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HIGH LEVELS OF INTERFERON ALPHA IN THE SERA OF CHILDREN WITH DENGUE VIRUS INFECTION

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Abstract. We measured the levels of interferon alpha (IFNα) in the sera of Thai children hospitalized with dengue hemorrhagic fever (DHF) or dengue fever (DF) to examine the role of IFNα in dengue virus infections of humans. The percentage of patients who had detectable levels of IFNα (≥ 3 U/ml) was higher in patients with DHF (80%, $P < 0.001$) and in patients with DF (60%, $P < 0.001$) than in healthy Thai children (7%). The levels of IFNα were higher in patients with DHF and in patients with DF on the first few days after the onset of fever than in healthy Thai children. The average levels of IFNα in patients with DHF were high two days before defervescence, decreasing gradually until the day of defervescence. There was a subset of patients with DHF who had increasing levels of IFNα after defervescence. However, the levels of IFNα in patients with DF were not high after fever subsided. The levels of IFNα were not different among children with DHF grades 1, 2 and 3. Among patients with DHF, T lymphocytes were activated to a higher degree in high IFNα producers than in low IFNα producers. These results indicate that similarly high levels of IFNα are produced in vivo during the acute stages of DHF and DF, and that high levels of IFNα remain after fever subsides in some patients with DHF, but not in patients with DF.

Dengue virus infections are a major cause of morbidity in tropical and subtropical areas of the world. Dengue virus infection causes two forms of illness; dengue fever (DF) and dengue hemorrhagic fever (DHF).[1] Dengue fever is a self-limited febrile disease, while DHF is a severe, sometimes fatal syndrome characterized by hemorrhagic manifestations and plasma leakage that may lead to shock. The mechanisms of recovery from dengue virus infection and the pathogenesis of DHF are not clearly understood.

We have reported that dengue virus–infected cells are lysed by natural killer cells and by antibody-dependent cell-mediated cytotoxicity.[2] We have also reported the presence of dengue virus–specific, cytotoxic T lymphocytes that lyse dengue virus-infected cells in a major histocompatibility complex–restricted fashion.[3,4] Antibodies to dengue viruses neutralize the virus and can prevent infection.[5] These immune responses may be important in prevention and recovery from dengue virus infection, and may contribute to the pathogenesis of DHF. We have detected higher levels of activation of T lymphocytes in patients with DHF than in those with DF, and have hypothesized that dengue virus–specific T cells play an important role in the pathogenesis of DHF.[6] Another host defense mechanism that should be considered is interferon (IFN) production. It has been reported that IFN has an important role in controlling viral infections.[7] We have reported that dengue virus–infected monocytes produce IFNα,[8] and that dengue virus–infected monocytes also induced IFNα from autologous nonimmune lymphocytes.[9] Interferon alpha produced by these two mechanisms protects uninfected monocytes from dengue virus infection.[8,9] In addition to antiviral effects, IFNα has immunoregulating effects on natural killer cells,[10] T cells,[11] and B cells.[12] Thus, IFNα may have important regulatory roles in the pathogenesis of DHF.

In this study, we examined the levels of IFNα in the sera of patients hospitalized with DHF and those hospitalized with DF, and compared those data with levels of IFNα in the sera of healthy Thai children. The results show that significantly higher levels of IFNα are detected in

the sera of patients with DHF or DF before and on the day of defervescence than in the sera of healthy Thai children. The average levels of IFNα are still high 7–19 days after defervescence in some patients with DHF, while the levels are not high after fever subsides in patients with DF.

PATIENTS AND METHODS

Patients and normal control donors

We examined serial serum specimens from 45 children with an age range of 5–14 years (35 children with DHF and 10 children with DF) who were hospitalized with dengue virus infections during 1987 and 1988 in the hemorrhagic fever unit of the Bangkok Children's Hospital. These sera were obtained from a randomly selected group of sequential patients whose sera were submitted for evaluation of suspected dengue virus infection. Specimens were collected for diagnostic studies by nurses participating in the project within 24 hr of admission to the hospital and daily until discharge; a convalescent specimen was also collected from each child 7–10 days after hospital admission. A portion of each specimen was kept at −70°C and was available for analysis.

