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Elevated Plasma Interleukin-10 Levels in Acute Dengue Correlate With Disease Severity

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INTRODUCTION

Dengue viruses, of which there are four serotypes, are the most important arthropod-borne viral infections in the world, accounting for more than 250,000 cases of dengue hemorrhagic fever (DHF) and 10,000 deaths annually [Monath, 1994]. Infection with dengue viruses can yield different clinical syndromes, including (1) undifferentiated febrile illness, seen more commonly in children; (2) dengue fever (DF), a flu-like syndrome characterized by high fever, headache, retroorbital pain, myalgias, abdominal pain, nausea, and vomiting; and (3) dengue hemorrhagic fever (DHF), a plasma leak syndrome that, in its most severe form, can be life-threatening [Nimmannitya, 1987].

Plasma leakage is a major clinical feature of DHF and tends to occur around the time of defervescence. We have been interested in the events that precede the period of plasma leakage to better define its etiology. We have found that dengue virus-specific $CD4^+$ and $CD8^+$ cytotoxic T cells (CTL) are generated after primary dengue virus infections and that these CTL can produce cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL-2), tumor necrosis factors α and β (TNF-α, TNF-β) in response to exposure to heterologous dengue viruses in vitro, and lyse dengue virus-infected autologous cells [Kurane et al., 1989, 1990; Mathew et al., 1996; Gagnon et al., 1999]. $CD4^+$ T cells are classified as T-helper (Th) type 1 or 2, based on their ability to produce certain cytokines. Th1 cells produce IFN-γ, IL-2, and lymphotoxin, whereas Th2 cells produce IL-4, IL-5, IL-10, and IL-13 [O'Garra, 1998]. Plasma levels of IFN-γ are elevated in dengue infection and other markers of T-cell activation, such as soluble IL-2 receptors and soluble CD8, are higher in children with DHF than DF [Kurane et al., 1991; Green et al., 1999]. These findings have suggested a role for Th1-mediated immunopathology in the pathogenesis of DHF.

IL-12 is a heterodimer of 70 kD (p70) and is made up of a p35 and a p40 chain [Trinchieri, 1995]. The p70 heterodimer is the active molecule. The p40 molecule alone is inactive but is inducible [Ma et al., 1995]. IL-12 has been shown to be a potent inducer of IFN-γ and enhances the cytotoxic activity of both natural killer (NK) and T cells [Trinchieri, 1994]. As IFN-γ levels have been found to be elevated in acute dengue [Kurane et al., 1991; Green et al., 1999], investigation of IL-12 production in DHF was warranted.

Previous studies of DHF in children have found that levels of TNF-α, soluble TNF receptors, and IFN-γ are elevated in DHF [Hober et al., 1993, 1996; Bethell et al., 1998; Green et al., 1999]. IL-10 has been found to be a potent inhibitor of proinflammatory cytokines, such as IL-12, IFN-γ, and TNF-α [Trinchieri, 1995]. Therefore, the goal of this study was to investigate further the role of Th1 cytokines, such as IL-12, and that of counterregulatory Th2 cytokines, such as IL-10, to further elucidate the possible immunological mechanisms responsible for the development of the plasma leakage seen in DHF.

MATERIALS AND METHODS

Study Design

Details are described elsewhere in a summary of the clinical results from the first year of data from the Dengue Hemorrhagic Fever Project, which has enrolled pa-

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TABLE I. Serologic Responses and Virus Serotypes* in Subjects With Dengue Fever and Dengue Hemorrhagic Fever

	DF			DHF		
Dengue type	Primary infection	Secondary infection	Total DF	Primary infection	Secondary infection	Total DHF
1	5	4	9	0	6	6
2	0	2	2	0	9	9
3	4	4	8	2	2	4
4	0	3	3	0	1	1
Total	9	13	22	2	18	20

*Dengue viruses were isolated from the blood in 22 of 22 patients with dengue fever (DF) and in 20 of 20 patients with dengue hemorrhagic fever (DHF).

tients with suspected dengue from April 1994 through December 1998 [Kalayanarooj et al., 1997]. Briefly, Thai children were enrolled with a history of fever for less than 72 hours and no obvious source of infection. A venous blood sample was drawn daily until the day after defervescence, and a follow-up blood sample was obtained at an outpatient visit between study days 8–13 for serologic testing. On the day after defervescence, a right lateral decubitus chest radiograph was performed. Informed consent was obtained from parents or guardians of patients. This protocol was approved by the Institutional Review Boards of the Thai Ministry of Public Health, the Office of the U.S. Army Surgeon General, and the University of Massachusetts Medical Center.

