Rediscovering Red Blood Cells: Revealing Their Antigens Store

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Abstract: This paper describes a newly observed phenomenon related to red blood cells (RBCs). We found that plasma from a healthy individual immune-reacted with hemolysates from the same person and from other individuals. This strongly suggested presence of antigens in RBCs and corresponding antibodies in plasma. Those RBCs' antigens are different from RBCs proteome. Those antigens can be separated using plasma / serum of blood from which RBCs were taken. It is found that those antigens consist of HLA antigens, tissue specific antigens, and foreign antigens. The foreign antigens can be fetus antigens in pregnant females, microorganisms' antigens, food, insects or other antigens from environment. The collection of those transported antigens represents a dynamic store. Consequently, RBCs may play role in tolerance through transporting those antigens to central organs of the immune system. The experiments, which have been done, reveal some of the antigens of the store, and show how this phenomenon can be exploited, for instance, in diagnosis of human tuberculosis (TB). In effect, this work opens a new avenue of research and hopes.
[Mahmoud Rafea, Serhiy Souchelnytsky and Saleh El-Ayouby. Rediscovering Red Blood Cells: Revealing Their Antigens Store. Journal of American Science 2011;7(6):220-226]. (ISSN: 1545-1003).
http://www.americanscience.org.

Key words: Red Blood Cells, Erythrocytes, Immune Tolerance, Self-Antibodies, Erythrocytes Antigen Store, Erythrocytes Functional Proteome, Protection of Fetus as Allograft

1. Introduction:

We found that plasma from a healthy individual immune-reacted with hemolysates from the same person and from other individuals. This strongly suggested presence of antigens in RBCs and corresponding antibodies in plasma. Figure 1(a) shows Ouchterlony immuno-precipitation test of normal serum against self and other normal hemolysate. We confirmed this finding by using Western Blot technique, and showed that serum from one individual recognized antigens in hemolysate from two normal persons, Figure 1(b). Further confirmation was obtained by using two-dimensional gel electrophoresis (2-DE) of co-immunoprecipitated hemolysate antigens using self-serum, Figure 1(c). Antigenicity of the separated proteins was confirmed by immune-blotting proteins separated by 2-DE with the same self-serum. This excluded co-precipitation of non-antigens, as they would not be detected in immune-blotting. Notice that the number of the immunoprecipitated antigens is numerous and many spots were enriched by immune-precipitation because those antigens were not detected in 2DE gel of hemolysate, Figure 1(d). It should be remarked that this phenomenon is not reported before.

This observed phenomenon can be exploited in many directions. The proposed direction is to exploit functional proteomics approach with the following three crucial aspects of the experimental design (Thompson, *et al.*, 2008):

1) The strategy used for the selection, purification and preparation of the antigens to be analyzed by mass spectrometry
2) The type of mass spectrometer used and the type of data to be obtained from it
3) The method used for the interpretation of the mass spectrometry data and the search engine used for the identification of the proteins in the different types of sequence data banks available

Consequently, using a subset of antibodies which are specific against a subset of antigens of a particular disease will enable the use of those antibodies and those antigens in beneficial applications. This approach has been used, in this article, to identify *Mycobacterium tuberculosis* bacilli protein antigens (MTPAs) in TB-patient hemolysate.

In the next section, the motivation and the aspects of experimental design are described. Experiments for identifying MTPAs in TB-patient hemolysate are detailed in section 3. Section 4 contains discussion arguments. The last section is the conclusion and future work.

