SHORT COMMUNICATION

Detection of Circulant Tumor Necrosis Factor-α, Soluble Tumor Necrosis Factor p75 and Interferon-γ in Brazilian Patients with Dengue Fever and Dengue Hemorrhagic Fever

Elzinandes LA Braga, Patrícia Moura*, Luzia MO Pinto, Sonia RN Ignácio, Maria José C Oliveira, Marly T Cordeiro**, Claire F Kubelka/+**

Departamento de Virologia, Instituto Oswaldo Cruz-Fiocruz, Av. Brasil 4365, 21045-900 Rio de Janeiro, RJ, Brasil *Departamento de Patologia, Instituto de Ciências Biológicas, Universidade de Pernambuco, Recife, PE, Brasil **Departamento de Virologia, Laboratório Central, Fusam, Recife, PE, Brasil

Pro-inflammatory cytokines are believed to play an important role in the pathogenesis of dengue infection. This study reports cytokine levels in a total of 54 patients examined in Recife, State of Pernambuco, Brazil. Five out of eight patients who had hemorrhagic manifestations presented tumor necrosis factor-α (TNF-α) levels in sera which were statistically higher than those recorded for controls. In contrast, only one out of 16 patients with mild manifestations had elevated TNF-α levels. The levels of interleukin-6 (IL), IL-1β tested in 24 samples and IL-12 in 30 samples were not significantly increased. Interferon-γ was present in 10 out of 30 patients with dengue. The data support the concept that the increased level of TNF-α is related to the severity of the disease. Soluble TNF receptor p75 was found in most patients but it is unlikely to be related to severity since it was found with an equivalent frequency and levels in 15 patients with dengue fever and another 15 with dengue hemorrhagic fever.

Key words: dengue - cytokines - dengue hemorrhagic fever

Dengue has been claimed to be an immunopathological disease (Halstead 1980). Patients with dengue hemorrhagic fever (DHF) initially show typical systemic manifestations of dengue fever (DF) such as fever, retroorbital headache, severe myalgia and rash. Thereafter, they rapidly develop a more severe and life-threatening syndrome with thrombocytopenia, diffuse capillary leakage, hemoconcentration and hypotension, which may be followed by hemorrhage. In the most severe form of the disease, dengue shock syndrome (DSS), the pulse pressure narrows and circulatory collapse leading to shock occurs (Halstead 1990, PAHO 1994). DHF/DSS is most often associated with a secondary immunological reaction caused by a dengue virus serotype different from that originating the primary infection, in which the immune response is crossreactive but not protective (Pang 1987, Kliks et al. 1989).

Monocytes/macrophages are considered target cells for dengue infection (Halstead 1980, Anderson et al. 1997). These cells may act as antigen-presenting cells and secrete monokines, which participate in T cell activation and production of macrophage activating cytokines. During a secondary immune response, dengue virus activated mononuclear phagocytes are thought to release pro-inflammatory cytokines. Tumor necrosis factor-α (TNF-α) would act by feedback on these cells and also on vascular endothelial cells stimulating secretion of interleukin-1β (IL) and IL-6 into circulation (Yang et al. 1995, Hober et al. 1996, 1998, Bethell et al. 1998). These three cytokines have similar functions and are able to induce an acute phase response which can result in fever, liberation of chemoattractant factors, altered vascular permeability (Dinarelo 1996) resembling features present in DHF/DSS and therefore suggesting their involvement in the pathological features of dengue infection.

Pro-inflammatory cytokines have been detected in patients from Asia and Central America (Hober et al. 1993, Kuno & Bailey 1994) and also in Brazilian patients (Kubelka et al. 1995, Pinto et al. 1999). The association of cytokine production and severity has been suggested.

Financial support: Fiocruz, Facepe, CNPq and Colab
+Corresponding author. Fax: +55-21-598.4491. E-mail: claire@gene.dbbm.fiocruz.br
Received 7 January 2000
Accepted 5 October 2000

Recent studies in different viral infections and inflammatory diseases provide evidence that soluble tumor necrosis factor receptors (sTNFRs) may modulate an excess of biological TNF-α activity (Van Zee et al. 1992, Dinarello 1996). Alternatively soluble receptors could stabilize TNF molecules acting thus as agonists (Leuwenberg et al. 1994). Indeed, Bethell et al. (1998) related sTNFRs levels with disease severity. Most Brazilian patients with exanthematic dengue fever show elevated sTNFRs levels even in the absence of hemorrhagic manifestations (Pinto et al. 1999).

