High Levels of Plasma Dengue Viral Load during Defervescence in Patients with Dengue Hemorrhagic Fever: Implications for Pathogenesis

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Received April 25, 2002; returned to author for revision June 18, 2002; accepted July 31, 2002

Studies of the pathogenesis of dengue hemorrhagic fever (DHF), a potentially life-threatening disease, have revealed the importance of initial high levels of virus replication. However, the possible involvement of virus during the transition from fever to defervescence, a critical stage in determining the severity of disease, has not been appreciated. Using quantitative reverse transcription-polymerase chain reaction, we examined the levels of plasma dengue viral load during both fever and defervescence periods in patients from a DEN-3 outbreak in southern Taiwan in 1998. Higher levels of plasma dengue viral RNA were found in DHF patients than in DF patients. During defervescence, while the level of plasma dengue viral RNA was undetectable in most DF patients, it remains high in all DHF patients. Using a modified immunoprecipitation assay, we demonstrated for the first time that the plasma dengue viruses persisting during defervescence were in the immune complexes for most DHF patients. These findings suggest that continued active viral replication or delay in the clearance of viremia contributes to the pathogenesis of DHF. Moreover, high levels of plasma dengue viral RNA during defervescence may serve as a disease marker for DHF.

Key Words: dengue virus; pathogenesis; quantification; immune complex; RT-PCR.

INTRODUCTION

Among the 70 or so arthropod-borne flaviviruses, outbreaks of the four serotypes of dengue virus (DEN-1, DEN-2, DEN-3, and DEN-4) continue to be a major public health problem in tropical and subtropical areas (Monath, 1994; Innis, 1995; Gubler, 1998). While most dengue virus infections develop as asymptomatic or mild, self-limited illness, in the form of dengue fever (DF), some patients may develop severe and potentially life-threatening diseases, dengue hemorrhagic fever—dengue shock syndrome (DHF/DSS) (Innis, 1995; WHO, 1997; Gubler, 1998). It has been estimated that approximately 100,000,000 dengue infections occur annually throughout the world (Halstead, 1988; Monath, 1994; Gubler, 1998).

The initial clinical presentations of DF and DHF/DSS are similar, both including a sudden onset of fever and a variety of nonspecific symptoms and signs (Innis, 1995; WHO, 1997; Gubler, 1998). It is at the time of defervescence that severe hemorrhage and circulatory failure occur in DHF/DSS patients. Without early diagnosis and prompt treatment, patients will die quickly (Innis, 1995; WHO, 1997; Gubler, 1998).

Along with other valuable insights, two major hypotheses, the immune and the viral hypotheses, have been proposed regarding the pathogenesis of DHF/DSS (Innis, 1995; Gubler, 1998). The immune hypothesis attempts to explain the epidemiological observations that individuals experiencing secondary infections with a heterologous dengue serotype had a significantly higher risk of developing DHF/DSS (Halstead, 1988; Burke et al., 1988; Thein et al., 1997). Dengue viruses have been shown to replicate to higher titers in human monocytes in the presence of cross-reactive nonneutralizing antibodies in vitro (Halstead and O'Rourke, 1977; Brandt et al., 1982). The antibody-dependent enhancement (ADE) phenomenon is believed to augment viral replication in vivo by increasing the numbers of dengue-infected antigen-presenting cells. The increase results in profound activation of preexisting cross-reactive cytotoxic T-lymphocytes and the release of cytokines and cellular mediators, thus leading to subsequent immunopathological processes and DHF/DSS (Innis, 1995; Gubler, 1998; Green et al., 1998; Rothman and Ennis, 1999). The viral hypothesis proposes that the severity of the disease is related to the strains of the viruses; some virus strains may have evolved to replicate faster and to higher levels.
with greater epidemic potential, thus causing more severe diseases (Rosen, 1977; Gubler et al., 1978; Leitmeyer et al., 1999).

As suggested by both hypotheses, the outcome of disease severity correlates directly with the magnitude of viral replication. Investigations of various clinical, virological, and immunological parameters during the course of infection have revealed the contribution of initial high levels of virus replication to DHF/DSS (Vaughn et al., 1997, 2000; Murgue et al., 2000; Libraty et al., 2002). However, the involvement of viruses in the later process, particularly during the transition from fever to defervescence that is a critical stage in determining the severity of diseases, has not been examined thoroughly.

