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Shift from a Th1-type response to Th2-type in dengue haemorrhagic fever

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Dengue virus causes a mild febrile illness, dengue fever (DF) and at times a severe illness, dengue haemorrhagic fever (DHF), the pathogenesis of which is not fully known. The present study was undertaken to investigate the profile of Th1- and Th2-type cytokines in the sera of 117 patients of various grades of dengue illness and 21 normal healthy controls. Commercial sandwich ELISA kits were used to assay the serum levels of tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin (IL)-2, IL-4, IL-6, and IL-10. Serum levels of IFN-γ and IL-2 were the highest in DF while in the most severe cases of DHF (i.e. grade IV) serum levels of IL-6 and IL-10 were maximum. Levels of IL-10 were negligible in patients with DF and levels of IFN-γ were lowest in patients with DHF grade IV. The levels of TNF-α were higher in cases of DHF grades II, III, and IV and did not show the clear association pattern shown by IFN-γ, IL-2, IL-4, IL-6, and IL-10. The levels of IFN-γ, IL-6 and TNF-α increased first while IL-4 and IL-10 levels increased during the 4th to 8th day of the illness. The most significant finding of the present study was a shift of the predominant Th1-type response observed in 66% of DF patients to the Th2-type response seen in the 71% of DHF grade IV patients, thus indicating a possible role for Th2 cells in the pathogenesis of DHF.

DENGUE virus, prevalent in over 100 tropical and sub-tropical countries with about two billion people at risk[1], produces a mild self-limiting acute febrile illness, dengue fever (DF), and a life threatening severe illness, dengue haemorrhagic fever (DHF). DHF has emerged as the most important arbovirus disease in man in the last two decades. It has been estimated that about 100 million cases of DF occur every year with about 250,000 cases of DHF[2]. The frequency of dengue epidemics has markedly increased with hyperendemic transmission and expansion to newer geographical areas. In a number of dengue endemic countries such as Bangladesh, Sri Lanka, and India where DHF was previously unknown, severe epidemics of DHF have occurred[1], (U. C. Chaturvedi, unpublished).

DHF has been classified into four grades on the basis of the clinical presentation and laboratory findings; the mildest is grade I and the most severe is grade IV[3].

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The pathognomonic features of DHF are increased capillary permeability, cerebral edema, altered number and functions of leucocytes, increased haematocrit and thrombocytopoenia[4,5]. Extensive plasma leakage in various serous cavities of the body including the pleura, pericardium and peritoneal cavities in DHF grades III and IV may result in profound shock. Despite extensive studies, the pathogenesis of DHF is still not fully understood, though various suggestions have been made to explain the plasma leakage[2,6,7].

Dengue virus infection induces the production, by $CD4^+$ T cells, of a unique cytokine, cytotoxic factor (CF), in mice (mCF) and in man (hCF). The amino-terminal sequence of mCF has no homology with any of the known proteins or cytokines (as of December 1996) (refs. 8–12). mCF and hCF appear to be pathogenesis-related proteins, capable of reproducing DHF-like pathological lesions in mice, such as increased capillary permeability, cerebral edema, and blood leukocyte changes[9–15].

Cytokine secretion profiles correlate well with the distinctive functions of helper T(Th)1 and Th2 cells which are the major subsets of fully differentiated $CD4^+$ Th cells. Th1 cells secrete interferon-gamma (IFN-γ), interleukin-2 (IL-2) and tumour necrosis factor-beta (TNF-β) and are responsible for cell-mediated inflammatory reactions, delayed type hypersensitivity and tissue injury in infections and autoimmune diseases. Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 and are associated with help for B cell antibody production. These cytokines can also be produced by Th0 cells. Cross-regulation of the two clones is mediated by IL-10 and IFN-γ. Furthermore, TNF-α and IL-10 form an autoregulatory loop, in which TNF-α is an inducer of IL-10, and IL-10 is a down-regulator of TNF-α. Infections eliciting a dominant humoral immune response induce a higher expression of Th2-related cytokines and are associated with low levels of IFN-γ and IL-2, whereas those characterized by delayed-type hypersensitivity response show a higher expression of Th1 cytokines IFN-γ and IL-2 and low levels of IL-4 (refs 16–19). In a number of parasitic, fungal, bacterial and viral infections such as human immunodeficiency virus (HIV), herpes simplex and influenza viruses, a Th1 response is linked to recovery from infection while a Th2-type response tends to lead to severe pathology and exacerbation of the disease (reviewed in ref. 19). A few studies have reported the levels of IFN-γ, TNF-α, IL-1β, IL-2 and IL-6 in cases of DHF[20–22]. Information on the Th1-type of cytokines in DHF is inadequate and no information on the Th2 response is available in the literature. Since cytokines are so crucially involved in resistance to, and exacerbation of infectious diseases, this study was undertaken with a view to unravel possible pathogenic mechanisms in DHF. The findings presented here indicate a shift from Th1- to Th2-type response correlating with increasing severity of the illness, thus indicating a possible role for Th2-type response in the pathogenesis of DHF.

