum	with the studied RBCs		
	Agglutination	Diameter (μM)	N
Anti-A heated 2.5 hrs at 13 °C	neg	↑	
Anti-A,B after B RBCs + AGS 13 hrs at 13 °C	neg		normal
Anti-A		↑	↓
Anti-B	3^+	5.9 ±0.5	
Indirect Coombs' test: citrate anti-A 1 hr at 37 °C	neg		
Serum from AB	Rouleaux formation	5.37±0.94	
Anti-A,B after B RBCs + AGS	neg		
Plasma from BA+ 13 hrs at 37 °C		↑ 4.54±0.36-6.48±0.46	
Anti-A,B after B RBCs 1 hr at 37°C water bath 1 hr at 37 °C	neg		
Anti-A + Complement		↑4.0±0.1-4.38±0.16	
Anti-A 1 day	2^+	5.83±0.86	
Anti-A 1 hr at 13 °C	Tendency to w^{+hi}		
Anti-A,B after B RBCs at 20 °C	Tendency to w^+		
Anti-A at 37 °C 1 day	neg	5.65±0.45	
Anti-A,B after A,B RBCs at 37 °C		↑	
Anti-A,B + Lincomycin		3.87±0.9-3.9±0.18	
Anti-A,B + Nystatin		4.16±0.06	
Anti-A,B + Fenbendazole		4.13±0.43	

Note: d: diameter, N: quantity of RBCs, AGS: antiglobulin serum, neg: negative

serum increased the diameter of A RBCs from 5.16±0.5 μM in 1:32 titer to 6.33±0.61 μM in 1:2 titer, as well as the heated one: from 5.56±0.7 μM in 1:32 titer to 5.83±0.86 μM in 1:2 titer. Anti-A serum after 1 hr contact with the studied RBCs at 13 °C showed a slight tendency to agglutination contrary to the contact at 20 °C.

Study of the person's plasma.

The plasma agglutinated A RBCs at 4 °C and 37 °C and B RBCs at 37 °C. Fungi growth was detected. However, the heated to 60 °C serum for 30 mins did not agglutinate B RBCs at 37 °C. EDTA plasma from the studied person after incubation with his own RBCs at 37 °C for 13 hrs led to the increased diameter of RBCs from 4.54±0.36 μM to 6.48±0.46 μM with shades of the objects on the contrary to the contact with normal B RBCs (diameter 4.94±0.13 μM) (Table 3). The plasma from the studied person agglutinated A RBCs in 1:16 titer and increased their diameter (from 4.3±0.52 μM to 5.93±0.49 μM), as well as the heated plasma (at 60 °C for 30 mins) (from 5.51±0.28 μM to 5.86±0.43 μM).

The studied plasma did not increase the diameter of O RBCs after the contact at 20°C for 3 hrs (4.28±0.66 μM-4.02±0.07 μM) and A RBCs (4.0±0.1 μM-3.96±0.22 μM). The studied plasma agglutinated A RBCs weaker at 20 °C as compared to 13 °C. The plasma did not agglutinate B RBCs, however increased the diameter of the one-third of RBCs (from 4.05±0.26 μM to 4.86±1.0 μM).

Three-hour incubation of the studied plasma did not induce agglutination of B RBCs at 20 °C, however, increased their diameter (4.05±0.26 μM to 4.86±1.05 μM). The studied plasma weaker agglutinated A RBCs and decreased their diameter after the use of the complement at 20°C contrary to B RBCs. The plasma from the studied person agglutinated B RBCs at 37 °C in 1:2 titer after 7 hrs of incubation at 20 °C contrary to incubation at 10 °C. The studied plasma strongly agglutinated A RBCs (the highest titer) at 4 °C and weakly at 20 °C (the lowest titer). On the contrary, at 4 °C the studied plasma showed the absence of anti-B antibody activity, which was revealed at 37 °C in 1:2 titer. The studied plasma did not agglutinate B RBCs at 13 °C for 2 hrs contact, as well as the heated one, and did not increase the diameter of B RBCs on contrary to the heated plasma (5.75±0.7 μM in 1:32 titer to 6.02±0.4 μM in 1:2 titer).

