Chapter 2

Rediscovering Red Blood Cells: Revealing Their Dynamic Antigens Store and Its Role in Health and Disease

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Additional information is available at the end of the chapter

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1. Introduction

The only identifiable function of Red Blood Cells (RBC) is the delivery of Oxygen. In mammals, RBC is a unique cell because:

- It does not have cellular organelles like any other cells in the body.
- It has a very especial protein: Hemoglobin which plays the role of carrying Oxygen to tissues and carries back carbon dioxide to lungs.
- It has a very especial cell membrane which carries a number of blood groups antigens' systems. Their functions include transporting other proteins and molecules into and out of the cell, maintaining cell structure, attaching to other cells and molecules, and participating in chemical reactions [1]. Those systems are genetically controlled with blood groups determining genes.

The work described in this chapter is based on the function carried out by the cell membrane antigens which are transporting other proteins and molecules into and out of the cell. The question is what are those proteins that are transported? In fact, this question identifies the knowledge gap about RBC role in health and disease. In the next section, some hypotheses will be inducted and deduced through analysis of available background knowledge. The experiments that can proof those hypotheses are described in section 3. This is followed by describing a theory about the role of RBC in health and disease based on the proved hypotheses and how we can benefit from this theory in diagnosing and treating of patients.

2. Knowledge analysis and hypotheses induction

Basically, when an antigen is introduced into a body, the immune system (IS) does either one of two reactions: immune tolerance (IT) or immune response (IR). IT-reaction is never

absolute [2]. It is usually accompanied by a weak IR. In normal IR, one cannot identify if there is a degree of IT, because there is no defined laboratory method/test that can measure the degree of IT. Meanwhile, by logical implication, some degree of IT should exist with the normal IR. This entails that there is an equivalence relation between IT and IR.

Hypothesis I: There is no absolute immune tolerance, if and only if there is no absolute immune response

In central IT, immature self-reactive T lymphocytes recognize antigens in the thymus and undergo negative selection (deletion) [3]. Consequently, in normal IR against a particular antigen, measuring the concentration of this antigen in the thymus can be correlated to the degree of the accompanied IT. The transport mechanism of antigens to the thymus is a critical issue because of the remarkable capacity of IS which can recognize any antigen [4]. In [5], authors claim that Dendritic Cells (DCs) have several functions, not only, in innate and adaptive immunity, but also there is increasing evidence that DCs in situ induce antigen specific unresponsiveness or tolerance in central lymphoid organs and in the periphery. The evidence that DCs transport antigens to thymus in central tolerance is very weak while the evidence that DCs have role in peripheral tolerance is more acceptable based on the review article [6]. In conclusion RBC may be vehicles which transport self antigens to induce central IT.

The role of RBC in transporting antigens has not been investigated before. If RBC are capable of antigen transport to induce IT, this will unveil important knowledge. For instance, in hemolytic disease of fetus and newborn (HDFN), maternal anti D alloantibody and feto-maternal ABO incompatibility are the two major causes of HDFN, Meanwhile, with the implementation of Rhesus D immunoprophylaxis, hemolytic disease due to ABO incompatibility and other alloantibodies have now emerged as major causes of this condition. [7].

In pregnancy, most of delivered infants are normal when there is no anti D alloantibody which means that there is an efficient mechanism that can handle the other incompatibilities. The mechanisms explained in literature explain why ABO incompatibilities, only, do not occur [8], [9] and [10], but these mechanisms do not explain why those incompatibilities occur. The mechanism may be based on trapping those antibodies in placenta through RBC catering of ABO and other incompatible blood groups antigens. Consequently, the occurrence of HDFN may be due to depletion of those antigens' store from RBC. Also, if this RBC transport function is the mechanism a body tolerates his self antigens, this will explain how a pregnant woman is able to tolerate her fetus and placenta, assuming that they are part of self.

Hypothesis II: RBC hide antigens to transport them to target organs.

From these *hypotheses I & II*, if RBC play role in antigen transport, one can deduce that in any mammal, blood circulating antibodies against self and foreign, either antigens or tolergens, will react with hemolysate.

Hypothesis III: There is an injection function (one-to-one) between circulating antibodies and RBC's hemolysate antigens.

To proof that RBC have role in immune reactions (IR and IT), one need to proof that there is an inverse correlation between antibodies concentration in plasma and antigens concentration in RBC.

Hypothesis IV: In immune response, antibodies concentration in plasma against a particular antigen in hemolysate is higher than this antigen concentration in hemolysate. Meanwhile, in immune tolerance, antibodies concentration in plasma against a particular antigen in hemolysate is lower than this antigen concentration in hemolysate.

