transplacentally transferred maternal-infant antibodies to dengue virus

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Abstract. Antibodies of all four dengue virus serotypes were detected by hemagglutination inhibition (HI) in 97% of 2,000 infants’ cord sera at the time of delivery. In comparison with 250 mother–infant’s paired sera, we found that 53% of the infants’ serum HI titers were higher than those of the mother’s. The mother/infant IgG subclasses 1, 2, 3, and 4 titers were 53.1/87.0, 8.4/11.7, 0.14/0.11, and 1.1/1.0 mg/dL, respectively. In 18 months of follow-up of 100 infants studied, we observed that antibody to dengue virus disappeared in 3% by two months of age, in 19% by four months of age, in 72% by six months of age, in 99% by nine months of age, and in 100% by 12 months of age, with a half-life of 41 days. We conclude that the antibodies to dengue virus disappeared in the first year of life. We suggest that the most appropriate age for vaccination with a live-attenuated dengue vaccine in an endemic area is one year of age.

Introduction

Dengue hemorrhagic fever (DHF) is an acute, potentially life-threatening, capillary leak syndrome caused by the dengue viruses. Dengue viruses are composed of four distinct serotypes: dengue 1, 2, 3, and 4 are the most prevalent flavivirus infections of humans.1,2 Frequent epidemics occur in the tropical and subtropical areas of Asia, the Pacific Rim, and the Americas. Thailand has a half-century of continuous dengue virus transmission and human disease, with a particularly large epidemic in 1998 (incidence = 201.4 cases per 100,000).3 Dengue hemorrhagic fever as a distinct clinical entity was first described in Southeast Asia during the 1950s. The pathogenesis of DHF is not clearly understood. Early observations that infants less than 12 months of age in Bangkok, Thailand infected with dengue viruses were at high risk for DHF if maternal antibodies to dengue virus were present at subneutralizing levels led to the theory that DHF is caused by antibody enhancement of viral infection.4–6 Virus enhancement occurs when subneutralizing antibody to dengue virus is present, either from a previous dengue virus infection or from maternal placentally transferred antibody that cross-links virus and Fc receptor-bearing cells such as macrophages and dendritic cells.7 This cross-linking subsequently enhances virus uptake and results in immune activation leading to reversible tissue damage and plasma leakage. Currently, little is known about the subclass isotype patterns of dengue-enhancing antibody or its specificity for the dengue virus structural and non-structural proteins.

The role of IgG antibodies in various viral infections depends on their subclass types. For example, IgG3 levels increase more rapidly than IgG1 levels during an acute phase viral infection.8 In infection with human immunodeficiency virus (HIV), HIV-specific antibody-dependent cellular cytotoxicity is mediated mainly by IgG1. IgG1 is stored by memory-plasma cells in the bone marrow during the convalescent period of viral infections.9,10 After vaccination or during infection with the bacteria Neisseria meningitidis, IgG2 exhibited the greatest neutralizing activity and bactericidal activity of all the IgG subclasses.11,12 Previous studies have determined transplacentally transferred antibody half-lives for a variety of viruses. Maternal antibodies disappeared from infant circulation as early as six months for respiratory syncytial virus, 10–12 months for varicella zoster virus, and from four months to years for HIV.13–15

The purpose of this study was to assess the kinetics of maternally-acquired antibody to dengue virus in infants and to further characterize the type of transferred maternal antibody in terms of IgG subclasses and its specificity to the dengue virus structural and non-structural proteins. The information from this study is needed to better understand the pathogenesis of DHF, as well as to determine the optimal age for routine dengue vaccination with a live-attenuated dengue vaccine.

