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# Estimation of the Erythrocytes Diameter for the Detection of the Specific Binding with Blood Group Antibodies

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## ABSTRACT

Different methods of the detection of blood group antigens exist in hematology. Nevertheless hemolytic reactions still occur despite an accurate performance of standard methods of blood group detection. Binding of the antigen with antibody is fixed by the phenomenon of agglutination of erythrocytes. Since we measured the erythrocyte diameter size we calculated Relative blood cell Distribution Size (RDS) by dividing the Standard Deviation (SD) of erythrocyte size from the Mean Corpuscular Size (MCZ). The result of this straightforward equation is then multiplied by 100 to express results in percentage (%).The increase of the mean value of the erythrocytes diameter on 16.6 % with appearence of agglutinated microcytes and macrocytes testify to the specific binding of absorbing and agglutinating determinants of the erythrocytes with IgG antibodies.

## INTRODUCTION

Nevertheless hemolytic reactions still occur despite an accurate performance of standard methods of blood group detection [1]. Binding of the antigen with antibody is fixed by the phenomenon of agglutination of erythrocytes. The aim of the study was to reveal the conformational changes of erythrocytes agglutinated by group specific antibodies. The incorporation of biomarkers in the actually used risk scores seem to be helpful for early identifying cases of incompatible hemotransfusion. The aim of this study is to investigate the potential clinical significance of Red blood cell Distribution Size (RDS) in incompatible hemotransfusion cases [2].

## MATERIALS AND METHODS

Following informed consent whole blood was drawn from normal healthy volunteers into heparin vacutainer tubes. O, A, B and O washed erythrocytes with anti-A or anti-B antibody absorbing abilities were included in the investigation.

Erythrocytes were obtained by washing three times with saline-suspended in normal saline and centrifuged at 1000 g for 10 minutes. The supernatant was removed from the suspension and the same amount of normal saline was added to the suspension. The procedure was repeated three times in order to suspend only Red Blood Cells (RBCs) in normal saline, removing the other components such as leukocytes, platelets and plasma.

To obtain IgG antibodies the serum was heated for 30 minutes at 60 °C. IgG antibodies with complement of guinea pig were added to the reaction for 1 hour incubation. Briefly, 50 µl of RBCs (blood type A) were added to 100 µl of anti-A serum with complement and incubated at 20°C, as well as to 100 µl of anti-B typing serum and serum from AB blood group type (negative controls).

The photos of the RBCs were obtained by MicroMed XS-3330 microscope with a digital camera Sigeta DCM - 900 9.1 Mpixels, recorded on a hard disk and diameters of 20 RBCs were fixed in micrometers.

*In vitro* and *in vivo* RBCs were reported to self-associate; giving rise to RBC stacks [3]. In turn, this Rouleaux formation underlies the sedimentation rate used clinically. The standard deviation was smaller than 3 %. Statistical analysis was performed using Student's criterion.

The initial diameter of A erythrocytes 6.09 ± 1.14 µm was increased after the contact with anti-A heated serum and complement to 6.9 ± 1.06 µm, whereas under the influence of anti-B heated serum and complement: to 6.36± 1.04 µm. The initial diameter of B erythrocyte from 6.04 ± 0.95 µm under the influence of anti-A heated serum and complement was not changed: 5.95 ± 0.96 µm. However under the influence of anti-B heated serum and complement the diameter of B erythrocytes was significantly increased to 7.5 ± 0.4 µm. B erythrocytes without serum were highest in number and discoidal in size, whereas cells with anti-B serum were fewer in number as well as abnormal in size depicting abrupt morphological changes. The diameter of O erythrocytes with anti-A absorbing ability increased in a less degree under the influence of anti-A heated serum and complement: from 6.06 ± 0.75 µm to 6.33 ± 0.75 µm as compared to the influence of anti-A heated serum and complement on A erythrocytes. The similar less increase of the diameter was observed after the contact of anti-B heated serum with complement with O erythrocytes with anti-B absorbing ability: from 6.05 ± 1.01 µm to 6.56 ± 0.79 µm as compared to the effect of anti-B heated serum with complement on B erythrocytes. The similar less increase of the diameter was determined under the influence of anti-B heated serum and complement on both A erythrocytes with anti-B absorbing ability (from 6.1 ± 0.85 µm to 6.44 ± 0.7 µm) and O erythrocytes with anti-B absorbing ability (from 4.65 ± 0.7 µm to 6.64±0.92 µm). The diameter of AB erythrocytes increased under the influence of anti-A heated serum with complement from 5.65±0.75 µm to 7.62 ± 1.1 µm. Importantly, the mean value of the initial diameter of erythrocytes did not differ between groups with different ABH characteristics–in a range of 6.0 µm. The presence of both agglutinating and absorbing determinants (as in the case of A and B erythrocytes) led to the significant modification of the erythrocyte diameter in a case of the specific binding with anti-A and anti-B antibodies – to 6.9 ± 1.06 µm–7.5 ± 0.4 µm. The presence of only absorbing determinants (anti-A or anti-B absorbing ability) or so-called week variants of A and B antigens led to the less modification of erythrocyte diameter (6.33 ± 0.75; 6.44 ± 0.7; 6.56 ± 0.79; 6.62 ± 1.1; 6.64 ± 0.92 µm) under the influence of specific anti-A and anti-B antibodies with

