Increased phagocytosis of platelets from patients with secondary dengue virus infection by human macrophages.

Author(s)
Honda, Shoko; Saito, Mariko; Dimaano, Efren M; Morales, Philip A; Alonzo, Maria T G; Suarez, Lady-Anne C; Koike, Natsuki; Inoue, Shingo; Kumatori, Atsushi; Matias, Ronald R; Natividad, Filipinas F; Oishi, Kazunori

Citation
The American journal of tropical medicine and hygiene, 80(5), pp.841-845; 2009

Issue Date
2009-05

URL
http://hdl.handle.net/10069/23207

Copyright © 2009 by The American Society of Tropical Medicine and Hygiene
Increased Phagocytosis of Platelets from Patients with Secondary Dengue Virus Infection by Human Macrophages

Shoko Honda,† Mariko Saito,† Efren M. Dimaano, Philip A. Morales, Maria T. G. Alonzo, Lady-Anne C. Suarez, Natsuki Koike, Shingo Inoue, Atsushi Kumatori, Ronald R. Matias, Filipinas F. Natividad, and Kazunori Oishi*†

Department of Clinical Medicine and Virology, Institute of Tropical Medicine Nagasaki University, Japan; Department of Virology, Graduate School of Medicine, Tohoku University, Japan; Department of Disaster Prevention System, Faculty of Risk and Crisis Management, Chiba Institute of Science, Japan; Laboratory for Clinical Research on Infectious Diseases, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Japan; Department of Blood Borne Diseases, San Lazaro Hospital, Manila, Philippines; Research and Biotechnology Division, St. Luke’s Medical Center, Quezón City, Philippines

Abstract. The relationship between the percent phagocytosis of platelets by differentiated THP-1 cells was examined using flowcytometry and the peripheral platelet counts as well as platelet-associated IgG (PAIgG) in 36 patients with secondary dengue virus (DV) infections. The percent phagocytosis and the levels of PAIgG were significantly increased in these patients during the acute phase compared with the healthy volunteers. The increased percent phagocytosis and PAIgG found during the acute phase significantly decreased during the convalescent phase. An inverse correlation between platelet count and the percent phagocytosis (P = 0.011) and the levels of PAIgG (P = 0.041) was found among these patients during the acute phase. No correlation was found, however, between the percent phagocytosis and the levels of PAIgG. Our present data suggest that accelerated platelet phagocytosis occurs during the acute phase of secondary DV infections, and it is one of the mechanisms of thrombocytopenia in this disease.

INTRODUCTION

Dengue virus (DV), a mosquito-borne human viral pathogen, belongs to the genus Flavivirus of the family Flaviviridae, and has four serotypes (DEN-1, DEN-2, DEN-3, and DEN-4). Dengue virus types 1–4 induce a wide spectrum of clinical manifestations, including hemorrhagic manifestations associated with thrombocytopenia and increased vascular permeability. Secondary DV infections, which are commonly observed in dengue-endemic areas, are more likely to constitute a risk factor for dengue hemorrhagic fever (DHF). The disease is now highly endemic in more than 100 tropical countries. The number of cases has rapidly increased during the past three decades, and it has become a major public health concern particularly in tropical and subtropical countries.

Although DV-induced bone marrow suppression decreases platelet synthesis, an immune mechanism of thrombocytopenia resulting in increased platelet destruction appears to be operative in patients with DHF. An increased level of platelet-associated IgG (PAIgG) is frequently observed in patients with chronic idiopathic thrombocytopenic purpura (ITP), but it also is found in a variety of other diseases. Secondary DV infections, and it is one of the mechanisms of thrombocytopenia in this disease.

MATERIALS AND METHODS

Patients and study design. Forty-two patients clinically suspected of having a DV infection were enrolled at San Lazaro Hospital between September 2006 and February 2007. Of these subjects, 40 were diagnosed with an acute phase of DV infection based on the results of a particle agglutination test for dengue IgM or reverse transcription-polymerase chain reaction (RT-PCR). Of these patients, 37 were diagnosed with an acute phase of a secondary DV infection based on the results of a hemagglutination inhibition (HI) test. Among the 37 patients with a secondary DV infection, we evaluated 36 patients who were examined for the peripheral platelet count, PAIgG levels, the frequency of platelet phagocytosis at the time of enrollment (acute phase), and 4 days after the first test (convalescent phase) in this study. One patient withdrew from the study following transferral to another hospital. Thirty-six healthy volunteers (HVs), who were age-matched, were also enrolled as control subjects at St. Luke’s Medical Center during the same period. These HVs also received a particle agglutination test for dengue IgM, a platelet count, and an examination for PAIgG levels at the time of enrollment. Ethylenediaminetetraacetic acid (EDTA) and 3.8% sodium citrate blood were drawn from these patients and from HVs for these tests. The platelet counts were determined using an automatic hemocytometer (Sysmex, Hyogo, Japan). The PAIgG levels were determined using a competitive enzyme-linked immunosorbent assay (ELISA), as previously described. The DHF was diagnosed by World Health Organization (WHO) criteria; a platelet count nadir of less than 100,000/µL,
hemorrhagic manifestations, and an increased hematocrit equal to or greater than 20% above the average or the presence of either pleural effusion or ascites fluid.14 Cases of DHF were further graded on a scale of I–IV. Dengue fever (DF) was defined as an increase in hematocrit of less than 20% and no detectable pleural effusion on the right lateral decubitus chest radiograph.

