MAJOR ARTICLE

Slower Rates of Clearance of Viral Load and Virus-Containing Immune Complexes in Patients with Dengue Hemorrhagic Fever

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Background. Although previous studies have revealed the contribution of an initial high level of dengue virus replication to the severe and potentially life-threatening diseases dengue hemorrhagic fever (DHF) and dengue shock syndrome, the involvement of dengue virus in the immuopathological processes during the transition from fever to defervescence, which is a critical stage in determining the progression to DHF, has not been appreciated. Previously, we reported that dengue virus can be detected in the immune complexes of patients with DHF during this period.

Methods. We investigated plasma dengue viral load, virus in immune complexes, antibody response, complements, and cytokines for 54 patients with dengue fever (a relatively mild form of disease) and 49 patients with DHF. The patients had confirmed secondary infection with dengue virus type 2 from a large outbreak in southern Taiwan in 2002.

Results. Patients with DHF had a significantly higher viral load and a slower rate of clearance than patients with dengue fever. For viral loads >5.7 log RNA copies/mL on the day of defervescence, the positive and negative predictive values for DHF are 0.88 and 0.95, respectively. A higher level and slower decline of dengue virus–containing immune complexes (and a subsequently higher elevation of C5a and soluble interleukin 2 receptor) were found in patients with DHF, compared with patients with dengue fever.

Conclusions. These findings indicate that slower rates of clearance of viral load and virus-containing immune complexes are associated with subsequent immune activation and contribute to the progression of DHF at this critical stage. Moreover, viral load on the day of defervescence can predict cases of DHF.

The 4 serotypes of dengue virus (dengue virus serotypes [DEN] 1–4) cause the most important arboviral disease in tropical and subtropical areas [1–3]. It has been estimated that >50 million dengue virus infections occur annually worldwide [1–3]. The clinical presentations range from asymptomatic, to a relatively mild illness (dengue fever [DF]), to severe and potentially life-threatening diseases (dengue hemorrhagic fever [DHF] and dengue shock syndrome [DSS]) [2–5].

The pathogenesis of DHF and DSS has been one of the major issues in dengue virus research. Along with the risk factors reported for DHF and DSS, 2 major factors—the viral strain and the immune status of the host—have been identified [2, 3, 5]. Based on the observation that individuals experiencing secondary infection had a higher risk of developing DHF and/or DSS, the immune hypothesis states that cross-reactive, nonneutralizing antibodies from previous infection may enhance dengue virus infection [5]. This was supported by the antibody-dependent enhancement in vitro [6, 7] and confirmed by several cohort studies [8–10]. Because different dengue viral strains have been reported to link to outbreaks of very mild or very severe disease [11–13], the viral hypothesis contends that severe dengue disease is the result of infection with a more virulent strain [14]. Both hypotheses predict that a higher magnitude of viral replication will be seen in patients with more severe disease, as has been demonstrated by a

Received 22 March 2006; accepted 5 July 2006; electronically published 14 September 2006.

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Clinical Infectious Diseases 2006; 43:1023–30

1058-4838/2006/4308-0012$15.00

positive correlation between peak viral load and disease severity in studies focusing on the early stage of infection [15–17].

Clinically, a critical stage in determining the progression to DHF and/or DSS is the transition from fever to defervescence, during which severe hemorrhage, plasma leakage, and/or circulatory failure occur. Studies of immune responses have reported circulating immune complexes and complement activation in patients with DHF and/or DSS during this period [18–22]. In addition, several cytokines and cytokine receptors, such as TNF-α, IL-2, IL-10, IFN-γ, IL-6, IL-1β, IL-13, IL-18, soluble IL-2 receptor (sIL-2R), and soluble TNF receptor II (sTNFR-II), have been reported to be elevated in patients with DHF and/or DSS [17, 23–26]. However, the involvement of the virus in the immunopathological processes at this critical stage remains unknown. Previously, we reported that dengue virus can be detected in the immune complexes of patients with DHF during defervescence [27]. In the current study, we examined viral load, virus in the immune complexes, antibody responses, complements, and cytokines in both patients with DF and patients with DHF during this period. The patients were from the 2002 outbreak of DEN-2 infection in Kaohsiung, Taiwan, which was the largest outbreak in Taiwan since World War II.