To study healthy children (controls), we examined aliquots of single serum specimens from a random sample of healthy Thai children (n = 30, age range 6–11 years) obtained in an earlier cross-sectional study of hepatitis antibody prevalence. These sera were stored at −70°C from the time of collection until assay.

A diagnosis of dengue hemorrhagic fever was assigned to children with dengue infection when the level of thrombocytopenia and signs of hemorrhage and plasma leakage met established criteria.[13] Hospitalized patients were followed with frequent determinations of blood pressure and pulse. Measurements of hematocrit in blood obtained by fingerprick were recorded at 3–4-hr intervals, according to vital signs. Physical findings of plasma leakage (pleural effusion, ascites) and indications of circulatory collapse (cyanosis, cold extremities) were recorded in the clinical record. Whenever feasible, chest radiographs including decubitus views were performed to document the presence of pleural fluid. Hemorrhagic manifestations (positive tourniquet test result for capillary fragility, skin hemorrhages, epistaxis, and gingival, gastrointestinal, or urinary tract hemorrhage) were also recorded. Without knowledge of IFNα levels, three of the investigators (S. N., B. L. I., and A. N.) reviewed every record, including radiographs, to assign a diagnosis. Cases of dengue infection that did not meet the World Health Organization (WHO) definition of DHF[13] were classified as dengue fever (n = 10). The severity of cases of DHF was categorized according to the WHO grading scheme:[13] grade 1 = fever accompanied by nonspecific constitutional symptoms (the only hemorrhagic manifestation is a positive tourniquet test result); grade 2 = spontaneous bleeding, in addition to the manifestations of grade 1 patients, usually in the form of skin and/or other hemorrhages; grade 3 = circulatory failure manifested by rapid and weak pulse, narrowing of pulse pressure (≤ 20 mm Hg) or hypotension, with the presence of cold, clammy skin and restlessness.

Dengue virus infections were confirmed by the detection of antiviral IgM or increasing titers of antiviral hemagglutination-inhibiting antibodies or by virus isolation from plasma, according to previously published methods.[14] Cases of dengue infection were categorized as secondary (dengue infection in a child previously infected with a heterologous flavivirus) or primary (no prior flavivirus infection) according to the presence or absence of an anamnestic antiflavivirus antibody response.[14]. Table 1 shows the age and sex distribution and serologic data of patients and control subjects.

Assay for IFNα

The levels of IFNα were measured in sandwich-type enzyme-linked immunosorbent assays (ELISA) as previously described.[15, 16] Fifty microliters of purified monoclonal antibodies to human IFNα[16, 17] at a concentration of 10 μg/ml was coated on U-bottomed wells of polyvinyl chloride microtiter plates (Dynatech, McLean, VA) for 1 hr at 37°C. The remaining sites in the wells were blocked overnight with 150 μl of blocking buffer (2% bovine serum albumin–phosphate-buffered saline [PBS]) at 4°C. Excess antibodies and blocking buffer were removed and the wells were washed four times with 0.05% Tween 20-PBS. Following the last wash, serial dilutions of human IFNα standard (first international reference preparation for human leukocyte interferon, 69/19, 5,000 IU/ampule) or serum samples were added (50 μl/well) and incubated at 37°C for 1 hr. The wells were then

TABLE 1

*Age and sex distribution and dengue antibody responses of the patients and control subjects**

Subjects	No.	Sex		Average ± SD, years (range)	Dengue serology	
		Male	Female		Primary	Secondary
DHF	35	19	16	9.1 ± 2.9 (5–14)	3	32
DF	10	6	4	9.9 ± 2.9 (5–14)	4	6
Healthy children	30	13	17	7.9 ± 1.4 (6–11)	0	0

* DHF = dengue hemorrhagic fever; DF = dengue fever.

washed four times with Tween 20-PBS. Horseradish peroxidase–linked calf antihuman IFNα immunoglobulin at a 1:4,000 dilution in 10% fetal calf serum-PBS (50 μl/well) was added and incubated for 30 min at 37°C. Finally, the wells were washed three times with Tween 20-PBS and twice with 0.1 M citrate phosphate buffer, pH 5.0, followed by the addition of *o*-phenylenediamine substrate at a concentration of 1 mg/ml in 0.1 M citrate phosphate buffer containing 0.006% hydrogen peroxide. Color was developed in the dark for 30 min at room temperature and the reaction was terminated by the addition of 50 μl of 1 M H_2SO_4 to each well. Optical densities were read at 492 nm in a Titertek Multiskan plate reader (Flow Laboratories, McLean, VA). The levels of IFNα in serum samples were interpolated from the IFNα standard calibration curve, using the 1st international reference preparation for human leukocyte interferon, 69/19, 5,000 IU/ampule. The detection limit of the assay was 3 IU/ml.

