Levels of Circulating Tumor Necrosis Factor-α in Children with Symptomatic Dengue Evaluated by ELISA and Bead-Based Assays

Federico Perdomo-Celis,¹ Doris M. Salgado,¹,² and Carlos F. Narváez¹

Abstract

Tumor necrosis factor (TNF)-α is a key cytokine in the pathogenesis of dengue virus infection, and its accurate detection in several types of human samples is critical. The enzyme-linked immunosorbent assay (ELISA) is the gold standard for the detection of TNF-α, but multiplexed bead-based assays such as cytometric bead array (CBA) are now frequently used. Here, using ELISA and two CBAs commercially available, we measured TNF-α concentrations in plasma and serum from children with acute dengue virus infection and healthy controls. To evaluate the detection efficiency and factors affecting it, spiked recovery and immune complex dissociation assays were also performed. The levels of TNF-α evaluated by ELISA in paired serum and plasma samples from children with dengue positively correlated ($\rho = 0.99$, $p < 0.0001$). Children with dengue had higher levels of plasma TNF-α than those of healthy children ($p = 0.004$). The ELISA detected TNF-α in a higher number of plasma samples than the CBA ($p < 0.0001$), and both methods only correlated when TNF-α was evaluated in buffer-based solutions but not in plasma, indicating the presence of a factor interfering with the detection of TNF-α in plasma. The recovery of several types of human recombinant TNF-α was dramatically decreased in plasma but not in tissue culture media ($p \leq 0.01$), and this effect was similar in the plasma obtained from the children with dengue or the healthy controls. The dissociation of immune complexes did not improve TNF-α recovery. Dilution of the plasma samples increased the recovery of TNF-α, but at high concentrations of the cytokine. In short, plasma affects the efficiency of TNF-α detection, and this effect should be considered in the measurement of this cytokine.

Keywords: dengue virus, tumor necrosis factor-α, ELISA, cytometric bead array, plasma, serum

Introduction

TUMOR NECROSIS FACTOR (TNF)-α is a major cytokine (CT) implicated in anti-pathogen immunity and is secreted by a large range of immune cells, such as monocytes-macrophages, dendritic cells, T cells, and others (8). TNF-α has two known receptors (TNF receptor I and II) that activate intracellular signaling pathways leading to the recruitment of nuclear factor-kB and cysteine-aspartic proteases, which trigger inflammatory responses and apoptosis, respectively (28). During the immune response, TNF-α induces the expression of adhesion molecules by leukocytes and endothelial cells, the production of prostaglandins with subsequent vasodilatation and increased vascular permeability, the activation of antigen presenting cells with increased expression of major histocompatibility complex molecules, the production of other inflammatory CTs such as interleukin (IL)-1β and IL-6, and increasing phagocytosis (8,45). Due to its involvement during infection and inflammation, TNF-α is often measured in human samples.

Several factors affect the efficiency of the detection of CTs in biological samples, and in some cases, the results obtained may not reflect the true concentrations (38). Some biological factors that affect detection include the CT kinetics or the binding of proteins or soluble decay receptors that could be increased during the acute phase of infections (7). Technical factors include the type of the sample and its handling, ability of the operator, and type of assay used (53).

For the measurement of TNF-α, the enzyme-linked immunosorbent assay (ELISA) is the most widely used method and is considered the gold standard (19). However, bead-based assays, such as the cytometric bead array (CBA),
allow for the analysis of a larger number of analytes in a smaller sample volume with a reported higher sensitivity, and thus the use of CBA has increased in recent years (37).