PATIENTS AND METHODS

Study participants. From June to December 2002, during a DEN-2 infection outbreak in Kaohsiung, a metropolis in southern Taiwan, 120 adult patients who were admitted to either Yuan General Hospital or Huei-Te Hospital and received a diagnosis of either DF and DHF [4] were included in the study. The severity of disease was classified by the World Health Organization grading system [4, 27]. None of the participants were found to belong to the previously described intermediate category [17]. Illness day 1 was defined as the day of onset of fever (oral temperature, $\geqslant$38°C). Fever day 0 was defined as the day of defervescence (oral temperature, <38°C), and days prior to and after this time point were designated consecutively [15, 28]. With informed consent, sequential blood samples were collected, and plasma specimens were prepared [27]. All patients were confirmed to have infection due to DEN-2 by a nested RT-PCR assay [27, 29]. An envelope/membrane IgM/IgG Elisa was performed using convalescent serum samples to determine whether the patient fulfilled the definition primary or secondary infection [30]. A Japanese encephalitis virus nonstructural protein 1 IgM was used to exclude Japanese encephalitis virus infection [30].

Dengue viral RNA in plasma and in immune complexes. Dengue viral RNA in plasma or immune complexes was isolated through a modified immunoprecipitation assay [27] and quantified by a real-time RT-PCR assay with the sensitivity of 357 RNA copies per mL of plasma [31].

Duration and clearance rate of viral load. The duration of detectable viral load was estimated by assuming that the period during which viral RNA was detectable began on the day prior to onset of illness and ended on the last day that viral RNA was detected [15]. The rate of clearance of viral load was determined by calculating the slope of viral load decrease between fever day -2 and fever day +3.

Antidengue IgM and IgG. Antidengue IgM and IgG antibodies were detected using a commercial IgM/IgG capture ELISA (Dengue Duo; PanBio) with the levels determined as the percentage reactivity of the positive control samples [32]. Serial dilutions of the IgM positive control sample (100- to 800-fold dilutions) and of IgG positive control sample (25,600- to 409,600-fold dilutions) were subjected to IgM and IgG ELISA, respectively, to obtain a linear curve between absorbance (450 nm) and dilutions. Diluted serum samples (100-fold dilutions for IgM and 25,600-fold dilutions for IgG) were subjected to IgM and IgG ELISA simultaneously with respective positive control samples and cutoff samples (100-fold dilution). A ratio of the absorbance of serum to that of a cutoff sample of $\geqslant$1 was defined as positive [27,33]. The IgM and IgG ELISA scores of positive samples were the percentage of absorbance compared with that of the IgM positive control sample (100-fold dilution) and that of the IgG positive control sample (25600-fold dilution), respectively. The same batch of positive control samples and cutoff samples (provided by manufacturer) was used for all samples tested.

Western blot analysis. Lysates of DEN-2 New Guinea C strain–infected and mock-infected C6/36 cells were electrophoresed in an SDS–12% polyacrylamide gel. After transfer and blocking, the nitrocellulose membrane was cut into strips and incubated with serum (5000-fold dilution), followed by peroxidase-conjugated antihuman IgG (Pierce Biotechnology). The signals were detected by chemiluminescence reagent (Perkin-Elmer Lifesciences).

ELISA. IFN-γ, IL-10, and sIL-2R in plasma were determined by ELISA kits from Pierce Endogen, sTNFR-II was determined by a kit from R&D systems, and C3a and C5a were determined by kits from BD Biosciences. The upper limits of normal values for IFN-γ, IL-10, sIL-2R, and sTNFR-II are 2.6 pg/mL, 15.6 pg/mL, 1521 U/mL, and 2262 pg/mL, respectively.

Statistical analysis. The Mann-Whitney *U* test was used to compare parameters between the 2 groups for all variables except sex, which was compared using Fisher's exact test.

RESULTS

Patient characteristics. Of the 120 study participants, 17 fulfilled the definition of primary infection, and 103 fulfilled the definition of secondary infection. Because primary infection is thought to have a different pathogenic mechanism from secondary infection [2, 3], and because the case number was small,

Table 1. Characteristics of 103 patients with secondary dengue virus serotype 2 infection.