Adsorption tests

Anti-A,B serum after adsorption by the studied RBCs for 13 hrs at 15 °C increased the diameter of A RBCs after the contact for 4 hrs (from 4.47±0.19 μM in 1:64 titer to 4.81±0.45 μM in 1:16 titer), however in a higher concentration the serum decreased A RBCs diameter (4.28±0.31 μM in 1:2 titer, 4.32±0.37 μM in 1:4 titer) (Table 4). Moreover, the serum did not lose the ability to agglutinate RBCs. Anti-A,B serum after adsorption by B RBCs increased the RBCs diameter as well (from 4.51±0.35

Table 3. Incubation of the studied plasma with A and B RBCs.

The contact of	Cells A		Cells B	
	Agglutination	Diameter (µM)	Agglutination	Diameter (µM)
Plasma from BA^+ at 4 °C	+ (highest titer)		neg	
Plasma from BA^+ at 20 °C 3 hrs	+ (lowest titer)	4.0±0.1-3.96±0.22	neg	↑4.05±0.26-4.86±1.05
Plasma from BA^+ at 37 °C			+ (1:2)	
Plasma from BA^+ 2 hrs at 13 °C			neg	
Anti-A+ plasma from BA^+ Complement at 20 °C'	↓			
Anti-A + plasma from BA^+	w^+		neg	
Plasma from BA^+ at 10 °C			neg	
Plasma from BA^+ 7 hrs at 20 °C			+	
Plasma from BA^+ 3 hrs at 20 °C		↑1:16 4.3±0.52-5.93±0.49		Normal 4.28±0.66-4.02±0.07
Heated plasma from BA^+ 2 hrs at 13 °C		5.51±0.28-5.86±0.43	neg	↑ 5.75±0.7-6.02 ±0.4 (1:32-1:2)
Anti-A,B after BA^+	↓ (1:32-1:16)			

Note: BA^+: the studied RBCs, w: weak, neg: negative

Table 4. Incubation of A RBCs with sera after adsorption by the studied RBCs.

The contact of the serum	Cells A	
	Agglutination	Diameter (µm)
Anti-A,B after BA^+RBCs 13 hrs at 15 °C		↑ 4.47±0.19-4.81±0.45 (1:64-1:16)
Citrate anti-A after unwashed BA^+RBCs	↓	↑ 5.03±0.81-5.5±0.86 (1:32-1:4)
Anti-A,B after unwashed BA^+RBCs 13 hrs at 10 °C	↓ (1:32-1:16)	↑ 4.34±0.23-4.48±0.59 (1:16-1:2)
Anti-A,B after unwashed BA^+RBCs13 hrs at 20 °C	↓↓ (1:16)	
Anti-A after BA^+RBCs 13 hrs at 20 °C	↑	4.14±0.24-4.2±0.21 (1:32-1:2)
Anti-A after BA^+RBCs+ Fenbendazole	↓ (1:8)	
Anti-A,B after BA^+RBCs at 37 °C	↓ (1:16)	
Anti-A,B after BA^+RBCs 13 hrs at 10 °C	↓ (1:32-1:16)	↑ 4.4 ±0.32-4.51±0.45
Anti-A,B after BA^+RBCs 13 hrs at 20 °C	↓ (1:16)	4.13±0.24-4.45±0.35 (1:16)
Anti-A after BA^+RBCs loaded by AB serum 5 hrs at 10 °C	Neg (1:2 initially)	
Anti-A after BA^+RBCs loaded by AB serum 5 hrs at 20 °C	neg	
Anti-A,B after BA^+RBCs1 day at 10 °C	↓ from 1:16-1:8	
Anti-A,B after BA^+RBCs + Fenbendazole	↑ from 1:16-1:32	
Anti-A,B after BA^+RBCs + Lincomycin	↓ from 1:16-1:8	
Anti-A after BA^+RBCs 14 hrs at 4 °C	neg	
Anti-A,B after BA^+RBCs at 37 °C	↓ (1:16)	
Anti-A,B after BA^+RBCs 1 day	↓ (1:16-1:8)	
Anti-A,B after BA^+RBCs + Fenbendazole	+(1:8)	↑
Anti-A,B after BA^+RBCs	↓ from 1:8-1:4	
Anti-A,B after BA^+RBCs + Nystatin	↓ (1:4)	
Anti-A 1:2 after 2 volumes of BA^+ RBCs 14 hrs at 4 °C	↓ (1:8-1:4)	
Anti-A,B after unwashed BA^+RBCs	Normal	
Anti-A,B after unwashed BA^+RBCs+ Fenbendazole	↓ (1:8)	
Anti-A,B after unwashed BA^+RBCs + Nystatin	+(1:8)	
Anti-A after BA^+RBCs(6:1) 23 hrs	↓	4.38±0.2-4.05±0.2 ↓ (1:32-1:16)