complement. The diameter of O erythrocytes with both anti-A and anti-B absorbing ability increased from 5.86 ± 1.14 μm to 6.62 ± 1.1 μm under the influence of anti-A heated serum and complement and to 6.62 ± 1.2 μm under the influence of anti-B heated serum and complement. Interstingly, the binding of B determinants with anti-B heated serum led to the more expressed increase of erythrocyte diameter of B erythrocytes: from 6.04 ± 0.95 μm to 7.5 ± 0.4μm, of A erythrocytes with anti-B absorbing ability: from 6.1 ± 0.85 μm to 6.44 ± 0.7 μm and of O erythrocytes with anti-B absorbing ability: from 4.65 ± 0.7 μm to 6.64 ± 0.92 μm on the contrary to the influence of anti-A heated serum with complement on O and B erythrocytes with anti-A absorbing ability: from 5.86 ± 1.14 μm and 6.05 ± 1.01 μm to 6.36 ± 1.1 μm and 6.56 ± 0.79 μm accordingly. Importantly, the specific binding of anti-A antibodies with erythrocytes with the only absorbing anti-A ability led to the appearence of separate microcytes (4.8 ± 0.5 μm) and agglutinated macrocytes (8.0 ± 0.7 μm). The same anisocytosis was observed in the reaction of anti-B heated serum with complement and erythrocytes with the only absorbing anti-B activity – with appearence of separate microcytes–echinocytes (6.4 ± 0.7 μm) and macrocytes with rouleaux formation (8.0 ± 0.7 μm). Meanwhile, both agglutination of A erythrocytes with anti-A heated serum and complement and B erythrocytes with anti-B heated serum and complement led to the appereance of agglutinated both macrocytes and microcytes. The reports of the presence of inhibitory substance able to prevent the absorbtion of group specific antibodies by erythrocytes in the serum of some persons encouraged to investigate the influence of the sera of the persons of O blood group with anti-A and anti-B absorbing ability on RBCs [4]. Thus, the serum from O blood group person with anti-A, anti-B absorbing ability agglutinated A erythrocytes, however did not enlarge their diameters - 6.3 ± 0.69 μm and 4.96 ± 0.64 μm - the diameters of intact erythrocytes and RBCs with the heated serum accordingly.

The studies on the morphology of erythrocytes in different pathologies are becoming more popular [5-7]. The presented method showed the potential of the investigation of the erythrocyte diameter for the detection of blood group type after the contact with complement dependent IgG antibodies. The increase of erythrocytes diameter and appearence of anisocytosis with separate microcytes-echinocytes and agglutinated macrocytes after the contact of the specific IgG antibodies with absorbing determinants of erythrocytes have not been mentioned before [8]. The study also revealed the enlargement of the erythrocytes and agglutination of both microcytes and macrocytes in a case of the specific binding of antibodies with erythrocytes carrying both agglutinating and absorbing determinants.

## RESULTS AND DISCUSSION

Since we measured the erythrocyte diameter size we calculated relative blood cell distribution size by dividing the Standard Deviation (SD) of erythrocyte size from the Mean Corpuscular Size (MCZ). The result of this straightforward equation is then multiplied by 100 to express results in percentage (%).

