Hemolysis, Platelet Aggregation and Antibacterial Activities of Human Antiphospholipid Antibody

Farzaneh Ahmadi Shapoorabadi¹, Maryam Sadat Mirbagheri Firoozabad¹,²*, Neda Habibi³,⁴ and Giti Emtiazi¹

¹Department of Biotechnology, Faculty of Biological Sciences and Technology, Shahid Ashrafi Esfahan University, Esfahan, Iran; ²Department of Biology, Faculty of Science, Yazd University, Yazd, Iran; ³Department of Biomedical Engineering, College of Engineering, The University of Texas at San Antonio, San Antonio, TX, USA; ⁴Advanced Materials Technology Program, Northwest Vista College, San Antonio, TX, USA

Abstract: Background: Anti-phospholipid antibodies have the potential to become an alternative to conventional antibiotics for humans. The Antiphospholipid Syndrome (APS) is an autoimmune disease where the body’s defense system incorrectly reacts against its own phospholipids. APS is distinct through the existence of venous and arterial thromboses, frequently multiple and recurring fetal losses, commonly accompanied by moderate thrombocytopenia. Anti-phospholipid antibodies include lupus anti-coagulant, anti-cardiolipin, anti-beta 2 glycoprotein I, and anti-prothrombin antibodies.

Methods: In this study, the mechanism of action of Anti-phospholipid antibodies against Klebsiella pneumonia and Staphylococcus aureus was investigated in great detail using a unique combination of imaging and biophysical techniques. Antibacterial activity of antiphospholipid antibodies was detected by a diffusion method and the investigation of the complexity of antibody-antigen was done by spectroscopic examination, scanning electron microscopy (SEM), and transmission electron microscopy (TEM) imaging.

Results: There was a profound change in the bacteria treated with healthy and patient serum in the optical microscopic study. In all of the studied fields, bacterial treatment with patient serum immediately induced bacterial swelling and cumulative accumulation of the bacteria while no changes were observed in the healthy serum. Anti-bacterial activities of patient serum were detected on the plate. The result of this study showed that after platelet activation by thrombin and incubation with antiphospholipid antibodies, the platelet was aggregated. The transmission electron microscopy (TEM) image showed that the cell wall of Klebsiella pneumonia and Staphylococcus aureus incubated with antiphospholipid had a bizarre shape and antiphospholipid antibodies bound to bacterial membranes.

Conclusion: The data indicated that antiphospholipid antibodies with hemolysis activities have an effect on Gram-positive and negative bacteria and these antibodies have the potential to become antibiotic for human.

Keywords: Antiphospholipid syndrome, autoimmune disease, electron force microscopy, bacteria, platelet, transmission electron microscopy.

1. INTRODUCTION

Antiphospholipid Antibody Syndrome (APS) is an autoimmune syndrome which manifests due to recurring venous, arterial or small vessel thrombosis, and pregnancy sickness in the presence of antiphospholipid antibodies. APS may occur in association with other autoimmune disorders called secondary APS. The discovery of this syndrome can be dated back to the 1950s with the finding of prolonged activated partial thromboplastin [1, 2].

APS can be caused by the lupus anticoagulant, anticardiolipin antibodies, or other antiphospholipid antibodies. Despite what the name suggests, clinically applicable APS antibodies bind to phospholipid-binding proteins, rather than to phospholipids themselves. Antigens for APS antibodies include β2 glycoprotein I (β2GPI), prothrombin, annexin V, tissue factor, and protein C. Patients have socializing antibodies against proteins that bind to anionic phospholipid surfaces such as β2 glycoprotein I and prothrombin [2, 3].

