Flow cytometry detection of platelets autoantibodies in children with idiopathic thrombocytopenic purpura

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Abstract Immune thrombocytopenic purpura is an acquired disorder, in which accelerated platelet consumption is due to platelet autoantibodies. The aim of this study was to investigate the clinical value of platelet autoantibodies assay in children with ITP and to evaluate flow cytometry in the detection of platelet autoantibodies in comparison with monoclonal antibody specific immobilization of platelet antigen (MAIPA) assay. We measured platelet autoantibodies by flow cytometry and MAIPA in 18 children with ITP (6 acute, 7 chronic and 5 in remission), in addition to 5 healthy children with matched age and sex as a control group. Significant elevation of platelet-associated immunoglobulin G (PAIgG), PAIgM and PAIgA was demonstrated in children with acute ITP compared to controls and children with chronic ITP ($P < 0.05$). There was significant elevation of PAIgG and PAIgM in children with acute ITP compared to children with ITP in remission ($P < 0.05$). There was significant negative correlation between platelet count and PAIgG levels in ITP children ($r = -0.717$; $P = 0.001$). Flow cytometry found PAIgG in 94.4% of ITP children. MAIPA has detected platelet specific IgG autoantibodies in 83.3% of ITP children. ROC analysis revealed sensitivity of 94%, specificity of 57% with overall accuracy of 83% for detection of PAIgG by flow cytometry compared to MAIPA.

Conclusions Platelet autoantibodies testing in ITP can discriminate acute from chronic forms of the disease and is helpful in follow up of patients. Determination of PAIgM in combination of PAIgG could be of interest in the investigation of ITP. Flow cytometry is a sensitive method of detection of platelet autoantibodies that could be used in screening of suspected ITP and should be followed by MAIPA assay in positive cases to establish the diagnosis.

Keywords ITP · Flow cytometry · MAIPA

Introduction

Immune thrombocytopenic purpura is an acquired disorder, in which accelerated platelet consumption is due to platelet autoantibodies. Immune thrombocytopenic purpura is classified as “primary” or “idiopathic” when occurring without any underlying disease or considered as “secondary” when associated with various dysimmune disorders, such as systemic lupus erythematosus or lymphoproliferative diseases. According to the American Society of Hematology guidelines, the diagnosis is based principally on the history, physical examination, complete blood count, and examination of the peripheral smear, which should exclude other causes of thrombocytopenia and not on the detection of platelet autoantibodies [1]. Recent studies, however, suggest that laboratory tests could be incorporated in current guidelines [2]. Nevertheless, the diagnosis of idiopathic thrombocytopenic purpura continued to be one of exclusion and the clinical utility of platelet autoantibodies assay in the clinical course of ITP is still a matter of debate.

The detection of platelet autoantibodies may be of use in separating immune from non-immune thrombocytopenia. Furthermore, significant higher frequency of platelet autoantibodies were demonstrated in children with ITP who

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developed the acute form of the disease than in those who developed the chronic form. Interestingly, there was no switch from IgM, to IgG antibodies over time. The finding that there is difference in platelet autoantibodies prevalence in acute and chronic childhood ITP suggests that the pathogenesis of the two forms the disease may be different. Moreover, platelet autoantibodies testing could be used to discriminate between acute and chronic forms of ITP. This could be reflected on patients management since over 70–80% of cases of acute ITP in children will resolve spontaneously, with hemostatic platelet counts often attained within weeks, and the risk of acute intracranial hemorrhage is exceedingly low (0.1–1.0). There is no formal evidence that the incidence of this rare event is lessened by treatment and therapy is often inconvenient, expensive, and in the case of high-dose corticosteroids, often accompanied by adverse effect, i.e. in mood and behavior [3, 4]. There are various methods for detection of platelet autoantibodies. The earliest were platelet function-dependent endpoints such as alpha granule release, aggregation, or agglutination. They were succeeded by methods that measure platelet-associated immunoglobulins (PAIgs) like platelet immunofluorescence test, radioimmunoassay and flow cytometry. Recently, methods that measure platelet-specific autoantibodies were then developed including monoclonal antibody-specific immobilization of platelet antigen (MAIPA), antigen capture ELISA, modified antigen capture ELISA (MACE) and immunobead assay [3].

