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Immunoglobulin G- and M-Specific Enzyme-Linked Immunosorbent Assay for Detection of Dengue Antibodies

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An enzyme-linked immunosorbent assay for the detection of antibodies to dengue virus is described. This method correlates well with a hemagglutination inhibition technique. The enzyme-linked immunosorbent assay can also be specific for human immunoglobulin M antibodies when a μ-chain-specific antiglobulin-enzyme conjugate and fractionated serum are employed. By using this technique, dengue immunoglobulin M antibodies were demonstrated in an infant suspected of having a recent dengue infection.

Dengue virus is a mosquito-borne virus belonging to the flavivirus group (6). There are four dengue virus serotypes, and these share antigenic characteristics with other members of the flavivirus group which include St. Louis encephalitis virus and yellow fever virus.

Infections caused by any one of the dengue serotypes can result in a self-limiting, febrile illness which is often referred to as dengue fever or breakbone fever. Dengue virus infections occur commonly in subtropical and tropical regions where *Aedes aegypti* vectors exist. The epidemiology of dengue infections in the Caribbean area was reviewed by Ehrenkrantz et al. (4). In recent years, major outbreaks of dengue infections were reported throughout the islands of the Caribbean and in several northern countries of South America (11).

Various serological tests have been used to detect dengue antibodies, and they include hemagglutination inhibition (HI), complement fixation, and neutralization tests. These tests are of diagnostic importance if one can demonstrate a seroconversion of fourfold rise in antibody titer between acute and convalescent serum samples (9).

Recently, an enzyme-linked immunosorbent assay (ELISA) procedure has been developed for the serological diagnosis of various infectious diseases (5, 13). This assay is proving to be a simple, rapid, and reliable means for detecting humoral antibodies to disease agents. Additionally, immunoglobulin M (IgM)-type antibodies can be detected when a specific anti-IgM conjugate is used.

We report the development of an ELISA system for the detection of dengue antibodies. By using specific immunoglobulin conjugates, we were able to study the serological response in a mother-infant pair.

MATERIALS AND METHODS

Sera. Samples of serum were obtained from specimens submitted to the Viral Diagnostic Laboratory, Jackson Memorial Hospital, Miami, Fla., and from samples obtained from individuals in the Dominican Republic. All sera were maintained at −20°C. Several samples were fractionated by applying 0.10 ml to a Sephadex G-200 column (1 by 30 cm). The serum was eluted with 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0)-0.3 M NaCl in 1-ml fractions. The absorbance at 280 nm was determined for each fraction as an estimate of relative protein content.

Serological procedure. The HI test for dengue was conducted by the method of Clarke and Casals (2), adapted for use in a microtiter system.

Antigens. The following virus serotypes were used: dengue 2 (TR 1751-Trinidad) obtained from the Trinidad Regional Virus Laboratory and dengue 3 (Puerto Rico) obtained from Philip Russell (Walter Reed Army Medical Center, Washington, D.C.). Antigens for the HI test were prepared by a sucrose-acetone extraction of infected mouse brain as described by Clarke and Casals (2). Antigens for the ELISA tests were prepared by fractionation of 1.0 ml of the HI antigen by rate zonal centrifugation in a 5 to 25% sucrose gradient (1). Fractions that contained antigen as determined by hemagglutination were pooled and then dialyzed against the buffer used for coating the microtiter plates (see ELISA procedure). The dialyzed antigen material was stored at −20°C. Antigen controls for the ELISA test were prepared from uninfected mouse brain.

Reagents. Two enzyme-labeled immunoglobulin solutions containing immunospecifically purified goat anti-human immunoglobulin labeled with calf intestine alkaline phosphatase were obtained from Cordis Laboratory, Miami, Fla. One preparation of enzyme-labeled reagent was a mixture of anti-IgG and anti-

IgM (polyspecific conjugate), whereas the other preparation was anti-IgM. The reagents contained 0.0025 M tris(hydroxymethyl)aminomethane–0.2% bovine albumin–1.0 mM magnesium chloride–0.15 M sodium chloride–0.03 M sodium phosphate and were preserved with 0.02% sodium azide (pH 7.4 to 8.0).

The *p*-nitrophenyl phosphate solution (Sigma 104 phosphate substrate) consisted of 1 mg of the disodium salt per ml dissolved in 10% diethanolamine buffer, pH 9.8 (17).