Dengue is a disease in which TNF-α is critical. This disease is caused by infection with the dengue virus (DENV) and is an important viral vector-borne disease worldwide (4). During the acute phase of infection, high serum and plasma levels of TNF-α, such as IL-2, IL-6, IL-8, IL-10, interferon-γ, and TNF-α have been reported (21, 34). Moreover, these CTS have been implicated in the development of vascular leakage and bleeding, which are the hallmarks of this disease (10, 47), and in some instances, the plasma or serum levels of these CTS have been associated with clinical severity (29, 33, 42). However, the case of TNF-α is peculiar. Although TNF-α levels have been reported to increase in patients with dengue in comparison with those of healthy controls (12, 35, 49), the plasma and serum concentrations of TNF-α are typically very low (3, 39, 43). In other cases, no differences were found in the plasma levels of TNF-α between the acute and convalescent phases of the disease (46). Likewise, the relationship between the levels of TNF-α and clinical severity has been inconsistently reported, and positive (2), negative, or no associations have been shown (31). These discrepancies are less dramatic when TNF-α is evaluated in cell culture supernatant or other buffers (11, 25). As the analysis of TNF-α in dengue and other infections and autoimmune diseases is an essential requirement, studies that evaluate the factors affecting its detection are necessary.

Here, the efficiency of TNF-α measurement in plasma and serum obtained from children with confirmed DENV infection and healthy controls was evaluated using commercially available ELISA and bead-based assays. The recovery of human recombinant TNF-α from plasma and tissue culture media was also assessed. Together, the results demonstrate a low efficiency in the detection of TNF-α in plasma obtained from children with dengue disease controls, particularly when it is evaluated using CBA. This effect should be taken into account when using this type of sample.

Materials and Methods

Ethics statement

This study was approved by the Committee on Ethics of the Hospital Universitario de Neiva. Informed consent and assent (for children over 6 years of age) were obtained from all the subjects included. All experiments followed the principles expressed in the Declaration of Helsinki.

Patients and samples

This study was conducted from April 2009 to August 2015 in the Laboratorio de Infecció & Immunidad of the Facultad de Salud, Universidad Surcolombiana. Children between 1 and 14 years old with confirmed acute DENV infection (n = 94) on the fourth to eighth day of fever onset and healthy children who served as controls (n = 20) were included in this study. For the inclusion of the control group, a medical examination was performed for the pediatric department of the Hospital Universitario de Neiva, and any ongoing disease was ruled out. For the diagnosis, classification, and clinical monitoring of the dengue patients, the revised guidelines of the World Health Organization 2009 (1) were followed. The diagnosis of infection was confirmed by plasma evidence of the viral nonstructural protein (NS1) (Dengue Early ELISA, Ref. E-DENG202, and Dengue-specific immunoglobulin (IgG) W Dengue IgG Capture ELISA, Ref. E-DENG101, both from Penton, Marlton, MA), which were detected using ELISA and following all manufacturer's instructions. The ratio of serum IgM primary or secondary was determined using the dengue-specific IgG IgM ratio and the latter evaluated with the Penton Dengue IgG Capture kit (Ref. E-DENG202), where a ratio > 1 represented a secondary infection (24).

Two to four milliliters of venous blood was collected in tubes containing ethylene-diamine-tetra-acetic acid (EDTA) and BD Vacutainer, San Jose, CA, Cat. No. 367812. To obtain paired serum samples, venous blood was taken in tubes without anticoagulants from some children (n = 30, BD Vacutainer, Cat. No. 367812). Within 1 h of collection, the tubes were centrifuged at 300 g for 10 min, and the plasma and serum were immediately collected and stored at -70°C until the time of analysis. All of the plasma and serum samples were obtained in the morning hours. The median (range) storage time of samples from children with dengue and the healthy controls were 25 (5-85) and 25 (14-79) months, respectively (p = 0.1, Mann-Whitney test). Due to limited sample volume, some of the children were not included in all the analyses.

Additionally, adult patients with lung diseases such as suspected pulmonary tuberculosis, bacterial pneumonitis, or tumors, (n = 50) who underwent a diagnostic bronchoscopy with bronchoalveolar lavage BAL, were also included. All of these participants were sero-negative for human immunodeficiency Virus infection. To obtain the BAL, 80 mL of sterile normal saline solution (Baxter, Deerfield, IL, Cat. No. 4889102) was added through a flexible bronchoscope Fujinon, Saitama, Japan, Ref. EB-470S), retained during the procedure and at least 5 mL was collected, centrifuged at 300 g for 10 min, filtered through a 0.22 μm filter (Merck Millipore, Billerica, MA, Cat. No. 3002025) before storage at -70°C. The bronchoscopy procedure was completed by a trained pulmonologists.

