

Dengue Specific Immunoglobulins M, A, and E in Primary and Secondary Dengue 4 Infected Salvadorian Children

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El Salvador is a Central American country that has been affected by several dengue outbreaks. This study investigated the levels of IgM, IgA, and IgE anti-dengue antibodies in serum samples from children in El Salvador, with a clinical and serological diagnosis of dengue infection during the dengue 4 outbreak in 2002–2003. Seventy one serum samples were tested by ELISA and cases were classified in three groups: 13 primary dengue fever (PDF), 21 secondary dengue fever (SDF), and 37 secondary dengue hemorrhagic fever (SDHF). Also, the specificity of anti-dengue IgM for the different serotypes was tested. No significant differences in the IgM response were found between PDF and SDF, but these were detected between PDF and SDHF ($P=0.0053$) and between SDF and SDHF ($P=0.0003$). The IgA and IgE values showed a statistically significant difference between primary and secondary groups. The highest positivity percentage of IgA was between 95% (SDF) and 100% (SDHF) towards day 7 of onset of fever. All secondary cases were positive for IgE antibodies. The specificity of IgM was determined for DENV-4 virus in primary and secondary DF groups. This is the first study on dengue cases in Salvadorian children related to the immune response of different immunoglobulins to the type of infection and the clinical picture. Further prospective studies are needed to define if the pattern of immunoglobulins can determine early dengue infection and/or severity. **J. Med. Virol.**

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KEY WORDS: IgM; IgA; IgE; dengue infection; Salvadorian children

INTRODUCTION

Dengue is one of the most important human arboviral infections in terms of morbidity and mortality, causing a spectrum of illness from the classical dengue fever (DF) characterized by fever, headache, myalgia, and arthralgia to the more severe forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [Guzman and Kouri, 2002]. Most of the clinical descriptions of DHF refer to children's disease considering that since its recognition in the 1950s, it was observed mainly in children. However, DHF in the American region has affected frequently both children and adults [Guzman and Kouri, 2003].

El Salvador is a Central American country that has been affected by several dengue outbreaks. This country reported its first dengue epidemic in 1980 with 2,060 cases. Since then dengue epidemics have been observed in cycles of increasing and decreasing number of cases. In 1993 and 1995, there were 9,015 and 9,658 notified cases, respectively. In 2000, the largest dengue epidemic was reported, dengue virus 2 being the etiological agent [PAHO, 2000; Hayes et al., 2003]. Two years later, at the end of the 2002, dengue 4 virus was isolated in patients from San Salvador (Virology Laboratory, National Institute

Conflict of interest statement: none.

Ethical consideration: The protocol was in accordance with the ethical standards of the Institutional Ethics Committee, the Helsinki Declaration and the CIOMS international guidelines.

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The humoral immune response in dengue cases in relation to IgM, IgA, or IgE antibodies has been studied and compared in some works [Cuzzubbo et al., 1998; Talarmin et al., 1998; Groen et al., 1999; Koraka et al., 2001, 2003; Balmaseda et al., 2003] but few studies have included all these antibody types. The objective of this present study is to know the behavior of specific anti-dengue IgM, IgA, and IgE in serum samples from Salvadorian children during a DENV-4 outbreak in the city of El Salvador during 2002–2003, with different clinical pictures and type of infections (primary or secondary cases). The specificity of the IgM antibodies to the different serotypes has also been determined.

MATERIALS AND METHODS

Serum Specimens

A total of 71 serum samples from children—diagnosed clinically as dengue—during the DENV-4 outbreak in the city of San Salvador in 2002–2003 were studied. DENV-4 virus was isolated and identified by indirect immunofluorescence test and/or detected by RT-PCR in samples from some of these patients. The samples were received at the laboratory of the National Institute of Public Health “Max Bloch,” San Salvador, through the national dengue surveillance system. Dengue diagnosis was confirmed by IgM detection using Capture IgM ELISA (MAC-ELISA) [Hayes et al., 2003] in samples collected at days 5–7 of the onset of fever (day 5 $n=10$, day 6 $n=24$, and day 7 $n=37$). Thirty-four patients were classified as DF and 37 as DHF according to PAHO/WHO Guidelines for the Control and Prevention of Dengue [PAHO, 1994].