It should be remarked that Humans expressing a defective form of the transcription factor AIRE (autoimmune regulator) develop multi-organ autoimmune disease (autoimmune polyendocrinopathy syndrome type 1) [11]. Liston et all [12] prove that this autoimmune syndrome is caused by failure of a specialized mechanism for deleting forbidden T cell clones, establishing a central role for this tolerance mechanism.

3. Experiments

The methodology applied will demonstrate the existence of particular self tolerogens and particular foreign antigens in RBC (Hypothesis I & II) and show that innumerable antigens exist in RBC which react with innumerable antibodies that exist in plasma. This partially proves that RBC play a role in immune reaction. To proof Hypothesis IV, it will be demonstrated that the concentration of foreign antigens in RBC varies by time in relation to IR known behavior. The experiments done are the following:

1. RBC of pregnant females transport male spouse ABO blood group antigens
2. RBC of pregnant females transport male spouse HLA antigens
3. RBC transport self HLA antigens
4. RBC transport self Tissue Specific Antigens (TSAs).
5. RBC hemolysate antigens are precipitated by plasma obtained from the same individual and cross reacted with plasma from different individuals.
6. RBC transport bacterial antigens.
7. RBC antigens and plasma antibodies concentration vary with time.

3.1. Materials for experiments 1, 2, and 3

Couples that have children, pregnant females, and single females were selected from relatives and friends. The purpose of the experiments was explained to them. Not all the combinations could be found, after blood grouping. The combinations presented, in Table-1, were used to conduct the experiments. Blood samples were taken on heparin. Some of the blood samples were used to prepare RBC and plasma and the rest was used to prepare lymphocytes using the Ficoll hypaque technique [13].

RBC were washed several times using phosphate buffer saline (PBS). The male RBC were divided into two tubes. The first tube was divided into small aliquots that were frozen to rupture RBC. The second tube was used to prepare a 5% suspension. The female RBC were divided into small aliquots that were frozen to rupture RBC. Notice that we do not need female intact-RBC.

Female ABO group	Male ABO group
O	A
O	B
O	AB
A	O
A	B
B	A
O (SINGLE)	-

Table 1. The ABO blood groups of couples used in the experiments

3.2. RBC of pregnant females transport male spouse ABO antigens

To test RBC transport of male spouse ABO antigens, a technique based on competitive inhibition of RBC agglutination was followed. If the hemolysate contains ABO specific antigens, then those antigens will compete with RBC and prevent their agglutination. Figure 1 illustrates a schematic description of the experiment.

Method

The experiment was performed, for each couple, as follows:

- In positive control tubes which represent also reference tubes for comparison with test tubes, serial dilutions (up to 1/128) of female spouse plasma were made using normal saline. A drop of a male spouse's hemolysate was added before adding his RBC's suspension.
- In test tubes, serial dilutions of the female spouse's plasma were made using normal saline. A drop of the female spouse's hemolysate was added before adding a drop of her male spouse's RBC suspension.

Results

Whenever there is ABO incompatibility and the male spouse is not 'O', agglutination was inhibited by the female spouse hemolysate and was not inhibited by male spouse hemolysate. In most cases, agglutination was inhibited in the first tube. However, agglutination was never observed in subsequent tubes. The single virgin female RBC do not contain any ABO antigens.

3.3. RBC of pregnant females transport male spouse HLA antigens

This experiment was performed using commercial HLA Typing Trays for the identification and definition of HLA Class I Antigens using the microlymphocytotoxicity assay [14]. It is

also based on competitive inhibition. Consequently, if typing wells that show positive reaction were inhibited in corresponding testing wells by adding hemolysate, this proves the existence of specific competing antigens. Figure 2 illustrate the experiment steps.

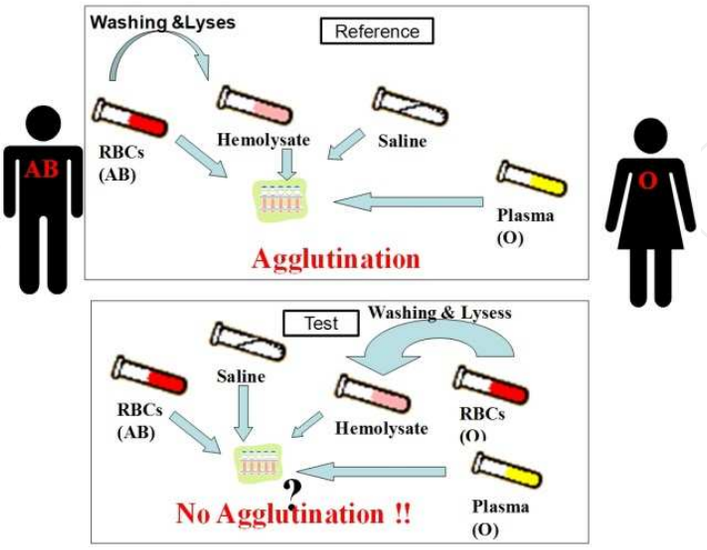

Figure 1. Schematic drawing of ABO antigen transport experiment, the upper part shows how the reference positive control is conducted, while the lower part shows how the test is conducted.

