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Role of interleukin-12 in patients with dengue hemorrhagic fever

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Abstract

Interleukin (IL)-12 has a broad range of activities including regulation of cytokine synthesis and selective promotion of Th1-type cell development. A shift from a Th1-type response to Th2-type has been suggested to be important in the pathogenesis of dengue hemorrhagic fever (DHF). This study was undertaken to investigate the possible role of IL-12 in this shift. A total of 76 patients with various grades of dengue illness and 21 normal healthy controls were tested for IL-12 levels in serum samples and IL-12 mRNA in their peripheral blood mononuclear cells. The results showed that the levels of IL-12 were the highest in patients with dengue fever (270 ± 102 pg ml^{-1}) followed by decreasing levels in the patients with DHF grade I (198 ± 86 pg ml^{-1}; $P < 0.05$) and DHF grade II (84 ± 52 pg ml^{-1}; $P < 0.001$). Neither IL-12 nor its mRNA could be detected in the patients with DHF grades III and IV. The cytokine appeared and reached peak levels during the first 4 days of illness, started to decline by day 5–8 and disappeared by day 9 onwards. The absence of IL-12 during severe illness and late phases of the disease may be responsible for the shift to a Th2-type response and thus for the pathogenesis of DHF.

Keywords: Dengue; Dengue hemorrhagic fever; IL-12; Cytokine; Pathogenesis

1. Introduction

Dengue virus produces either a mild, self-limiting febrile illness, dengue fever (DF), or a severe, often fatal illness, dengue hemorrhagic fever (DHF). The characteristic pathological features of DHF are increased capillary permeability, cerebral edema, altered number and functions of leucocytes, increased hematocrit and thrombocytopenia [1,2]. Extensive plasma leakage into various serous cavities of the body may result in profound shock and death. Despite extensive studies, the pathogenesis of DHF is still not fully understood. We have recently observed a shift from a Th1-type cytokine response in DF to a Th2-type cytokine response in DHF that correlates with increasing severity of the illness [3]. Dengue virus-infected human peripheral blood leucocyte cultures also show a similar cytokine response [4]. This indicated a possible role of Th1-type cytokines in protection and Th2-type cytokines in pathogenesis of DHF.

Interleukin (IL)-12, mainly produced by monocytes/macrophages in response to infectious agents, has a profound effect on the levels of Th1 cells and Th1-type cytokines. The presence of IL-12 upregulates Th1-type cytokines while its absence shifts the balance towards Th2-type cytokines (reviewed in [5]). Thus, it was possible that higher levels of Th1- and Th2-type cytokines in DF and DHF grades III and IV, respectively, were in response to differences in the levels of IL-12 in these patients. To determine if such a possibility existed, we have determined the serum levels of IL-12 and IL-12 mRNA in the peripheral blood mononuclear cells (PBMC) of patients with DF and DHF. Our results show that the lack of IL-12 in patients with DHF grades III and IV may play a role in shifting the response towards Th2-type cytokines in these patients.

2. Materials and methods

2.1. Patients

Northern India was gripped in an extensive epidemic of

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dengue and DHF during August–November 1996. The patients suffering from typical dengue-like illness admitted to the Gandhi Memorial and Associated Hospitals, Lucknow, and the Pediatrics Department of the All India Institute of Medical Sciences, New Delhi, were studied during this epidemic. Clinicians examined the patients thoroughly and laboratory investigations were carried out. At the time of reporting to the hospital, the clinical presentation of every patient was recorded, the Hess test was done and hematocrit values and platelet counts were measured; the last two tests were repeated daily during the course of their stay in the hospital. In the present study, the grade of the illness at the time of admission, when the blood was collected, has been taken into consideration. The day of the onset of fever was considered as day 0 of the illness and thus the day of sample collection was calculated for each patient accordingly. Depending upon the severity of the illness (clinical presentation) and the findings of hematocrit and platelet count, they were classified as DF or DHF grades I, II, III or IV according to the criteria of the World Health Organization [6]. A patient was labeled as of grade I when his hematocrit values were increased by more than 20%; grade II when he had, in addition, spontaneous bleeding in skin or other sites; grade III had hypotension and/or narrowing of pulse pressure to 20 mm Hg or less, with cold clammy skin and restlessness (shock); and grade IV had undetectable blood pressure or pulse (profound shock). A total of 76 patients were included in the present study. Diagnosis of dengue virus infection was established either by virus isolation [7] or by detection of virus-specific IgM in the sera using standard protocols [8]; in a number of cases, dengue IgM capture enzyme-linked immunosorbent assay (ELISA) was also done using commercial kits (Pan BIOS, East Brisbane, Australia). As controls, 21 normal age-matched healthy individuals, without history of any febrile or other illnesses in the previous 3 months, were included. Among the patients, 16 were classified as DF, 10 as DHF grade I, 24 as grade II and 13 each as grade III and grade IV. Sera collected from the patients (on the first to the 18th day of illness) and the controls were divided into aliquots (to avoid repeated freezing and thawing) and quickly frozen and stored at −60°C. For the determination of cytokine concentration, sera were transported to Kuwait on dry ice and stored at −70°C until tested.