According to Henry's Clinical Diagnosis and Management by Laboratory Methods), the conventional reference range of Red blood cell Distribution Width (RDW) is roughly comprised between 12% and 15%[9].

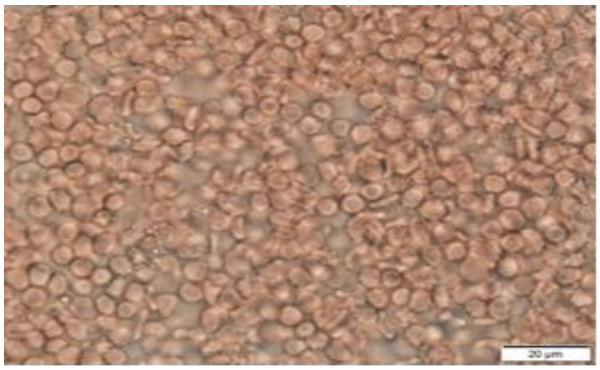
IgG antibodies increased the mean value of erythrocyte diameter while increasing concentration of IgG antibody, meanwhile RDS was increased at low concentrations of IgG antibody due to the appearence of enlarged erythrocytes among erythrocytes with diameter of intact ones and while increase of IgG concentration RDS was decreasing due to the bigger quantity of erythrocytes with increased diameter.

The appearence of the expressed anisocytosis after the contact of erythrocytes with groupspecific serum and complement corresponding to the agglutinating and absorbing determinants of erythrocytes was verified by the increase of the value of RDS from 13-15% (without specific antibodies) to 18-24% (after the contact with specific complement dependent antibodies). Thus, the contact of anti-A heated serum with complement increased RDS of A RBCs from 13.1% to 20.2%, O with anti-A absorbing ability: from 14.3% to 19.6%, B with anti-A absorbing ability: from 16.3% to 20.12%. The contact of anti-B heated serum with complement increased RDS of B RBCs from 15% to 21.3%, O with anti-B absorbing ability: from 14.3% to 20.1%, A with anti-B absorbing ability: from 16.3% to 23.2%. The significance of RDS was assessed by other authors [10]. Erythrocytes with RDS values exceeding the local reference range after the contact with groupspecific antibodies may be more aggressively investigated and managed, in order to identify and attenuate the impact of possible underlying incompatibilities causing both anisocytosis and hemotransfusion complications. IgM CIC is one of the mainstays in inducing RBC damage in severe conditions. The infuence of IgM antibodies on erythrocyte morphology was reported before; however the effect of IgG antibodies has been discussed in detail in the conducted study [11].

Further, the role of IgM Circulating Immune Complexes (CICs) in inducing morphological changes of RBC was confirmed through flow cytometric studies, which demonstrated a significant shift towards a lower forward-scatter when RBC were sensitized with IgM CICs. The RBC cell size of sick donors was typically more altered than RBC of healthy donors, due possibly to the IgM CICs interaction with RBC during the severe condition. Similar alterations in RBC membrane organization have been documented in acute childhood lymphoblastic leukaemia and leishmaniasis [12,13]. As IgM CICs primarily bind with CR1/DAF/glycophorin-A on RBC, they might also influence ROS generation. Prolonged exposure to ROS can thus induce oxidative stress leading to damage of the RBC membrane. It is well known that RBC not only counteract oxidative stress, but also play an important role in maintaining immunological homeostasis in infectious diseases. More studies have demonstrated recently that higher oxidant exposure causes Hb and spectrin aggregation, leading to a change in RBC membrane structure. Mohanty et al. have demonstrated that increased levels of ROS affect the discoidal structure of RBC [14]. Thus, the potency of IgM-bound CICs to trigger ROS in RBC was also quantified by measuring the amount of intracellular free radicals generated. Interestingly, the study revealed 2•67- and 2•84-fold increased ROS in RBC of SD compared to with mild symptoms and without symptoms patients, which was statistically significant (P=0•0004). Additionally, the studies demonstrated that the capacity of IgM-bound CIC purified from secondary dengue patients to trigger ROS generation was greater compared to that purified from primary dengue, demonstrated that macrophages are key players for the clearance of damaged RBC. Interestingly, this was reflected in erythrophagocytosis levels of both diseased and dengue IgM CICs-sensitized RB healthy donors.