Materials and Methods

Study site and specimen collection. Phramongkutklao Hospital is a 1,200-bed government-run tertiary care center that serves both the military and civilian populations in the greater Bangkok, Thailand urban area. Approximately 350 deliveries are performed per month in the Department of Obstetrics. Two thousand pregnant women with uncomplicated pregnancies gave informed consent to have their own venous and umbilical cord blood specimens collected from March 1998 to October 1999. A subset of 250 mothers and their infants were enrolled by simple random sampling using a random number table to compare the degree of dengue-specific antibody transfer and to characterize maternal antibodies. This was also the estimated number for having 100 mothers and their infants consent to further follow-up study with serial serum sampling up to 18 months of age. All infants’ serum samples at the age of disappearance of hemagglutination inhibition (HI) antibody titer were confirmed by dengue 50% plaque-reduction neutralizing titer (PRNT50). Children in the follow-up period did not receive vaccination with the Japanese encephalitis B vaccine until completion of the study. Serum samples were stored in aliquots at −70°C until analysis.

The study was reviewed approved by the Ethical Review Subcommittee of the Phramongkutklao Hospital (Bangkok, Thailand) and the Human Subjects Research Review Board of the Office of the U.S. Army Surgeon General (Washington, D.C.).
ton, DC). Written informed consent was obtained from parents or guardians.

**Hemagglutination inhibition.** The HI titers to all four dengue virus serotypes were determined at the Department of Virology, Armed Forces Research Institute of Medical Sciences (Bangkok, Thailand). The HI serologic analysis was performed according to the method of Clarke and Casals as modified for the microtiter system and previously described. Briefly, all sera were extracted with acetone and absorbed with goat erythrocytes before testing. The antigens used were sucrose- and acetone-extracted pooled infected suckling mouse brains. The virus strains and suckling mouse passage levels used as routine antigens were dengue type 1, Hawaii strain, passage 23; dengue type 2, New Guinea C strain, passage 38; dengue type 3, H87 strain, passage 38; dengue type 4, H241 strain, passage 38; and a Japanese encephalitis strain, passage 20. An HI titer ≥ 1:10 to at least one serotype was considered seropositive and a titer < 1:10 to all four serotypes was considered seronegative.

**Plaque-reduction neutralization titer.** The PRNT<sub>50</sub> uses prototype dengue seed viruses, monkey sera controls, LLC-MK2 cell lines, and an agar overlay media with neutral red staining as previously described. A probit analysis was performed to measure plaque reduction and titer was reported as the titer required to reduce dengue viral plaques by 50% (PRNT<sub>50</sub>).

**Dengue virus-specific IgG subclass assay.** Human IgG isotype assays using dengue virus types 1, 2, 3, and 4 were modified from a protocol provided by Dr. Kent Kester (Department of Immunology, Walter Reed Army Institute of Research, Silver Spring, MD). Briefly, Immunun-2, flat-bottom, 96-well plates (Dynatech Laboratories, Chantilly, VA) were coated with a 1:400 dilution of goat anti-mouse IgG in bicarbonate buffer, pH 9.0, and kept at 4°C overnight. The plates were washed with phosphate-buffered saline (PBS) and filled with 350 μL of 1% bovine serum albumin (BSA) in PBS, pH 7.4. A second antibody, 50 μL of a 1:1000 dilution of mouse monoclonal antibody to the flavivirus group (4G2), was added in 0.5% BSA in PBS, pH 7.4, and incubated at room temperature for two hours. Pooled dengue virus antigen consisting of 50 μL of 16 hemagglutination units of polyvalent dengue virus type 1, 2, 3, and 4 were diluted in 0.5% BSA in PBS, pH 7.4, added to each well, and incubated overnight at 4°C. The test sample, 50 μL (two wells per sample) at a 1:100 dilution in 0.5% BSA in PBS, pH 7.4, was added and incubated at room temperature for two hours. Standard curves were generated for each specific IgG isotype using the following subclass standards (Binding Site, San Diego, CA): IgG1, 4 μg/mL; IgG2, 4 μg/mL; IgG3, 4 μg/mL; and IgG4, 25 μg/mL. Fifty microliters of 0.5% BSA in PBS, pH 7.4, was added to the last two wells as a negative control. Fifty microliters of a specified subclass of either horseradish peroxidase (HRP)–labeled anti-IgG1 at a 1:500 dilution, HRP-labeled anti-IgG2 at a 1:600 dilution, HRP-labeled anti-IgG3 at a 1:2,000 dilution, or HRP-labeled anti-IgG4 at a 1:1,500 dilution (all from Binding Site) in 0.5% BSA in PBS, pH 7.4, was added per well and incubated at room temperature for four hours. The wells were then washed nine times with PBS, pH 7.4, 100 μL of peroxidase substrate (1:1 2,2'-azino-di(3-ethyl benzthiazoline sulfonic acid:hydrogen peroxide [ABTS:H₂O₂], Catalog No. 50-2-01; Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well, and the wells were incubated at room temperature for 15–30 minutes. Color development was stopped by adding 100 μL of 1% sodium dodecyl sulfate (SDS) per well and the optical density was read at an absorbance of 414 nm on an automatic plate reader (Titertek, Huntsville, AL).