Study Definitions

Study day 1 represents the day a child was enrolled and fever day 0 represents the day of defervescence, with days before this day designated fever day −1, −2, and so on. A clinical diagnosis of DHF and severity grading (grades 1–4) were assigned according to World Health Organization criteria [WHO, 1986]. Pleural effusion index was defined as [(width of effusion)/(width of hemithorax) × 100]. All patients with DF or DHF had evidence of acute dengue virus infection by dengue IgM enzyme-linked immunosorbent assay (ELISA), HAI antibody responses, and/or dengue virus isolation in *Toxorrhyncites splendans* mosquitoes as described [Vaughn et al., 1997]. Other febrile illnesses (OFI) were presumed to represent self-limited non-dengue viral infections, as these patients had no dengue virus-specific IgM or HAI antibody responses, no dengue virus isolated from their plasma, and no obvious bacterial, rickettsial, or protozoal etiology for their illness.

Sample Processing

Blood was drawn into EDTA tubes (Becton Dickinson, Franklin Lakes, NJ), maintained at 4°C, and centrifuged at 300*g* for 10 minutes. The plasma was then divided into aliquots and frozen at −70°C until analysis.

Assays for IL-10, IL-12 p70, and IL-12 (p40 + p70)

Plasma IL-10, IL-12 p70, and IL-12 (p40 + p70) levels were measured using commercial ELISA (Endogen, Cambridge, MA) according to the manufacturer's recommendations. The lower limits of detection were <3 pg/ml for IL-10, <3 pg/ml for IL-12p70, and <5 pg/ml for IL-12 (p40 + p70).

Sample Selection

A subset of study subjects was selected from each of the three diagnostic categories—DHF, DF, and OFI—for immunoassay testing. Because the volume of plasma obtained was limited, the same patients' specimens could not be assayed for all immune response parameters. These subset populations were selected without knowledge of clinical data other than final diagnosis. We selected all available samples from each subject selected (range: 2–6 samples). Plasma samples from the 6-month follow-up visit from study subjects with acute dengue virus infection were tested in each immunoassay as healthy controls (n = 15). All samples were tested under code.

Statistical Analysis

Mean plasma levels of the measured parameters were compared by means of independent sample *t*-tests using final diagnosis (DF vs. DHF, or dengue vs. OFI) and fever day. Comparisons were made between all subjects with acute dengue virus infection (combined DF and DHF) and those with OFI, as well as between subjects with DHF and those with DF. We used both Pearson and Spearman correlations. Analyses were done using computer software (SPSS). $P < 0.05$ was considered significant.

RESULTS

Study Population Demographics

The population studied included 20 children with DHF, 22 children with DF, and 19 children with OFI. Of the 20 children with DHF, 8 were classified as grade 1 DHF, 7 as grade 2 DHF, and 5 as grade 3 DHF. Primary versus secondary infections and the serotypes of dengue viruses that were isolated are shown in Table I.

Plasma Levels of IL-10

Mean plasma IL-10 levels in children with dengue rose during illness and peaked on fever day 0 (Fig. 1). Mean plasma levels of IL-10 were significantly higher