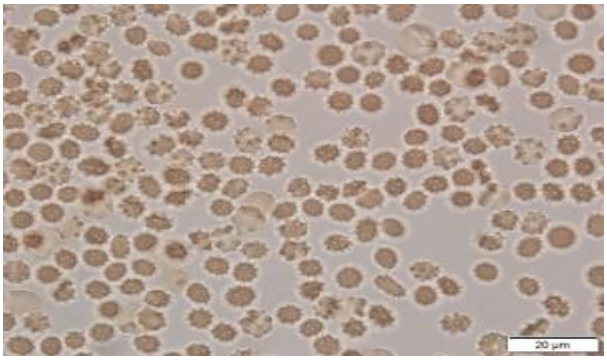
The contact of IgM anti-A antibody with B erythrocytes did not gradually increase the diameter of RBCs with the increase of antibody concentration, however the gradually enhanced diameter of RBCs was observed after the contact with IgM anti-B antibody, with increased expression of anisocytosis, verified by the raised values of RDS and decreased concentration of RBCs (mean diameter from 6.33 ± 0.5 μm - to 8.62 ± 1.99 μm (RDS 23%), 8.16 ± 0.98 μm (RDS 12%), 7.66 ± 1.0 μm (RDS 13%), 6.53 ± 0.77 μm (RDS 11.7%) in 1:2 1:4 1:8 1:16 1:32 dilutions of the serum accordingly (Figure 1).

**Figure 1.** OA+B+ erythocytes (4.97 ± 0.53 μm).



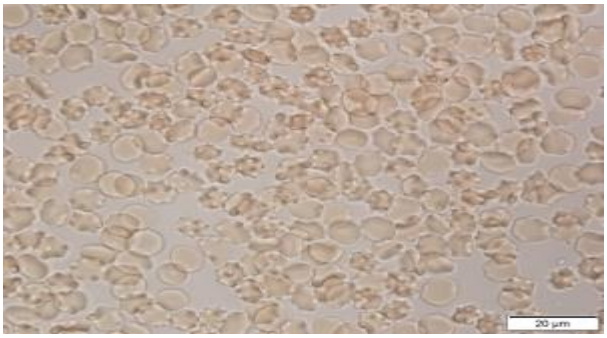
The same increase of RDS was observed after the contact of IgM anti-A antibodies and A erythrocytes (7.9 ± 1.85 μm mean diameter): 11.5%, 9.8%, 8.8% in 1:2, 1:16, 1:64 dilutions. After absorbtion by A erythrocytes anti-A IgM antibodies caused a less increase of anisocytosis: 9.3%, 10.88%, 6.1% in 1:4, 1:8, 1:64 dilutions accordingly (Figure 2).

**Figure 2.** OA+B+ erythrocytes with anti-A heated serum and complement (5.36 ± 1.0 μm).



After the contact of the heated anti-A serum with A RBCs RDS decreased: 21%, 23.6%, 16.8% in 1:4, 1:16, 1:64 dilutions accordingly. After absorbtion by A erythrocytes RDS was gradually decreased: RDS 16%, 19.8%, 16.2% in 1:4, 1:16, 1:64 dilutions of serum accordingly (Figure 3).

**Figure 3.** OA+B+ erythrocytes (5.41 ± 1.39 μm).



The contact of anti-A heated serum with A erythrocytes gradually increased the diameter of RBCs with the increase of antibody concentration with the raised values of RDS (mean diameter from 6.35 ± 0.56 µm - to 8.6 ± 1.69 µm (RDS 19.72%), 8.12 ± 2.8 µm (RDS 34%), 8.0 ± 1.85µm (RDS 23.2%), 7.2 ± 1.2 µm (RDS 16.7%), 7.31 ± 1.25 µm (RDS 17.14%), 6.8 ± 0.93 µm (RDS 13.7%) in 1:2 1:4 1:8 1:16 1:32 1:64 dilutions of the serum accordingly. After absorbtion by A erythrocytes anti-A IgG antibodies caused a less increase of erythrocyte diameter and anisocytosis: to 8.09 ± 1.72 µm (RDS 21.3%), 7.76 ± 1.45 m (RDS 18.76%) (Figures 4 and 5).