In the present study, we investigated levels of TNF-α, IL-1-β, IL-6, IL-12, IFN-γ and TNF-Rp75 in sera from Brazilian patients with dengue attended in Recife, State of Pernambuco, Brazil. For TNF-α, IL-1-β and IL-6 determinations we used sera from 16 individuals (two children) attended in 1997 who had DF with usual clinical manifestations (fever, headache, myalgia and rash) devoid of hemorrhagic manifestation. Five other cases also classified as DF presented hemorrhagic manifestations such as epixtasis, gingival or abdominal bleeding. Three adults had confirmed DHF, following WHO criteria (PAHO 1994), with thrombocytopenia (<100.000 platelets/mm^3) and hemoconcentration. Fifteen apparently healthy individuals were used as controls.

For IL-12, IFN-γ and TNF-Rp75 determinations, we used sera from 15 individuals attended in 1999 who had DF without hemorrhagic manifestations and an additional 15 individuals with hemorrhagic manifestations and confirmed DHF with thrombocytopenia and hemoconcentration.

Dengue infection was confirmed serologically in all patients by IgM positivity using a Mac ELISA test (Nogueira et al. 1992).

Serum samples were obtained during appointment and were stored in aliquots at -20°C until use in the different assays. Levels of TNF-α and sTNF-Rp75 in sera were determined using commercial ELISA kits according to the manufacturer's prescriptions. Predicta from Genzyme was used for TNF-α, IL-1-β and IL-6. R&D Systems Quantikine was used for IFN-γ and sTNF-Rp75 and High Sensitivity Quantikine for IL-12. The following sensitivity limits were achieved in standard curves: TNF-α, 18.75 pg/ml; IL-6, 28.125 pg/ml; IL-1β, 4 pg/ml; IFN-γ, 15.6 pg/ml; IL-12, 0.78 pg/ml sTNF-Rp75, 7.8 pg/ml.

Statistical analyses were performed by Student's *t*-Distribution ($t_{n-1=8,\ \alpha=0.025} = 2.262$, $t_{n-1=9,\ \alpha=0.025} = 2.306$ or $t_{n-1=14,\ \alpha=0.025} = 2.145$) calculating a referential limit value for positivity, according to the following formula: *Average of values from control samples + [Standard Deviation of values from control samples X* $t_{n-1;\alpha=0.025)}$].

Determinations above referential limit values were considered positive.

TNF-α was detected in 6 out of 24 (25%) patients above the referential limit value for positivity, 41 pg/ml (Figure). IL-6 and IL-1β were not detected in any patient (referential limit values for positivity were respectively 207,04 and 16,3 pg/ml).

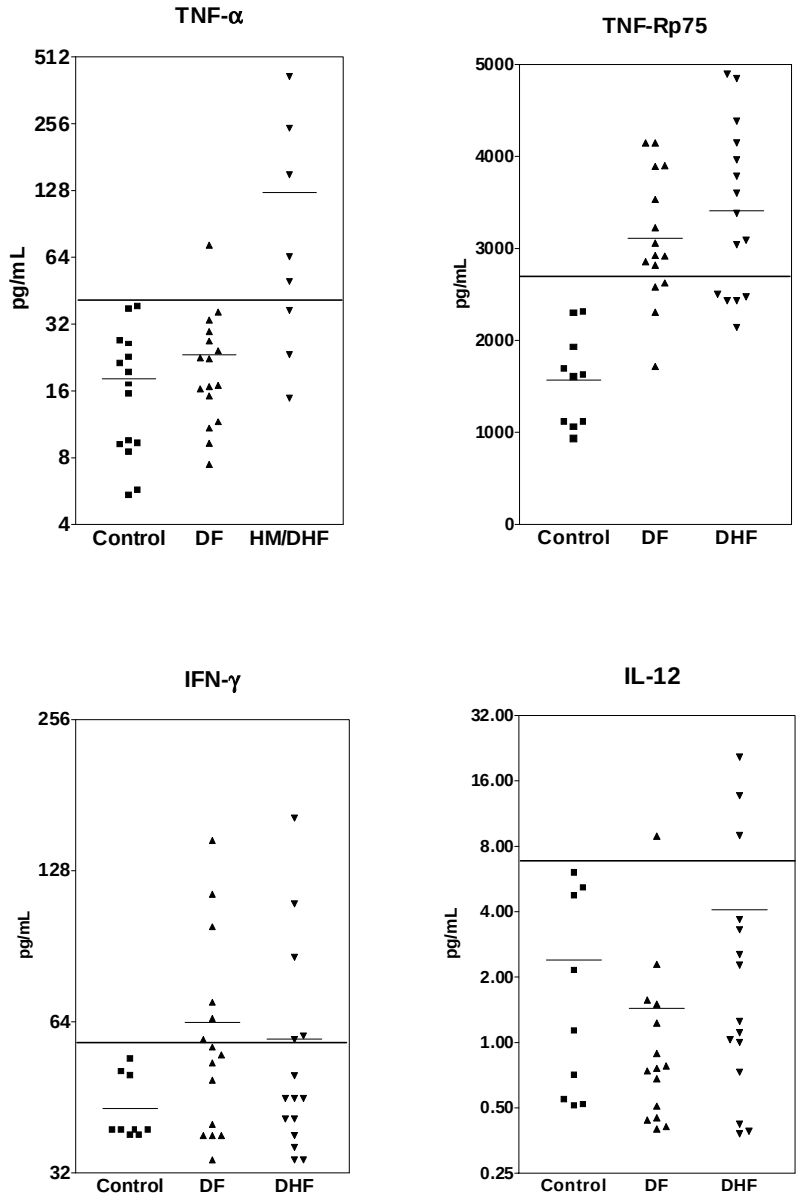
Soluble TNF-Rp75 was detected in 21 out of 30 patients (73% above the referential limit value for positivity, 2715,1 pg/ml, Figure). IL-12 was detected in 4 out of 30 (13% above 7,66 pg/ml) and IFN-γ was detected in 10 out of 30 patients (33% above 58,03 pg/ml).