APS antibodies deregulate normal cellular activities and are associated with recurrent thrombosis, pregnancy complications like an obstetric failure, pre-eclampsia and eclampsia, and non-specific manifestations disease, chorea and nephropathy [4]. Patients with APS have an increased risk of transient ischemic attack, pulmonary embolism, and extreme thrombosis [5]. The well-studied antigen, β2GPI, is a highly abundant plasma protein [6]. β2GPI is a phospholipid-binding protein of about 50kDa weight with a high plasma concentration of 50–400 μg/mL. de Laat et al. in 2009 showed the connotation between beta2-glycoproteinI plasma levels and the risk of
myocardial infarctions in older men. β2GPI has been recognized as the major target for APS antibodies. β2GPI changes conformation after binding to anionic phospholipids, revealing an epitope domain I. Antibodies against this domain appear to be more commonly related to thrombosis. β2GPI must be immobilized on a negatively charged surface, such as the anionic phospholipid phosphatidylserine [7, 8].

Normally, phosphatidylserine is limited to the inner surface of the plasma membrane and thus circulating β2GPI and APS antibodies do not interact [3]. The standardization of platelets as a phospholipid surface in APS research has been challenging. When a cell is disturbed or activated, phosphatidylserine is manifested on the outer layer of the plasma membrane and thus circulating phosphatidylserine [7, 8].

Phosphatidylserine enables circulating APS antibodies to bind to the cells and exert their pathogenetic effects [9].

Antiphospholipid antibodies can interact with a range of different cell types through cell surface receptors, as well as TLR2, TLR4, and apolipoprotein E receptor 2 [10, 11].

These physiological changes allow for the effective exchange of nutrients and wastes between the mother and the fetus. Highlighting the importance of syncytialization, the syncytiotrophoblast constitutively represents phosphatidylserine on its cell surface [12, 13]. There is also some recommendation to suggest that extravillous trophoblasts exteriorize phosphatidylserine [13]. Trophoblasts also synthesize endogenous β2GPI proposing that there may be many areas for β2GPI binding on the surface of the human placenta [6].

Schneider et al., 2016 investigated the mechanism of action of Host Defense Peptides (HDPs) like CATH2 (The HDP chicken cathelicidin-2) against Escherichia coli (E. coli) using a unique combination of imaging and immuno-gold TEM showed CATH-2 binding to bacterial membranes [14].

In this study, we focused on the impact of APS antibodies on the bacterial cell wall and platelet phospholipids. The possibility of visible structures might be identified if the APS plasma phospholipid layer interaction with platelet and bacterial cell wall was imaged with high-resolution microscopy techniques - specifically Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). Also, the possible anti-bacterial activity of this antibody was studied.

2. MATERIALS AND METHODS
2.1. Antiphospholipid Antibody

Antiphospholipid antibody serum was obtained from patients in clinical labs in Isfahan city, who were diagnosed with APS, according to the Iranian Rheumatism association criteria. The healthy serum was used as a negative control.

2.2. Platelet and Bacteria

Platelets were obtained from the Blood Bank of Al-Zahra Hospital Isfahan University of Medical Sciences. Two species of bacteria Staphylococcus aureus ATCC6538 and Klebsiella pneumonia ATCC13883 were used for the investigation. Media and chemical material were purchased from Merck Company.

2.3. Agar-well Diffusion Method

Antibacterial activity was checked by the agar-well diffusion method with bacteria grown on Luria broth agar (Merck, Germany). 100μl suspension of the bacteria (10^8CFU/ml) was plated on the agar layer in Petri dishes (10 cm in diameter). Five wells per dish were prepared, each 5 mm in diameter overloaded with hundred microliters of each patient serum. The antibacterial activity was estimated by the diameter of inhibitory zones in the agar layer after incubation at 37° C for 24 h as the experiments were carried out in triplicate. Control experiments were carried out with the negative plasma and antibiotic for positive control.

2.4. Hemolysis Test

Citrated blood (3ml) washed three times with phosphate buffer was centrifuged each time the supernatant dispersed from the pellets. 200μl of washed blood was mixed entirely with 4 liters of washed and diluted phosphate buffer. 400 μl was added along with 800 μl of different dilutions of the serum and then were vortexed slowly. Pure water and phosphate buffer were used as a positive and negative control, separately.

The tubes were kept at room temperature for 2 hours and then centrifuged at 1600 for 5min. The supernatant liquid was read at 541nm by spectrophotometric to define hemolysis. The percent of hemolysis was then calculated.