Serum samples were kept at -20°C . All samples were tested by an ELISA inhibition method [Vazquez et al., 2009] for the classification in primary or secondary cases. Thirteen DF cases were due to primary dengue infection and 21 cases were due to secondary infection. All DHF cases experienced a secondary infection.

For this study three groups were considered: Primary DF (PDF), Secondary DF (SDF), and Secondary DHF (SDHF).

Virus and Antigens

Dengue antigens used in the serological studies were obtained from the brain of infected suckling mice, extracted by the sucrose–acetone method [Clark and Casals, 1958] and tested by hemagglutination [Casals and Brown, 1954]. Viruses employed were dengue 1 (Hawaii strain), dengue 2 (New Guinea C strain), dengue 3 (H-87 strain), and dengue 4 (H-241 strain).

Conjugate of Human Anti-Dengue Immunoglobulin (IgG) With Peroxidase

To prepare the conjugate, IgG anti-dengue from human sera collected from convalescent dengue cases were used with the following characteristics: negative for IgM and IgA anti-dengue and with a titer $\geq 10,240$ against four dengue virus antigens as determined by ELISA inhibition method [Vazquez et al., 2009]. These sera were negative for hepatitis B and C viruses and human immunodeficiency virus. Horseradish peroxidase, type VI, (Sigma, Saint Louis, MO) was conjugated to human IgG anti-dengue by the periodate method [Nakane and Kawoi, 1974]. The conjugate used in all ELISA tests was kept at -20°C in glycerol and 1% bovine serum albumin (BSA) [Vazquez et al., 2003].

Capture IgM, IgA, and IgE Dengue Antibodies by ELISA (MAC-ELISA, AAC-ELISA, and EAC-ELISA)

Serum samples were tested by MAC-ELISA, AAC-ELISA, and EAC ELISA assays following protocols previously described [Vazquez et al., 2005, 2007]. Briefly, NUNC Maxisorp plates were coated with goat IgG anti-human IgM (Sigma), goat IgG anti-human IgA (Immune assay Center, Havana, Cuba), and goat IgG anti-human IgE (Sigma), depending on immunoglobulin isotype to be determined and blocked with BSA to 1%. Fifty microliters of 1/20 serum dilution to detect IgM and IgA dengue antibodies and 1/5 serum dilution to detect IgE dengue antibodies in PBS plus 0.5% BSA were added. Positive and negative controls were included in each test. Samples were incubated for 2 hr at room temperature for IgM and IgA and at 37°C for IgE antibody detection. A mix of the four dengue antigens at 16 hemagglutinant units (HU) was added. After 18 hr incubation at 4°C , human conjugate IgG anti-dengue at 1/4,000 dilution was added in each assay. Orthophenilendiamine (OPD) and hydrogen peroxide in buffer phosphate citrate pH 5 was used as substrate. After 30 min incubation at room temperature, the reaction was stopped using a solution of H_2SO_4 . Plates were read at 492 nm.

Optical density ratio (OD ratio) = P/N was calculated in all samples. P represents the OD of each serum sample and N represents the ODs mean of the negative control. All samples with an OD ratio ≥ 2 were considered positives by MAC and AAC-ELISA tests. For EAC-ELISA, an OD ratio ≥ 1.4 was applied as criterion for positivity.

MAC-ELISA using single dengue antigens at 16 HU was used to determine the serotype specificity of the IgM response to each dengue serotype. OD ratio ≥ 2 to each serotype was applied as positive criteria.

Statistical Analysis

The GraphPad Prim 5.0 program (2007) was used for data analysis. In order to compare the OD ratio

of each detected antibody for DF and DHF cases, and for primary and secondary infection, the Mann–Whitney test was used and p values of <0.05 were considered statistically significant. In order to compare the positive percentages of serum samples in each group, the chi-square test ($P < 0.05$) was used.