Evaluation of TNF-α concentrations by ELISA and CBA

At the time of analysis, each plasma, serum or BAL sample was thawed at room temperature and centrifuged at 2,000 rpm for 10 min. The concentration of TNF-α was evaluated by ELISA using a commercial kit (DuolSet Human TNF-α, R&D, Minneapolis, MN, Cat. DY-210) following all of the manufacturer’s recommendations. The reported detection limit was 3 pg/mL. The mean optical density (OD) of the negative controls (diluent medium alone) at 450 nm was 0.006. TNF-α concentrations were calculated by interpolation of OD of the samples to a standard curve using 4-parametric logistic regression with GraphPad Prism® software (GraphPad Software, La Jolla, CA). In the case of CBA, the Human TNF-α N 혹은 Kit II (BD, Cat. No. 551800) and the human TNF Enhanced Sensitivity Flex Set (BD, Cat. No. 561516) were used following all of the manufacturer’s recommendations. The assay's limit of detection for TNF-α was 2.8 pg/mL for the BD Flex Set II and 0.007 pg/mL for the Enhanced Sensitivity Flex Set. The FACSA Diva software (v. 6.1.3) and FCAP (v. 3.0.14) software, both from BD, were used for bead acquisition and calculation of CT levels, respectively. The ELISA and CBA analyses of plasma and serum were simultaneously completed using the same samples. The samples were analyzed in duplicate, with a
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variability of less than 10% was noted. Only ELISA and CBA experiments with standard curves with R² > 0.98 and significant p values were included.

Human recombinant TNF-α spike recovery assays

Known amounts of human recombinant TNF-α (hrTNF-α, R&D; Cat: 840121 and BD; Cat: 51-9003509) were added to plasma (from children with dengue and healthy controls) or to sterile tissue culture media (RPMI 1640; Gibco, Carlsbad, CA; Cat: 11875-085). After incubation for 30 min at 37°C, the samples were analyzed by ELISA and/or CBA as described above. Additionally, in some experiments, two-fold serial dilutions of plasma samples after the addition of the hrTNF-α were performed using 1% Bovine Serum Albumin (BSA, Sigma-Aldrich, St. Louis, MO; Cat: A7906) in 1xDPBS (Gibco; Cat: 21600-069), pH 7.3, filtered, as a diluent medium. The concentrations of hrTNF-α in the diluted samples were interpolated from the ELISA standard curve (as described in the Evaluation of TNF-α concentrations by ELISA and CBA section) and then multiplied by the dilution factor. The percentage of hrTNF-α recovery was obtained as previously reported (41):% Recovery = (spiked sample result−unspiked sample result)/known spike added concentration × 100.

Dissociation of immune complexes

A previously described protocol for the dissociation of immune complexes in plasma was applied (27,30). Briefly, 100 µL of plasma with and without hrTNF-α were mixed with 50 µL of 0.5 N hydrochloric acid (Merck, Darmstadt, Germany; Cat: K21327871 488; pH 2.5−3), incubated for 60 min at 37°C, and neutralized with 50 µL of 0.5 N sodium hydroxide (Merck; Cat: B676398 522; pH 6.8−7.2). The concentrations of hrTNF-α were measured by ELISA in the treated and untreated pared samples. To prove the efficiency of the protocol used here, we evaluated the level of dissociation of DENV-specific IgM - DENV complexes in plasma from three children with secondary acute DENV infection. After the pH treatment, four 10-fold serial dilutions of the samples were performed, and the DENV-specific IgM was semi-quantified in duplicate using a commercial ELISA (Dengue IgM Capture ELISA, Panbio, Alere, Waltham, MA; Ref: E-DEN01 M). The dissociation of the immune complexes was demonstrated by increased absorbance at OD450 nm after treatment.