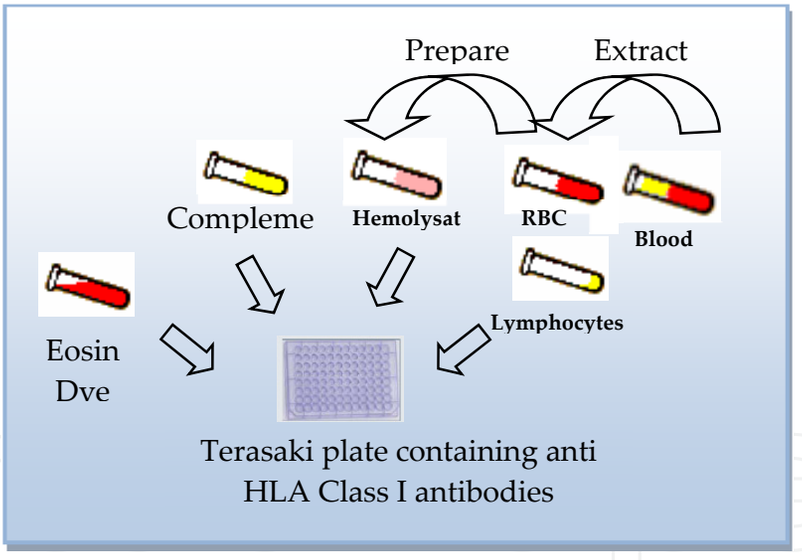

Figure 2. Re-typing of male spouse but using his female spouse hemolysate to compete with his lymphocytes

Method

First, each couple was HLA typed, and then the following was done:

- A hemolysate from a third person was added to control wells. The positive control should give positive reaction. In this way, we excluded inherent errors or non-specific reaction.
- Female spouse hemolysate (diluted 1/16) was added to typing wells

- Male spouse lymphocytes was added and followed by the complement and eosin dye as usual.
- Wells that gave positive reaction in typing were examined by contrast microscope.

Results

It was observed that female spouse hemolysate inhibited the typing reaction while the third person hemolysate did not. This indicates the existence of male spouse HLA antigens in female spouse hemolysate.

3.4. RBC transport self HLA antigens

This experiment is similar to the previous one. The only difference is the use of the male's own hemolysate instead of his female's spouse hemolysate. It was observed that a male hemolysate inhibited the typing reaction of his lymphocytes indicating the existence of self HLA antigens.

3.5. RBC transport Tissue Specific Antigens (TSAs)

If RBC transport antigens to central organs of the immune system to induce tolerance, then RBC will definitely transport TSAs. Otherwise this transport function has nothing to do with tolerance. Consequently, the objective of this experiment was to demonstrate that antibodies against TSAs can be prepared through injecting RBC of white mice into rabbits. Figure 3 illustrates the experiment.

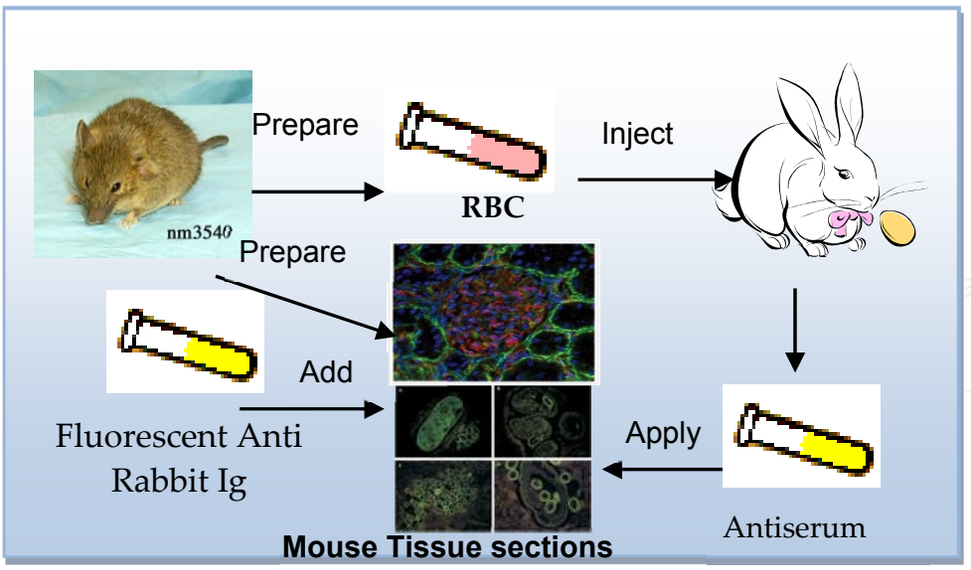

Figure 3. Preparing antibodies against white mice TSAs in rabbit

Materials

A number of white mice were slaughtered to collect their blood on sodium citrate and their organs (liver, kidney and spleen) were preserved on 10 % formalin. The separated RBC were

washed many times with sodium citrate and then diluted with 3% formol-saline to kill any bacterial contamination. An ordinary rabbit was selected to prepare the antibodies.

