Circulating levels of tumour necrosis factor-α & interferon-γ in patients with dengue & dengue haemorrhagic fever during an outbreak

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Background & objectives: Dengue haemorrhagic fever (DHF) is a severe illness caused by dengue viruses, the pathogenesis of which is not fully understood. Immune enhancement caused by cytokines, interferon and activated complement system is one of the hypotheses proposed to explain the haemorrhagic form of disease. This study was undertaken to investigate the role of pro-inflammatory cytokines tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) in dengue infection, and their possible association with disease severity.

Methods: Blood samples were collected from 60 patients; 28 patients of DF and 32 of DHF (clinically categorized into DF or DHF as per WHO criteria) from September to November 2003 at Delhi. Fifteen healthy individuals were included as control. All the patients were confirmed positive for dengue infection by serology or by reverse transcriptase-polymerase chain reaction (RT-PCR). Serum levels of TNF-α and IFN-γ were determined by ELISA.

Results: The levels of both the cytokines were significantly elevated in the disease group as compared to the control group. Significantly higher levels of TNF-α; 258.02 pg/ml (P<0.005) were seen in patients having secondary infection, while patients with primary infection had higher level of IFN-γ; 29.47 pg/ml (P<0.005). TNF-α was elevated in the later phase of illness, while IFN-γ was elevated in early phase also.

Interpretation & conclusion: Overproduction of TNF-α during secondary infection may have a role in immunopathogenesis of DHF, however, study of other cytokines produced along with IFN-γ during the course of dengue infection is required to know the specific role of IFN-γ in DI.

Key words Cytokines - DHF - dengue fever - IFN-γ - TNF-α
Dengue viruses (DV) belong to the family *Flaviviridae*, and have four distinct antigenic types (serotypes Den 1 to 4). Worldwide, more than 50 million cases of dengue fever (DF), 500,000 cases of dengue haemorrhagic fever (DHF) occur annually1,2. Most persons infected with dengue viruses remain asymptomatic or develop DF, a mild febrile illness of 2-7 days with manifestations like headache, retro-orbital pain, myalgia/arthralgia, rash and leukopenia. DHF, a more severe life threatening form is evidenced by one or more of the symptoms like thrombocytopenia, positive tourniquet test, petechiae or purpura, bleeding from mucosa, injection sites or other sites, haematemeses, melena and/or increased capillary permeability. DHF and dengue shock syndrome (DSS), which can be fatal, develop in a minority of infected persons3. Since 1967, many outbreaks of dengue have been investigated from India, with two major outbreaks of dengue reported from Delhi in 1996 and 20034. Frequency of dengue infection (DI) has increased in India and it has now become a major public health problem of concern5,6.

The life threatening severe form of dengue infection, DHF has been postulated to result from immune enhancement after a second (heterotypic) DI by the proposed mechanism of antibody-dependent enhancement7. Mononuclear macrophages and T cells are activated during DI and cytokines released by them are proposed to be involved in the pathogenesis of DHF8,9. We undertook this study to investigate the role of pro-inflammatory cytokines tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) in patients with DI. The levels of these cytokines in patients having DF and DHF were further studied to correlate with the disease severity.

**Material & Methods**

The present study was conducted on patients attending Lok Nayak Hospital Delhi, who were clinically suspected to have DI, during the dengue outbreak between September and November 2003. Blood samples were collected from 60 patients of DI in the age group 8-52 yr (mean age 27 yr); including 28 patients of DF and 32 patients of DHF selected according to the WHO criteria10. A case was included if he/she had high fever with clinical symptoms suggestive of dengue infection. A case was excluded, if routine laboratory testing suggested bacterial or any viral infection other than dengue infection or any other disease. With all the aseptic precautions, venous blood samples collected from each patient were processed for the separation of serum and immediately transferred to -70°C freezer till processed further. DI was confirmed by detection of dengue specific antibodies (IgM and IgG) or by RNA detection using reverse transcriptase polymerase chain reaction (RT-PCR). Fifteen healthy individuals were included as controls.

