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Incomplete Antibodies May Reduce ABO Cross-match Incompatibility. A Pilot Study

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Short Title: ABO blood group and Fab antibodies

Incomplete Antibodies May Reduce ABO Cross-match Incompatibility. A Pilot Study

Abstract:

Objective: Any erythrocyte transfusion among humans having type A or B blood groups is impossible due to antibodies causing fatal transfusion complications. A cross-match test is performed to prevent immune transfusion complications before transfusion. Our hypothesis is that the Fragment antibody (Fab) part of antibody (incomplete antibody) may be used to prevent an immune stimulus related to complete antibody. Therefore, we designed a pilot study to evaluate the effectiveness of these incomplete antibodies with using cross-match tests.

Materials and Methods: Pepsin enzyme and staphylococcal protein A columns were used to cut Anti-A and Anti-B monoclonal antibodies and purify the Fab(2) fragments of them, respectively. A Rh positive erythrocyte suspension with purified anti-A Fab(2) solution and B Rh positive erythrocyte suspension with purified anti-B Fab(2) solution were combined, correspondingly. Cross-match tests were performed by tube and gel centrifugationmethods. Then, agglutination level due to the anti-A and anti-B Fab(2) antibodies and their effect on the agglutination normally observed with complete antibodies were measured.

Results: No agglutination for the purified incomplete anti-A Fab(2) with A Rh + erythrocyte and anti-B Fab(2) with B Rh + erythrocyte combinations were observed in the tube cross-match test. These agglutination levels were 1 positive in two wells in the gel centrifugation cross-match test. Fab(2) treated erythrocytes were also resistant to the agglutination which normally occurs with complete antibody.

Conclusion: We evaluated that the Fab(2) fragments of antibodies not only may be used to obtain a mild or negative reaction when compared to complete antibodies, but also they might be used for decreasing ABO incompatibility. The incomplete antibodies might be a therapeutic option in autoimmune hemolytic anemia and also they may be used in solid organ or hematopoietic stem cell transplantation. Therefore, we have planned an in vivo study to prove these in vitro findings.

Keywords: Transfusion Medicine, Red Blood Cells, Complications, The Humoral Immune Response

İnkomplet Antikorlar ABO Çapraz Karşılaştırma Uyumsuzluğunu Azaltabilirler. Bir Başlangıç Çalışması

ÖZET:

Amaç: Tip A ve B kan grubuna sahip insanlar arasında herhangi bir eritrosit nakli öldürücü transfüzyon komplikasyonlarına neden olan antikorlar nedeniyle imkansızdır. İmmün transfüzyon komplikasyonlarını önlemek için transfüzyondan önce çapraz karşılaştırma testi yapılır. Hipotezimiz komplet antikorla ilişkili bağışıklık yanıtını önlemekte antikorun Fragman antikor (Fab) parçasının (inkomplet antikor) kullanılabileceğidir. Bu inkomplet antikorların etkinliğini değerlendirmek için de çapraz karşılaştırma testlerini kullanarak bir başlangıç çalışması tasarladık.

Materyal ve Metotlar: Anti-A ve Anti-B monoklonal antikorlarını kesmek ve saflaştırmak için sırasıyla pepsin enzimi ve stafilokokal protein A kolonları kullanıldı. A Rh pozitif

eritrosit süspansiyonu ile saflaştırılmış Anti-A Fab (2) solüsyonu ve B Rh pozitif eritrosit süspansiyonu ile saflaştırılmış Anti-B Fab (2) solüsyonu sırasıyla birleştirildi. Çapraz karşılaştırma testleri tüp ve jel santrifügasyon yöntemleri kullanılarak çalışıldı. Sonrasında Anti-A ve Anti-B Fab (2) antikorlara bağlı aglütinasyon düzeyi ve bunların komplet antikorlarla normalde gözlenen aglütinasyon üzerine etkileri ölçüldü.

