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PAIgG and PAIgM levels in secondary dengue virus infections lead to thrombocytopenia in patients from KP, Pakistan

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ABSTRACT

Objective: To understand the impact of platelet associated immunoglobulin G (PAIgG)/ platelet associated immunoglobulin M (PAIgM) on severity of dengue virus infection leading to thrombocytopenia.

Methods: In this study we examined a total of 52 patients who were having secondary infection of dengue in acute phase by using competitive ELISA.

Results: A decrease in the platelet count was observed at the acute phase of infection while all along the recovery stage the count of platelet was significantly increased. A significant decrease was observed in PAIgG and PAIgM in these subjects. Inverse correlation was found between platelets count and PAIgG/PAIgM among the subjects studied. In the platelets elution from ten subjects, anti-dengue virus immunoglobulin G and immunoglobulin M were observed. PAIgG and PAIgM with inclined levels were higher in dengue hemorrhagic fever than the classical dengue fever. In the development of dengue hemorrhagic fever PAIgM inclined level was independently associated with high specificity, showing a possible indication of dengue hemorrhagic fever.

Conclusions: This study suggests that in secondary dengue virus infection, the PAIgG and PAIgM levels, and the activity of anti-dengue virus play key roles, both in the development and severity of the disease.

1. Introduction

The dengue-virus is a human viral pathogen, that is basically mosquito borne, affecting about 2.5 billion peoples in tropical and sub-tropical regions of the world. Dengue virus belongs to genus *Flavivirus*, family Flaviviridae having 4 serotypes (DEN-1, 2, 3, 4) [1,2]. The transfer of virus often involves the viremic blood ingestion by mosquitos' *Aedes*, then for viral replication, an 8–10 days period of incubation occurs, virus appears in the saliva and from here may transfer to a human that is susceptible. Oviposition is stimulated by the female mosquito as blood meal, during the extrinsic incubation period this undergoes one or more reproductive cycles, in this way virus enter the egg and passed to the next generation of mosquitoes [3]. Type 1 and type 4 of dengue virus has clinical manifestation with a wide spectrum, these manifestations includes "thrombocytopenia" and "increased vascular permeability" [4–7]. Severe dengue-virus infection complexity may occur that causes dengue hemorrhagic fever (DHF), which induces thrombocytopenia, abnormalities in coagulation and leakage of plasma in children and worse results in adults showing increased occurrence of bleeding, shocks and organs failure [2,3,8,9].

In the dengue endemic areas secondary dengue virus infection is commonly observed, and probably constitute a risk factor for

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DHF. More than in hundred of tropical countries, the disease is highly endemic now. During the past three decades the number of dengue patients has rapidly increased, and in tropical and sub-tropical countries now dengue infection is a most important community health concern [4,10]. Dengue is endemic in Pakistan in the post-monsoon period with its usual peak. In the year 1985 for the first time in Pakistan dengue was reported as undifferentiated fever in children under 16 years of age. In 2011 Pakistan had the worst strike of dengue, in which 2 300 cases were reported [11]. Although platelets synthesis decreases with dengue induced bone marrow suppression, in patients with DHF thrombocytopenia an immune mechanism is operative that results in increased platelets destruction. In patients with chronic idiopathic thrombocytopenia purpura an increased level of platelets associated immunoglobulin G (PAIgG) has been observed, but in a variety of other diseases it has been founded. Oishi *et al.* studied that throughout the acute phase of secondary dengue virus infection, there is an inverse correlation between level of PAIgG and platelets count [12]. As the dengue virus binds directly to the platelets, with this they speculated that on the platelets the immune complex of the dengue virus with anti-dengue virus are located. While formation of PAIgG may play a part in the initiation of thrombocytopenia via both of fragment crystallizable receptors- and complement receptors-mediated platelets removal by the macrophages and complement mediated platelets lysis, development of platelets associated immunoglobulin M (PAIgM) can also play a part in the initiation of thrombocytopenia of thrombocytopenia through the same mechanism (excluding the use of FC receptors) based on immunoglobulin M (IgM) pentamer's function. As in DHF thrombocytopenia is more common than in dengue fever so we therefore assumed that increase in the level of PAIgG may be linked with disease's severity, by developing thrombocytopenia in dengue patients. Furthermore, although with secondary dengue infection the number of IgM that are associated with platelets, is also increased in this disease but the role of PAIgM remains uncertain [12,13]. In secondary dengue virus infection, no studies have been conducted in less developed country like Pakistan to determine the levels and role of PAIgG/PAIgM for disease severity and inducing thrombocytopenia. So, we in this hospital based study determined the levels and correlation of PAIgG/PAIgM with thrombocytopenia as well as the disease severity.