Patients and methods

Patients

An extensive epidemic of dengue haemorrhagic fever occurred in Northern India during August–November 1996. The study population consisted of 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 during this epidemic. All the patients were examined thoroughly by a clinician and the laboratory investigations were done. At the time of reporting to the hospital, the clinical presentation of every patient was recorded, a Hess test was done and their 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 the 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 dengue fever (DF) or dengue haemorrhagic fever (DHF) grades I, II, III or IV according to the criteria of the World Health Organization[3]. A total of 117 cases were included in the present study, among them 72 (62%) were below 15 years of age, the youngest being 8 months old and the oldest being 55 years old. Diagnosis of dengue virus infection was established either by virus isolation or by detection of virus-specific IgM in the sera. The virus was isolated by ic inoculation in 1–3 day old infant mice followed by titration of the isolates and typing by neutralization test as described earlier[23–24], and the virus-specific IgM were measured using standard protocol[25]; in a number of cases dengue IgM capture ELISA was also done using commercial kits (Pan Bio, East Brisbane, Australia). For controls, 21 age-matched normal healthy individuals, without history of febrile or any other illnesses in the previous three months, were included. Among the patients, 35 were classified as DF, 12 as DHF grade I, 35 as grade II, 21 as grade III and 14 as grade IV. Sera collected from the patients (on the 1st to the 18th day of illness) and controls were divided in aliquots (to avoid repeated freezing and thawing) and quickly frozen and stored at $-60°C$. For the cytokine estimation, sera were transported to Kuwait on dry ice and stored at $-70°C$ until further tested.

Assay of cytokines

Serum cytokine levels were assayed by commercial ELISA kits purchased from Immunotech (Coulter Co. France). Assays were carried out according to the instructions of the manufacturer without diluting the sera. All the tests were set-up in duplicate and the data was analysed by Genesis Windows Software for micro-plate-based assays (Labsystems). The minimum detectable concentrations by the present assay were 5 pg/ml of TNF-α, 8 pg/ml of IFN-γ, 5 pg/ml of IL-2, 5 pg/ml of IL-4, 3 pg/ml of IL-6, and 5 pg/ml of IL-10. The mean value of the cytokine in the control sera plus 3 SD was used as the cut-off value for designation of a patient's sera as positive or negative. The data was analysed statistically using Students *t*-test. A p value of less than 0.05 was considered significant.

Results

Tumour necrosis factor-alpha

The data summarized in Figure 1 *a* show the mean values of the concentration of TNF-α in the sera of the patients with different grades of the illness. The maximum mean value of 155 ± 30 pg/ml was seen in cases of DHF grade IV with a peak value of 1393 ± 243 pg/ml in one case while the minimum value (78 ± 19 pg/ml) was seen in cases of DF ($p = < 0.001$). In a 52-year-old patient with DHF grade II, the serum concentration of TNF-α on the 8th day of the illness was 1880 ± 36 pg/ml. His recovery from the illness was uneventful. Among the controls, values ranged from 0 to 18 pg/ml with a mean value of 2.83 ± 5 pg/ml. A maximum of 85% of the sera of grade IV were positive for TNF-α while 68% of the DF sera were positive (Figure 1 *a*). An analysis of the sera positive for TNF-α vis-a-vis the day of illness presented in Figure 1 *b* shows that among the samples collected within the first three days of the illness, 83% were positive.

Interferon-gamma

The data presented in Figure 2 *a* show a peak mean value of 119 ± 28 pg/ml of IFN-γ in cases of DF with the highest value of 1200 ± 310 pg/ml in one case. The cases of DHF grade IV had the lowest mean value of 41 ± 15 pg/ml ($p = < 0.001$), with mean value in the control sera being 7 ± 6 pg/ml. The maximum number of IFN-γ positive cases were seen in DF (83%) and the lowest number of positive cases (42%) were seen in DHF grade II (Figure 2 *a*). A day-wise distribution of IFN-γ positive cases presented in Figure 2 *b* shows maximum number (87%) among the patients seen during the first three days of illness.