Bulgular: Tüp yöntemi ile yapılan çapraz karşılaştırma testinde saflaştırılmış inkomplet Anti-A Fab (2) ile A Rh pozitif eritrosit ve Anti-B Fab (2) ile B Rh pozitif eritrosit kombinasyonlarında aglütinasyon gözlenmedi. Jel santrifügasyon yöntemi ile yapılan çarpraz karşılaştırma testinde bu aglütinasyon düzeyleri her iki kuyucukta da 1 pozitifti. Fab (2) ile muamele edilen eritrositler komplet antikorla normalde oluşan aglütinasyona da dirençliydiler.

Sonuç: Antikorların Fab (2) fragmanlarının sadece komplet antikorlara kıyasla daha hafif veya negatif reaksiyonu elde etmekte değil, aynı zamanda ABO uyumsuzluğunu azaltmakta da kullanılabileceğini değerlendirdik. İnkomplet antikorlar otoimmün hemolitik anemide bir tedavi seçeneği olabileceği gibi aynı zamanda solid organ veya hematopoeitik kök hücre naklinde kullanılabilir. Bu nedenle in vitro bulguları doğrulamak için in vivobir çalışma planladık.

Anahtar kelimeler: Transfüzyon Tıbbı, Alyuvarlar,Komplikasyonlar, Hümöral Bağışıklık Yanıtı

Introduction:

There are many blood groups in the human population including ABO, Rh, Kidd, Kell, Duffy, MNS and Lewis. The ABO system is the most important of all blood groups in transfusion practice due to the reciprocal antibodies [1]. These antibodies consistently and predictably present in the sera of normal people whose erythrocytes lack the corresponding antigen(s) [2]. These antibodies may cause immediately lysis of donor Red Blood Cells (RBCs) during ABO incompatible transfusion and initiate fatal hemolytic transfusion reactions [1].

The type and screen are the first step of the pretransfusion compatibility tests. These tests used to define the patient's ABO group and Rh type and to detect expected and unexpected antibodies in the patient's serum. The cross-match is the final step of pretransfusion testing [3]. In this test, donor cells are combined with patient's serum and checked for agglutination, which would signify incompatible blood [4]. This process, also known as major crossmatch, serves as the last guard to ensure a safe transfusion [4-6].

Antibodies are also essential for humoral immunity [7]. Many antibodies have been shown to be primarily related to autoimmune diseases and such diseases are referred to as "antibody-related autoimmune diseases" [7-9]. Many of these diseases may disappear by the absence of certain antibodies [9].

All antibodies have two fragments. The fragment antigen-binding (Fab) binds to an antigen, and the the fragment crystallizable (Fc) stimulates the immune system by activating the complement [10]. Additionally, macrophages or lymphocytes detect the Fc fragment of antibodies [11,12]. Therefore, the Fab fragment detects antigens, and the Fc fragment stimulates the immune system. The Fc part removed antibody, which only Fab fragment of antibody exists, may be called as "incomplete antibody". Papain or pepsin enzymes can be used in fragmentation of the antibodies and produce Fab or Fab (2) fragments of the antibodies, respectively [13]. The effectiveness of Fab and Fab (2) fragments of an antibody

are similar and interchangeable [14]. Our hypothesis is that incomplete antibodies may be used to prevent an immune stimulus. Moreover, we designed a pilot study to examine the effectiveness of these incomplete antibodies in incompatible cross-matches due to ABO antibodies, and we are presenting it here. Local ethical committee approval has been obtained for this study.

Material and Methods:

Anti-A, Anti-B monoclonal antibodies (Eryclone, Verna Ind. Estata, India) were used for this study. Firstly, pepsin enzyme was used to cut these monoclonal antibodies and staphylococcal protein A columns was used to purify the Fab (2) fragments of them. The Pierce™ F(ab')2 Preparation Kit (Thermo Fisher Scientific, Rockford, IL, USA) was used to produce the Fab (2) fragments from complete antibodies. This process was composed according to the manufacturer's instructions.