Method:

- A rabbit was injected subcutaneously with one ml of white mice RBC for four times on weekly intervals.
- Blood was collected from ear-vein after 35 days from the first injection.
- The serum was examined for antibodies against mice RBC using direct agglutination slide test.
- The serum was examined for antibodies against TSAs of white mice (liver, kidney and spleen) using the sandwich technique in histo-pathology sections.

Results:

All sections showed florescence. Figure 4 illustrates some of the histopathology sections taken from a white mouse's organs.

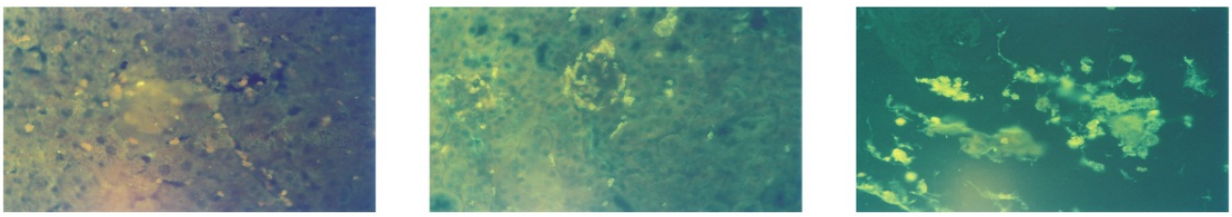

Figure 4. Histopathology sections from a white mouse's organs examined by florescent microscope showing florescence due to antigen-antibody reaction, A: kidney tissue, B: liver, and C: spleen.

3.6. RBC hemolysate antigens are precipitated by plasma

Ouchterlony immuno-precipitation test of normal serum against self and other normal hemolysate was conducted, Figure 5(a). 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 5(b). Further confirmation was obtained by using two-dimensional gel electrophoresis (2-DE) of co-immunoprecipitated hemolysate antigens using self-serum, Figure 5(c). Notice that the number of the immune-precipitated antigens is numerous and many spots were enriched by immune-precipitation because those antigens were not detected in 2-DE gel of hemolysate, Figure 5(d). Antigenicity of the separated proteins was confirmed by immune-blotting proteins separated by 2-DE with the same self-serum, Figure 5(e). This excluded co-precipitation of non-antigens, as they would not be detected in immune-blotting.

3.7. RBC transport bacterial antigens

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. The approach taken follows the following steps Figure 6:

1. The study resources are:
   - [A] Patients
   - [B] Mycobacterium tuberculosis (H37Rv)
2. For each patient:
   - Collect blood sample on anticoagulant (step 1)
   - Separate RBC and wash many times with saline (step 2)
3. Hemolysate [C] is prepared by rupturing RBC 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)

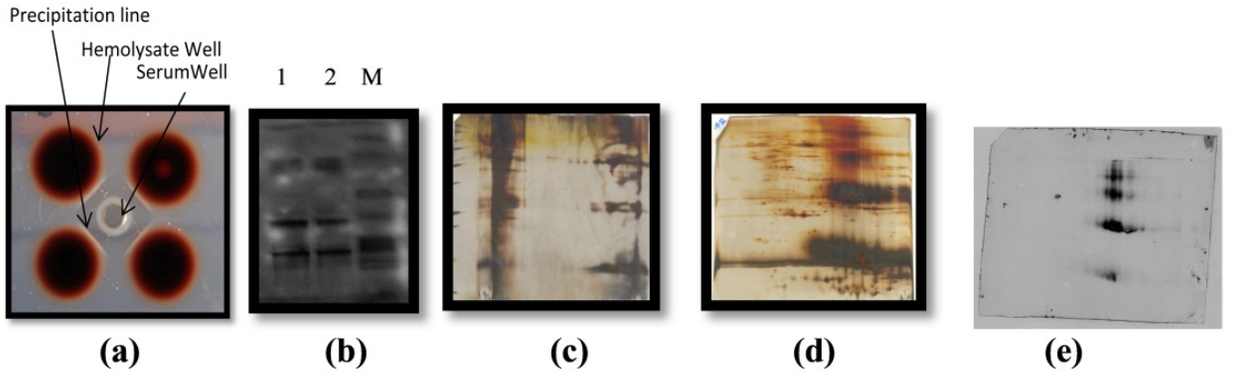

Figure 5. Detection of innumerable antigens in Red Blood Cells. (a) Ouchterlony test showing serum of normal against hemolysate of self and others (b) Western Blot using two normal hemolysate propped with serum of one of them (c) Silver stain of 2-DE of immune-precipitated hemolysate antigens (d) Silver stain of 2D electrophoresis of hemolysate (e) Western Blot of 2-DE of hemolysate propped with serum.

