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The potency of erythrocytes investigation in revealing anti-A and anti-B antibodies absorbing and agglutinating epitopes

Mykola Korzh [1], Pavlo Grigorievich Kravchun [2], Frida Solomonivna Leontieva [1], Danyshchuk Zinayda [1], Nataliya Ashukina [1] & Dielievska Valentyna Yuriivna [2,]*

[1] Sytenko Institute of Spine and Joint pathology National Academy of Medical Sciences of Ukraine, Kharkiv, Ukraine. [2] Department of Internal Medicine No. 2 Clinical Immunology and Allergology, named after academician L.T. Malaya, Kharkiv, Ukraine.

*Corresponding author: valentinka_1987@ukr.net.

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ABSTRACT

Different methods of erythrocyte antigens detection exist in hematology. However, weak variants of blood group antigens are still difficult to reveal. The study aimed to determine erythrocytes' diameter after the specific incubation of O, A, B, and O-washed erythrocytes with anti-A, anti-B antibody absorbing ability with IgM and IgG complement-dependent antibodies. Thus, the contact of anti-A serum with A erythrocytes at 20 °C resulted in an increase of their diameter (from 6.19±0.55 μm to 6.28±1.48μm, from 5.41±0.71 μm to 6.02±1.2 μm) with a less increase of erythrocytes diameter in a less titer (1:2) of the serum. A similar increase in erythrocyte diameter was observed in A.B. erythrocytes after contact with anti-A, B serum for one-day incubation. At the same time, the contact of anti-B serum with A erythrocytes did not increase erythrocyte diameter. At 37°C the contact of anti-A serum and A erythrocytes for 1-day incubation resulted in a less increase of erythrocytes diameter (5.27±1.5 μm) as compared to the one observed at 20°C. IgG antibodies increased the diameter of erythrocytes more significantly than IgM antibodies. Incubation of IgG antibodies with complement at 4°C leads to the hemolysis of erythrocytes of the appropriate specificity. The hemolyzing activity of the complement was decreased in the alkaline medium at 37°C and appeared to be highest in the acid medium at 4°C. Fenbendazole decreased erythrocytes' anti-A antibody absorbing ability, whereas itraconazole interfered with anti-B antibody absorbing ability. The presence of the serum from the person with additional absorbing antigens led to less hemolyzing activity of the complement and increased the number of erythrocytes in the case of the specific antigen-antibody binding. Therefore, evaluating the erythrocytes' diameter after contact with IgG complement-dependent antibodies at 20°C may be applied to determine the type of blood group antigens.

Keywords: Antigen, Antibody, Diameter, Determination, Erythrocyte.

INTRODUCTION

Detection of blood group antigens is based on hemagglutination reaction and is widely used in hematology [1]; despite an accurate performance of the methods of blood group detection by monoclonal antibodies, hemolytic complications while hemotransfusions still occur [2]. The erythrocyte diameter measurement in revealing hemolysis in vitro under the influence of complement-dependent blood group-specific antibodies might improve the diagnostics of early hemotransfusion reactions. The revealing and understanding of the pathogenetic mechanisms of the influence of group-specific antibodies on the erythrocytes membrane in specific antigen-antibody interactions are important in searching the laboratory method for hemolysis detection. The erythrocytes' diameter was reported to be from 7.2 μm to 7.9 μm (from 7.5 μm to 8.7 μm in diameter and 1.7 μm to 2.2 μm in thickness) [3]. The biconcave shape and deformability of the erythrocytes are important features of their function [4]. The biconcave erythrocytes have a flexible membrane facilitating deformation of the erythrocytes while passing through capillaries. This feature of erythrocytes can be affected by hemolytic conditions [5], which may be detected by the significant alterations of the erythrocytes' shape [6]. The studies reported the presence of an erythrocyte surface layer [7]. The

erythrocytes parameters were studied in various conditions. Thus, after 6 days in water and air, erythrocytes retained their integrity and were measured 5.48μ-5.88 μm in diameter, whereas the erythrocytes from the bloodstains stored for 14 years showed irregular shape and deformations of the membrane [8].

For revealing hemolysis, a reaction is needed in which the antibody reacts with all the cells. Since the single antibody is known to be unable to penetrate the cell glycocalyx and cause membrane deformation, adding a second antibody or complement might be promising - they were reported to cause erythrocytes charge decrease, responsible for the hemolysis of the erythrocytes [9]. The present study intended to reveal the ability of the complement to destroy erythrocytes membrane in a case of antigen-antibody specific binding [10,11].

The study aimed to assess the possibility of erythrocyte diameter measurement for blood group determination after the contact of the erythrocytes with IgM and IgG complement-dependent anti-A and anti-B antibodies.

METHODS

Blood was drawn from 270 normal healthy volunteers, examined at Sytenko Institute of Spine and Joint pathology, into heparin tubes (Zhejiang SKG Medical Technology Co., Ltd, China). The mean age of the participants was 64.0±7.70 years, 106 men and 164 women. O, A, B, AB, and O-washed erythrocytes with anti-A or anti-B antibody absorbing ability were included in the investigation.

The erythrocytes were obtained thrice with saline and centrifuged at 1000 g for 10 minutes. To reveal IgM antibody influence on erythrocytes diameter, anti-A, anti-B, and anti-A, B sera were used at 20°C, 37°C, and 4°C temperatures for one-day incubation with the erythrocytes. To obtain IgG antibodies, the serum was heated for 30 minutes at 60 °C. 50 μl of control erythrocytes (blood type A) were added to 100 μl of anti-A serum, following adding of the complement and incubation at 20°C, as well as to 100 μl of anti-B serum and serum from A.B. blood group type (negative controls). 100 μl of a guinea pig complement was added to the reaction and incubated for 1 day.

Statistical analysis was performed by Statistics 10.0 software using Student's coefficient and Friedman's criterion [12].

RESULTS

The effect of polyclonal serum on erythrocytes at 20°C

The erythrocytes did not increase their diameter immediately after contact with the specific polyclonal serum, whereas prolonged (one day) incubation led to the modified diameter. The diameter of A erythrocytes after the contact with anti-A serum was modified (6.78±1.01 μm - 7.1±0.96 μm, 5.41±0.61 μm - 6.02±0.7 μm ($p<0.05$), 6.19±0.55 μm - 6.28±1.48 μm (Fig.1), 6.67±0.95 μm - 7.75±0.82 μm ($p<0.05$), 7.17±0.74 μm - 7.75±0.82 μm ($p<0.05$), less increase was observed with the same antibody in 1:2 titer (7.0±1.22 μm) ($p<0.05$). The diameter of B erythrocytes after the reaction with anti-B serum was increased (from 7.36±0.43 μm to 7.63±0.55 μm, from 7.73±0.92 μm to 8.43±0.82 μm, $p<0.05$), the diameter of unwashed A erythrocytes after the contact with anti-A serum was increased from 7.17±0.74 μm to 7.75±0.82 μm ($p<0.05$). Thus, only the specific binding led to the increased diameter of erythrocytes: anti-B serum did not increase the diameter of A erythrocytes (7.28±0.95 μm - 7.12±0.73 μm), as well as the diameter of O erythrocytes was not increased after the contact with anti-A serum (8.08±0.94 μm - 7.76±0.54 μm), (7.63 μm - 7.7±1.2 μm)($p>0.05$).