ELISA procedure. The ELISA procedure used for the detection of dengue antibodies was a modification of the indirect technique described by Voller et al. (17). Polystyrene microtiter plate wells (Cooke Laboratory Products, Alexandria, Va.) were first coated with antigen by incubation of 0.25 ml of each antigen preparation diluted in carbonate-bicarbonate buffer (0.05 M, pH 9.6) in the microtiter plate for 24 h at 4°C. The plates were stored at 4°C for not longer than 2 weeks. Before use, the plates were washed three times with phosphate-buffered saline–Tween 20 solution (PBS–Tween), 0.05 M phosphate (pH 7.4), 0.15 M NaCl, and 0.02% Tween 20. Each wash was in contact with the wells for 3 min and then was removed by inverting the plate. Patients' sera were diluted in PBS–Tween containing 4% bovine serum albumin to a final dilution of 1:100 or greater. A 0.25-ml sample was added to each well and incubated at room temperature for 2 h. The plates were then washed as before, and 0.25 ml of anti-human immunoglobulin conjugated to alkaline phosphatase diluted in PBS–Tween–4% albumin was added to each well. This mixture was incubated at room temperature for 2 h and then washed as before. To detect the bound enzyme activity, *p*-nitrophenyl phosphate solution was added to each well and incubated for 1 h at room temperature. The reaction was stopped by adding 0.10 ml of 1.5 M NaOH, and the absorbance was read in a spectrophotometer at 405 nm. The ELISA value reported was the difference between the mean of triplicate determinations of wells containing dengue antigen and the mouse brain control. Use of direct visual interpretation of the wells was not studied; however, it was noted that most positive sera could be identified in this manner.

Optimum antigen and conjugate concentrations. Optimal reagent concentrations were determined by the method of Voller et al. (17). The optimum antigen concentration was determined with a polyspecific conjugate. The optimum antigen concentration determined, generally a 1:20 dilution, was used in assays involving either the polyspecific or anti-IgM conjugate.

The optimal conjugate dilutions were determined by using microtiter wells coated with 0.25 ml of either 100 ng of human gamma globulin per ml or 100 ng of human IgM (Kallestad Inc., Chaska, Minn.) per ml. Diluted conjugate was added to these wells, and after 3 h of incubation at room temperature the plate was washed and then assayed for bound enzyme activity. The conjugate dilution giving an absorbance value of approximately 1.0 was used for all subsequent tests.

RESULTS

Preliminary studies demonstrated that the dengue ELISA could distinguish between HI-negative control sera and dengue-positive sera. Plots of the ELISA absorbance versus the serum dilutions tended to flatten out at low and high serum dilutions but had linear central portions. Leinikki et al. (8) had reported that derived data from the linear portion of such a curve can be used as a method of quantitation of results. A serum dilution of 1:200 was used in all subsequent testing because the resulting ELISA absorbance fell in the linear range of our assay system and, therefore, could be used to quantitate the results. Additionally, the background absorbance values at this dilution were minimal.

A comparison of the dengue ELISA test with a polyspecific immunoglobulin conjugate and the HI test is shown in Fig. 1. The sera used were from an area endemic for both dengue serotypes 2 and 3 (16). Such sera are known to be highly cross-reactive in the HI test. Therefore, the ELISA was run with both serotypes, since most of the serum samples had HI titers to both. These data demonstrate that the ELISA absorbance values increase as the HI titer increases for both of the serotypes tested. The correlation coefficients obtained from a linear

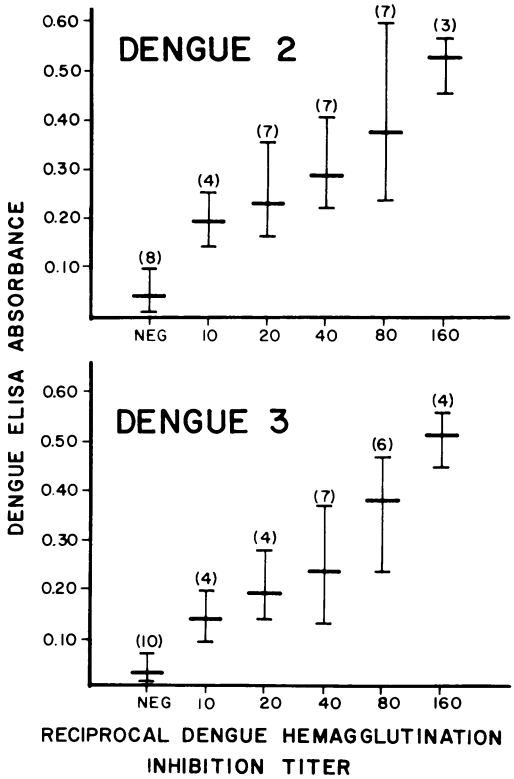

FIG. 1. *Comparison of the dengue ELISA and the HI test with dengue types 2 and 3. The mean ELISA values (horizontal bars) and the range (vertical bars) are expressed; number of samples is in parentheses.*

regression analysis were $r = 0.73$ for the dengue 2 antigen and $r = 0.86$ for the dengue 3. Serum samples that were negative by HI gave absorbance values less than 0.1. All positive HI sera had absorbance values greater than 0.1. The coefficient of variation of the ELISA absorbance of a strong positive sample assayed on 5 different days was 14%.