2. Materials and methods

2.1. Patients and specimen

At Hayat Abad Medical Complex (Peshawar, Pakistan), a total of 122 clinically suspected patients of having dengue virus infection were enrolled between August 2013 and December 2014. Of all these subjects, the number of diagnosed subjects having acute phase dengue virus infection (3–7 days after the onset of disease) was 83, as verified by screening tests *i.e.* IgM capture ELISA and RT-PCR [14–16]. By using hemagglutination inhibition test 75 subjects with the acute phase of secondary dengue infection (3–7 days just after the onset of disease) were diagnosed. By using the hemagglutination inhibition test, 7 subjects were diagnosed with a primary dengue infection. In this study we evaluated platelet count, PAIgG and PAIgM at the time of enrollment (acute phase) and then after 4 days from the 1st test (convalescent phase) out of 52 patients. During the same period at Hayat Abad Medical Complex, Peshawar as a control group 55 healthy volunteers with same age were screened for IgM, platelets number and PAIgG/PAIgM levels [17]. Ethylene diamine tetraacetic acid tubes were drawn from the infected subjects and healthy volunteers.

2.2. Platelets count

With automatic hemocytometer the platelet count was determined. Through World Health Organization criteria DHF was determined *i.e.* the platelets count must be 100 000 cells/mm^3, hematocrit elevation 20% above the average age group and hemorrhagic manifestation [7,17]. All the DHF cases were further grouped as I–IV. Dengue fever was defined as an elevation in hematocrit of $< 20\%$ and on the right lateral decubitus chest radiograph with no detectable pleural effusion [18]. Procedure for this study was approved by the Bioethics Committee of Hayat Abad Medical Complex, Peshawar Pakistan. Written informed consent was provided to parents and guardians of all patients.

Hemagglutination inhibition test was performed according to the method of Clarke and Casals [19], a paired plasma sample with an interval longer than 7 days was used. When the hemagglutination inhibition titer was 1:2 560 in the plasma of subjects having acute/convalescent phase, it was considered to be a secondary infection [17,20].

2.3. Competitive ELISA for PAIgG/PAIgM

The 96-wells flat-bottomed microtitre polystyrene plates were previously coated with standard-human immunoglobulin M (IgG) (Inter. Cell Tech.) (400 ng/well) or with human-IgM (ChemiCon Inter. USA). By following the protocol used by Saito *et al.*, the values of PAIgG/PAIgM were recorded as the IgG/IgM values in ng/10^7 platelets count [17].

2.4. Activity of anti-dengue virus IgG/IgM in the samples of elute platelets

The platelets were separated from plasma samples of 10 subjects having acute phase of secondary infection (eight having dengue fever while two were with DHF) and from the plasma of six healthy volunteers. The activity of IgG/IgM (anti-dengue virus) in the elute platelets samples was measured. Antibodies were eluted from the separated platelets. At 4 °C overnight the elute was dialyzed against phosphate buffered saline, and were concentrated to a final volume of 1 mL containing 3×10^9 platelets by using a concentrator. Until use these samples were stored at −80 °C. The 96 wells flat-bottomed microplates previously coated with 100 mL of a dengue-virus antigens mixture (2.5 μg/mL) was used in the IgG/IgM indirect ELISA for the detection of anti-dengue virus antigens [8,17]. For antigen preparation dengue-type I (Hawaii Strain), type II (THNH7/93 strain), type III (PHNH4/84) and type IV (CT93-158) viruses were used. After washing the plates, 50 mL of undiluted elutes were added to the duplicate wells, following 30 min incubation at room temperature [17]. The plate was washed and then it was reacted with 100 mL alkaline phosphatase conjugated anti-human IgG goat serum (1:5 000 dilution, Biosource Int., USA) or anti-human IgM goat serum (1:2 500 dilution, Biosource Int.), and then finally at 405 nm the optical density (OD) was measured [17].

2.5. Statistical analysis

All the data were expressed as mean ± SD. We used statistical software, JMP, 10.0 SAS, USA for the analysis of platelets count and PAIgG/PAIgM levels during the period between the acute and convalescent phase. Platelets count and PAIgG/PAIgM levels were analyzed between all healthy volunteers *vs.* patients with dengue virus infection, and between patients with dengue fever *vs.* DHF by the Mann–Whitney *U*-Test. On the severity of disease a multivariate logistic regression was used to assess the association of PAIgG/PAIgM and platelet count. Using the Spearman's rank correlation the significance of correlation was estimated. The level of significance was measured as $P < 0.05$.