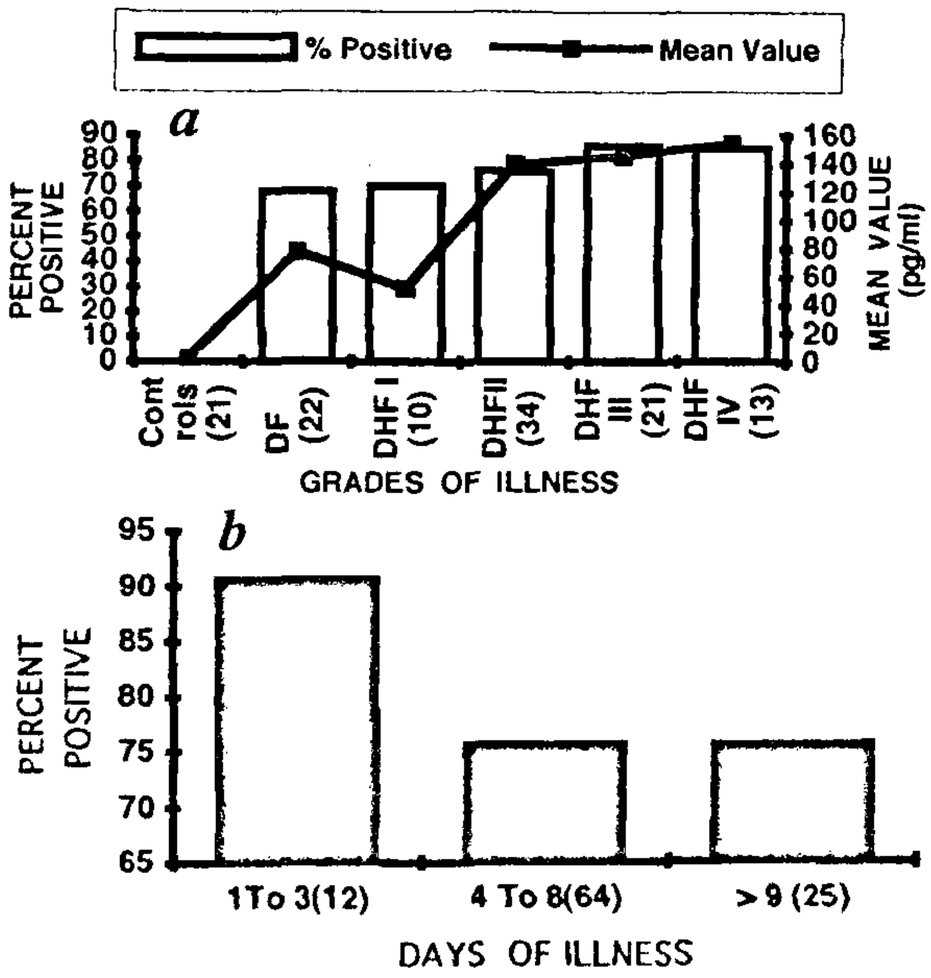

Figure 1. Levels of tumour necrosis factor-alpha (TNF-α) in cases of dengue. Sera collected from the patients of various grades of the illness were screened for TNF-α concentration by sandwich ELISA using commercial kits. The mean value of the data has been presented. Mean value from the 21 normal healthy control sera plus three standard deviation was taken as the cut-off value to designate a serum sample from a case as positive. *a*, Mean value of TNF-α in the sera and the percentage of the sera having TNF-α levels above the cut-off value in various grades of illness. *b*, Percentage of TNF-α positive patients as a function of the stage of dengue illness. The figures in the parenthesis represent total number of the cases in each group.

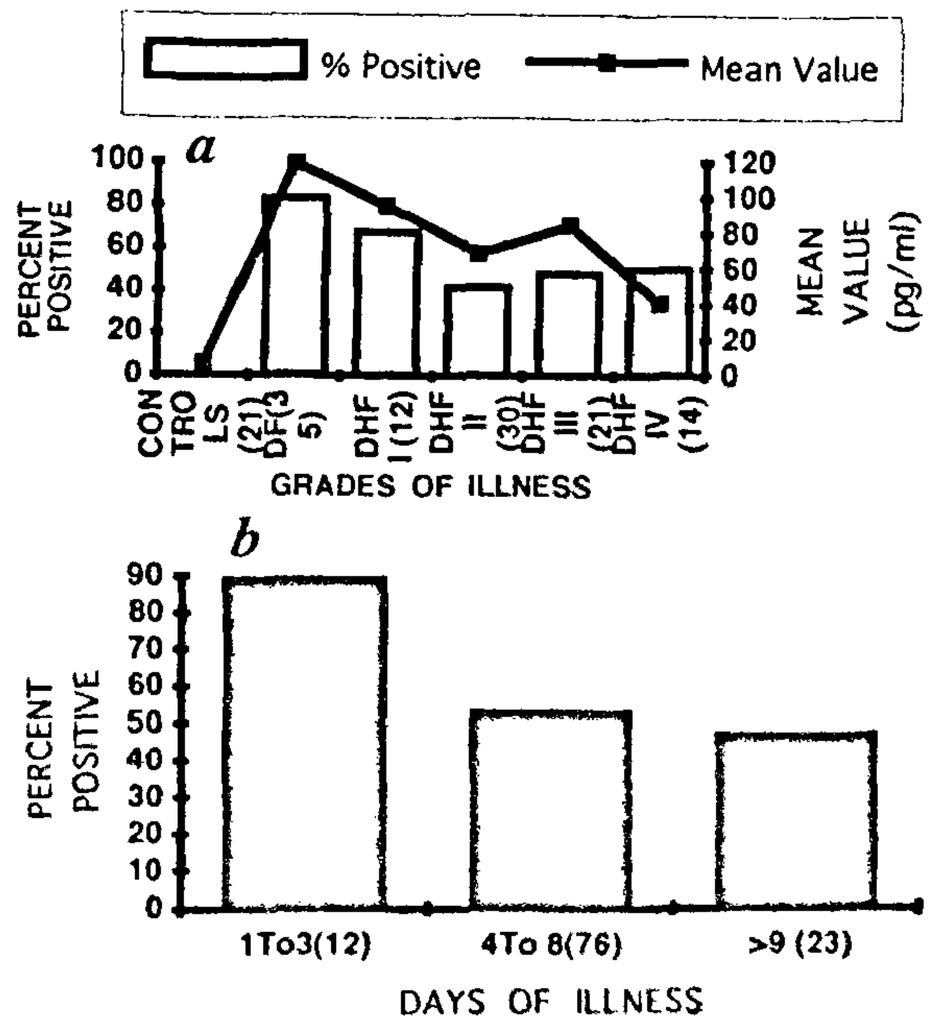

Figure 2. Levels of interferon-gamma (IFN-γ) in cases of dengue. Sera collected from the patients of various grades of the illness were screened for IFN-γ concentration by sandwich ELISA using commercial kits. *a*, Mean value of IFN-γ in the sera and the percentage of the sera having IFN-γ levels above the cut-off value in various grades of illness. *b*, Percentage of IFN-γ positive patients as a function of the stage of dengue illness.

Interleukin-2

The mean values of IL-2 in the control sera was 2.6 ± 3 pg/ml with a range of 0 to 13 pg/ml. The data of the mean value presented in Figure 3 *a* show a peak of 70 ± 30 pg/ml in cases of DF, with the highest concentration of 328 ± 115 in one case. In the case of DHF grade II it was minimum, the mean value being 13 ± 7 pg/ml ($p = < 0.001$) while in DHF grade IV the mean value was 44 ± 18 pg/ml ($p = < 0.001$). The percentage of the sera positive for IL-2 was higher in patients of DF than those of DHF grade IV ($p = < 0.02$) (Figure 3 *a*). A day-wise distribution of the IL-2 positive cases presented in Figure 3 *b* shows maximum number (78%) among the sera collected within the first three days.