Unfortunately, there is a lack of a sensitive and specific single immunoassay for the detection of platelet autoantibodies that may support a clinical diagnosis of immune thrombocytopenia and the question of what is the most appropriate assay for diagnosis of immune thrombocytopenia remained unanswered.

The present study aimed at assessment of the clinical utility of platelet autoantibodies assay in the clinical course of ITP and evaluation of flow cytometry in the detection of platelet autoantibodies in comparison with MAIPA.

Subjects and methods

Selection of patient

The present study was conducted on 23 children categorized into the following groups:

Group 1: Included 18 patients with ITP (6 acute, 7 chronic and 5 in remission). They were 13 males and 5 females. Their ages ranged from 2–15 years (mean = 8.5 ± 2.6). They were selected from those attending the hematology outpatient clinic and inpatient department, Mansoura University Children Hospital. The diagnosis was based on the American Society of Hematology guidelines [1].

Group 2: Comprised 5 control children with matched age and sex. They were 4 males and 1 female. Their ages ranged from 3–12 years (mean = 6.6 ± 3.5).

Informed consents were obtained before inclusion in the study.

Methods

Specimen collection

Venous blood samples were collected by clean venepuncture and dispensed as follows:

1. Five milliliter were mixed with the dipotassium salt of ethylene-diamine-tetra-acetate (EDTA) at a concentration of 1.2 mg of the anhydrous salt per milliliter of blood. This specimen was used to carry out the complete blood count and the flow cytometric assay for the detection of PAIgs.
2. Three milliliter were allowed to clot and the yielded serum was utilized for the identification of platelet antigen specific autoantibodies by MAIPA.

Flow cytometric assay for the detection of PAIgs

It was done using commercial PAIg kit (Chemicon, USA). The kit provides platelet gating reagent phycoerythrin-conjugated-CD41 monoclonal antibody to GPIIb, FITC-conjugated polyclonal antibody to total human Ig, FITC-conjugated polyclonal antibody to human IgG, FITC-conjugated polyclonal antibody to human IgM, FITC-conjugated polyclonal antibody to human IgA, FITC-conjugated polyclonal antibody to total rabbit Igs (negative control). A direct immunofluorescence assay adapted from Rosenfeld et al. [4] was used. The whole procedure was completed within 4 hours of blood collection.

Preparation of platelet suspensions

EDTA-blood was initially centrifuged at $150 \times g$ for 10 minutes to obtain a platelet-rich plasma sample, which was transferred in a polypropylene tube. A platelet pellet was obtained by centrifuging the PRP at $2,300 \times g$ for 5 minutes and then resuspended in ammonium oxalate (1%) for red cell lysis. After centrifugation at $1,100 \times g$ for 5 minutes to discard supernatant, the pellet was washed twice in phosphate-buffered saline (PBS) containing EDTA (0.009 mol/l Na₂EDTA, 0.0264 mol/l Na₂HPO₄, 0.07 mol/l NaCl) and resuspended at the concentration of 5×10^6 platelets/ml in PBS-EDTA buffer.

Fluorescent staining of platelets

Five flow cytometric tubes were labeled as negative control, total Ig, IgG, IgM and IgA. Ten microliter of platelet gating reagent and the appropriate FITC-labeled immunoglobulin were added to each tube. Then 100 μ l of the platelet suspension was pipetted into each tube, mixed gently and

incubated in the dark at room temperature for 15 minutes. The platelets were then washed twice with PBS-EDTA buffer and resuspended in 0.5 ml of PBS-EDTA buffer and immediately analyzed on a flow cytometer.

Flow cytometric analysis of stained platelets

EPICS XL, Beckman Coulter (Paris, France) was utilized in analysis. Platelet population was gated with platelet forward and side scatter characteristics. Acquisition and processing data from 10,000 platelets were performed and analyzed with computer software (Expo 32, Beckman Coulter).

