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Galectin-9 plasma levels reflect adverse hematological and immunological features in acute dengue virus infection☆

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ABSTRACT

Background: Dengue virus (DENV) infection remains a major public health burden worldwide. Soluble mediators may play a critical role in the pathogenesis of acute DENV infection. Galectin-9 (Gal-9) is a soluble β-galactoside-binding lectin, with multiple immunoregulatory and inflammatory properties.
Objective: To investigate plasma Gal-9 levels as a biomarker for DENV infection.
Study design: We enrolled 65 DENV infected patients during the 2010 epidemic in the Philippines and measured their plasma Gal-9 and cytokine/chemokine levels, DENV genotypes, and copy number during the critical and recovery phases of illness.
Results: During the critical phase, Gal-9 levels were significantly higher in DENV infected patients compared to healthy or those with non-dengue febrile illness. The highest Gal-9 levels were observed in dengue hemorrhagic fever (DHF) patients (DHF: 2464 pg/ml; dengue fever patients (DF): 1407 pg/ml; non-dengue febrile illness: 616 pg/ml; healthy: 196 pg/ml). In the recovery phase, Gal-9 levels significantly declined from peak levels in DF and DHF patients. Gal-9 levels tracked viral load, and were associated with multiple cytokines and chemokines (IL-1α, IL-8, IP-10, and VEGF), including monocyte frequencies and hematologic variables of coagulation. Further discriminant analyses showed that eotaxin, Gal-9, IFN-α2, and MCP-1 could detect 92% of DHF and 79.3% of DF, specifically ($P < 0.01$).
Conclusion: Gal-9 appears to track DENV inflammatory responses, and therefore, it could serve as an important novel biomarker of acute DENV infection and disease severity.
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1. Background

Dengue is caused by the dengue virus (DENV), which belongs to the family Flaviviridae, genus *Flavivirus*, and is now emerging as one of the most rapidly spreading mosquito-borne viral diseases worldwide.[1,2] DENV has an incubation period of 3–7 days, where after the symptoms suddenly appear. Clinically, the onset of symptoms is rapid and follows 3 distinct phases: (1) an initial febrile phase on days 1–3 of illness; (2) a critical phase on days 4–6 of illness, which coincides with defervescence; and (3) a spontaneous recovery phase on days 7–10 of illness. Dengue fever (DF) is accompanied by a high fever, headaches, severe myalgia, and rash. Severe DENV infection complications can occur resulting in dengue hemorrhagic fever (DHF), which is characterized with clinical and laboratory features of thrombocytopenia, coagulation abnormalities, and plasma leakage in children and worse outcomes in adults

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presenting with increased incidences of bleeding, shock and organ failure.[3,4]

It is thought that following acute DENV infection, the high viral load triggers an activated immunological state, resulting in the release of inflammatory cytokines, chemokines, immune complexes, and other inflammatory mediators.[5] During the evolution of DENV infection, both pro-inflammatory and anti-inflammatory cytokines and chemokines are induced, suggesting that multifactorial mediators are also involved in DENV-induced pathogenesis.[6–8]

Galectins constitute a family of mammalian lectins that have an affinity for β-galactoside. These proteins are released into the extra-cellular environment under stress conditions such as infectious, during which they serve as "danger signals" or exert their actions on other cells.[9] Galectin-9 (Gal-9) was first described as an eosinophilic chemoattractant.[10,11] Since then, Gal-9 is reported to be produced by both T and endothelial cells,[12,13] and its functions as a bidirectional immunoregulator was recently described.[14,15] We previously described increases in Gal-9 and histamine levels in an allergic patient and suggested that the activation of mast cells is associated with elevation in Gal-9 levels.[16] We also reported a marked elevation of Gal-9 in acute human immunodeficiency virus (HIV) infection and a rapid decrease after anti-retroviral therapy, and our data from that study suggested that Gal-9 could be a potential danger signal biomarker of acute virus infection.[17,18]

2. Objectives

To examine the kinetics and activities of Gal-9 in DENV infection and its association with other circulating plasma mediators during the course of acute DENV infection.

