

Serum Levels of Cytokines in Two Ethnic Groups with Dengue Virus Infection

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Abstract. This study compared the serum levels of tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), and interferon gamma (IFN- γ) in 78 Colombian patients, from two ethnic groups, with dengue virus infection. TNF- α levels were significantly higher in Afro-Colombians than in Mestizos and IL-6 levels were significantly higher in Mestizos than in Afro-Colombians, during the acute phase. IFN- γ levels were similar in both ethnic groups. Significantly higher TNF- α levels were found in Afro-Colombians than in Mestizos in both dengue fever (DF) and dengue hemorrhagic fever (DHF). The IL-6 levels were higher in Mestizos than in Afro-Colombians among patients with DF, but levels of this cytokine were higher in Afro-Colombians than in Mestizos among patients with DHF. Levels of IFN- γ were higher in patients with DHF than DF. Higher levels of these cytokines were observed in secondary infection. These results suggest that ethnicity may contribute to differences in immune responses to dengue infections.

INTRODUCTION

Dengue is an acute febrile disease caused by a virus of the genus *Flavivirus*, family *Flaviviridae*, with four serotypes (DENV-1 through DENV-4) that are transmitted by *Aedes* mosquitoes. Dengue is one of the most serious public health problems in tropical and subtropical countries. In Colombia, according to the Ministry of Health, there were 31,362 reported cases of dengue fever (DF), and 5,379 reported cases of dengue hemorrhagic fever (DHF) in 2006. All four DENV serotypes are actively circulating in many parts of the country throughout the year.

Clinical symptoms of DENV infection vary from asymptomatic infection to fatal disease. The most common clinical form is DF, which is characterized by fever, headache, retro-ocular pain, muscle and joint pain, and rash. Disease can evolve into DHF, or the extreme form, dengue shock syndrome (DSS).^{1,2} The DHF is characterized by plasma leakage resulting from increased vascular permeability, hemorrhagic manifestations, and thrombocytopenia.³

Various mechanisms have been proposed to explain the severe clinical forms of dengue, including virulence of the viral strain.^{4,5} In secondary DENV infections, antibody-dependent enhancement of DENV infection has been proposed as a cause of severe disease.^{6–8} Furthermore, it has been postulated that the production of cytokines by DENV infected cells plays an important role in the pathogenesis of the severe dengue syndromes.^{9–13} This later mechanism has been extensively studied.¹⁴ Cytokines are involved in the modulation of the immune responses to pathogens, but they have been proved to mediate immunopathology in some situations. At present, there is clear evidence confirming that monocytes/macrophages respond to infection by DENV by stimulating memory cells T (CD4+ cells) to produce diverse cytokines.¹⁵ Predominant Type 1 helper (Th1) responses resulting in IFN- γ , and TNF- α production are responsible for cell-mediated inflammatory reactions. Meanwhile, the production of IL-6, a Th2-type cytokine, can enhance antibody production via B cell activation.¹⁶ However, cytokine re-

sponses as well as susceptibility to DENV infection, and the resulting severity of disease, might be strongly influenced by the hosts genetic background. This has been suggested by studies in Haiti, where DHF/DSS is not reported,¹⁷ and Cuba, where African descendants exhibited less severe forms of dengue.^{18–21} In this study, we compared the serum levels of TNF- α , IL-6, and IFN- γ in DENV-infected patients from two ethnic groups.

MATERIALS AND METHODS

Patient enrollment. A prospective study was performed from May 2005 to March 2007 in the State of Antioquia. The population consisted of 43 Mestizos and 35 Afro-Colombians with clinical and laboratory diagnosis of dengue according to the Pan-American Health Organization.²² Ethnic classification was made according to their type of hair, facial features, and skin color. All participants were recruited from local health centers. Patients with malaria were not included. The project was approved by the Ethics Committee of the Instituto Colombiano de Medicina Tropical, and informed consent was obtained from each patient before inclusion in the study.