3.8. RBC antigens and plasma antibodies concentration vary with time

The objective of this experiment is to investigate the dynamics of foreign antigens in RBC. In effect, antibodies are taken at one instance of time, while RBC are taken at different instances.

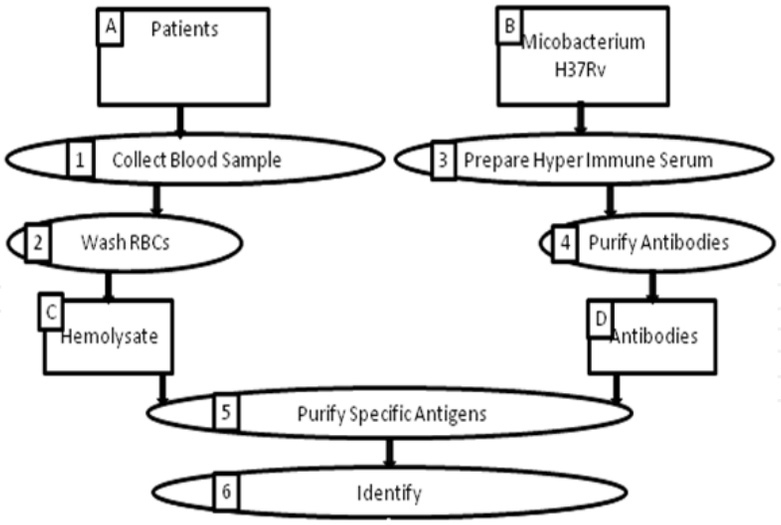

Figure 6. Flowchart depicting the resources and steps for identification of hemolysate antigens related to *Mycobacterium tuberculosis* (H37Rv)

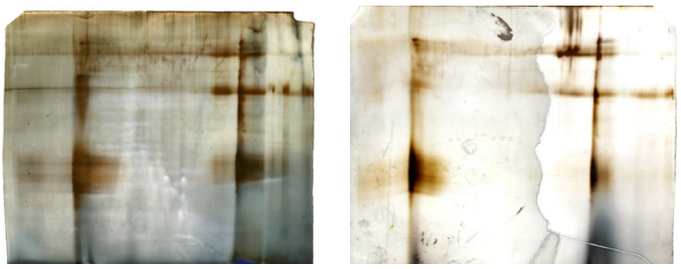

Figure 7. Precipitated Antigens separated using 2D electrophoresis Gel

Materials

- **Bacteria:** ***Escherichia coli*** O157:H7 strain[1] was inoculated onto SMAC agar (Oxoid). Colonies were tested by ***E. coli*** O157 latex kit (Oxoid DR 620) and confirmed biochemically. A single colony of ***E. coli*** bacterial growth from the plate was inoculated into Brain heart infusion broth (Oxoid) and incubated overnight at 37 ˚C and adjusted to a concentration of approximately 10^{10} CFU.
- **Animals:**
  1. A rabbit
  2. A baladi sheep between 8 to10 months.

The rabbit and sheep were tested serologically, to be negative, for ***Escherichia coli*** O157:H7.

Methods:

The first experiment method was done as follows, Figure 8:

1. Rabbits were vaccinated by Escherichia coli. Rabbits were injected subcutaneously with one ml on weekly basis for three weeks.
2. Blood was collected from the ear-vein after 21 days from the first injection.

[1] This strain is kindly provided by the serology department, Animal Health Research Institute (AHRI), Giza, Egypt.

3. Rabbits sera were separated and examined for antibodies against E. coli O157 using direct bacteria slide agglutination test.
4. A sheep was infected by oral administration of bacterial suspension.
5. Red blood cells were prepared from anti-coagulated sheep blood collected at 0 time (i.e., before inoculation), 1st week, 2nd week, and 3rd week. The collected blood was centrifuged at 4 ˚C for 25 minutes at 1170 g. Plasma and Buffy coat from each sample were removed. RBC were washed twice in normal saline solution by centrifugation at 4 ˚C for 5 minutes at 2000 g, and then re-suspended in Tris/Saline buffer pH 7.5 and subjected to lyses by freezing.
6. Nobel agar 1% in Tris/Saline was used as a supportive media for antigen-antibody precipitation, where the central well contained rabbit serum and peripheral wells contained sheep RBC hemolysate.