Identification of platelet antigen specific autoantibodies by MAIPA

MAIPA was performed using platelet screening panel kit and platelet identification panel kit procured from Diamed (Switzerland), according to the method previously described by Kiefel et al. [5].

Procedure

The flat plate (Maxisorb microtiter plate, Nunc Inc, 551730) was coated with 100 μ l of the optimal concentration of goat anti-mouse IgG (1:300). For coating a whole plate 40 μ l of antibody and 12 ml coating buffer was used for 2-hour at 37°C. Plate was then washed 4 times with TBS washing buffer and blocked with 200 μ l PBS with 2% BSA for 30 minutes at room temperature.

Platelets from healthy blood donors (blood group O and HPA1a) were added to each well in a U-well microtiter plate, centrifuged at $900 \times g$ using Sigma 2–16 centrifuge (Sigma Diagnostics, USA) and the supernatant was discarded and 30 μ l PBS with 2% BSA were added and mixed on the ELISA shaker for 15 seconds. Then 50 μ l of patient serum were added, mixed and incubated for 40 minutes at 37°C, centrifuged at $900 \times g$ for 3 minutes, flicked off, mixed with 200 μ l of 0.3% EDTA/PBS with 2% BSA wash solution, then it was spinned and flicked off and washed 2 times. Then 40 μ l of platelet screening panel and platelet identification panel (1:20 dilution in PBS with 2% BSA) were added, incubated for 40 minutes at 37°C and mixed thoroughly before next step.

Samples were then applied on top of 180 μ l of 10% sucrose in a new U-well microtiter plate and centrifuged for 3 minutes at $900 \times g$, flicked off and mixed on the ELISA shaker.

Pellet was resuspended immediately in 130 μ l solubilization solution and kept for 15 minutes at room temperature before centrifugation for 15 minutes at $900 \times g$. Then the plate was washed 4 times in TBS washing buffer and blocked with 200 μ l PBS with 2% BSA for 30 minutes at room temperature.

For the flat coated plate, the blocking solution was removed and 100 μ l of supernatant were added to the desired well (100 μ l TBS washing solution is added to blank position) then it was incubated 40 minutes at 37°C. The plate was then washed 6 times with TBS wash solution. Then 100 μ l HRP conjugated goat anti-human IgG were added (for the whole plate 4 μ l HPR goat anti-human IgG + 12 ml TBS washing buffer), and incubated in the dark for 40 minutes at 37°C. Then the plate was washed 6 times with TBS wash solution and 100 μ l substrate solution were added (for the whole plate 12 ml D.W. + 4 OPD tablets + 5 μ l H_2O_2 mix water) and the plate was incubated 10–15 minutes in the dark at room temperature. Then 50 μ l of 1 M H_2SO_4 were added to stop reaction. Then the antibody concentrations were calculated using ELISA reader. Tables of HPAs types provided with platelet panel kits were utilized for identification of glycoprotein specificities.

Statistical analysis

Statistical analysis of data was performed using SPSS (statistical package for social science) computer program version 10. Kolmogorov Smirnov test was used to evaluate the distribution of data. Qualitative data were presented as number and percentage while qualitative data were presented as mean, median and standard deviation. The Chi-squared test was used to test the association between raw and column variables of qualitative data. For parametric variables, student's "t" test was used to compare means and standard deviations of two groups and the one-way ANOVA test was used to compare means and standard deviations of more than two groups. Mann-Whitney U test and Kruskal-Wallis test are the non-parametric tests equivalent of the "t" test and ANOVA. Correlation between variables was done using Pearson correlation for parametric data and Spearman rank correlation for non-parametric. The receiver operating characteristic (ROC) curve was used to evaluate the accuracy of flow cytometry in detecting platelet autoantibodies. $P < 0.05$ was considered significant. Sensitivity was defined as the proportion of subjects with the disease who have a positive test for the disease and is calculated as true positive/true positive + false negative. Specificity was defined as the proportion of subjects without the disease who have a negative test and is calculated as true negative/true negative + false positive.