Previously, we established a convenient and sensitive quantitative competitive-reverse transcription-polymerase chain reaction (QC-RT-PCR) assay for quantification of dengue viruses (Wang et al., 2000). In this study, we investigated the levels of plasma dengue viral load during both fever and defervescence periods in patients from a DEN-3 outbreak in southern Taiwan in 1998. We report here that DHF patients continue to have high levels of plasma dengue viral RNA during defervescence and demonstrate that plasma dengue viruses persisting in the DHF patients during defervescence were in the immune complexes.

**RESULTS**

**Study participants**

The basic demographic, clinical, and virological information on the 20 DEN-3 patients, including 11 DF and 9 DHF, are summarized in Table 1. The male-to-female ratio was 1. The ages of the participants ranged from 23 to 70 years. There were no statistical differences in gender, age, duration of fever, or sampling time between the DF and DHF groups (P > 0.5, Fisher’s exact two-tailed test, P = 0.33, P = 0.41, and P = 0.82, Mann–Whitney test, respectively).

**Quantification of plasma dengue RNA by RT-PCR**

We first employed a QC-RT-PCR assay, modified from a previously established method (Wang et al., 2000), to investigate the levels of plasma dengue viral RNA. Figure 1 illustrates the results of QC-RT-PCR of two patients, ID 22 and ID 24. For ID 22, addition of increasing amounts of the competitor RNA, Ci40-RNA, to replicate reactions containing identical amounts of target RNA resulted in a gradual increase in the intensity of the 210-bp product and a gradual decrease in the intensity of the 170-bp product (Fig. 1B, left). The point where the ratio of Cor. Int. 210 to Int. 170 equals 1 (molar equivalence) represents the amount of target RNA, which was determined

<table>
<thead>
<tr>
<th>Diseasea</th>
<th>IDb</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Fever periodc</th>
<th>Sampling timec</th>
<th>Primary or secondarye</th>
<th>Dengue RNA (copies/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF</td>
<td>ID2</td>
<td>60</td>
<td>F</td>
<td>d1–d5</td>
<td>d4</td>
<td>S</td>
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<td>M</td>
<td>d1–d5</td>
<td>d4</td>
<td>P</td>
<td>3,710,000</td>
</tr>
<tr>
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<td>ID5</td>
<td>54</td>
<td>M</td>
<td>d1–d2</td>
<td>d2</td>
<td>S</td>
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<td>DF</td>
<td>ID6</td>
<td>51</td>
<td>F</td>
<td>d1–d4</td>
<td>d4</td>
<td>P</td>
<td>3,440,000</td>
</tr>
<tr>
<td>DF</td>
<td>ID8</td>
<td>36</td>
<td>F</td>
<td>d1–d9</td>
<td>d7</td>
<td>P</td>
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<td>49</td>
<td>F</td>
<td>d1–d5</td>
<td>d4</td>
<td>S</td>
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<tr>
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<td>ID13</td>
<td>69</td>
<td>M</td>
<td>d1–d3</td>
<td>d7 (+4)</td>
<td>S</td>
<td>&lt;600</td>
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<tr>
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<td>S</td>
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<td>P</td>
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<td>P</td>
<td>3900</td>
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<td>M</td>
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<td>d3</td>
<td>S</td>
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<td>ID20</td>
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<td>F</td>
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<td>d4</td>
<td>P</td>
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<tr>
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<td>ID21</td>
<td>23</td>
<td>F</td>
<td>d1–d5</td>
<td>d4</td>
<td>P</td>
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<tr>
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<td>ID22</td>
<td>67</td>
<td>F</td>
<td>d1–d5</td>
<td>d8 (+3)</td>
<td>S</td>
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<tr>
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<td>ID23</td>
<td>27</td>
<td>M</td>
<td>d1–d7</td>
<td>d8 (+1)</td>
<td>P</td>
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<td>ID24</td>
<td>63</td>
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<td>d5 (+1)</td>
<td>S</td>
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<td>d1–d6</td>
<td>d8 (+2)</td>
<td>P</td>
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<td>70</td>
<td>M</td>
<td>d1–d4</td>
<td>d7 (+3)</td>
<td>S</td>
<td>46,700</td>
</tr>
</tbody>
</table>