Assays for soluble interleukin-2 receptor (sIL-2R) and soluble antigens of CD4 and CD8 cells (sCD4 and sCD8)

The levels of sIL-2R, sCD4, and sCD8 were measured using commercial ELISAs (cell-free IL-2 receptor test kit, cell-free CD4 test kit, and cell-free T8 test kit, respectively; T Cell Sciences, Inc., Cambridge, MA). The results are expressed as units per milliliter based on the standard provided by the manufacturer.

Assay for IL-2

The levels of IL-2 were measured using a commercial ELISA (Intertest-2; Genzyme, Boston, MA). The results are expressed as units per milliliter.

Assay for IFNγ

Levels of IFNγ were determined because they are one of the markers that reflect T cell activation in patients with DHF. They were measured using a commercial radioimmunoassay (Centocor Diagnostics, Malvern, PA). The results are expressed as international units per milliliter.

Statistical analysis

Differences between values were examined using the Student's *t*-test and the chi-square test. The levels of IFNα were log-transformed for statistical analysis. Undetectable levels of IFNα (< 3 IU/ml) were considered to be 1 IU/ml for log-transformation. Differences yielding *P* values of ≤ 0.05 were regarded as significant.

RESULTS

Levels of IFNα in the sera of patients with DHF or DF

The sera from patients with DHF or DF were examined for levels of IFNα. These levels were then compared with levels in the sera of healthy Thai children. The day of onset of fever was defined as day 0 (Figure 1). Interferon alpha was detected in 80% (28 of 35) of the patients with DHF and in 60% (6 of 10) of the patients with DF during days 1–20 after the onset of fever, while only 7% (2 of 30) of the sera of healthy Thai children contained detectable levels of IFNα ($P < 0.001$ for DHF and $P < 0.001$ for DF, by chi-square test) (Figure 1).

The levels of IFNα in the sera of patients with acute DHF were higher than those in the sera of healthy Thai children ($P < 0.001$ on day 3 and $P < 0.05$ on days 2 and 4), and the levels were also high on days 6–20 after onset of fever ($P < 0.05$ on days 6, $P < 0.02$ on day 7, and $P <$

