**Original Article**

**A Clinical Case of Weak A Antigen on the Erythrocytes in a Person with Coexistent Anti-A Antibodies**

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**ABSTRACT**

This study investigated a person with an AB0 discrepancy. Her blood group initially typed at the birth as AB Rh+ (positive); however, it was B Rh+ (positive) or Rh- (negative) when she was in her teens. At room temperature, her erythrocytes were agglutinated by anti-B, and the agglutination was significantly weaker at 37 °C. As a result, her erythrocytes did not absorb anti-B but anti-A. Furthermore, her erythrocytes were agglutinated by anti-A at 37 °C with signs of hemolysis in the presence of complement. The unwashed erythrocytes were also agglutinated in an antiglobulin test by polyclonal anti-A at 37 °C and by heated polyclonal anti-A and anti-A MAB 2-8 at room temperature. Moreover, her serum agglutinated A erythrocytes at room temperature with less activity at 37 °C; however, it agglutinated B erythrocytes at 37 °C. The ability of the erythrocytes of this person to absorb anti-A came along with the agglutination of her erythrocytes at 37 °C by polyclonal serum and decreased activity of the serum to agglutinate A erythrocytes at 37 °C, compared to room temperature. The absence of anti-B absorbance by the person’s erythrocytes was accompanied by the presence of anti-B in the serum, which was active at 37 °C. The incubation of the person’s serum with 0 erythrocytes induced the ability of erythrocytes to absorb anti-A and to be hemolyzed by anti-A in the presence of complement in accordance with the person’s characteristics of erythrocytes. The reaction of absorption and agglutination at room temperature and 37 °C by heated serum with the use of complement may help to reveal both weak A and B antigens and anti-A and anti-B antibodies while AB0 blood typing.

**Keywords:** Absorption, Agglutination, A-transferase, Hemolysis, Weak A antigen

**Un cas clinique d’un taux faible d’antigène A sur les érythrocytes d’une personne avec des anticorps anti-A coexistants**

**Résumé:** Cette étude a examinée une personne présentant un écart AB0. Son groupe sanguin a d'abord été typé à la naissance comme AB Rh+ (positif); cependant, celui-ci a été rapporté B Rh+ (positif) ou Rh- (négatif) lors de son adolescence. À température ambiante, ses érythrocytes ont été agglutinés par des anti-B, et l'agglutination était significativement plus faible à 37 °C. En conséquence, ses érythrocytes n'ont pas absorbé les anti-B mais les anti-A. En outre, ses érythrocytes ont été agglutinés par anti-A à 37 °C avec des signes d'hémolyse en présence de complément. Les érythrocytes non-lavés ont également été agglutinés lors d’un test à l'antiglobuline par un anticorps polyclonal anti-A à 37 °C et par un anticorps polyclonal anti-A et anti-A MAB 2-8 chauffé à température ambiante. De plus, son sérum a agglutiné les érythrocytes A à température ambiante avec moins d'activité à 37 °C; cependant, il a agglutiné les érythrocytes B à 37 °C. La capacité des érythrocytes de cette personne à absorber les anti-A s'est accompagnée de l'agglutination de ses érythrocytes à 37 °C par le sérum polyclonal et d'une diminution de l'activité d'agglutination du sérum vis-à-vis des érythrocytes A à 37 °C, comparée aux analyses effectuées à température ambiante. L'absence d'absorbance anti-B par les érythrocytes
INTRODUCTION