- Disease includes dengue fever (DF) and different grades of dengue hemorrhagic fever (DHF) (WHO, 1997).
- ID, identification.
- Fever period is defined as from the first day of fever (d1) to the last day of fever.
- Sampling time d1 is the first day of fever. Numbers in parentheses indicate the day of defervescence, +1 being the first day of defervescence.
- Primary (P) or secondary (S) infection is determined by the IgM and IgG capture ELISA and the HI titer at convalescence.
to be 998 copies, corresponding to 594,000 copies of RNA per milliliter plasma. For ID 24, the amount of RNA was determined to be 2206 copies per reaction, corresponding to 1,310,000 copies of RNA per milliliter plasma (Fig. 1C). Of note was that for reactions near the equivalent points, there was a third band migrating slower than the 170- and 210-bp products (Fig. 1B, lanes of 100 to 5000 copies competitors, and Fig. 1C, lanes of 500 to 10,000 copies competitors). The slower migration pattern and the prominence of the bands seen only in reactions near the equivalent points suggest that these bands are heteroduplex complexes between the two differently sized products. Such heteroduplexes would not affect the accuracy of our quantification, since it was based on the relative amount of the product of the target to that of the competitor RNA, and both products will in principle be similarly affected by the formation of the heteroduplex complex (Piatak et al., 1993; Freeman et al., 1999; Wang et al., 2000).

High levels of plasma dengue RNA persisting in DHF patients

The levels of plasma dengue RNA of the 20 patients thus determined range from less than 600 copies to 14,900,000 copies of RNA per milliliter plasma (Table 1). There is no significant difference in the levels of plasma dengue RNA with regards to age, gender, or primary
versus secondary antibody response ($P = 0.93$, ANOVA, $P = 0.39$, and $P = 0.53$, Mann–Whitney test, respectively). Compared with those in the DF patients, the levels of dengue RNA in the DHF patients were significantly higher (Fig. 2A, $P = 0.025$, Mann–Whitney test). We next analyzed the samples collected during the fever and defervescence periods separately. When only febrile patients were compared, the levels of plasma dengue RNA in the DHF group, ID18 to ID21 (geometric mean: 6.19 log copies/ml), were higher than those in the DF group, ID2 to ID9 (geometric mean: 5.08 log copies/ml), though this has not reached statistical significance ($P = 0.48$, Mann–Whitney test) (Fig. 2B). During defervescence, all DF patients except one have dengue RNA below the detection limit. In contrast, all DHF patients retain high levels of dengue RNA (geometric mean: 5.64 log copies/ml) (Fig. 2C). The difference was highly significant ($P = 0.008$, Mann–Whitney test).

**Analysis of sequential plasma samples**

The differences in plasma dengue RNA observed between DF and DHF patients suggest that there are different patterns of viremia during the transition from fever to defervescence. To investigate such possibility, we examined sequential plasma samples from six patients, three DHF and three DF. One of the DHF patients was ID 18, from whom the first sample was taken on day 3 and the second on day 9. The other two DHF patients (ID30 and ID31, both DEN-2) and the three DF patients (ID 27 and ID 28, DEN-3; and ID29, DEN-4) were identified between 1999 and 2001. The clinical course and plasma dengue RNA level are shown in Fig. 3. For all three DF patients, the levels of dengue RNA declined to the level below detection when the fever subsided (Figs. 3A–3C). In contrast, dengue RNA remains in plasma for up to 6 days after defervescence in DHF patients (Fig. 3D–3F). These results, though based on a small sample size examined, are in agreement with the cross-sectional observations and indicate that DF and DHF patients have different patterns of viremia during the course of infection. Moreover, these findings suggest that high levels of plasma dengue RNA persisting after defervescence may serve as a marker for DHF.

**Dengue viruses in the immune complexes**

It has been reported that anti-dengue IgM antibody developed within 5 days of onset and were detectable in

![FIG. 2. The relationship between plasma viral load and disease severity. (A) The levels of plasma dengue viral RNA (in log scale) of 11 DF and 9 DHF patients. (B, C) Subset analysis of samples obtained during the fever (B) and the defervescence (C) periods. Horizontal lines indicate geometric means. Dashed lines indicate the limit of detection of the assay, i.e., 600 copies/ml plasma.](image)