Consistent with data obtained by the HI test, the ELISA system with the polyspecific immunoglobulin conjugate is unable to distinguish between antibody types. However, the ELISA system has the potential to detect specific immunoglobulin types. The specificity of our goat anti-human IgM conjugate (μ-chain specific) was determined in the ELISA system by using purified IgG and IgM as the antigen (Fig. 2). At various conjugate dilutions, the absorbance values with the IgG antigen were insignificant when compared to the high absorbance value found with the IgM-specific antigen. A 1:20 dilution of this conjugate was used in subsequent assays because it gave an approximate absorbance value of 1.0.

When the IgM-specific conjugate was used to assay sera for the presence of dengue IgM antibodies, there was a high degree of nonspecific conjugate binding to the negative control serum sample. The high background recordings were serum related; if sera were added to uninfected mouse brain tissue, the background readings were high. When saline was substituted for sera in this control, the background reading was markedly reduced. We were able to remove the nonspecific factor in the serum which interfered with the assay by fractionating patients' sera on a Sephadex G-200 column. The presence of dengue IgM antibodies was determined on serial samples from a child suspected of having a dengue infection within 4 months of birth (Fig. 3).

The HI titers were also determined on the infant's serum and on serum obtained from the mother at parturition. The maternal serum was negative for dengue 2 antibodies, whereas the cord sample had a titer of 1:40. Although the HI test on the mother's serum was negative, an ELISA test with the polyspecific conjugate gave an absorbance value of 0.2. This value is within the positive range of the assay (Fig. 1), which indicated to us that the dengue HI titer found in the cord sample is of maternal origin. Previous investigators have found that cord titers for dengue antibodies are often higher than the corresponding maternal titer (16). At 4 months of age the infant's serum had an HI titer of 1:160, which suggested a recent infection with dengue virus. A serum sample obtained at 8 months had an HI titer of 1:80.

When the ELISA with a polyspecific conjugate was used to examine the infant's sera, all specimens had absorbance values that corresponded to their respective HI titer. When an ELISA with a μ-chain-specific conjugate was used to assay Sephadex G-200-fractionated sera, the cord sample had no detectable IgM. Several fractions of the 4-month sample contained significant levels of dengue IgM. These levels were correspondingly reduced in the 8-month sample.

DISCUSSION

Of the four dengue serotypes, types 1, 2, and 3 are known to be present in the Caribbean area

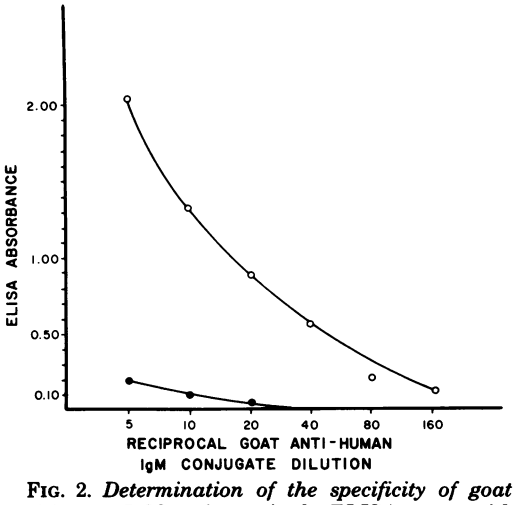

FIG. 2. *Determination of the specificity of goat anti-human IgM conjugate in the ELISA system with either* (●) *IgG or* (○) *IgM.*

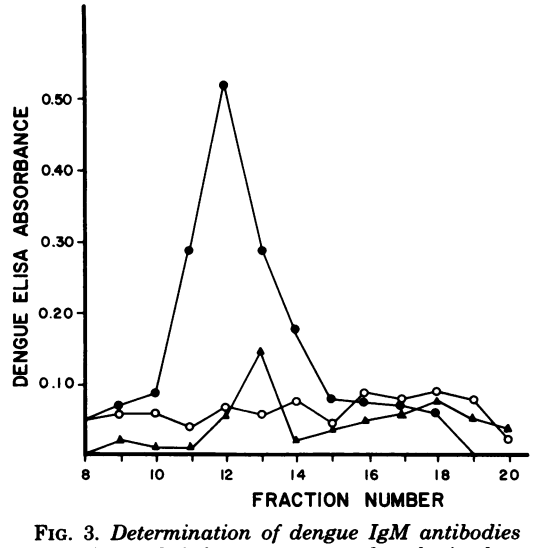

FIG. 3. *Determination of dengue IgM antibodies on fractionated, infant serum samples obtained at birth* (○) *4 months* (●) *and 8 months* (▲).

