Detection of Anti-Platelet Glycoprotein Antibodies Using MAIPA Method

Mohsen Hamidpour[1,*], Ghader Khalili[2], Mahin Kamali[1], Mohammad Hasan Arzani[3], Farzaneh Jadali, [4] Hamid Alavi Majd[5], Navid Khojasteh[3]

[1] Department of Hematology, Faculty of Paramedical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2] Departments of Immunology, The Iranian Institute of Pasture, Tehran, Iran
[3] Departments of Hematology, Mofid Pediatric Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[4] Department of Pathology, Mofid Pediatric Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[5] Departments of Biostatistics, Faculty of Paramedical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran

*Corresponding Author: e-mail address: mohsenhp@SBMU.ac.ir (M. Hamidpour)

ABSTRACT

AITP mostly occur in children accompanied by variable clinical sings including petechiae, purpura, ecchymosis and severe bleeding. This study has determined and characterized the anti-platelet glycoproteins in children with ITP. The aim of this study was to determinate anti-platelet glycoproteins (GPs) using MAIPA method. During 18 months 38 children with clinical signs of AITP were studied in Mofid children hospital. To determine anti-platelet antibodies by ELISA technique, washed O negative platelets were used as a source of platelet antigens. MAIPA method was used to detect antibodies against individual platelet membrane glycoprotein. The anti-platelet antibodies level above mean+ 3SD of control group was assumed as positive. The results indicated that the platelet count ranges was between $2\times109/L$ and $95\times109/L$. 63.5 % out of 38 patients were anti-platelet antibodies positive with ELISA method. The correlation between the above patients with anti-platelet antibody positive and clinical signs was 0.4. Results for determination of antibody against platelet GPIIb/IIIa, GPIb/IX and GPIa/IIa using MAIPA method were 44%, 51% and 25% respectively. In conclusion the preference of MAIPA method is the detection of very small amount of antibody. Since MAIPA is the specific method for the detection of antibody against glycoprotein antigens, it has the advantage of differentiating immune and non-immune thrombocytopenia.

Keywords: Platelet; Anti-Glycoprotein Antibodies; MAIPA

INTRODUCTION

Autoimmune thrombocytopenia purpura (ITP) is an autoimmune disease characterised by loss of self tolerance leading to the production of autoantibodies directed against platelet antigens [1,2,3]. The autoantibodies produced against platelet glycoproteins are able to bind to platelet membranes, initiating pathways that result in dysfunction and destruction of platelets and clinical singes. These include pethechiae, ecchimosis, and bleeding in some patients [3, 4]. Around 85% of platelet autoantigens lie on the platelet GPIIb/IIIa, GPIb/IX or GP Ia/IIa complexes, and 15% of them are found on other membrane glycoproteins [4]. Antiplatelet antibodies have been found in the sera of up to 80% of chronic ITP patients. However, despite many studies on antiplatelet antibodies, characterization of binding and evaluation of antiplatelet glycoproteins utoantibodies remain poor [5] and many questions remain unanswered [3].This study has determined and characterized the antiplatelet GPs in children with ITP.

MATERALS AND METHODS

Patients

47 children who were hospitalized with clinical signs of ITP in Mofid children hospital during 18 months, 38 of them were under study in our project. The physician in clinic recorded the patients' information including age, gender, history and clinical signs such as petechiae, purpura and ecchymosis in a standard format. All the relatives gave us consent to take blood sample from their children.

Samples preparation

For platelet count, 2ml anticoagulated [Ethylen Diamine Tetraacetic Acid (EDTA)] blood was collected and counted by a cell counter (Sysmex). To detect anti-platelet glycoproteins antibodies, 3 ml serum was obtained from patients' clotted blood and aliquoted into two tubes and stored in freezer.