RESULTS

IgM, IgA, and IgE Dengue Antibodies Response in Primary DF (PDF), and Secondary DF (SDF) and DHF (SDHF) Patients

Figure 1 shows the OD ratio values with the arithmetic means of IgM, IgA, and IgE anti-dengue antibodies of the all serum samples from PDF and SDF and SDHF patients. The IgM OD ratio arithmetic means were 13.82, 12.78, and 7.35 for PDF and SDF and SDHF patients, respectively. No significant differences were found when PDF and SDF cases were compared ($P = 0.52$) however a significant statistical difference was observed when OD ratios of anti-dengue IgM from SDHF was compared with the PDF cases ($P = 0.0053$) and the SDF cases ($P = 0.0003$). The values for IgA were 2.85, 7.03, and 5.02 founding significant statistical differences among the groups: PDF and SDF ($P = 0.0028$); PDF and SDHF ($P = 0.0029$), however between secondary cases was not significant ($P = 0.282$). The response of specific anti-dengue IgE obtained showed (Fig. 1) arithmetic mean ratios values of 1.73, 3.72, and 3.65, respectively. Significant differences between PDF and SDF and SDHF were observed ($P < 0.0001$). The comparison between secondary groups have not demonstrated significant differences ($P = 0.83$).

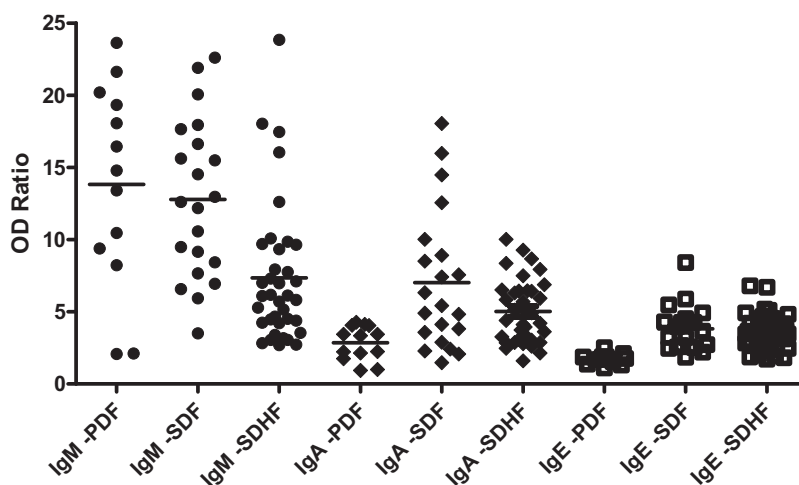


Fig. 1. Comparison of the different immunoglobulins: IgM, IgA, and IgE antibodies obtained by MAC-ELISA (●), AAC-ELISA (◆), and EAC-ELISA (□) tests in primary DF (PDF), secondary DF (SDF), and DHF (SDHF). Showing the OD arithmetic means ratio in each group. Significant differences ($P < 0.05$) were observed in: IgM PDF/SDHF $P = 0.0053$, SDF/SDHF $P = 0.0003$; IgA PDF/SDF $P = 0.0028$, PDF/SDHF $P = 0.0029$, and IgE PDF/SDF $P < 0.0001$, PDF/SDHF $P < 0.0001$.

Comparison of the Positivity of IgA and IgE Serum Samples in PDF, SDF, and SDHF Patients in Relation to Sample Time Collection

Figure 2 shows the OD ratio values with the arithmetic means of IgA anti-dengue antibodies of serum samples from PDF, SDF, and SDHF patients collected on day 5, 6, or 7 after onset. The OD ratio arithmetic means for IgA in PDF cases were 0.94, 2.24, and 3.39, for SDF 4.84, 5.07, and 9.27 and for SDHF 2.83, 5.02, and 5.59, respectively. An increment of OD ratio arithmetic means was observed for all infection types in function of the day of serum sample collection.

Figure 3 shows the OD ratio values with the arithmetic means of IgE anti-dengue antibodies of serum samples from PDF, SDF, and SDHF patients collected on day 5, 6, or 7 after onset. The OD ratio arithmetic means for IgE in PDF cases were 1.10, 1.85 and 1.74, for SDF 3.18, 3.32 and 4.30, and for SDHF 2.61, 3.24 and 4.21, respectively. An increment of OD ratio arithmetic means was observed for both secondary dengue infection groups in function of the day of serum sample collection. In PDF cases an increase between days 5 and 6 was observed.

