Humoral Immune Response of Dengue Hemorrhagic Fever Cases in Children from Tegucigalpa, Honduras

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Abstract. The humoral immune response in Honduran dengue hemorrhagic fever (DHF) hospitalized pediatric cases from the epidemics of 2004 and 2005 was studied in sera collected from 5 to 7 days of fever onset. A total of 145 cases were included in the study: 40 classified as primary with DHF Grade I or II and 86 classified as secondary; from them, 73 were DHF Grade I or II and 13 were dengue shock syndrome (DSS) Grade III or IV. The highest number of primary cases was found in children < 1 year of age. The highest number of secondary cases was observed in children between 5 and 10 years of age. The IgA values showed a statistically significant difference between primary and secondary groups. The relationship between antibody responses and severity grade is discussed. This is the first study related to the humoral immune response and severity grade in DHF cases in Honduran children.

INTRODUCTION

Dengue fever (DF) and the severe clinical forms of dengue virus (DENV) infection, dengue hemorrhagic fever including dengue shock syndrome (DHF/DSS) are the principal arthropod-borne viral diseases in humans.1 The World Health Organization (WHO) reports that DF/DHF occurs in > 100 countries and as many as 2.5 billion people are living in places that have a high risk for transmission of DENV. It is estimated that at least 100 million cases of DENV infection occur worldwide annually, which result in 250,000–500,000 cases of dengue shock syndrome (DHS/DSS) cases occurred during 1981 in Cuba, with 10,312 cases and 18,843 clinical cases and 636 confirmed DHF cases in 2004 and 19,971 DF and 351 confirmed DHF cases in 2005 when the serotypes 1, 2, and 4 were circulated.7–9 Since the 1960s, > 4 million people, mostly children, have been hospitalized and >65,000 have died of DHF and DSS.5

The prevalence of dengue has increased dramatically in recent decades. The first epidemic in the Americas with DHF/DSS cases occurred during 1981 in Cuba, with 30,12 cases reported.6 From 1981 to 1997, 24 countries in the Americas reported laboratory-confirmed cases of DHF.1 In Honduras, DHF was first observed in 1991, and since then, it has been endemic, especially during the rainy season (June to October).2 During 2002, there were 32,269 DF cases reported and 4,033 suspected cases of DHF, with 863 confirmed DHF cases and 17 deaths. This constituted the largest epidemic registered in the country to date,4 with recurrent epidemics every year with 19,971 DF and 351 confirmed DHF cases in 2004 and 18,843 clinical cases and 636 confirmed DHF cases in 2005 when the serotypes 1, 2, and 4 were circulated.7–9

The aim of this work was to study the humoral immune response of Honduran DHF hospitalized pediatric cases from the epidemics of 2004 and 2005 in serum samples collected from 5 to 7 days of fever onset in Tegucigalpa, the capital city of Honduras. The relationship between antibody responses and severity grade is discussed in this study.

MATERIALS AND METHODS

Serum specimens. A total of 145 serum samples (collected from 5 to 7 days of fever onset) from pediatric patients hospitalized with a clinical diagnosis of DHF from March 2004 to November 2005 in Tegucigalpa, Honduras, were studied.7–9 These samples were collected from hospitalized patients at the Social Security Honduras Hospital (Hospital Hondureño de Seguridad Social [HHSS]) during 2004 and 2005; in addition, in 2005, patients from the Maternal–Children School Hospital (Hospital Materno Infantil, HMI) also were included. All samples were kept at −20°C until use. ELISA tests to detected IgM, IgA, and IgG were used in this study.10,11 All patients were classified according to Pan American Health Organization (PAHO)/WHO Guidelines for the Control and Prevention of Dengue and Dengue Hemorrhagic Fever in the Americas (1994). Parents or guardian consent was obtained for their participation, and local internal review board (IRB) approval was obtained previous to the initiation of the study.

Virus and antigens. Dengue antigens used in the serologic studies were obtained from sucking mice brains by the sucrose-acetone method.12 The prototype strain dengue viruses used were dengue 1 (Hawaii strain), dengue 2 (New Guinea C strain), dengue 3 (H-87 strain), and dengue 4 (H-241 strain).