3. Results

Out of the 52 patients that were having secondary dengue virus infection, 27 were diagnosed with dengue fever and 25 were diagnosed as DHF. The DHF patients were further grouped into DHF I ($n = 7$) and DHF II ($n = 18$). Platelets-count, and PAIgG/PAIgM were significantly different ($P < 0.001$) in patients having acute-phase of secondary infection and healthy individuals (Table 1). Statistically significant ($P < 0.001$) variation was observed in the maximum percent hematocrit increase, PAIgG/PAIgM among subjects with dengue fever and dengue hemorrhagic fever, while difference in subjects age, period after onset and platelets count was not significant between these two groups. We detected a significant correlation ($P < 0.0001$, $r = -0.241$) between platelets count and PAIgG level in total 52 patients having secondary dengue virus infection (Figure 1A). We also observed a strong correlation ($P < 0.0001$, $r = -0.335$) among these patients at the time of enrollment (Figure 1B). In 52 patients having secondary dengue infection, the change in platelets count and PAIgG/PAIgM was compared between acute and convalescent phases. In these patients platelets count with low baseline [(45.6 ± 23.1) × 10^3/mL] during the acute phase, increased significantly ($P < 0.001$, Figure 2A) and improved to normal count during the period of convalescing [(242.7 ± 52.4) × 10^3/mL]. On the other hand, in the same subjects during the acute phase the increased baseline PAIgG [(28.3 ± 17.0) ng/10^7 platelet] or PAIgM [(21.3 ± 15.3) ng/10^7 platelet] decreased significantly [$P < 0.001$ for PAIgG (Figure 2B); $P < 0.001$ for PAIgM (Figure 2C)] during the convalescent phase to a normal level for PAIgG [(5.04 ± 2.3) ng/10^7 platelet] and [(3.1 ± 2.0) ng/10^7 platelet] for PAIgM. In the elutes of the platelets samples the levels of antidengue virus IgG or IgM were determined. In elutes from six healthy volunteers the OD at 405 nm for antidengue virus IgG and IgM were 0.20 ± 0.10 and 0.09 ± 0.05, respectively. In contrast, in elutes from the subjects in the acute phase of secondary infection, an increased activity of anti-dengue virus IgG/IgM (OD 405 nm; 1.54 ± 0.35 for anti-dengue virus IgG, 0.35 ± 0.20 for anti-dengue virus IgM) was found. In the patients with acute phase of secondary infection we then examined whether the PAIgG/PAIgM levels correlated directly with the hematocrit increase, as for vascular permeability it is a critical indicator [21]. PAIgG level and hematocrit percent increase correlates non-significantly ($n = 52$, $r = 0.061$, $P > 0.05$). A weak correlation ($n = 52$, $r = 0.21$, $P = 0.038$) between the level of PAIgM and percentage increase in hematocrit was observed, while no significant correlation was observed between subjects with dengue fever ($n = 27$, $r = -0.11$, $P > 0.05$) and ($n = 25$, $r = -0.29$, $P > 0.05$) in patients with DHF. PAIgM was associated independently with DHF among parameters of platelets count, PAIgG and PAIgM ($P < 0.001$) as demonstrated by a logistic regression. As an interpreter for the subsequent progression of DHF a level of PAIgM higher than 20 ng/10^7 platelets with a