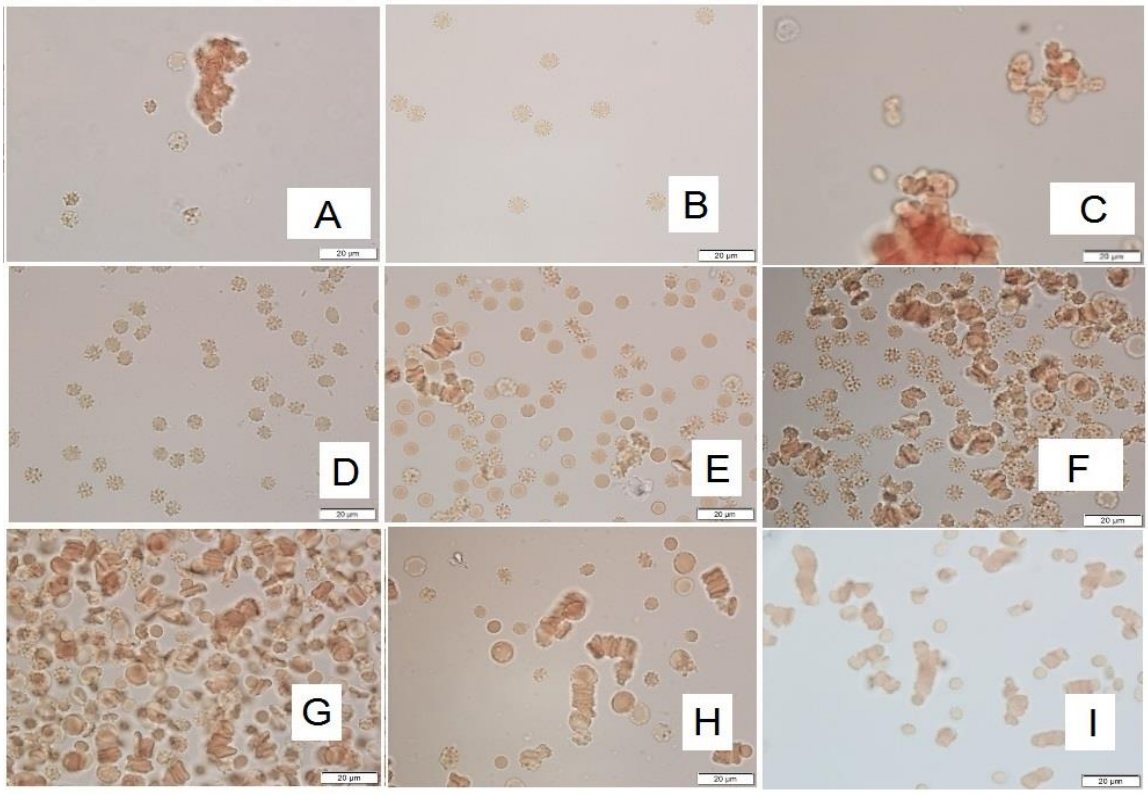

Figure (1):

A. A erythrocytes and anti-A serum at room temperature for one day
B. A.B. erythrocytes
C. A.B. erythrocytes with anti-A and B antibodies at room temperature for one-day incubation
D. B erythrocytes with anti-A antibody absorbing ability;
E. B erythrocytes with anti-A antibody absorbing ability with serum from A.B. person for one-day incubation
F. O erythrocytes with serum from A.B. person for one-day incubation
G. O erythrocytes with anti-A and anti-B antibody absorbing ability with serum from A.B. person for one day incubation
H. O erythrocytes with anti-A and anti-B antibody absorbing ability with anti-A serum at room temperature for one-day incubation
I. Anti-B serum with B erythrocytes for one day.

A similar increase of erythrocytes diameter was observed in reaction with A.B. erythrocytes after contact with anti-A, B serum at 20°C for one day incubation (from 6.32±0.62 μm to 8.0±0.8 μm) ($p<0.05$) (Fig.1B, 1C) and with A erythrocytes (6.32±0.62 μm - 8.0±0.8 μm) ($p<0.05$). A.B. erythrocytes, after contact with anti-A serum at room temperature for one day, increased their diameter as well (6.1±0.78 μm - 6.54±0.73 μm) ($p<0.05$).

The effect of antibodies on absorbing ant gens at 20°C

The serum from the A.B. blood group person did not increase the diameter of B erythrocytes with an anti-A absorbing ability (5.88±0.56 μm - 5.92±1.2 μm) ($p>0.05$)(Fig.1D, 1E) and did not decrease the quantity and diameter of O erythrocytes (6.35±1.8 μm - 5.6±1.3 μm) nor cause their hypocrisy, with O blood group erythrocytes with anti-A, B antibody absorbing ability led to the appearance of the rouleaux formation and separate erythrocytes (Fig.1G), with O erythrocytes with anti-A absorbing ability (7.65±0.42 μm, RDS 5.4%) showed the presence of dissociates and normochromic.

Anti-A serum increased the diameter of nonheparinized B erythrocytes with an anti-A absorbing ability (5.68±0.25 μm - 5.88±0.59 μm, $p<0.05$) of agglutinated cells), O erythrocytes with an anti-A absorbing ability

(8.22±0.39 μm, p<0.05) with no agglutination at pH 6.0 and rouleaux formation at pH 8.0, of O erythrocytes with anti-A antibody absorbing ability (4.36 ±1.22 μm - 7.86±0.32 μm, p<0.05, red blood cells distribution size (RDS) 4%) with the appearance of hypochromic spherocytes, O erythrocytes with anti-A, anti-B antibody absorbing ability (3.53±0.27 μm - 3.76±0.21 μm, p<0.05), (after three times loading 5.58±0.62 μm - 7.77±0.6 μm, p<0.05) (Fig.1H), as well as anti-B serum (4.37±0.83 μm, p<0.05). At the same time, the diameter of O erythrocytes was not increased (7.54±0.78 μm - 5.68±0.59 μm, 4.57±0.35 μm)(p<0.05).

Anti-A plasma after contact with O erythrocytes with anti-A absorbing ability showed rouleaux formation (7.73±0.4 μm), as well as at pH 6.0, the decreased quantity of erythrocytes (6.72±0.71 μm) was observed, whereas at pH 8.0 an increased size with a decreased quantity of erythrocytes were determined (8.0±1.4 μm) (p<0.05).

Anti-B serum contacted with O erythrocytes with anti-B absorbing ability at 20°C led to the rouleaux formation and increased diameter of B erythrocytes with anti-A antibody absorbing ability (5.55±0.5 μm - 5.94±0.71 μm) (p<0.05).

However, the diameter of anti-B antibody absorbing erythrocytes was not increased after contact with serum from the A.B. blood group person (5.67±1.05 μm, RDS 18.7%) (p>0.05).

The effect of polyclonal serum on erythrocytes at 37°C

B erythrocytes, after contact with anti-A serum at 37°C for one day of incubation, showed a diameter of 5.17±0.36 μm, whereas A erythrocytes contacted with anti-A serum showed a diameter of 5.27±1.14 μm, that is less than observed at 20°C (8.19±0.47 μm) (p<0.05).

Anti-B serum increased B erythrocytes diameter (5.55±0.5 μm - 5.94±0.61 μm, Fig.1I) (p<0.05).