The research proposal for this study was approved by both the Bioethics Committees of San Lazaro Hospital and by St. Luke's Medical Center. Parents or guardians of all patients provided written informed consent. An interim target sample size of 62 was chosen to ensure that there would be at least a 70% chance for detecting a difference of 30% (50% versus 20%), with a one-sided alpha level of 0.05, in the percent phagocytosis of platelets between patients with an acute phase of secondary DV infection and HVs.

**Platelet preparation for platelet phagocytosis assay.** Platelet-rich plasma (PRP) was separated from 5 mL of 3.8% sodium citrate blood drawn from patients with secondary DV infection and from HVs by centrifugation of 200 x g for 10 min at room temperature. After removing PRP with washing buffer [140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM sucrose, and 1 µg/mL PGE2 (pH 6.0) (Cayman Chemical, Ann Arbor, MI)], 2 x 10^9 washed platelets were suspended with 60 µL of physiologic buffer (PB) (140 mM NaCl, 3 mM KCl, 0.5 mM MgCl2, 5 mM NaHCO3, 10 mM glucose, and 10 mM HEPES, pH 7.4) with or without 6.0 µg of anti-human platelet monoclonal antibody (MAb) (mouse IgG1, Immuno-Biological Laboratories Co. Ltd, Takasaki, Japan) for 30 min at 37°C, and washed with washing buffer. Washed platelets were then stained with 20 µM of CellTracker Orange CMTMR (CTO; Molecular Probes, Inc., Eugene, OR) for 30 min at 37°C.15 The stained platelets were washed and suspended in 0.5 mL of PB, and then incubated another 30 min at 37°C to remove excess dye. Washed platelets were resuspended in 0.7 mL of PB, the number of the platelets were counted using an automatic hematocytometer. The efficiency of platelet labeling with CTO was determined to be 94.2% using flowcytometry. In preliminary experiments, a negligible fluorescent signal of CTO was detected in the supernatants of washing buffer. However, no direct staining of differentiated THP-1 cells with CTO in the supernatants of wash buffer for CTO-stained platelets was confirmed by flowcytometry (data not shown).

**Flowcytometry analysis.** The frequency of platelet phagocytosis by differentiated THP-1 cells was determined by the frequency of CTO positive and platelet–specific marker CD61 negative cells. The differentiated THP-1 cells were gated and 10,000 events were acquired from each sample. For the standardization of the values for platelet phagocytosis, the percent phagocytosis was expressed using the following formula: the frequency of phagocytosis of test platelets divided by the frequency of phagocytosis of the positive control platelets (pre-treated with anti-platelet MAb) x 100.

**Statistical analysis.** All data were expressed as the mean ± SD. Platelet counts and PAIgG levels during the period between the acute and convalescent phase were tested using a Wilcoxon signed rank test. The levels of platelet phagocytosis, PAIgG, platelet count between HVs and patients with DV infections, and platelet count between patients with DF and DHF were analyzed using the Mann–Whitney U test. The significance of the correlations was estimated using the Pearson correlation; P < 0.05 was considered to be significant. The SPSS statistical software, version 13.0 (SPSS Inc., Chicago, IL) was used for data analysis.

**RESULTS**

Of the 36 patients with secondary DV infections, 24 and 12 were diagnosed as DF and DHF, respectively (Table 1). Twelve patients with DHF were further classified into DHF I (N = 2) and DHF II (N = 10). No cases of DHF III or IV were included. The peripheral platelet counts were significantly lower in patients with DV infection than those in HVs (P < 0.05). Although the platelet counts and the increase in the
hematocrit were significantly different in patients with DHF from patients with DF ($P < 0.05$), no significant difference was found between those two subgroups with respect to the demographic data, which includes age and days after onset. The levels of PAIgG were significantly higher in patients with DV infection than those in HVs ($P < 0.05$). Although the levels of PAIgG were higher in DHF patients than those in DF patients, no significant difference was found between those two subgroups, which is in disagreement with our previous report.9

Representative data of the frequency of platelet phagocytosis in a single experiment are shown in Figure 1. The frequencies of phagocytosis were 20.8% for platelets from an HV that were pretreated with anti-platelet MAb (Figure 1A), 3.2% for untreated platelet from an HV (Figure 1B), and 16.8% for untreated platelets from a patient with DF (Figure 1C). The values of percent phagocytosis were 15.4% for an HV and 80.8% for the patient with DF, respectively. The percent phagocytosis was significantly higher in the total number of patients with secondary DV infections than in the total number of HVs ($P < 0.05$, Table 1). The percent phagocytosis was similarly higher in DHF patients than in DF patients, but no significant difference was found between those two subgroups.