Comparison of Positivity Percentage of IgA and IgE Serum Samples in PDF, SDF, and SDHF Patients in Relation to Sample Time Collection

In PDF cases 76.9% (10/13) were positive for IgA and IgE dengue antibodies. In SDF cases 95.2% (20/21) and SDHF cases 97.3% (36/37) were positive for IgA dengue antibodies. All SDF (21/21, 100%) and SDHF (37/37, 100%) cases were positive for IgE antibodies (Fig. 4). The comparison of IgA or IgE

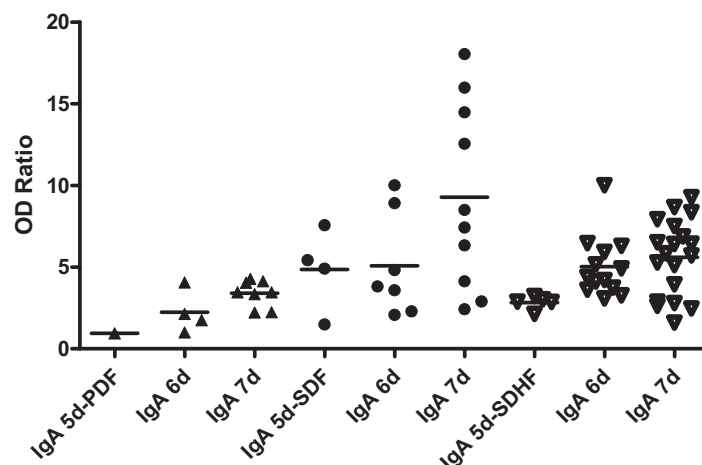


Fig. 2. Comparison of the optical density (OD) ratio IgA antibodies in relation to sample time collection (between 5 and 7 days), in primary DF (PDF) ($\blacktriangle$), secondary DF (SDF) ($\bullet$), and DHF (SDHF) (∇). Showing the OD arithmetic means ratio in each group.

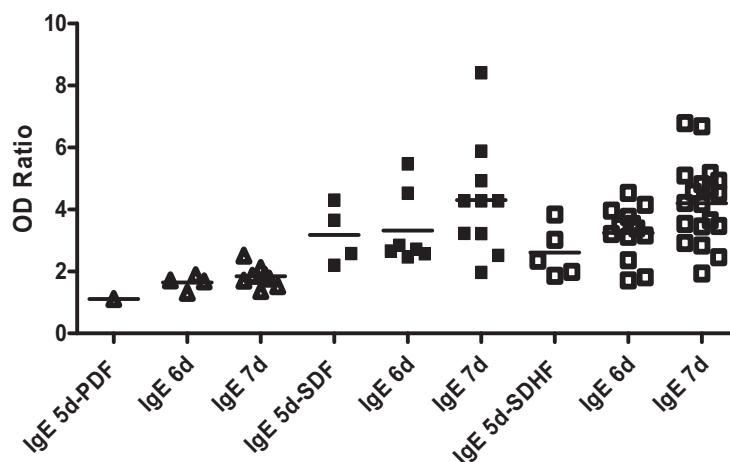


Fig. 3. Comparison of the OD ratio IgE antibodies in relation to sample time collection (between 5 and 7 days) in primary DF (PDF) ($\triangle$), secondary DF (SDF) ($\blacksquare$), and DHF (SDHF) ($\square$). Showing the OD arithmetic means ratio in each group.