![FIG. 3. Changes in the plasma dengue viral load during the transition from fever to defervescence. (A, B, C) DF patients, ID 27, 28, and 29. (D, E, F) DHF patients, ID 18, 30, and 31. The levels of sequential plasma dengue RNA (in log scale) plotted by the day, hatched bars indicating the fever period. Dashed lines indicate the limit of detection, 600 copies/ml plasma.](image)
more than 90% of the patients from day 6 to day 10 (Gubler, 1998; Vaughn et al., 1999). In this study, all five defervescence samples from DHF patients (ID22 to 26) had detectable anti-dengue antibodies (Table 1 and data not shown). To investigate whether the dengue viruses persisting at defervescence in the DHF patients were contained in the immune complexes, we employed a modified immunoprecipitation assay. In the reconstitution experiment, equal amounts of a DEN-2 virus were preincubated with or without anti-dengue mAb before incubation with immobilized protein G. Dengue viral RNA isolated from the unbound and the bound fractions were subjected to quantitative RT-PCR. A band of the expected size of 170 bp was detected in the bound fraction of the reaction containing a DEN-2 specific anti-envelope mAb, 3H5, but not in those containing no antibody or containing 5D4, a DEN-3-specific anti-NS1 mAb (Fig. 4A). The proportion of bound fraction by 3H5 was calculated to be 33.3%.

We next incubated with immobilized protein G the plasma from five DHF patients (ID 22 to 26) at defervescence as well as plasma from five DF patients and examined the amounts of dengue RNA in the unbound and the bound fractions. With the exception of ID 25, considerable amounts of dengue RNA, corresponding to 7.2 to 42.5% of the total, were detected in the bound fractions of the defervescence plasma of the other four DHF patients (Fig. 4B). In contrast, no dengue RNA was found in the bound fractions of the control DEN-3 H87 virus or that of the plasma from DF patients, who have either detectable anti-dengue antibodies (ID4, 8, 9, and 17) or have not yet developed antidengue antibody (ID6) (Fig. 4B and data not shown).

**DISCUSSION**

In this study, we used the quantitative RT-PCR method, which is more sensitive and convenient than virus isolation, to investigate the relationship between plasma dengue viral load and disease severity during the course of infection (Piatak et al., 1993; Freeman et al., 1999; Murgue et al., 2000; Wang et al., 2000; Sudiro et al., 2001). Consistent with the outcome predicted by both immune and viral theories, higher levels of dengue RNA were seen in DHF patients than in DF patients, when comparing all acute plasma samples together (Fig. 2A) (Rosen, 1977; Halstead, 1988; Innis, 1995; Gubler, 1998; Thein et al., 1997). Uniquely, we found that DHF patients, in contrast to DF patients, continue to have high levels of plasma dengue viral RNA during defervescence, a critical stage in determining the severity of disease. Moreover, we demonstrated for the first time that the plasma dengue viruses persisting during defervescence were in the immune complexes for most DHF patients. These findings illustrate the importance of both viral and host factors during the transition from fever to defervescence and would provide new insights into our understanding of the pathogenesis of dengue (Innis, 1995; Gubler, 1998; Thein et al., 1997).

Using the PanBio IgM and IgG capture ELISA and the HI tests, 10 primary infections were found among the 20 patients in this study (Table 1). The possibility of Japanese encephalitis virus (JEV) infection was excluded, since all cases were negative for the JEV IgM-ELISA (Shu et al., 2000). Moreover, dengue viral signals were detected by the two-round RT-PCR assay and confirmed by DNA sequencing in all cases (data not shown). The observation of a high proportion of primary infection is in agreement with a recent report of the same outbreak by another medical center in Taiwan (Huang et al., 2001). This is probably due to the absence of dengue outbreak since 1943 in the central district of Tainan city, in which most cases of this study were found (King et al., 2000). A strong association of secondary infection with older age (older than 55 years old) through stratification analysis further supports this interpretation (Fisher’s exact test, \( P = 0.005 \)). These findings suggest that the epidemiology of dengue in Taiwan is different from that in Southeast Asia, where predominantly secondary infections were reported by pediatric studies (Gubler et al., 1981; Vaughn et al., 1997, 2000). With the small sample size studied here, secondary infection was not found to be associated with DHF (Table 1, 5 secondary infections of 9 DHF cases versus 5 secondary infections of 11 DF cases, \( P = 0.5 \), Fisher’s exact two-tailed test).

