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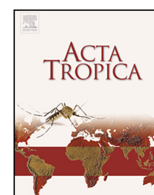

Magnitude of viremia, antigenemia and infection of circulating monocytes in children with mild and severe dengue

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ABSTRACT

Dengue is a major public health problem in tropical regions around the world. Viral and immune host factors determine the clinical courses of the infection. We analyzed the dynamics of viremia (by real-time polymerase chain reactions), antigenemia (through detection of the viral non-structural protein [NS]-1 by enzyme-linked immunosorbent assays) and the frequency of virus-infected peripheral blood mononuclear cells (PBMCs) (by multiparametric flow cytometry) in children with primary or secondary dengue virus (DENV) infection in mild to severe cases. Additionally, we evaluated the association of these factors with clinical severity and laboratory parameters. The levels of viremia and antigenemia peaked during the early days of illness and these viral parameters were correlated (rho = 0.37, P = 0.003). Circulating monocytes were the most naturally infected subset within the PBMCs population, with kinetics similar to those of viremia and antigenemia. The levels of viremia and antigenemia were higher in children with primary infections than in those with secondary infections ($P \leq 0.04$). Although there were no associations between the three evaluated factors and clinical severity, the levels of plasma NS1 and the frequency of dengue virus-infected monocytes correlated with prolonged coagulation times. In short, the viremia, antigenemia and infected monocytes were detected early and were not related to clinical severity. The magnitude of antigenemia and infected circulating monocytes was associated with coagulation disorders.

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1. Introduction

Despite substantial efforts to reduce the high burden of dengue, it remains an important viral vector-borne disease worldwide (Bhatt et al., 2013). This disease is caused by an infection with any of four dengue virus (DENV) serotypes (DENV 1–4) and is transmitted to humans by *Aedes* genus mosquitoes. Dengue constitutes a clinically dynamic disease and is characterized by febrile, effervescence and recovery phases (Guzman and Harris, 2015). In addition to the absence of a specific treatment, the high number of cases of severe dengue is attributable to the lack of viral and host markers that efficiently predict the clinical outcome (John et al., 2015); therefore, the search for severity-associated factors is necessary.

Dengue virus infection is characterized by an early peak of viremia that decreases after the fourth day of fever onset and by the early presence of high levels of circulating non-structural protein (NS)-1, which is actively secreted from infected cells (Muller and Young, 2013; Simmons et al., 2012). The virus has tropism for several cell types such as endothelial cells, hepatocytes, dendritic cells and monocytes (Clyde et al., 2006), the latter being the most affected subset within the peripheral blood mononuclear cells (PBMCs) population (Kou et al., 2008).

The severe forms of dengue develop once the viremia and NS1 levels have decreased, suggesting that plasma leakage (the hallmark of severe dengue) is a consequence of virus-induced immune response. However, the greater the viremia and infection of target cells, the greater the production of expected inflammatory mediators (Srikiatkhachorn, 2009). Endothelial dysfunction in dengue is at least partially due to the direct effect of NS1 through complement activation (Avirutnan et al., 2006) and glycocalyx disruption (Puerta-Guardo et al., 2016). These parameters (viremia, antigenemia and natural infection of PBMCs) have been proposed as severity markers in DENV infection (Durbin et al., 2008; Libraty et al., 2002b; Srikiatkhachorn et al., 2012; Wang et al., 2003). However, this finding is highly modified by the dynamics of the disease and the study design (Singla et al., 2016). Thus, other reports did not find a relationship between these factors and clinical outcomes (Duong et al.,

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2011; Fox et al., 2011). In this study, we analyzed 59 children acutely infected with DENV, focusing on the dynamics and magnitude of viremia, antigenemia and infection of PBMCs as well as the association of these virological and host factors with clinical severity and laboratory parameters.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Ethics Committee at the Universidad Surcolombiana and the Hospital Universitario de Neiva. Written parental informed consent and assent (for children >6 years old) were obtained for each of the children included. This study was conducted in accordance with all Helsinki statements.

2.2. Study design

This study was conducted between May 2012 and September 2015 in the Laboratorio de Infección & Inmunidad of the Facultad de Salud, Universidad Surcolombiana. We included children between 1 month and 14 years of age with a clinical diagnosis of DENV infection who were treated either at the E.S.E Hospital "San Carlos" of Aipe (a primary health care hospital) or the Hospital Universitario de Neiva (a tertiary health care hospital), both located in the department of Huila in southern Colombia, an endemic area for DENV infection (http://www2.paho.org/col/dmdocuments/Guiadengue210310.pdf). Dengue infection was confirmed by the detection of plasma NS1, DENV-specific immunoglobulin (Ig) M and/or circulating DENV RNA in all the children included as described below.