Results

The results of the present study are summarized in Tables 1–5 and Figs. 1 and 2.

Table 1 shows the characteristics of the studied groups.

Table 2 shows the comparison of PAIg levels between the studied groups. There is significant elevation of

total PAIGs, PAIgG, PAIgM and PAIgA in children with acute ITP compared to controls and children with chronic ITP ($P < 0.05$). Furthermore, there is significant elevation of PAIgG and PAIgM in children with acute ITP compared to children with ITP in remission ($P < 0.05$).

Table 3 shows the correlation between platelet count and PAIg levels in ITP patients. There is significant negative correlation between platelet count and each of total PAIGs ($r = -0.757$, $P = 0.000$) and PAIgG ($r = -0.717$, $P = 0.000$) in ITP cases.

Table 4 shows the frequency of platelet-specific autoantibodies among ITP patients. HPA1 (61.1%) and GPIIb-IIIa (38.8%) are the most frequent target platelet antigens for platelet-specific autoantibodies.

Table 5 shows the comparison between flow cytometry and MAIPA for the detection of PAIgG in ITP patients. Flow cytometry found PAIgG in 94.4% of ITP children. MAIPA has detected platelet specific IgG autoantibodies in 83.3% of ITP children.

Figure 1 illustrates representative flow cytometric histograms of PAIGs in a case of acute ITP. Panel A represents the internal negative control. Panel B represents total PAIGs

(92%). Panel C represents PAIgG (83%). Panel D represents PAIgM (7.5%). Panel E represents PAIgA (0.5%).

Figure 2 depicts ROC analysis of PAIgG by flow cytometry compared to MAIPA in ITP cases. At a cut-off value of 4.5% (99th percentile of PAIgG percents in controls) for PAIgG, ROC analysis revealed sensitivity of 94%, specificity of 57% with overall accuracy of 83% for the detection of PAIgG by flow cytometry compared to MAIPA in ITP cases.

Discussion

Idiopathic thrombocytopenia purpura is the most common cause of immune-mediated thrombocytopenia. The primary mechanism of thrombocytopenia in ITP is that platelets opsonized by platelet autoantibodies are destroyed by the reticuloendothelial system. Also autoantibodies activate the complement pathway to a similar degree, and platelet lysis may occur in some ITP patients. Platelet autoantibodies may also impair megakaryocyte production and platelet turnover [6].

To define the immune nature of a thrombocytopenia, the platelet-bound autoantibody as well as its antigen must be

Table 1 Characteristics of the studied groups

Parameters	Acute ITP n = 6	Chronic ITP n = 7	ITP in remission n = 5	Control n = 5	P value
Age (year) median (range)	4.0 (2–9)	10.0 (8–15)	10.0 (8–15)	4.5 (3–12)	0.635
Sex (M/F)	2/4	1/6	2/3	4/1	0.264
Pallor (+ve)	4/6	2/7	0/5	0/5	0.001
Petechial bleeding (+ve)	4/6	3/7	0/5	0/5	0.000
Orificial bleeding (+ve)	2/6	1/7	0/5	0/5	0.000
Hb (g/dl) mean $\pm$ SD	9.0 $\pm$ 1.06	9.7 $\pm$ 1.5	11.1 $\pm$ 0.9	12.3 $\pm$ 0.5	0.089
TLC ($\times 10^9/l$) mean $\pm$ SD	3.8 $\pm$ 0.45	4.4 $\pm$ 1.5	4.2 $\pm$ 0.6	3.9 $\pm$ 0.7	0.970
Platelet ($\times 10^9/l$) median (range)	23.5 (13–68)	46 (31–61)	210 (156–295)	233 (199–320)	0.000