Note: BA^+: the studied RBCs.

μM in 1:64 titer to 4.62±0.42 μM in 1:2 titer), as well as without adsorption (4.53±0.25 μM in 1:64 titer to 4.65±0.3 μM in 1:2 titer).

After adsorption of citrate anti-A plasma by unwashed studied RBCs light decrease of agglutination of A citrated RBCs was noticed in 1:32 titer with increased RBCs diameter (5.03±0.81 μM in 1:32 titer to 5.5±0.86 μM in 1:4 titer). This effect was not observed with nonadsorbed citrate anti-A plasma (4.76±0.85 μM in 1:32 titer and 5.1±0.78 μM in 1:2 titer). Anti-A,B serum after adsorption by the studied unwashed RBCs for 13 hrs at 10 °C revealed the titer of agglutination of A citrated RBCs from 1:32 to 1:16 and increased their diameter (4.23±0.45 μM in 1:16 titer to 4.48±0.59 μM in 1:2 titer after 5 hr incubation at 10 °C) as well as without adsorption (4.16±0.25 μM in 1:16 titer to 4.42±0.5 μM in 1:2 titer). Anti-A,B serum after adsorption by unwashed studied RBCs for 13 hrs at 20 °C significantly lost the agglutinating ability of A RBCs: weak agglutination was detected in 1:16 titer. An interesting effect was noticed: RBCs diameter 4.01±0.18 μM in 1:16 titer and 4.3±0.48 μM in 1:2 titer.

Two samples of anti-A,B sera after adsorption by washed studied RBCs for 13 hrs at 10 °C decreased the titer of agglutination of A RBCs from 1:32 to 1:16 and increased RBCs diameter (4.4±0.32 μM in 1:32 titer and 4.51±0.45 μM in 1:2 titer, 4.58±0.41 μM in 1:32 titer and 4.67±0.34 μM in 1:4 titer, 4.4±0.56 μM in 1:2 titer).