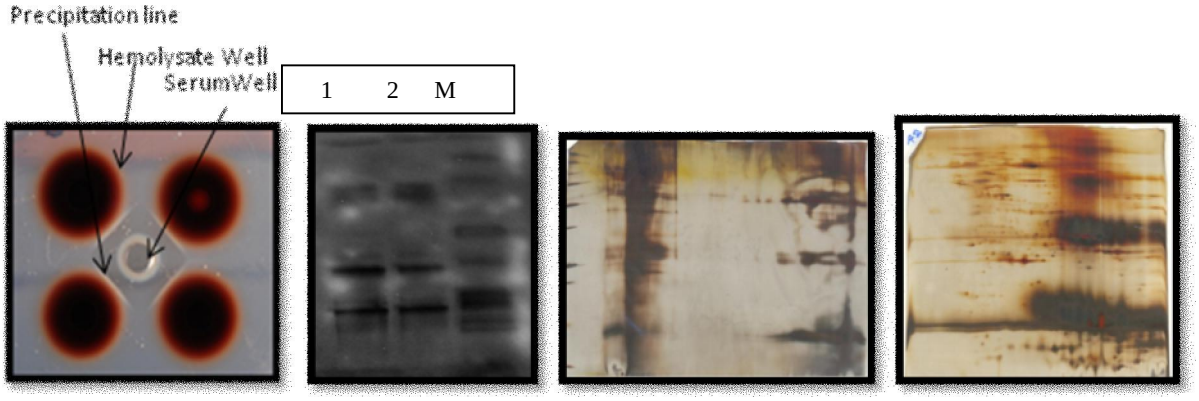


Figure 1: Detection of antigens in Red Blood Cells. (a) Ouchterlony test showing serum of normal against hemolysate of self and others (b) Western Blot using two normal hemolysate propped with serum of one of them (c) Silver stain of 2D electrophoresis of immune-precipitated hemolysate antigens (d) Silver stain of 2D electrophoresis of hemolysate.

2. Motivation and aspects of experimental design

Before trying to detect hemolysate antigens using self antibodies[1], there were some observations which lead to the induction of a number of hypotheses. Using background knowledge, some simple experiments were performed to prove or disprove those hypotheses. The observations start with a question related to Hemolytic Disease of Fetus and Newborn (HDFN). HDFN occurs sometimes due to ABO blood group incompatibility (Cariani, *et al.*, 1995). The incidence of HDFN does not exceed 5% (Cariani, *et al*., 1995). In pregnant women with blood group 'O', ABO antibodies are mainly IgG in nature (Daniels & Bromilow, 2006). IgG antibodies usually pass the placenta barrier (Leach, *et al.*, 1996). In most pregnancies (> 95%), delivered infants are normal which means that there is an efficient mechanism that can handle this incompatibility. The question is: *"How do circulating antibodies against blood group ABO in pregnant women who have blood group "O" not harm the fetus whose blood group is not "O"?"*

It is claimed that the low prevalence of clinically significant ABO HDFN is due to the expression of ABO by fetal cells and tissues other than RBCs, i.e., ABO is a histo-blood group system (Mollison, *et al.*, 1997) and (Tawfik, 2005). In addition, it is also stated that A and B red cell antigens are not fully developed in the fetus (Hadley & Soothill, 2002) and (Tawfik, 2005). In fact, this may explain the mechanism of why HDFN does not occur in 95% of pregnancies but it does not explain the mechanism of why it occurs in the 5%. Furthermore, neutralizing the effect of ABO antibodies on the fetus side is acceptable as a part of a mechanism, but not accepted as the full mechanism due to: a) the tiny size of a fetus in relation to the volume of blood of his mother, and b) the possible destructive effects of the consequences of antigen-antibody immune complex reactions. This neutralization effect should occur in the placenta by catering for the needed antigen similar to HLA antibodies (Koch & Platt, 2003). The placenta is designed for such reactions. This raises the question: *"How are ABO antigens delivered to the placenta without being trapped by the ABO circulating antibodies and lymphocytes?"*

For ABO antigens to be transported, a possible way is by RBCs because RBCs are specialized for transport. RBCs pinocytic activity has been demonstrated very early (Orci & Perrelet, 1973). Consequently, the occurrence of HDFN is due to the depletion of the RBCs antigen store. The sources of those antigen(s) may be the fetus, the environment, and/or the male spouse. A simple experiment that shows that hemolysate contains ABO antigens different from the person's ABO group antigens is described in Figure 2. It was observed that whenever there is ABO incompatibility and male spouse is not 'O', male spouse RBCs agglutination by female spouse plasma, was inhibited by female spouse hemolysate and was not inhibited by male spouse hemolysate.

[11] Self antibodies are antibodies of blood from which RBCs are taken. This term is used because autoantibodies are just a fraction of self antibodies.