Preparation of whole platelets

Anti-coagulated [Acid Citrate Dextrose (ACD)] blood was collected from healthy O negative volunteers and centrifuged at 200 g for 10 min. The platelet-rich plasma (PRP) was removed and recentrifuged at 1200 g for 10 min. After four time washing with phosphate buffer saline (PBS) pH 7.4, the sedimented platelets were resuspended in buffer containing 10% dimethylethyl sulfoxide (DMSO) and aliquoted into a cryotubes and stored in liquid nitrogen.

Anti-platelet antibodies detection

ELISA plate was coated with 50 μL of washed platelet suspension containing 3×10^8 platelets in bicarbonate buffer (pH 8.6) in each well or 100 μl of platelet lysate (200 μg/ml) and incubated at 4°C overnight. The following day plate was centrifuged at 2000 g for 5 minutes. After washing and blocking with 3% BSA in PBS and incubating for an hour at room temperature (RT), 100 μL of diluted patients and controls sera (1:10 diluted in PBS) were added and the plate was incubated for an hour at 37 °C. The unbound anti-platelet antibodies were removed by washing four times with PBS 0.05% Tween 20 (PBST washing buffer). 100 μL Goat antihuman HRP-conjugated antibody (1: 50 000 diluted in PBS) ((Serotech Co. UK)) was added to each well and incubated at RT for an hour. The plate was washed five times with washing buffer then 100 μL of substrate [Tetramethylbenzidine (TMB) sigma] was added and the plate incubated for 30 min in a dark place. The reaction was stopped by adding 100 μL of 3 M HCL, and the OD was measured at 650 nm.

MAIPA (Monoclonal Antibody Immobilization of Platelet Antigens)

ELISA plate was coated with 100 μL of Goat anti mouse IgG [final concentration 5 ug/ml diluted in Carbonate Bicarbonate Buffer (CBB) pH 8.6] in each well and incubated overnight at 4°C. After washing with Tris Buffer Saline (TBS), the plate was blocked with 200 μL of 3% BSA in PBS and incubated for an hour at room temperature (RT). 100 μL of washed O negative platelets suspension containing 5×10^{11}/ L were added to the tubes which were blocked with 50 μL 3% BSA and incubated for an hour with 100 μL of patients and controls serum for an hour at 37 °C. 40 μL of each monoclonal mouse anti platelet-glycoproteins [IIb/IIIa, Ib/IX and IIa/Ia (Novacastra UK)] with final concentration of 20 μg/ml diluted in PBS were added to the individual tubes containing mixture of washed platelets and patients or controls sera and incubated at 37 °C for 30 minutes. The unbound human anti platelet-glycoproteins were removed by washing three times with TBS 0.05% Tween 20 (TBST washing buffer). 100 μL of lysis buffer (TBS containing 0.05% Nonidet P-40 Sigma) were added to each tubes and incubated for 30 minutes at 37 °C.

To prevent protein destruction, anti-protease agents was added to lysis buffer. After centrifuging all tubes at 14000 g for 30 minutes at 4 °C, 100 μL of supernatant was added to each well which were coated with anti-mouse antibody and incubated at 4 °C for 90 minutes. The plate was washed four times with washing buffer and 100 μL of Goat antihuman HRP-conjugated antibody (1: 50 000 diluted in PBS) (Serotech Co. UK)) was added to each well and incubated at RT for an hour. The plate was washed five times with washing buffer. 100 μL of substrate (TMB) was then added and the plate incubated for 30 min in a dark place. The reaction was stopped by adding 100 μL of 3 M HCL, and the OD was measured at 650 nm.

RESULTS

In this project the goal of our study is to determine anti-platelet GPs antibody in children with ITP. 38 patients and 12 healthy individuals were included in our study as negative controls. 68% of patients were girls and 32% boys and their age ranges between less than one year and up to sixteen years. The clinical signs were variable including 15 individuals only with petechiae, 11 of them not only with petechiae, but also with purpura and 8 of them with ecchymosis signs, and finally 4 of them were found out with severe bleeding from gums and urethral tract.