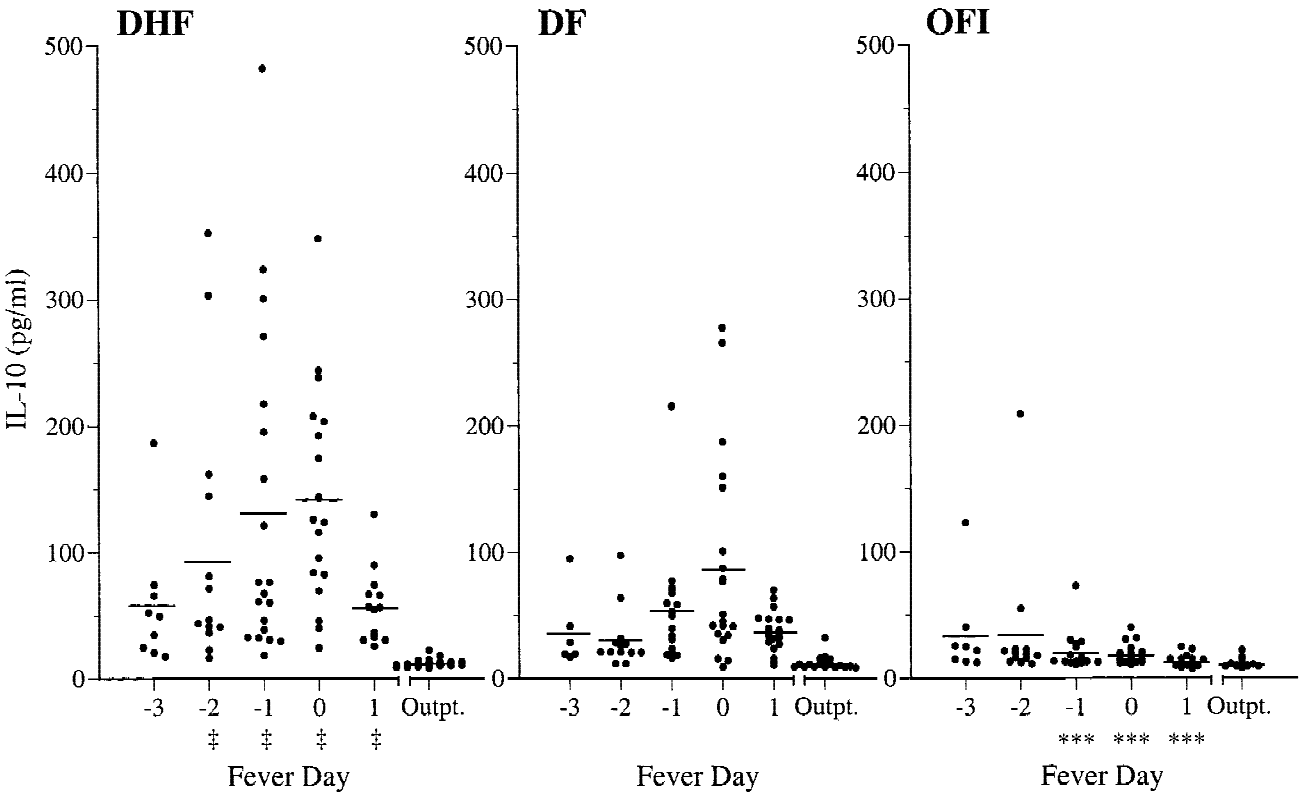

Fig. 1. Plasma interleukin-10 (IL-10) levels in dengue hemorrhagic fever (DHF), dengue fever (DF), and other febrile illnesses (OFI). Fever day 0 represents day of defervescence. DHF vs. DF: ‡$P < 0.05$; OFI vs. dengue (DHF plus DF): ***$P < 0.001$. Mean IL-10 levels in healthy controls: 10.0 pg/ml. Outpt., outpatient visit on study days 8–13.

in children who later developed DHF, compared with those with uncomplicated DF as early as 2 days before defervescence ($P < 0.05$). Similarly, children with a diagnosis of dengue had higher mean IL-10 levels than were noted in children with OFI as early as 1 day before defervescence ($P < 0.001$). Maximal plasma IL-10 levels measured from fever day –2 through fever day 0 correlated with the degree of plasma leakage as determined by the pleural effusion index measured 1 day after defervescence (Fig. 2).