2.2. Assay of IL-12

Commercial ELISA kits (purchased from R&D Systems, Minneapolis, MN, USA) were used to measure IL-12 levels in the sera of patients and controls according to the instructions of the manufacturer. All the tests were set up in duplicate and the data were analyzed by Genesis Windows Software for microplate-based assays (Labsystems, Finland). By this assay, the minimum detectable concentration of IL-12 was 5 pg ml^{-1}. The mean value of the cytokine in the control sera plus 3 S.D. was used as 'cut-off' value for designation of patients sera as positive or negative. The data were analyzed using Student's t test. A P value of less than 0.05 was considered significant. The findings are presented as mean value ± S.D.

2.3. Preparation of PBMC, mRNA extraction and reverse transcription (RT-) PCR

PBMC consisting of monocytes and lymphocytes were separated from the heparinized peripheral venous blood on Lymphoprep (1.077 g ml^{-1}; Nyegaard and Co. As., Oslo, Norway) as described elsewhere [9,10]. mRNA was extracted from 10×10^6 PBMC of the patients and the controls and used in RT-PCR for human cytokines as described earlier [11]. Briefly, mRNA from the PBMC were extracted by using the Quick Prep Micro mRNA purification kit (Pharmacia Biotech, Sweden) according to the manufacturer's instructions. cDNA was synthesized from the mRNA by using a first strand cDNA synthesis kit (Pharmacia Biotech, Sweden) according to the protocol of the kit manufacturer. PCR was performed using the first strand cDNA as the template and the primers specific for the cytokine IL-12 (sense 5′-CCCTGACACCTGGAGTACTC-3′ and antisense 5′-CAGTTAGGTTCTGATCCAGGA-3′). As a positive control, the primers specific for the house keeping gene, β-actin (sense 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ and antisense 5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′), were used. The reagents of the DNA PCR kit (Perkin-Elmer, Cetus) were used in amplification reactions according to the manufacturer's instructions. For PCR, the cycling parameters were denaturation at 95°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 45 s. PCR was performed for 35 cycles followed by an additional step of extension at 72°C for 7 min and the amplified DNA was analyzed by gel electrophoresis. The DNA bands for β-actin (661 bp) and IL-12 (227 bp) were size-identified by comparing with the bands of molecular mass marker DNA. In all the specimens, the DNA corresponding to β-actin was amplified suggesting that purified mRNA was suitable for RT-PCR. Depending upon the presence or absence of a DNA band of 227 bp expected size, a specimen was considered positive or negative for IL-12 mRNA.

3. Results

3.1. Serum levels of IL-12

The mean value of IL-12 in the control sera was 2.6 ± 2.4 pg ml^{-1} with a range of 0–10 pg ml^{-1}. The findings presented in Fig. 1 show that IL-12 was present in the sera of the patients with DF (70%), DHF grade I (40%) and DHF grade II (28%), while it was not detectable in

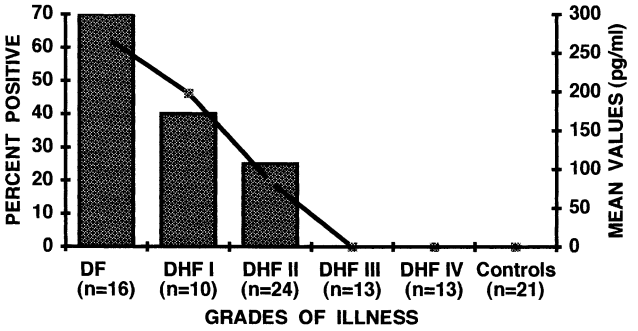

Fig. 1. Levels of IL-12 in cases of dengue. Sera collected from the patients with various grades of the illness were screened for IL-12 concentration by sandwich ELISA using commercial kits. The mean values (line) of the data (pg ml^{-1}) and the percentages of the patients positive for IL-12 (columns) have been presented. The figures in parentheses represent the total number of the cases in each group.

any of the patients with DHF grade III and DHF grade IV. As compared to patients with DF (mean value of 270 ± 102 pg ml^{-1}), lower levels of IL-12 were detected in the sera of patients with DHF grade I (198 ± 86; $P < 0.05$) and substantially lower levels in patients with DHF grade II (84 ± 52; $P < 0.001$). The highest IL-12 value (670 pg ml^{-1}) was seen in one male patient aged 25 years, with DF, on the second day of the illness.