**Figure 4.** OA+B+ erythrocytes with anti-B heated serum and complement (7.05 ± 1.51 µm).

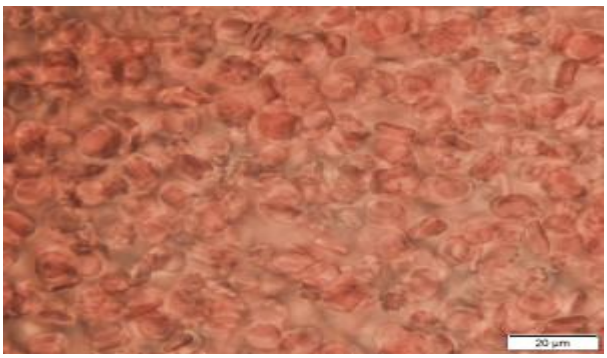
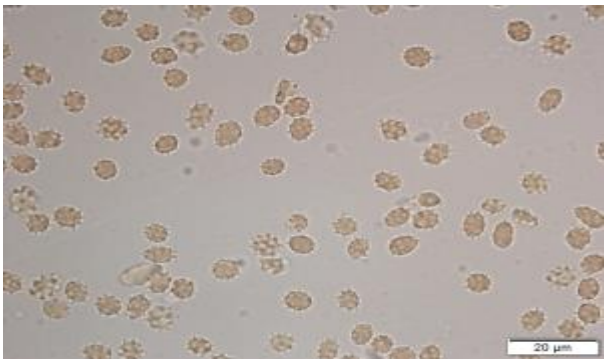
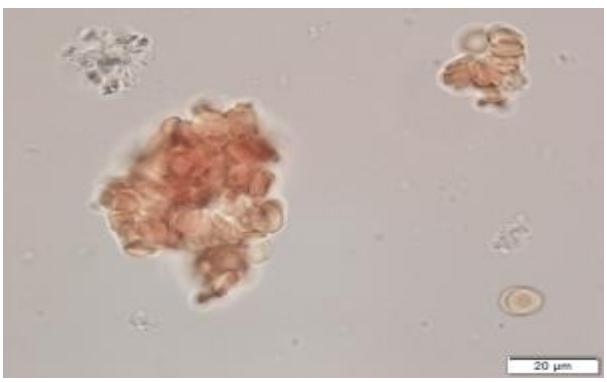


**Figure 5.** A erythrocytes (5.8 ± 0.74 µm).



6.89 ± 0.94 µm (RDS 13.6%), 7.3 ± 1.56 µm (RDS 21.33%), 6.72 ± 0.76 µm (RDS 10.7%), 6.6 ± 0.32 µm (RDS 4.96%) in 1:2 1:4 1:8 1:16 1:32 1:64 dilutions of the serum accordingly (Figure 6).

**Figure 6.** A erythrocytes with anti-A heated serum and complement (7.3 ± 0.51 µm).



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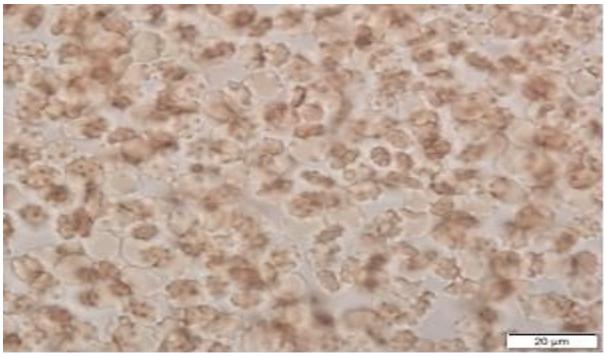
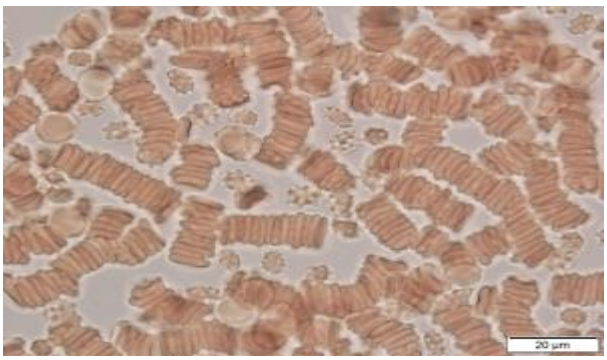
The contact of the heated anti-A,B serum with complement much more increased both the diameter and degree of anisocytosis of RBCs at 4°C as compared at 20°C: from 6.6 ±0.37 μm (RDS 5.6%) to 7.93 ± 1.99 μm (RDS 25%) and 7.66 ±0.99 μm (12.9%) accordingly (Figure 7).