After obtaining purified Fab (2)s, we began the second part of the study. During purification of Fab (2)s, the volume of the products have changed. The ratios of the complete monoclonal antibodies to the standard erythrocyte solution for an optimal cross-match test were calculated according to themanufacturer's instructions. We used these ratios for the anti-A or -B Fab (2) to the A or B Rh positive erythrocyte solutions for the cross-match tests, respectively.

After calculation, Anti-IgG cross-match card (Ortho-Clinical Diagnostics, High Wycombe, UK) has been used for the compatibility tests. We combined 10 µL of A Rh positive 5% erythrocyte suspension with 150 µL of purified anti-A Fab (2) solution in the same well to conduct a cross-match test in order to proof that the erythrocytes are covered with anti-A Fab (2). We also used 150 µL of complete anti-A antibodies for the positive control and 150 µL of Phosphate-buffered saline (PBS) for the negative control. We incubated all cards at 37 °C for 10 minutes and then centrifuged them for 5 minutes. The negative controls lacked the complete and incomplete antibodies. We repeated the same process with complete and incomplete anti-B antibodies and B Rh positive erythrocyte suspension. In addition, we repeated these tests with using Across Gel® Anti Human Globulin IgG+C3d cross-match cards (Dia Pro, Istanbul, Turkey). We have also evaluated agglutination levels when complete and incomplete antibodies were put in same well in same time which mentioned the amounts for A and B erythrocyte suspensions. We conducted an antibody titration test and repeated this last test with severalratios (32/1, 8/1, 4/1, 1/1, ¼, and 1/16) for complete to incomplete antibodies when used simultaneously. Finally, we evaluated the reactions in all wells.

We also performed a tube test to confirm the results of the card tests and to show if incomplete antibodies inhibit normal agglutination with complete antibodies or not. Firstly, we treated A Rh + erythrocytes with anti-A Fab (2) and B Rh + erythrocytes with anti-B Fab (2) in two separate tubes. Then we added complete anti-A and anti-B antibodies to the respective tubes and then we mixed them. As a positive control, A Rh + erythrocytes treated only with complete anti-A antibodies and B Rh + erythrocytes only with complete anti-B antibodies in a tube without adding incomplete fragments.Consequently, there were no incomplete antibodies in positive control tubes. Then, we evaluated agglutination levels in the tubes.

In addition, we performed a flow cytometric analysis to prove the results of all these tests. B Rh + erythrocyte sample was transferred to a tube containing K3 EDTA and the

tube divided into four tubes. We mixed the tubes with PBS or alone Anti-B complete antibodies or alone Anti-B incomplete antibodies or mix Anti-B antibodies (1 to 1 ratio for incomplete to complete). To label the erythrocytes, CD235a FITC (Glycophorin A, BD Pharmingen USA) and cytoplasm-staining nucleic acid dye 7- Amino-Actinomycin (7 ADD) (BD Pharmingen, USA) was added to the tubes. The samples were analyzed with using the FACSDiva software of FACSCanto II model flow cytometry (BD Biosciences, San Jose, CA, USA). Viable erythrocytes were identified as cells stained positive with CD235a FITC and negative with 7 ADD. We evaluated 100 000 events per sample to showthe erythrocyte agglutination levels in the tubes. Agglutination levels were calculated with SCA and FSC gating strategy [15].

Results:

For the card test, we observed 1 + reaction for the purified incomplete anti-A Fab (2) and A Rh + erythrocyte combination. However, weobserved 4 + reactions for the complete anti-A antibody with A Rh + erythrocyte combination. No positive reactions were observed in the negative control wells. The test results were similar for the B Rh+ erythrocyte and complete anti-B or incomplete anti-B Fab (2) antibody combinations and negative controls (Figure 1 and 2).

The antibody titration test results are given at Table 1. Higher concentrations of complete antibodies (from 8 to 32 times more than incomplete antibodies) were associated with 4+ agglutination levels in simultaneous using on the cross-match card tests (Table 1). Lower ratios than 8/1 showed double population results when both complete and incomplete antibodies were simultaneously added to the wells before erythrocytes (Table 1 andFigure 2). Increasing amounts of incomplete antibodies did not cause any 4+ results if complete antibodies were not added to the wells.