The contact of anti-B serum and A erythrocytes with anti-B antibody absorbing ability at 37°C led to decreased erythrocytes with a diameter of 6.1±1.63 μm. A similar decrease in the erythrocytes quantity was observed after the contact of anti-A serum with O erythrocytes with anti-A antibody absorbing ability (diameter 6.08±2.16 μm).

The effect of polyclonal serum on erythrocytes at 4°C

The binding of absorbing erythrocyte antigens with corresponding antibodies led to increased erythrocyte diameter at 4°C (1-week incubation) with rouleaux formation. The contact of anti-B serum and O erythrocytes with anti-A, B antibody absorbing ability led to the increased erythrocytes diameter (4.71±0.39 μm - 5.35±0.76 μm, 6.03±1.0 μm), and RDS (8.2% -23.5%, 16.5%, accordingly) (p<0.05). Anti-A, B serum contacted with A.B. erythrocytes at 4°C increased the erythrocytes diameter as well (6.32±0.6 μm to 7.84±0.66 μm) (p<0.05).

The effect of the own serum on erythrocytes at 20°C, 37°C, and 4°C

Erythrocytes with anti-B antibody absorbing ability (Fig.2B) after contact with their serum showed the tendency to agglutination (diameter 7.6±1.8 μm) (Fig.2C). In contrast, at pH 5.8, an expressed agglutination appeared (Fig.2D), and at pH 8.0 increased quantity of erythrocytes without agglutination was observed (Fig.2E).

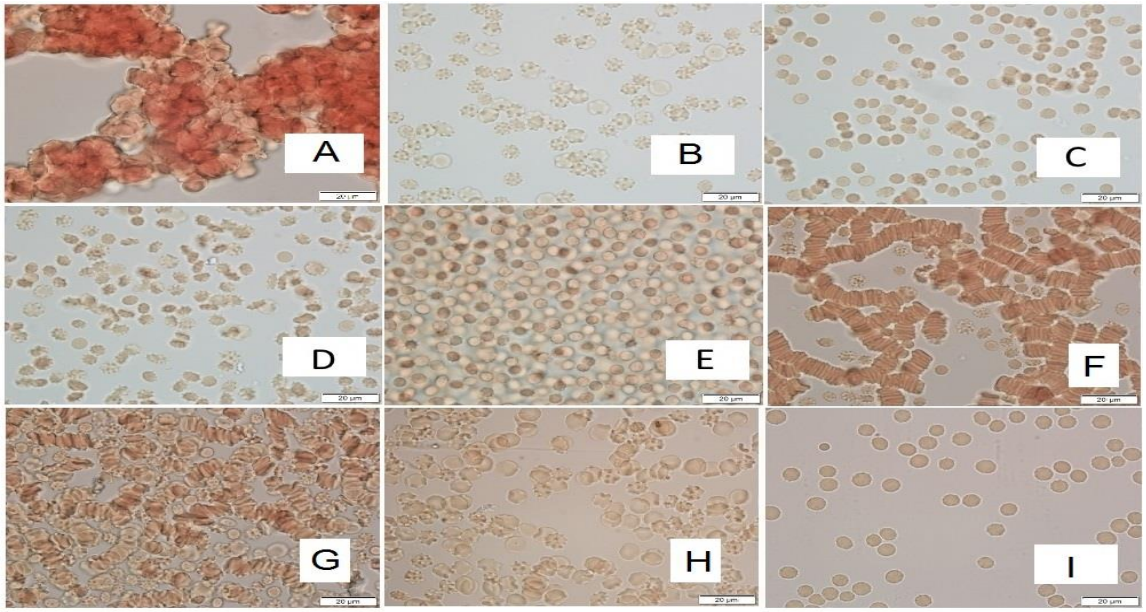

Figure (2):

A. Anti-A, B serum with A.B. erythrocytes at 4°C for one day
B. Erythrocytes with the anti-B absorbing ability for one-day incubation
C. Erythrocytes with the anti-B absorbing ability with their anti-B serum for one day incubation
D. Erythrocytes with the anti-B absorbing ability with their anti-B serum at pH 5.8 for one-day incubation
E. Erythrocytes with the anti-B absorbing ability with their anti-B serum at pH 8.0 for one-day incubation
F. Unwashed O erythrocytes with anti-A and anti-B antibody absorbing ability with their serum for one-day incubation
G. Unwashed O erythrocytes with anti-A and anti-B antibody absorbing ability with own serum and fenbendazole for one day incubation
H. Unwashed O erythrocytes with anti-A and anti-B antibody absorbing ability with their serum and itraconazole for one day incubation
I. A erythrocytes

Unwashed O erythrocytes with anti-A, anti-B antibody absorbing ability formed rouleaux formation with their serum after one-day incubation at room temperature (diameter 6.32±1.02 μm, Fig.2F). However, rouleaux formation disappeared after adding fenbendazole to the reaction: (diameter .67±1.52 μm, Fig.2G), whereas after adding itraconazole (Fig.2H) the erythrocytes modified their shape to the shape observed without antibodies: 7.44±0.92 μm. The same rouleaux formation was observed with anti-A serum after one-day incubation, as well as increased erythrocytes diameter (7.66±0.26 μm - 7.83±0.41 μm) ($p<0.05$).

The contact of own serum and unwashed A erythrocytes at 37°C showed the tendency to agglutination (diameter 5.3±0.61 μm).

The contact of O erythrocytes with their serum at 37°C showed no agglutination with a diameter of 6.57±0.77 μm.

B erythrocytes with their serum at 37°C showed the tendency to agglutination with a diameter of 4.74±0.27 μm.

The inhibitor of complement showed activity in the alkaline medium. Thus, A erythrocytes with anti-B antibody absorbing ability with their serum and complement showed the tendency to agglutination; at pH 5.8, agglutination was absent, and at pH 8.0, agglutination was not determined. At room temperature for one day of incubation, the contact of unwashed erythrocytes with their serum caused rouleaux formation with a diameter of erythrocytes 6.32±1.02 μm; after adding fenbendazole fewer rouleaux formation was ob-

served with diameter 7.12±1.53 μm, after adding gentamycin the diameter increased to 7.73±0.4 μm ($p<0.05$), after adding itraconazole fewer rouleaux formation with anisocytosis were determined (diameter 7.4±1.5 μm). Washed erythrocytes with their serum did not lead to rouleaux formation.

Absorption of the serum from the O blood group with anti-A, B antibody absorbing ability by own washed erythrocytes led to the less expressed agglutination of B erythrocytes (diameter 5.74±0.7 μm) as compared to the one observed after the incubation of unabsorbed serum with B erythrocytes (diameter 5.56±1.28 μm).

The effect of the heated serum on the erythrocytes

The comparison of A erythrocytes contacted with usual anti-A serum (fig,2I) (6.4±0.8 μm, fig. 1A), anti-A heated serum (5.9±0.55 μm, fig.3A), and heated serum with complement (6.2±1.2 μm, fig.3B) showed the presence of differences. Thus, the usual serum at one-day incubation at room temperature caused agglutination and hemolysis of erythrocytes; meanwhile, the heated serum caused agglutination of increased erythrocytes without signs of the hemolysis, whereas adding the complement caused hemolysis with less quantity of increased agglutinated hypochromic erythrocytes.