Using the Fisher exact test (α=0.05; one sided), patients with hemorrhagic manifestations or DF/DHF showed a significantly higher frequency of positivity for TNF-α when compared with DF patients devoid of hemorrhagic manifestations (P= 0.0069). The sTNF-Rp75 elevation in patients was highly significant (P=0.0002) but the frequency of this factor was not significantly different between the two forms of the disease (DF had 11 out of 15 positive sera and DHF had 10 out of 15).

Hober et al. (1993) have detected the pro-inflammatory cytokines TNF-α and IL-6 in Asiatic patients with DHF/DSS during Dengue-3 infection. Moreover, the follow up of a child infected by Dengue-4 demonstrated that an increase in severity was followed by elevation of serum levels of TNF-α, IL-6 and IL-1β (Iyngkaran et al. 1995). The only study previously performed in Central America correlated higher cytokine levels with child hospitalization (Kuno & Bailey 1994) but no other severity parameter was described. We observed in an earlier work, one fatal case in Rio de Janeiro with a serum level of 900 pg/ml TNF-α (Kubelka et al. 1995). In our present study in Brazil, all three dengue patients with TNF-α levels higher than 100 pg/ml showed an elevated grade of severity, supporting the concept that this factor is produced in greater quantities during DHF or DF with hemorrhagic manifestations.

Soluble TNF-Rp75 was detected in the majority of patients tested but it was not possible to associate its presence with severity in contrast to the situation described by Bethell et al. (1998). In that work DHF patients had altered sTNF-Rp75 levels in sera but only patients with fatal shock had marked raise of the soluble receptor. We have only witnessed one case of recovery from shock; it may be the case that the association between elevated sTNFRp75 and severity is only true in very severe cases or even fatal ones. However, we should emphasize that most Brazilian patients are adults whereas the bulk of Asiatic cases involve children. Furthermore, genetic and geographical factors may influence this association.

High TNF-α levels have been more clearly correlated with hemorrhagic manifestations and/or confirmed DHF. TNF has many effects on the endothelium and stimulates the production of vasodilating substances such as prostaglandin I_2 and nitric oxide. Also, TNF-α has a powerful antifibrinolytic action, by decreasing the production of plasminogen activators and increasing that of plasminogen activators inhibitor 1 (Vassali 1992, Mantovani et al. 1997). Nevertheless, investigations are far from elucidating the complex mechanisms of immunopathology during dengue infection; more cases in different countries and several immunological parameters remain to be studied. Understanding the cellular and molecular regulation mechanisms of pro-inflammatory cytokines has a potential therapeutic significance by providing strategies for inducing or inhibiting desired cytokine profiles in response to dengue virus as it has recently been suggested (Eigle et al. 1997).



Tumor necrosis factor-α (TNF-α), soluble tumor necrosis factor receptor p75 (sTNFRp75) interferon-γ (IFN-γ), and interleukin-12(IL-12) levels in sera from dengue infected patients. DF: patients with dengue fever; HM: patients with dengue fever with hemorrhagic manifestations; DHF: patients with confirmed dengue hemorrhagic fever. Dots represent determinations for single individuals. Short lines represent mean of each group values. Long line represent *referential limit value for positivity* calculated according to Student's T distribution as described in text.

ACKNOWLEDGMENTS

To Drs Hermman MG Schatzmayr and Marilda M Siqueira for their continuous encouragement.

REFERENCES

Anderson R, Wang S, Osiowy C, Issekutz AC 1997. Activation of endothelial cells via antibody-enhanced dengue virus infection of peripheral blood monocytes. *J Virol 71*: 4226-4232.