Statistical analysis

GraphPad Prism® 7.0 software for Mac was used for the statistical analysis. The data are presented as medians and ranges. For statistical purposes, a value equal to half of the reported limit of detection of the assays was assigned to samples with TNF-α levels below the cutoff. The Mann−Whitney and Wilcoxon tests were used to analyze two independent or dependent groups, respectively. To analyze more than two independent or dependent groups, the Kruskal−Wallis and Friedman tests were used, respectively. If the Kruskal−Wallis or Friedman p value was <0.05, Dunn’s post hoc test was used. The degree of correlation between variables was determined using the Pearson (r) test. Fisher’s test was used for frequency analysis. A p value <0.05 was considered significant.

Results

Patients included

This study included 94 children with confirmed acute DENV infection (20 primary and 74 secondary infections, as determined by the dengue-specific IgM/IgG ratio) and 20 healthy children as controls. The median (range) day of illness when the children with dengue were included was 5 (4−8). Table 1 shows the age and the laboratory characteristics of the children included. As expected, the children with dengue had lower leukocyte and platelet counts than those of the healthy controls (p=0.003 and p=0.001, respectively, Mann−Whitney test, Table 1), but there were no differences in the levels of hemoglobin and hematocrit (p=0.3 and p=0.08, respectively, Mann−Whitney test, Table 1). In the adult patients with lung diseases (age, median [range]: 45 years [18−88]), 7 patients (14%) had active pulmonary tuberculosis (confirmed by mycobacterial culture and/or real time polymerase chain reaction), 33 patients (66%) had bacterial pneumonia, 5 patients (10%) had tumors, and 5 patients (10%) had pulmonary tuberculosis sequelae (data not shown).

The level of TNF-α measured in plasma is comparable to that in serum

Although serum is frequently used for the measurement of CTs, its use does not allow for the isolation of peripheral blood mononuclear cells or other cellular analyses for which anticoagulated blood is required. Therefore, plasma in those cases is preferred over serum, and the comparison of its performance with that of serum is necessary. To determine whether plasma is comparable to serum for the detection of TNF-α, the concentration of this CT was determined by ELISA in both paired types of samples from the children with dengue. As is shown in Figure 1A, the levels of TNF-α evaluated in serum and plasma strongly correlated (r=0.99,

<table>
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<th>Parameter</th>
<th>Healthy (n=20)</th>
<th>Dengue (n=94)</th>
<th>Mann−Whitney test p-value</th>
</tr>
</thead>
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<tr>
<td>Age, months, median (range)</td>
<td>96 (48−132)</td>
<td>52 (13−172)</td>
<td>0.2</td>
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<tr>
<td>Leukocytes, cells/µL×10⁹, median (range)</td>
<td>7.6 (5.6−10.9)</td>
<td>4.6 (2.4−10.1)</td>
<td>0.003</td>
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<tr>
<td>Hemoglobin, g/dL, median (range)</td>
<td>12.1 (11.2−15.7)</td>
<td>12 (10.6−14.7)</td>
<td>0.3</td>
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<tr>
<td>Hematocrit, %, median (range)</td>
<td>36.6 (33.8−47.3)</td>
<td>38.5 (31.5−40)</td>
<td>0.08</td>
</tr>
<tr>
<td>Platelets, cells/µL×10³, median (range)</td>
<td>290 (262−460)</td>
<td>87 (42.0−179.9)</td>
<td>0.001</td>
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</table>

Not all of the children with dengue or all of the healthy children were included in each reported experiment.
FIG. 1. Comparison of plasma and serum for the detection of TNF-α (A) The levels of TNF-α in the plasma and serum from children with dengue were measured by ELISA, and a correlation between both types of samples was determined. Pearson’s test (r and p-value) is shown. (B) The levels of TNF-α in the plasma from the children with dengue and the healthy controls were measured by ELISA. The r and p-value of the Mann-Whitney test is shown. The dotted lines indicate the median for each group. The dashed line indicates the detection limit of the assay. The number of samples analyzed is shown at the top. TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay.

p < 0.0001, Pearson test), indicating that the detection of the CT in both types of samples is comparable. The evaluation of TNF-α in plasma also allowed for the determination of logistical differences between the children with dengue and the healthy controls. Similar to the results of previous reports (2,39), low but detectable levels were obtained in the DENV-infected children when compared with those of the healthy controls (median pg/mL, range 4.6 [1.5-40] and 1.5 [1.5-41.2], respectively, p = 0.004, Mann-Whitney test, Fig. 1B). Similar results were also obtained when the samples were isolated from these two groups, was completed (p = 0.004, Mann-Whitney test, data not shown).