Interleukin-4

The mean value of IL-4 in the control sera was 2.7 ± 3 pg/ml with a range of 0 to 11 pg/ml. The findings presented in Figure 4 *a* show significantly higher amounts ($p = 0.001$) of IL-4 in the sera of DHF grade IV (176 ± 45 pg/ml) with the maximum value of 1725 ± 508 pg/ml in one case. This patient, aged 26 years, was in deep shock and bleeding from all over the body when the blood sample was collected on the 5th day of the illness and she died the same evening. 71% of DHF grade IV patients and 38% of grade III patients were positive for IL-4 (Figure 4 *a*).The day-wise distribution of IL-4 positive sera presented in Figure 4 *b* shows maximum positives during the 4th to 8th day of illness while the minimum occurred during the first three days.

Interleukin-6

The mean serum levels of IL-6 in the controls was 1 ± 2 pg/ml with a range of 0 to 7 pg/ml. The mean concentration of IL-6 was 203 ± 65 pg/ml in DHF grade IV with a peak value of 2163 ± 417 in a patient while the minimum was 24 ± 8 pg/ml ($p = < 0.001$) in a patient with DHF grade I (Figure 5 *a*). A distribution of IL-6 positive sera presented in Figure 5 *a* did not show marked differences in the various grades of illness ($p = > 0.05$). A day-wise analysis of IL-6 positive sera shows 72% in the first three days and 77% during the 4th to 8th day (Figure 5 *b*).

Interleukin-10

IL-10 was absent in all the control sera. The data summarized in Figure 6 *a* show that IL-10 was present mainly in the sera of the cases of DHF grade IV and the mean value was 72 ± 34 pg/ml with a peak value

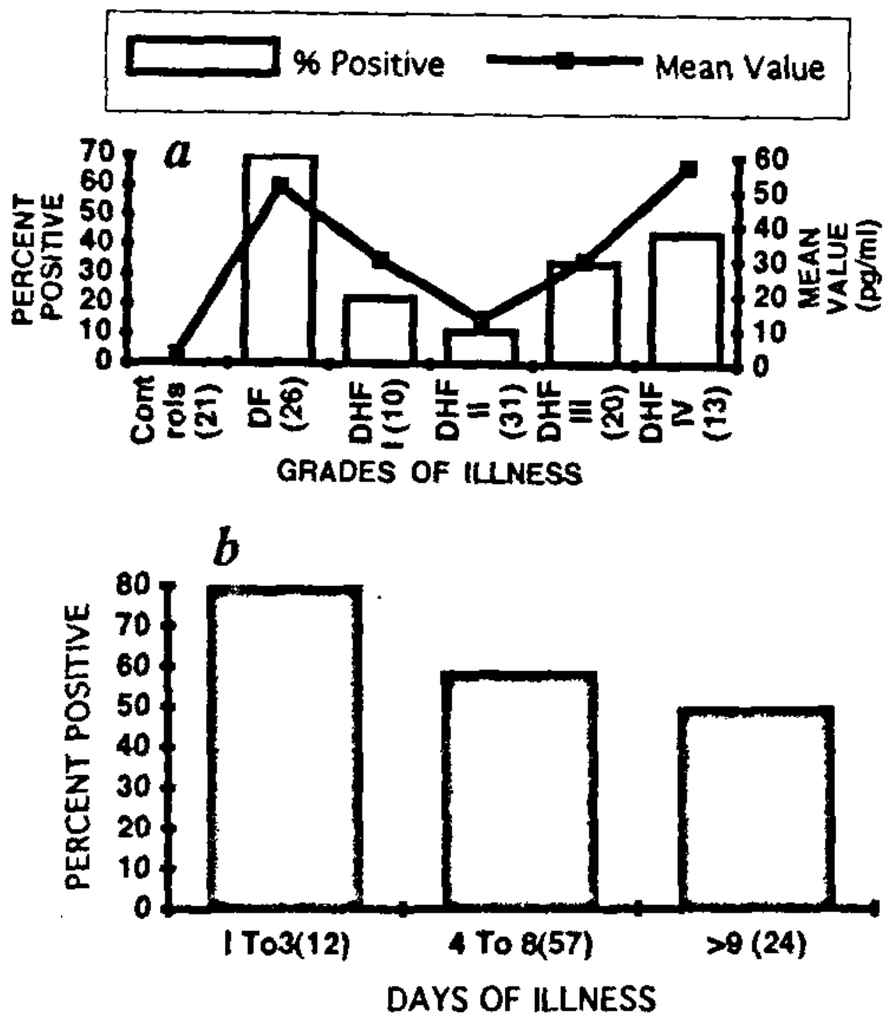

Figure 3. Levels of interleukin-2 (IL-2) in cases of dengue. Sera collected from the patients of various grades of the illness were screened for IL-2 concentration by sandwich ELISA using commercial kits. *a*, Mean value of IL-2 in the sera and the percentage of the sera having IL-2 levels above the cut-off value in various grades of illness. *b*, Percentage of IL-2 positive patients as a function of the stage of dengue illness.