**Serodiagnosis:** Dengue specific IgM and IgG antibodies were detected by rapid immunochromatographic test according to the manufacturer’s instructions (Pan Bio, Windsor, Australia). The presence of dengue specific IgM antibodies alone was indicative of primary infection, while the presence of IgM and IgG antibodies was suggestive of secondary DI11.

**Detection of viral RNA by RT-PCR:** RNA extraction was carried out by the modified method of Chomczynski & Sacchi12. Dengue viral RNA was detected by RT-PCR as per the method of Harris *et al*13 with modifications, using type specific consensus primers from capsid- premembrane region. Primers were purchased from Invitrogen Corp. USA. Briefly, the master mix of RT-PCR contained 5 mM dithiothreitol, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris (pH 8.5), four dNTPs at the concentration of 200 µM each, 50 pmol each of D-1 upstream and D-2 downstream consensus primer, 200 units of Superscript II RNase H reverse
Transcriptase (RT) (Invitrogen Corp., USA) and 1 unit of Taq DNA polymerase (Invitrogen Corp., USA) per reaction. Reverse transcription was carried out at 42°C for 50 min followed by 40 cycles of PCR; each cycle consisting of denaturation at 94°C for 30 sec, primer annealing at 55°C for 1 min and primer extension at 72°C for 2 min with a final extension at 72°C for 10 min. Amplified DNA product of 511 b.p. was visualized on 1.5 per cent agarose gel by ethidium bromide staining.

Cytokine detection: Serum levels of TNF-α and IFN-γ were detected by ELISA (BD Biosciences, USA) according to the manufacturer’s instructions. The TNF-α concentration was calculated in the test samples on the basis of the curve produced by plotting the optical density values of the known standards (range 7.5 - 500 pg/ml) on log-log graph paper. The level of IFN-γ in patients and controls sera was similarly detected using IFN-γ ELISA kit. The detection limits of the kit for TNF-α and IFN-γ were 2 and 1 pg/ml respectively.

Statistical analysis: The statistical significance of difference in the levels of TNF-α and IFN-γ between the controls, DF and DHF groups were analyzed using Kruskal-Wallis test, P<0.005 was considered to be statistically significant. Correlation between TNF-α and IFN-γ levels among different groups was evaluated using Spearman’s correlation test.

Results

Dengue infection was confirmed by serology or detection of RNA by RT-PCR (Table I). The levels of both the cytokines were elevated in the disease group as compared to the control group (P<0.001) (Table II). However no significant difference was observed in the levels of TNF-α and IFN-γ when compared within the three groups; controls, DF and DHF groups. The level of TNF-α was observed to have no correlation with IFN-γ in either control group (r=0.3417, P=0.21) or disease group; DF (r=0.1691, P=0.37) and DHF group (r=0.1244, P=0.512).

The level of TNF-α was found to be significantly elevated in patients of DF as well as DHF when compared with controls (P<0.001). The level of

| Table I. Seropositivity and RT-PCR profile amongst patients of DF and DHF |
|--------------------------------|-----|-----|------|
| Laboratory diagnosis | DF patients (n=28) | DHF patients (n=32) | Total DI patients (n=60) |
| IgM positive | 5 | 10 | 15 |
| IgM + IgG positive | 16 | 18 | 34 |
| RT-PCR positive | 7 | 4 | 11 |

DF, Dengue fever; DHF, dengue hemorrhagic fever; DI, dengue infection; RT-PCR, reverse transcriptase-polymerase chain reaction