For the tube test, we observed no agglutination for the A Rh + erythrocytes and incomplete anti-A Fab (2) antibodies combination and B Rh + erythrocytes and incomplete anti-B Fab (2) antibodies combination in two separate tubes. There were no agglutination also when complete anti-A and anti-B antibodies were added to the respective tubes. No agglutination was continuing when two tubes were mixed (Figure 3). Agglutination was present in positive control tube that contains complete antibodies (Figure 4).

Flow cytometric analysis showed also similar results (Figure 5). Agglutinated erythrocytes expressed brighter CD235a positivity than non-agglutinated erythrocytes. Almost all erythrocytes were viable in the tubes. Erythrocyte agglutination levels were calculated as 0.9 % for PBS tube, 0.1 % for Fab (2)tube, 7.1 % for complete Anti-B antibody tube and 2.9 % for mix tube (1 to 1, complete to incomplete antibody).

Discussion:

Although complete Anti-A and –B antibodies cause strong agglutination, Fab (2) parts of these antibodies caused minimal or no agglutination on thecard and tube cross-match tests. Minimal agglutination with Fab (2) parts was similar to negative controls. These results come from the characteristicfeatures of an antibody. The Fab part of an antibody binds to antigen, and the Fc part of the antibody both starts agglutination and stimulates the immune system via activating the complement system and/or binding to Fc receptors of macrophages or lymphocytes [10-12]. Fc and its interaction with Fc receptor of macrophage has a critical role and is required for antibody response [16,17]. Hemolytic disease of newborn is a good example for this pathologic mechanism of antibody response. In this antibody related disease,

Anti D antibody treatment are used to prevent hemolytic disease of newborn [18]. This Anti D antibody drugs should be composed by complete antibodies to prevent competitive binding of Fab fragments [16]. In past, purified Fab fragment of Anti D antibody were studied for hemolytic disease of newborn because of its binding to Rh + erythrocytes [19,20]. However, anti D Fab treatment was not sufficient for being used for hemolytic disease of newborn due to its ineffectiveness [16]. This situation comes from the Fc part of the antibody. Removal of Fc part of an antibody may results ineffectiveness of the antibody when stimulating immune system even if it binds to an antigen. Similarly, digoxin specific incomplete Fab antibody effectively binds to its antigen (Digoxin). However, no significant immune reaction was reported in patients who use this agent probably due to the absence of Fc part of the antibody [21].

ABO incompatibility is an unavoidable clinical issue, and complications associated with ABO incompatibility should be managed and treated appropriately [22]. Hemovigilance procedures are recommended and used because of the potential for fatal complications following blood transfusion [23]. Although some procedures for treating ABO incompatible blood transfusions are used, to the best of our knowledge, none of them are specific [24]. In our study, we showed that if erythrocytes are exposed to Fab (2) and complete antibodies simultaneously, complete antibody associated agglutination ratios may decrease via coating some erythrocytes with Fab (2) and others with complete antibodies. Therefore, we hypothesized that anti-A and anti-B Fab (2) antibodies might be a useful treatment for these patients and may reduce fatal complications. Competitive binding between complete and incomplete antibodies may reduce or eliminate the effects of complete antibodies [25]. No strong agglutination with high amounts of Fab (2) and insufficiency of low amounts of Fab (2) in preventing agglutination related to complete antibodies support also our hypothesis. Similarly, anti Rh antibodies are considered to use for preventing transfusion reactions in hemolytic disease of newborn and their effect is superior when it is usedearly after birth [26]. Our results have also been explained with epitope masking hypothesis [27]. In other words, an epitope on an antigen when coated with an antibody other antibodies cannot bind the same epitope. Therefore, if the first antibody did not start immune response and occupy the epitopethe following antibodies will also be not able to cause an immune response. Our hypothesis may be stated that Fab (2) parts of the same antibodies may be used for masking the epitopes instead of other antibodies. Moreover, our findings may also help universal group O red blood cell studies [28].Instead of polyetilen glycol, Fab (2)s may be used in order to cover erythrocytes via coating surface antigens. However, our in vitro study needs to be supported with future in vivo animal studies for this using. Therefore, we are planning to conduct an in vivo study to prove the results of our pilot study.