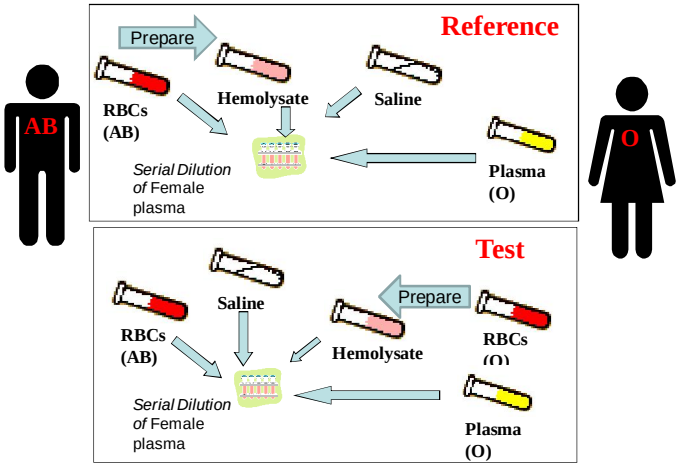


Figure 2: Detection of ABO antigens in RBCs' hemolysate. The experiment is performed, for each couple, by making two serial dilution of female plasma. In the first serial dilution (Reference), a drop of male spouse hemolysate is added to all tubes followed by a drop of his intact RBCs. In the second serial dilution (Test), a drop of female spouse hemolysate is added followed by a drop of male spouse intact RBCs.

If RBCs are capable of ABO antigen transport to the placenta, they may be also capable of transporting male spouse genetic antigens (e.g., HLA antigen) to thymus and bone marrow to induce tolerance to those antigens. RBCs may deliver also self antigens (e.g., HLA and Tissue Specific Antigens (TSAs)) to those central organs of the immune system to maintain self tolerance. Especially, this transport mechanism is important because the probability for any antigen to reach the thymus without being trapped by the immune system is zero. Consequently, the mechanism by which a pregnant woman is able to tolerate her fetus and placenta is the same mechanism a body tolerates his self antigens. The simple experiment that demonstrates the existence of self and spouse HLA antigens is, also, based on competitive inhibition and was performed using commercial HLA Typing Trays for the identification and definition of HLA Class I Antigens using the microlymphocytotoxicity assay (Hopkins, 1990). It was observed that female and male spouse hemolysate inhibited the typing reaction while a third person hemolysate did not. This indicates the existence of male HLA antigens in his hemolysate and in his female spouse hemolysate, Figure 3.

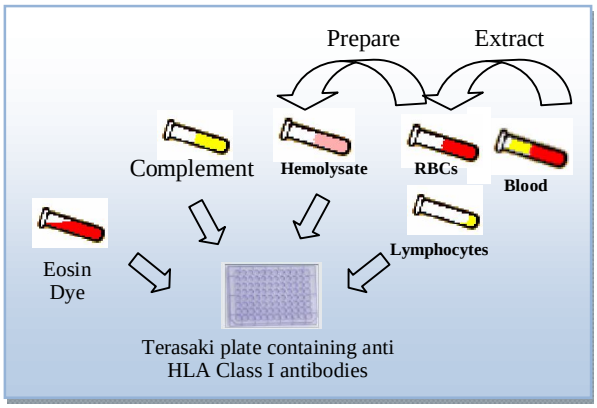


Figure 3: Detection of HLA antigens in RBCs' hemolysate. First, a couple is HLA typed and a suitable control person that does match the couple is selected. Three Terasaki plates are used for each couple. In each plate a different hemolysate (diluted 1/16) is used while male lymphocytes are used in the three plates.

If RBCs hide tolerated antigens (tolerogens) but antibodies still exist for those tolerogens because there is no absolute tolerance (Burek, 1998), then by implication some antigens, which induce immune response, may exist in RBCs, i.e., there is no absolute immune response, too. Consequently, if this is true, then blood circulating antibodies in any individual will react with his RBCs' hemolysate antigens. When

it was found that this hypothesis is true, searching for specific bacterial antigens is needed to confirm the hypothesis. As TB is a priority disease, trying to find *Mycobacterium tuberculosis* bacilli protein antigens (MTPAs) in TB-patient hemolysate was conducted through 2D electrophoresis, and then identifying gel spots with mass spectrometry. Fortunately, we discovered four MTPAs. This motivated us to do the experiments of the next section to identify more MTPAs in hemolysate of TB patients.