The patients were grouped according to the day of the illness at the time of collection of the sera, as between days 1 and 4, between days 5 and 8 and day 9 onwards. It was observed that IL-12 levels were highest during the first 4 days of illness and the mean values were 265 ± 110 pg ml^{-1}, a lower level (116 ± 60 pg ml^{-1}) was found on days 5–8 and was then not detectable on day 9 onwards (Fig. 2). The data were further analyzed with respect to the distribution of the patients with various grades of illness and the days of illness with detectable IL-12. It was observed that during the first 4 days of the illness, the patients with DF had the highest levels (325 ± 110 pg ml^{-1}) of IL-12 while the patients with DHF grades I and II had 240 ± 95 pg ml^{-1} and 100 ± 46 pg ml^{-1} of IL-12, respectively. The number of DHF grade III patients ($n = 2$) was too small and no patient of DHF grade IV in this group was available. During the 5–8 day period, the levels of IL-12 declined in all the patient groups with a

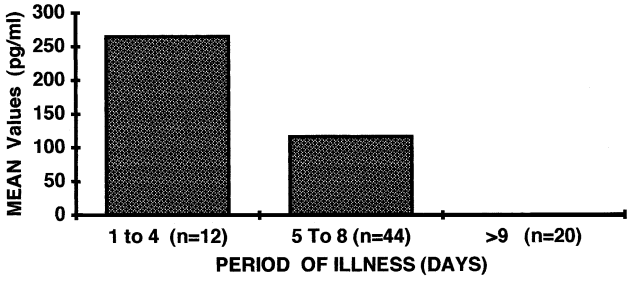

Fig. 2. Amount of IL-12 in patients sera (pg ml^{-1}) as a function of the stage of dengue illness. The figures in parentheses represent the total number of patients in each group.

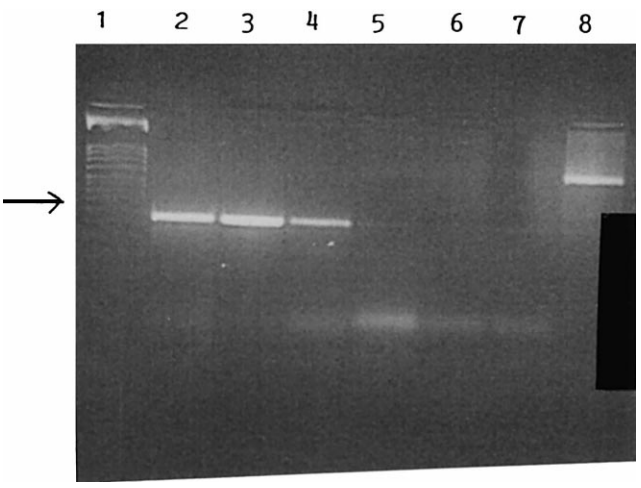

Fig. 3. Ethidium bromide-stained agarose gel of PCR products. Detection of the IL-12 mRNA (arrow corresponding to 227 bp) amplified from the PBMC of the cases of DF (lane 2), DHF grade I (lane 3); grade II (lane 4); grade III (lane 5); grade IV (lane 6) and; absence of IL-12 mRNA in the PBMC from a normal healthy control (lane 7); the mRNA band for β-actin (lane 8) and they were size-identified by comparing with the bands of molecular mass marker DNA (lane 1).

maximum amount of IL-12 detected in the sera of the patients with DF (215 ± 93 pg ml^{-1}) which were significantly higher ($P < 0.001$) than the levels in cases with DHF grade II.

3.2. RT-PCR for IL-12 gene expression

IL-12 gene expression in PBMC of healthy control subjects and in all the 76 patients with dengue was studied by