Anti-A,B serum after adsorption by the studied RBCs for 13 hrs at 20 °C significantly decreased the titer of agglutination of A RBCs: less expression of agglutination in 1:16 titer with increased RBCs diameter (from 4.13±0.24 μM in 1:16 titer to 4.45±0.35 μM in 1:4 titer, 4.28±0.24 μM in 1:2 titer). Anti-A serum after adsorption by the studied RBCs at 7 °C for 5 hrs, loaded previously with serum from AB person (1:3 ratio), stopped agglutinating A RBCs (initial titer 1:2) as well as after adsorption at 20 °C. Anti-A,B serum after adsorption by the studied RBCs at 37 °C decreased the expression of agglutination of A RBCs in 1:16 titer. Anti-A,B serum after adsorption by B RBCs contacted with the studied RBCs for 13 hrs at 37 °C, and antiglobulin serum did not lead to agglutination. Anti-A,B serum did not decrease the titer of agglutination of A RBCs after adsorption by unwashed studied RBCs (1:16 and 1:32), however, decreased the titer to 1:8 after adsorption by the studied RBCs with fenbendazole and nystatin. Adsorption of anti-A,B serum with the studied RBCs for one day at 10 °C the titer of A RBCs decreased from 1:16 to 1:8. Interestingly, from two anti-A sera after adsorption by the studied RBCs for 23 hrs (6:1 ratio of the sera and RBCs) only one serum decreased the titer of agglutination of A RBCs (from 1:32 to 1:16 titer) and decreased the diameter of A RBCs (4.38±0.2 μM in 1:32 titer to 4.05±0.4 μM in 1:2 titer). The nonadsorbed serum increased the diameter of A RBCs (3.96±0.1 μM in 1:32 titer to 4.33±0.68 μM in 1:2 titer).

Adsorption by washed and unwashed RBCs anti-A,B serum decreased the titer of agglutination of A RBCs from 1:32 to 1:16 titer. Adsorbed by washed and unwashed RBCs with saline in 2:1 ratio anti-A,B serum decreased the titer of agglutination of A RBCs. Adsorption by washed RBCs appeared more successful as compared to adsorption by unwashed RBCs. The most optimal adsorption appeared to be at 4 °C for 13 hrs with anti-A,B serum. Thus, anti-A,B serum after adsorption by the studied RBCs as well as RBCs combined with Nystatin decreased the expression of agglutination of A RBCs contrary to the adsorption by the studied RBCs combined with Fenbendazole.

The adsorption of the studied RBCs by anti-A serum was better at 4 °C as compared to 37 °C. Anti-A,B serum increased the diameter of A RBCs s from 5.08±0.6 μM in 1:32 titer to 6.5±0.4 μM in 1:4 titer. However, did not increase the diameter of A RBCs after adsorption by the studied RBCs and e RBCs combined with fenbendazole, however, increased the RBCs diameter after adsorption by RBCs combined with nystatin (from 5.33±0.81 μM in 1:32 titer to 5.47±0.33 μM in 1:8 titer).

Anti-A serum continued agglutinating A RBCs at 1:8 titer after adsorption by the studied RBCs without previous loading with serum from AB person and increased A RBCs diameter (4.16±0.41 μM in 1:64 titer to 4.6±0.28 μM in 1:2 titer). The anti-A,B serum decreased the titer of agglutination of A RBCs s from 1:16 to 1:8 after adsorption by the studied RBCs for one day contrary to adsorption by the studied RBCs with fenbendazole (1:32 titer), however, the titer of the other anti-A,B serum also decreased after adsorption by the studied RBCs with Lincomycin (from 1:16 to 1:8).

The supernatant after washing the studied RBCs in 1:2 and 1:4 titer decreased the expression of agglutination of A RBCs by anti-A,B serum. Anti-A,B serum after adsorption by the studied RBCs decreased the titer of agglutination of A RBCs from 1:32 to 1:16 contrary to the serum after adsorption by the studied RBCs with Ofloxacine: the titer 1:32 (sensitive to *Proteus vulgaris* of the person's blood culture of the person appeared to be sensitive to Ofloxacine).