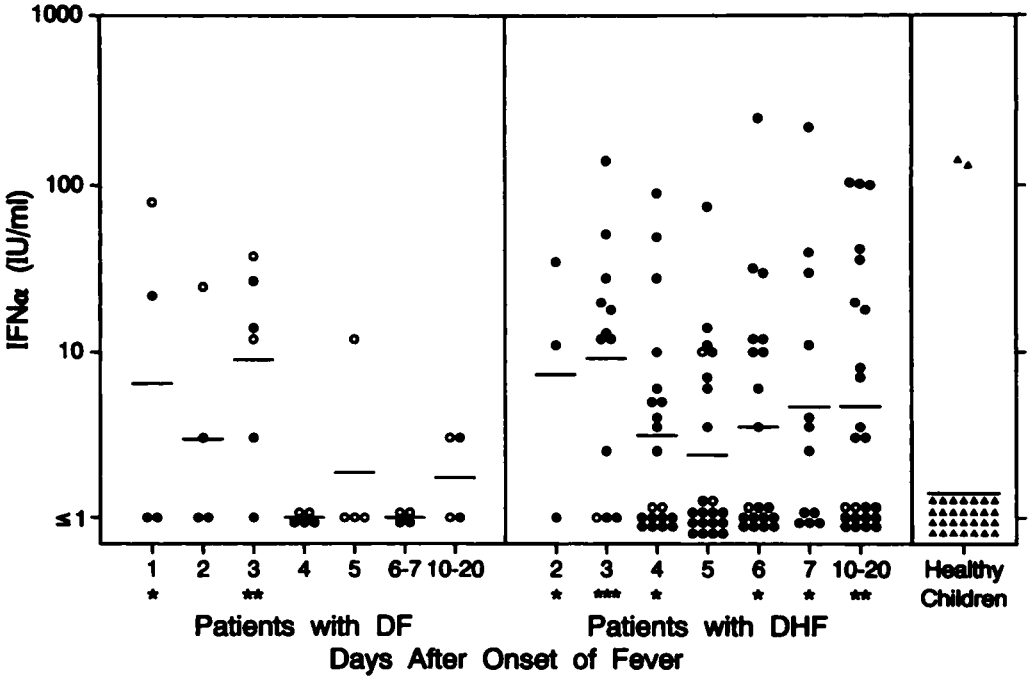

FIGURE 1. Levels of interferon alpha (IFNα) in the sera of patients with dengue hemorrhagic fever (DHF) and those with dengue fever (DF) on days after the onset of fever. The geometric mean titers (bars) of IFNα were 1.39 IU/ml (n = 30) in healthy Thai children, 7.28 IU/ml (n = 3) on day 2, 9.14 IU/ml (n = 12) on day 3, 3.10 IU/ml (n = 20) on day 4, 2.37 IU/ml (n = 22) on day 5, 3.49 IU/ml (n = 20) on day 6, 4.65 IU/ml (n = 12) on day 7, and 4.68 IU/ml (n = 25) on days 10–20 in the sera of patients with DHF, and 6.47 IU/ml (n = 4) on day 1, 2.94 IU/ml (n = 4) on day 2, 8.95 IU/ml (n = 6) on day 3, ≤ 1 IU/ml (n = 5) on day 4, 1.86 IU/ml (n = 4) on day 5, ≤ 1 IU/ml (n = 4) on days 6–7, and 1.73 IU/ml (n = 4) on days 10–20 in the sera of patients with DF. ○ = primary, hospitalized; ● = secondary, hospitalized; ▲ = healthy Thai children. Levels of IFNα were compared with the levels in the healthy Thai children by Student's *t*-test after log-transformation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

0.005 on days 10–20). The levels of IFNα in the sera of patients with acute DF were higher than those in the sera of healthy Thai children ($P < 0.05$ on day 1 and $P < 0.005$ on day 3); however, the levels were not high on days 4–20 after onset of fever (Figure 1).

Levels of IFNα in the sera of patients before and after the day of defervescence

The timing of plasma leakage in patients with DHF is predictable; circulatory collapse occurs as fever subsides. Therefore, we evaluated the levels of IFNα in the patients with DHF or DF, defining the day of defervescence as day 0 (Figure 2). The average levels of IFNα in patients with DHF were highest two days before defervescence, and decreased gradually until the day of defervescence. However, the average levels of IFNα did not change during days 0–19. The levels of IFNα in patients with DF were high one day before and on the day of defervescence, but these levels were not high after the fever subsided.

Changes in the serum levels of IFNα in each patient with DHF

We attempted to determine the changes in the levels of IFNα in each patient during the course of DHF. Patients with DHF were separated into two groups, based on the levels of IFNα on days 7–12 after defervescence: 11 subjects who had detectable levels of IFNα (> 3 IU/ml) (Figure 3A) and 10 patients who did not have detectable levels of IFNα (Figure 3B). Most of the patients who had detectable levels of IFNα on days 7–12 after defervescence had lower levels of IFNα dur-