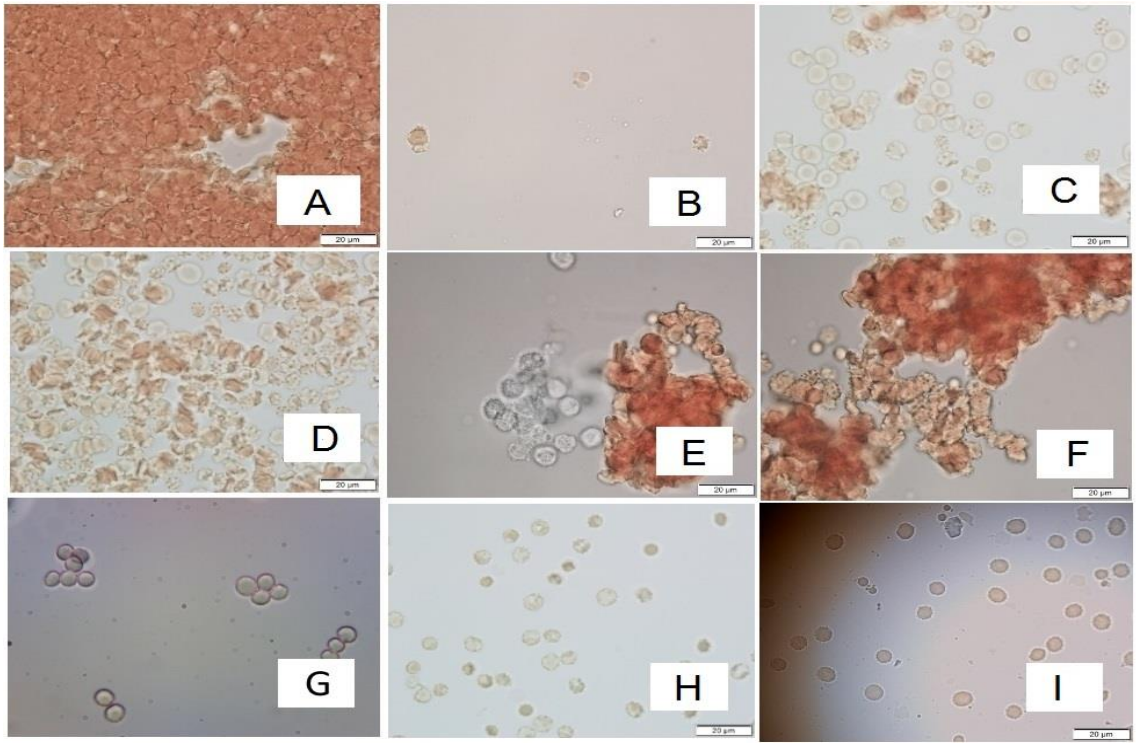

Figure (3):

A. An erythrocyte and anti-A heated serum at room temperature for one day
B. An erythrocyte and anti-A heated serum and complement at room temperature for one day
C. Anti-A serum, unwashed erythrocytes, compliment at room temperature.
D. Anti-A serum, A erythrocytes, compliment, and serum from A blood group with the anti-B absorbing ability for one-day incubation
E. Anti-A, B heated serum with A.B. erythrocytes, complement at 4°C for one day
F. Anti-A, B heated serum with A.B. erythrocytes, compliment, and plasma from A blood group with anti-B absorbing ability at 4°C for one day
G. Anti-B serum 1:10 with B erythrocytes at room temperature for 1 hour.
H. Anti-B serum with A erythrocytes and anti-B absorbing ability and antiglobulin serum for one day
I. Anti-A heated serum after absorption by O erythrocytes and anti-A, B antibodies absorbing ability and further contact with A erythrocytes for one hour.

At 4°C the diameter of unwashed O erythrocytes with anti-B antibody absorbing ability was not increased after the contact with anti-B IgG and complement (7.93±0.61 μm, RDS 7.6% - 6.4±1.65 μm, RDS 25.7%), ($p<0.05$); however, erythrocytes diameter was increased after the contact of washed erythrocytes with anti-B IgG antibodies (5.35±0.36 μm - 6.03±0.60 μm, RDS 9.2%) ($p<0.05$) and IgG anti-B antibodies with complement (4.72±0.39 μm - 5.53±0.7.2 μm) ($p<0.05$). Microcytes, schistocytes, and bite cells appeared after O blood group erythrocytes with anti-A antibody absorbing ability contacted with an anti-A heated serum with complement (diameter 7.4±1.44 μm, RDS 19.4%).

O blood group erythrocytes with anti-A antibody absorbing ability increased their size after contact with anti-A heated serum with complement (7.42±0.54 μm, RDS 7.2% - 9.1±0.83 μm, RDS 9.1%), (from 5.52±0.47 μm, RDS 21% to 6.61±0.45 μm, RDS 21%), (from 7.55±0.48 μm, RDS 12.9% to 7.78±0.5.0 μm, RDS 12.8%) ($p<0.05$); however, the diameter was not increased with anti-B heated serum: 7.14±1.72 μm (RDS 24%) ($p>0.05$).

B blood group erythrocytes with anti-A antibody absorbing ability modified their diameter after contact with anti-A heated serum and complement (4.7±0.59 μm, RDS 12.5% - 6.76±1.44 μm, RDS 21.3%) ($p<0.05$) with rouleaux formation (after the threefold loading).

The diameter of A erythrocytes (5.87±0.74 μm, RDS 12.6%)) was increased after the contact with anti-A heated serum and complement (7.3 ±0.51 μm, RDS 6.9%, 7.86±0.93 μm, RDS 11.8%), as well as after the contact with the heated anti-A,B serum and complement (7.8±1.02 μm, RDS 13%, with the presence of ring cells and signs of hemolysis) ($p<0.05$), whereas after the contact with anti-A heated serum from B blood group person with anti-A antibody absorbing ability the erythrocytes diameter was not increased (5.62±0.41 μm, RDS 13.7%) ($p>.05$). The diameter of A erythrocytes was increased after the contact with anti-A heated serum (6.19±0.55 μm, RDS 8.7% - 6.41±0.43 μm, RDS 17.6%) ($p<0.05$) with decreased quantity of erythrocytes, as well as after the contact with the heated anti-A,B serum (6.62±0.36 μm, RDS 5.4%) ($p<0.05$) with appearance of stomatocytes, whereas the contact with the heated serum from A.B. blood group person did not lead to the increased A erythrocytes diameter (5.42±0.66 μm, RDS 12.1%) ($p<0.05$).

The diameter of A.B. erythrocytes under the influence of anti-A heated sera and complement was modified from 4.83±0.15 μm (3.1%) to 5.05±0.37 μm (7.3%) and 5.67±0.53 μm (9.3%) ($p<0.05$) with the presence of spherocytes, from 6.32±0.62 μm, RDS 9.8% to 8.57±0.57 μm, RDS 6.65% ($p<0.05$) at room temperature under the influence of anti-A, B heated serum, and complement.

The diameter of B erythrocytes after the contact with IgG anti-B antibodies and complement was modified from 7.3±1.93 μm to 8.73±0.93 μm (RDS 10.6 %), from 5.0±0.54 μm (RDS 1.08%) to 7.72±1.19 μm (RDS 15.4%) ($p<0.05$), whereas with the heated serum without anti-B antibodies and complement the erythrocytes diameter was not increased (7.16±1.34 μm) ($p>.05$).