Anti-A serum did not decrease the titer of agglutination of A RBCs after adsorption by washed studied RBCs for 13 hrs at 20 °C and increased the RBCs diameter (4.14±0.4 μM in 1:32 titer and 4.2±0.33 μM in 1:2 titer) as well as nonadsorbed serum (4.32±0.37 μM in 1:32 titer and 4.42±0.43 μM in 1:2 titer). However, anti-A,B sera with a higher quantity of IgG antibodies showed inhibition of agglutination after adsorption with the studied RBCs. After the contact with Fenbendazole the adsorption of anti-A antibodies by the studied RBCs was increased: the titer of agglutination of A RBCs was decreased to 1:8 (with smaller RBCs diameter 4.01±0.33 μM in 1:2 titer and increased RBCs diameter in 1:4 titer: from 4.02±0.18 μM in 1:16 titer to 4.24±0.29 μM). One sample of anti-A,B serum showed the absence of agglutination of A RBCs after adsorption by the studied RBCs with fenbendazole (1:32 initial titer).

The supernatant from washed studied RBCs did not decrease agglutination of B RBCs by citrate anti-B plasma and somewhat increased the expression of agglutination as compared with the same concentrations of anti-A citrate plasma (control) and increased RBCs diameter (from 4.96±0.95 μM in 1:16 titer and 6.05±0.49 μM in 1:2

titer). The serum decreased agglutination of A RBCs in 1:16 titer after adsorption by unwashed studied e RBCs with fenbendazole, as well as after adsorption by the studied RBCs with nystatin. Anti-A,B serum increased the diameter of A RBCs (from 4.62±0.29 μM in 1:32 titer to 4.95±0.57 μM in 1:2 titer, and somewhat less, increased the diameter of A RBCs after adsorption by the studied RBCs s (from 4.32±0.28 μM in 1:32 titer to 4.65±0.21 μM in 1:2 titer, after adsorption by the studied RBCs with Nystatin: from 4.06±0.11 μM in 1:32 titer to 4.9±0.65 μM in 1:2 titer). Anti-A,B serum after adsorption by the studied RBCs with fenbendazole decreased the titer of agglutination of A RBCs from 1:32 to 1:2. Anti-A,B serum after adsorption by the studied RBCs decreased the titer of agglutination of A RBCs from 1:8 to 1:4 (the diameter was decreased with decreasing concentration of the serum) on the contrary to the adsorption with fenbendazole (the titer appeared 1:8) and the RBCs diameter increased with decreased concentration of the serum. The adsorption of the serum by the studied RBCs combined with nystatin decreased the titer of agglutination of A RBCs to 1:4 (as well as after adsorption by the studied RBCs without nystatin). After adsorption of the studied RBCs with fenbendazole the titer of anti-A,B serum with A RBCs was not decreased (1:16 and 1:32). After adsorption by the studied RBCs with Lincomycin the titer of anti-A,B serum decreased from 1:16 to 1:8.

Indirect Coombs' test

Indirect Coombs' test with anti-A serum at 37 °C did not show agglutination of the studied RBCs. Citrate anti-A plasma was incubated with the studied RBCs for 1 hr at 37 °C, the supernatant was removed and antiglobulin serum (AGS) was added, however, agglutination was not determined, as well as with anti-A,B serum after adsorption by B RBCs. Anti-A,B serum without anti-A and anti-B antibodies (being adsorbed by A and B RBCs) in a water bath with AGS and incubation at 20°C for 13 hours did not decrease the quantity of the studied RBCs contrary to the sample adsorbed only by B RBCs with the presence of anti-A antibodies, that led to the significant decrease of the quantity of the RBCs with a tendency to agglutination.

The study of the transfer of weak A antigen expression from the person's plasma to normal B RBCs

Anti-B serum containing A transferase for one day contact with B RBCs at 4 °C induced anti-A antibody adsorbing ability of B RBCs. Thus, the titer of agglutination of A RBCs by anti-A,B serum adsorbed by decreased from 1:32 to 1:8 after adsorption by B RBCs previously incubated with anti-B serum the on the contrary to the titer of anti-A,B serum adsorbed by usual B RBCs.

