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ASSOCIATION BETWEEN SEX, NUTRITIONAL STATUS, SEVERITY OF DENGUE HEMORRHAGIC FEVER, AND IMMUNE STATUS IN INFANTS WITH DENGUE HEMORRHAGIC FEVER

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Abstract. The association between sex, nutritional status, and the severity of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), and immune status was investigated in 245 Vietnamese infants with predominantly primary infections with dengue virus. Male and female infants were at equal risk of developing DHF/DSS. However, infants of low height and weight for age were under-represented among DHF/DSS cases compared with 533 healthy baby clinic infant controls. Acute illness phase blood levels of selected cytokines (interferon-γ and tumor necrosis factor-α) and serum levels of antibodies to dengue virus were elevated in the same range in male and female infants with DHF/DSS, as well as in infants with and without malnutrition.

INTRODUCTION

Dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) is an important public health problem in southeast Asian and western Pacific countries. It is one of the leading causes of hospitalization and death among children in many tropical Asian countries.[1–3] Patients with DHF may die of prolonged shock, massive bleeding (usually gastrointestinal bleeding), respiratory failure, and dengue encephalopathy.[3] The vast majority of cases, nearly 95%, are among children less than 15 years of age; while infants comprise 5% or more of all DHF/DSS cases.[4,5]

A preponderance of DHF/DSS in Thai females ≥ 4 years old has been documented.[6] Rates of infection with dengue (DEN) virus among boys and girls have been shown to be identical in countries endemic for DHF/DSS.[6] The integrity and strength of the cell-mediated immune response should be correlated with the severity of DHF/DSS.[7] It is known that malnutrition suppresses cellular immune responses. Anto and others[8] and Thiasykorn and Nimmannitya[9] have shown that even mild degrees of protein-calorie malnutrition serve to spare children from severe DHF/DSS. The immunopathogenesis of dengue virus infection, with an emphasis on the immune deviation, autoantibodies, and cytokine production has been proposed.[10] In our previous report as part of study project of DHF in infants, we demonstrated the production of high levels of both proinflammatory cytokines (interferon-γ [IFN-γ] and tumor necrosis factor-α [TNF-α]) and anti-inflammatory cytokines (interleukin-10 [IL-10] and IL-6) in infants with DHF/DSS that was consistent with infection-enhancement due to acquired maternal antibody.[11] Primary dengue virus infections in infants less than one year of age comprise a unique subgroup of patients with DHF/DSS.[5] Here we further characterize attributes of the DHF syndrome observed in infants less than one year of age experiencing predominantly primary dengue virus infections.

MATERIALS AND METHODS

Patients. Two hundred seventy-two infants < 12 months old admitted to the Department of Dengue Hemorrhagic Fever of Children's Hospital No. 1 (Ho Chi Minh City, Vietnam) from August 1997 to December 2002 with a clinical diagnosis of DHF according to the criteria of the World Health Organization (WHO) (1997) were enrolled in the study after parental or guardian consent was obtained.[3] Dengue virus infections in the patients were confirmed by 1) a viral envelope and membrane (E/M)–specific capture IgM enzyme-linked immunosorbent assay (ELISA) and/or a nonstructural protein 1 (NS1) serotype-specific IgG ELISA at the Center for Disease Control in Taipei, Taiwan,[12] or 2) a capture IgM ELISA at the Pasteur Institute in Ho Chi Minh City.[13] Patients were under routine care of one or more of the authors. Basic demographic data and medical history were obtained and a detailed physical examination including measurement of weight and height was conducted by the authors, and subsequent progress was recorded on a standard data form. Five hundred thirty-three healthy infants who visited the outpatient department to receive vaccinations during the period 2001–2002 were examined as a control group for assessment of nutritional status. Dates of birth were recorded and measurements of weight and height were obtained. The mean age of control infants was seven months (range = 2–11 months). Ethical approval of the study was obtained from the Scientific and Ethical Committee of Children's Hospital No. 1 in Ho Chi Minh City.