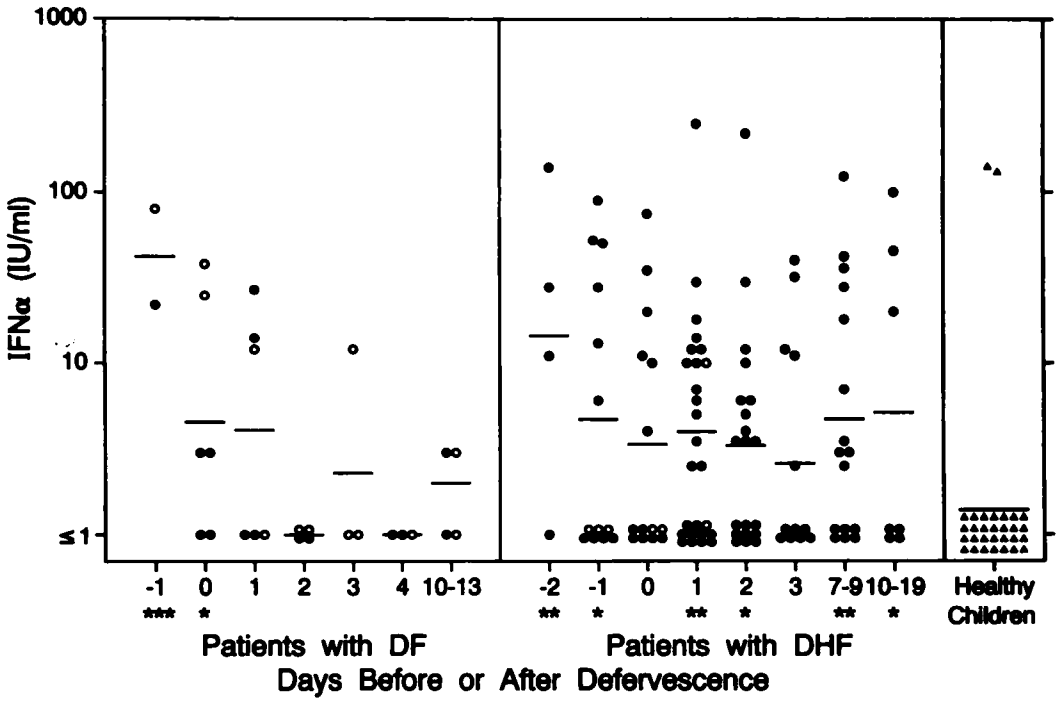

FIGURE 2. Levels of interferon alpha (IFNα) in the sera of patients with dengue hemorrhagic fever (DHF) and those with dengue fever (DF) on days before or after defervescence. The day of defervescence is defined as day 0. The geometric mean titers (bars) of IFNα were 1.39 IU/ml (n = 30) in healthy Thai children, 14.4 IU/ml (n = 2) on day −2, 4.68 IU/ml (n = 13) on day −1, 3.36 IU/ml (n = 14) on day 0, 3.97 IU/ml (n = 27) on day 1, 3.30 IU/ml (n = 20) on day 2, 2.59 IU/ml (n = 11) on day 3, 4.68 IU/ml (n = 16) on days 7–9, and 5.11 IU/ml (n = 7) on days 10–19 in the sera of patients with DHF, and 41.9 IU/ml (n = 2) on day −1, 4.52 IU/ml (n = 6) on day 0, 4.07 IU/ml (n = 6) on day 1, ≤ 1 IU/ml (n = 4) on day 2, 2.29 IU/ml (n = 3) on day 3, ≤ 1 IU/ml (n = 3) on day 4, and 1.73 IU/ml (n = 4) on days 10–13 in the sera of the patients with DF. ○ = primary, hospitalized; ● = secondary, hospitalized; ▲ = healthy Thai children. Levels of IFNα were compared with the levels in the healthy Thai children by Student's *t*-test after log-transformation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

ing days 0-4 after defervescence (Figure 3A). Most of the patients who did not have detectable levels of IFNα during days 7–12 had detectable levels of IFNα during days 0–4 after defervescence (Figure 3B). It is of interest that high levels of IFNα were detected on days 7–19 after defervescence in some patients who had lower levels of IFNα during the early stages of illness.

Comparison of the levels of IFNα among patients with DHF grades 1, 2, and 3

The levels of IFNα were compared among patients with DHF grades 1, 2, and 3 from one day before defervescence to nine days after defervescence (Figure 4). They did not differ among these three groups.

Levels of T cell activation in high IFNα producers and low IFNα producers among patients with DHF

We have previously reported that T lymphocytes are highly activated in patients with DHF by determining the high serum levels of sIL-2R, sCD4, sCD8, IL-2, and IFNγ.[6] The levels of these soluble cell-surface proteins and lymphokines were compared between high IFNα producers and low IFNα producers among patients with DHF during days 2–8 after onset of fever (Table 2). Patients who had serum IFNα levels higher than 30 IU/ml at least one day during days 2–8 after onset of fever were defined as high IFNα producers. Patients who did not have serum IFN levels higher than 10 IU/ml during this period were defined as low IFNα producers. The levels

of sIL-2R were significantly higher in high IFNα producers than in low IFNα producers ($P < 0.001$). The levels of sCD4, IL-2, and IFNγ were higher in high IFNα producers than in low IFNα producers, but the differences were not statistically significant. These results suggest that T lymphocytes are activated to a higher degree in high IFNα producers than in low IFNα producers.