To reveal the activity of A and B transferase in the studied plasma A and B RBCs were contacted with the studied plasma for two days at 37 °C. Thus, A RBCs contacted with the studied plasma showed anti-B antibody adsorbing ability: the titer of agglutination of B RBCs by anti-A,B serum was decreased from 1:4 to 1:2. Similarly, the titer of agglutination of A RBCs by anti-A,B serum adsorbed by B RBCs after contact with the studied plasma was decreased from 1:32 to 1:16.

Discussion

Weak subgroups are detected, if there is a discrepancy between RBCs and serum grouping and are often found when unexpected anti-A or anti-B activity of the serum is determined.[12,13] Weak subgroups of A blood type are reported by the researchers. Thus, a patient with a discrepancy in forward and reverse blood typing was studied by adsorption elution tests and saliva tests. The RBCs were similar to A_3 subtype. The person appeared to be a secretor (A and H substances were detected in the saliva). Anti-A_1 antibody was not revealed in the serum. The weak A antigen showed serological characteristics of A_3.[14]

Weak A antigen may be differentiated by various methods. These weak antigen appear due to weak expression of weak allele at the ABO loci. The subgroups are weakly agglutinated by anti-A sera (A_3, A_x, and A_{end}) or do not react (A_m, A_y, and A_{el}). Saliva hemagglutination inhibition test and adsorption-elution procedures are used to confirm the presence of weak antigen. While saliva investigation was reported to reveal specific substances, adsorption-elution showed A antigen specificity in different of A_m, A_y, and A_mB antigen.[15] A antigen subgroups have been classified into two main classes. Approximately, 20% of individuals having A antigen in blood belonging to A_2 antigen were identified in 20% of persons with A blood type thus, forming A_2 or A_2B subgroups. Weaker than A_2 subgroups are seldom detected. A_1 and A_2 antigen differ by the reaction with lectin: anti-A_1 (a cold agglutinin) agglutinates A_1 RBCs. A_2 and A_2B subgroups show the presence of anti-A_1 antibodies reacting at 37 °C.[16,17]

The distinction between A_1 and A_2 was made by testing red cells with the lectin from Dolichos biflorus. The A_2 gene has two nucleotides different from A_1 gene and has diminished enzymatic activity.[13,18,19] Scanning immune electron microscopy with monoclonal anti-A antibodies showed A antigen on less than 5% of A_m and A_{el} cells with strong labeling, therefore Am and A_{el} cells are able to adsorb significant amounts of anti-A antibodies without visible agglutination. Thus, the method of adsorption, but not agglutination is preferred for revealing weak A antigen, that showed effectiveness in the conducted study.

A_3 is considered as a heterogenous subgroup, A antigen were found on 82 and 58% of the v of A_3 persons. A receptor was detected on 75% of A_x RBCs. Weak A antigen are thought to be better detected if anti-A antibodies react with other A oligosaccharide chains than type 2 is used. From hyperimmune pregnancy sera A_x, $A_{m,}$ and A_{el} cells adsorbed antibodies that had other characteristics than antibodies adsorbed by A_2 RBCs. We conclude that weak subgroups of A may deviate from A_2 both by the number of RBCs expressing A antigen and the biochemical nature of the antigen.[20]

The discrepancy between forward and reverse blood typing often occurs due to the low expression of antigens on RBCs. Neutralization agglutination is used for

the detection of group antigens in saliva. Surface plasmon resonance imaging is also used for antigen identification on RBCs and in saliva. Antigen are detected by anti-A, anti-B, and anti-H antibodies, the array is immobilized on the sensor surface. RBCs and saliva specimens are analyzed by passing them over the antibody array, where the secretor status and blood group may be identified.[19]

ABO discrepancy cases were described when RBCs from blood group A persons come into contact with certain gram-negative bacteria of colonic origin. These bacteria carry an enzyme, that cleaves a part of A antigen (acetyl group from the last sugar on A chain, N-acetylgalactosamine), leaving galactosamine, that resembles the B antigen. Acquisition of A and B antigen are widely discussed, especially in ABO-incompatible hematopoietic stem cell transplantation (HSCT). The studies investigated the weak blood group A or B antigen expression by donor-derived group O RBCs observed following transfusion or minor ABO-incompatible HSCT.[21]