Plasma Levels of IL-12 (p40 + p70) and IL-12p70

Mean plasma IL-12 (p40 + p70) levels declined over the period of observation (Fig. 3). IL-12 (p40 + p70) levels were elevated in children with DF, compared with those with DHF from fever day –2 ($P < 0.05$). IL-12 (p40 + p70) levels were also higher in children with dengue than in healthy controls, as early as fever day –3 ($P < 0.001$) through fever day +1 ($P < 0.05$). Mean plasma IL-12 p70 levels did not change over the period of observation (data not shown). IL-12 p70 levels were found to be lower in children with dengue than those with OFI from fever day –2 (mean ±SD: 3.8 ± 1.1 vs. 4.8 ± 1.0; $P < 0.01$) through fever day +1 (3.7 ± 0.8 vs. 4.6 ± 0.6; $P < 0.001$). There was no difference in IL-12 p70 levels between children with DF and those

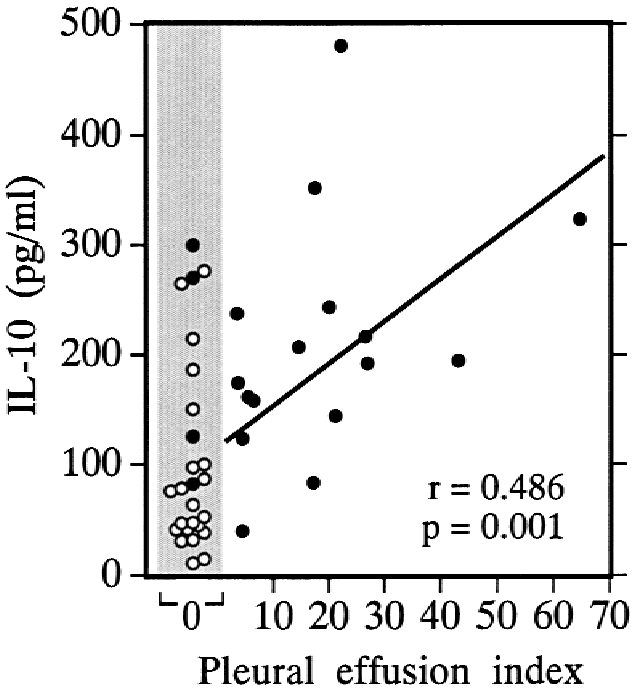

Fig. 2. Correlation between maximal plasma interleukin-10 (IL-10) levels during acute illness and pleural effusion index (PEI) 1 day after defervescence. PEI = 100 × [(width of pleural effusion/width of hemithorax)] on decubitus chest radiograph. *Open symbols,* dengue fever; *closed symbols,* dengue hemorrhagic fever.