Assessment of nutritional status of DHF patients and healthy control infants. Epi-Info 2000 1.1 software (Centers for Disease Control and Prevention, Atlanta, GA) was used to calculate the anthropometric indices such as weight-for-age (WA), height-for-age (HA), and weight-for-height (WH) of infants with DHF and healthy control infants. These nutritional measurements were converted to z-scores (weight-for-

age Z-score [WAZ]; height-for-age Z-score [HAZ]; and weight-for-height Z-score [WHZ]), also referred to as SD units, based on the National Center for Health Statistics/World Health Organization reference. The Z-score cutoff point to classify low anthropometric levels is 2 SD units below the reference median for the three indices. The cutoff for very low anthropometric levels is more than 3 SD units below the median.

Sample collection. Paired blood samples were collected from each patient in the study: one acute-phase and one convalescent-phase. An acute-phase blood sample (2–3 mL) was obtained at admission (days 3–7 after the onset of fever). A convalescent-phase blood sample (2–3 mL) was obtained in the convalescent phase (days 8–19 after the onset of fever). Sera were separated as quickly as possible and stored at −70°C until used.

Assessment of immune response of the patients. Immune response of the patients was assessed by determining the capacity to produce cytokines, IgM antibody to dengue virus, and/or NS1 serotype-specific IgG antibodies.

Capture IgM and IgG ELISAs. Capture IgM and IgG ELISAs using diluted pooled virus antigens from culture supernatants of DEN 1-, DEN 2-, DEN 3-, DEN 4-, and Japanese encephalitis (JE) virus-infected Vero cells as antigens were performed to measure the IgM and IgG antibodies from paired sera of 118 infants at the Center for Disease Control in Taipei, Taiwan. The optical densities of culture supernatants of Vero cells with and without dengue virus infection were designated the test absorbance and negative control value, respectively, for each sample in the ELISA. Positivity was determined by comparison with individual negative controls. A positive sample had a ratio of test absorbance to negative control ≥ 2.0, and a negative sample had a ratio < 2.0. For serum samples with positive results in the capture IgM and IgG ELISAs, a ratio of IgM to IgG ≥ 1.2 was defined as a primary dengue virus infection, and a ratio < 1.2 was defined as a secondary dengue virus infection.[12] A capture IgM ELISA using diluted dengue or JE virus antigens from infected suckling mouse brain extracted by the sucrose-acetone method and monoclonal antibody SLE 6B6C-1/HRP conjugate was performed to measure the IgM antibodies in 154 infants following the protocol of the Centers for Disease Control and Prevention (Fort Collins, CO) at the Pasteur Institute in Ho Chi Minh City.[13]

NS1 serotype-specific IgG ELISA. An NS1 serotype-specific IgG ELISA using diluted NS1-containing culture supernatants of DEN 1-, DEN 2-, DEN 3-, DEN 4-, or JE virus-infected Vero cells as antigens was performed to measure the NS1-specific IgG antibody from the sera of the patients. The enzyme activity was developed and the optical density was determined.[12]

Cytokine assays. The plasma levels of six cytokines (IFN-γ, TNF-α, IL-10, IL-6, IL-4, and IL-2) of the patients were measured simultaneously by the BD Human Th1/Th2 Cytokine Cytometric Bead Array Kit-II (BD Biosciences, Pharmigen, CA) in a 50-μL sample according to the instructions of the manufacturer.[11]

Data and statistical analysis. Analysis of variance was used to compare the statistical significance of differences in normally distributed data, while the Kruskal-Wallis test for two groups was used if the variances in the samples differed. Statistical analyses were performed with Epi-Info 2000 version 1.1 software. Differences with P values < 0.05 were considered significant.

