Use of Four Dengue Virus Antigens for Determination of Dengue Immune Status by Enzyme-Linked Immunosorbent Assay of Immunoglobulin G Avidity

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We used an enzyme-linked immunosorbent assay (ELISA) of immunoglobulin G avidity to determine the dengue immune status of 105 pairs of serum samples from patients infected with dengue virus. This study shows that a simple avidity test, for which only one acute-phase serum sample is required, is potentially more useful than the hemagglutination inhibition test for the discrimination of primary from secondary dengue virus infection, whatever the type of dengue antigen used.

Dengue is a mosquito-borne viral disease in humans. It occurs in tropical areas and affects up to 100 million people each year. Initial infection with one of the four serotypes of dengue virus may lead to a self-limiting, febrile illness: dengue fever. In some cases, the disease may be associated with more severe manifestations, such as dengue hemorrhagic fever (DHF) and/or dengue shock syndrome. The pathogenesis of DHF is unclear, but it is thought that secondary infection with a different serotype in patients with heterologous dengue antibodies increases the risk of DHF (9, 10). Immune enhancement by primary infection has been proposed as an explanation for this observation (7, 8, 10). If this antibody-dependent enhancement mechanism is confirmed, it could be important for the establishment of an early diagnostic test to distinguish primary from secondary infection in patients infected with dengue virus. The hemagglutination inhibition (HI) test is used to discriminate between primary and secondary dengue virus infections (18). However, this serological test cannot provide an early diagnosis and requires paired serum samples. An enzyme-linked immunosorbent assay (ELISA) based on the immunoglobulin M (IgM)/IgG ratio has been proposed to circumvent these limitations (13). Nevertheless, as IgM persists for more than 8 months, this test may lead to errors in interpretation of the immunological status of the patient (2, 3).

IgG avidity tests have been shown to be useful for distinguishing primary from chronic or recurrent infections for a few infectious diseases (6, 11, 12, 17), including dengue virus infection (5). We previously demonstrated that the dengue IgG avidity index, determined from a single acute-phase serum sample, discriminates between primary and secondary dengue virus infections (15). We developed this test with the DEN-2 antigen, prepared by the sucrose acetone method (4). Here we extended this test for use in any laboratory using one of the four types of dengue antigen by carrying out the previously described dengue IgG avidity test (15) with all four dengue antigens (DEN-1 to DEN-4) simultaneously.

Sera from the collection of the Centre National de Référence des Arbovirus from the Institut Pasteur de la Guyane were used for this study. We tested 105 pairs of serum specimens from patients infected with the DEN-3 serotype. All sera were collected from patients living in French Guiana with basic clinical symptoms of dengue (fever, headache, myalgia, arthralgia) associated or not with rash and minor hemorrhagic manifestations. A first serum sample was collected during the acute phase (day 1 to day 5), with the first day of fever considered the first day of the disease. Dengue virus infection was diagnosed virologically, based on the acute-phase serum samples, by means of culture from AP 61 mosquito cells (Aedes pseudocutellaris) or reverse transcription-PCR by routine methods (14, 16). A second sample was collected during the convalescent phase, which was 7 days or more after the onset of fever. The HI test was also performed with all of the serum samples analyzed by using our IgG avidity test. According to the HI test criteria (18), 59 pairs of serum specimens were classified as having primary dengue virus infection and 46 were classified as having secondary dengue virus infection. We then compared the IgG avidity index for each type of dengue antigen (DEN-1 to DEN-4) by testing 105 pairs of serum samples. Using the DEN-1 antigen, we assessed the levels of avidity of 27 pairs of

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Mean AI* for primary dengue virus infection (n)</th>
<th>Mean AI* for secondary dengue virus infection (n)</th>
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</thead>
<tbody>
<tr>
<td>DEN-1</td>
<td>24.9 (180)</td>
<td>76.2 (144)</td>
</tr>
<tr>
<td>DEN-2</td>
<td>25.2 (192)</td>
<td>68.7 (144)</td>
</tr>
<tr>
<td>DEN-3</td>
<td>30.9 (168)</td>
<td>82 (132)</td>
</tr>
<tr>
<td>DEN-4</td>
<td>29.9 (168)</td>
<td>73 (132)</td>
</tr>
</tbody>
</table>

* AI, avidity index.
** n, number of avidity measures.
serum samples (15 primary infections and 12 secondary infections). In the same way, using the DEN-2 antigen, we assessed 28 pairs of serum samples (16 with primary infections and 12 with secondary infections). Using the DEN-3 and DEN-4 antigens, we tested 14 pairs of serum samples from primary infections and 11 pairs of serum samples from secondary infections, respectively. Six measures of avidity were performed with each serum sample for each antigen used.

The IgG avidity test was performed with 105 pairs of serum samples, as described previously (15). The sera were first diluted (1:100) in phosphate-buffered saline (PBS) supplemented with 0.1% Tween 20 and 5% nonfat milk powder (the reagents were from Sigma Laboratories l'Isle d'Abeau Chesnes, France) and then added to two separate microplates previously coated with one of the four dengue antigens (DEN-1, DEN-2, DEN-3, or DEN-4). The plates were incubated for 2 h at 37°C. One of the plates was washed with 0.1% Tween 20 (PBS-T) in PBS, and the other was washed with 8 M urea in PBS-T (Sigma Laboratories). Bound IgG was detected at 650 nm ($A_{650}$), and an avidity index corresponding to ($A_{650}$ assay with urea/$A_{650}$ assay without urea) × 100 was calculated for each sample. Normal mouse brain antigen was used as a control in all tests. Sera from patients being vaccinated against yellow fever (six serum samples from patients vaccinated against yellow fever with no history of dengue virus infection) were included as a negative control to rule out the possibility of cross-reaction with other flaviviruses, such as the virus that causes yellow fever (1).

In a first step, we compared for each dengue antigen the levels of avidity of the antibodies according to the patient’s immune status using the Student’s $t$ test. Thus, in order to prove that the level of avidity is related for each patient to the day that blood was drawn and to the immune status, we performed a covariance analysis (15). The avidity was significantly higher ($P < 0.001$) for secondary dengue virus infections than for primary dengue virus infections for the four antigens tested (Table 1). Analysis of covariance further showed that the avidity increased significantly ($P < 0.001$) in relation to the time that blood was drawn and was significantly higher ($P < 0.001$) for secondary dengue virus infections than for primary dengue virus infections (Fig. 1). Thus, discriminatory analysis is possible regardless of the dengue antigen used for the determination of avidity index.

In conclusion, this study shows that a simple avidity test, for which only one acute-phase serum sample is required, is potentially more useful than the HI test for the discrimination of primary from secondary dengue virus infection, whatever the type of dengue antigen used. It is our expectation that such an analysis technique could become widely distributed.

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REFERENCES