ABO incompatibility between the donor and the recipient can cause hemolysis in the recipient, especially when performing hematopoietic and solid organ transplantations [22,29]. It also presents several challenges for hematopoietic stem cell transplantation [29].During hematopoietic stem cell transplantation, transfused erythrocytes and other blood products change based on the donor's and recipient's blood groups, and such changes are not stable [30]. Irradiated, filtered and leukocyte-depleted blood products are commonly used for blood transfusions [31]. Some hemolytic anemia patients have also auto-anti-A or auto-anti-B antibodies [32-34]. We hypothesize that the Anti-A and anti-B Fab (2) antibody fragments presented here may be used to prepare suitable or alternative blood products for such patients in the future. Using Fab (2) fragments of antibodies, including other blood groups, may simplify current antibody screening and identification tests. Although the importance of these tests, due to problems which originated from technical procedures and evaluation methods, these tests take time, postpone blood products using in patients and sometimes turn into an

inconclusive event [35]. As we showed, seeing a double population at a well may help the identification process of antibodies.

ABO incompatible solid organ transplantation presents other challenges, and some such transplants are currently impossible due to ABO incompatibility [22,36]. Solid organs contain ABO antigens that can cause incompatibility [22,36]. Immunoadsorption techniques are used to prevent the antibody related immune response and to extend survivals of grafts and transplant recipients having ABO incompatibility [37]. Hyperacute rejectionin solid organ transplants may also be reversed by using Fab fragments [38]. We hypothesized that the intravenous administration of anti-A and anti-B Fab (2) antibody fragments may also be used in solid organ transplantation.

Study Limitations:

Our study has some limitations. Our sole aim was to test our hypothesis that Fab (2) antibody fragments can be used to prevent an immune stimulus. All of the funds for this project are provided by the authors. However our funds were not sufficient to complete the project. Although the results of cross-match tests and flow cytometric analysis were consistent, we were not able to evaluate all possible immune stimulus mechanisms associated withincomplete antibodies. In addition, we would have preferred to measure the levels of the Fab (2) antibody fragments and pepsin after completing the reaction, and also to measure the reaction in various environmental conditions but we did not have sufficient funds to perform all these tests. It should also be noted that the weak positive reactions in group A or B erythrocytes with incomplete anti-A or anti-B antibodies on IgG crossmatch card tests, respectively, may have been originated from inadequate Fab (2) antibody fragment yields with pepsin and protein A columns in our study [39]. However, we cannot state a definite reason explaining these mild agglutinations in card test by the cause that we could not measure the levels of complete and incomplete antibodies in the products used for card tests. Higher agglutination results related to Fab (2) fragments on gel centrifugationtechnique than tube test may also have been originated from its higher sensitivity in detecting agglutination [40].

Conclusion:

In this in vitro study, we showed that ABO incompatibility can be minimized by using Fab (2) antibody fragments of anti-A and anti-B antibodies. In vivo studies are needed to explore the potential therapeutic effects of these agents. Therefore, we have planned to start an in vivo study to prove these in vitro findings.