Results

The rabbit serum showed high titer (1/160) of antibodies against E. coli. Antigens of E. coli could be precipitated from sheep RBC of the 1st and 2nd week after infection, only, Figure 9.

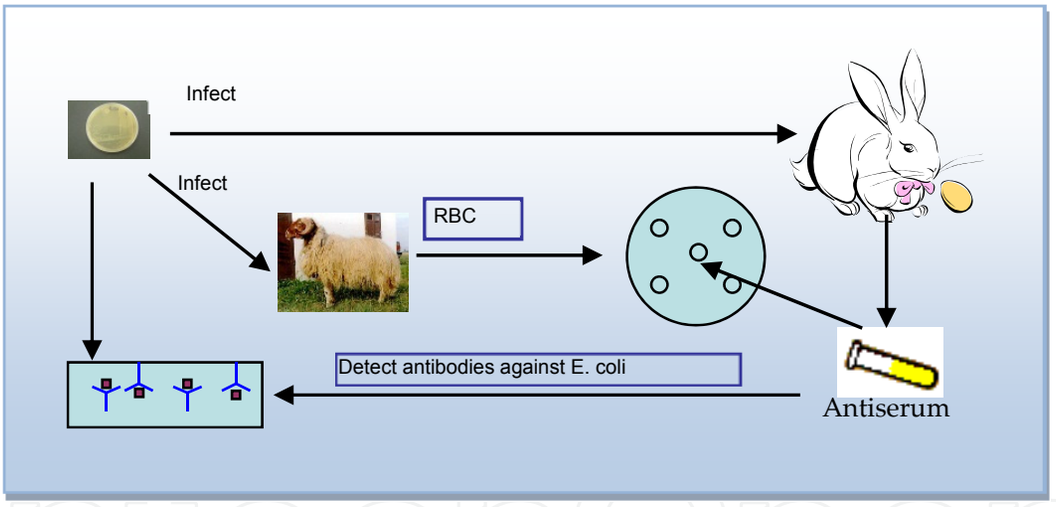

Figure 8. Preparing Antibodies against bacteria and preparing RBC carrying antigens of this bacteria. The purpose is to precipitate Bacteria antigens from RBC of infected animals using the prepared antibodies against those antigens.

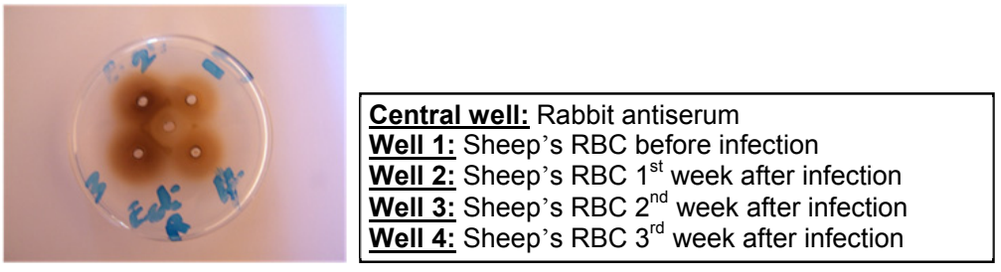

Figure 9. Illustrates the dynamics of RBC's antigens

4. The role of RBC antigens transport in health and disease

The RBC transport function maintains tolerance to self antigens. This function is exploited positively to protect a fetus from the immune system attack using the same mechanism of protecting the self. In effect, a fetus, which is an allograft, is considered part of self.

In humans and animals, not all microorganisms are capable of causing disease. Some of those microorganisms are equipped with the machinery that can overcome biological barriers and can cause disease in animals but not in humans and vice versa [15]. The role of RBC antigens transport in inducing tolerance to self-antigens is a feature that can be considered as a security-hole, as invaders can exploit this process to escape from the response of the immune system by disguising themselves as self. Tumors and parasites are negative examples.

Notice that this mechanism of tolerance induction does not contradict with all what we know about tolerance. Further, it explains the documented properties of tolerance. For instance, some of the properties that can be explained are:

- Artificially induced tolerance is of finite duration because antigen stores get depleted.
- Tolerance to self antigens is a process that continues throughout life but begins during fetal development because RBC are transporting self antigens all the time.

Notice that the discovered function of RBC fills a gap in the understanding of tolerance. Part of this gap can be expressed in the following questions:

1. Why soluble antigens administered intravenously favor tolerance while particulate antigens injected into the skin favor immunity.
2. Why ingested large doses of soluble proteins induce systemic T lymphocyte tolerance, whereas the components of vaccines such as the Sabin polio vaccine induce an effective local immune response.
3. Why tolerance is easier to induce in prenatal rather than postnatal life.

Answer of Question 1 and 2: RBC can easily absorb soluble antigens through pinocytosis while a particulate antigen needs receptor sites on RBC in order to be absorbed, which is the RBC membrane antigens function. Notice that the probability that the immune system will react to some processed antigens still exists. That is why the dose of antigens plays an important role. As far as there are enough stores of antigens in RBC, they are effectively tolerated.