Laboratory tests. Dengue infection was confirmed by the presence of IgM antibodies to DENV in serum obtained during the acute phase. Samples were processed using a commercial test (Dengue IgM Capture ELISA, Panbio, Sinnamon Park, Australia). Furthermore, IgG antibodies to DENV were measured in the acute phase serum sample using a commercial test (UMELISA Dengue IgG, Centro de Inmunoen-sayo, La Habana, Cuba), to distinguish primary or secondary DENV infection. Sera from patients were rapidly separated, frozen at -20°C , and stored at -70°C at the Instituto Colombiano de Medicina Tropical until use.

For cytokine detection (TNF- α , IL-6, and IFN- γ), serum samples were obtained daily during five days after enrollment. These five days of study occurred during Day 1, and Day 13 of the onset of fever, and one sample from the convalescent period (after Day 13). Samples were analyzed using commercial enzyme-linked immunosorbent assay kits (Quantikine or Quantikine HS, R&D Systems, Minneapolis, MN), according to the manufacturer's protocols. Clinical information was recorded in a questionnaire, which included demographic data, symptoms, and laboratory data.

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Statistical analysis. This was performed using the Statistical Package for the Social Sciences, SPSS version 15 (SPSS, Chicago, IL). Data were expressed as medians and quartiles. Differences in proportions between ethnic groups were analyzed by the χ^2 test. Statistical comparison of the cytokine production between ethnic groups and clinical diagnoses was performed by the non-parametric Mann-Whitney test, and comparison of cytokines between acute and convalescent phase by the Wilcoxon test. Significance was set at P value < 0.05 .

RESULTS

Comparisons between ethnic groups. The serum levels of TNF- α , IL-6, and IFN- γ were measured in 78 dengue patients: 43 Mestizos and 35 Afro-Colombians. No significant difference was found in the mean number of days after the onset of fever of the first serum collection between Mestizos (6.50 ± 2.47 days), and Afro-Colombians (6.49 ± 3.51 days). There was, however, a significant difference in age between Mestizos (30.4 ± 10.64 yr) and Afro-Colombians (21.48 ± 11.48 years). Five Afro-Colombian patients were younger than 10 years of age; the remainder was adults. No significant difference was found according to gender (Male/Female = 0.9 in both ethnic groups). Fourteen patients (17.9%) had DHF, and 64 (82.1%) had DF. Although a larger proportion of cases with DHF was observed in Mestizos than in Afro-Colombians, 10 (23.2%) versus 4 (11.4%), the difference was not statistically significant.

Clinical findings. The most common symptoms and signs were fever (100%), headache (97.4%), and myalgias/arthralgias (87.2%). It was noteworthy to observe 17 patients (21.8%) with peripheral edema and three patients with anasarca. The most frequent hemorrhagic manifestations observed were petechiae (48.7%), gingival bleeding (20.5%), epistaxis (16.7%), and positive tourniquet test (35.3%). Most symptoms and signs were similar in frequency in both groups, but rash, edema, and petechiae were significantly more common in Mestizos, whereas hepatomegaly was more common in Afro-Colombians (Table 1). A higher proportion of Mes-

tizos required hospitalization than Afro-Colombians (88.4% versus 58.8%, $P = 0.003$ χ^2 test). The mean platelet count was lower in Mestizos than in Afro-Colombians ($78,564 \pm 67,495$ versus $105,497 \pm 72,519$ per mm^3 ; $P = 0.003$). The hematocrit was higher in Mestizos than in Afro-Colombians ($43.1 \pm 4.6\%$ versus $38.5 \pm 6.3\%$; $P < 0.001$). No fatalities were observed in either group.

Kinetics of cytokine responses in the acute phase. Median levels of the three cytokines were significantly higher in the acute phase serum than in the convalescence phase serum for the whole cohort, 1.23 versus 0.054, ranges 0.0–4.24 and 0.0–3.45, respectively; for TNF- α ($P < 0.001$), 6.18 versus 1.74, ranges 0.0–15.05 and 0.0–8.73, respectively; for IL-6 ($P < 0.001$), 0.0 versus 0.0, ranges 0.0–1.99 and 0.0–0.0, for IFN- γ ($P = 0.002$).