(11). Infections caused by one of the serotypes does not confer lasting immunity to infection by other serotypes. A reinfection by a different serotype is possible 2 months after a primary attack (4). This situation often complicates serological studies with the HI test, due to the presence of cross-reactive IgG-type antibodies in most individuals in these areas, including infants.

The detection of IgM-type antibodies might be a valuable aid to the interpretation of these sera or sera from other patients. After a primary flavivirus infection in humans or other primates, IgM antibodies are initially produced and then followed by IgG antibodies. A second infection with a heterologous serotype results mainly in the production of IgG antibodies which are highly cross-reactive. The IgM response is usually low or sometimes absent (3). Serodiagnosis of most patients by the IgG response requires the use of paired acute-convalescent serum to obtain meaningful results. Detection of IgM antibodies, on the other hand, could provide information from a single serum sample (10). Since the IgM antibodies are produced first and are of short duration, their presence is the earliest serological evidence of an ongoing infection. Additionally, the IgM antibodies show little cross-reactivity with other dengue serotypes (7, 12). The clinical application of these advantages, however, still awaits further testing.

The first studies involving the dengue ELISA were done to determine whether it would be a feasible alternative to the HI test for the detection of dengue antibodies. The data clearly indicate that dengue antibodies can be detected in whole serum, even when the HI titers are low. The correlation with the HI procedure indicates that the mean ELISA values increase as the HI titer increases.

The ELISA offers several advantages over the HI test. First, the ELISA can use whole serum, except for detection of IgM antibodies, whereas the HI test requires serum that has been extracted with acetone or kaolin and then absorbed with goose erythrocytes to remove nonspecific inhibitors. The ELISA is also more sensitive than the HI test, since a serum sample with an HI titer of 1:10 still gave a positive ELISA value at a 1:200 dilution.

The flavivirus serotype specificity is an area that needs further evaluation. The ELISA correlated well with the HI assay when sera that were cross-reactive in the HI assay were used. The serum from the infant in the IgM studies also gave HI and ELISA with the polyspecific conjugate results that closely correlated. This suggests that the ELISA presently has a cross-reactivity for dengue serotypes similar to that of the HI assay. It might be possible to make the ELISA more type specific if further purification of the antigen material is done. Trent (14) reported that a difference between dengue serotypes can be demonstrated when purified DEN-2gp58 is used in a solid-phase radioimmunoassay system.

The ability of the ELISA to be made immunoglobulin type specific made it an attractive method for detection of dengue IgM antibodies. As suggested earlier, the detection of dengue IgM antibodies might provide information from a single serum sample. In our study of the infant serum samples, the appearance of IgM antibodies at 4 months was distinct. However, the HI titer and the ELISA value when using a polyspecific conjugate on these samples were more difficult to evaluate, since both methods also demonstrated antibodies in the cord serum. Additionally, convalescent serum taken from an adult individual who had traveled to an endemic dengue area was also found to contain IgM antibodies to dengue 2 (data not shown). This serum was obtained within 1 month after the onset of symptoms clinically diagnosed as a dengue infection. The HI titer was 1:20 for the acute serum and 1:1,280 for the convalescent serum. Although this is a significant rise in titer, there were still detectable levels of antibody in the acute serum. We were not able to detect dengue IgM antibodies in the acute serum. The value of dengue IgM determinations, therefore, might be to support data obtained from dengue IgG determinations, especially if only one serum sample is available.

Most existing methods, such as HI, do not distinguish immunoglobulin type and can only detect IgM antibodies after separation from other immunoglobulins. Immunofluorescent antibody technique has been reported to detect specific IgM antibodies in whole serum. Vathanophas et al. (15) detected dengue IgM antibodies by immunofluorescent antibody technique, but the fluorescence was often weak and the assay was not dengue serotype specific. We are presently investigating several ways to improve the ELISA to permit detection of IgM-type antibodies in whole serum.

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