Efficiency of CBA and ELISA for the detection of TNF-α in plasma and buffer solutions

Plasma was compared to serum for the detection of TNF-α by ELISA. Because CBA offers a large number of advantages over ELISA, we next determined whether CBA is comparable to ELISA for the measurement of TNF-α in plasma. For this purpose, the levels of the TNF-α were determined in the plasma from the children with dengue by ELISA and two available CBA formats (Th1/Th2 cytokine kit II and TNF-α Enhanced Sensitivity Flex Set). As shown in Figure 2A, the levels of TNF-α evaluated by the CBA Th1/Th2 kit II were below the assay’s limit of detection in 93% of the samples, whereas 46% of the samples were undetectable by ELISA (p = 0.0001, Fisher’s test), indicating a higher detection rate of TNF-α by ELISA. When the second CBA format was used in some samples, detectable but very low amounts (in femtogram range) of TNF-α were noted in 100% of the samples analyzed (Fig. 2B). However, there was no correlation between the levels of TNF-α in plasma detected by ELISA and the CBA Th1/Th2 kit II (r = 0.03, p = 0.8, Pearson test, Fig. 2A) or between ELISA and the CBA Enhanced Sensitivity Flex Set (r = 0.2, p = 0.5, Pearson test, Fig. 2B), indicating a lower comparability between both assays for the measurement of this CT in plasma.

CBA was less efficient than ELISA for the detection of TNF-α in plasma, and we determined whether this efficiency depends on the assay or the type of sample used. TNF-α levels in the BAL from the adult patients with lung diseases were measured by ELISA and CBA Th1/Th2 kit II. As shown in Figure 2C, high levels of the CT were obtained by both methods in the BAL samples, with a positive correlation (r = 0.72, p = 0.0001, Pearson test, Fig. 2C). Together, these results indicate that CBA

FIG. 2. Comparison of ELISA and CBA by the detection of TNF-α in plasma and buffer solutions. Correlation between the levels of TNF-α in the plasma from children with dengue determined by ELISA from R&D and CBA Th1/Th2 kit II (A) and ELISA and CBA Enhanced Sensitivity Flex Set (B). (C) Correlation between the levels of TNF-α in bronchoalveolar lavage fluid (BAL) from the adult patients with lung diseases, determined by ELISA and CBA Th1/Th2 kit II. For all cases, Pearson’s determination coefficient (r and p-value) and the number of samples analyzed are shown at the top. The dashed lines indicate the detection limits of the assays. CBA, cytometric bead array.

A.V. Pastrana Borrero - Cra 1a. PBX: 8754753 FAX: 8758890 - 8759124
Edificio Administrativo Cra. 5 No. 23 - 40 PBX: 8753686
Linea Gratuita Nacional: 01800968722
www.usco.edu.co Neiva - Huila

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were obtained in healthy controls (Fig. 3A). Previously, it was shown that TNF-α sTNFRs complexes affect TNF-α quantification (14,36). Additionally, the levels of IL-1β in plasma have been found to be unmodified in DENV or L. mexicana infections due to the increase of the antagonist IL-18 binding protein (35). Thus, although we did not directly explore this issue, the interactions of CTs with their soluble receptors or binding proteins should be contemplated, and the simultaneous assessment of both is advised.