2.5. Electron Microscopy

APS plasma-treated with bacteria were imaged with Scanning Electron Microscopy (SEM). For this, the cells and antiphospholipid were embedded on silicon and were imaged. Also, the cells were incubated with antibody the cells pellet, removed from the culture medium and dehydrated by alcohol. Then the cells were fixed by adding 1 volume of 25% glutaraldehyde to 9 volumes of cell suspension in the original culture medium. Then the pellet was embedded in epoxy resin for a few days. The fixed cells were cut with ultra mictotome equipped with a diamond knife. Ultramicrotomy is a method for cutting specimens into extremely thin slices, called ultra-thin sections, and after slicing, the sample embedded on the grid is fixed and then examined by Transmission Electron Microscopy (TEM).

Bacterial samples were imaged with SEM by mounting the pellet on lamella or silicon, dry and fixed with hot glue. The samples were coated for 30 seconds. The samples were imaged using a Leo Stereoscan 360 scanning electron microscope at a voltage of 15 kV and an average viewing distance of 12 mm.

3. RESULTS

The bacterial cells were treated with patient serum and compared with healthy serum as control. The preliminary microscopic examination showed that there was a profound change in the bacteria treated with patient serum. In all of the studied fields of bacterial treatment with patient serum, immediately bacterial swelling and cumulative accumulation of the bacteria were observed, while no changes were seen in the healthy serum treatments. Also, anti-bacterial activities of patient serum were detected on the plate.
Researchers believe that the cell wall of bacteria has a specific structure and function. The presence of these structures is important for bacterial cells and has made them exceptional targets for the development of antibiotics. By using bacteria for the productivity of some of the synthesized building blocks, researchers have gained knowledge into how they can change antibiotics. The bacterial cell wall is the target of some of the most potent antibiotics ever discovered. Some antibiotics targeting the cell wall may include some commonly approved treatments such as beta-lactam antibiotics. Medicines affecting the cell wall are on the list of the safest antimicrobial drugs, which is because human cells lack a cell wall and thus do not receive antibiotic treatment. In this study, the bizarre shapes of bacterial cells treated with this antiphospholipid have been shown in Figs. (1 and 2). These data indicated that the antiphospholipid antibody has antibacterial activities probably through cell wall destruction.

The bizarre shape of treated bacteria with antiphospholipid antibody clearly was different compared to the healthy serum treatment. This damage was clearly obvious in all fields compared to healthy serum treatment. Both samples were dehydrated, fixed and cut with the same method, however, the bizarre shape was only observed in the antiphospholipid antibody treatment. This observation was also seen by Schneider et al, when the bacterial cells were treated with cathelicidin-2 [14].

Also, the aggregation of bacteria in antiphospholipid fiber is seen in the SEM image in Fig. (3). The anti-bacterial activities of this antibody are reported for the first time. The antiphospholipid with its effect on Gram-positive and negative bacteria requires more research to understand the exact mechanism of antibacterial activity, which might help in the development of a new drug. The effect of this antibody on Gram-positive bacteria is shown in Fig. (1).

The phospholipid antibody also aggregated the platelets. The SEM image of the aggregation of platelets by the antiphospholipid fiber is shown in Fig. (4).
In Fig. (5), the results displayed the antibacterial activities of antiphospholipid on the agar diffusion plate. The hemolysis of healthy serum compared to the antiphospholipid antibody positive serum showed that the positive serum has more effect on hemolysis in comparison to healthy serum (Fig. 6).

Fig. (5). The antibacterial activities of antiphospholipids on the agar diffusion plate.

In the model and drawing based upon SEM in Fig. (7), the plasma membrane of platelets interact with APS antibodies (especially β2GPI) and the interaction of bacterial surfaces and APS antibodies has been shown schematically.

4. DISCUSSION

Antibacterial resistance in bacteria has commonly caused the development of new drugs for the treatment of infections. Meanwhile, to reduce medical expenses, research in the field of antibacterial drugs and the development of new drugs in the world is under development. Currently, more than 40,000 antibodies have been introduced and more than 100 kinds of antibiotics are commercially available. In the past 30 years, antimicrobial peptides have also been reported [15].