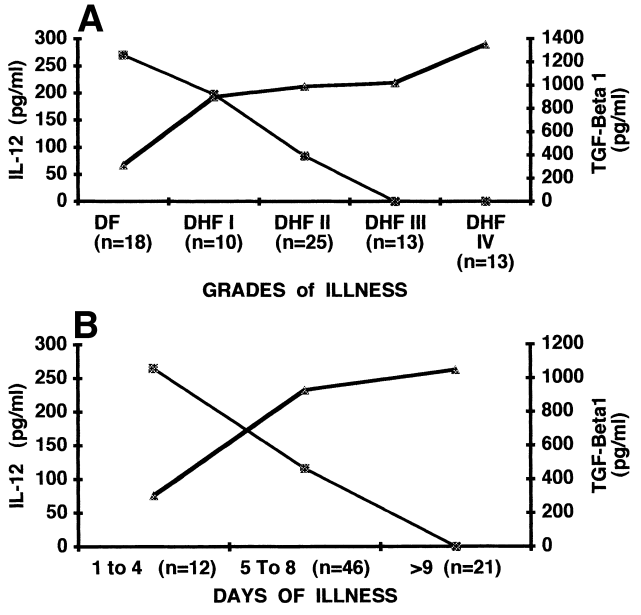

Fig. 4. Comparison of the levels of IL-12 (rectangles) with those of TGF-β1 (triangles) (see [16]) in the sera of the patients with dengue in relation to the grades of the illness (A) and the duration of the illness (B).

detection of IL-12 mRNA using RT-PCR. The results of representative experiments with controls, DF and DHF grade I, II, III and IV patients are given in Fig. 3. The overall results showed that PBMC from none of the healthy control subjects and patients with DHF grades III and IV expressed IL-12 mRNA, whereas 70% of the patients with DF showed evidence of IL-12 gene expression in their PBMC.

4. Discussion

IL-12 is primarily produced by monocytes/macrophages in response to infectious agents (reviewed in [5]). This cytokine has been associated with protection in a number of viral infections (reviewed in [5,12]). The results of this study demonstrate the activation of the IL-12 gene and elevated levels of IL-12 protein in the patients with milder dengue illness (DF) and in early time periods. The levels of IL-12 and mRNA decreased in patients with DHF grades I and II and disappeared from the patients with DHF grades III and IV. These findings suggest that IL-12 is produced early in response to dengue virus infection and may have a protective role against severe dengue disease.

IL-12 is an important immunoregulatory cytokine and has been shown to enhance host cellular responses, including the ability to shift the response in favor of Th1-type and generally promotes clearance of virus and host recovery from infection (reviewed in [12]). Our previous studies on the same group of dengue patients showed a shift from the predominant Th1-type cytokine response observed in 66% cases of mild illness (DF) to a Th2-type response in 71% of the patients with severe DHF grade IV which has a high fatality rate [3]. Studies carried out in the mouse model support the above findings (reviewed in [2,3]). On the basis of these results, it has been proposed that dengue virus induces the production of a cytokine cascade that shifts a Th1-dominant response to a Th2-biased response, resulting in an exacerbation of dengue disease and possibly death of the patients [2,3]. The results of this study are consistent with the hypothesis that the presence of IL-12 is associated with Th1-type response and its absence with the Th2-type response. Thus IL-12 appears to play a role in preventing the severe dengue disease by maintaining the Th1-type response. If this is true, IL-12 therapy may have a profound beneficial effect on the outcome of severe dengue disease as is seen in a number of other viral infections (reviewed in [12]).

Similar to the findings of the present study, rapid induction of IL-12 gene expression and production of IL-12 has been reported in a number of viral infections (reviewed in [5,12]). However, recent studies show that IL-12 is not essential for the generation of a Th1-type cytokine response in mouse hepatitis virus [13] and lymphocytic choriomeningitis virus [14] infections. What factors regulate Th1 cell differentiation in such situations is not clearly known.

Macrophages are the principal cells to replicate dengue virus and present its antigen to immunologically competent cells (reviewed in [2]), therefore, in dengue virus infection macrophages could be the primary source of IL-12. Macrophages also produce the cytokine transforming growth factor-β1 (TGF-β1) which is an important immunoregulatory cytokine and has multiple roles to play in the pathogenesis of viral diseases. TGF-β1 may act as a proinflammatory or anti-inflammatory cytokine, decreases the production of free radicals, inhibits receptor expression and functions of IFN-γ, IL-1α, IL-2 and TNF-α, inhibits Th1-type cytokines and enhances production of Th2-type cytokines such as IL-10 (reviewed in [15]). Romani et al. [5] have suggested that TGF-β is probably the most effective inhibitor of the IL-12 system, by inhibiting both IL-12 production and its effects on T and NK cells. Our previous study on the same group of patients has shown that the levels of TGF-β1 in patients with dengue correlated with the severity of the disease and the duration of illness, i.e. the maximum levels of TGF-β1 were detected in patients with DHF grade IV and those who had the disease for more than 9 days. In more severely sick patients of DHF grade III and IV, the levels of TGF-β1 showed a persistent increase at all time points [16]. The results of serum levels of TGF-β1 and IL-12, when analyzed together (Fig. 4A,B), show an inverse relationship in patients with DF and with various grades of DHF and duration of disease. These findings warrant an in-depth investigation to determine the exact role of IL-12 and TGF-β1 in protection versus pathogenesis of severe dengue disease.

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