RESULTS

Clinical findings of DHF/DSS patients in the study. Based on the capture IgM ELISA, a dengue virus infection was confirmed as the etiology for 245 of 272 infants hospitalized with DHF. Among these infants, 182 were categorized as nonshock DHF (grade I, 1 infant; grade II, 181 infants) and 63 as DSS (grade III, 54 infants; grade IV, 9 infants). The mean age of the patients was 6.8 months (range = 1–11 months). The clinical and cytokine profiles of 107 of these patients have been published, and serologic testing showed that almost all (95.3%) of the patients had primary dengue virus infections.[11] All patients had high continuous fever that lasted from 2 to 13 days, with a mean 5.2 days. Petechiae on the skin and hepatomegaly were observed in 244 (99.6%) and 238 (97.1%) patients, respectively. DSS was recorded in 63 (25.7%) patients in whom there were 6 cases complicated with prolonged shock. Gastrointestinal (GI) bleeding and respiratory failure were noted in 14 (5.7%) and 13 (5.3%). Eighteen (7.3%) patients had neurologic signs (dengue encephalopathy) manifested by convulsions (12 cases), lethargy (7 cases), coma (6 cases), and focal neurologic sign (1 case).

Hemoconcentration, as shown by a $\geq 20\%$ increase in the hematocrit in reference to a convalescent value, was observed in 224 (91.4%) patients. The remaining 21 (8.5%) patients had a $\geq$ 10–19% increase in the hematocrit. Thrombocytopenia (platelet count $\leq 100 \times 10^3/mm^3$) was found in 230 (93.8%); the remaining 15 (6.1%) had platelet counts of $104–190 \times 10^3/mm^3$.

Association between sex, nutritional status, and the severity of DHF in infants. The male/female ratio for all DHF infants was 138:107 (1.29:1); for DSS cases, it was 40:23 (1.73:1). Among 533 healthy control infants, the male/female ratio was 276:257 (1.07:1). The distribution of sex in infants with DHF or DSS in the study is not different from that of healthy control subjects (odds ratio [OR] = 1.20, 95% confidence interval [CI] = 0.88–1.65, $P = 0.2$) (Table 1). There was no sex bias associated with prolonged shock, GI bleeding, respiratory failure, or encephalopathy (Table 2).

Of infants with DHF/DSS, only17 (6.9%) were malnourished (underweight) as assessed by WA on admission: 16 with moderate malnutrition ($-3 <$ WAZ <-2), and 1 with severe malnutrition (WAZ ≤ -3). Height was measured in 218 of 245 infants. When assessed by HA, 17 (7.7%) infants had malnutrition (stunting) on admission, including 14 with moderate malnutrition and 3 with severe malnutrition. When assessed by WH, 31 (14.2%) infants were malnourished (wasting): 25 with moderate malnutrition and 6 with severe malnutrition. Among 533 control infants, 62 (11.6%), 6 (1.1%), and 121 (22.7%) infants had malnutrition as assessed by WA, WH, and HA, respectively. Compared with healthy controls, malnourished infants, as assessed by WA and HA, occurred less frequently among DHF cases (6.9% versus 11.6%; $P = 0.03$; 7.7% versus 22.7%; $P < 0.001$, respectively). In contrast, infants with DHF had a higher percentage of malnutrition (wasting) as assessed by WH (14.2% versus 1.1%; $P < 0.001$). With smaller numbers, no statistically significant differences

TABLE 1
Association between sex, nutritional status, and the severity of dengue hemorrhagic fever (DHF) in infants*

Findings	All patients (n = 245)	Nonshock DHF (n = 182)	DSS (n = 63)	Healthy controls (n = 533)	OR (95% CI), P†	OR (95% CI), P‡
Sex, number (%)						
Male	138	98 (71)	40 (28.9)	276	1.20 (0.88–1.65), 0.2	1.49 (0.79–2.81), 0.2§
Female	107	84 (78.5)	23 (21.4)	257		
Nutritional status, number (%)						
Assessed by weight-for-age	(n = 245)	(n = 182)	(n = 63)	(n = 533)		
Normal mutritional	228 (93.1)	169 (92.8)	59 (93.6)	471 (88.3)	0.55 (0.30–1), 0.03	0.88 (0.20–3.00). 1¶
Malnourished	17 (6.9)	13 (7.1)	4 (6.3)	62 (11.6)		
Assessed by height-for-age	(n = 218)	(n = 171)	(n = 47)			
Normal nutritional	201 (92.2)	160 (93.5)	41 (87.2)	412 (77.2)	0.29 (0.16–0.50), P < 0.001	2.13 (0.61–6.71), 0.2§
Malnourished	17 (7.7)	11 (6.4)	6 (12.7)	121 (22.7)		
Assessed by weight-for-height	(n = 218)	(n = 171)	(n = 47)			
Normal nutritional	187 (85.7)	149 (87.1)	38 (80.8)	527 (98.8)	3.20 (2.65–3.85), P < 0.001	1.60 (0.63–4.04), 0.3§
Malnourished	31 (14.2)	22 (12.8)	9 (19.1)	6 (1.1)		