Capture anti-DENV IgM (MAC-ELISA) and anti-DENV IgA (AAC-ELISA). The MAC-ELISA and AAC-ELISA were preformed according to Vázquez and others11 with minor modifications. Briefly, Immunol 2HB strips were coated with 10 μg/mL of anti-human IgM or anti-human IgA at the same concentration (Sigma Chemical, St. Louis, MO) diluted in sodium carbonate-bicarbonate buffer (pH 9.5) and were incubated overnight at 4°C. After the strips were blocked with 150 μL of 1% bovine serum albumin (BSA) for 1 hour at 37°C, 50 μL of each serum sample diluted 1:20 in PBS-Tween 20 was added to the wells and incubated for 2 hours at room temperature. Next, 50 μL of antigen mixture from four DENV serotypes at 16 hemagglutinate units (HUs), were added and incubated at 4°C overnight. Peroxidase-conjugated human anti-DENV IgG diluted 1:4,000 was added to each well and incubated for 1 hour at 37°C. After the addition of 100 μL of substrate (OPD plus hydrogen peroxide at 3% in citrate-phosphate buffer) and 30-minute incubation at room temperature, the reaction was stopped with 1 N H2SO4 and read at OD 492 nm/OD 630 nm. All samples with an OD ratio (OD sample/OD mean negative control) ≥ 2.0 were considered positive by MAC-ELISA and AAC-ELISA.

ELISA inhibition method. The detection of IgG Antibod-
ies was performed by the ELISA inhibition method (EIM) described by Vázquez and others \(^1^0\) with minor modifications. In brief, 96-well Costar plates were coated with 100 µL per well of human IgG at 10 µg/mL and incubated overnight at 4°C. The plates were blocked with 150 µL of 1% BSA followed by incubation for 1 hour at 37°C. Subsequently, 100 µL of DENV serotype 2 antigen was added to each well, diluted 1:20 in PBS-Tween 20, and incubated for 1 hour at 37°C, followed by the addition of 100 µL of the patient serum samples serially diluted from 1:20 to 1:10,240 and incubated 1 hour at 37°C. Next, 100 µL of peroxidase-conjugated human anti-DENV IgG diluted 1:4,000 was added to each well with another incubation of 1 hour at 37°C. The substrate (OPD-hydrogen peroxide at 3%) was added (100 µL per well), and the plates were held at room temperature for 30 minutes. The reaction was stopped with 100 µL of 1 N H2SO4 and read at 492 nm/630 nm. The titer of each sample was the last dilution for which the percentage of inhibition was ≥ 50%.

A serum sample with an IgG antibody titer ≤ 20 and that was IgM positive was considered as a primary infection case and a serum sample with an IgG antibody titer ≥ 1,280 and that was IgM positive was considered as a secondary infection case. \(^1^1\)

**Statistical analysis.** The GraphPad Prism 5.0 program (2007) was used for data analysis. To compare the OD ratio of each detected antibody for primary and secondary infection, a parameter t test was used. \(P < 0.05\) was considered a statistically significant difference.

## RESULTS

From 145 patients, 130 were classified as DHF without shock (Grades I and II) and 15 as DHF with shock (Grades III and IV). They were no fatalities.

**Primary and secondary DENV infection cases classified by EIM.** Table 1 shows the distribution of the 126 cases in function of antibody response and the severity grades. From 145 dengue cases, 40 (27.6%) were primary DENV infections with DHF Grade I or II. Eighty-six cases (59.3%) were classified as secondary infection; 13 were DHF Grade I or II and 13 were DSS Grade III or IV. Three cases from the total cases (2.1%) could not be classified as primary or secondary cases by EIM criteria, because the serum samples showed an IgG titer between 160 and 640. These cases were DHF Grade I or II. Eleven percent of the cases (16/145) were considered negative in relation to the antibody response; however, they presented a clinical picture of DHF or DSS. All cases were negative for either IgM and IgA antibodies, and only two cases with DHF had IgG titers of 40 and 80, respectively; the rest were IgG negative (EIM titer < 20).

**Primary and secondary DENV infection cases in relation with ages.** Figure 1 shows the distribution of the 126 cases, classified as primary (40 cases) and secondary (86 cases) DENV infections in relation to the child’s age. The group of children < 1 year old made up 45% (18/40) of the primary cases. The remaining 22 primary cases (55%) were observed in almost all the age groups with a lower percentage, from 2.5% for ages 1, 2, 4, and 12 years to 10% in those 3 and 6 years of age. The highest number of secondary cases (76.7%; 66/86) was observed in the group between 5 and 10 years of age; in this group, 12 of 13 DSS cases were also found. The modal age found in this study was 7 years of age.