DISCUSSION

In this study, we examined the levels of IFNα in the sera of patients hospitalized with DHF or DF. The percentage of subjects who had detectable levels of IFNα and the levels of IFNα on days 1–3 after the onset of fever were higher in patients with DHF or DF than in healthy Thai children. These results indicate that high levels of IFNα are produced in vivo during the acute stage of dengue virus infection.

The average levels of IFNα in the sera of patients with DHF were also high on days 4–20 after the onset of fever, while the levels of IFNα in the sera of patients with DF were not high after day 4. The levels of IFNα in patients with DHF were high two days before defervescence and decreased gradually until the day of defervescence, defined as day 0. The average levels of IFNα did not change between days 0 and 19. Approximately half of the patients with DHF had detectable levels of IFNα on days 7–19 after defervescence, and the levels of IFNα were higher on days 7–19 than on days 0–4 after defervescence in these patients. In contrast, the average levels of IFNα in patients with DF were high one day before defervescence and on the

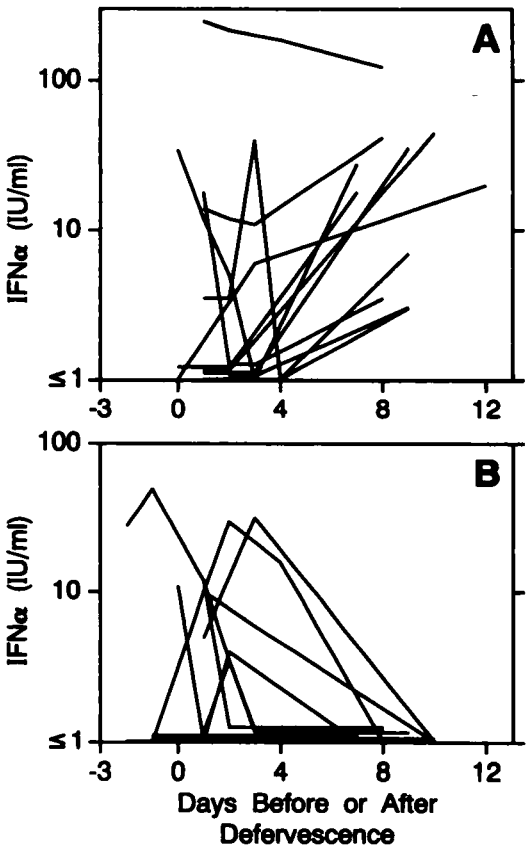

FIGURE 3. Changes in the levels of interferon alpha (IFNα) in each patient with dengue hemorrhagic fever during the course of illness. Each line represents the changes in the levels of IFNα in one patient. **A**, patients who had detectable levels of IFNα (≥ 3 IU/ml) in their sera on days 7–12 after defervescence. **B**, patients who did not have detectable levels of IFNα on days 7–12 after defervescence.

TABLE 2

*Levels of soluble cell-surface proteins and lymphokines in high interferon alpha (IFN α) producers and low IFNα producers among the patients with dengue hemorrhagic fever (DHF)**

Markers	High IFNα producers (>30 IU/ml)		Low IFNα producers (<10 IU/ml)		P
	Titers, U/ml (mean ± SD)	No. of samples	Titers, U/ml (mean ± SD)	No. of samples	
sIL-2R	5,921 ± 781	4	2,079 ± 261	7	<0.001
sCD4	48.4 ± 9.5	5	31.2 ± 3.0	9	<0.1†
sCD8	1,270 ± 225	7	1,355 ± 164	9	<0.8†
IL-2 ($\log_{10}$)	1.654 ± 0.372	7	1.391 ± 0.272	9	<0.8†
IFNγ ($\log_{10}$)	0.200 ± 0.244	7	−0.201 ± 0.186	9	<0.5†

* Patients with DHF who had serum IFNα levels higher than 30 IU/ml at least one day during days 2–8 after the onset of fever were defined as high IFNα producers. Patients with DHF who did not have serum IFNα levels higher than 10 IU/ml during days 2–8 were defined as low IFNα responders. The highest levels of soluble interleukin-2 receptor (sIL-2R), sCD4, sCD8, IL-2, and IFNγ during days 2–8 after the onset of fever were compared between high IFNα producers and low IFNα producers by Student's *t*-test. The titers of IL-2 and IFNγ were log-transformed for analysis.
† Not significant.