* DSS = dengue shock syndrome; OR = odds ratio; CI = confidence interval.
† *P* values of comparison between all infants with DHF and healthy control infants by Yates' corrected chi-square test.
‡ *P* values of comparison between nonshock DHF and DSS groups by §Yates' corrected chi-square test or ¶two-tailed Fisher exact test.

were observed in the distribution of various parameters of undernutrition in the DSS and nonshock DHF group (Table 1).

Further analysis indicated that there was no association between malnutrition status as assessed by WA and severe complications (prolonged shock, GI bleeding, respiratory failure, and encephalopathy) in DHF infants (Table 2).

Association between sex, nutritional status, and production of cytokines and antibodies to dengue virus in infants with DHF/DSS. Serum concentrations of IFN-γ, TNF-α, IL-10, and IL-6 measured at one interval during the acute stage of illness in 62 infants with DHF/DSS were higher than those in control group.[11] No significant differences were observed in serum levels of IFN-γ, TNF-α, IL-10, and IL-6 in male DHF/DSS patients compared with female DHF/DSS patients (Table 3).

Levels of IgM antibody to dengue virus were measured in acute- and convalescent-phase serum samples, while NS1 serotype-specific IgG antibodies were measured in convalescent-phase serum samples. Serologic testing showed that 107 of 118 infants analyzed at the Center for Disease Control in Taipei and 138 of 154 infants analyzed at the Pasteur Institute in Ho Chi Minh City were positive for IgM antibodies to dengue virus by the capture IgM ELISA. Results for NS1 serotype-specific IgG were positive in 36 infants in the study. The results showed that male infants did not differ from female infants in their titers of IgM antibodies to dengue virus and to NS1 serotype-specific IgG antibodies (Table 4).

Similarly, there were elevated serum levels of IFN-γ, TNF-α, IL-10, and IL-6 in infants with DHF/DSS with or without malnutrition as assessed by WA (Table 3). However, except for TNF-α, the serum levels of IFN-γ, IL-10, and IL-6 were not different between infants with and without malnutrition. Further analysis also showed that the titers of IgM antibody to dengue virus and NS1 serotype-specific IgG antibodies in infants with DHF/DSS with or without malnutrition as assessed by WA were not significantly different (Table 4).

DISCUSSION

This study confirms the observation of a normal male:female ratio observed among Thai infants with a primary dengue virus infection complicated by DHF/DSS.[14,15] The present study is the first in Vietnam to assess the relationship in infants with a confirmed primary dengue virus infection between sex and the severity of DHF (nonshock DHF versus DSS) and severe complications. There was no significant difference between the percentage of male and female infants with DSS (28.9% versus 21.4%; OR = 1.49, 95% CI = 0.79–2.81, *P* = 0.2) (Table 1). The results of this study also showed that there was no relationhip between sex and severe complications of DHF, such as prolonged shock, GI bleeding, respiratory failure, and encephalopathy, in infants.

Assessment of the nutritional status of infants with DHF/DSS in the present study showed that only 6.9%, 14.2%, and 7.7% of the patients were malnourished as assessed by WA, HA, and WH, respectively. Infants with DHF had lower per-

TABLE 2
Association between sex and malnutrition as assessed by weight-for-age and severe complications in dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) in infants*