Figure 2 shows the distribution of 18 cases of those < 1 year of age classified as primary; of them, 17 patients were between 5 and 11 months of age. Only one case was 3 months old.

**IgM and IgA antibody response in primary and secondary cases.** Figure 3 shows the OD mean ratio and 95% confidence intervals to IgM and IgA antibodies of serum samples from primary DHF in children < 1 or ≥ 1 year of age and secondary DHF or DSS infection cases. The comparison between IgM values of primary DHF in children < 1 year of age with primary DHF cases in children ≥ 1 year of age, with secondary DHF cases, and with secondary DSS cases was not statistically significant \((P = 0.52, P = 0.71,\) and \(P = 0.06,\) respectively). The comparison between primary cases in children ≥ 1 year of age and secondary DHF cases was statistically significant. However, there was no statistically significant difference between primary DHF ≥ 1 year of age and secondary DSS cases or between secondary cases (DHF and DSS). The comparison of IgA values between those < 1 and ≥ 1 year of age with primary DHF cases did not show a statistically significant difference; however, there was a statistically significant difference when primary cases < 1 and ≥ 1 year of age was compared with the secondary groups \((P < 0.0001)\) and secondary DHF with DSS \((P = 0.02)\).

The percentage of positive cases to IgA antibody in the different dengue type infections were: 67% in primary cases < 1 year (12/18) and 77% for ≥ 1 year of age (17/22). In secondary DHF cases the percentage was 95.5% (70/73) and 100% in DSS (13/13).

## DISCUSSION

Several hypotheses for the pathogenesis of dengue virus infection have been proposed. \(^1^3–1^6\) One of them, antibody-dependent enhancement (ADE), explains that the severe manifestations of DHF/DSS could occur in a patient who suffered a secondary infection with a dengue serotype different to that which caused the first infection. \(^1^3\) Another one is related with the virologic factors involved in the pathogenesis of DENV infection. \(^1^4\) DHF occurs in both primary and secondary infections, although more cases are seen in secondary infections. \(^1^5–1^7\) Another is the integral hypothesis that relates epidemiologic, viral, and individual factors. \(^1^5,2^0\)

<table>
<thead>
<tr>
<th>Severity grade</th>
<th>Patients</th>
<th>Primary cases</th>
<th>Secondary cases</th>
<th>Indeterminate cases</th>
<th>Negative cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHF (Grades I and II)</td>
<td>130</td>
<td>40 (30.8%)</td>
<td>73 (56.1%)</td>
<td>3 (2.3%)</td>
<td>14 (10.8%)</td>
</tr>
<tr>
<td>DSS (Grades III and IV)</td>
<td>15</td>
<td>—</td>
<td>13 (86.7%)</td>
<td>—</td>
<td>2 (13.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>145</td>
<td>40 (27.6%)</td>
<td>86 (59.3%)</td>
<td>3 (2.1%)</td>
<td>16 (11.0%)</td>
</tr>
</tbody>
</table>
There are reports of the occurrence of DHF during primary DENV infection in the first year of life in children born from a mother immune to DENV, therefore acquiring maternal antibodies, supporting the hypothesis of ADE.\textsuperscript{17,21,22} It is noteworthy that, in our study, in the group of children < 1 year of age, none showed a secondary response; however, the DHF picture in these children could still be related with the ADE because of the presence of heterotypic maternal dengue antibodies passively acquired and not detected.

At birth, maternal antibodies protect infants from dengue infection, but as IgG antibodies are catabolized, a period of risk to enhanced infection could take place.\textsuperscript{23} Halstead\textsuperscript{23} observed few cases with DHF/DSS in infants younger than 3 months of age, and the largest numbers were in infants 6–8 months old; Hongsiriwon\textsuperscript{24} found that most DHF/DSS in the first year of life in Thailand occur in children who are 5–9 months old. Similar results were found by Pengsaa and others.\textsuperscript{25} In our study, we did not find any cases in children < 3 months old, observing the largest numbers of cases in children who were 5–6 and 10 months old.

In contrast, other risk factors related to the virus or to individual factors could have determined the occurrence of DHF or DSS in the 22 primary cases \(\approx 1\) year old.