False positive or negative results can be caused by the presence of heterophilic- and auto-antibodies in the plasma or serum, which can cross-link capture and detection of anti-TNF-α. False positive results have been confirmed in acutely DENV infected samples (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could mainly in secondary infections (27), these molecules could main...
has a low efficiency for the detection of TNF-α in plasma but not in BAL (a buffer-based solution). As ELISA was more efficient for detection of TNF-α in plasma, this assay was used for the subsequent set of experiments.

Plasma affects the recovery of hTNF-α

To evaluate the effect of plasma in the detection of TNF-α, known concentrations of the hTNF-α were added to plasma samples or tissue culture media and then measured by ELISA, and the percentage of TNF-α recovery was determined. As shown in Figure 3A, in comparison with tissue culture media, a significantly lower percentage of hTNF-α recovery was obtained in plasma (mixed children with dengue and healthy controls) when low (10–100 pg/mL), intermediate (500–1,000 pg/mL), and high (5,000 pg/mL) concentrations of the recombinant CT were added (p < 0.01, Mann–Whitney test: Fig. 3A), indicating that plasma largely affects the measurement of TNF-α. Similar results were obtained with CBA, using the hTNF-α from both R&D and BD (n = 10, p < 0.01, plasma versus tissue culture media, Mann–Whitney test, data not shown). However, it is possible that the effect is not due to the plasma itself but is due to the condition of the individual from whom the sample was taken. For example, in acute DENV infection, increased levels of the soluble TNF receptors (sTNFRs) are found (2), and these proteins could act as decoys for the soluble TNF-α and interfere with its detection (14). Hence, the percentage of hTNF-α recovery (at the previously described concentrations) in the plasma from the children with dengue and the healthy controls was compared. As shown in Figure 3B, a higher percentage of hTNF-α recovery was obtained in tissue culture media when compared with the plasma from the children with dengue and the healthy controls (p < 0.0001, Dunn’s post-hoc test: Fig. 3B), but no difference was found between both pediatric groups (p = 0.9, Dunn’s post-hoc test, Fig. 3B). In summary, plasma affected the detection of hTNF-α, but this effect was independent of the condition of the individual from whom the sample was obtained.

The low detection of TNF-α is not due to the presence of circulating immune complexes

In addition to the presence of circulating decoy receptors, the low detection of TNF-α in plasma could be explained by the binding of antibodies (such as low affinity natural antibodies) to TNF-α. To establish a mechanism explaining the low detection of TNF-α in plasma, we hypothesized that the presence of circulating immune complexes (e.g., increased auto-antibodies) in the plasma obtained during DENV infection could interfere with the measurement of the CT (51). Thus, the recovery of TNF-α in plasma after the dissociation of immune complexes was evaluated. As shown in Figure 4A, the pH-based protocol for immune complexes dissociation was efficient, as higher DENV-specific IgM in the plasma from the children with secondary DENV infection was obtained when the samples were pH-treated in comparison with the untreated samples (p < 0.0005, Wilcoxon test). However, the pH-based treatment did not improve the hTNF-α recovery at both concentrations of 100 and 1,000 pg/mL of the added recombinant CT (p ≥ 0.3, Wilcoxon test, Fig. 4B). Therefore, the presence of circulating immune complexes does not explain the low detection of TNF-α in the plasma from the children with dengue.

Plasma dilution improves the detection of hTNF-α, but only at high concentrations of the CT

Together, the previous results showed that plasma affects the efficiency of TNF-α detection independent of the assay used or the condition of the individual, even after the addition of high concentrations of the human recombinant CT. The dilution of the sample, and thus the proteins and other soluble factors interfering with the TNF-α measurement, would improve the detection of this CT. Therefore, known concentrations of hTNF-α were added to the plasma from the children with dengue and the healthy controls, two-fold serial dilutions of the samples were completed, and the TNF-α levels were measured by ELISA. As shown in Figure 5, there were no differences in the detection of hTNF-α.
FIG. 4. Effect of circulating immune complexes in the detection of TNF-α in plasma. (A) Plasma samples from three children with secondary acute DENV infection were treated or not with a pH-based protocol for dissociation of immune complexes. Then, the DENV-specific IgM was evaluated by ELISA. All of the OD readings at 450 nm of four 10-fold serial dilutions (each one by duplicate) of the samples are shown. The p-value of the Wilcoxon test is shown. (B) Known concentrations of hTNF-α (100 and 1,000 pg/mL) were added to the plasma samples with dengue, followed by the dissociation of immune complexes using pH-based treatment. The levels of the CT were determined by ELISA. Box and whiskers plot is shown. NT, no treatment; pH, pH-based treatment; ns, not statistically significant by Mann–Whitney test.