The persons typed as A, B, and AB and transfused from group O donors show the presence of A antigen on donor-derived RBCs (A- A_x - profiles). After group O donor RBCs were incubated with group A/B secretor/non-secretor donor plasma or RBCs adsorption of A/B antigen-bearing glycolipids from secretor plasma pointed to a secretor-independent mechanism for A/B antigen acquisition. Conversion of donor-derived blood group O RBCs to ABO subgroup-similar RBCs *in vivo* after transfusion or minor ABO-incompatible HSCT shows the necessity to reveal and control weak antigen expression.[21]

Some reports of A subgroup with acquired B antigen were described in urinary tract infections. The RBCs showed mixed field agglutination with anti-A, anti-AB and microscopic agglutination with anti-B antibodies and anti-B antibodies on serum grouping. There was a strong reaction with A_1 Lectin and anti-H, a positive auto-control, and direct antiglobulin. A subgroup blood transfusion was considered to be avoided since the patient demonstrated the presence of autoantibodies.[22]

The cases of weak A antigen appear rather often. Thus, the RBCs of type O persons with *Proteus mirabilis* infection were agglutinated by an adsorbed anti-A antibody. The reaction with anti-A antibody was inhibited by blood group A substance. Acquisition of A-like antigen was considered to be induced by *Proteus* infection but, the changes persisted after the bacterial infection was cured. Various abnormalities of the RBCs membrane were noticed. Therefore, the presented study confirms the data of the association of anti-A antibody adsorbing ability with *Proteus* infection. Other researchers report the person with acquired A antigen. The patient's RBCs were agglutinated by anti-A antibodies and less strongly by anti-B antibodies with mixed field agglutination and serum anti-A and anti-B antibodies were found in serum. The antiglobulin test was positive. The person's serum contained hemolytic anti-A antibodies and saline agglutinating anti-B antibodies. The person's serum agglutinated but never hemolyzed his own cells. Neither freezing in liquid nitrogen nor in glycerol had any effect on the acquired A-like antigen of the person's cells. Eluates made on several occasions displayed weak antibody activity. The eluate agglutinated A_1 RBCs. The weak positive direct antiglobulin test was seen in the same specimen in which the patient's serum lacked auto-agglutinating anti-A. Adsorption with a person's cells did not significantly lower the titer of anti-A antibodies. A substance caused total inhibition of anti-A agglutination while B and H substances had no effect. When total neutralization of the anti-A antibody was achieved, as evidenced by its failure to agglutinate normal A_1 and A_2 cells, some neutralized sera still reacted weakly with the person's cells. The reactions between five different commercial anti-A or anti-A_1 reagents and a person's cells were totally inhibited by A substance. An activity of RBCs was absent after ficin treatment. The enzyme activity of the supernatant was destroyed by boiling. Bacterial or viral infections seem likely to initiate the erythrocyte changes, the Ogata phenomenon was also reported. Lack of a particular fraction of anti-A and anti-B antibodies may be the factor, that allows an individual to acquire A and B antigen.

In a person with acquired A antigen a difference between A antigen on RBCs and antigen on polyagglutinable cells is that the person's anti-A antibody could be inhibited by A substance while anti-A reaction could be inhibited only with N-acetyl-D-galactosamine. Further evidence of bacterial association is provided by the numerous studies that have convincingly demonstrated the presence of both A- and B-like substances in *Escherichia* and *Proteus* organisms. A marked difference between acquired A and B antigen in recently described cases is that the acquired A antigen of the person survived freezing in glycerol and liquid nitrogen while the acquired B antigen in patients with polyagglutinable cells did not. The mechanism of A antigen acquisition is discussed. RBCs with decreased sialic acids have occurred due to the inherited variance or an acquired alteration. Thus, if a portion of the glycolipid and/or glycoprotein chain is removed, an A-like antigen is exposed, possibly following the reorientation of the remaining portions of the chains. Acquisition of an A-like antigen might be independent of the *Proteus* infection, since the acquired A antigen persisted for six months after the disappearance of *Proteus* infection. The *Proteus* infection may have initiated, an alteration in the antigenic expression of RBCs.