For the development of new antibiotics, in order to deal with microbial resistant, extraction from nature by chemical means has been proposed. Therefore, more than 1000 types of antimicrobial peptides are isolated from eukaryotes and prokaryotes and hundreds of chemical forms are synthesized [16].

Between anti-β2GP1 antibodies, β2GP1, and cells like platelets, endothelial cells and monocytes have been suggested as a means for potential mechanisms of interaction [7]. However, studies studying the effects of anti-β2GP1 antibodies and β2GP1 on platelets may help cause an im-
proved understanding of their interactions, thus, their impact on the haemostatic system. Clot formation initiated thrombosis, and pregnancy disorders could activate platelet receptors or metabolic pathways through anti-β2GP1 antibodies and β2GP1. Available data show that many of the autoantibodies related with APS are directed against a number of plasma proteins and proteins stated on or bound to the surface of vascular endothelial cells or platelets. The participation of APS antibodies in clinically important normal procoagulant and anticoagulant reactions and on definite cells altering the expression, and secretion of various molecules may offer a basis for definitive research of possible mechanisms through which APS antibodies may develop thrombotic trials in patients with APS. These ultra-large complexes are unchanging, mainly antigenic, bind multiple IgG antibodies per complex, and promote platelet activation. It is not known whether similar complexes between cell-surface glycosaminoglycan and PF4 are formed on the surface of platelets or how heparin affects the surface complex formation and antigenicity [16].

There was a profound change in the bacteria treated with healthy and patient serum in the optical microscopic study. In all of the studied fields, bacterial treatment with patient serum immediately induced bacterial swelling and cumulative accumulation of the bacteria while no changes were observed in the healthy control bacterial treatment with healthy serum. The bizarre shape of treated bacteria with antiphospholipid antibody clearly was different compared to healthy serum treatment. This damage was clearly obvious in all fields compared to healthy serum treatment. Both samples were dehydrated, fixed and cut with the same method, however, the bizarre shape was only observed in antiphospholipid antibody treatment. The morphological changes in bacterial cell wall were reported by Schneider et al., when the bacterial cells were treated with cathelicidin-2. They used live-imaging microscopy demonstrating the real-time attack of this peptide on E. coli. The morphological changes of the cell after peptide treatment and bizarre cell wall were observed with transmission electron microscopy [14].

Rauova et al. in 2006 investigated that antibodies recognizing complexes of the polyanions opsonize PF4-coated bacteria and chemokine platelet factor 4 (PF4/CXCL4) thereby mediating the bacterial host defense. A subset of these antibodies may activate platelets after binding to PF4.
or heparin complexes, causing the prothrombotic adversarial drug reaction heparin-induced thrombocytopenia [17].

Taatjes et al. 2017 focused on the interaction between components from APS plasmas and phospholipid layers, which have not previously been reported. They investigated the image of the interaction with AFM and Scanning Electron Microscopy (SEM) and demonstrated how high-resolution microscopic techniques can contribute to advancing the understanding of an enigmatic disorder [18].

CONCLUSION

In the present study, the results of the hemolysis effect of positive antiphospholipid serum compared to healthy serum show that the antiphospholipid positive serum has more effect on hemolysis compared to healthy serum. The effect of antibodies was investigated in the bacterial surface and phospholipids of the platelet membrane. Images of electron microscopy TEM and SEM showed bizarre shapes of bacteria and agglutination of platelets. The antiphospholipid antibodies affected the eukaryotic cell membrane and changed gram-negative and positive bacterial surface as well as blood cells and platelets. The electron microscopic study showed that this antibody is fiber and aggregates bacterial and human cells. The results of this study suggested that this antibody could be applied as a new generation of antibiotics since the extraction and purification of this antibody are easy and the titrations in patients are high.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Sayed Hamid Emami and Dr. Sayed Mahdi Ghasemi, assistant professors from Shahid Ashrafi Esfahani University, Reihane Amini and Dr. Daryush Shokri for their guidance and for supporting this project. We thank Farbod Shirvanifar for plagiarism editing.

REFERENCES