**Dengue virus-specific Western blot.** A dengue virus-specific Western blot was performed by methods adapted from Churdboonchart and others and Towbin and others. Briefly, pooled prototype dengue viral antigens 1 through 4 (Den-1, Hawaii; Den-2, New Guinea-C; Den-3, Hawaii; Den-4, Hawaii 241) were obtained from both suckling mice brain after sucrose gradient extraction and from an infected C6/36 cell lysate. Pooled dengue antigen was dissolved in 100 μL of Tris-glycine-SDS buffer (catalog no. LC2676; Novex, Inc., San Diego, CA) and 92 μL of PBS. Dengue proteins were subjected to electrophoresis at 20 mA on a 4–20% gradient Tris-glycine gel in an Xcell II Mini-Cell gel electrophoresis system (Novex, Inc.) along with MultiMark standards (Novex, Inc.). The gel was equilibrated in Towbin’s buffer (containing 25 mM Trizma base, 192 mM glycine, and 20% [v/v] methanol). A pre-soaked polyvinylidene fluoride membrane (Novex, Inc.) was placed on the gel, and the gel was placed in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Inc., Hercules, CA) and run at 65 mA for 16 hours. After trans-blotting, the membrane was blocked with blocking buffer (0.5% casein, 0.5% goat serum in PBS, pH 7.4) and cut into strips. The strips were placed into eight-lane incubation trays with diluted (1:500) test sera and rocked on a rocker tray at room temperature for 12 hours. The strips were washed three times with PBS and incubated with alkaline phosphatase–labeled goat anti-human IgG (Bio-Rad, Inc.) for one hour at room temperature. A 5-bromo, 4-chloro, 3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) (Kirkegaard and Perry Laboratories) solution was added for two minutes and the reaction was stopped by washing with distilled water. Blots were read immediately after drying and compared with positive and negative controls that were run simultaneously with all sera. Positive controls contained high titer, pooled human dengue IgG that identified envelope (E) protein, pre-membrane (pre-M) protein, and nonstructural (NS) NS1 and NS3 proteins, monoclonal antibody 4G2 specific for dengue E protein, and monoclonal antibody D2-7E11-1 specific for NS1 protein. Monoclonal antibody D2-7E11-1 was a gift from Dr. Robert Putnak and Jeanne M. Burrous (Walter Reed Army Institute of Research, Silver Spring, MD). Human sera from non-endemic areas and screened for HI antibody to dengue virus were used as negative controls.

**Statistical analysis.** The sample size was calculated to identify differences in dengue antibody prevalence ≥ 1.6% (α = 0.05, prevalence of 85% in a pilot study in 1998). The data were stored in the Epi-Info database (Epidemiologic Information, Version 6; Centers for Disease Control and Prevention, Atlanta, GA) and analyzed by percentage calculation. The reciprocal mean dengue HI titer was calculated from the results of all four dengue virus serotype HI titers. A paired t-test was used to determine significant differences in antibody levels between mothers and their infants. The level of significance was set at 0.05. The half-life of antibody to dengue virus was calculated by plotting the mean antibody titer versus age out to 18 months of age on both linear and logarthm scales. The line of best fit was calculated by exponential regression using Microsoft (Redmond, WA) Excel® 98. The
RESULTS