Throughout the acute phase, a dominant TNF- α response was more consistently observed in Afro-Colombians, with the highest peak on Day 2. A second peak was observed on Day 4 in DHF cases only; additional peaks were detected on Day 8 and Day 12. In Mestizos the peak levels were detected on Days 3 and 5, and the highest levels were seen on Day 12 for DHF and DF patients combined. (Figure 1A, D, and G). On the other hand, a dominant IL-6 response was more consistently observed in Mestizos during the acute period, with the highest peak on Day 3 and stable levels thereafter. In Afro-Colombians two peaks were detected on Day 3 and Day 12. Among patients with DF, the production of IL-6 was higher in Mestizos than in Afro-Colombians throughout the illness, although in Afro-Colombians the levels of this cytokine were highest early in DHF (Figure 1B, E, and H). A peak of IFN- γ was observed on Day 1 for Mestizos only; imperceptible levels were found in both ethnic groups thereafter. The levels of IFN- γ were higher early in DHF in both ethnic groups until Day 6 (Figure 1C, F, and I). In DHF the highest peaks of production for TNF- α and IL-6 were on Day 1 and Day 4 in Afro-Colombians, and for IFN- γ were on Day 4 in both ethnic groups.

Serum levels of cytokines according to ethnic group and severity. By ethnic group, and pooling both clinical forms, during the acute phase TNF- α levels were significantly higher in Afro-Colombians than in Mestizos, whereas IL-6 levels were significantly higher in Mestizos than in Afro-Colombians. On the other hand, no significant difference was found in the levels of IFN- γ between the two ethnic groups (Table 2).

By clinical presentation, significantly higher TNF- α levels were found in Afro-Colombians than in Mestizos in both DF and DHF. Meanwhile, levels of IL-6 were significantly higher in Mestizos than in Afro-Colombians in DF but higher in Afro-Colombians in DHF cases, especially at the beginning of the acute phase. However, no significant difference was detected pooling the data from all five days of study. IFN- γ levels were similar in both ethnic groups (Table 2).

The comparison of median cytokine levels between the clinical forms within each ethnic group showed that in Mestizos TNF- α was significantly higher in DHF than DF. The contrary was observed for IL-6, which was significantly higher in DF than in DHF. In Afro-Colombians, TNF- α and IL-6 levels were higher in DHF than in DF, but this difference was statistically significant for IL-6 only. For both races the median IFN- γ levels were undetectable in both clinical forms;

TABLE 1

Signs and symptoms in patients with dengue according to ethnic group

Signs and symptoms	Mestizos		Afro-Colombians		Total		P value
	No.	%	No.	%	No.	%	
Fever	43	100	35	100	78	100	–
Headache	43	100	33	94.3	76	97.4	0.198
Myalgias/arthralgias	38	88.4	30	85.7	68	87.2	0.493
Retro-orbital pain	36	83.7	28	80.0	64	82.1	0.446
Abdominal pain	29	67.4	23	65.7	52	66.7	0.531
Anorexia/asthenia	23	53.5	25	73.5	48	62.3	0.058
Rash	33	76.7	10	28.6	43	55.1	0.000
Peripheral edema	14	32.6	3	8.6	17	21.8	0.010
Hepatomegaly	0	0.0	5	14.3	5	6.4	0.015
Anasarca	3	7.0	1	2.9	4	5.2	0.401
Petechiae	27	62.8	11	31.4	38	48.7	0.005
Gingival bleeding	7	16.3	9	25.7	16	20.5	0.228
Epistaxis	10	23.3	3	8.6	13	16.7	0.075
Purpura/ecchymosis	4	9.3	6	17.6	10	13.0	0.229
Melena	4	9.3	6	17.1	10	12.8	0.245
Hematuria	5	11.6	4	11.4	9	11.5	0.631
Metrorrhagia	7	31.8	2	11.8	9	11.5	0.275
Hematemesis	5	11.6	1	2.9	6	7.7	0.155