3. Study design

3.1. Patients and specimens

We conducted a study at the San Lazaro Hospital in Manila, Philippines, which included 65 serially recruited patients with a clinical diagnosis of DF and DHF.[19] In 2010, there were consecutive cases of dengue in this hospital, and we enrolled patients who met the study's inclusion criteria. None of the patients included in our study died, and all of them were discharged from the hospital when their condition improved. EDTA plasma and serum were obtained by centrifugation of peripheral blood at 3000 rpm for 10 min, and were aliquoted into 1.2 ml micro tubes and stored at −80 °C until use. Specimens were collected at 2 time points during illness of the critical phase (on days 4–5) and the recovery phase (on days 7–8). All enrolled patients underwent laboratory tests, their medical histories were recorded, and they were physically examined by resident clinicians. Plasma was also obtained from 30 demographically matched healthy controls (HCs). HCs were donors who came to the Hospital for annual health checks or who volunteered at the Hospital. In addition, 90 patients with non-dengue febrile illness, who had visited San Lazaro Hospital, were enrolled. These patients were clinically diagnosed with leptospirosis, confirmed by serological analysis and/or microscopic agglutination test.[20] Plasma from patients with non-dengue febrile illness was collected at the time of admission.

3.2. Serological analysis

Primary and secondary DENV infections were confirmed by determining antiviral IgM and/or IgG antibodies levels using sera (The Panbio Duo Dengue IgM and IgG Capture enzyme-linked immunosorbent assay (ELISA), Panbio, Queensland, Australia).[21]

3.3. RNA extraction

Genomic viral RNA was extracted from 140 μl of each patient serum (critical phase, $n = 65$) using the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany). The extracted RNA was stored at −80 °C until further use.

3.4. DENV genotyping

DENV genotyping was performed by the dengue genotype-specific reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) method.[22] The RT-LAMP reaction was carried out in a 25 μl reaction mixture with the use of the Loopamp RNA Amplification Kit (Eiken Chemical Co., Ltd., Tokyo, Japan), and it was performed with 1 μl of template RNA. The reaction mix was incubated at 60 °C for 60 min in a Loopamp real-time turbidometer LA-320C (Teramecs, Kyoto, Japan). Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were taken. The results were confirmed by LA-320C software.

3.5. Real-time RT-PCR and DENV quantification

The DENV copy number in plasma was measured by a TaqMan® one-step real-time RT-PCR as described previously.[23] The real-time RT-PCR primers and hydrolysis probe specific to the 3′ untranslated region (UTR) of the four-dengue genotypes were described previously.[24] In this study, hydrolysis probe was labeled by FAM at the 5′ end and BHQ-1 at the 3′ end. The real-time RT-PCR assay was performed using the SuperScript® III Platinum One-Step qRT-PCR Kit (Invitrogen, USA), according to the manufacturer's instructions. Quantitative standard RNA or each DENV genotype was performed using the *in vitro* transcription of the pCR®2.1-TOPO® vector (Invitrogen, USA), which was cloned at the 3′ UTR for each DENV genotype: genotype 1 (strain 99St-12A; GenBank accession no GU377286), genotype 2 (00St-22A; GU377287), genotype 3 (SLMC50; GU377288), and genotype 4 (SLMC318; GU377289), respectively. The target RNA copy number was calculated, and 10-fold serial dilutions ranging from 10^2 to 10^5 RNA copies per microliter were used for quantification standards. One microliter of RNA standard or extracted RNA was used as template per reaction. Virus titer in each reaction was calculated using 7500 System Software (Applied Biosystems, USA).

3.6. Galectin-9 and cytokine/chemokines detection assay

Plasma Gal-9 was quantified by means of ELISA, as previously described.[17] Briefly, the sandwich ELISA consists of anti-human Gal-9 monoclonal antibodies (clone 9S2-3; GalPharma, Takamatsu, Japan) and biotinylated-anti-human Gal-9 polyclonal antibodies (GalPharma, Takamatsu, Japan) as a coating and detection antibodies, respectively. Colorimetric analysis was carried out using streptavidin-conjugated horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA, USA) and tetramethyl benzidine (KPL, Gaithersburg, MD, USA). Gal-9 concentration was quantified using a standard curve constructed with recombinant human Gal-9 (GalPharma, Takamatsu, Japan). Plasma samples were also assayed for 29 selected cytokines and chemokines (EGF, eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IL-1ra, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β, and VEGF) using a Milliplex Human Cytokine and Chemokine multiplex assay Kit (Merck Millipore, Billerica, MA, USA). The experiments were performed according to the manufacturers' instructions using a Luminex 200 System (Luminex Corporation, Austin, USA).[25]

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