Detection of Anti-Platelet Glycoprotein Antibodies Using MAIPA Method

Mohsen Hamidpour1,*, Ghader Khalili2, Mahin Kamali1, Mohammad Hasan Arzani3, Farzaneh Jadali,4 Hamid Alavi Majd5, Navid Khojasteh3

1Department of Hematology, Faculty of Paramedical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran
2Departments of Immunology, The Iranian Institute of Pasteur, Tehran, Iran
3Departments of Hematology, Mofid Pediatric Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4Department of Pathology, Mofid Pediatric Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran
5Departments 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 2×109/L and 95×109/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 petechiae, ecchymosis, 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 antibodies remain poor [5] and many questions remain unanswered [3]. This study has determined and characterized the antiplatelet GPs in children with ITP.

MATERIALS 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% dimethylsulfoxide (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 3×10⁸ 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 of Goat antihuman HRP-conjugated antibody (1: 50 000 diluted in PBS) (Novocastra UK) was added to each well and incubated for 90 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-platelet antibodies 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: 50000 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

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 5×10¹¹/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/IIa (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.

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MAIPA (Monoclonal Antibody Immobilization of Platelet Antigens)
ELISA plate was coated with 100 µL of Goat anti mouse IgG [final concentration 5 µg/ml diluted in Carbonate Bicarbonate Buffer (CBB) pH 8.6] in each well and incubated overnight at 4°C. After washing with Tris
ranges from less than $3\times10^9/L$ up to $95\times10^9/L$.

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

<table>
<thead>
<tr>
<th>Platelets count</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>$51-95\times10^9/L$</td>
<td>7</td>
</tr>
<tr>
<td>$31-50\times10^9/L$</td>
<td>5</td>
</tr>
<tr>
<td>$10-30\times10^9/L$</td>
<td>18</td>
</tr>
<tr>
<td>$&lt;10\times10^9/L$</td>
<td>8</td>
</tr>
</tbody>
</table>

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

$$\text{Percent change} = \frac{\text{OD sample} - \text{OD control}}{\text{OD control}} \times 100$$

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 $41.5\times10^9/L$ 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.

**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 $38\times10^9/L$. 54% were anti-GP Ib/IX positive with mean platelet count of $50\times10^9/L$. The correlation between anti-GP Ib/IX positive and clinical signs was 0.2. Out of 38 patients only 25% were anti-GP-Ia/Ia positive with mean platelet count of $48\times10^9/L$. Using non parametric spearman coefficient, it was statistically shown that a significant correlation was seen between increased anti-GP Ia/Ia level and the platelet count in patients ($r= 0.35$, $P<0.05$).

Figure 3 shows the anti-Gp antibodies in ITP patients.

**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.
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-GPla/IIa in sera and correlation between level of anti-GPla/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.

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