| Table II. Cytokine profile in patients with dengue infection and controls |
|-----------------|-------|------|
| Cytokine level (pg/ml) |
| Study group | TNF-α | IFN-γ |
| Control group (N=15) | 10.12 | 7.4 |
| DI (n=60) | 230 ± 192.2** | 25.28 ± 33.53** |
| DF (n=28) | 220.65 ± 212.95** | 27.67 ± 31.3* |
| DHF (n=32) | 239.4 ± 173.37** | 22.9 ± 30.80* |
| Primary infection (n=15) | 240.33 ± 191.43 | 29.47 ± 40.69 |
| Secondary infection (n=34) | 258.02 ± 193.5+ | 24.15 ± 30.4+ |
| Days of illness (1-4 days) | 172.56 ± 175.37 | 33.2 ± 39.07 |
| Days of illness (5-8 days) | 278.43 ± 190.56++ | 34.26 ± 56.8 |
| DF, Dengue fever; DHF, dengue hemorrhagic fever; DI, dengue infection |
| P<0.005; **<0.001 compared to controls |
| P<0.005 compared to primary infection |
| P++<0.001 compared to group with illness of 1-4 days |
IFN-γ was also significantly elevated in these groups in comparison with controls (Table II).

Fifteen (25%) and 34 (57%) patients had primary and secondary infection respectively. TNF-α; levels were significantly higher \( (P<0.005) \) in patients having secondary infection, while IFN-γ levels were significantly higher in patients with primary infection \( (P<0.005) \) (Table II). Analysis of cytokine levels on the basis of days after the onset of illness showed comparatively higher mean value of TNF-α; \( (P<0.001) \) in patients with illness between 5-8 days. However, there was no statistically significant difference in the levels of IFN-γ when compared between patients with fever for 1-4 and 5-8 days, respectively (Table II).

**Discussion**

Dengue virus infection leads to production of various cytokines by infected monocytes, B-lymphocytes and mast cells. Recently the focus of research on the pathogenesis of DHF has greatly shifted to cell mediated immune response. It is reported that the DV can infect both CD4+ and CD8+ cells. During secondary infection serotypes cross-react with CD4+ and CD8+ cells and augment infection by producing various cytokines\(^{14}\). In response to DV antigens CD4+ and T cells produced TNF-α, IFN-γ and TNF-β, which might be contributing to pathogenesis of secondary DI\(^{15}\). Higher activation of CD8+ and natural killer (NK) cells has been observed in children having DHF as compared to DF\(^{16}\). Both these cells are the potential source of early IFN-γ production. An increase of IFN-γ can lead to higher T cell activation consequently increasing TNF-α, IFN-γ secretion along with interleukin-2 (IL-2) release, through its effects on dendritic cells or other antigen presenting cells. The cascade of events generated due to disregulation of type-1 response (pro-inflammatory) may thus be contributing to multiple aspects of DHF pathogenesis.

It is apparent from the present findings that the levels of both the pro-inflammatory cytokines increased significantly during DI compared with healthy controls. The elevation of TNF-α was observed to be more in cases having DHF. Prolonged elevation of this pro-inflammatory cytokine during secondary infection, mainly in DHF cases, suggested an association of TNF-α with disease severity. The role of this cytokine in increasing the severity of disease has also been suggested by others\(^{17,18}\), who have reported a significant increase in the levels of TNF-α in patients with DHF. In two different studies on the effect of cytokines in DF vs. DHF patients, higher levels of TNF-α, IL-6, IL-13 and IL-18 have been reported in DHF as compared to DF patients suggesting a role in causing increased vascular permeability and shock during DI\(^{19,20}\).

Levels of IFN-γ has been observed to be significantly elevated in infants having DF/DHF when compared to controls as observed by Hung et al\(^{21}\). In the present study, concentration of IFN-γ was found to be more in DF cases, as compared to DHF, as has also been reported in previous studies\(^{17,18}\). The level of IFN-γ was observed to be more in primary cases with almost the same concentration in patients having illness of 1-4 days and 5-8 days. Green et al\(^{22}\) suggested that IFN-γ is produced early in the course of infection and peak levels occur on or before the day of defervescence thus coinciding with disappearance of viraemia\(^{22}\). It was suggested that though IFN-γ is secreted as a pro-inflammatory cytokine by T helper cells and has a role to play in acute inflammatory process, on its own it may not be responsible for the pathogenesis of DHF.

In conclusion, our findings showed serum concentration of TNF-α and IFN-γ to be significantly elevated in patients as compared to normal controls. However, further studies need to be done to know the specific role of these cytokines in deciding whether the infection will be simple DF or may lead to DHF.
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References


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