Characteristics	Patients with dengue fever (n = 54)	Patients with dengue hemorrhagic fever (n = 49)	P
Age, mean years (range)	51.0 (18–82)	52.1 (18–76)	.84
Sex, M/F	15/39	19/30	.30
Illness day at study entry, days[a]	3.3 ± 1.6	3.4 ± 1.5	.60
AST level at study entry, U/L	99.9 ± 68.9	173.3 ± 127.2	.001
ALT level at study entry, U/L	81.4 ± 54.0	133.3 ± 103.5	.01
WBC count at study entry, 10^3cells/mm^3	4.1 ± 1.7	4.5 ± 2.0	.38
Platelet count at study entry, 10^3 platelets/mm^3	90.7 ± 51.8	80.7 ± 57.8	.21
Platelet count nadir, 10^3 platelets/mm^3	42.8 ± 36.4	20.8 ± 9.7	<.001

NOTE. Data are mean ± SE, unless otherwise indicated. Dengue fever and dengue hemorrhagic fever are classified according to the World Health Organization case definition [4]. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

[a] Illness day 1 is defined as the first day of fever.

we focused our analysis on the 103 cases of secondary infection, including 54 cases of DF and 49 cases of DHF. As shown in table 1, there were no statistically significant differences in sex (P = .30, by Fisher's exact 2-tailed test), age, or day of entry (P = .84 and P = .60, respectively, by Mann-Whitney U test) between the DF and DHF groups. Although there was no statistically significant difference in WBC and platelet counts at entry between 2 groups, the nadir platelet counts were lower in the DHF group than in the DF group (P < .001, by Mann-Whitney U test). Moreover, aspartate aminotransferase and alanine aminotransferase levels at entry were higher in the DHF group than in the DF group (P = .001 and P = .01, respectively, by Mann-Whitney U test). These findings were generally in agreement with those reported for pediatric cases [17, 34].

Higher dengue viral load in patients with DHF than in patients with DF during the fever-to-defervescence transition. To investigate the relationship between viral load and disease severity, the levels of dengue viral RNA in plasma from 2 groups were compared for each day from fever day −2 to fever day +4 [31] (figure 1). On fever day −2 and −1, the levels of dengue viral RNA in plasma samples from patients with DHF were higher than those in plasma samples from patients with DF (P = .05 and P = .009, respectively, by Mann-Whitney U test). A similar trend was observed during defervescence. Patients with DHF had higher dengue viral RNA levels than did patients with DF on fever day 0, +1, +2, and +3 (P < .001, P < .001, P = .001, and P = .03, respectively, by Mann-Whitney U test).

Virus-containing immune complexes persist at defervescence for patients with DHF. To further investigate dengue virus in the immune complexes during this period, we focused on 26 patients (12 patients with DF and 14 patients with DHF) who had ⩾3 sequential plasma samples examined [27, 31]. The results of 4 representative cases (2 cases of DF and 2 cases of DHF) are shown in figure 2. Consistent with the cumulative data in figure 1, viral load dropped to a level below 3.7 log RNA copies/mL on fever day +1 for patients with DF and remained >3.7 log RNA copies/mL in patients with DHF. Interestingly, dengue virus–containing immune complexes were found in both patients with DF and patients with DHF during the late fever period. At defervescence, they dropped to very low or undetectable level in patients with DF, but remained high in patients with DHF.

Antibody responses. In agreement with the report that IgG antibody responses predominated in cases of secondary infection, antidengue IgG increased more rapidly and to a higher