Answer of Question 3: If antigens are introduced to a fetus while the immune system is still incapable of respond, there is a good chance for those antigens to be processed by the Antigen-Presenting-Cells (APC) and then absorbed by RBC. When mature lymphocytes production starts, later in life, antigen stores of RBC are used to induce tolerance. This may explain why tolerance is easier to induce in prenatal life.

Further, a pathogenesis mechanism of some autoimmune disease can be postulated. If RBC antigen-transport function is impaired for a particular self-antigen, for some reason, the

tolerance to that antigen will eventually vanish. Consequently, an autoimmune response will be provoked to that antigen and autoimmune disease is established.

5. How this RBC antigens transport function can be exploited

This observed RBC antigens transport function creates an antigens' store. This store can be exploited in many directions. The proposed direction is to exploit functional proteomics approach [16] with the following three crucial aspects of the experimental design to produce products which are among diagnostic kits, vaccines or treatment components:

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

The aim of this approach is to identify antigens which are relevant to a particular disorder.

5.1. Direct approach for products development

This approach is based on using a subset of antibodies which are specific against a subset of antigens of a particular disease to enable the use of those antibodies and those antigens in preparation of beneficial products.

Diagnostic kits can be prepared for all infectious microorganism and all tumors. In such disorders, simple kits can be prepared using the following steps:

1. Extract antigens from microorganism/tumor-cell-line cultures in coupling buffer
2. Prepare hyper immune serum using extracted antigens
3. Build an affinity column
4. Antibodies purification: Use affinity column containing antigens to separate their related antibodies from hyper immune serum
5. Adsorb purified antibodies to latex beads

A more advanced kits based on selection of antigen-determinant sites (epitopes) can be prepared. The problem of such kits, which uses a particular antigen, is in its validation which will be more sophisticated. One can expect that this particular antigen may not exist in RBC antigens' store of some population who are genetically different from the population used in preparation of the kit.

Active vaccines against all infectious microorganism and all tumors can be prepared by using the purified antibodies prepared for diagnostic kits in identifying related antigens existing in RBC antigens' store. The identified antigens can be prepared using the technology of recombinant proteins purification.

5.2. Bioinformatics approach for products development

The proposed mathematical model and a data mining algorithm will not only help in identifying proteins (antigens) that can be used in diagnosis and treatment of difficult disorders, but also will help in etiological diagnosis of idiopathic disorders and their treatment. This approach is based on building large databases of RBC antigens' store for patients and normal individuals. Consequently, a patient sample is collected on anticoagulant. RBC and plasma are separated. The plasma IgG is separated and then used as ligand in immunoaffinity chromatography to separate hemolysate antigens. The collected antigens are identified by mass spectrometry. The database record consists of the diagnosis and the set of identified antigens.

5.2.1. Mathematical model

It consists of four main parts; definitions of symbols, model of diseases caused by microorganisms, tumors, or foreign proteins; model of autoimmune diseases which result as a consequence of missed tissue proteins from RBC antigens' store; and model of diseases of unknown cause (Idiopathic).

Definitions

Let the assumption of this work be as the following:
p_i: protein amino acid sequence, where i = 1 .. n
d_j: health state, i.e., normal or disease name, where j = 1 .. m
$P = \{p_1, \ldots, p_n\}$, Set of all proteins of RBC antigens' store
$D = \{d_1, \ldots, d_m\}$, Set of all diseases
P_p: patient proteins where $P_p \subset P$ where p is the patient ID
O_p: (p_i , d_j), ordered pair of patient presented by protein sequence (i) and health state (j).

a. Model of Diseases caused by microorganisms, tumors, or foreign proteins

$$P_{dj} = \cap \{P_p\}_{dj}$$

Where P_{dj} is the set which contains all common proteins associated with d_j.

$$P_{normal} = \cup \{P_p\}_{normal}$$

Where P_{normal} is the set which contains proteins associated with normal.

P'_{normal} such that $\forall$ p in P_{normal} if the number of occurrence of $p \in P_{normal}$ is less than 5% of the total number of p in P_{normal} then remove p from P_{normal}.

$$P'_{dj} = P_{dj} - P'_{normal}$$

Where P'_{dj} is the set which contains proteins that can be used as biomarker or vaccines, Figure 10.