The most interesting observation of this study was that while most DF patients had undetectable plasma dengue RNA during defervescence, all DHF patients continued to have high levels (Fig. 2C). This striking difference could not be attributed to earlier sampling for the DHF
In summary, our findings suggest that high levels of plasma dengue RNA during defervescence serve as a disease marker of DHF. Based on our analysis, plasma dengue RNA levels higher than $10^4$ copies per milliliter at day 2 of defervescence are able to distinguish DHF from DF patients (Fig. 2C). Nonetheless, due to the dynamic nature of viremia in dengue infection, further analysis of more cases at comparable and multiple time points during the transition from fever to defervescence is required to define a cutoff value as the marker or predictor for the development of DHF.
MATERIALS AND METHODS

Study participants

The diagnoses of DF and DHF followed the WHO clinical definition (WHO, 1997). Detection of dengue genomic sequences in plasma by a previously described two-round RT-PCR assay, which included RT-PCR and a second round PCR, was the laboratory criteria of confirmation for all cases (Lanciotti et al., 1992; WHO, 1997). Between November and mid-December 1998, during an outbreak in southern Taiwan, there were 20 dengue patients at three hospitals (Chi-Mei Foundation Medical Center, Kuo General Hospital, and Sin-Lau Christian Hospital) confirmed at our laboratory (King et al., 2000). The day of onset of fever (oral temperature ≥38°C) is defined as day 1 of illness (d1). Defervescence is the period that oral temperature is below 38°C without further elevation, and the first day of defervescence (+1) is thus defined. Acute blood samples were collected in EDTA-containing tubes between day 2 and day 8 of illness. Plasma was prepared within 6 h of collection and stored at −80°C until use (Wang et al., 2000). The patients were closely observed during hospitalization and monitored with routine laboratory tests. After careful review of the charts by infectious disease physicians, the severity of disease was assessed by the WHO grading system (WHO, 1997; Murgue et al., 2000; Vaughn et al., 2000). The serotype of the 20 dengue patients was determined to be DEN-3 using the two-round RT-PCR assay, which can distinguish the four dengue serotypes by the size of the products (Lanciotti et al., 1992).

To determine primary or secondary dengue virus infection, two methods were used in testing the convalescent sera. One is the commercial IgM and IgG capture ELISA (PanBio Dengue Duo, Brisbane, Australia), which contained stabilized dengue antigens (DEN-1 to DEN-4) in one plate and anti-human IgM or anti-human IgG in another plate (Vaughn et al., 1999). The ratio of the absorbance at 450 nm of the patients’ sera (1:100 dilution) to that of the reference sera provided (cutoff calibrators, CO) were determined, and a ratio ≥1 was defined as positive. An IgM/CO ≥ 1 and IgG/CO < 3 are considered primary infection, and an IgG/CO ≥ 3 is considered secondary infection as described previously (Vaughn et al., 1999). The other method is the traditional hemagglutination inhibition (HI) test, in which primary (HI titer ≤ 1280) or secondary (HI titer > 1280) infection was classified according to the WHO definition (WHO, 1997). Both methods showed consistent results. In addition, all serum samples were tested with a previously described JEV-NS1 IgM ELISA to exclude JEV infection (Shu et al., 2000), and all were negative.

Sequential samples, collected from 1999 to 2001, taken at two to three time points, from three DF patients and two DHF patients at the Kaoshiung Veterans General Hospital and the Yuan General Hospital in southern Taiwan are also included in the analysis.

Isolation of viral RNA from plasma

Dengue viral RNA was isolated from plasma using the QIAamp viral RNA mini kit (Qiagen, Germany) as described previously (Wang et al., 2000).

RT-PCR primers

The primer pair, d3C14A and d3C69B, was modified from a previously described primer pair, which can detect all four dengue serotypes but not other flaviviruses (Wang et al., 2000). The sequence of d3C14A was 5’-AATATGCTGAAACGCGTGAGAAACCG-3’ (genome positions 134 to 159 of the DEN-3 H87 strain) (Osatomi and Sumiyoshi, 1990), and the sequence of d3C69B was 5’-CCCCATCTAGCCAGACCTCTGCTG-3’ (positions 278 to 302 of the H87 strain). They were designed to amplify a 170-bp product for dengue RNA or a 210-bp product for competitor RNA in the capsid (C) region.