Bethell DB, Flobbe K, Cao XT, Day NP, Pham TP, Buurman WA, Cardosa MJ, White NJ, Kwiatkowski D 1998. Pathophysiologic and prognostic role of cytokines in dengue hemorrhagic fever. *J Infect Dis 177*: 778-782.

Dinarello CA 1996. Cytokines as mediators in the pathogenesis of septic shock. In ET Rietschel, H Wagner (eds), *Pathology of the Septic Shock*, Springer-Verlag, Heidelberg, Germany, p. 134-165.

Eigle A, Sinha B, Hartmann G, Endres S 1997. Taming TNF: strategies to restrain this proinflammatory cytokine. *Immunol Today 18*: 487.

Halstead SB 1980. Immunological parameters of togaviruses disease syndromes. In RW Schlesinger, *The Togaviruses. Biology, Structure, Replication*, Academic Press, New York, p. 107-174.

Halstead SB 1990. Dengue and dengue hemorrhagic fever. *Curr Scie 3*: 434-438.

Hober D, Delannoy AS, Benyoucef S, De Groote D, Wattre P 1996. High levels of sTNFR p75 and TNF alpha in dengue infected patients. *Microbiol Immunol 40*: 569-573.

Hober D, Nguong VT, Shen L, Há DQ, Benyoucef S, Nguong TH, Bui TM, Loan HK, Le BL, Bouzidi A, De Groote D, Drout MT, Deubel V, Wattre P 1998. Tumor necrosis factor alpha levels in plasma and whole-blood culture in dengue-infected patients: relationship between virus detection and pre-existing specific antibodies. *J Med Virol 54*: 210-218.

Hober D, Poli L, Roblin B, Gestas P, Chungue E, Granic G, Imbert P, Pecarere JL, Vergez-Pascal R, Wattre P, Maniez-Montreuil M 1993. Serum levels of tumor necrosis factor-a (TNF-α), interleukin-6 (IL-6) and interleukin-1b (IL-1β) in Dengue-infected patients. *Amer J Med Hyg 48*: 324-331.

Iyngkaran N, Yadav M, Sinniah M 1995. Augmented inflammatory cytokines in primary dengue infection progressing to shock. *Singapore Med J 36*: 218-221.

Kliks SC, Nisalak A, Brant WE, Wahl L, Burke DS 1989. Antibody-dependent enhancement of Dengue virus growth in human monocytes as a risk factor for Dengue hemorrhagic fever. *Amer J Trop Med Hyg 40*: 444-451.

Kubelka CF, Borges PA, vonSydow FOF, Lampe E 1995. Analysis of tumor necrosis factor-α serum level in Brazilian patients with Dengue-2. *Mem Inst Oswaldo Cruz 90*: 741-742.

Kuno G, Bailey RE 1994. Cytokine responses to Dengue infection among Puerto Rican patients. *Mem Inst Oswaldo Cruz 89*: 179-182.

Leuwenberg JFM, Dentener MA, Buurman WA 1994. Lipopolysaccharide-mediated soluble TNF receptor release and TNF receptor expression by monocytes. *J Immunol 152*: 5070-5076.

Mantovani A, Bussolino F, Introna M 1997. Cytokine regulation of endotelial cell function: from molecular level to the bedside. *Immunol Today 18*: 231-240.

Nogueira RMR, Miagostovich MP, Cavalcanti SMB, Marzochi KBF, Schatzmayr H 1992. Levels of IgM antibodies against Dengue virus in Rio de Janeiro, Brasil. *Res Virol 143*: 423-427.

PAHO-Pan American Health Organization 1994. Dengue and dengue hemorrhagic fever in Americas: guidelines for prevention and control. PAHO/WHO, Scientific Publication no. 548.

Pang T 1987. Dengue haemorrhagic fever: virus or response? *Bio Essays 6*: 141-143.

Pinto LMO, Oliveira SA, Braga ELA, Nogueira RMR, Kubelka CF 1999. Increased pro-inflammatory cytokines (TNF-α And IL-6) and anti-inflammatory compounds (sTNFRp75 and sTNFRp55) in Brazilian patients during exanthematic dengue fever. *Mem Inst Oswaldo Cruz 94*: 1-8.

Van Zee KJ, Kohno T, Fischer E, Rock CS, Moldawer LL, Lowry SF 1992. Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor-α *in vitro* and *in vivo*. *Proc Natl Acad Scie 89*: 4845-4849.

Vassali P, 1992. The pathophysiology of tumor necrosis factors. *Annu Rev Immunol 10*: 411-452.

Yang KD, Lee CS, Hwang KP, Chu ML, Shaio MF 1995. A model to study cytokine profiles in primary and heterologously secondary Dengue-2 virus infections. *Acta Virol 39*: 19-21.