Weak subgroups of A can be defined when erythrocytes give weaker reactions or are nonreactive serologically with anti-A than do those of subjects with A_2 erythrocytes. These weak phenotypes result from the expression of an alternate weak allele present at the ABO loci. Weak subgroups of A can be divided into 2 categories depending upon whether the cells are agglutinated with anti A or not. In this regard, A_3, A_end, and A_x are agglutinated, whereas A_m, A_y, and A_el cells are not. The phenotypes can be serologically differentiated from each other using the following methods, namely the agglutination of cells by anti-A, anti-B, anti-A, B, and anti-H; serum grouping of ABO antibodies and the presence of anti-A_1; adsorption elution experiments with polyclonal anti-A from group B and group O individuals; and secretor status for the presence of H and/or A antigen (Heier et al., 1994; Pennec et al., 1996; Thakral et al., 2005). Moreover, the researchers have recently discovered that the presence of phenotype of cis-AB is A_2B_3 when it can express various phenotypes as paired with an A or B allele. Insufficient investigation can lead to misclassification in the ABO grouping and consequently to adverse hemolytic transfusion reactions (Chun et al., 2019). Samples from cis-AB subjects present forward or reverse ABO blood typing as ABO discrepancy. It may be suspected when there is weak agglutination of erythrocytes with the anti-B reagent in cell typing and weak agglutination with B cells in serum typing. Weak agglutination with B cells can be enhanced when the reaction is incubated at room temperature for 15 min. In contrast to cis-A_1B_3, the trans-A_1B_3 blood group originated from heterozygosity of A_1 and B_3 shows no agglutination with B cells in serum typing despite prolonged incubation. In addition, a measurable amount of H antigen is suggestive of the cis-AB blood group (Chun et al., 2019). The authors have presented cases when for a person cis-A_1B_3 interpreted as type A and the transfusion of four units of A erythrocytes and four units of A fresh frozen plasma (FFP) caused delayed transfusion adverse effects since the results of pre-transfusion cross-match had not been properly interpreted (Woo et al., 2006). Although the reaction between the B antigen of the cis-AB patient and anti-B antibodies from the A-type FFP is theoretically possible, the authors could not draw a definitive conclusion on the cause of hemolysis (Woo et al., 2006). Another group of researchers reported a case of transfusion of A erythrocytes, FFP, and platelets to a 14-year-old male with cis-A_2B_3 blood type. Reverse typing showed that his serum contained anti-B, but no anti-A antibodies. Moreover, the patient showed no adverse reactions, which can be explained by the weak B antigen on his erythrocytes without anti-A antibodies against the transfused A erythrocytes. The same patient had been transfused with typical AB blood at the age of 13 months without any adverse reaction, which can also be explained by the fact that he may have had no or low anti-A and anti-B antibodies in his serum against the transfused AB blood during this first transfusion (Yoon et al., 2005). The Cis-AB is difficult to determine since it presents more than one phenotype;
moreover, the various phenotypes make quick blood group determination difficult. Therefore, this study aimed to determine the AB0 blood group type phenotype in a person with AB0 discrepancy.

MATERIAL AND METHODS

The determination of the blood group of the AB0 system using various methods was conducted in this study. Serological reactions were performed using standard protocols (Denomme et al., 2000; Brecher, 2002). Moreover, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$ was used for pH 6.0 and pH 8.0. The agglutination of erythrocytes by polyclonal serum was performed at room temperature of 37 ºC. In addition, the antiglobulin test and agglutination with complement were used for AB0 typing. Totally, two volumes of serum (unheated or heated at 60ºC for 30 min) were mixed with one volume of 3% suspension of erythrocytes (washed or unwashed) and incubated at 37 ºC to stimulate agglutination for 1 h. Subsequently, samples were examined macroscopically and microscopically for agglutination. The presence of agglutinating anti-A or anti-B antibodies was fixed, and antiglobulin serum was used for the antiglobulin test. The preparation of glycoconjugates from erythrocyte membranes was described before (Delevsky, 1999)(i.e., glycolipid fraction of erythrocytes [Alp], and glycoprotein fraction of erythrocytes [Apr]). Furthermore, monoclonal antibody MAB 2-8 anti-A IgG$_3$ (clone GAMMA-120, mouse monoclonal culture supernate, and Gamma Biologicals, Inc, USA) were utilized to detect A antigen on erythrocytes. The complement of guinea pig (C') in dilution with 0.9% NaCl 1:5 was used for agglutination in the presence of complement. Moreover, 0 erythrocytes, serum of A (II) Rh+positive, and B (III) Rh+positive persons were used for the experiments.

RESULTS

A student was included in this study for the detection of her blood group type. She was typed as AB Rh+ after birth; however, she was typed as B Rh- (negative) and B Rh+ (positive) when she was in her teens. Several investigations were conducted on her blood group detection. At room temperature, her erythrocytes were agglutinated by monoclonal anti-B antibody, not monoclonal anti-A antibody. Moreover, her serum agglutinated A erythrocytes at room temperature and did not agglutinate B-erythrocytes. At 37 ºC, the washed and not washed erythrocytes were also agglutinated by polyclonal anti-B. Interestingly, the erythrocytes did not absorb polyclonal anti-B but did absorb anti-A. Furthermore, the unwashed erythrocytes at 37 ºC were found to be agglutinated by polyclonal anti-B in the presence of the complement as well as by the heated polyclonal anti-B and adding the complement; however, there was no decrease in the number of erythrocytes. At room temperature, the washed erythrocytes were also agglutinated in the presence of polyclonal anti-B and complement, as well as by the heated polyclonal anti-B in the presence of the complement (Figure 1).