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Table legends

Table 1. Antibody titration test on the IgG crossmatch cards according to the ratios for complete to incomplete antibodies which added in same time

Figure legends:

Figure 1. A. Group A erythrocytes

Ai. With incomplete anti-A antibody (1+ reaction)

A+. With complete anti-A antibody. (4+ reaction)

A-. With negative control (no reaction)

B. Group B erythrocytes

Bi. With incomplete anti-B antibody (1+ reaction)

B+. With complete anti-B antibody. (4+ reaction)

B-. With negative control (no reaction)

Figure 2. B. Group B erythrocytes

T. With complete (total) antibody (4+ reaction)

PBS. With PBS (- reaction)

Fab. With Anti-B Fab (2) (- reaction)

Fab+T: With Anti-B complete (total) and Anti-B Fab (2) simultaneously, 1/1 dilution (Double reaction with 4+ and -)

Figure 3 . Group A and Group B erythrocytes in same tube incubated with incomplete anti-A and –B Fab fragments and after added complete anti-A and –B to the medium. No agglutination

Figure 4 . Group A andGroup B erythrocytes in same tube incubated with complete anti-A and –B antibodies. Positive agglutination

Figure 5. Flow Cytometric Analysis with Group B erythrocytes

5a. With PBS, 0.9% agglutination

5b. With Anti-B Fab (2), 0.1% agglutination

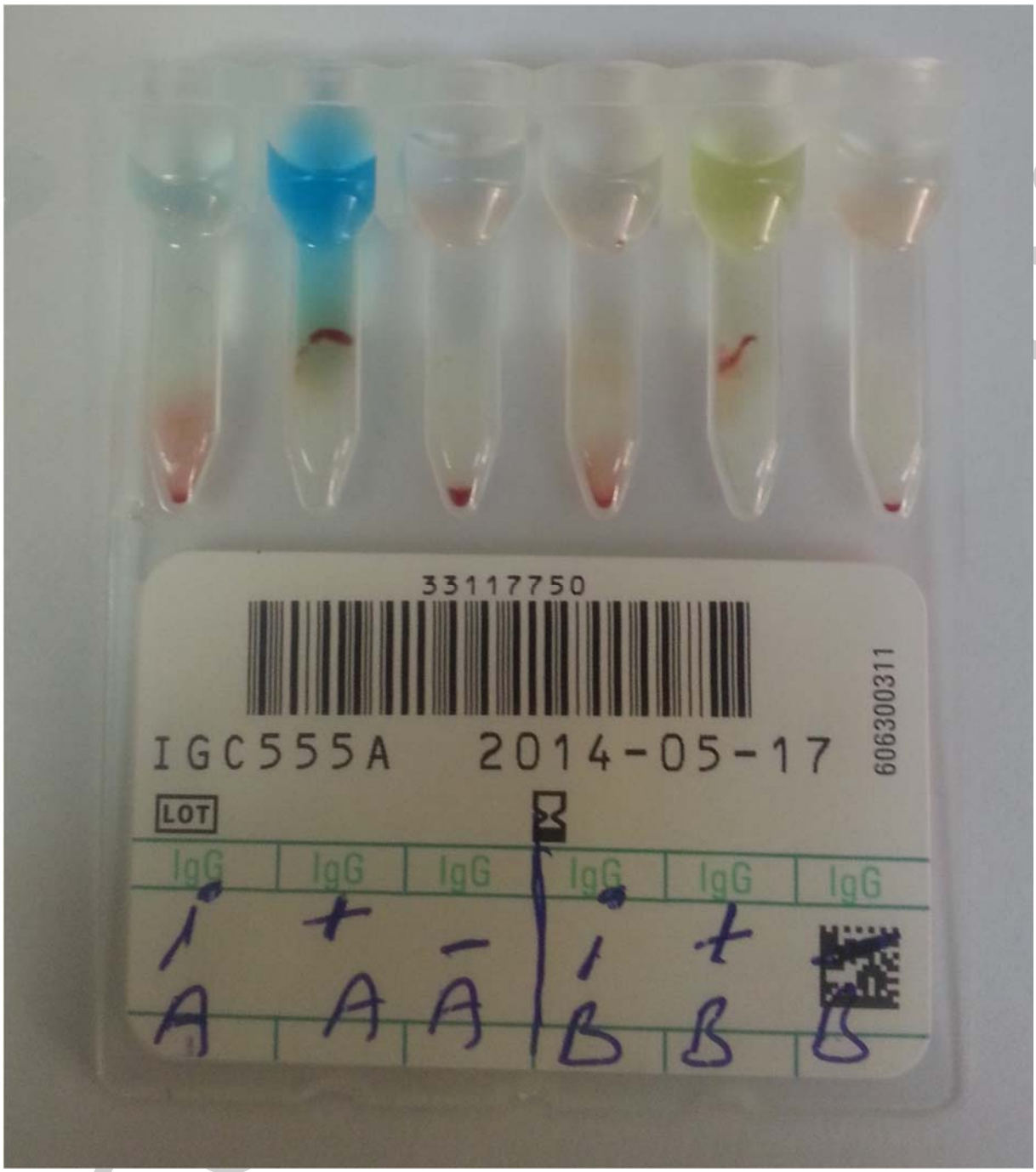
5c. With Complete Anti-B, 7.1% agglutination