IgG antibodies (anti-B heated serum) increased the diameter of B erythrocytes without anticoagulant (8.44±1.0 μm - 13.8±1.37 μm) more than IgM anti-B antibodies (6.33±1.0 to 9.5±1.1μm) ($p<0.05$).

The effect of the serum from the person with additional absorbing antigens on size modification of erythrocytes

At room temperature, the diameter of A erythrocytes was modified after contact with anti-A heated serum and complement from 4.97±0.29 μm (RDS 5.8%) to 6.9±1.14 μm (RDS 18.4%) and 5.28±0.56 μm (RDS 10.7%) after the contact with anti-A heated serum, complement, and serum with an inhibitor of complement ($p<0.05$).

The addition of the serum from the A blood group with anti-B antibody absorbing ability to the reaction of anti-A serum, A erythrocytes, and complement diminished the erythrocytes size modification under the influence of specific complement-dependent anti-A antibodies (6.86±1.43 μm, Fig.3C) as compared to the diameter of A erythrocytes after the contact with anti-A serum and complement (8.06±0.43 μm, Fig.3D) ($p<0.05$).

The contact of the heated anti-A, B serum with A.B. erythrocytes and complement at 4°C increased diameter (Fig.3E) from 6.32±0.62 μm to 7.95±1.42 μm (8.57±0.57 μm at room temperature) ($p<0.05$), whereas adding the plasma from A blood group person with anti-B antibody absorbing ability decreased the erythrocytes diameter (7.0±1.11 μm), (7.54±0.62 μm at room temperature, Fig. 3F) ($p<0.05$).

The inhibitor of complement did not abolish erythrocytes size increase if the specific antigen-antibody binding was absent: the heated serum from the A.B. blood group contacted with O erythrocytes and complement at 4°C led to the erythrocytes diameter 5.4±1.19 μm, and after adding plasma from A blood group with anti-B antibody absorbing ability there was no erythrocytes diameter decrease (6.4±1.04 μm) ($p>0.05$).

Adding the complement to anti-A heated serum and A erythrocytes at room temperature decreased the number of enlarged erythrocytes (7.03±1.17 μm) with the appearance of their shadows and less expressed agglutination as compared to the contact of anti-A heated serum without complement (6.8±0.98 μm).

After the contact of anti-A serum with A erythrocytes at room temperature, more increased quantity and agglutination of erythrocytes were observed.

Thus, complement led to decreased erythrocytes due to the stimulation of the hemolytic effect of heat-stable specific antibodies.

Meanwhile, the inhibitor of complement decreased the diameter (5.6±1.38) and increased the number of erythrocytes after adding to the anti-A heated serum and A erythrocytes.

The effect of IgM antibodies on erythrocytes

IgM anti-B antibodies did not increase the diameter of washed B erythrocytes after one-hour incubation at room temperature (6.6±0.98 μm, RDS 14.7% - 6.0±0.36 μm, RDS 6.1% at 1:10 titer, 6.25±0.45 μm at 1:100 titer RDS 7.2%, $p<0.05$). Nevertheless, some peculiarities have been observed. At higher antibodies, concentration erythrocytes were mostly agglutinated and hypochromic with less quantity. At lower concentrations, IgM anti-B antibodies neither caused agglutination and hypochromic nor decreased the number of erythrocytes.

Whereas IgG anti-B antibodies with complement increased the erythrocytes diameter (10.96±0.88 μm) ($p<0.05$).

The diameter of B erythrocytes with anti-A antibody absorbing ability was modified after contact with anti-A IgM antibodies in 1:10 titer (7.8±0.66 μm, RDS 8.4% - 8.36±1.12 μm, RDS 13.4%), after the contact in 1:100 titer - to 7.87±0.9 μm (RDS 11,4%) ($p<0.05$). The diameter of B erythrocytes was increased after contact with anti-B IgM antibodies in 1:10 titer (6.0±0.36 μm - 7.5±0.46 μm, Fig.3G) ($p<0.05$).

The effect of antibody titer increase on erythrocytes

Various antibodies were diluted into contact with erythrocytes to reveal IgG antibody influence on the morphology of erythrocytes. Thus, the diameter of erythrocytes was increased after contact with specific IgG antibodies and gradually decreased with the antibody titer increase (Table 1).

Table (1): Diameter of erythrocytes after contact with IgG antibodies of various titers (μm).

	1:2	1:4	1:8	1:16	1:32	1:64
Anti-B heated and B er. (8.44±1.0, 8-9)	11.04±1.09 + 9.6-12	10.4±1.04 +9.6-11.2	9.92±0.96 -8.8-12.0	9.2±1.01 -8.0-10.4	7.36±0.88 -6.4-10.4	-
Anti-A heated and A er. (8.58±1.0, 8-10)	10.48±1.09 +8.8-11.2	9.28±0.62 +7.2-11.2	9.44±0.8 +8-11.2	8.09±0.96 -8.0-10.4	6.72±0.54 6.4-8.0	6.88±0.48 6.4-8.0
Anti-B heated after absorption by B er. and B er.(8.44±1.0, 8-9)	10.73±1.6 -8.8-12.0	10.56±1.04 -9.6-11.2	10.08±1.04 -9.6-12.4	9.72±1.04 -8.8-12.4	6.96±0.8 -5.6-8.8	6.16±0.36 -5.6-6.4
IgM Anti-B and B er. (6.33±1.0, 6-7)	7.6±0.88 6.4-8.0	7.44±0.88 5.6-8.0	6.66±0.88 6.4-6.88	5.44±0.88 4.0-7.2	5.01±0.8 4.8-7.2	
IgM Anti-A and B er. (6.33±1.0, 6-7)	5.97±0.8 4.8-7.2	5.44±0.8 4-8	6.18±0.8 4-8	5.2±0.8 4-6.4	6.6±0.88 4.8-8	
Anti-A heated after absorption by B er. with anti-A absorbing ability and A er. (8.58±1.0, 8-10)	9.41±0.8 +8.8-10.4	8.35±0.88 +6.4-9.6	6.4±.0.66 -5.6-7.2	-	6.92±0.72 -6.4-8.8	6.72±0.32 -6.4-7.2
Anti-A heated with A er. 5 min.	7.08±0.96 +6.4-9.6	7.76±1.04 +6.4-12.0	6.51±0.29 +6.4-7.2	6.84±0.9 +5.6-8.8	7.0±1.04 +5.6-9.6	6.56±0.5 +5.6-7.2
Anti-A heated after A er. with A er. 5 min.	6.86±0.92 +5.6-8.8	7.46±1.0 +6.4-8.8	7.66±1.12 +5.6-11.2	6.32±1.04 -4.8-9.6	7.44±0.8 -5.6-10.4	6.54±1.04 -5.6-8.8
IgM anti-A with A er. 5 min.	5.8±0.7 +4.8-7.2	6.05±0.9 +4.0-7.2	6.85±0.96 +5.6-8.0	6.12±0.4 +5.6-6.4	6.48±0.48 +5.6-7.2	6.12±0.4 +5.6-6.4
IgM anti-A after A er. with A er. 5 min.	6.12±0.45 +5.6-.64	5.76±0.5 +4.8-6.4	6.24±0.5 +5.6-7.2	6.29±0.56 +5.6-7.2	5.75±0.29 -4.9-6.4	6.4±0.64 -5.6-7.2

Anti-B IgG antibodies showed the potency of increasing erythrocytes' diameter depending on the antibody's titer. Thus, IgG anti-B antibody at 1:2 titer increased the diameter of B erythrocytes to 11.04±0.9 μm, and less increase was observed at 1:32 tired (7.36±0.88 μm) ($p<0.05$). Being absorbed with B erythrocytes, anti-B IgG antibodies increased the diameter of B erythrocytes less (10.73±1.6 μm

in 1:2 titer and 6.96±0.8 μm in 1:32 titer) as compared to the nonabsorbed serum ($p<0.05$).