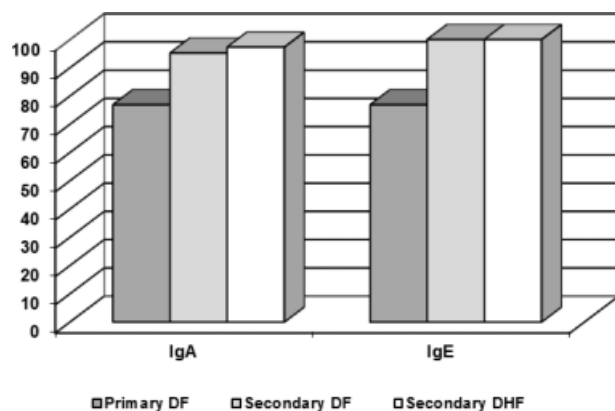


Fig. 4. Percentages of positive samples to IgA and IgE dengue antibodies in primary and secondary dengue cases. The comparison of IgA or IgE were significantly different between primary and secondary cases (DF or DHF), $P < 0.05$.

positive percentages was significantly different between PDF and secondary cases (DF or DHF) ($P < 0.05$). When comparing to sample time collection corresponding to day 6 and 7 after onset in PDF, 50% and 100% were positive for IgA, respectively and, 75% and 87.5% for IgE. The highest positivity percentage of IgA in secondary cases was between 95% and 100% towards day 7.

IgM Serotype Dengue Antibody Response in Primary DF, Secondary DF, and DHF Patients

Figure 5 shows the OD mean ratio values $\pm$ SD obtained for each dengue serotype in serum samples from primary and secondary DF and DHF patients. The highest levels were observed for dengue 4 virus in primary and secondary DF groups. The OD ratio values against dengue 4 serotype in the primary

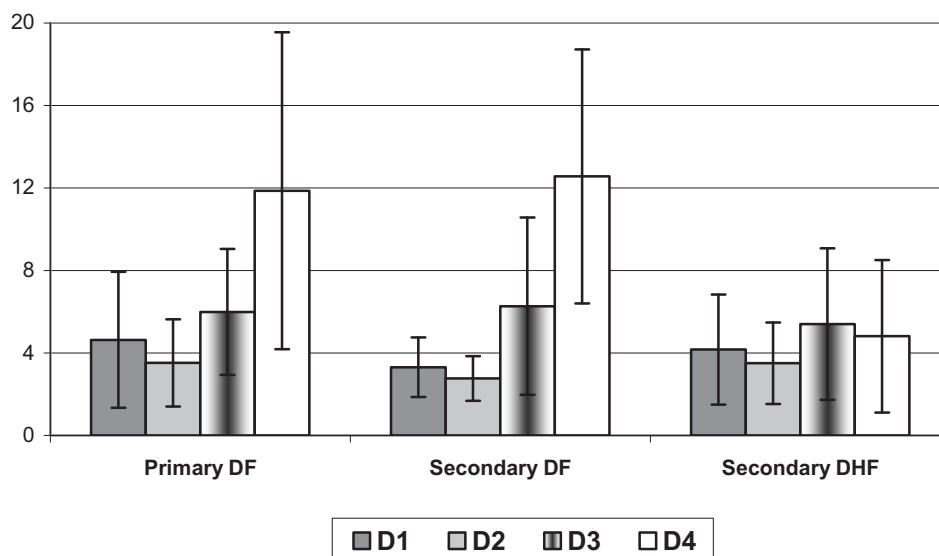


Fig. 5. Specific IgM antibody reactivity to the four dengue virus serotypes in each group studied. OD means ratio $\pm$ SD. The identification of DENV-4 serotype was observed in primary or secondary DF groups ($P < 0.001$). In secondary DHF there was not significant differences among serotypes ($P > 0.05$).

cases as well as secondary DF cases were statistically significant when compared with the other three serotypes ($P < 0.001$). In secondary DHF cases, DENV-4 serotype OD ratio values were not statistically significant when compared to the other three serotypes ($P > 0.05$).

DISCUSSION

Some studies have been performed to examine the response of either IgM or IgA or IgE from patients with primary or secondary infection or from patients with different clinical pictures [Groen et al., 1999; Balmaseda et al., 2003; Vazquez et al., 2005; Lorenzana de Rivera et al., 2008], but few studies included all these antibodies [Vazquez et al., 2005, 2007].