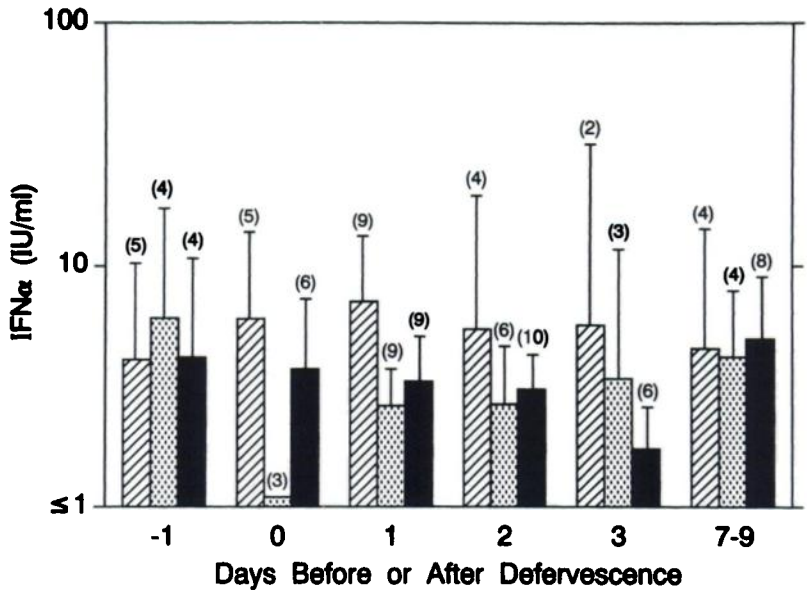

FIGURE 4. Levels of interferon alpha (IFNα) in patients with dengue hemorrhagic fever grades 1, 2, and 3. Levels are shown as mean titers + SEM. Values in parentheses above the bars are the number of samples. Hatched bars = grade 1; dotted bars = grade 2; solid bars = grade 3.

day of defervescence, but decreased after the fever subsided.

The origins of IFNα detected in the sera are not clear. We have reported that dengue virus–infected monocytes produce IFNα ,[8] and that these dengue virus–infected monocytes induce IFNα from autologous HLA-DR+ lymphocytes.[9] We assume that the IFNα detected in these children was released by these mechanisms; however, other mechanisms of IFNα production cannot be ruled out. The finding that some patients with DHF had increasing levels of IFNα during convalescence may suggest that the infection was not completely eradicated even 7–19 days after defervescence. We did not find increasing levels of IFNα after defervescence in patients with DF, which possibly indicates that the termination of virus replication is more rapidly achieved in DF.

Burke and Morrill reported that high levels of IFNα were detected in the plasma and cerebrospinal fluids of patients with Japanese encephalitis, and that there was a positive correlation between the levels of IFNα in cerebrospinal fluids and in fatal outcome.[18] They interpreted that the correlation between high IFNα levels and fatal outcome reflects a higher degree of virus replication in the brain. Our observation that similar levels of IFNα were detected in patients with DHF and in patients with DF at least during the early stage of illness suggests that the degree of infection may be similar between patients with DHF and patients with DF whose symptoms are severe enough to require hospitalization. The patients with DF examined in this study were hospitalized and they had more severe symptoms than many children with DF who usually do not need to be hospitalized. Therefore, the sera of nonhospitalized children with less severe DF need to be examined in a future study.

Among patients with DHF, the levels of sIL-2R, sCD4, IL-2, and IFNγ were higher in subjects who had high serum levels of IFNα than in those who had low serum levels of IFNα, although the differences were not statistically significant for sCD4, IL-2, and IFNγ. These results suggest that the levels of T cell activation are higher in high IFNα producers than in low IFNα producers. This difference may reflect the difference in the levels of dengue virus infections; i.e., higher levels of dengue virus replication induce higher levels of IFNα and also induce higher levels of T cell activation.

Although this study does not suggest a direct role of IFNα in the pathogenesis of DHF, it is possible that IFNα has an important role in the

control of dengue virus infections. Thus, the role of IFNα in recovery from dengue virus infection is an important subject for further investigation.

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