Seroprevalence of antibody to dengue virus and transplacental transfer. The mean maternal age was 26.4 years (range 15–45 years). Most (79.9%) of the volunteers lived in Bangkok and 1,937 (96.9%) of 2,000 mothers were seropositive for antibody to dengue virus. All mothers greater than 35 years of age gave birth to infants who were seropositive for antibody to dengue virus (Figure 1). Antibodies to dengue virus detected by the HI technique were observed in 93.3%, 93.9%, 95.5%, and 95.1% for dengue virus serotypes 1, 2, 3, and 4, respectively. The degree of transfer of antibody to dengue virus to infants was determined in 250 randomly selected maternal-infant pairs, with 242 (96.8%) pairs seropositive. All of the seropositive mothers transferred antibodies to dengue virus to their babies. We compared the reciprocal mean dengue HI titer between the mothers and the cord sera (Table 1) and found that approximately 53% of the cord sera had a higher titer than those of the maternal sera. Dengue virus-specific IgG subclasses and Western blot. The dengue virus-specific IgG subclass levels for IgG 1, 2, 3, and 4 of the maternal sera were 53.1, 8.4, 0.14, and 1.1 mg/dL, respectively, while those of the cord sera were 87.0, 11.7, 0.11, and 1.0 mg/dL, respectively. A paired t-test showed a significant difference for the dengue-specific IgG subclass 3 levels and no differences for the other subclasses (Table 2). Dengue-specific Western blots were performed on all mother-infant sera for antibody against the structural and nonstructural dengue viral proteins (E, pre-M, NS1, NS3). Antibodies to both structural and nonstructural dengue viral proteins were detected in maternal antibody in infants, and no difference in the frequency of antibody to these proteins were found between mother and infant pairs.

Kinetics of maternal antibody to dengue virus in infants. Of the 242 dengue-seropositive mother-infant pairs, 101 mothers consented for further follow-up study and their infants completed follow-up at 1, 2, 4, 6, 9, 12, 15, and 18 months of age or until seronegative by HI. One infant was excluded from the analysis because of an increasing antibody titer to dengue virus following an acute dengue virus infection while on the study protocol. Three percent of these infants lost their dengue antibody by two months of age, 19% by four months, 72% by six months, 99% by nine months, and 100% by 12 months. Ninety-eight infants’ serum samples after the age of 12 months were available for testing the dengue virus neutralizing activity and all were negative for antibody to dengue virus by PRNT$_{50}$. The half-life of antibody to dengue virus was calculated from the 18 months of follow-up by HI titer in the infants’ sera. The antibody level was plotted against age in months on both linear and logarithm scales and the line of best fit were found as calculated by exponential regression (Figure 2). From this curve, the half-life of the maternal antibody to dengue virus in infants was 41.2 days.

DISCUSSION

Our study has demonstrated the followings. 1) In a country endemic for dengue virus transmission, nearly all pregnant women had detectable levels of antibodies to dengue virus and all who were seropositive for these antibodies transplacentally transferred them to their infants. 2) Maternally derived antibody showed a higher titer in infants, suggesting an active transport of antibody across the placenta. 3) Maternally derived antibody to dengue virus in infants was primarily of the IgG subclass 1 and retained its specificity to both structural and nonstructural dengue viral proteins. 4) The half-life of maternally derived antibody to dengue virus in infants was approximately 42 days, with all infants losing their antibody by 12 months of age.

Previous studies of maternal-fetal transfer of immunoglobulins have demonstrated that all four maternal IgG subclasses were actively and selectively transported across the placenta in an affinity-restricted process and that this process was Fc-
gamma receptor mediated. Less than 10% of the immunoglobulins were synthesized in the fetus. Thus, antibodies to dengue virus detected in the umbilical cord blood were transferred from their mothers and were selected for their Fc-gamma receptor affinity. We demonstrated that the current seroprevalence of antibodies to dengue virus in pregnant Thai women was as high as 97%. This reflected the high amount of circulating dengue virus that is present in Thailand and the large amount of human dengue virus infections that occurred in this population up to the time of the study. The seroprevalence of each dengue virus serotype determined by HI reflected the constant circulation of each dengue virus serotype in Thailand, as well as antibody cross-reactivity among the dengue virus serotypes in this assay.