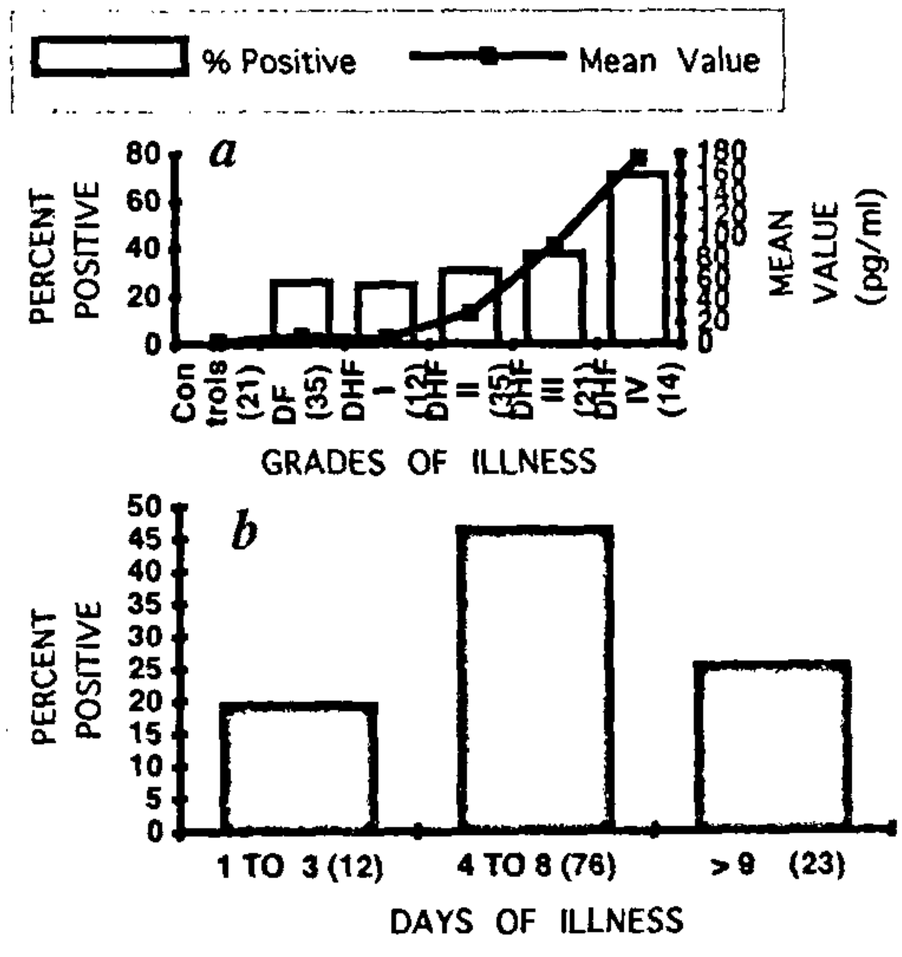

Figure 4. Levels of interleukin-4 (IL-4) in cases of dengue. Sera collected from the patients of various grades of the illness were screened for IL-4 concentration by sandwich ELISA using commercial kits. *a*, Mean value of IL-4 in the sera and the percentage of the sera having IL-4 levels above the cut-off value in various grades of illness. *b*, Percentage of IL-4 positive patients as a function of the stage of dengue illness.

of 359 ± 128 pg/ml in one case, while the mean value was 1 ± 3 pg/ml in DF ($p = < 0.001$). Similarly, 64% of the sera of DHF grade IV were positive for IL-10 (Figure 6 *a*). A higher percentage were positive for IL-10 during the 4th to 8th day of illness (Figure 6 *b*).

Correlation of cytokine profile with severity of illness

The data were analysed to ascertain whether the cytokine response in an individual patient could be categorized as Th1-biased, Th2-biased or indeterminate (i.e. having a mixed response). The cytokine profile in the case of a DF presented in Figure 7 *a* shows increased levels of IFN-γ and IL-2 and absence of IL-4, IL-6 and IL-10, a typical Th1 response while that of DHF grade IV presented in Figure 7 *b* shows increased levels of IL-4, IL-6 and IL-10 and lower levels or absence of IL-2 and IFN-γ, a typical Th2-type of response. An analysis of the total number of cases is summarized in Figure 8. It was observed that 66% of the cases with DF had Th1-type of response. As the severity of the illness increased the response shifted to Th2-type which was seen in 71% of the cases with DHF grade IV. Some of the cases had a mixed response and were categorized as 'indeterminate' group and varied from 6 to 28% in different grades of illness (Figure 8).