**Figure 1.** The studied washed erythrocytes and heated anti-B and C' at room temperature.

The method of agglutination in the presence of the complement was used as it was previously proposed (Kravchun et al., 2019) for the detection of weak antigens on the erythrocytes. Therefore, at 37 ºC, the washed erythrocytes were weakly agglutinated by polyclonal anti-A and were not agglutinated in the reaction with the complement and polyclonal anti-A;
however, the amount of the erythrocytes diminished. At 37 ºC, the unwashed erythrocytes were agglutinated by polyclonal anti-A (Figure 2) and were also agglutinated with the decrease of their amount in the presence of the heated polyclonal anti-A and complement (Figure 3), compared to the heated polyclonal anti-B and complement (Figure 4).

At room temperature, the washed erythrocytes were not agglutinated by polyclonal anti-A and complement. Nevertheless, they were agglutinated in the presence of the heated polyclonal anti-A and complement (Figure 5) no less than by anti-B in the same reaction (Figure 1).

In the reactions at 37 ºC with antiglobulin serum (AGS), the washed erythrocytes were not agglutinated by anti-A. However, both washed and unwashed erythrocytes were agglutinated by the heated anti-A at 37 ºC, the heated anti-A, and AGS. A special monoclonal antibody anti-A IgG3 MAB 2-8 was used for the analysis of the presence of A antigen on the erythrocytes. Accordingly, at room temperature, the unwashed erythrocytes were also agglutinated by MAB 2-8 in the antiglobulin test. Moreover, the unwashed erythrocytes were not agglutinated at 37 ºC in the presence of MAB 2-8 and AGS; nevertheless, they were agglutinated at pH 6.0 and 8.0. The studied serum of the student agglutinated washed A erythrocytes at 37 ºC (Figure 6). Moreover, the unwashed erythrocytes were not agglutinated at 37 ºC in the presence of MAB 2-8 and AGS; nevertheless, they were agglutinated at pH 6.0 and 8.0. The studied serum of the student agglutinated washed A erythrocytes at 37 ºC (Figure 6). At 37 ºC, the serum weakly agglutinated washed B erythrocytes; however, it did not agglutinate in the presence of the complement. The anti-B was considered as complement independent; nonetheless, it was hemolysis since after adsorption of her anti-B by B erythrocytes, the serum hemolyzed B erythrocytes at 37
ºC in the presence of complement with less activity (Figure 7), compared to the serum without adsorption (Figure 8). Furthermore, anti-B antibodies that are active at 37 ºC and reactive with complement might cause hemolysis of own erythrocytes carrying B antigen. Moreover, at 37 ºC, the serum agglutinated and hemolyzed the unwashed erythrocytes in the presence of the complement (Figure 9).

The substance in the serum caused less hemolysis at 37 ºC by anti-A and complement of own unwashed erythrocytes, compared to the reaction with washed erythrocytes (Figure 10). Immunosuppressive α-globulin present in the serum may inhibit this reaction (Heier et al., 1994; Kravchun et al., 2019). It is worth mentioning that the serum at 37 ºC did not agglutinate B erythrocytes in the antiglobulin test.
It was suggested that anti-A antibodies might be bound to the weak A antigens on the erythrocytes and in the presence of the complement may cause hemolysis (Figure 9). Therefore, the previous adsorption of anti-A by A erythrocytes allowed to neglect such hemolysis in the presence of own serum, erythrocytes, and the complement (Figure 11).

Description of the Results of AB0 Typing of the Studied Sample.

Weak A antigen
- absorbs anti-A at 37 °C
- is revealed at room temperature by the heated polyclonal serum with complement and the increased size of erythrocytes (d=7.6 µm), compared to the erythrocytes with not heated serum (d=7.1 µm) and complement.
- is weakly agglutinated by polyclonal anti-A and complement at room temperature
- is revealed by polyclonal serum at 37 ºC with washed erythrocytes
- is revealed by MAB 2-8 with unwashed erythrocytes and AGS at room temperature
- is revealed by MAB 2-8 at 37 ºC at pH 6.0 and pH 8.0 in the reaction with AGS
- is weakly revealed at 37 ºC by heated anti-A with unwashed erythrocytes and complement
- erythrocytes are hemolyzed at pH 6.0 at 37 ºC by complement and polyclonal serum
- washed erythrocytes are hemolyzed at 37 ºC by the heated polyclonal anti-A and AGS (unwashed are not hemolyzed)
- is not revealed at room temperature with washed erythrocytes as well as with complement