In the present study the IgM antibody response in PDF and SDF patients has not shown statistically significant differences; however, when SDHF cases were compared to DF cases (primary or secondary) statistically significant differences were found. These results were similar to those obtained in serum samples collected from Cuban DF or DHF adult patients with a primary or secondary dengue 3 infection [Vazquez et al., 2005]. On the other hand, Lorenzana de Rivera. [2008] did not find differences in the IgM response between primary and secondary cases with same clinical picture (DHF).

In dengue primary infection, the antibody response depends on the activation of naive B cells producing primarily IgM and later IgG anti-dengue specific antibodies. Alternatively, during a secondary infection, the sources of the major antibodies production are the memory B cells, however, IgM antibodies seem to be produced by the specific naive cells. That

could explain the similar levels of IgM antibodies found in PDF and SDF cases. In SDHF cases the lowest response could be due to the formation of immune complexes related to the presence of a higher viral load observed in individuals suffering from the severe forms of the illness [Vaughn et al., 2000; Libraty et al., 2002; Wang et al., 2006] and these antibodies as immune complexes cannot be detected by ELISA.

In the present study the detection of IgA and IgE antibodies to DENV were included, considering its utility as possible serological markers of active infection as shown by previous studies [Talarmin et al., 1998; Groen et al., 1999; Balmaseda et al., 2003; Koraka et al., 2003; Vazquez et al., 2007].

Isotype switching from IgM to other Ig isotypes typically occurs subsequent to a first exposure to antigen. At the molecular level this process involves the somatic rearrangement of immunoglobulin heavy chain constant region genes and/or alternative RNA splicing of germline transcripts. Isotype switching is regulated by T-helper cells through physical contact with B-cells and some cytokines produced by these cells. TGF-beta appears to specifically induce B cells to switch to IgA production, while IL-4 and IL-13 is involved in isotype switching to IgE production [Poulsen and Hummelshoj, 2007; Cerutti, 2008; Stavnezer and Kang, 2009]. Both cytokines, TGF beta1 and IL-13, have been reported as increased in dengue patients [Agarwal et al., 1999; Mustafa et al., 2001].

Koraka et al. [2001] found in the acute phase of the illness that patients who developed shock had significantly higher levels of DEN-specific IgA when compared with DF or DHF patients, in samples collected between 2 and 4 days of the onset of fever, however

in those collected between 5 and 7 days there were no observed differences of IgA levels between DHF and DSS cases, but there were differences to DF cases. In the present study, when comparing the IgA antibody response between PDF with SDF or SDHF cases statistically significant differences in OD ratio values were found, being higher in SDF cases (Fig. 1). The difference between our results with those found by Koraka et al. [2001] could be due to the classification of the cases used in each study.

A higher percentage of IgA antibody positivity was obtained in SDF and SDHF cases than in PDF cases, showing that the IgA response may be more related to secondary infection than to severity of disease. Other authors have also found a higher IgA response in secondary cases [Groen et al., 1999; Balmaseda et al., 2003; Vazquez et al., 2005; Lorenzana de Rivera et al., 2008]. In a previous kinetic study of IgA antibodies it was found that 100% of the cases were positive towards the 7th day of onset in both primary and secondary cases [Vazquez et al., 2007]. Lorenzana et al. [2008], studying the presence of IgA in pediatric patients from Tegucigalpa, Honduras with primary DHF, secondary DHF, or DSS infection found percentages of 72.5%, 95.9%, and 100%, respectively, in samples collected principally between days 6 and 7 of onset. However in an earlier study [Vazquez et al., 2005] percentages of 23.8%, 85.4%, and 56.8% were obtained for PDF, SDF, and SDHF, respectively, in serum samples from adult Cuban patients collected principally at day 5 of fever onset. In general, in this present study the highest IgA OD ratio values were found towards day 7 of fever onset, this also representing the day with the highest percentage of positivity (Fig. 2). IgA antibodies could be used as a serological marker for dengue diagnosis, keeping in mind that this antibody is detected predominantly in secondary cases; day 7 after onset of fever seems to be the best day for collection, especially for primary cases.

