Detection of Anti-Platelet Glycoprotein Antibodies Using MAIPA Method

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ABSTRACT

AITP mostly occur in children accompanied by variable clinical signs 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 signs. These include petechiae, ecchymosis, and bleeding in some patients [3, 4]. Around 85% of platelet autoantigens lie on the platelet GPIIb/IIIa, GPIb/IX and 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 autoantibodies 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, 2 ml anticoagulated [Ethylene 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 lysis buffer (PBS 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 glycoproteins [IIb/IIIa, Ib/IX and IIa/IIa (Novacstra 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.

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 $3 \times 10^9/L$ up to $95 \times 10^9/L$.

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>
<thead>
<tr>
<th>Platelets count</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>$51-95 \times 10^9/L$</td>
<td>7</td>
</tr>
<tr>
<td>$31-50 \times 10^9/L$</td>
<td>5</td>
</tr>
<tr>
<td>$10-30 \times 10^9/L$</td>
<td>18</td>
</tr>
<tr>
<td>$&lt;10 \times 10^9/L$</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$+3\text{SD}$) 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 $41.5 \times 10^9/L$ 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.

**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 < 30x10^9/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 41.5x10^9/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 signs. 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 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-GP Ib/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 has reported that 75% of ITP patients have anti-GP IIb/IIIa and Ib/IX [14-16]. In 25% of patients we have found anti-GP Ia/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 supernmatant 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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