Figure 1 shows more details about clinical signs in ITP patients. Peripheral platelet count

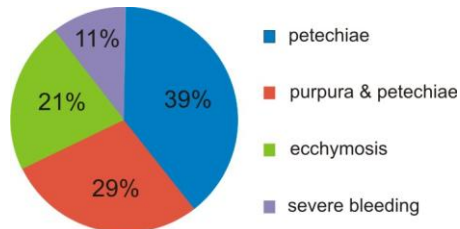ranges from less than 3×10^9/L up to 95×10^9/L.

Figure 1. A pie chart of the ITP Patients' Clinical signs

Table No 1 shows more details about platelet count in patients. The results with ELISA indicated that anti-platelet antibodies in sera of all patients either with clinical signs and low platelets count or just low platelets count reacted strongly with whole platelet antigens.

Table 1. The platelet count in patients

Platelets count	Number of patients
51-95x10^9/L	7
31-50x10^9/L	5
10-30x10^9/L	18
<10x10^9/L	8

It should be mentioned that we used peroxidase activity to calculate level of antibody instead of OD by following formula;

Percent change = OD sample – OD control / OD control $\times$ 100

The mean controls absorbance was used as normal value. We also used the mean controls antibody level with three standard deviation (mean+3SD) as the cut-off. The anti-platelet antibodies level of each sample above the cut off was assumed as antibody positive.

Out of 38 patients, 63.5% had anti-platelet antibody positive with mean platelet count of 41.5×10^9/L using non parametric spearman coefficient, it was statistically shown that there was a significant correlation between clinical signs and antibodies level in patients (r = 0.45, P<0.05).

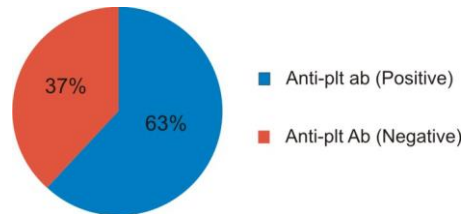Figure 2 shows the anti- platelet antibodies in ITP patients.

Figure 2. ITP Patients who were Anti-platelet antibodies positive against whole platelet (Anti-platelet antibodies against platelet antigens of 63.5% of patients were detected (green color).

The determination of anti-platelet GP antibodies

Using MAIPA method, antibodies in patients' sera were analyzed against three most important platelet glycoproteins (GP IIb/IIIa, IIa/Ia, Ib/IX). Result was shown that 48% of patients were anti-GP IIb/IIIa antibody positive with mean platelet count of 38×10^9/L. 54% were anti-GP Ib/IX positive with mean platelet count of 50×10^9/L. The correlation between anti-GP Ib/IX positive and clinical signs was 0.2. Out of 38 patients only 25% were anti GP-Ia/IIa positive with mean platelet count of 48×10^9/L. Using non parametric spearman coefficient, it was statistically shown that a significant correlation was seen between increased anti-GP Ia/IIa level and the platelet count in patients (r= 0.35, P< 0.05).
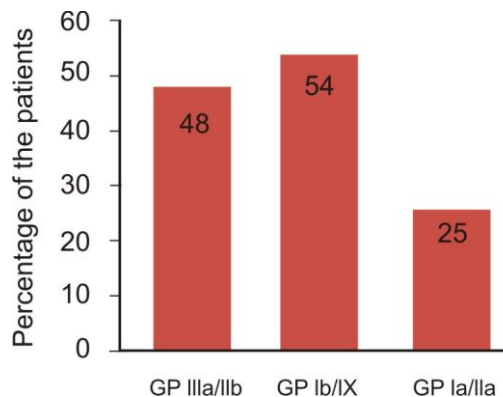
Figure 3 shows the anti-Gp antibodies in ITP patients.

Figure 3. ITP Patients who were shown antibodies positive against platelet glycoproteins.