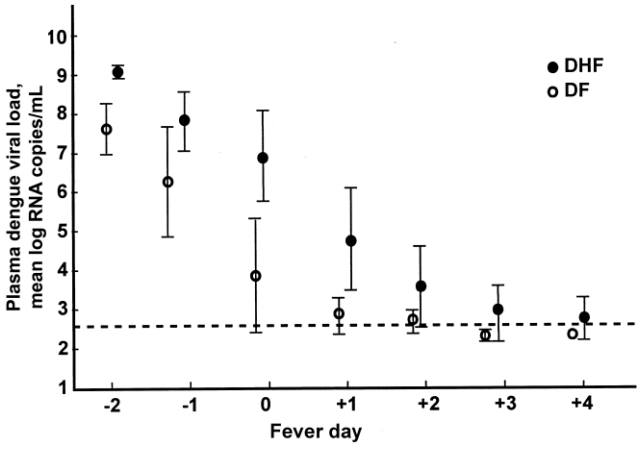

Figure 1. Plasma dengue viral load in patients with secondary dengue virus serotype 2 infection during the transition from fever to defervescence. Levels of dengue viral RNA in plasma of patients with dengue fever (DF) and patients with dengue hemorrhagic fever (DHF) were determined as described in Patients and Methods. Fever day 0 is the first day of defervescence. The dashed line indicates the limit of detection of the assay (i.e., 357 RNA copies per mL of plasma). Error bars indicate SE.

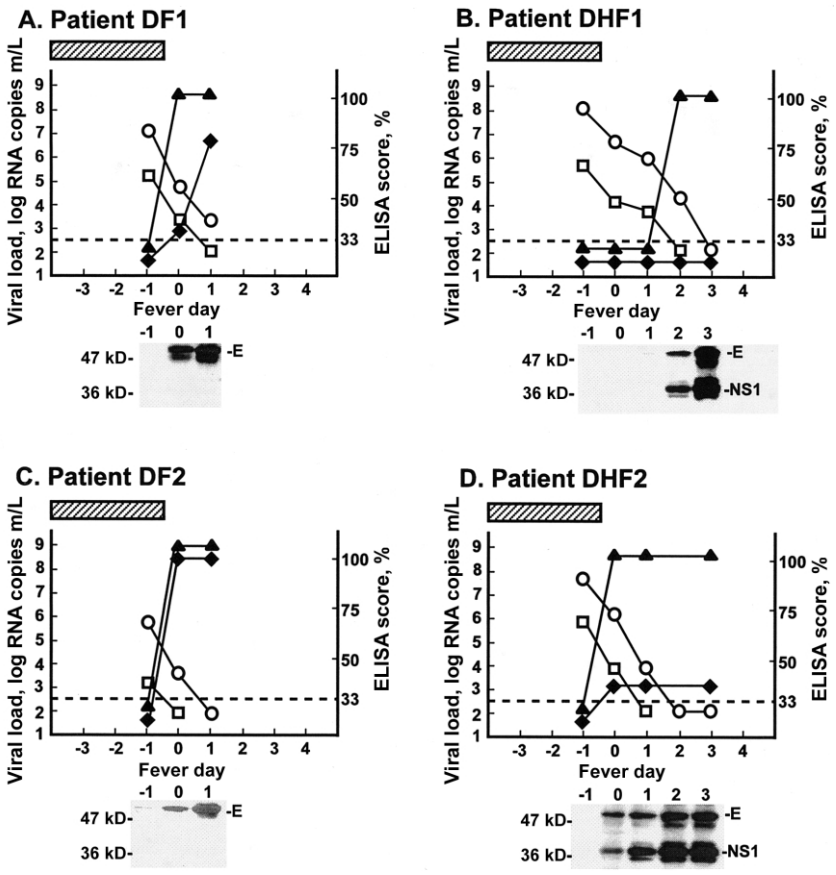

Figure 2. Relationships between plasma dengue viral load, virus in the immune complexes, and antidengue antibody responses during the transition from fever to defervescence in representative patients with dengue fever (DF) *(A, C)* and patients with dengue hemorrhagic fever (DHF) *(B, D)* who had secondary dengue virus serotype 2 infection. The levels of dengue viral RNA in plasma *(open circles)* and in the immune complexes *(open squares)* and the levels of antidengue IgG *(closed triangles)* and IgM *(closed diamonds)* antibodies were determined as described in Patients and Methods. Dashed lines indicate the limit of detection of the assay (357 RNA copies per mL of plasma) and of the ELISA score (33%). Hatched bars indicate the fever period. Fever day 0 is the first day of defervescence. The Western blot analysis is described in Patients and Methods, with the fever day shown above each lane and the molecular weight marker shown on the left. For simplicity, IgG ELISA scores >100% were presented as 100%. E, envelope; NS1, nonstructural protein 1.