Severe complications	Male DHF/DSS infants (n = 138)	Female DHF/DSS infants (n = 107)	Malnourished DHF/DSS infants (n = 17)	Normal nutritional DHF/DSS infants (n = 228)	OR (95% CI), P†	OR (95% CI), P‡
GI bleeding	9	5	1	13	1.42 (0.41–5.57), 0.7§	1.03 (0.0–8.61), 1
Prolonged shock	3	3	1	5	0.55 (0.07–4.46), 0.6¶	3.49 (0.06–54.54), 0.3
Respiratory failure	6	7	1	12	0.65 (0.18–2.26), 0.6§	1.13 (0.0–9.47), 1
Hepatic failure	8	4	3	9	1.55 (0.48–5.01), 0.6§	3.47 (0.54–21.63), 0.1
Encephalopathy	9	9	2	16	0.76 (0.26–2.20), 0.7§	1.77 (0.0–9.35), 0.3

* GI = gastrointestinal; OR = odds ratio; CI = confidence interval.
† *P* values of comparison between male and female infants with DHF/DSS by §Yates' corrected chi-square test and ¶two-tailed Fisher's exact test.
‡ *P* values of comparison between infants with DHF/DSS with and without malnutrition by two-tailed Fisher's exact test.

TABLE 3

Comparison of serum levels of cytokines in acute-phase serum samples between male and female infants with dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) and between infants with DHF/DSS with and without malnutrition as assessed by weight-for-age*

Cytokine, mean ± SD pg/mL (range)	All patients (n = 62)	Male DHF/DSS infants (n = 35)	Female DHF/DSS infants (n = 27)	Malnourished infants (n = 6)	Normal nutritional infants (n = 56)	Controls (n = 6)	p†
IFN-γ	56.2 ± 115.4 (0–690.8)	46.7 ± 76.8 (0–351.3)	68.6 ± 152.5 (0–690.8)	237.7 ± 275.5 (0–690.8)	36.8 ± 62.4 (0–351.3)	4.1 ± 5.8 (0–13.9)	0.01‡, 0.7§ 0.07¶
TNF-α	9.0 ± 13.2 (0–77.6)	9.5 ± 11.6 (0–44)	8.4 ± 15.4 (0–77.6)	19.2 ± 11.4 (2.5–31.4)	7.9 ± 13.0 (0–77.6)	0.8 ± 1.2 (0–2.1)	0.01‡, 0.5§ 0.01¶
IL-10	73.8 ± 69.8 (2.8–405.1)	87.8 ± 83.4 (2.8–405.1)	55.6 ± 41.7 (5.4–135.4)	84.8 ± 50.2 (20.8–160.2)	72.6 ± 71.9 (2.8–405.1)	0.3 ± 0.9 (0–2.3)	<0.001‡, 0.1§ 0.3¶
IL-6	28.2 ± 41.7 (0–210)	33.4 ± 48.0 (0–210)	21.4 ± 31.3 (2.1–123.2)	26.2 ± 20.2 (2.9–56.1)	28.4 ± 43.5 (0–210)	1.4 ± 2.2 (0–4.9)	<0.001‡, 0.09§ 0.5¶
IL-4	2.0 ± 3.2 (0–17)	2.3 ± 3.6 (0–17)	1.5 ± 2.7 (0–10.3)	5.4 ± 3.7 (0–10.3)	1.6 ± 2.9 (0–17)	0.2 ± 0.5 (0–1.4)	0.2‡, 0.4§ 0.003¶
IL-2	2.6 ± 4.7 (0–30)	3.5 ± 5.7 (0–30)	1.5 ± 2.4 (0–8.6)	4.1 ± 4.3 (0–10.4)	2.5 ± 4.7 (0–30)	1.8 ± 2.4 (0–6)	0.9‡, 0.1§ 0.1¶

* IFN = interferon; TNF = tumor necrosis factor; IL = interleukin.
† *P* values of comparison between ‡all DHF/DSS infants and controls, §male and female infants with DHF/DSS and ¶infants with DHF/DSS with malnutrition and those with normal nutritional status by the Kruskal-Wallis test.