DISCUSSION

Anti-platelet antibodies react against platelet glycoproteins and cause autoimmune thrombocytopenia purpura (ITP). ITP is an autoimmune disease characterized by platelet destruction, accompanied by increased bleeding

and haemorrhage [3, 4, 7]. In order to further characterize the function of autoantibodies against platelet glycoproteins in ITP patients, this study has investigated the following: first the determination of anti-platelet antibodies using ELISA method, next detection of these antibodies by flow cytometry and compares their results with ELISA assay and finally detection of anti-GPs antibodies by MAIPA.

The most clinical signs were petechiae and purpura and in 4 individuals severe bleeding. The platelet count was shown that more than 50% of patients had platelet count $< 30 \times 10^9$/L which correlates with the severity of clinical signs. In some patients not only the platelet count was decreased but they also had anemia due to low hemoglobin concentration and RBC count. Normochromic normocytic anemia in these patients may be due to blood loss as a result of hemorrhage and massive ecchymosis or probably they had ITP with autoimmune hemolytic anemia(Evans syndrome)[8].

The mean result of controls anti-platelet antibodies+3SD (Mean+3SD) was used as the cut off, and the patients with anti-platelet antibodies above the cut off were assumed as antibody positive. 63.5% out of 38 patients in our assay were anti-platelet antibodies positive with the mean platelet count of 41.5×10^9/L which shows a good correlation between decreased circulating platelets and increased antibodies levels in comparison with anti-platelet negative patients. There was a positive correlation between increased serum anti-platelet antibodies and clinical signs. In some patients there was neither correlation between anti-platelet antibodies and platelet count nor clinical sings. It could be due to acute phase of disease which the antibodies absorbed on platelets membrane and removed from circulation by phagocyte system [8]. On the other hand, detection of auto antibodies on platelet surface due to platelet structure is not easy [9]. Cordiano and et al. has reported that 56% of 32 ITP patients were anti-platelet antibodies positive by ELISA method [12]. The results showed that 48% of patients were anti-GPIIb/IIIa positive by MAIPA method. The correlation between anti-GPIIb/IIIa antibody and antibodies against whole platelet antigens was 0.4. Van Leeuwen has reported that 80% of 42 ITP patients were anti-GPIIb/IIIa positive [13].

In our study 54% of patients were anti-GPIb/IX positive. It should be mentioned that 35% of patients had both anti-GPIIb/IIIa and Ib/IX antibodies in their sera. Fuji Sawa and colleagues have reported that 75% of ITP patients have anti-GP IIb/IIIa and Ib/IX [14-16]. In 25% of patients we have found anti-GPIa/IIa in sera and correlation between level of anti-GPIa/IIa and low platelet count was 0.4. However the determination of antibodies against the platelet glycoproteins by MAIPA is very sensitive though it is time consuming [17-21].

Conclusions

Since coating platelets at the bottom of ELISA plate is difficult, we have modified the method by centrifuging the plate and fixing them with Glutaraldehyde.

Instead of using optical density (OD) for analysis of the data which can not differentiate low absorption, we used percentage of peroxidase activity.

The preference of MAIPA method is due to the large amount of Ag-Ab complex in the supernatant of platelet lysate (the second process) and the detection of a trace amount of antibody.

Since MAIPA is the specific method for detection of antibody against glycoprotein antigens, it has the advantage of differentiating immune from non-immune thrombocytopenia.

ACKNOWLEDGEMENT

REFERENCES

1- Bloom A.L, Thomas D. P. Haemostasis and thrombosis. London: Churchill Livingstone; 1981.

2- Freedman J, Garvey M.B, Elinder G. Blanchette V.S. Directions for research in autoimmune thrombocytopenic purpura (ITP). Acta Paediatr Suppl 1998; 424: 82-84.

3- Victor Blanchette, Paula Bolton-Maggs. Childhood Immune Thrombocytopenic Purpura: Diagnosis and Manageme. Hematol Oncol Clin N Am 2010; 24: 249–273.