**Figure 7.** B erythrocytes (7.23 ± 1.47 μm).



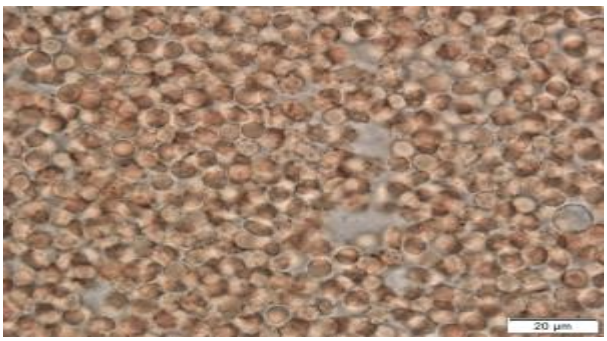
Addition of the serum with inhibitor of complement (from O blood group person with anti-B absorbing ability)caused less increase of AB erythrocyte diameter and at 4°C 6.88 ± 1.42 μm (RDS 20.6%) and at 20°C: 7.42 ± 0.95 μm (RDS 12.8%) (Figure 8).

**Figure 8.** B erythrocytes with anti-B heated serum and complement (7.95 ± 0.86 μm).



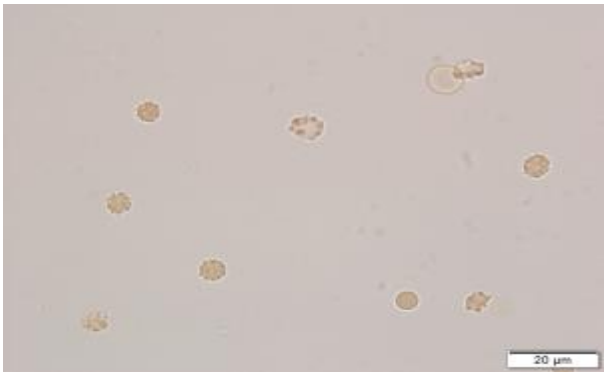
The contact of the heated anti-A,B serum without complement also caused less increase of the diameter and anisocytosis of RBCs at 4°C: 7.82 ± 1.47 μm (RDS 18.7%) and at 20°C: 7.0 ± 1.36 μm (RDS 18%) (Figure 9).

**Figure 9.** B erythrocytes with anti-A absorbing ability (5.8 ± 0.33 μm).



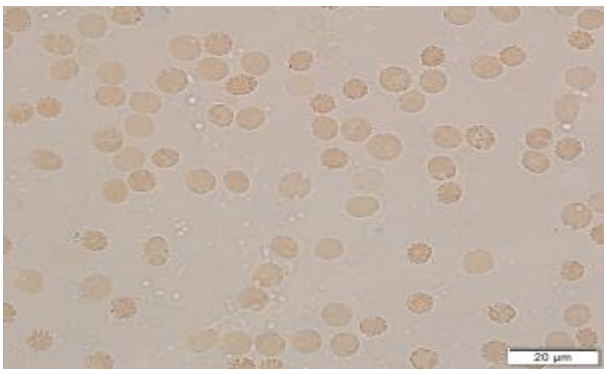
The contact of IgM anti-A,B antibodies (not heated anti-A,B serum) without complement also caused less increase of the diameter and anisocytosis of RBCs both at 4°C: 7.7 ± 1.02 μm (RDS 13.2%) and at 20°C: 7.2 ± 1.12 μm (RDS 15.5%) (Figure 10).