An investigation of various titers of IgG antibodies on the size and RDS of erythrocytes was rather interesting. Thus, IgG anti-A antibodies with A erythrocytes increased RDS (27.5%) at low titers (1:64): normal size and increased size erythrocytes were present in the sample. Moreover, a higher concentration of IgG antibodies led to the decreased RDS due to the increased portion of enlarged erythrocytes and decreased quantity of normal-sized erythrocytes (from 17.1% at 1:32 titer to 10.4% at 1:2 titer, $p<0.05$).

The same ability of the influence of IgG antibodies on RDS was noticed for anti-B IgG antibodies. The highest RDS was determined at their highest titer (18.4% at titer 1:32), and their lowest RDS was noticed at the lowest titer (6.5% at 1:2 titer) ($p<0.05$).

Absorption of IgG antibodies by B erythrocytes caused a parabola curve of RDS modification after the contact of IgG anti-B antibodies with B erythrocytes (at the highest titer 1:64 the low value of RDS 5.8 % was detected since the most of the erythrocytes demonstrated normal size due to the absorbed IgG antibodies). On the other hand, increasing the concentration of absorbed IgG RDS showed the tendency to increase (16.2% at 1:32 titer) due to the appearance of enlarged erythrocytes among erythrocytes of normal size. However, a further increase in antibody concentration decrease in RDS was developed (7.6% at 1:2 titer) since most erythrocytes were enlarged.

IgM anti-A antibodies did not increase erythrocytes size after the 5-minute incubation (6.48±0.26 μm, RDS 4% at 1:64 titer and 7.08±0.85μm, RDS 12% at 1:8 titer), ($p<0.05$), however, caused anisocytosis with the appearance of deformed cells already at 1:64 titer. Absorption of IgM anti-A antibodies by A erythrocytes did not significantly influence the size of erythrocytes, nevertheless affected the expression of agglutination and influence on the erythrocyte shape (6.88±0.43 μm, RDS 6.2% at 1:64 titer and 7.2±0.63 μm, RDS 8.7%). Moreover, absorbed IgM antibodies at high titer 1:32 did not modify the shape of erythrocytes after the 5-minute incubation.

Interestingly, anti-A heated serum after 5-minute incubation with A erythrocytes showed a gradual increase of the size and RDS of erythrocytes (RDS 19.3%, 7.5±1.45 μm at 1:64 titer and 8.0±1.76 μm, RDS 22% at 1:4 titer), ($p<0.05$). In contrast, absorbed by A erythrocytes anti-A heated serum abolished agglutination already at 1:16 titer and diminished modification of the size of the erythrocytes and RDS (14.02%, 7.2±1.01 μm at 1:64 titer and 6.97±1.06 μm, 15.2% at 1:4 titer) ($p<0.05$).

IgG antibodies against helminths in persons with anti-A, B antibody absorbing ability

Since the persons of O blood group type with ant-A, B antibody absorbing ability showed increased IgG antibodies to Ascaris Lumbricoides (1.23 units of optical density (O.D.)), Toxocara canis (1.77 OD), Strongyloides (5.03 OD), persons with B blood group and anti-A absorbing ability demonstrated increased IgG antibodies to Ascaris Lumbricoides (1.37 OD), Toxocara canis (3.28 OD), Opistorchis felines (1.21 OD), the analysis of the levels of IgG antibodies to Ascaris Lumbricoides and Toxocara canis was performed.

Investigation of the level of IgG antibodies to Ascaris Lumbricoides and Toxocara canis showed that persons with anti-A absorbing ability tended increased antibodies to Ascaris Lumbricoides, whereas persons with anti-B antibody absorbing ability showed the tendency of an increased level of IgG antibodies to Toxocara canis. Thus, the O blood group phenotype with anti-B antibody absorbing ability showed 0.72±0.11 O.D. of IgG antibodies to Toxocara canis over 0.65±0.02 O.D. of IgG antibodies Ascaris Lumbricoides ($p<0.05$). Similarly, A blood group phenotype with anti-B antibody absorbing ability had increased values of IgG antibodies to Toxocara canis 0.5±0.12 O.D. over 0.33±0.06 O.D. of IgG antibodies to Ascaris Lumbricoides ($p<0.05$).

The O blood group phenotype with anti-A antibody absorbing ability showed increased IgG antibodies to Ascaris Lumbricoides - 0.18±0.07 O.D. over 0.12±0.06 O.D. of IgG antibodies Toxocara canis ($p<0.05$). The same tendency was observed in the B blood group phenotype with anti-A absorbing ability - 0.34±0.05 O.D. to Ascaris Lumbricoides over 0.28±0.04 O.D. to Toxocara caniss($p<0.05$) (Table 2).

Table (2): IgG antibodies to Ascaris Lumbricoides (first column) and Toxocara canis (second column) depending on absorbing properties of erythrocytes (in units of optical density).

O with anti-B AAS		A with anti-B AAS		B with anti-A AAS		O with anti-A AAS		O with anti-A, B AAS		O without anti-A, B AAS	
IgG to Ascaris Lumbricoides	IgG to Toxocara canis	IgG to Ascaris Lumbricoides	IgG to Toxocara canis	IgG to Ascaris Lumbricoides	IgG to Toxocara canis	IgG to Ascaris Lumbricoides	IgG to Toxocara canis	IgG to Ascaris Lumbricoides	IgG to Toxocara canis	IgG to Ascaris Lumbricoides	IgG to Toxocara canis
0.65	0.72	0.34	0.35	0.34	0.28	0.7	0.3	0.7	0.3	0.39	0.06
0.7	0.3	0.27	0.28			0.18	0.12	0.14	0.12		
0.14	0.12	0.4	0.87			0.14	0.12				

Note: AAS - antibody absorbing ability.

Interestingly, the O blood group phenotype with anti-A and anti-B antibody absorbing ability showed a higher level of IgG antibodies to Ascaris Lumbricoides (0.34±0.31 O.D.) and Toxocara canis (0.21±0.12 O.D.) ($p<0.05$).as compared to O blood group phenotype with the absence of anti-A and anti-B antibody absorbing abilities (0.39±0.17 O.D. and 0.06±0.002 O.D. accordingly).

The effect of antiglobulin serum on erythrocytes size

The contact of antiglobulin serum (AGS) after the incubation of anti-B serum and A erythrocytes with anti-B antibody absorbing ability led to the appearance of both small and enlarged erythrocytes: 6.21±1,24 μm (Fig. 3H), in the case of IgG anti-B antibodies, use - an increased quantity of smaller cells was observed (5.04±0.89 μm), after adding LISS the quantity of the cells was increased. The use of AGS following incubation of IgG anti-B antibodies with B erythrocytes led to the erythrocytes diameter of 5.71±1.66 μm and in the case of IgG anti-A antibodies with A erythrocytes - 5.84±0.75 μm, and after LISS adding the number of erythrocytes was decreased.