5d. With Anti-B complete and Fab (2) simultaneously, 2.9% agglutination

Table 1. Antibody titration test on the IgG crossmatch cards according to the ratios for complete to incomplete antibodies which added in same time

Complete/incomplete Ratio	**Group AErythrocytes**	**Group BErythrocytes**
From 8/1 to 32/1	4+	4+
4/1	-/4+*	-/4+*
1/1	-/4+*	-/4+*
¼	-/4+*	-/4+*
1/16	-/4+*	-/4+*

*Double population


33117750
IGC555A 2014-05-17
606300311
LOT
IgG IgG IgG IgG IgG IgG
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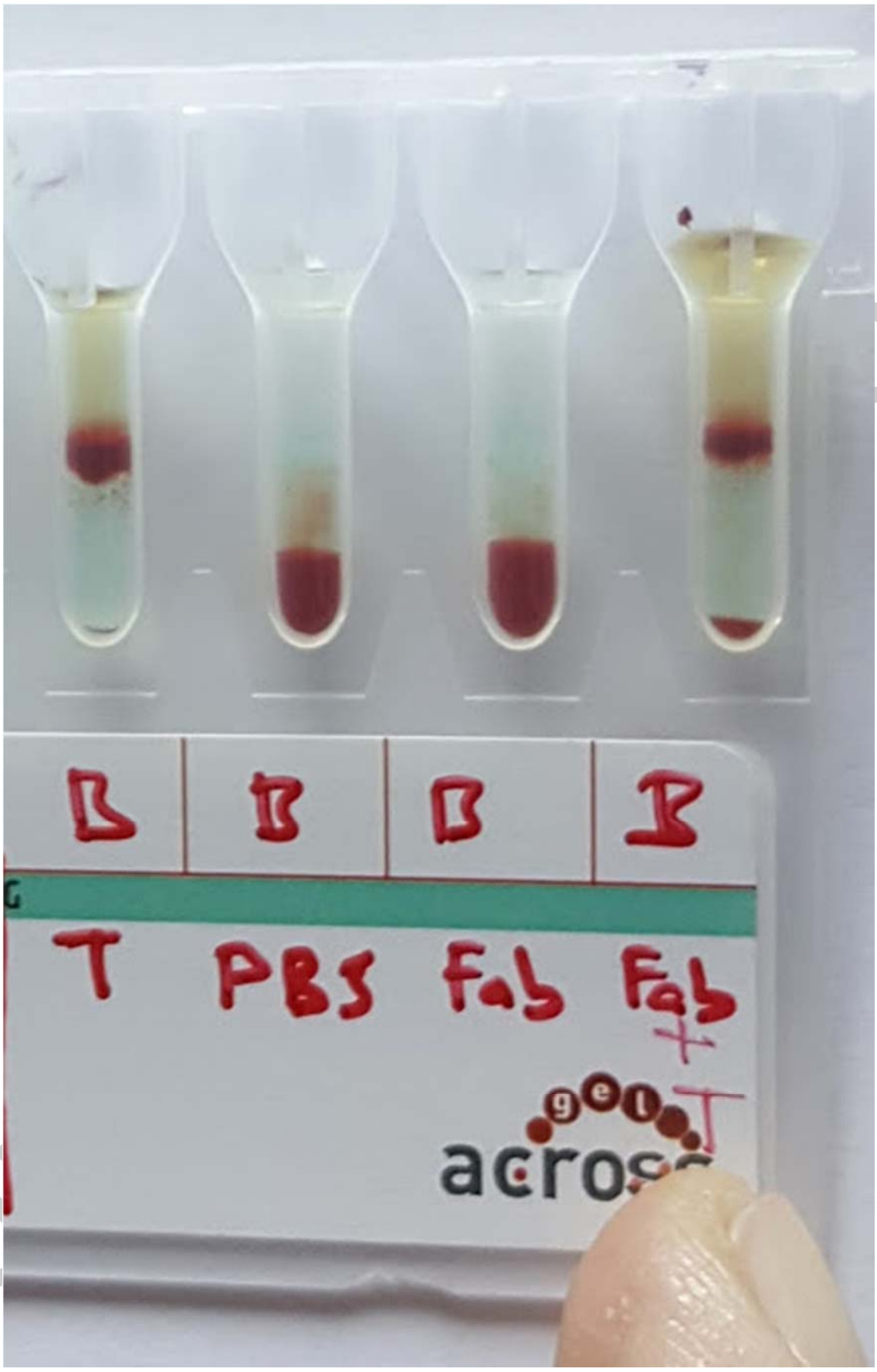
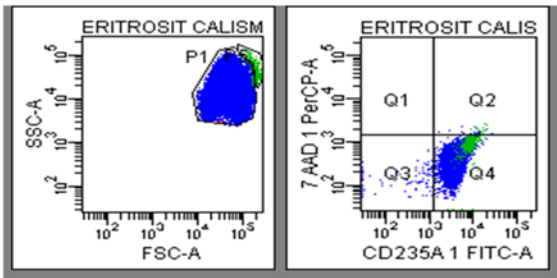
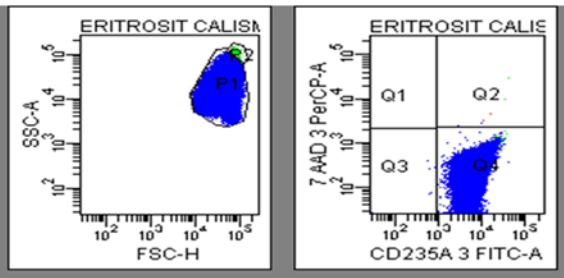
A A Ā B B B

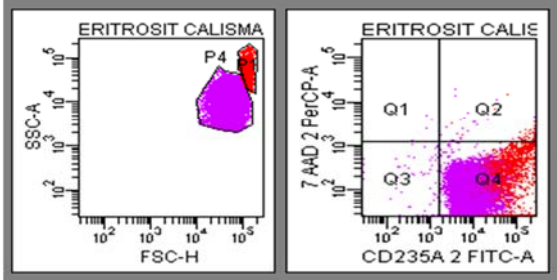

T
PBS
Fab
Fab
+



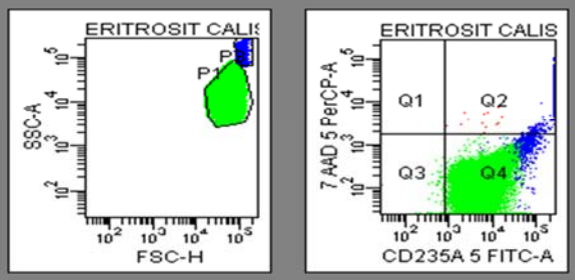
5a. With PBS, 0.9% agglutination



5b. With Anti-B Fab (2), 0.1% agglutination



5c. With Complete Anti-B, 7.1% agglutination



5d. With Anti-B complete and Fab (2) simultaneously, 2.9% agglutination