Anti-A
- is active at room temperature (1:16)
- is active at room temperature at pH 8.0; however, it is less active at 6.0

B antigens
- do not absorb polyclonal anti-B at 37 ºC
- are revealed at room temperature by polyclonal serum and polyclonal serum with complement
- are revealed at room temperature by heated polyclonal anti-B and complement use, the size of erythrocytes decreases (d=7.6 µm) as compared to erythrocytes after the contact with unheated polyclonal serum and complement (d=8.1 µm)
- are revealed by heated polyclonal anti-B at 37ºC and complement with less agglutination at room temperature
- bind complement at room temperature with washed erythrocytes both with polyclonal and the heated serum, as well as with unwashed erythrocytes at 37ºC

Weak Anti-B
- is active at 37 ºC
- is not active in the presence of complement and AGS

The biosynthesis of weak A antigens was performed on 0 erythrocytes by the serum of a person with A weak antigen following the method of Delevsky (1974) on the biosynthesis of glycolipid and glycoprotein determinants on erythrocytes. Moreover, the incubation of 0 erythrocytes with the studied serum for 48 h at 37 ºC with medium 199 and antibiotic-induced the ability of 0 erythrocytes to absorb polyclonal anti-A. Furthermore, the incubation at pH 6.0 led to the same strength of 0 erythrocytes to absorb anti-A, whereas the incubation at pH 8.0 diminished; however, it did not abolish the induced anti-A absorbance by the erythrocytes.

It is suggested that the A-transferase present in the serum of the person with A weak antigen-induced synthesis of weak A antigens on 0 erythrocytes, which then inhibited hemagglutination of anti-A and A erythrocytes at 37 ºC. According to the present activity at pH 6.0 and reduced activity at pH 8.0, this transferase is active in the acid medium.

Interestingly, the incubation of 0 erythrocytes with glycolipid fraction of A erythrocytes Alp (Delevsky, 1974; Delevsky, 1999) did not induce anti-A absorbance of the erythrocytes. Nevertheless, the incubation with glycoprotein fraction of membranes of A erythrocytes Apr induced anti-A absorbing ability of 0 erythrocytes. Erythrocytes acquired the ability to absorb anti-A and to be agglutinated by anti-A at pH
6.0. In addition, 0 erythrocytes after incubation in the medium of serum with A-transferase activity appeared to have non-agglutinating complement binding glycotopes of A specificity in accordance with the characteristics of the donor’s serum (Table 1, Table 2).

Table 1. A-agglutinogenic activity of 0 erythrocytes at 37 ºC after incubation with a serum of the donor with A weak antigen

<table>
<thead>
<tr>
<th>Anti-A</th>
<th>Anti-A+C'</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 erythrocytes after incubation</td>
<td>-</td>
</tr>
<tr>
<td>0 erythrocytes after incubation at pH 6.0</td>
<td>+</td>
</tr>
<tr>
<td>0 erythrocytes after incubation at pH 8.0</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. Inhibition of hemagglutination by 0 erythrocytes and glycoconjugates of lipid and protein origin

<table>
<thead>
<tr>
<th>Dilution of the serum</th>
<th>1:2</th>
<th>1:4</th>
<th>1:8</th>
<th>1:16</th>
<th>1:32</th>
<th>1:64</th>
<th>1:128</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-A+A erythrocytes (control)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>anti-A+A (not incub)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+m</td>
<td>+m</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>anti-A2+A (incub)</td>
<td>+</td>
<td>+</td>
<td>+m</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-A3+A (pH 6.0)</td>
<td>+</td>
<td>+</td>
<td>+m</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-A4+A (pH 8.0)</td>
<td>+</td>
<td>+</td>
<td>+m</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-A5+A (Apr)</td>
<td>+</td>
<td>+</td>
<td>+m</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-A6+A (Alp)</td>
<td>+</td>
<td>+</td>
<td>+m</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: anti-A1: anti-A after absorption with 0 erythrocytes
anti-A2: anti-A after absorption with 0 erythrocytes incubated with A weak antigen serum
anti-A3: anti-A after absorption with 0 erythrocytes incubated with A weak antigen serum at pH 6.0
anti-A4: anti-A after absorption with 0 erythrocytes incubated with A weak antigen serum at pH 8.0
anti-A5: anti-A after absorption with 0 erythrocytes incubated with Apr
anti-A6: anti-A after absorption with 0 erythrocytes incubated with Alp