The effect of the eluted antibodies from absorbing antigens on erythrocytes

The contact of eluted anti-A and anti-B antibodies obtained by the heating of erythrocytes after absorption led to the increased size (5.4±1.02 μm) ($p<0.05$) (Fig. 3I) and decreased quantity of A erythrocytes and B erythrocytes (4.6±0.52μm). It was important, adding fenbendazole to the reaction of absorption of anti-A antibodies by O erythrocytes with anti-A, anti-B antibody absorbing ability led to the elution of only agglutinating, but not hemolyzing anti-A antibodies detected by an increased quantity of agglutinated A erythrocytes after the contact with eluted anti-A antibodies (Fig.4A). In contrast, after adding itraconazole, no difference was noticed.

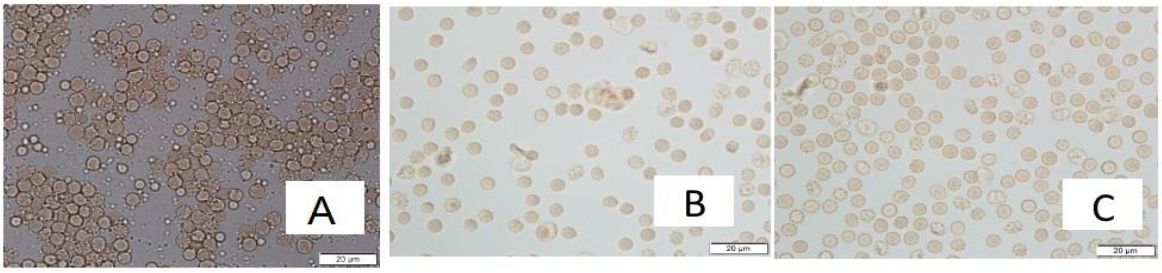

Figure (4):

A. Anti-A heated serum after absorption by O erythrocytes with anti-A, B antibodies absorbing ability and fenbendazole and further contact with A erythrocytes for one hour
B. Anti-A heated serum with B erythrocytes and anti-A antibody absorbing ability, complement at 37°C for one day
C. Anti-A serum with B erythrocytes with anti-A antibody absorbing ability, complement at 37°C for one day.Whereas adding itraconazole to the absorption of anti-B antibodies by O erythrocytes with anti-A, anti-B antibody absorbing ability led to the decreased 1quantity of the eluted anti-B hemolyzing antibodies.

The effect of IgG complement-dependent antibodies on erythrocytes at 37°C

At 37°C, anti-A heated serum agglutinated A erythrocytes in the presence of complement with a diameter of 4.9±0.59 μm. The contact of anti-A heated serum with O erythrocytes with anti-A antibody absorbing ability in the presence of complement at 37°C led to agglutination and decreased erythrocytes.

After the contact of anti-B heated serum with B erythrocytes and complement at 37°C (from 5.0±0.54 μm to 6.26±1.17 μm), fewer erythrocytes diameter was observed as compared to the one measured at 20°C (7.45±1.51 μm) ($p<0.05$). Anti-A, B heated serum at 37°C decreased the quantity of washed O erythrocytes with anti-A antibody absorbing ability (5.4±0.4 μm) and slightly increased the diameter of unwashed erythrocytes in the presence of complement (6.43±1.34 μm) ($p<0.05$).

A 37°C anti-B heated serum showed slight agglutination of O erythrocytes with anti-B antibody absorbing ability and complement. In contrast, at pH 6.0, an increased quantity of erythrocytes was observed, contrary to pH 8.0, when the serum decreased the quantity of A erythrocytes with anti-B absorbing ability in the presence of the complement. Interestingly, the A blood group serum with anti-B antibody absorbing ability at 37°C agglutinated B erythrocytes did not decrease their quantity, whereas, at pH 6.0, the serum decreased the number of erythrocytes contrary to pH 8.0.

At 37°C anti-A heated serum after contact with B erythrocytes with anti-A antibody absorbing ability and complement showed less quantity of erythrocytes (diameter 6.4±2.1 μm - 5.27±0.5 μm, Fig. 4B).

The effect of complement-dependent antibodies of polyclonal serum on erythrocytes

The contact of anti-A serum with A erythrocytes and complement at 37°C showed the presence of agglutination with erythrocytes diameter 4.78±0.36 μm.

Anti-B serum after contact with B erythrocytes and complement at 37°C led to 5.11±0.33 μm of erythrocytes diameter, whereas anti-A serum with A erythrocytes: to 5.15±0.37 μm and agglutination, anti-A, B serum with B erythrocytes: to 4.57±0.59 μm. Anti-B serum with A erythrocytes and complement led to the diameter 5.27±0.77 μm. Anti-A serum with B erythrocytes with anti-A absorbing ability and complement at 37°C led to the erythrocyte diameter of 4.62±0.31 μm (Fig. 4C).

DISCUSSION

Different methods of erythrocyte survival analysis exist in laboratory medicine: measurement of lactate, hematocrit, and hemoglobin levels, radioactive labeling methods with 51Cr, and other isotopes [13, 14, 15, 16, 17, 18, 19]. Differential agglutination is widely used when in the 24 h post-transfusion blood sample, erythrocytes from the donor or the recipient are agglutinated by an appropriate antiserum, and the remaining erythrocytes are counted [20]. An automated technique was described: agglutinates are removed automatically, and the remaining hemoglobin is quantified colorimetrically [21]. Erythrocytes from a donor may be labeled with biotin before injection into the recipient [16].

Studies using the 51Cr technique demonstrated that morphological modifications of erythrocytes correlate with transfusion recovery [15]. The proportion of erythrocytes with a discoid shape was reported to correlate with a positive outcome, and the morphology index after rejuvenation correlated with the outcome [22,23]. An estimation of erythrocyte morphology was reported as a marker of transfusion recovery since the erythrocyte shape can be categorized reliably. Imaging flow cytometry may also help concur this problem when a subpopulation of small spherocytic erythrocytes may be identified during storage with wide variations between donors [11]. The spherocytic shift might be a relevant parameter for identifying a subpopulation of erythrocytes expected to be cleared rapidly after transfusion.

In the minor antigen mismatch method, an antibody to a minor antigen, different between the donor and recipient, is used to detect by flow cytometry the percentage of transfused erythrocytes in the sample one day after transfusion. The conducted study showed the diameter of erythrocytes and RDS were higher after the contact with IgG antibodies (from 6.32±0.62 μm to 10.4±1.09 μm) ($p<0.05$) as compared to the one observed after IgM

(7.5±0.46 μm). Moreover, IgG antibodies predominantly led to the appearance of burr cells, whereas IgM antibodies modified the erythrocytes into bite cells. IgG antibodies increased the diameter of erythrocytes by 4-5 degrees, whereas IgM antibodies decreased erythrocytes' diameter by 3-4 degrees.