Identifying MTPAs in TB patients hemolysate

The goal is to find the set of antigens, in TB patients' hemolysate, which is related to *Mycobacterium tuberculosis* bacilli. This set of antigens can be used in preparation of: 1) a kit for TB diagnosis, and vaccine. The approach taken follows the following steps Figure 4:

1. The study resources are:
   - [A] Patients
   - [B] *Mycobacterium tuberculosis* (H37Rv)
2. For each patient:
   - Collect blood sample on anticoagulant (step 1)
   - Separate RBCs and wash many times with saline (step 2)
3. Hemolysate [C] is prepared by rupturing RBCs with low isotonic solution which is the binding buffer in affinity chromatography
4. Prepare hyper immune serum for M. *tuberculosis* (step 3)
5. Purify antibodies using Protein A Sepharose beads (step 4)
6. The purified antibodies are then used to separate antigens from hemolysate (step 5)
7. The disease related antigens are identified using in gel trypsin digestion and MALDI TOF mass spectrometry (step 6)

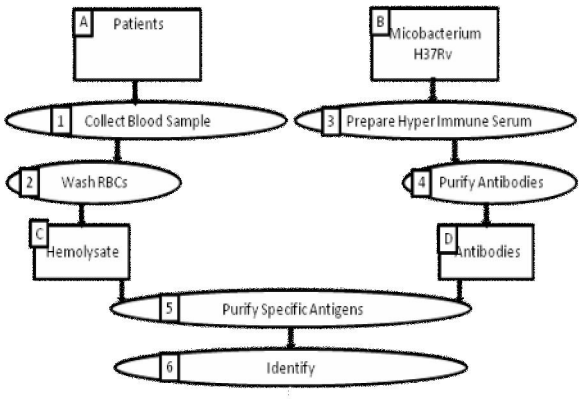


Figure 4: Flowchart depicting the resources and steps for identification of hemolysate antigens related to *Mycobacterium tuberculosis* (H37Rv)

2.1. *Mycobacterium tuberculosis* (H37Rv) Antigens Extraction

H37Rv strain was obtained from Veterinary Serum and Vaccine Research Institute (VSVRI), Tuberculosis Department. The protocol applied is a modified version of (Wood, *et al.*, 1988). Mycobacterium were grown on modified Middlebrook 7H11 medium (Gallagher & Horwill, 1977). The bacterial suspension was adjusted, approximately, to 10^9 bacterail ml^{-1}, and killed in 80^oC waterbath for 2h followed by sonication for 10 min. The suspension was centrifuged at 4000g for 15 min. The supernatant was filtered sequentially through 0.45 *u*m and 0.22 *u*m cellulose acetate filters. The total protein content was estimated by measuring UV absorbance at 280 nm. This supernatant is referred to as extracted antigens.

2.2. Preparation of hyper immune serum

Female rabbits weighting 1.5- 2.0 kg were injected subcutaneously on 3 occasions at 2 week intervals with 0.5 ml suspension of the extracted antigens (100 *u*g of protein) in oily adjuvant (Jansen & Knoetze, 1979) and 150 *u*g of H7 Flagellin derived from E. *coli* O157:H7 (McNeilly, *et al.*, 2008) while Flagellin preparation was done according to (El Ayouby, *et al.*, 2008). Blood was collected 2 weeks after the last injection. Serum was separated immediately after clot retraction and stored as 2 ml aliquots at -70 ^oC.