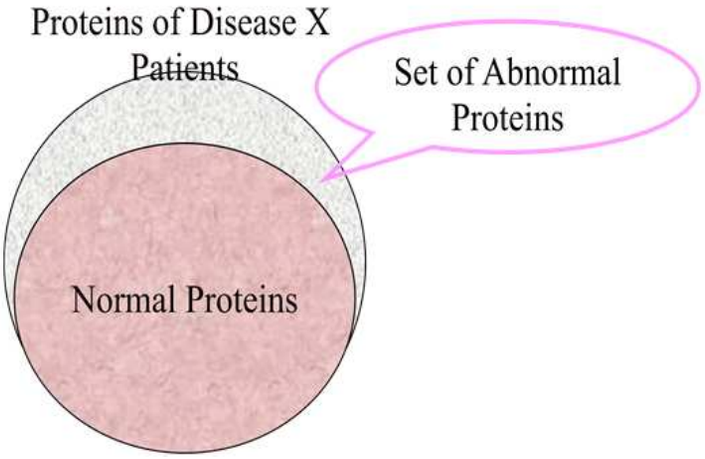

Figure 10. Venn diagram depicting set of abnormal protein of disease X (P'_{dj})

b. Model of Diseases caused as a result of missed tissue proteins

$$P^{u}_{dj} = \cup\{P_p\}_{dj}$$

The result is the set which contains all proteins associated with d_j

$$P''_{dj} = P_{normal} - P^{u}_{dj}$$

The result is the set which contains proteins that can be used to diagnose patients through detecting circulating auto-antibodies and to treat those patients through desensitizing them with the proteins that give positive reaction, Figure 11.

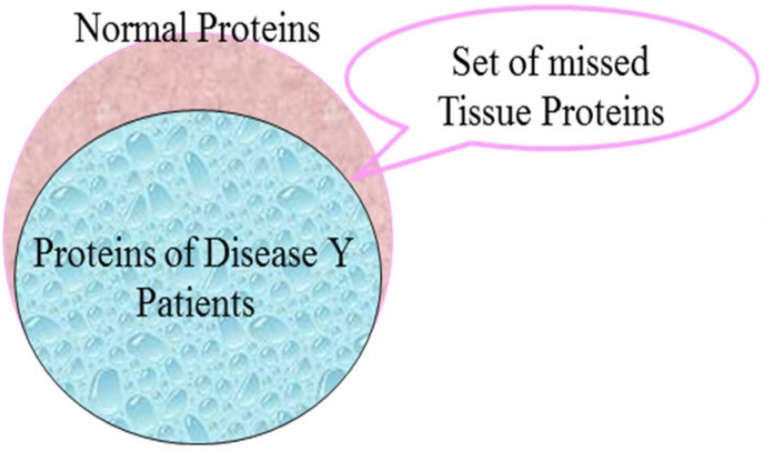

Figure 11. Venn diagram depicting set of missed normal proteins of disease Y (P''_{dj})

c. Model of Diseases of unknown cause (Idiopathic)

- There are many diseases that are identified as idiopathic.
- Those diseases can be caused due to existence of abnormal protein or absence of tissue proteins.

- Applying data mining methods (A and B) can help to identify new diseases and treat patients appropriately.

5.2.2. Scenario of the system in clinical environment

Patients' blood samples will be collected on anticoagulant. RBC and plasma are then separated in different tubes. Plasma is used as a ligand in immune-affinity chromatography to separate hemolysate antigens that can bind to plasma antibodies. The separated antigens are identified by MS and stored in the database indexed by the patient disorder.

In the same time, queries are done to verify the diagnosis and get a prognosis and a recommended treatment component. The following formulas describe the usage of this model in clinical practice.

Let Dp' is the set of all discovered P'_{dj}
Let Dp'' is the set of all discovered P''_{dj}

Then

$$\forall P'_{dj} \in Dp', \text{ if } P'_{dj} \subset P, \text{ then patient is diagnosed to have } d_j$$

Else

$$P''_{dj} \in Dp'', \text{ if } P''_{dj} \not\subset P, \text{ then patient is diagnosed to have } d_j$$

6. Conclusions

All the previous work in RBC proteomics neither has identified another function nor has mentioned the finding of: 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 RBC antigens' store is little. This makes those antigens invisible and hence easily missed.

The work described is just a pilot study that throws some light on a new theory related to RBC. This theory is based on finding antigens' store consisting of self and non-self antigens. Although this theory can be related to immune tolerance by logical induction, the concrete evidence and mechanism need further research. Mainly, the logical induction is based on 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.

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 mechanism by which a pregnant woman is able to tolerate her fetus and placenta. Simply, it 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 2-DE of co-immunoprecipitated antigens demonstrated that RBC have an antigens' store. Mass spectrometry of spots obtained from 2-DE 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 RBC's 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.

In the experiment which investigates the dynamics of foreign antigens in RBC using sheep RBC which has been infected with *E. coli*, it was shown that the concentration of foreign antigens in RBC varies by time in relation to IR known behavior. This proves that RBC have role in immune reactions (IR and IT).

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.

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developed a computer program that implements the mathematical model to help in its verification.

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