The contact of erythrocytes with appropriate antiserum is well known to result in agglutination. The present study showed that IgM and IgG antibodies have peculiar effects on the erythrocyte membrane. Thus, IgM antibodies agglutinated erythrocytes, whereas IgG antibodies both agglutinated and increased the diameter of erythrocytes in a case of appropriate specificity. Both the erythrocytes in normal saline and erythrocytes contacted with serum without anti-A and anti-B antibodies (from A.B. blood group person) are morphologically and functionally normal. Meanwhile, the contact with appropriate antibodies modified the erythrocyte structure.

Our results are in agreement both with the notion of the different (glycolipid and glycoprotein) binding sites of IgG and IgM antibodies and with the different nature of ABO determinants on the erythrocyte membrane; thus, measurement of erythrocytes diameter might help extend the list of laboratory methods for specific antigen-antibody interactions and hemolysis detection [24]. A 5-minute incubation of anti-A IgG and IgM antibodies did not reveal a gradual decrease of the erythrocyte diameter while increased antibody titer, whereas absorption of IgG antibodies by A erythrocytes showed a decrease of the erythrocytes diameter.

Interestingly, immediately after contacting IgM antibodies with erythrocytes, the diameter of erythrocytes decreased compared to those not contacted with antibodies.

Importantly, with the increase of antibody dilution, the mean value of the diameter of erythrocytes and standard deviation decreased, thus leading to the decreased relative blood cell distribution size. The erythrocyte diameter size RDS was calculated by dividing the standard deviation (S.D.) of erythrocyte size by the mean corpuscular size (MCZ). The result of this straightforward equation was then multiplied by 100 to express results in percentage (%) (Table 3). According to Henry's Clinical Diagnosis and Management by Laboratory Methods, the conventional reference range of RDW (thus, RDS) comprises between 12% and 15% [6].

Table (3): Red blood cell distribution size caused by the contact with antibodies of various titers.

	1:2	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**
A er. (10%) with anti-A IgG	10.4%	14.65%	16.4%	18.9%	17.2%	27.5%
B er. (5.8%) with anti-B IgG	6.5%	6.3%	7.1%	8.2%	18.4%	
A er.(8.8%) with anti-A IgM	11%	12%	8.55%	5.7%	12.9%	4%
B er. (7.8%) with anti-B IgM 5 min incubation	8.7%	6.1%	3%	14%	16.3%	
B er. With anti-B IgG after absorption by B er.	7.6%	5.9%	7.9%	8.8%	16.2%	5.8%
A er. with anti-A IgM after absorption by A er.	7.5%	8.7%	8.6%	4.9%	6.7%	6.2%
A er. with anti-A IgG 5 min	26.5% +	22% +	7.6% +	15.14% +	17.5% +	19.3% +
A er with anti-A IgG absorbed by A er. 5 min	25.9% +	15.2% +	23.8% +	23% -	16.7% -	14.02% -

Note: er – erythrocytes, + - the presence of agglutination.

IgG antibodies gradually increased the mean value of erythrocyte diameter according to their increasing concentration, while RDS decreased due to the increased quantity of erythrocytes with enlarged diameter. At the same time, increased RDS was observed at low concentrations of IgG antibodies due to the start of enlarged erythrocytes appearance among normal diameter erythrocytes. On the other hand, IgM antibodies did not significantly increase the erythrocyte diameter, and the values of RDS of erythrocytes, contacted with low and high concentrations of IgM antibodies, appeared to be similar.

The diameter of washed O erythrocytes with anti-B antibody absorbing ability after contact with IgG anti-B antibodies and complement at 4°C was increased from 4.72±0.39 μm (RDS 8.2%) to 5.53±0.52 μm (RDS 21.6%), as well as after the contact with anti-B IgG antibodies 5.35±1.26 μm (RDS 23.5%) and 6.03±1.0 μm (RDS 16.5%) ($p<0.05$). The complement inhibitor on the surface of unwashed erythrocytes possibly inhibited the modification of erythrocyte diameter under the influence of specific antibodies and complement.

Fenbendazole and itraconazole abolished the agglutination of O erythrocytes with anti-A, anti-B antibody absorbing ability caused by their serum. The use of specifically absorbed antibodies (anti-B heated serum by B erythrocytes) with appropriate erythrocytes increased the number of erythrocytes with normal size (at 1:32 titer) as compared to nonabsorbed anti-B serum).

The polyclonal serum did not increase the diameter of erythrocytes immediately after the contact and following one-hour incubation, whereas using the heated serum for one-hour incubation caused a clear increase in erythrocyte diameter. Incubation for one day for polyclonal serum appeared to be enough to cause expressed changes in the erythrocyte structure. In the acid medium (at pH 5.8), stronger agglutination was observed compared to the alkaline medium (at pH 8.0), and higher activity of the complement was noticed at acid pH. At 4°C, IgG antibodies, in cooperation with complement the most actively modified and hemolyzed erythrocytes as compared to 37°C.

LIMITATIONS

This study has limitations as the investigation included persons without complaints of parasite infection.

CONCLUSIONS

The serum containing anti-A and anti-B antibodies (contrary to the serum from A.B. blood type) contacted with appropriate by blood group type erythrocytes led to the increased diameter of erythrocytes with higher values at 20°C as compared to the ones at 37°C. An anti-B serum with O erythrocytes with anti-B antibody absorbing ability at 20°C showed rouleaux formation.

AGS added to anti-A serum after the incubation with unwashed B erythrocytes with anti-A antibody absorbing ability at 37°C caused agglutination at 20°C contrary to 37°C temperature. Meanwhile, erythrocytes increased after contact with anti-A heated serum and AGS. IgG anti-A monoclonal antibody (MAB) 2-8 was contacted with B erythrocytes with the anti-A absorbing ability, and AGS did not cause agglutination; however, agglutination appeared at pH 6.0 and 8.0. At 37°C, anti-A IgG contacted with unwashed erythrocytes led to decreased erythrocytes.

The contact of anti-A serum with O erythrocytes with anti-A antibody absorbing ability after three times loading at 20°C led to the increased diameter of erythrocytes.

Anti-A serum with erythrocytes with anti-A absorbing ability after two times loading led to the hypocrisy of erythrocytes.

Contact of own anti-B serum with A erythrocytes with anti-B antibody absorbing ability led to the tendency of agglutination and presence of agglutination at pH 5.8 at 37°C contrary to pH 8.0 (with increased quantity of erythrocytes).

Estimating the increased diameter of erythrocytes after the specific binding with blood group antibodies as the main finding of the present article might be used in the clinical practice of hematologists. The differences in antibody titers to helminths in persons with anti-A or anti-B antibody absorbing abilities, elucidated in the study, are the perspective for future research. Moreover, the limitation of the study was an investigation of the persons without complaints of parasite infection.

Ethical approval was obtained by the expert commission of the National Medical University, confirming that the article's materials can be published in the open press (21.01.22 N 2). Informed consent was obtained from all the participants.

Authors contribution

Korzh M. O.: conceptualization, data curation, investigation. **Kravchun P.G**.: conceptualization, data curation, funding acquisition. **Leontieva F.S.:** formal analysis, funding acquisition, investigation. **Danyshchuk Z.M.:** investigation, methodology, software, visualization. **Ashukina N.O.:** investigation,

methodology, resources, software, visualization. **Dielievska V. Yu.:** writing review & editing, writing-original draft, data curation.

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Conflict of interest

The authors declare that they have no conflict of interest.

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