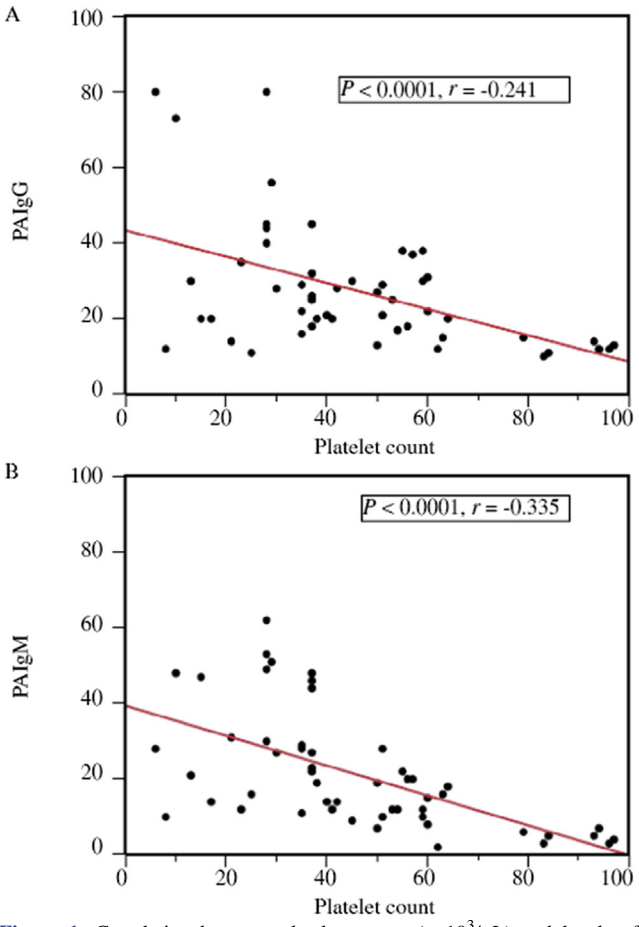

Figure 1. Correlation between platelets count (× 10^3/μL) and levels of PAIgG (ng/10^7 platelet) ($n = 52$) (A) /PAIgM (ng/10^7 platelet) ($n = 52$) (B) in patients having acute secondary phase dengue virus infection.

Table 1
Laboratory data on patients with acute phase of secondary dengue virus infection and healthy volunteers.

Diagnosis	Number	Age (years)	Days after onset	% Increase in hematocrit	PLT count (10^3/μL)	PAIgG (ng/10^7 PLT)	PAIgM (ng/10^7 PLT)
HV	55	18.3	–	–	261.1 ± 68.2	12.2 ± 3.1	5.8 ± 1.1
DV Infection	52	17.8	5.9 ± 1.1	21.3 ± 5.6	45.6 ± 23.1*	28.3 ± 17.0*	21.3 ± 15.3*
DF	27	17.7	6.1 ± 0.1	10.7 ± 8.1	62.9 ± 17.5	23.0 ± 11.7	11.6 ± 6.6
DHF	25	18.1	6.4 ± 1.1	25.5 ± 7.9**	27.0 ± 10.2	34.1 ± 20.0***	31.8 ± 15.2****

HV: Healthy volunteer; DF: Dengue fever; PLT: Platelet; Data represent as mean ± SD; *: $P < 0.001$ versus HV; **: $P < 0.001$ versus DF; ***: $P < 0.01$ versus DF; ****: $P < 0.001$ versus DF.

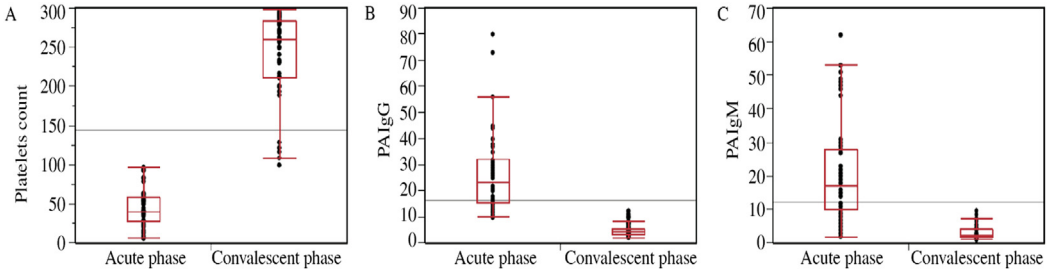

Figure 2. Comparing the peripheral platelets count ($\times 10^3/\mu L$) ($n = 52$) (A), level of PAIgG (ng/10^7 platelet) ($n = 52$) (B) and level of PAIgM (ng/10^7 platelet) ($n = 52$) (C) between the acute (the first test) and convalescent phase (4 days after the first test) of secondary dengue virus infections.
*: $P < 0.001$.