2.3. MTPSs separation using affinity column

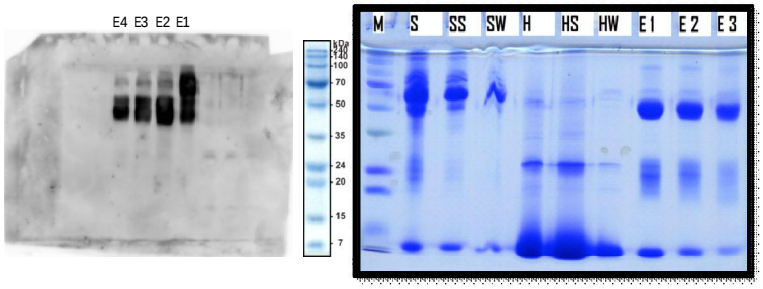
The reagents used are Binding Buffer (BB): 0.02 M sodium phosphate, pH 7.0, Elution Buffer (EB): 0.1 M citric acid, pH 3.0, Neutralization Buffer (NB): 1 M Tris-HCl, pH 9.0, and Desalting buffer (DB): 8M urea. A syringe (5 ml) is packed with a small wad of cotton wool in the bottom which is thick enough to prevent the passage of solid support but not too thick that a large amount of air is trapped. The syringe was filled three-fourths full of EB and a small amount was drained. To remove any air bubbles from the cotton wool, a glass rod was used to tamp it. The solid support was prepared from 1 ml Protein A Sepharose 4 Fast Flow slurry by washing it 3 times with EB. The slurry is then slowly added after draining some of EB from the syringe.

The rabbit serum and hemolysate are centrifuged at 10 000 g for 10 minutes to remove cells and debris. Both serum and hemolysate are diluted 1: 5 in binding buffer (BB) to adjust pH conditions and ionic strength. The diluted serum and hemolysate are used, in electrophoresis, as a reference to compare with the affinity column output. Figure 5 shows Western Blot of column elution fraction and the SDS-PAGE of all outputs of affinity

column. The labels shown in the figure are explained in the following column separation steps:

1. Equilibrate the column with 5 column volumes of binding buffer (BB).
2. Apply serum, and collect the output in a tube and labeled “Serum Sample (SS)”.
3. Wash with 5 column volumes of BB to remove impurities and unbound material. Collect output in a tube labeled “Serum Wash (SW)”.
4. Apply hemolysate, and collect the output in a tube and labeled “Hemolysate Sample (HS)”.
5. Wash with 5 column volumes of BB to remove impurities and unbound material. Collect output in a tube labeled “Hemolysate Wash (HW)”.
6. Elute with 5 column volumes of EB. Since elution conditions are quite harsh, collect fractions of 1 ml into NB (100 µl per fraction). Label tube fractions: E1, E2, E3, E4, E5.
7. Immediately re-equilibrate with 10 column volumes of BB.
8. The total protein content was estimated by measuring UV absorbance at 280 nm. Fractions were desalted with DB using a desalting column.

In Figure 5(b), notice the heavily stained 50 KDa bands which represent the concentrated antigens from the hemolysate. Meanwhile, Western Blot, **Figure 5**Figure 5(a), shows other larger and smaller proteins that appear hazy in the SDS-PAGE. Western Blot is done to compare human patient antibodies against MTPAs with those prepared in rabbits.



(a) **(b)**

Figure 5: Verification of affinity column output. a) Western Blot of column elution fraction E4, E3, E2, and E1 which are transferred from SDS-PAGE to nitrocellulose membrane and then probed by human TB patient plasma. b) Coommassie Blue stain of affinity column output compared to serum (S) and hemolysate (H)

2.4. Antigen Identification

The affinity column separated hemolysate antigens from two patients were used in 2D-electrophoresis, Figure 6. Notice that the number of spots has decreased and become better manageable when compared with Figure 1(c). Spots were cut and proteins were extracted and digested according to the trypsin digestion protocol mentioned in (Shevchenko, *et al.*, 2002). We could identify 11 proteins belonging to H37Rv from 60 spots. Other proteins were related to bacterial commensals, e.g., bacteriods.

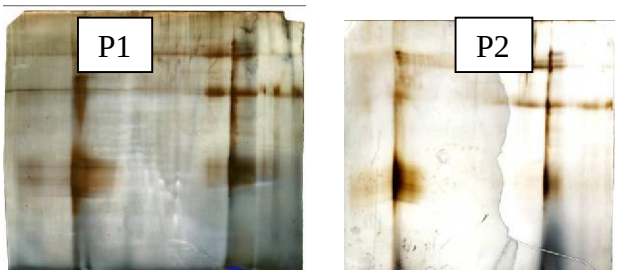


Figure 6: 2D-Electrohoresis of patients hemolysate antigens separated by affinity column chromatography containing Protein A Sepahrose beads and the ligand is Ig of rabbit hyper immune serum against M. *tuberculosis*. Eleven proteins at pI: 4.8 and KDa: 219.14, 219.16, 219.27, 218.27, 54.23, 54.52, 54.62, 54.63, 54.65, 54.68, and 54.75. The rest are proteins related to bacterial commensals, mainly bacteriods.