4- Harrington W.J, Minnich V, Hollingsworth J.W & Moore C.V. Demonstration of a thrombocytopenic factor in the blood of patients

with thrombocytopenic purpura. J Lab Clin Med 1951; 38: 1-10.

5-McMillan R. the pathogenesis of chronic immune (Idiopathic) thrombocytopenic purpura. Semin Haematol 2000; 37: 2-9

6- Escher A.R, Muller D, Vogel M, Miescher S. Recombinant human Natural autoantibodies against GPIIb/IIIa inhibit binding of autoantibodies from patients with AITP. Br. J. Haematology 1998; 102: 820-828.

7- McMillan R, Tani P, Millard F. (1987) Platelet associated and plasma anti-glycoprotein autoantibodies in chronic immune thrombocytopenic purpura. Blood 1987; 70: 1040-1045.

8-Kokawa T, Nomura S, Yanabu M & Yasunaga K. Detection of platelet antigen for anti-platelet antibodies in idiopathic thrombocytopenic purpura by flow cytometry, antigen capture ELISA, and immunoblotting: a comparative study. Eur J Haematol 1993; 50: 74-80.

9- Faberis F, et al. Anti-platelet antibodies in patients with autoimmune disease with and thrombocytopenia**.** Hematological 2002; 20: 340-347.

10- Meyer O, Agaylan A, Bombard S. Kiesewetter H, Salama A. A novel antigen-specific capture assay for the detection of platelet antibodies and HPA-1a phenotyping. Vox Sanguinis 2006; 91: 324–330

11- He R, Reid D.M, Jones C.E & Shulman N.R. (1994) Spectrum of Ig classes, specificities, and titters of Serum anti-glycoproteins in chronic idiopathic thrombocytopenic purpura. Blood 1994; 83:1024-1032.

12-Beardsley DS, Ertem M. Platelet autoantibodies in immune thrombocytopenic purpura. Transfus Sci 1998; 19: (3) 237-44.

13- Cordiano I, Salvan F, Randi M.L, Ruffatti M.A, Steffan A, Girolami A & Fabris F. Anti-platelet glycoprotein autoantibodies in patients with autoimmune disease with and without thrombocytopenia. J Clin Immunol 1996; 16: 340-347.

14- Van Leeuwen E.F, van der Ven J.Th M, Engelfriet C.P & von dem Borne E.G. Kr. Specificity of auto antibodies in autoimmune thrombocytopenia. Blood 1982; 59:23-26.

15- Fujisawa K, Tani P, O'Toole T.E, Ginsberg M.H & McMillan R. Different specificities of platelet associated and plasma autoantibodies to platelet GPIIb/IIIa in patients with chronic immune thrombocytopenic purpura. Blood 1992; 79: 1441-1446.

16- Michele P. Lambert. Childhood ITP: knowing when to worry? Blood 2009; 114 (23).

17- Van Leeuwen E.F, van der Ven J, Th M, Engelfriet C.P & von dem Borne E.G. Kr. Specificity of autoantibodies in autoimmune thrombocytopenia. Blood 1982; 59:23-26.

18- Lubenko A, Savage J. Antigen captures ELISA for platelet antibody detection. Transfusion Medicine 2000; 10, 213- 218.

19- Douglas B. Cines and James B. Bussel (2006). How I treat idiopathic thrombocytopenic purpura (ITP). Blood 2005; 106(7)**.**

20-Kiefel V, Santoso S, Weisheit M & Mueller-Eckhardt C. Monoclonal antibody-specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. Blood 1987; 70: 1722–1726.

21 - Lucas G. F. and Rogers S. E. Evaluation of an enzyme-linked immunosorbent assay kit (GTI PakPlus) for the detection of antibodies against human platelet antigens. Transfusion Medicine 1999; 9: 63-67

22**-** Brighton TA, Evans S, Castaldi PA, Chester man CN, Chong BH. Thrombocytopenia pura and other immune thrombocytopenias. Blood 1996; 88: 194-201.