The obtained data suggest that the anti-A absorbing ability of 0 erythrocytes is generated by A transferase. Moreover, it is active in the acid medium and responsible for the production of glycoprotein determinants on erythrocytes. This supports the earlier discussed notion of two types of ABH antigenic marking on erythrocytes, namely agglutinating and absorbing (Delevsky, 1974; Delevsky, 1999; Delevsky, 2013). Therefore, the serum of the person with the anti-A absorbing ability of erythrocytes induced the same ability in 0 erythrocytes. Lack of detecting anti-A absorbing ability of erythrocytes might lead to transfusion of plasma from B group person containing anti-A antibodies and possible homotransfusion complications. The present case helps to reveal weak antigens on erythrocytes in AB0 typing to prevent possible homotransfusion complications.

**DISCUSSION**

The present case of the weak A antigen revealed in the reaction of agglutination at 37 ºC with polyclonal serum in a person with B antigen (revealed at room temperature with standard monoclonal antibodies) presents a necessity of thorough investigation of any case of AB0 discrepancy with use of additional methods allowing to detect weak antigens and antibodies of AB0 system. The concomitant ability of the person to absorb anti-A and not to absorb anti-B caused to perform a series of experiments in AB0 typing. Therefore, the following list of methods helped to reveal the weak antigen:

1. Agglutination with heated polyclonal serum at room temperature and complement
2. Agglutination at room temperature with unwashed erythrocytes and polyclonal serum
3. Agglutination with washed erythrocytes at 37 ºC and polyclonal serum
4. Agglutination with washed and unwashed erythrocytes at 37 ºC and heated polyclonal serum
5. Antiglobulin test with unwashed erythrocytes and heated polyclonal serum at 37 ºC
6. Antiglobulin test with monoclonal antibody anti-A 2-8 and unwashed erythrocytes at room temperature

The method allowed knowing that the weak anti-B antibodies were prolonged incubation of the serum with test erythrocytes at 37 ºC. The coexistence of B antigen and anti-B antibodies was described by other authors (Mohammadi et al., 2018). Moreover, the antiglobulin test helped detect the weak forms of A antigens rather than washed erythrocytes.
erythrocytes. Accordingly, these methods could be considered for use in laboratories in cases of AB0 discrepancies. It should be noted that the serum of the person containing transferase can stimulate the production of A glycoprotein determinants on the 0 erythrocytes, and different modes of incubation allowed to reveal the activity of transferase in acid medium but not alkaline. This supports the postulate of previous researchers of two types of transferases, namely acid and alkaline (Kishi et al., 1977). More importantly, erythrocytes were severely hemolyzed under the influence of the complement and group-specific antibody, especially in the acid medium but not in the alkaline. Our results are in line with the findings of previous studies when in cell electrophoresis δ-potential of erythrocytes decreased under the influence of nonagglutinating hemolysing antibodies and complement at pH 6.3-7.5 (Delevsky, 2008). As a result, transferases can produce glycoconjugates of alkaline (with an isoionic point at pH 8.1-7.9) and acid (pI 6.6-6.5) origins (Kishi et al., 1977). The characteristics of the electrostatic interrelation were discussed in our previous studies (Delevsky and Dielievska, 2016). Additionally, the interaction characteristics of the formed acid or alkaline types of glycotopes and the ability of antibodies to react with complement define the value δ-potential of erythrocytes and possible hemolysis. This study revealed that a donor absorbed anti-A and her cold anti-A antibodies were active at room temperature and pH 8.0; however, it was less active at pH 6.0. Moreover, this person did not absorb anti-B and showed the presence of her anti-B antibodies (active at 37 ºC, nonbinding complement, and AGS). The cis A\_xB\_y phenotype was suggested in this study. Moreover, weak anti-B antibodies were revealed with washed erythrocytes at 37 ºC and prolonged incubation.

**Conflict of Interest**

The authors declare that they have no conflict of interest.

**Authors’ Contribution**

Study concept and design:
Acquisition of data:
Analysis and interpretation of data:
Drafting of the manuscript:
Critical revision of the manuscript for important intellectual content:
Statistical analysis:
Administrative, technical, and material support:

**References**