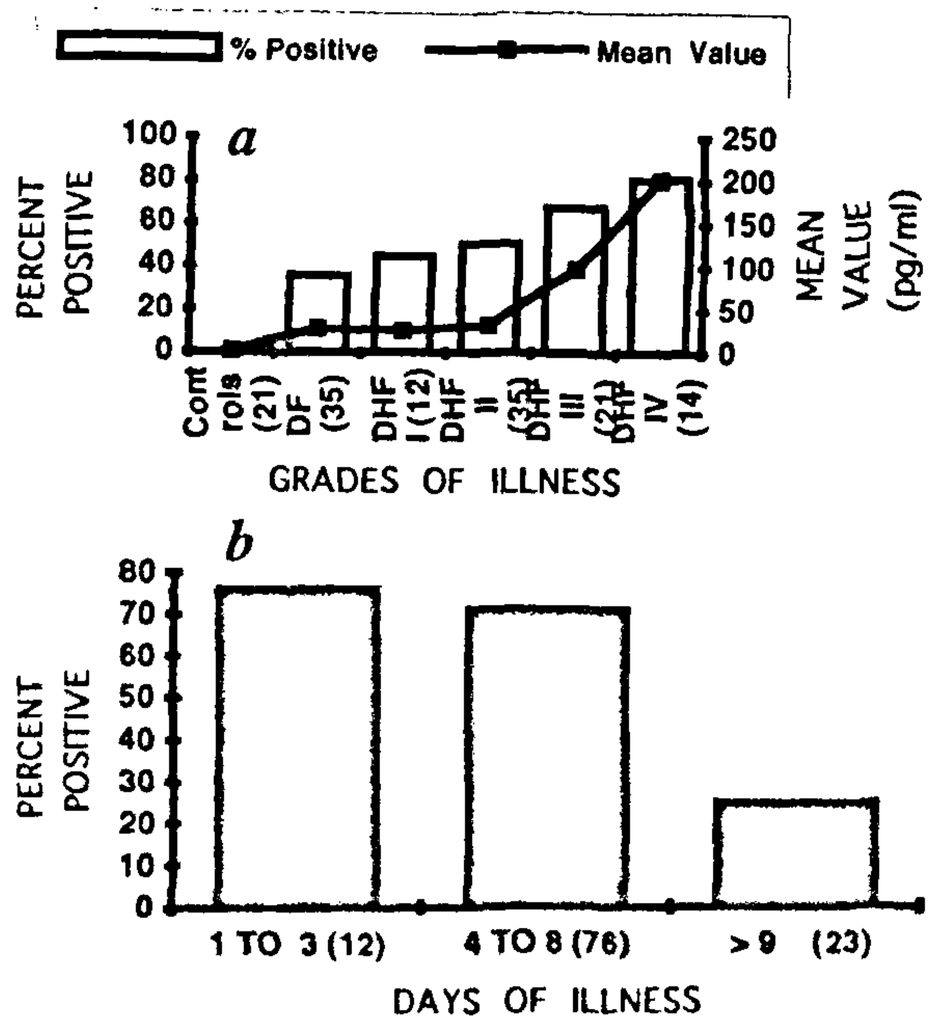

Figure 5. Levels of interleukin-6 (IL-6) in cases of dengue. Sera collected from the patients of various grades of the illness were screened for IL-6 concentration by sandwich ELISA using commercial kits. *a*, Mean value of IL-6 in the sera and the percentage of the sera having IL-6 levels above the cut-off value in various grades of illness. *b*, Percentage of the sera having IL-6 levels above the cut-off value in the sera collected at different periods of the illness.

Discussion

The most significant finding of the present study was a shift from the predominant Th1-type response observed in cases with DF to the Th2-type in severe cases with

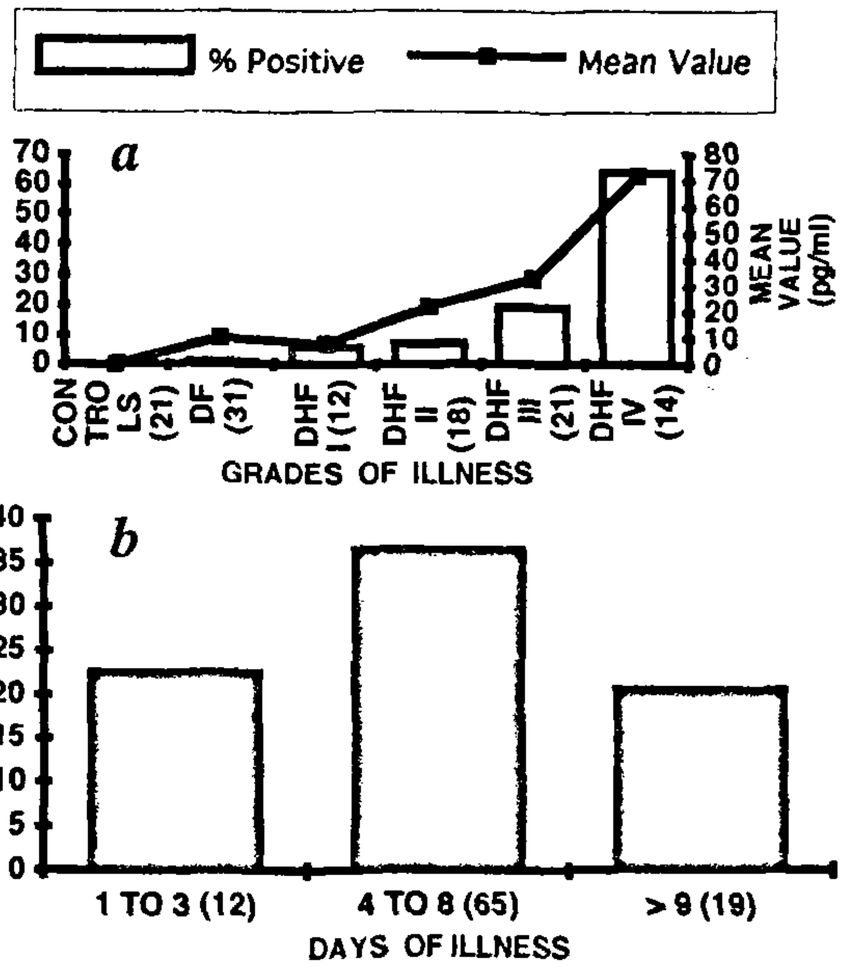

Figure 6. Levels of interleukin-10 (IL-10) in cases of dengue. Sera collected from the patients of various grades of the illness were screened for IL-10 concentration by sandwich ELISA using commercial kits. *a*, Mean value of IL-10 in the sera and the percentage of the sera having IL-10 levels above the cut-off value in various grades of illness. *b*, Percentage of the sera having IL-10 levels above the cut-off value in the sera collected at different periods of the illness.