Between the acute and convalescent phases, the changes in platelet counts and percent platelet phagocytosis or PAIgG were compared in 36 patients with a secondary DV infection. 8,9 Although no correlation between platelet count and percent phagocytosis of platelets among patients with an acute phase of secondary DV infection and healthy volunteers was noted, the presence of immune complexes on the platelets may contribute to the increased hematocrit, which is consistent with our previous results. 8,9 A significant inverse correlation was also found between the platelet count and the percent phagocytosis (Figure 3B) among these subjects. On the other hand, no significant correlation was found between the percent phagocytosis and the levels of PAIgG among these patients (Figure 3C).

**DISCUSSION**

In this study, we demonstrated that the percent phagocytosis of platelets from patients during the acute phase of secondary DV infection was significantly increased, compared with those from healthy volunteers, using an in vitro assay. The percent phagocytosis of platelets was significantly inversely correlated with platelet count during the acute phase among these patients, although no significant correlation was found between the percent phagocytosis of platelets and PAIgG levels. Because we previously detected anti-DV IgG and DV RNA on the platelets from patients with secondary DV infections, but not from healthy volunteers, the presence of immune complexes on the platelets may contribute to the increased phagocytosis of platelets among patients with an acute phase of secondary DV infection. 8,9 Although no correlation between the levels of PAIgG and platelet phagocytosis was found in this study, the values of PAIgG, which were determined by a competitive ELISA using anti-human IgG, may not reflect the amount of anti-DV IgG on the platelets in each individual case. A correlation between the levels of platelet-associated

---

**Table 1**

<table>
<thead>
<tr>
<th>Diagnosis (n)</th>
<th>Age (years)</th>
<th>Days after onset</th>
<th>% Increase in hematocrit</th>
<th>Platelet count ($×10^3$/µL)</th>
<th>PAIgG value (ng/10⁷ PLT)</th>
<th>Percent phagocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>HV (36)</td>
<td>20.1 ± 7.1</td>
<td>0</td>
<td>11.4 ± 10.1</td>
<td>322.7 ± 98.1</td>
<td>3.2%</td>
<td></td>
</tr>
<tr>
<td>DV infection (36)</td>
<td>20.2 ± 5.7</td>
<td>0</td>
<td>47.5 ± 70.7</td>
<td>53.1 ± 28.0*</td>
<td>55.5 ± 60.4*</td>
<td></td>
</tr>
<tr>
<td>DF (24)</td>
<td>20.1 ± 5.3</td>
<td>0</td>
<td>34.5 ± 47.8</td>
<td>60.0 ± 24.3</td>
<td>47.7 ± 52.8</td>
<td></td>
</tr>
<tr>
<td>DHF (12)</td>
<td>20.3 ± 6.8</td>
<td>0</td>
<td>71.5 ± 98.4</td>
<td>29.9 ± 9.9**</td>
<td>71.8 ± 73.2</td>
<td></td>
</tr>
<tr>
<td>HV (36)</td>
<td>20.1 ± 7.1</td>
<td>0</td>
<td>11.4 ± 10.1</td>
<td>322.7 ± 98.1</td>
<td>3.2%</td>
<td></td>
</tr>
<tr>
<td>DV infection (36)</td>
<td>20.2 ± 5.7</td>
<td>0</td>
<td>47.5 ± 70.7</td>
<td>53.1 ± 28.0*</td>
<td>55.5 ± 60.4*</td>
<td></td>
</tr>
<tr>
<td>DF (24)</td>
<td>20.1 ± 5.3</td>
<td>0</td>
<td>34.5 ± 47.8</td>
<td>60.0 ± 24.3</td>
<td>47.7 ± 52.8</td>
<td></td>
</tr>
<tr>
<td>DHF (12)</td>
<td>20.3 ± 6.8</td>
<td>0</td>
<td>71.5 ± 98.4</td>
<td>29.9 ± 9.9**</td>
<td>71.8 ± 73.2</td>
<td></td>
</tr>
</tbody>
</table>

$^*$PLT = platelets; HV = healthy volunteer; DV = dengue virus; DF = dengue fever; DHF = dengue hemorrhagic fever.  
$^{*P < 0.05}$ (versus HV), $^{**P < 0.05}$ (versus DF).