level than antidengue IgM in most cases [28] (figure 2). Because these were cases of DEN-2 infection and the ELISA kit contained a mixture of 4 dengue viruses as antigen, we used lysates derived from DEN-2 virus–infected cells in Western blot analysis to examine anti–DEN-2 antibody response. As shown in figure 2, detectable bands of anti-envelope antibody, which was the major antibody response [35], correlated with IgG ELISA scores of ⩾100%, indicating that IgG ELISA scores of 100% represent significant levels of anti–DEN-2 envelope antibody response. Of note, the absence of anti–nonstructural protein 1 antibody seen in the 2 patients with DF was not seen in other patients with DF; therefore, this was not pursued further.

Slower rate of clearance of viral load in patients with DHF. The mean levels of viral load, virus in the immune complexes, anti-dengue IgG and IgM antibodies in these 26 patients are summarized in figure 3*A* and 3*B*. Compared with patients with DF, patients with DHF had higher levels of viral load and virus-containing immune complexes ($P = .003$ and $P = .02$, respectively, by Mann-Whitney *U* test) (table 2). Further analysis revealed that the estimated duration of detectable viral load was longer for patients with DHF than for patients with DF (mean duration, 7.7 vs. 6.2 days; $P = .02$, by Mann-Whitney *U* test). Moreover, the slope of the decrease in viral load was less for patients with DHF than for patients with DF, indicating a slower rate of clearance for patients with DHF (mean clearance rate, −1.30 vs. −2.01 log RNA copies/mL per day; $P = .037$, by Mann-Whitney *U* test) (table 2). This was further supported by a slower increase of antidengue IgG and IgM antibodies for patients with DHF than for patients with DF ($P = .002$ and $P = .02$, for IgG and IgM levels, respectively, between fever day 0 and +1, by Mann-Whitney *U* test).

Complement and cytokine activation. Similar to the decrease in viral load, the slope of the decrease in virus-containing immune complexes was smaller for patients with DHF than