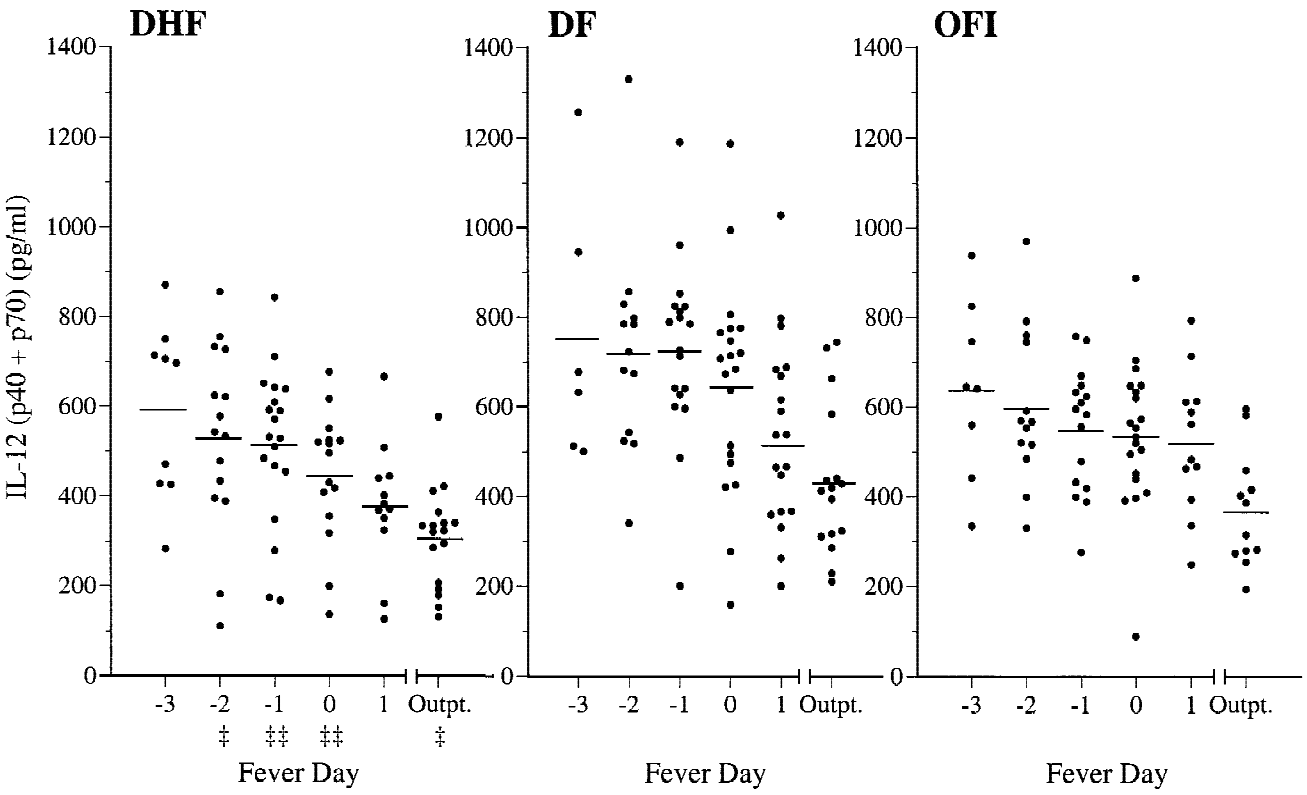

Fig. 3. Plasma IL-12 (p40 + p70) levels in dengue hemorrhagic fever (DHF), dengue fever (DF), and other febrile illnesses (OFI). DHF vs. DF: $^{‡}P < 0.05$; $^{‡‡}P < 0.01$. Mean IL-12 (p40 + p70) levels in healthy controls: 315.8 pg/ml. Outpt., outpatient visit on study days 8–13.

with DHF (data not shown). IL-12 p70 levels were lower in children with dengue on fever day +1 and on the nonfebrile follow-up visit (3.8 ± 0.7 vs. 4.3 ± 1.0, $P < 0.05$), compared with healthy controls.

DISCUSSION

This study demonstrated elevated levels of IL-10 in children with dengue infection, associated with disease severity (DF vs. DHF) and the degree of plasma leakage quantified by the size of the pleural effusions. IL-10 has been shown to inhibit TNF-α production by human monocytes [de Waal Malefyt et al., 1991] as well as TNF production in murine and monkey models of endotoxemia [Gerard et al., 1993; van der Poll et al., 1997]. Administration of IL-10 to healthy adults induces no clinically significant adverse reactions [Chernoff et al., 1995]; therefore, IL-10 itself is unlikely to be the cause of the plasma leakage seen in our population. We hypothesize that in DHF, IL-10 plays a role in the negative feedback of proinflammatory cytokines such as TNF-α and IFN-γ, but this anti-inflammatory response may be inadequate due to insufficient quantity or delayed timing of the response. Elevated IL-10 levels have been observed in other infectious diseases that have been associated with high levels of proinflammatory cytokine production. In a study of meningococcemia, elevated IL-10 levels were associated with the presence of shock and the likelihood of a fatal outcome [Lehmann et al., 1995]. In *Plasmodium falciparum* malaria, high levels of IL-10 were found in parasitemic patients, as compared with healthy controls [Wenisch et al., 1995].