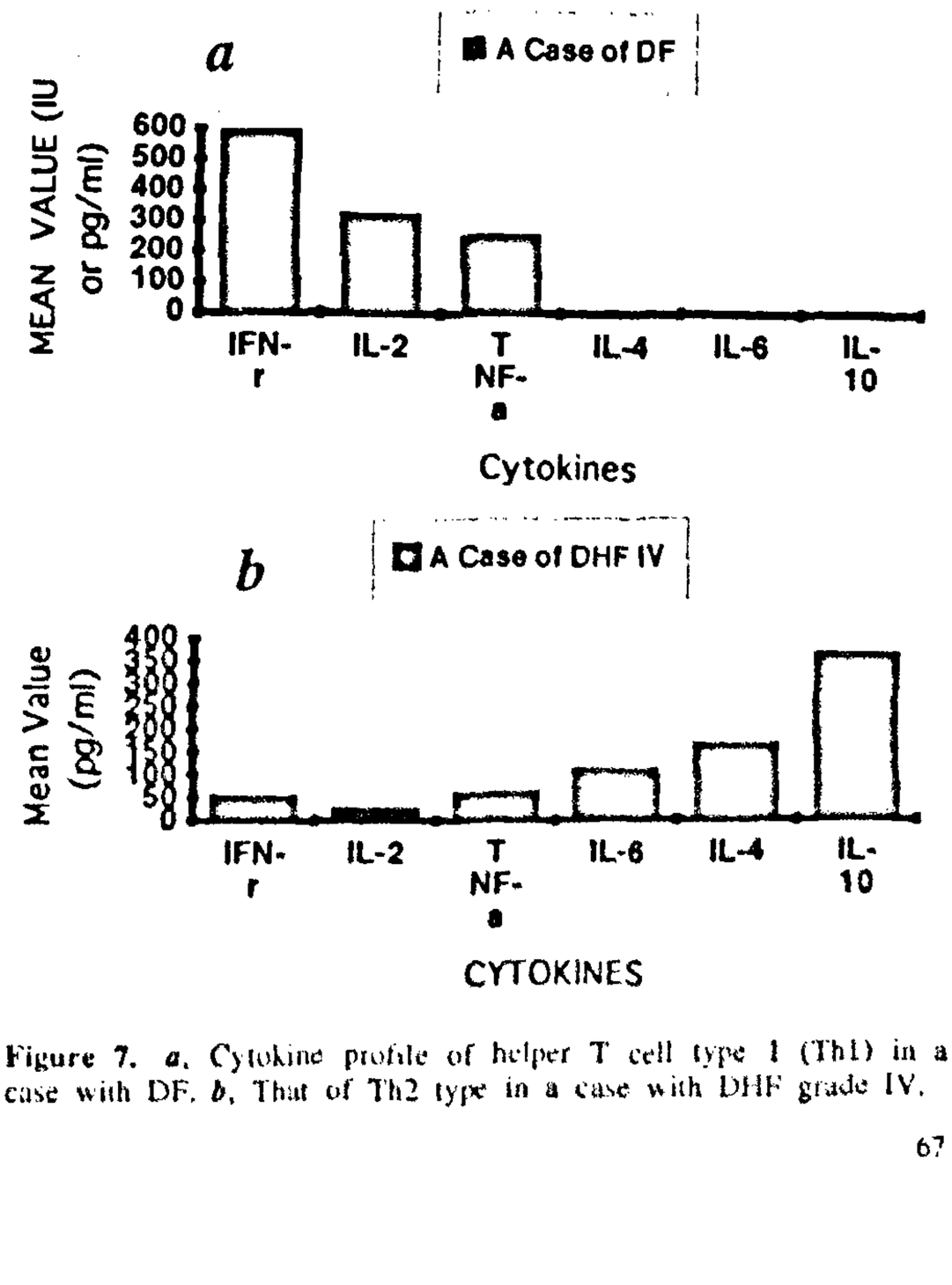

Figure 7. *a*, Cytokine profile of helper T cell type 1 (Th1) in a case with DF. *b*, That of Th2 type in a case with DHF grade IV.

DHF grade IV. Increased serum levels of IL-4 and IL-10 were observed mainly in cases with DHF grades III and IV. The number of IL-2 and IL-6 positive sera was similar in cases with DF and DHF grade IV, but the mean value of IL-6 was significantly higher in the latter group ($p = < 0.001$). In contrast, the levels of IFN-γ and IL-2 were highest in cases with DF and low in DHF grade IV. TNF-α levels did not show a definite associative pattern. The cytokine levels which increased first were IL-2, IL-6, IFN-γ and TNF-α, while IL-4 and IL-10 emerged during the 4th to 8th day of the illness. On the basis of similar elevation of serum levels of IL-2 and IFN-γ in both DF and DHF , it was previously concluded that these cytokines alone are not responsible for the pathogenesis of DHF[22]. A previous study reported inconsistencies in TNF-α, IL-6 and IL-1β production in dengue-infected patients[21] while others suggest in-depth study of TNF-α in patients of DHF[26,27].