**Figure 10.** B erythrocytes with anti-A absorbing ability with anti-A heated serum and complement (6.46 ± 1.17 μm).



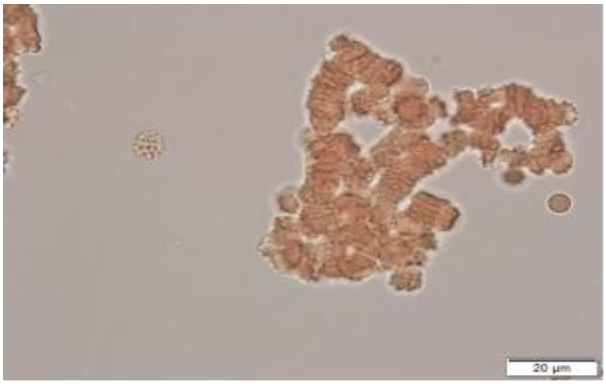

At 37°C erythrocytes with anti-A or anti-B absorbing ability did not increase theie diameter under the influence of Ig G anti-A and anti-B antibodies with complement. A and B erythrocytes at 37°C were agglutinated by Ig G anti-A and anti-B antibodies with complement (Figure 11).

**Figure 11.** A erythrocytes with anti-B absorbing ability (4.87 ± 1.26 μm).




B erythrocytes with anti-A absorbing ability modified their diameter from 6.24 ± 1.49 μm to 5.27 ± 0.5 μm, whereas at 20°C : 6.54 ± 1.67 μm (Figure 12).

**Figure 12.** A erythrocytes with anti-B absorbing ability with anti-B heated serum and complement (6.5 ± 0.51 µm).




The diameter of A erythrocytes with anti-B absorbing ability after the contact with anti-B heated serum and complement: 5.0 ± 0.49 µm, at 20°C: 7.22 ± 1.03 µm.

The diameter of O erythrocytes with anti-A absorbing ability after the contact with anti-A heated serum and complement: 5.64 ± 0.38 µm, at 20°C: 8.33 ± 0.82 µm.

The diameter of B erythrocytes after the contact with anti-B heated serum and complement: 7.06 ± 1.38 µm, at 20°C: 4.94 ± 0.57 µm.

The diameter of B erythrocytes (5.0 ± 0.54 µm) after the contact with anti-B heated serum and complement: 6.26 ± 1.17 µm, at 20°C: 7.72 ± 1.19 µm (Table 1).

**Table 1.** The diameters of erythrocytes under the influence of group specific antibodies (µm) with RDS (%).

| Erythrocytes | anti-A heated serum | anti-B heated serum | Without serum |
|---|---|---|---|
| A | 6.9 ± 1.4(20.2%) | 6.36 ± 1.04 | 6.09 ± 0.8(13.1%) |
| B | 5.95 ± 0.96 | 7.5 ± 1.6(21.3%) | 6.04 ± 0.9(14.9%) |
| O with anti-A absorbing activity | 6.33 ± 1.2(18.9%) | | 6.06 ± 0.85(14%) |
| B with anti-A absorbing ability | 6.56 ± 1.32(20.12%) | | 6.05 ± 1.0(16.3%) |
| A with anti-B absorbing ability | | 6.44 ± 1.58(23.2%) | 6.1 ± 1.0(16.3%) |
| O with anti-B absorbing ability | | 6.64 ± 0.92(19.6%) | 4.65 ± 0.7(15%) |
| AB | 6.2 ± 1.1(17.7%) | | 5.6 ± 0.75(13.3%) |
| O with anti-A and anti-B absorbing ability | 6.62 ± 1.3(19.6%) | 6.66 ± 1.34(20.1%) | 5.86 ± 0.84(14.3%) |

## CONCLUSION

The increase of the mean value of the erythrocytes diameter on 16.6 % with appearence of agglutinated microcytes and macrocytes testify to the specific binding of absorbing and agglutinating determinants of the erythrocytes with IgG antibodies. Meanwhile the increase of erythrocytes diameter on 8.3 % and presence of macrocytes with a rouleaux formation and separate microcytes testify to the presence of the specific binding of absorbing determinants of erythrocytes with IgG antibodies. Enhanced RDS value is not only a predictive factor and a marker of specific antigen-antibody binding, but its measurement may also be helpful for predicting the risk of developing many adverse complications in patients with hemotransfusions.

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