Table 2 Comparison of PAIg levels between the studied groups

Parameters	Total PAIGs %	PAIgG %	PAIgM %	PAIgA %
Acute ITP median (range)	82.8 (71–92)	68 (19–80)	8.7 (2.1–71)	2 (1–3.5)
Chronic ITP median (range)	45.2 (33.5–69.2)	35.2 (25–63)	5 (3–19.1)	1.5 (0.9–3.1)
ITP in remission median (range)	18 (9.8–23)	8.5 (2.8–19.5)	5.4 (2.5–11.5)	1 (0–2)
Controls median (range)	3.7 (0.9–6.2)	0.7 (0.9–4.5)	0.5 (0–2.9)	0 (0–1.3)
P1	0.000	0.000	0.000	0.000
P2	0.001	0.001	0.036	0.036
P3	0.004	0.009	0.042	0.082
P4	0.003	0.003	0.014	0.014

P1 = acute ITP vs controls; P2 = acute ITP vs chronic ITP; P3 = acute ITP vs remission; P4 = chronic ITP vs remission

Table 3 Correlation between platelet count and PAIGs levels in ITP patients

Parameter	Total PAIGs %	PAIgG %	PAIgM%	PAIgA%
Platelet	R	–0.757	–0.717	–0.131
($\times 10^9/l$)	P value	0.000	0.001	0.605
				0.121

Table 4 Frequency of platelet specific autoantibodies among ITP patients

ITP patients (n = 18)	
Autoantibodies	n (%)
Anti-GPIa	0 (0.0)
Anti-GPIb	3 (16.6)
Anti-GPIIb	1 (5.5)
Anti-GPIIIa	4 (22.2)
Anti-GPIIb-IIIa	7 (38.8)
Anti-HPA1	11 (61.1)
Anti-HPA2	2 (11.1)
Anti-HPA3	5 (27.7)
Anti-HPA4	2 (11.1)
Anti-HPA5	1 (5.5)
Anti-HPA15	0 (0.0)

Table 5 Flow cytometry compared with MAIPA for the detection of PAIgG in ITP patients

ITP patients	Flow cytometry	MAIPA
Acute ITP (n = 6)	6/6	5/6
Chronic ITP (n = 7)	7/7	7/7
ITP in remission (n = 5)	4/5	3/5
Total	17/18	15/18

characterized by means of sensitive and specific assays. Unfortunately however, the detection of platelet autoantibodies is problematic in many aspects. Platelets are relatively small, uncolored, not found in abundance from affected patients, tend to spontaneously agglutinate, are somewhat coated with IgG molecules, and absorb IgG molecules by non-specific mechanisms. These properties are also the reason for the difficulty and complexity of the tests used for the detection of platelet autoantibodies [7]. Consequently, platelet autoantibody assay was not implemented in the diagnostic work up of ITP patients. However, with the recent application of advanced immunological assays to platelet immunology, the detection of platelet autoantibodies is recommended in recent guidelines to be included in the evaluation of ITP patients [2].

We designed this study to investigate the clinical value of platelet autoantibodies assay in children with ITP and to evaluate flow cytometry in the detection of platelet autoantibodies in comparison with MAIPA assay.

We measured platelet autoantibodies by flow cytometry and MAIPA in 18 children with ITP (6 acute, 7 chronic and 5 in remission), in addition to 5 healthy children with matched age and sex as a control group.

We demonstrated significant elevation of PAIgG, PAIgM and PAIgA in children with acute ITP compared to controls and children with chronic ITP. There was significant elevation of PAIgG and PAIgM in children with acute ITP compared to children with ITP in remission (Table 2).

These findings suggest a pathogenetic role of platelet autoantibodies in ITP cases. The significant reduction of antibody level in selflimiting course of acute ITP may be attributed to the clearance of PAIGs by the spleen. However, the significant reduction of antibody levels in chronic ITP and ITP in remission could be attributed to the effect of therapy which suppress the antibody production, resulting in reduced antibody levels. Furthermore, the significant reduction of platelet autoantibodies in chronic ITP and ITP in remission compared to acute ITP makes the assay of platelet autoantibodies a useful tool for discrimination between acute and chronic ITP and in follow up of patients. This is in agreement with previous reported data [8, 9].