Some investigators have studied the response of total Immunoglobulin E levels in dengue cases, Miguez-Burbano et al. [1999] found that in acute dengue infection and with past exposure to dengue higher IgE levels were significantly more common, but there were no differences between primary and secondary dengue infection. The study concluded that abnormally high IgE levels in—individuals with current or past exposure to the dengue virus—may suggest that a balanced immune response leads to disease resolution, rather than the induction of a particular TH cell pathway. The increase in serum IgE during the acute stage of dengue infection and the persistence of elevated IgG levels after recuperation from dengue disease may indicate the presence of a specific IgE response and possibly reflecting immune memory [Miguez-Burbano et al., 1999]. In a later study similar results were found [Vazquez et al., 2007], observing high levels of IgE in both primary and secondary cases. Mabalirajan et al.

[2005] studied the relationship between total IgE levels and disease severity. This study could not detect differences among the patients, but those with milder illness (DF) had higher mean levels of IgE than those with severe illness (DHF/DSS) [Mabalirajan et al., 2005]. The authors pointed out that the increase of IgE levels in patients with dengue is probably due to a reversal of inhibition of IL-4-dependent IgE synthesis, resulting from decreased levels of IFN during defervescence (typically fever lasts for 5–7 days followed by defervescence).

In other study carried out by Koraka et al. [2003] it was observed that patients with secondary dengue virus infections had no significantly increased levels of both total and dengue virus-specific IgE in the acute phase of disease compared to patients with primary dengue virus infections. However dengue virus-specific IgE was significantly higher in DHF and/or DSS patients compared to patients with dengue fever and non-dengue patients ($P < 0.05$).

In the present study the dengue specific IgE response was different, being higher in secondary (SDF or SDHF) compared to primary infection. Perhaps the anti-dengue IgE response—in samples collected between days 5 and 7 of fever of onset observed in this study—is mainly reflecting if it was primary or secondary infection rather than reflecting the clinical picture. These results were similar to those obtained in previous studies [Vazquez et al., 2005, 2007].

During a secondary heterotypic dengue infection, the response of cross-reactive memory T and B cells predominates over the naive specific T and B cells response [Williams et al., 2013]. This fact, could explain the Ig class switch secreted by activated B cells is favoring the responses of anti-dengue IgA and IgE present in the serum of patients early during the second dengue infection.

It would be important to study further dengue virus-specific IgA and IgE to elucidate better its relation to the type of infection and clinical pictures including asymptomatic dengue infected individuals.

Earlier studies have reported on the detection of IgM antibodies to determine the dengue virus infecting serotype during an outbreak [Chungue et al., 1989; Nawa et al., 2000; Delgado et al., 2002; Vazquez et al., 2005, 2007]. MAC-ELISA allowed the identification of the dengue virus infecting serotype in groups with PDF or SDF. It was not possible to identify the specific serotype in secondary DHF, probably due to the low levels of IgM antibody detected. Similar results were found in a previous study [Delgado et al., 2002; Vazquez et al., 2005]. This method could be an alternative way to identify the virus in an outbreak in those laboratories without the virological or molecular methods. However, this method is only useful in those places where only one dengue serotype is circulating. In a previous study [Delgado et al., 2002] it was found that when more than one serotype is present at the same time it is

not possible to establish differences between IgM serotypes.

This is the first study on dengue cases in Salvadorian children related to the immune response of different immunoglobulins towards the type of infection and the clinical picture. It is important to point out that the results of the present work showed (1) a similar relation of higher IgM levels as a response in PDF and SDF cases and (2) a lower IgM response in SDHF cases; (3) there is no relationship between the level of IgA and the illness severity; (4) but there is in secondary cases and (5) also the day of collection of samples is important; (6) the IgE response mainly reflects if the infection is either primary or secondary rather than the clinical picture and (7) the use of MAC-ELISA to define the circulating serotype.

The results reported in the present study are comparable with those from other patients in different epidemiological conditions, with differences in ethnicity and different age.

It is important to know more about the epidemiological aspects of dengue in El Salvador, which could bring new knowledge concerning the pathogenesis of the disease, as well as the use of serological and virological markers related to the risk of the severe forms of dengue that could improve diagnostics and/or prognosis of the disease for better patient management.

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