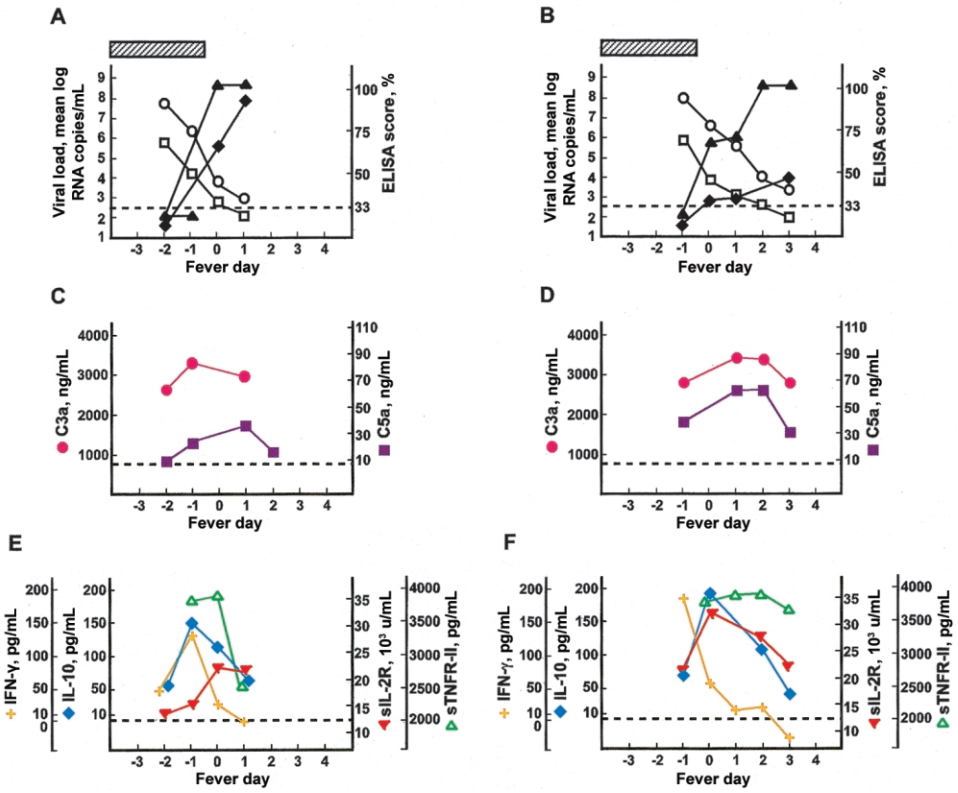

Figure 3. Relationships between the mean levels of dengue viral load, virus in the immune complexes, antidengue antibodies, C3a, C5a, cytokines, and cytokine receptors during the transition from fever to defervescence in 12 patients with dengue fever (DF) *(A, C, E)* and 14 patients with dengue hemorrhagic fever (DHF) *(B, D, F)* who had secondary dengue virus serotype 2 infection. *A* and *B* show the levels of dengue viral RNA in plasma *(open circles)* and in the immune complexes *(open squares)* and antidengue IgG *(closed triangles)* and IgM *(closed diamonds)* antibodies, determined as described in Patients and Methods. Dashed lines indicate the limit of detection of the assay (357 RNA copies per mL of plasma). Hatched bars indicate the fever period. Fever day 0 is the first day of defervescence. *C* and *D* show the levels of C3a *(pink closed circles)* and C5a *(purple closed squares)* in plasma, which were determined as described in Patients and Methods. The dashed lines indicate the upper limits of normal values for C3a (689.6 ng/mL) and C5a (7.8 ng/mL). *E* and *F* show the levels of soluble IL-2 receptor (sIL-2R; *red closed triangles*), soluble TNF receptor II (sTNFR-II; *green open triangles),* IL-10 *(blue closed diamonds),* and IFN-γ *(yellow crosses)* in plasma, which were determined as described in Patients and Methods. The dashed lines indicate the upper limit of normal values for IFN-γ (2.6 pg/mL).

for patients with DF, suggesting that circulating immune complexes had been present for a longer duration in patients with DHF than in patients with DF (figure 3*A* and 3*B*). To further investigate subsequent immunopathological events, 2 important complement mediators, C3a and C5a, were examined. As shown in figure 3*C* and 3*D*, elevation of C3a and C5a were found in both patients with DF and patients with DHF, with the peaks noted after virus-containing immune complexes decreased. Moreover, C5a was higher in patients with DHF than in patients with DF ($P = .009$, by Mann-Whitney *U* test).

We also examined the cytokines and cytokine receptors that have been reported to reach higher levels in patients with DHF than in patients with DF, including sIL-2R, sTNFR-II, IL-10, and IFN-γ [17, 23–25]. As shown in figure 3*E* and 3*F*, the peaks for these cytokines and cytokine receptors lagged behind the peaks for viral load for both patients with DF and patients with DHF, with the exception of IFN-γ, which peaked slightly earlier for patients with DHF. The level of sIL-2R was higher for patients with DHF than for patients with DF ($P = .01$, by Mann-Whitney *U* test), whereas there was no difference in sTNFR-II, IL-10, and IFN-γ between the 2 groups (table 2).

DISCUSSION

Previous studies of DHF have focused on the fever period and have reported the correlation of an initial high level of virus replication to DHF [15–17]. In this study, we focused on the fever-to-defervescence transition period and reported that patients with DHF had a higher viral load and slower rate of clearance than patients with DF. Moreover, a higher level and slower decrease of virus-containing immune complexes, as well as a subsequently higher elevation of C5a and sIL-2R, were found in patients with DHF, compared with patients with DF. To our knowledge, this is the first study demonstrating that

Table 2. Dengue viral load, virus in the immune complexes, and duration and clearance rate of viral load, complements, and cytokines during the transition to defervescence for patients with secondary dengue virus serotype 2 infection.