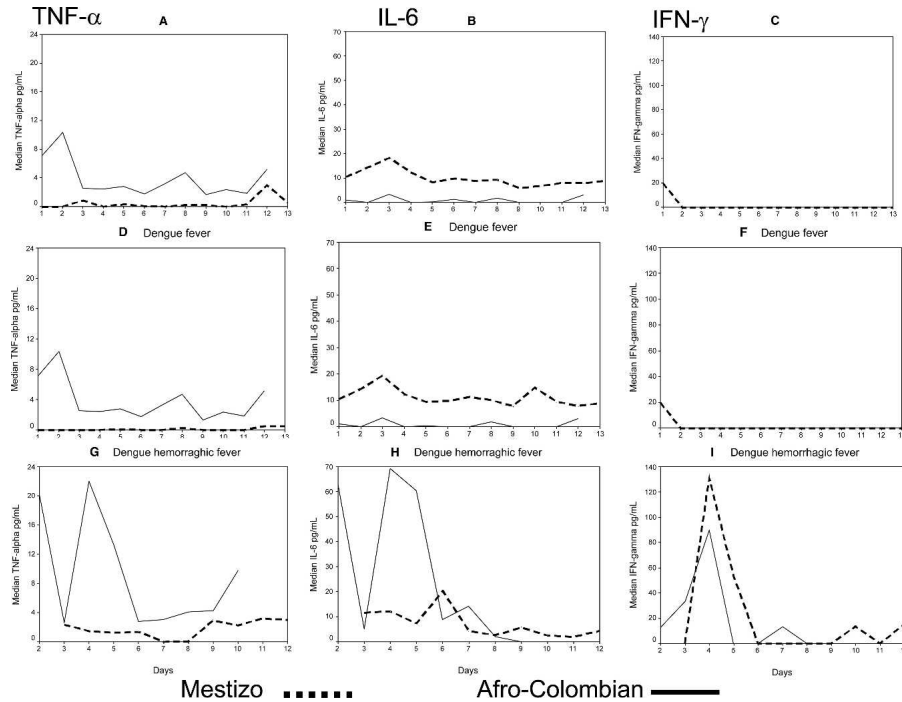


FIGURE 1. Median serum levels of TNF- α , IL-6, and IFN- γ in acute dengue according to clinical form and ethnicity.

there were detectable levels in some DHF cases, but the difference was not statistically significant (Table 2).

Similar levels of the three cytokines were detected in patients independent of their platelet count and whether they were hospitalized for both ethnic groups (data not shown).

The DENV IgG antibodies were assayed in 74 patients of which 33 of 33 Afro-Colombians and 29 of 41 Mestizos were positive. Higher levels of the three cytokines were observed in patients with secondary infection, but the differences were not statistically significant (data not shown).

According to the data of the Direccion Seccional de Salud de Antioquia during the study period the serotypes circulating were DENV-1, DENV-2, and DENV-3, with a predominance of DENV-3.

DISCUSSION

The present study aimed to compare the serum levels of TNF- α , IL-6, and IFN- γ in DF and DHF patients belonging to

two ethnic groups in Colombia. Important differences were observed when comparing the data obtained in the two ethnic groups. The DHF was more common in Mestizo than in Afro-Colombian patients (23.2% versus 11.4%), and more Mestizo patients required hospitalization. Furthermore, the platelet counts were lower and the hematocrits were higher in Mestizo individuals than in Afro-Colombians. These results are in agreement with reports in the literature in which people with African descent exhibit less severe clinical outcomes.^{17–23}

The finding of higher levels of cytokines during the acute phase of dengue compared with the convalescent phase suggests their involvement in the pathogenesis of the infection.

The monocyte/macrophage is the most important primary target of DENV infection.^{6,15,24} These cells, when infected, can produce diverse cytokines and further induce their secretion by stimulating memory T-cells.¹⁵ The TNF- α is mainly produced by monocytes, macrophages, and T-lymphocytes.¹¹ This cytokine has diverse actions including induction of other proinflammatory cytokines, such as IL-1, IL-6, and IFN- γ ,²⁴

TABLE 2
Cytokines levels according to clinical diagnosis and ethnicity

Cytokine pg/mL	Clinical form	Mestizo	Afro-Colombian	<i>P</i> value Mestizo/ AfroCol.	<i>P</i> value Mestizo DF/DHF	<i>P</i> value AfroCol. DF/DHF
		Median (range)	Median (range)			
TNF- α	DF*	0.0 (0.0–1.21)	2.92 (1.07–8.25)	0.000	0.000	0.829
	DHF†	1.37 (0.0–3.07)	3.64 (0.30–13.27)	0.022		
	Total	0.0 (0.0–1.68)	3.06 (1.04–8.45)	0.000		
IL-6	DF	10.24 (4.50–18.66)	0.0 (0.0–7.69)	0.000	0.001	0.007
	DHF	4.51 (1.14–9.99)	5.88 (0.0–14.48)	0.954		
	Total	9.13 (2.77–18.52)	0.0 (0.0–9.07)	0.000		
IFN- γ	DF	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.557	0.536	0.153
	DHF	0.0 (0.0–32.87)	0.0 (0.0–25.74)	0.738		
	Total	0.0 (0.0–0.56)	0.0 (0.0–0.0)	0.595		

* Dengue fever.