Studies carried out in a mouse model provide interesting clues. Mice inoculated intracerebrally with dengue type 2 virus (DV) develop illness around day 7–8 post-infection followed by a severe illness and death by day 11. Such mice develop immunosuppression towards the DV and heterologous antigens. They do not have cell-mediated immune responses as shown by (i) the absence of foot-pad swelling reaction (DTH), and (ii) the absence of a proliferative T cell response to challenge by DV, (iii) lack of protection against DV by adoptive transfer of DV-immune spleen cells, (iv) absence of graft-versus-host reaction, (v) depressed macrophage functions, and (vi) the development of an anti-SRBC antibody response with a concurrent absence of anti-SRBC DTH response[23,24,28,29]. With a retrospective analysis of these data obtained more than two decades ago from the view-point of the Th1–Th2 paradigm, we find a clear indication of a predominant Th2-type response during fatal dengue virus infection in mice. This supports the findings of the present study on humans showing the predominance of a Th1-type response in 66% cases of mild illness (DF) and a Th2-type response in 71% of severe DHF grade IV which has a high fatality rate. A similar conclusion was drawn in a report that progression of HIV infection to AIDS is dependent on a Th1 > Th2 dominance[30].

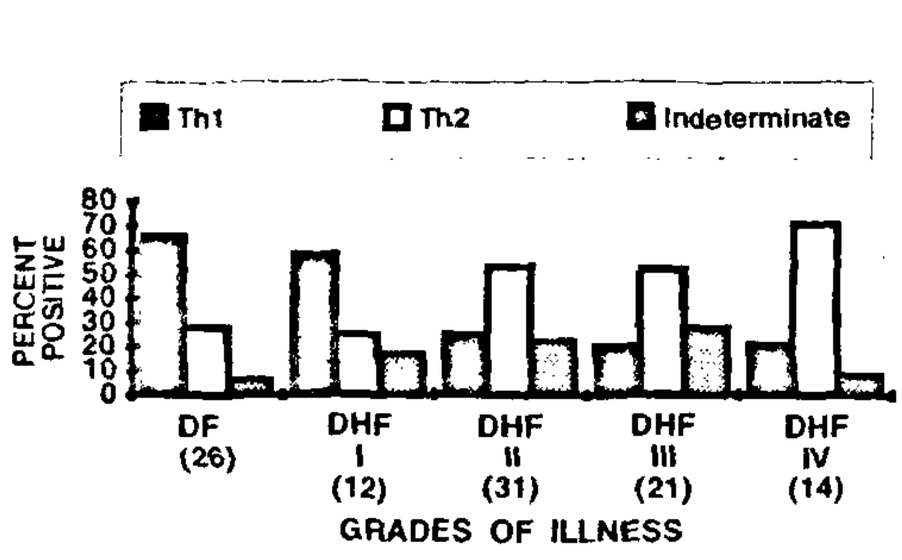

Figure 8. Analysis of total cases of different grades of illness on the basis of their cytokine profile. The figures in the parenthesis represent total number of the cases in each group.

The pathogenic role of mCF has been established in producing lesions in mice similar to those seen in DHF, for example, increased capillary permeability, cerebral edema and alterations in the number and functions of leucocytes[13–15]. The hCF purified from the sera (by ion-exchange and high pressure liquid chromatography) of DHF patients, when inoculated into mice brings about increased capillary permeability and damage to the blood–brain barrier indicating its pathogenicity[9]. During an extensive epidemic of DHF in northern India during 1996, the presence of hCF was shown in 90% of the 333 cases with peak amounts in the most severe cases of DHF grade IV[31]. The same lot of patients were investigated in the present study. Further, *ex vivo* culture of peripheral blood mononuclear cells (PBMC) of such cases showed production of hCF by $CD4^+$ T cells, but the number of hCF-producing cells did not correlate with the severity of the illness[32,33]. The number of such cells may not indicate the amount of hCF produced as it may depend upon factors (not yet known) that trigger or down-regulate the hCF-producing cells. It is not known whether hCF-producing cells are Th1 or Th2 or yet another subset of $CD4^+$ T cells. It would appear that hCF-producing cells are either Th2-type or are regulated by Th2-cytokines. The mechanism of action of mCF/hCF has been delineated. mCF selectively kills Th cells, $H\text{-}2A^+$ macrophages and the cells capable of liberating histamine via production of nitrite and respiratory burst leading to the formation of peroxynitrite which results in apoptosis of the target cells. This is a calcium-dependent process and induces influx of calcium in the target cells[11,34–37]. Liberation of histamine by the affected target cells increases vascular permeability causing plasma leakage and hemorrhages producing DHF[5,11]. The production of mCF/hCF precedes the clinical illness in mice and man[9,15,31,32]. The minimum amount of mCF required for detectable increase in capillary permeability is 150 µg/kg body weight in mice[38] (U. C. Chaturvedi, unpublished); translated in terms of a man weighing 60 kg, the amount of hCF required to induce DHF is estimated to be 9 mg. The free radicals, nitrite and peroxynitrite, directly up-regulate production of IL-1β, TNF-α, IL-8 and hydrogen peroxide in macrophages while TNF-α induces the production of IL-6, IL-8 and IL-10[39,40]. In another study we have shown that increased levels of IL-8 in the sera and IL-8-mRNA in the PBMC were associated with the increasing severity of DHF. About half of the patients of DHF grade IV who died had serum level of IL-8 above 200 pg/ml, the highest being 5568 pg/ml in one of them. These results suggest that IL-8 may have an important role in increasing the severity of the disease and death[41].

With the data available, it may be proposed that dengue virus induces the production of hCF which generates free radicals that in turn induce histamine production and Th-1 response followed by Th-2 response and IL-8 production, thus increasing vascular permeability and producing DHF. In conclusion, a shift from a Th1-dominant response to a Th2-biased response may be associated with an exacerbation of dengue virus infection reminiscent of the situation in HIV infection.

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