in any of the assessed conditions (undiluted, 1/2 and 1/4 diluted plasma) when the added concentration was low or intermediate (plasma without addition of hTNF-α, 10 or 100 pg/mL) in the plasma from the children with dengue and the healthy controls (p > 0.5, Friedman test, Fig. 5A). However, when 1,000 pg/mL of hTNF-α was added, higher levels were recovered in the 1/4 dilution when compared with undiluted plasma in the DENV-infected and healthy children (p = 0.0008, Friedman test, Fig. 5B). In agreement with our previous results, lower than expected concentrations of TNF-α were obtained in each condition [median percentage of recovery of 35% and 28% in the children with dengue and the healthy controls, respectively]. Thus, only in the presence of high TNF-α concentrations does plasma dilution improve its detection.

Discussion

Several factors affect the measurement of CTs. The type of sample is critical, and some aspects have to be kept in mind during the choice of method. For example, serum lacks coagulation proteins, fibrinogen, and platelets, which can interfere with the measurement, although during the process of coagulation, some CTs such as IL-1β could be released (9). In the case of plasma, the type of anticoagulant is also important, and the use of EDTA results in lower inter-assay variability versus heparin or citrate (20). Here, we observed that plasma strongly correlated with serum in the detection of TNF-α in children with dengue (Fig. 1A), and the evaluation of TNF-α in plasma revealed the biological differences classically reported during DENV infection (Fig. 1B). These data support the usefulness of plasma, mainly in laboratories where cellular analyses are also performed. The moment of the day in which the samples are taken and their storage and handling also alter the levels of CTs. In this regard, if it is advisable to take the samples at the same time of day, due to the circadian rhythm of CTs (48), to minimize the delay in sample processing, store the samples between −70°C and −80°C, and limit the freeze-thaw cycles (25).

The development of multiplexed bead-based assays has allowed the measurement of a high number of analytes in a low sample volume and a short time with acceptable inter- and intra-assay variability (37). Therefore, several reports have compared bead-based assays using the gold standard ELISA (17, 26, 32, 40). Although good degrees of correlation between both methods have been observed, variability is always present, mainly due to the use of assays from different manufacturers and thus distinct antibodies, buffers, and blockers (19). In most cases, the antibodies are raised against recombinant forms of the CTs, and their abilities to

FIG. 5. Effect of serial dilutions of plasma in the detection of TNF-α. (A) Using ELISA, the TNF-α levels in the plasma from the children with dengue (filled symbols) and the healthy controls (open symbols) without (circles) and with the addition of 10 (squares) and 100 (triangles) pg/mL of the CT were evaluated. (B) TNF-α levels in the plasma from the children with dengue (filled squares) and the healthy controls (open circles) after the addition of 1,000 pg/mL of the recombinant cytokine. In all the cases, undiluted and two-fold serial dilutions (1/2 and 1/4) of each condition were performed in diluent medium (n = 5 in each condition). The dashed lines indicate the detection limit of the assay. The median and ranges and the p-value of Friedman and Dunn’s post-hoc test are shown. *Significant differences in the concentration of TNF-α when undiluted and at 1/4 dilution are compared (p = 0.008).