Interestingly, we demonstrated high values of PAIgM and PAIgA in association with PAIgG. In some patients high levels of PAIgM compared to PAIgG were detected. These findings are in agreement with previous reported data that highlight the fact that IgM and IgA are frequently associated with IgG or even present alone for IgM [10]. The significance of these findings remained to be elucidated.

In the present study we found significant negative correlation between platelet count and each of total PAIGs and PAIgG levels in ITP cases (Table 3). This could be explained by the fact that reduction in platelet autoantibody level leads to decreased platelet destruction and so elevation of platelet count and vice versa. This is in agreement with Zhou et al. [11] who found that the platelet counts were negatively correlated with the levels of the autoantibody.

In the present study, MAIPA assay revealed that HPA1 (61.1%) and GPIIb-IIIa (38.8%) were the most frequent target platelet antigens for platelet-specific autoantibodies (Table 4). This is in agreement with previous reported data [12, 13].

In the present study flow cytometry found PAIgG in 94.4% of ITP children. MAIPA has detected platelet specific IgG autoantibodies in 83.3% of ITP children (Table 5). There are several possible reasons explaining the negative results in 16.7% of ITP cases by MAIPA: First all of the protein capture assays require that the target of the patients' autoantibody be known, so that the appropriate monoclonal antibodies can be used in the capture system. MAIPA assays routinely assay only for autoantibody against GPIIb/IIIa and GPIb/IX (and in some cases GPIa/IIa and GPIV), perhaps there are autoantibodies against other important antigens which are being missed. Some patients may also have only IgM or IgA autoantibodies [14]. Second, the practice American Society of Hematology guideline recommendations are not rigorous enough to make an accurate diagnosis of ITP, since they are based on the exclusion of other causes, or because the assays are deficient. Probably both possibilities are true to some degree. Some patients who satisfy the Practice Guidelines' criteria may not have ITP, other platelet surface antigens or Ig subtypes are involved. Third, Berchtold and weigner [14] suggested that ITP therapy has suppressed autoantibody production prior to assay. It has been

Fig. 1 Representative flow cytometric histograms of PAIgs in a case of acute ITP

well shown that autoantibody levels may become normal upon treatment with corticosteroids and other immunosuppressive agents. If assays are performed after therapy is introduced (which is often the case), false negative results may occur. Fourth, McMillan et al. [15] reported that there may be technical or sensitivity problems with the assays. It is possible that low-levels of autoantibody, which are undetected by MAIPA, may be sufficient to result in platelet destruction. It is also possible that the autoantibodies compete for the same antigenic site as the monoclonal antibodies used in the assays (steric hinderance). Fifth, Fujisawa et al. [16] have demonstrated that autoantibodies can disappear as patients respond to immunomodulatory

therapy. It is possible that patients who had borderline negative results could have positive results if tested on subsequent dates.

In the present study, ROC analysis revealed sensitivity of 94%, specificity of 57% with overall accuracy of 83% for detection of PAIgG by flow cytometry compared to MAIPA. These findings provide an evidence that flow cytometry is more sensitive in the detection of platelet autoantibodies when compared with MAIPA. This is in agreement with Tomer [17] who reported that in immune thrombocytopenia, flow cytometry has high sensitivity in the detection of platelet autoantibodies when compared with MAIPA, but it is non-specific. Whereas glycoprotein

Fig. 2 Receiver operating characteristic analysis of PAIgG by flow cytometry compared to MAIPA in ITP cases. The area under the curve is 0.859 which indicates high accuracy of flow cytometry compared to MAIPA in the detection of PAIgG in ITP cases

specific assays like MAIPA are highly specific but less sensitive. Furthermore, Hezard et al. [18] reported that flow cytometry for PAIgs assessment may be used to accurately decide whether or not MAIPA must be subsequently performed.

Conclusions

Platelet autoantibodies testing in ITP can discriminate acute from chronic forms of the disease and is helpful in follow up of patients. Determination of PAIgM in combination of PAIgG could be of interest in the investigation of ITP. Flow cytometry is a sensitive method of detection of platelet autoantibodies that could be used in screening of suspected ITP and should be followed by MAIPA assay in positive cases to establish the diagnosis.

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