2.3. Clinical samples

A blood sample was taken during the acute phase (2–7 days of the onset of fever); in a fraction of the patients (n = 15), a second sample in the convalescence phase (15–33 days after hospital discharge) was also obtained. Two to four milliliters of venous blood were collected in tubes containing ethylenediaminetetraacetic acid and centrifuged at 300 × g. The plasma was collected and stored at −70 °C until the time of analysis, and the cellular fraction was used for isolation of PBMCs. The diagnosis, classification and treatment of the patients were performed according to the revised guide of the World Health Organization (WHO) 2009, which classified the disease as dengue (including without warning signs [DNS] and dengue with warning signs [DWS]) and severe dengue (SD) (http://www.who.int/tdr/publications/documents/dengue-diagnosis.pdf). To determine the type of infection (primary or secondary), the ratio of DENV-specific IgM/IgG in the plasma was determined using a value ≤ 2 as a secondary infection, as previously reported (Castaneda et al., 2016; Innis et al., 1989). For this determination, the commercial enzyme-linked immunosorbent assay (ELISA) Dengue IgM Capture and Dengue IgG Capture kits were used (Panbio, Alere, Waltham, MA, Cats: E-DEN011 M and E-DEN02G, respectively) following the manufacturer's instructions.

2.4. Detection and quantification of DENV RNA

RNA purification was performed with the QIAmp viral RNA commercial kit (Qiagen, Valencia, CA, Cat: 52906) following the manufacturer's recommendations. The infecting serotype of DENV in plasma was initially identified through a conventional reverse transcription polymerase chain reaction (RT-PCR) following a previously described protocol (Lanciotti et al., 1992). The samples with detectable DENV RNA were then analyzed by real-time PCR (qPCR) for quantification of the absolute number of copies/mL using the Dengue Virus I, II and III Real Time RT-PCR Kits (Shanghai ZJ Bio-Tech, Shanghai, China, Cats: ER-0063-01, ER-0063-03 and ER-0064-01, respectively) and the Eco™ System (Illumina, San Diego, CA), as previously described (Perdomo-Celis et al., 2014). All the samples were analyzed in duplicate. The correlation coefficients of the standard curves were ≥98%, and the duplicate variability was ≤10%. The efficiency of the reactions was ≥87%. A Ct of 38 was selected as the cutoff. The number of DENV RNA copies/mL was obtained by interpolation of the Ct of the samples to the standard curve using a 4-parameter logistic regression using EcoStudy software v.5.0. The limit of detection of the assay was estimated at 50 copies/mL. For statistical purposes, for samples with undetectable viremia, a value equal to the half of the limit of detection was assigned.

2.5. Detection and semi-quantification of DENV NS1 in plasma

The Dengue Early ELISA kit (Panbio, Alere, Australia, Cat: E-DEN02P) was used for the semi-quantitation of DENV NS1 in plasma following the manufacturer's recommendations. Ten-fold dilutions starting from 1/2 dilution were performed. Samples with previously known undetectable NS1 by qualitative ELISA were included as negative controls, with mean OD 450 nm of 0.01 in the 1/2 dilution. Samples with at least double the value of the mean of the negative controls in the 1/2 dilution were reported as NS1 detectable. Because 45% of the samples analyzed did not reach the limiting dilution in the realized dilutions (until 1/20,000; data not shown), the value of DENV NS1 reported here was the OD 450 nm of the 1/200 dilution, where the linear phase was present in all samples and as previously used (de la Cruz-Hernandez et al., 2013).

2.6. Detection of DENV PreM protein in PBMCs

To preliminarily determine the optimum dose of the mouse anti-DENV PreM protein (clone D3-2H2-9-21, Millipore, Temecula, CA, Cat: MAB10217), Vero-76 cells (obtained from the ATCC, Cat: CRL-1587) treated with DENV-2 (kindly provided by Ivan Darío Velez, Universidad de Antioquia, Colombia) or with control for 48 h at 37 °C and 5% CO_2 were permeabilized with 300 μL of Cytofix/Cytoperm (BD, San Jose, CA, Cat: 554722). Five doses of the anti-DENV PreM antibody were tested, followed by the addition of 10 μL/10^6 cells of an allophycocyanin (APC)-labeled goat anti-mouse (GAM) IgG F(ab')2 (R&D Systems, Minneapolis, MN, Cat: F0101B) and incubated for 30 min. Finally, the cells were washed twice with 1X Perm/Wash solution (BD, San Jose, CA, Cat: 554723) and acquired in an FACS Canto II cytometer using FACS Diva v6.1.3 software (BD, San Jose, CA) within one hour of staining completion. A concentration of 0.04 μg/test of the mouse anti-DENV PreM antibody was chosen for subsequent experiments with PBMCs.

Peripheral Blood Mononuclear Cells were isolated through a ficoll density gradient and cryopreserved following a previously described protocol (Perdomo-Celis et al., 2016). The median [range] cryopreservation time was 17 [5–29] and 18 [5–31] months for acute and convalescence samples, respectively. At the time of analysis, the PBMCs were thawed at 37 °C and washed with RPMI-1640 supplemented with 10% FBS, penicillin 100 U/mL, streptomycin 100 μg/mL and L-glutamine 2 mM. They were then counted with Trypan blue exclusion staining, with a median [range] percentage of non-viable cells after cryopreservation of 15 [1–37] and 5 [5–31] for acute and convalescence samples, respectively (P = 0.0002, Mann-Whitney test, data not shown), which was consistent with our previous report (Perdomo-Celis et al., 2016). Then, the cells were washed with 3 mL of FACS buffer (0.5% bovine serum albumin [BSA] [Sigma-Aldrich, St. Louis, MO], 0.02% sodium azide [Merck,

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