IL-10 has numerous roles that affect the humoral immune response, including B-cell differentiation and amplification of immunoglobulin secretion of activated B lymphocytes [Rousset et al., 1992]. In our cohort, we had more primary cases of DF than DHF. To account for the potential interaction of antibody response upon our results, we performed linear regression analysis comparing maximal IL-10 levels with pleural effusion index and adjusted for maximal IgM, IgG, or hemagglutination inhibition antibody levels or serologic diagnosis (primary vs. secondary). We found that the relationship between IL-10 levels and pleural effusion index remained significant ($P < 0.01$, $P < 0.05$, $P < 0.01$, $P < 0.01$, respectively) (data not shown).

IL-10 also inhibits proliferation of T cells [Moore et al., 1993; Chernoff et al., 1995]. This T-cell unresponsiveness appears to be attributable to an effect of IL-10 on antigen-presenting cells, rather than to a direct effect on the T cell [Groux et al., 1998]. This is consistent with our finding of reduced in vitro proliferation of PBMC obtained during acute dengue infections [Mathew et. al., 1999].

We found that IL-12 (p40 + p70) levels are higher in children with DF than in children with DHF and that IL-12 p70 levels are not elevated in children with dengue. In the murine system, p40 has been found to be an

antagonist to the active p70 heterodimer [Gillessen et al., 1995]. The elevated levels of IL-12 (p40 + p70) in dengue may reflect an attempt to downregulate IL-12 activity. Our results suggest that the production of IL-12 p40 may be deficient in children with DHF, as levels are lower than in children with DF or OFI. IL-10 is also a potent downregulator of IL-12, and the kinetic results in our study suggest that IL-10 levels rise as the levels of IL-12 (p40 + p70) are decreasing. The levels of IL-12 (p40 + p70) were highest at the earliest time point we measured; therefore, it is possible that levels had been higher and were falling by the time the children entered our study.

DHF is much more commonly observed in cases of secondary infections than during primary infections. These secondary infections are caused by a different serotype of virus than the virus responsible for the primary infection, as primary infections are known to induce homotypic immunity [Sabin, 1952; Sangkawibha et al., 1984; Burke et al., 1988]. This epidemiologic finding has led to theories of immune enhancement and immunopathology as the basis for the plasma leakage. Dengue viruses are believed to bind to pre-existing heterologous dengue virus antibodies produced during primary infection and to form immune complexes that enable the virus to enter Fc receptor-bearing monocytes, a mechanism known as antibody-dependent enhancement [Halstead and O'Rourke, 1977]. Immune complexes inhibit IL-12 secretion by human monocytes in vitro. This inhibited secretion appears to be mediated by TNF-α-induced IL-10 and prostaglandin E_2 (PGE_2). It affects IL-12p70 production more than IL-12 p40 production [Berger et al., 1997]. This may explain why IL-12p70 levels are lower in children with dengue than in those with OFI. Impaired production of IL-12p70 is seen in other human viral diseases, such as measles [Karp et al., 1996] and human immunodeficiency virus (HIV) [Chehimi et al., 1995], which may be related to IL-10, or it may be attributable to a direct effect of viral infection of monocytes.

Our previous findings suggested that the immunopathogenesis of DHF is related to Th1-mediated immunopathology. Those studies demonstrated significantly increased levels of immune activation markers including sCD8, sIL-2R, TNF-α, and soluble TNF receptors during acute illness [Kurane et al., 1991; Green et al., 1999]. Plasma levels of IL-4 were not detectable [Green et al., 1999]. Similarly, attempts to generate dengue virus-specific $CD4^+$ T-cell clones of a Th2 phenotype from PBMC of dengue immune donors by the addition of IL-4 and anti-IL-12 to cultures have proved unsuccessful (Gigstad, Rothman, Ennis, et al., personal communication). The data presented in this report represent the first evidence of a Th2 component to the immune response in acute dengue infection, which is related to disease severity. There appears to be simultaneous immune activation as well as probable immunosuppression, as evidenced by elevated levels of IL-10, suppression of IL-12, and impaired T-cell proliferative responses [Mathew et al., 1999], similar to results reported with measles [Griffin et al., 1994; Karp et al., 1996]. Further studies on the mechanism and clinical relevance of this immunosuppression are warranted.

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