---

**Figure 1.** The frequencies of phagocytosis of platelets from a healthy volunteer (HV) with (A) or without anti-platelet monoclonal antibody treatment (B), or untreated platelets from a patient with dengue fever (DF) (C) are shown. The CellTracker Orange CMTMR (CTO) positive and CD61 negative cells (upper-left region) were considered to be the differentiated THP-1 cells that ingested platelets. The values of percent phagocytosis were determined to be 15.4% for an HV and 80.8% for a patient with DF according to the formula described in the Materials and Methods.
anti-DV IgG and the percent phagocytosis should be examined, although the assay for platelet-associated anti-DV IgG is not currently available.

Mitrakul and others\textsuperscript{17} reported that platelet survival was shortened in patients with an acute phase of DHF using radioactive platelets more than 30 years ago. They suggested that the damaged platelets were being trapped and sequestered in the liver rather than in the spleen, as is usually the case in this disease. Their finding of platelet clearance in the livers of patients with an acute phase of DHF could be explained, in part, by an increased phagocytosis of platelets as shown by the results of the present study.

We previously reported a lack of efficacy of a high dose of intravenous immunoglobulin for patients with secondary DV infection, and suggested that platelet clearance by macrophages through Fc\(\gamma\) receptors was not a primary mechanism in this disease.\textsuperscript{18} Furthermore, in a preliminary experiment on an inhibition assay of phagocytosis of platelets from a patient of DF, a partial inhibition of platelet phagocytosis by differentiated THP-1 cells was found with mouse anti-human complement receptor 3 (CR3) (CD11b) MAb, compared with control MAb. Because complement activation mediated by circulating viral antigen is involved in the pathogenesis of this disease,\textsuperscript{19,20} platelet clearance by macrophages through CR3 may be involved in this disease. Further studies of inhibition assays are required to draw conclusions for the precise molecular mechanisms of platelet phagocytosis in secondary DV infections.

Platelets, anucleated blood cells, may undergo an apoptotic program. Treatment of platelets with a variety of platelet agonists induces apoptosis and caspases, which are key effectors of apoptosis, are involved in this \textit{in vitro} phenomenon.\textsuperscript{21,22} Brown and others\textsuperscript{23} reported an increased expression of proapoptotic proteins by flowcytometry and morphologic changes similar to those of granulocyte apoptosis found by electron microscopy in aged platelets. Another possible mechanism for the increased phagocytosis of platelets from patients in this study could be the scavenger receptor-mediated phagocytosis.

In conclusion, this study demonstrated an increased phagocytosis of platelets freshly isolated from patients during the acute phase of secondary DV infection in an \textit{in vitro} assay employing differentiated THP-1 cells. Increased platelet phagocytosis was significantly associated with thrombocytopenia during the acute phase of this disease. Further studies are...
required to determine the molecular mechanisms of platelet phagocytosis by macrophages in a secondary DV infection.

Received July 9, 2008. Accepted for publication January 16, 2009.

Acknowledgments: We thank the staff of San Lazaro Hospital and the Research Biotechnology Division, St. Luke’s Medical Center.

Financial support: This study was supported by a Grant-in-Aid for Scientific Research (B: 16460609) from the Ministry of Education, Science and Culture, Japan and the 21st Century Center of Excellence (COE) Program of Nagasaki University.

Authors’ addresses: Shoko Honda and Shingo Inoue, Department of Internal Medicine and Virology, Institute of Tropical Medicine Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. Mariko Saito, Department of Virology, Graduate School of Medicine, Tohoku University, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. Efren M. Dimaano and Philip A. Morales, Blood Borne Diseases, San Lazaro Hospital, Manila, Philippines. Maria T. G. Alonzo, Ronald R. Matias, and Filipinas F. Natividad, Research and Biotechnology Division, St. Luke’s Medical Center, 279 E. Rodriguez Sr. Boulevard, Cathedral Heights, Quezon City, Philippines 1102. Lady-Anne C. Suarez, E-mail: suarezlacs@yahoo.com. Natsuki Koike, E-mail: nakkey2@yahoo.co.jp. Atsushi Kumatori, Faculty of Risk and Crisis Management, Chiba Institute of Science, Choshi 298-0025, Japan. Kazunori Oishi, Laboratory for Clinical Research on Infectious Diseases, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan, Tel: +81-6-6879-4253, Fax: +81-6-6879-4255, E-mail: oishik@biken.osaka-u.ac.jp.

Reprint requests: Kazunori Oishi, Laboratory for Clinical Research on Infectious Diseases, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Osaka 565-0871, Japan, Tel: +81-6-6879-4253, Fax: +81-6-6879-4255, E-mail: oishik@biken.osaka-u.ac.jp.

REFERENCES