All mothers who were dengue seropositive transferred specific antibody to their infants, and most cord sera contained higher levels of antibody to dengue virus than in maternal sera, which is consistent with an active transport mechanism across the placenta. Similar findings were reported in one previous study on transplacental antibody to dengue virus, as well as in a study on transplacental measles antibody. Higher concentrations of antibody titer in infants compared with their mothers has not been observed with all pathogens. Higher maternal levels of antibody were found for rubella, HIV and Plasmodium falciparum. All of these data suggest that the transplacental transfer of antibody from mother to infant is selective.

Previous studies of maternal-infant IgG subclasses to various pathogens demonstrated higher levels of IgG1, lower levels of IgG2, and equivalent levels of IgG3 and IgG4 in maternal sera compared with that of their infants. These differences may partly explain the susceptibility of newborns to various pathogens or antigens, such as anti-tetanus toxoid and anti-streptococcal carbohydrate, which bind predominantly to IgG1 and IgG2, respectively. In this study, transfer of dengue-specific IgG1 and IgG2 was found to be more efficient than that of IgG3 and IgG4; however, only IgG3 was shown to be significantly higher in the maternal sera than in the cord sera. Because IgG3 has higher synthetic and catabolic rates than other subclasses, we found that IgG3 could be detected in small amounts in the cord serum. The selectivity of this process could be influenced by the target epitope, rate of metabolization, and antibody avidity and its function.

The study of dengue IgG subclasses and its relationship to dengue disease severity is limited. Observations on the pathogenesis of DHF suggests a high degree of immune activation with extensive complement activation preceding the onset of shock, suggesting a role for complement split products such as C3a or C5a in endothelial damage and plasma leakage. This suggests that dengue IgG subclasses may be an important determinant of disease severity, since IgG1 and IgG3 are known to activate the complement system efficiently whereas IgG2 and IgG4 are poor complement activators. IgG4 may itself inhibit complement activation. Thein and others have demonstrated that acute phase sera in children with DHF have higher levels of dengue-specific IgG1 or IgG3 subclasses compared with children with dengue fever. This suggests that dengue-specific IgG1 may be important in complement activation in DHF and in the production of plasma leakage. Kliks and others demonstrated that subneutralizing levels of maternal antibody can enhance dengue virus replication in
monocytes and suggest a mechanism to explain the development of DHF in primary dengue in these infants.\(^7\) Our current findings suggest that this maternal antibody is selectively transported across the placenta by an Fc receptor-mediated process and is predominantly a complement fixing IgG subclass, IgG1. We have also demonstrated that the characteristics of the antibody in terms of its affinity for the structural and nonstructural dengue proteins are equivalent between mother and child.

While antibody to dengue virus following natural infection is long-lived, our study demonstrates that transplacentally transferred dengue antibody in infants does not last longer than one year after birth.\(^{45–47}\) As a future dengue vaccine may be a live-attenuated tetravalent vaccine and thus neutralized by maternally transferred dengue antibody in infants, our findings suggest that the appropriate age for dengue vaccination in dengue-endemic areas should be after 12 months of age or at nine months with additional doses after 12 months of age.\(^{48}\)

This study characterizes and quantifies maternal-fetal-transferred dengue-specific IgG and its possible role in pathogenesis of dengue hemorrhagic fever. Confirmation of these findings awaits further study and a prospective evaluation of infants and their risks for developing DHF upon their primary dengue infections. The half-life and disappearance of transplacentally transferred maternal-infant antibody to dengue virus increase our basic knowledge on dengue pathogenesis and the optimal age for dengue vaccination.

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