On the other hand, there have been observations that, in persons who have a second DENV infection, age is an intrinsic risk factor for vascular permeability.\textsuperscript{26} In 1981, during an epidemic of dengue 2 in Cuba, the greatest risk of DHF was observed in the youngest children.\textsuperscript{3} A study done by Halstead\textsuperscript{27} in children with DHF or DSS from Thailand during the 1980s and 1990s found that the highest rate was in children between 5 and 8 years of age. Balmaseda and others,\textsuperscript{28} studying the clinical manifestations in children and its relation with the dengue serotype-specific in two time periods, found that the children between 5 and 9 years of age displayed the highest burden of dengue diseases. A similar distribution of age was observed in our study, where the higher numbers of cases were found in children between 5 and 10 years of age (Figure 3). These observations could be related to the increase of dengue cases in Honduras between 1995 and 2002 and to several dengue serotypes in circulation during these years.\textsuperscript{9,29} Conversely, at 5 to 10 years of age, children already have a mature immune system, and the dengue immunopathogenesis phenomenon is associated with strong immune response.

In our study, only three patients that were considered positive to DENV infection (IgM and IgA positive) could not be classified as primary or secondary because they did not complete the criteria established by EIM. In other studies, there are cases reported with similar IgG response that have not been classified either.\textsuperscript{30} On the other hand, 16 patients, negative to IgM, IgA, and IgG antibodies, were found, despite the fact that they showed a classic clinical picture of DHF or DSS. It has been pointed out in some dengue kinetic studies that IgM immunoglobulin increases to high levels within 3 days of defervescence and peaks within 2 weeks in nearly all the patients with a primary infection,\textsuperscript{31–33} but it have also been observed that some patients may not develop IgM for 7–8 days after onset.\textsuperscript{1} In secondary infection, the kinetics of the IgM response is more varied, and the IgM to dengue virus appears later and is often preceded by IgG.\textsuperscript{31} In our cases, all these samples were collected between days 5 and 7 of fever onset, and none showed a secondary response. Despite classifying these cases as negative in relation to the antibody
response, we consider that they are more likely to be DENV infection cases considering the clinical diagnoses and the epidemiologic circumstances, especially those cases with DSS. On the other hand, it is known that an epidemic could occur with other viral or non-viral diseases such as leptospirosis, which could show a similar picture as DHF. These 16 cases were not confirmed to be other infections.

Previous studies pointed out possible use of IgA antibodies to DENV as a serologic marker to active infection.\(^\text{34-37}\) Anti-DENV IgA was reported to increase at the same time as IgM but persist for a shorter period of time.\(^\text{36}\) On the other hand, Koraka and others\(^\text{38}\) found significantly higher levels of dengue-specific IgA in acute sera from patients who developed shock compared with DF or DHF, correlating the levels of IgA with the severity of disease. In this study, a higher percentage of IgA antibody positivity was obtained in secondary DHF or DSS cases than in primary cases. Other authors have also found a higher IgA response in secondary cases.\(^\text{11,34,35}\) In a previous kinetic study of IgA antibody, it was found that 100% of the cases were positive by the seventh day of onset.\(^\text{37}\)

It is important to point out that antibody response to IgM and IgA in primary cases < 1 year of age had a similar response when they were compared with the primary group ≥ 1 year of age, despite the difference in age. In secondary cases, DHF and DSS also had a similar response between them, although there were statistically significant differences for IgA antibody responses.

In this study, the detection of antibodies IgM, IgA, and IgG in serum samples collected from 5 to 7 days allowed the confirmation of cases with a clinical diagnostic of DHF/DSS and their classification as primary or secondary DENV infection; it was also possible to relate them with the severity grade. This is the first study of the relation of the humoral immune response and severity grade in DHF cases in Honduran children.

It is evident that DF and DHF are important health problems in Honduras; since 1996, there have been recurring seasonal epidemics,\(^\text{5}\) and the severe forms can be fatal unless the patient receives opportune and adequate clinical management. There is an urgent need to further study the current and new laboratory methods to fortify the early diagnostic and prognostic tools, with the end result of providing valuable guidance to the medical doctors in the management of severe forms of DENV infections in the country.

It is important to know more about several behavioral aspects of dengue in Honduras that could bring new knowledge concerning the pathogenesis of this disease, as well of the use of the serologic, immunologic, and molecular markers related to the risk of DHF/DSS.

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