Generation of the constructs and RNA

C/pCRII-TOPO was a plasmid containing the entire C region and the N-terminal six amino acids of the precursor membrane (PrM) region of DEN-3 strain H-87 in the vector pCRII-TOPO (Invitrogen, San Diego, CA). The RNA of the C region, C-RNA, was generated by T7 in vitro transcription (Promega, Madison, WI) of the linearized C/pCRII-TOPO, purified, and quantified (Wang et al., 2000). The competitor construct, Ci40/pCRII-TOPO, was modified from C/pCRII-TOPO by digestion with the restriction enzyme, HincII, followed by filling both 3’ recessed ends and inserting a 40-bp adaptor (Fig. 1A). The competitor RNA, Ci40-RNA, was generated from the linearized Ci40/pCRII-TOPO and quantified (Wang et al., 2000).

Quantitative RT-PCR

To obtain accurate quantification, RNA eluates from some plasma samples were diluted 10- to 10,000-fold. For the QC-RT-PCR assay, equal amounts (6 μl) of the diluted RNA eluates were mixed with increasing copy numbers of Ci40-RNA and were subjected to RT-PCR. For the noncompetitive quantitative RT-PCR, equal amounts (6 μl) of the RNA eluates and increasing copy numbers of the C-RNA were subjected to RT-PCR simultaneously under the conditions described previously (Wang et al., 2000). The RT-PCR products were electrophoresed through 2% agarose gel, stained, and measured. RT-PCR products with the predicted size of 170 bp were seen in most samples, but not in reactions containing no RNA, or RNA from healthy donors, or when the RT step was omitted.

For QC-RT-PCR, the ratios of the corrected intensity of
RNA was calculated by the ratio of bound/bound competitive quantitative RT-PCR. The proportion of bound washing and elution. Dengue viral RNA, isolated from the incubated with protein G at 4°C for 4 h, followed by and of culture supernatants of a DEN-3 virus, H87, were in the circulating immunocomplexes, aliquots of plasma during washing (Fig. 4A). For detection of dengue viruses on dengue-2 virus using monoclonal antibodies. Am. J. Trop. Med. Med. Hyg. 31, 548–555.

Two murine antidengue monoclonal antibodies (mAbs), 3H5 (a DEN-2 specific anti-envelope mAb) and 5D4 (a DEN-3 specific anti-NS1 mAb), were derived from hybridomas, 3H5-1 and 5D4-11, respectively (American Type Culture Collection, Rockville, MD) (Gentry et al., 1982; Henchal et al., 1982). In the reconstitution experiment, equal amounts of DEN-2 New Guinea virus were incubated with or without antibody, at 4°C for 4 h, followed by incubation with immobilized protein G, which can bind human IgM and IgG, at 4°C for 4 h (Pierce, Rockford, IL). The unbound fractions were removed after centrifugation for 1 min. The immune complexes were washed six times with 0.5 ml binding buffer and subsequently eluted with the elution buffer to obtain the bound fractions (Pierce). Of note was that the amounts of dengue RNA derived from the unbound plus the bound fractions were comparable to the total dengue RNA at initial input, indicating that negligible amounts were lost during washing (Fig. 4A). For detection of dengue viruses in the circulating immune complexes, aliquots of plasma and of culture supernatants of a DEN-3 virus, H87, were incubated with protein G at 4°C for 4 h, followed by washing and elution. Dengue viral RNA, isolated from the unbound and bound fractions, was subjected to noncompetitive quantitative RT-PCR. The proportion of bound RNA was calculated by the ratio of bound/bound + unbound.

Statistical analysis

A nonparametric statistical method, the Mann–Whitney test in the software SPSS base 8.0 (SPSS Inc., Chicago, IL), was employed to compare age, fever duration, sampling time, and levels of viral RNA between groups. Fisher’s exact test was used to compare the difference in gender and primary versus secondary infection between DF and DHF patients.

ACKNOWLEDGMENTS

We thank Shu-Mei Chang at the Yuan’s General Hospital in Kaoshiung for kindly providing clinical samples, and Tzu-Ling Sung, Su-Ru Lin, Yu-Chen Tsai, Tsai-Yu Lin, and Pei-Yi Chu for technical assistance. We also thank Dr. D. J. Gubler for the DEN-2 New Guinea strain, and the DEN-3 H87 strain. This work was supported by the National Health Research Institute (NHRI-CN-CL8903P) and in part by the National Science Council (NSC91-2320-B-002-191), Taiwan, Republic of China.

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