Variable	Patients with dengue ever (n = 12)	Patients with dengue hemorrhagic fever (n = 14)	P
Viral load,[a] log RNA copies/mL	5.1 (3.7–6.2)	7.1 (6.5–8.0)	.003
Virus in immune complexes,[a] log RNA copies/mL	3.5 (2.5–4.0)	4.7 (4.0–5.8)	.02
Duration of detectable viral load, days	6.2 (6–7)	7.7 (7–8)	.02
Clearance rate of viral load, log RNA copies/mL per day	−2.01 (−1.78 to −2.08)	−1.3 (−0.88 to −1.77)	.037
C3a level,[b] ng/mL	3009.5 (2640.2–3014.5)	3205.0 (2242.2–3792.6)	.59
C5a level,[b] ng/mL	30.9 (16.8–40.7)	54.8 (36.4–73.1)	.009
Soluble IL-2 receptor level,[b] U/mL	18041.0 (14778.8–20000.0)	25476.2 (19247.8–28539.8)	.01
Soluble TNF receptor II level,[b] pg/mL	3590.7 (3225.0–3926.8)	3866.7 (3660.2–4007.8)	.08
IL-10 level,[b] pg/mL	106.5 (58.1–148.6)	111.2 (54.3–114.3)	.75
IFN-γ level,[b] pg/mL	44.1 (20.0–43.3)	50.0 (20.8–49.7)	.87

NOTE. Data are mean value (interquartile range). Twenty-six patients with secondary dengue virus serotype 2 infection, including 12 patients with dengue fever and 14 patients with dengue hemorrhagic fever [4] who had ⩾3 sequential plasma samples, were included in the analysis.

[a] Determined for fever day −1 to fever day 0.

[b] Determined from fever day −2 to fever day +2.

slower rates of clearance of viral load and virus-containing immune complexes are linked to subsequent immune activation and contribute to DHF. Studies of more cases of secondary infection in the future would validate these findings. Of note, analysis of 17 patients with primary infection also revealed higher viral load in patients with DHF than in patients with DF during this period (data not shown), although a detailed analysis of immune complexes and subsequent activation was not performed because of the small sample size. Future studies involving a larger number of cases of primary infection, such as cases involving infants <1 year old, would shed new light on the pathogenesis of DHF in cases of primary infection.

Since DHF and DSS are the leading causes of hospitalization and death among children in Asia [2, 3], several laboratory parameters, including aspartate aminotransferase, sTNFR (80 kD), and soluble nonstructural protein 1, have been proposed as predictors for DHF [17, 34, 36]. Because patients with DHF had a significantly higher viral load than did patients with DF between fever day −2 and fever day +3, we further examined whether viral load during this period could predict for DHF; the positive and negative predictive values are shown in table 3. Compared with the positive and negative predictive values of the other parameters reported thus far (which have ranged from 0.53 to 0.83) [17, 34, 36], the high positive and negative predictive values (0.88 and 0.95, respectively) for viral load >5.7 log RNA copies/mL on fever day 0 (the day of defervescence) indicate that this is a good predictor for DHF. Nonetheless, whether this predictor can be employed clinically in the future requires careful monitoring of defervescence and rapid measurement of viral load.

Table 3. Positive and negative predictive values of dengue viral load for patients with dengue hemorrhagic fever during the transition from fever to defervescence.

Variable	Positive predictive value	Negative predictive value
Fever day −1, viral load ⩾7.5 log RNA copies/mL	0.71	0.94
Fever day 0, viral load ⩾5.7 log RNA copies/mL	0.88	0.95
Fever day +1, viral load ⩾3.7 log RNA copies/mL	0.94	0.86

NOTE. Plasma dengue viral load was determined as described in Patients and Methods. A total of 103 patients with cases of secondary dengue virus serotype 2 infection, including 54 patients with dengue fever and 49 patients with dengue hemorrhagic fever [4], were included in the analysis. Fever day 0 is the first day of defervescence