Revealing of A antigen in person with B blood group was also reported. The B(A) phenotype showed the presence of anti-A antibodies in the serum and since A antigen on the RBCs were presented few, thus no hemolysis occurred. The cell grouping showed A weak B, while the serum grouping demonstrated B. A set of anti-A antibodies was showing weak agglutination. Thus, the case highlighted the serological characteristics of a B(A) phenotype.[23]

A blood type subgroups are known to secrete fewer antigen in saliva. They may adsorb naturally occurring anti-A_1 without hemolytic complications (cold antibodies), however, may develop anti-A_1 antibodies when exposed to A_1 antigen (reacting at 37°C), since they are of IgG

nature.[24,25] This explains the observed peculiarities in optimal temperatures for anti-A and anti-B antibodies of the studied person's plasma. The mechanisms of acquisition of A and B antigen deal with the glycosylation of complementary domains.[26] Thus, further studies on the mechanisms of A and B antigen acquisition are needed. This is the second case where we report a person with B blood group, weak A antigen expression, and anti-B antibodies reactive at 37 °C.[27]

Thus, a person's B blood group RBCs were agglutinated by anti-B antibody, and the agglutination was 1^+ at 37 °C. However, the RBCs did not adsorb anti-B but anti-A antibodies. The RBCs were agglutinated by anti-A at 37 °C with signs of hemolysis in the presence of complement. Meanwhile, the serum agglutinated A RBCs at room temperature with less activity at 37 °C and B RBCs at 37 °C. The RBCs were agglutinated at 37 °C by polyclonal serum, whereas serum weakly agglutinated A RBCs at 37 °C. The absence of anti-B antibody absorbance by the person's RBCs was accompanied by the presence of anti-B antibodies, active at 37 °C. The incubation of the person's serum with O RBCs induced the ability of RBCs to adsorb anti-A antibodies and to be hemolyzed by anti-A in the presence of complement. Adsorption and agglutination at room temperature and 37 °C by heated serum with the use of complement helped to reveal weak A antigen. However, the person was not studied bacteriologically.

The present study is in accordance with data of other researchers pointing to the possible associations between anti-A antibody adsorbing ability and *Proteus vulgaris* infection. Whether *Proteus vulgaris* infection induces A antigen expression or the presence of weak A antigen predisposes the development of *Proteus vulgaris* infection needs to be elucidated. Acquisition of A antigen was found long ago and reports of new cases stimulate the discussion and study of this phenomenon.[28,29] Thus, not only weak B antigen has been found to be associated with bacterial infection but A antigen acquisition as well.[30]

Conclusions

Subgroups are usually wrongly typed as O, which may develop into a hemolytic transfusion reaction. Adsorption tests of the subgroups could be helpful in confirming weak antigen. The presented case emphasizes the important role of cell and serum grouping in revealing such discrepancies, that may lead to complications during transfusion recipient, if not resolved. Any should be examined microscopically. The presented case emphasizes the important role of cell and serum grouping in revealing such discrepancies. If ABO discrepancy in forward and reverse typing is found, a high probability of weak antigen expression exists to reduce the incidence of transfusion-related reactions. The presence of weak antigen in the blood group should be accompanied by a thorough bacteriological investigation of the person.

Ethical approvals

The study was approved by the Kharkiv National Medical University ethics committee (protocol 4).

Conflict of interest

The authors declare that they have no conflict of interest.

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