centage of malnutrition as assessed by WA and HA (6.9% versus 11.6%; $P = 0.03$; 7.7% versus 22.7%; $P < 0.001$, respectively) compared with the healthy control subjects. The results of two surveys (500 patients for each survey) to assess the nutritional status of inpatients of other diseases at Children's Hospital No.1 in Ho Chi Minh City in 2001 and 2003 conducted by the Department of Clinical Nutrition showed that 31.4% and 29.4% of these patients had malnutrition as assessed by WA, respectively, while 24.6–34.9% of the infant group had malnutrition (Hoa NT and others, unpublished data). In humans, moderate/severe malnutrition is associated with a significant reduction in cell-mediated immunity, as indicated by a reduced number of $CD4^+$ T helper cells, and a lower $CD4^+/CD8^+$ratio. There is also a reduction in the production of secretory IgA antibody and various complement components (C3, C4, and factor B) and impairment of phagocytosis. The production of certain cytokines such as IL-2 and TNF is also decreased.[16] Anto and others[8] and Thiasykorn and Nimmannitya[9] have shown that even mild degrees of protein-calorie malnutrition are in children more than one year of age with severe DHF/DSS compared with controls. This suggests that malnutrition blunts the severity of secondary dengue infections by possibly reducing T cell activities. Here we show a similar effect on the severity of dengue infections in infants, with the difference being that nearly all these infants were experiencing a primary immune response. In this immunologic setting, cytokine production is not enhanced by a memory T cell phenomenon.

Nutritional status did not convey absolute protection from severe clinical dengue disease. Infants with moderate malnutrition (WA) and DHF/DSS had serum levels of cytokines (IFN-γ, TNF-α, IL-10, and IL-6), and antibodies to dengue virus that were not different from infants with DHF/DSS without malnutrition.

The present study showed that there was no association between sex, nutritional status, and the severity of DHF/DSS in infants with a primary type dengue antibody response (nonshock DHF versus DSS). The production of cytokines and antibodies to dengue virus was not different between male and female infants with DHF, as well as between infants with and without malnutrition.

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TABLE 4

Comparison of levels of anti-dengue IgM, NS1 serotype-specific IgG antibodies between male and female infants with dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) and between infants with DHF/DSS with and without malnutrition as assessed by weight-for-age*

IgM ELISA/NS1 serotype-specific IgG ELISA (highest OD), mean ± SD) (range)	All patients	Male DHF/DSS infants	Female DHF/DSS infants	Malnourished infants	Normal nutritional infants	P
IgM ELISA						
At Center for Disease Control, Taipei, Taiwan	(n = 107) 2.8 ± 1.1 (0.3–4.0)	(n = 57) 2.9 ± 1.1 (0.3–4.0)	(n = 50) 2.8 ± 1.0 (0.3–4.0)	(n = 8) 2.8 ± 0.8 (1.1–3.8)	(n = 99) 2.9 ± 1.1 (0.3–4.0)	0.6†, 0.4‡
At Pasteur Institute, Ho Chi Minh City, Vietnam	(n = 138) 1.8 ± 0.5 (0.4–3.2)	(n = 81) 1.8 ± 0.5 (0.4–3.2)	(n = 57) 1.7 ± 0.5 (0.4–2.2)	(n = 9) 1.7 ± 0.4 (0.4–2.8)	(n = 129) 1.8 ± 0.6 (0.4–3.2)	0.8†, 0.6‡
NS1 serotype-specific IgG ELISA	(n = 36) 1.2 ± 0.6 (0.4–3.1)	(n = 20) 1.1 ± 0.5 (0.4–2.1)	(n = 16) 1.3 ± 0.6 (0.4–3.1)	(n = 2) 1.5 ± 0.3 (1.3–1.7)	(n = 34) 1.2 ± 0.6 (0.4–3.1)	0.3†, 0.3‡

* NS1 = nonstructural protein 1; ELISA = enzyme-linked immunosorbent assay; OD = optical density. Levels of IgM antibody to dengue virus were measured in acute- and convalescent-phase serum samples, while NS1 serotype-specific IgG antibodies were measured in convalescent-phase serum samples.
† *P* values of comparison between male and female infants with DHF/DSS, with and without malnutrition by the Kruskal-Wallis test.
‡ *P* values of comparison between infants with DHF/DSS with and without malnutrition by the Kruskal-Wallis test.

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