† Dengue hemorrhagic fever.

activation and recruitment of neutrophils and eosinophils, co-stimulation of T-cells, promotion of the production of antibodies, and induction of the expression of Fc receptors for IgG (Fc γ R), which could act as an alternative DENV receptor in macrophages.²⁵ It has been shown that TNF- α may activate endothelial cells and thus participate in the clinical manifestations of DHF.¹² The IL-6 may be secreted by monocytes induced by other cytokines such as IL-1 and TNF- α .²⁴ Its major biologic activities include induction of acute phase proteins, promotion of terminal differentiation of B-cells, and activation of T-cells.^{16,24} It can also act as an endogenous pyrogen and affect the permeability of the endothelium.²⁶ The IFN- γ is mainly produced by Th1 type CD4+ T-cells and activated killer cells.²⁵ *In vitro*, IFN- γ can increase endothelial permeability and activate the expression of Fc γ receptors by monocytes/macrophages,^{15,26} which may result in a greater number of infected cells.

In our study, Afro-Colombians presented significantly higher levels of TNF- α than Mestizos. Levels of IL-6 were significantly higher in Mestizos than in Afro-Colombians, and similar IFN- γ levels were detected in both ethnic groups. Differences between ethnic groups in the immune responses to other viral infections have been reported by other authors. Sonnerborg and others²⁷ reported a higher level of TNF- α in Africans than in Caucasians in human immunodeficiency virus (HIV) infection. Similarly, Kimball and others²⁸ showed that African-Americans produced greater amounts of TNF- α than Caucasians in hepatitis C virus infection, in contrast to IFN- γ levels that were equivalent. These reports are in agreement with our results. There are few studies of IL-6 between racial groups. In a comparative study of women with spontaneous preterm birth, related with infectious, higher IL-6 concentrations were observed in European-American women than African-American women.²⁹ IFN- γ levels were similar between the two ethnic groups in our study, in opposition to other reports. De la Sierra and others,³⁰ showed higher release of IFN- γ in Caucasian patients with dengue infection, in contrast to Afro-descendants patients. The difference might be explained by the fact that these authors used an *in vitro* approach with peripheral blood mononuclear cells (PBMC) cultures, whereas our study was performed directly on the serum samples.

The comparison between the clinical forms within each ethnic group showed that in Mestizos and Afro-Colombians the levels of TNF- α were higher in DHF than DF. These results are in accordance with other authors.^{31–34} In our study, levels of IL-6 were significantly higher in the Mestizo patients with DF than those with DHF, although the converse was observed in Afro-Colombians. Other authors also reported opposite results. Bethell and others³⁵ reported low levels of IL-6 in patients with shock and Restrepo and others³⁶ observed IL-6 levels higher in DF than DHF, but Juffrie and others,²⁴ Hober and others,¹⁰ and Kuno and Bayley¹² reported higher levels of this cytokine in patients with severe forms of dengue.

In summary, we found a differential cytokine response according to ethnic background, with a more robust response in Afro-Colombians regarding the production of TNF- α in both clinical forms and of IL-6 in DHF cases. Blanton and others²³ reported that Afro-Brazilian ethnicity and African ancestry were protective for DHF. Sugimoto and others³¹ reported that African American ethnicity was associated with a more robust antiviral CD4 T-cell response than Caucasian Ameri-

can ethnicity among chronically HCV-infected patients. We also observed within each ethnic group greater production of TNF- α in DHF. These results suggest that the genetic background may be associated with variable immune responses to DENV infection in humans. However, the Afro-descendants, who have more immunological responses (highest levels of TNF- α), have less severe forms of dengue. Further studies of immunologic responses and their relationship with ethnicity and disease are therefore warranted.

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