3. Discussion

The study of RBC antigens was directed to cell membrane proteins, only. The differences in human RBCs are due to the presence or absence of certain protein molecules or antigens. The antigens are located on the surface of the red blood cells. Individuals have different types and combinations of these molecules. The blood group that one belongs to depends on what he has inherited from her/his parents. The antibodies against those molecules are in the blood plasma and depend on the type of antigens on the RBC membrane. There are more than 20 genetically determined blood group systems known today, but the AB0 and Rh systems are the most important ones used for blood transfusions. Nobel Laureate Karl Landsteiner was involved in the discovery of both the AB0 (Daniels & Bromilow, 2006) and Rh (Mollison, *et al.*, 1997) blood groups.

The only identifiable function of RBCs is the delivery of oxygen to tissues and return back of carbon dioxide to the lungs. Hemoglobin is the key player in this function. All the previous work in RBCs proteomics has not identified another function. Also, RBCs proteomics has not mentioned HLA, TSAs, or foreign proteins. The reasons are obvious. Firstly, it is not expected to find such proteins and consequently the method used for the interpretation of the mass spectrometry data, and the search engines used for the identification do not consider the right types of sequence data banks available. Secondly, the amount of most of the antigens which belong to the RBCs antigens' store is little. This makes those antigens invisible and hence easily missed. This is clear when one compares the 2DE gel of hemolysate immunoprecipitated antigens of Figure 1(c) and hemolysate of Figure 1 (d).

Although this phenomenon can be related to immune tolerance by logical induction, the concrete evidence and mechanism need further research. Mainly, the logical induction is based on the finding all kind of antigens in hemolysate, especially HLA antigens which are related to fetus. This existence of all kinds of antigens, definitely, plays some immunological role which may be immune tolerance.

Whatever the reason of this existence of antigens in hemolysate this existence can help in designing diagnostic kits for different types of diseases. Further, it will help in discovering, not only, new immunological disorders which are, now, categorized under idiopathic disease, but also, identifying the obscure cause of many immunological disorders, including cancer. The identification of the cause of a disorder will help in its treatment and prevention.

4. Conclusion

In this article, the work described is just a pilot study that throws some light on a new phenomenon related to RBCs. This phenomenon is the existence of antigens store consisting of self and non-self antigens. Most probably this antigens' store has some role in immune tolerance.

The initial experiment, which shows the existence of ABO antigens in hemolysate of pregnant females, explains the mechanism of how HDFN occurs. Meanwhile, the experiment which shows that HLA antigens exist in their hemolysates proposes a new hypothesis which is the mechanism by which a pregnant woman is able to tolerate her fetus and placenta is the same mechanism a body tolerates his self antigens.

The experiments which use hemolysate against self-serum: Ouchterlony immune-precipitation test, Western Blot, and 2DE of co-immunoprecipitated antigens demonstrated that RBCs have an antigens' store. Mass spectrometry of spots obtained from 2DE gel demonstrated the finding of all kind of antigens, self and non-self, in hemolysate. This indicates that blood circulating antibodies in any individual will react with his RBCs' hemolysate antigens. In effect, there is no absolute immune response, too.

This directed our attention to use hyper immune serum against Mycobacterium antigens. This will help to get rid of other proteins and do better separation; and hence better identification. Consequently, we could identify 11 proteins from 60 gel spots belonging to H37Rv strain. The rest of spots are proteins related to bacterial commensals. Consequently, purification of specific antibodies from hyper immune serum is recommended to get further better separation.

Acknowledgments

We would like to thank Dr. Essam Nasr and Professor Dr, Mervat El Anary. Dr. Nasr has provided the H37Rv strain and helped in the preparation of the antigen extract. Professor El Ansary has provided lab facilities and reagents for HLA typing and has examined the typing trays.

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5/6/2011