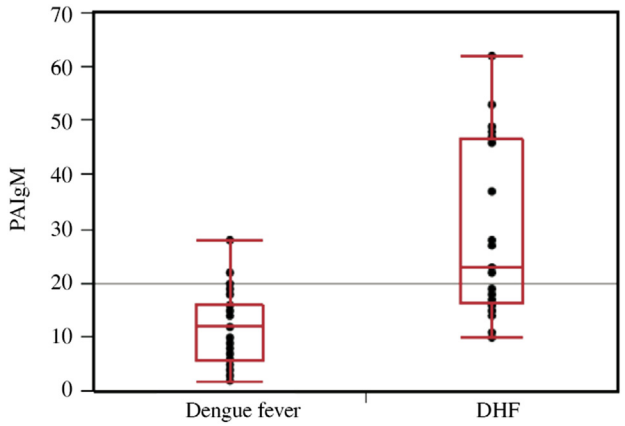

Figure 3. Comparison of the levels of PAIgM (ng/10^7 platelet) between patients with dengue fever ($n = 27$) and DHF ($n = 25$) during the acute phase of a secondary dengue virus infection.
The horizontal broken line represents the cut-off value (20 ng/10^7 platelets) for the prediction of DHF.

sensitivity of 32% (8/25) and a specificity of 92.5% (25/27) was observed during the acute phase of secondary infection (Figure 3).

4. Discussion

In this study we revealed that platelets count is significantly correlated with PAIgG as well as PAIgM levels in acute phase of secondary dengue virus infection. The PAIgG/PAIgM levels in acute phase were significantly higher than in the convalescent phase. Levels of PAIgG and PAIgM were found to be similar during acute and convalescent phases. Although antidengue virus IgG were relatively higher than antidengue virus IgM activities, we found that in elutes from patients in acute phase of secondary infection, the activity of both the anti-dengue virus IgG/IgM were increased. In the patients of acute phase of secondary dengue virus infection these data indicates that the PAIgM formation involving anti-dengue virus IgM may also contributes for inducing thrombocytopenia. By transient formation of PAIgG for thrombocytopenia as shown by Oishio *et al.* throughout acute phase of the secondary infection, development of PAIgM may also play a part for inducing thrombocytopenia [8]. While by formation of PAIgG may play a role in the induction of thrombocytopenia via both of fragment crystallizable receptors- and complement receptors-mediated platelets removal by the macrophages and complement mediated platelets lysis, development of PAIgM can also play a role in the induction of thrombocytopenia via the same mechanism (excluding the use of FC receptors) based on IgM pentamer's function [22,23]. Previously a study reported that in the existence of a virus-specific antibody, virus of dengue-2 sticks to the platelets [17,24]. By Satio *et al.* in a study among 21 patients with secondary dengue infection, observed a high positive rate of 42.85 with dengue-virus RNA in purified platelets samples through RT-PCR [17]. This data indicates that dengue-virus is present on the circulating platelets in subjects having secondary dengue infection, this supports our assumption of a procedure of inducing thrombocytopenia by involving the development of PAIgG/PAIgM. The increase in the PAIgG/PAIgM levels was significant, and we also observed that there was a significant correlation between PAIgG/PAIgM levels and platelets count in patients having acute secondary phase infection, the PAIgG in 8 patients (29.6%) and PAIgM in 17 patients (68%) levels persisted within normal range (0–14.9 ng/10^7 platelets in case of PAIgG, while 0–12 ng/10^7 platelets in case of PAIgM). So our data indicates that in these patients other methods may also be operative that are involve in the development of thrombocytopenia. The contribution of PAIgG appears to be much greater than that of PAIgM for induction of thrombocytopenia. As prognosticator of DHF other studies had recommended some host factors *i.e.* (a) soluble vascular cell adhesion molecule 1, (b) dengue-virus non-structural protein and (c) soluble tumor necrosis factor-receptors [25,26]. In this study PAIgM level increased was highly specific (92.5%) although its sensitivity was relatively low (32%), for the development of DHF throughout acute secondary phase infection. Now we could say that it is quite possible that development of PAIgM is contributing to the increasing vascular permeability. Krishnamurti *et al.* demonstrated the platelets binding to the endothelial cells that were infected with dengue-virus [27,28]. As vascular permeability increases with DHF, so for this mechanism active platelets that are associated with dengue-virus, anti-dengue virus IgM and endothelial cells that are infected with dengue-virus may be important [9,17,29]. In short, we have demonstrated that throughout the acute secondary phase dengue virus infection, the inclined PAIgG/PAIgM levels, anti-dengue virus IgG and IgM are closely linked with thrombocytopenia [17,30,31]. With development of DHF, PAIgM was independently associated throughout the acute secondary phase infection, and for DHF it was highly specific. In the mechanism of inducing thrombocytopenia via increasing vascular permeability, development of PAIgG/PAIgM may play a leading role. Further research and interpretations are required to illuminate and link the platelets associated immunoglobulins with the methods of development of thrombocytopenia via increase vascular permeability.

Conflict of interest statement

We declare that we have no conflict of interest.

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