AV. Pastrana Borrero - Cra 1a. PBX: 8754753 FAX: 8758890 - 8759124
Edificio Administrativo Cra. 5 No. 23 - 40 PBX: 8753686
Línea Gratuita Nacional: 018000968722
www.usco.edu.co
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FIG. 4. Effect of circulating immune complexes in the detection of TNF-α in plasma. (A) Plasma samples from three children with secondary acute HIV infection were diluted or not with a pH based protocol for dissociation of immune complexes. Then, the HIV specific IgM was evaluated by ELISA. All of the 91 samples at 490 nm of four 10 fold serial dilutions (two by duplicate) of the samples are shown. The p value of the Wilcoxon test is shown. (B) Known concentrations of HIV-α (100 and 1,000 pg/ml) were added to the plasma from children with dengue, followed by the dissociation of immune complexes using pH based treatment. The levels of the CT were determined by ELISA. Both box and whisker plots are shown. HI, not treatment; pH, pH based treatment; x, not statistically significant by Mann Whitney test.

in any of the assessed conditions (undiluted, 1/2 and 1/4 diluted plasma) when the added concentration was low or intermediate (plasma without addition of lHIV-α, 10 or 100 pg/ml) in the plasma from the children with dengue and the healthy controls (p < 0.5, Friedman test, Fig. 5A). However, when 1,000 pg/ml of lHIV-α was added, higher levels were recovered in the 1/4 dilution when compared with undiluted plasma in the HIV infected and healthy children (p = 0.0009, Friedman test, Fig. 5B). In agreement with our previous results, lower than expected concentrations of TNF-α were obtained in only 5 out of 20 children, a median percentage of recovery of 33% and 82% in the children with dengue and the healthy controls, respectively. These results vary in the presence of high TNF-α concentrations despite plasma dilution improves its detection.

Discussion

Several factors affect the measurement of CTs. The type of sample is critical, and some aspects have to be kept in mind during the choice of method. For example, serum lacks great substantive protein levels and plasmin, which can interfere with the measurement, although during the process of complement, some CTs such as H III could be released. In the case of plasma, the type of sample considered is more important, and the use of ELISA results in lower intra assay variability serum begins or controls (6). Here, we observed that plasma strongly correlated with serum in the detection of TNF-α in children with dengue (p 0.05) and the evaluation of TNF-α in plasma revealed the homogeneity of differences, basically repeated during HIV infection (p 0.05). These data supported the usefulness of plasma mainly in laboratories where cellular analyses are also performed. The moment of the day in which the samples are taken and their storage and handling also alter the levels of CTs. In this regard, it is advisable to take the samples at the same time of day, due to the circadian rhythm of Ct's (43), to minimize the delay in sample processing, since the samples between 06:00 and 24:00 avoid the circadian cycle (2).

The development of multiplex bead based assay has allowed the measurement of a high number of analytes in a low sample volume and a short time with acceptable intra and inter assay variability (5). Therefore, several reports have compared bead based assays using the gold standard ELISA (7,26, 32,40). Although good degrees of correlation between both methods have been observed, variability is always present, mainly due to the use of assays from different manufacturers and the distinct antibodies, buffers, and blockers (19). In most cases, the antibodies are raised against recombinant forms of the CTs, and their abilities to

FIG. 5. Effect of serial dilutions of plasma in the detection of TNF-α. (A) Using ELISA, the TNF-α levels in the plasma from the children with dengue (filled symbols) and the healthy controls (open symbols) without (circles) and with the addition of 100 (triangles) pg/ml of the CT were evaluated. (B) TNF-α levels in the plasma from the children with dengue (filled symbols) and the healthy controls (open circles) after the addition of 1,000 pg/ml of the recombinant cytokine. In all the cases, undiluted and two fold serial dilutions (1/2 and 1/4) of each condition were performed in diluent medium (n = 5 in each condition). The dashed lines indicate the detection limit of the assay. The median and ranges and the p value of Friedman and Dunn's post hoc test are shown. *Significant differences in the concentration of TNF-α when undiluted and at 1/4 dilution are compared (p = 0.008).

AV. Pastrana Borroto - Cra 1a. PBX: 8754753 FAX: 8758890 - 8759124
Edificio Administrativo Cra. 5 No. 23 - 40 PBX: 8753686
Linea Gratuita Nacional: 018000986722
www.usco.edu.co - Neiva - Huila
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Author Disclosure statement
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