By examining the slope of the decline in viral load, we showed that the clearance rate of viral load was slower for patients with DHF than for patients with DF. It is possible that this is an extension of higher peak viral load, resulting from larger numbers of virus-infected cells in patients with DHF. Because of the ubiquitous presence of RNAse, viral RNA detected in plasma most likely derived from virion, rather than from lysis of virus-infected cells. This finding was supported by a good correlation between the levels of viral RNA and the titers of viremia, determined by plaque assay using serial dilution of plasma for some of our patients (data not shown), as well as by cosedimentation of peak viral RNA and peak core antigen after sucrose-gradient ultracentrifugation of plasma containing another RNA virus, HIV-1 [37]. Alternatively, slower clearance of viral load is related to T cell dysfunction. Impaired T cell proliferation responses to dengue antigen have been reported in patients with acute dengue infection [38]. A recent study of T cell repertoire revealed apoptosis of dengue-specific T cell clones in patients with DHF between fever day −1 and fever day +1 and reported the "original antigenic sin" of T-cells [39], which has been shown to impair clearance of viremia [40, 41]. Because the timing of T cell apoptosis is after the peak of viral load, it is conceivable that dysfunction of dengue-specific T cells may account for the slower clearance of viral load observed in patients with DHF. Determining whether T cell apoptosis occurs earlier and contributes to the peak of viral replication requires sophisticated analysis of T cells at the early stage of infection. The finding of slower clearance of viral load in patients with DHF is in contrast to the findings of a previous study in which viremia decreased more quickly for patients with DHF than for patients with DF at defervescence [15]. Of note, the presence of viremia in that study [15] was determined by virus isolation, which could potentially be interfered with by the presence of antibodies or circulating immune complexes [20, 28, 42]. It is likely that greater amounts of immune complexes in patients with DHF at defervescence may cause a lower efficiency of virus isolation and, therefore, cause lower levels of viremia. Two recent studies using RT-PCR assay that showed a higher viral load until defervescence in patients with DHF, compared with patients with DF, support this interpretation [16, 17].

Previously, circulating immune complexes were reported in patients with DHF and/or DSS by detecting complement components associated with immune complexes [18–20]; however, the presence of dengue virus in the immune complexes remains unclear. In our study, we detected dengue virus in the immune complexes directly by a modified immunoprecipitation assay [27]. These virus and antibody complexes may augment virus replication in mononuclear cells through Fc receptor–mediated entry—the so-called antibody-dependent enhancement in vivo—which was supported by studies involving humans and rhesus monkeys [43, 44]. Alternatively, they may be involved in the clearance of viral load, presumably by phagocytic cells in the reticuloendothelial system [45]. Based on the timing of detection (i.e., shortly before or after defervescence) and the association with a decrease in viral load, the virus-containing immune complexes detected were most likely in the process of being cleared. Interestingly, the clearance of virus-containing immune complexes was also slower for patients with DHF than for patients with DF (figure 3*A* and 3*B*). This would lead to higher levels and a longer presence of circulating immune complexes, which would subsequently lead to more-profound complement activation in patients with DHF. Consistent with this prediction, both C3a and C5a peaked after virus-containing immune complexes decreased, and a higher level of C5a, one of the most potent inflammatory peptides, was found in patients with DHF, compared with patients with DF (table 2).

In agreement with previous reports, IFN-γ peaked slightly earlier in patients with DHF (figure 3*E* and 3*F*). In addition, sIL-2R and sTNFR-II were elevated in patients with DHF, with the level of sIL-2R higher in patients with DHF than in patients with DF (table 2) [17, 23, 25]. IL-10 and IFN-γ levels were also elevated in patients with DHF, but they were not higher in patients with DHF than in patients with DF. This could be due to the small sample size and/or due to the day-to-day variation of IFN-γ [26]. The observations that peak levels of cytokines and cytokine receptors lag behind peak levels of viral load (figure 3) and that viral loads are higher in patients with DHF, compared with patients with DF, indicate that higher magnitudes of viral replication lead to stronger T cell activation and greater cytokine release in patients with DHF [26]. Higher levels of T cell activation and cytokines, together with the stronger complement activation after sustained formation of immune complexes, would contribute to the immunopathogenesis of DHF.

Acknowledgments

We thank Su-Ru Lin, Min-Huei Wu, Mei-Ying Liao, I-Jung Liu, and Hsien-Ping Hu for technical assistance.

Financial support. The National Health Research Institute (NHRI-CN-CL9302P) and the National Science Council, Taiwan (NSC94-2320-B-002-065).

Potential conflicts of interest. All authors: no conflicts.

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