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Immunoglobulin A-Specific Capture Enzyme-Linked Immunosorbent Assay for Diagnosis of Dengue Fever

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Dengue fever (DF) is usually diagnosed by testing for dengue virus immunoglobulin M (IgM) by a capture enzyme-linked immunosorbent assay (ELISA) (MAC-ELISA). However, IgM can last for months, and its presence might reflect a previous infection. We have tested the use of anti-dengue virus IgA capture ELISA (AAC-ELISA) for the diagnosis of DF by comparing the results of MAC-ELISAs and AAC-ELISAs for 178 serum samples taken from patients with confirmed cases of DF. IgM appears more rapidly (mean delay of positivity, 3.8 days after the onset of DF) than IgA (4.6 days) but lasts longer; the peak IgA titer is obtained on day 8. The specificity and the positive predictive value of AAC-ELISA are 100%; its sensitivity and negative predictive value (NPV) are also 100% between days 6 and 25 after the onset of DF, but they decrease drastically when data for tests conducted with specimens from the first days of infection are included, because the IgA titers, like the IgM titers, have not yet risen. AAC-ELISA is a simple method that can be performed together with MAC-ELISA and that can help in interprating DF serology.

Dengue fever (DF) is a tropical mosquito-borne viral infection caused by four serotypes, dengue virus type 1 (DEN-1), DEN-2, DEN-3, and DEN-4. DF is a major public health problem that is responsible for millions of cases of illness and thousands of deaths in tropical countries every year (8). The increasing importance of DF and dengue hemorragic fever in Asia, South America, and the Caribbean (8) underlines the importance of early detection in controlling the spread of the disease. The hemagglutination inhibition assay (HI) has been the reference test for a long time (6), but the simple immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (ELISA) (MAC-ELISA) that is now available allows poorly equipped laboratories to perform confirmatory laboratory diagnoses (2).

The presence of specific IgM to dengue viruses in patients with acute DF is useful for detecting the numbers of recently infected individuals during an epidemic (1). However, IgM can persist for more than 8 months (4, 5). Therefore, in countries where DF is endemic and where many serotypes of dengue viruses are cocirculating, as in southeast Asia and French Guiana, it is sometimes very difficult to interpret a positive result for patients presenting with febrile illness, since the presence of IgM might reflect infection up to 8 months previously.

It was reported that IgA increases at the same time as IgM in patients with DF but persists for a shorter period of time (7). In order to test the usefulness of specific IgA for diagnosing dengue virus infection, we compared the results of MAC-ELISA and IgA capture ELISA (AAC-ELISA) with reference sera from patients confirmed to have DF, sera from patients with febrile illnesses due to other causes, sequential sera from patients confirmed to have DF, and sera sent to our laboratory for the diagnosis of dengue virus infection.

MATERIALS AND METHODS

Sera. (i) Reference sera. Sera from the collection of the Centre National de Référence pour la Surveillance de la Dengue et de la Fièvre Jaune (CNR), Institut Pasteur, Cayenne, French Guiana, were used as reference sera when seroconversion or a fourfold rise in the HI titers was observed between sera from the acute phase and sera from the recovery phase and when dengue virus was detected in an acute-phase sample either by isolation on *Aedes pseudoscutellaris* cell culture (AP 61) or by reverse transcription-PCR by previously described methods routinely used in our laboratory (11, 12). We thus tested 178 serum samples from 80 patients, with 45 serum samples being from patients infected with DEN-1, 123 being from patients infected with DEN-2, and 10 being from patients infected with DEN-4. Thirty-seven patients had primary infections, 25 patients had secondary infections, and the infections of the other patients were unknown on the basis of the criteria established by the World Health Organization (14).

(ii) Sera from patients with other febrile illnesses. Sera taken during the recovery phase from 112 patients who showed no increase in HI titers over that in acute-phase sera were tested. Ten patients were known to be infected with *Coxiella burnetii*, six were known to be infected with Mayaro virus, one was known to have yellow fever, and two were known to be infected with West Nile virus; the causes of infection in the other patients were unknown.

(iii) Sequential sera. Sixty-one sequential serum samples from day 1 or 2 of infection to day 6 or more of infection from 14 patients infected with dengue virus confirmed by virus detection and seroconversion were obtained from a previous study. They were used to evaluate the delay of positivity of the AAC-ELISA.

(iv) Other sera. A total of 442 patient serum samples sent to CNR for the diagnosis of dengue virus infection were tested for the presence of specific IgM and IgA. When paired samples were available, an increase in HI titers was observed, but the virus could not be identified in the acute-phase sample. Four serum samples collected in Tahiti during the DEN-3 epidemic in 1992 were included in the study.

MAC-ELISA and AAC-ELISA. The best dilutions of the different reagents for the MAC-ELISA and the ACC-ELISA were determined in preliminary tests with positive and negative reference sera. The method used was a modification of that described previously (10). Briefly, each well of flat-bottom microplates was coated with 100 μl of either goat anti-human IgA diluted 1:250 in phosphate-buffered saline (PBS) or goat anti-human IgM diluted 1:500 in PBS (both antibodies were from Sigma Laboratories, L'Isle d'Abeau Chesnes, France). The microplates were incubated for 1 h at 37°C and 1 h at 25°C in a humidified box and were washed with PBS containing 0.05% Tween 20 (PBS-T).

The sera were diluted at 1:100 in PBS containing 0.5% Tween 20 and 5% nonfat dried milk (PBS-T-NDM) and dispensed at 100 μl per well in duplicate. Six negative and two positive reference serum samples were included on each plate as controls. The plates were then incubated for 1 h at 37°C, and after washing with PBS-T, 100 μl (16 hemagglutination units) of tetravalent dengue virus antigen or an uninfected control sample diluted in PBS-T-NDM was dispensed into one of a pair of wells. The antigens were prepared by extraction of the antigens from the brains of suckling mice with sucrose-acetone. After incubation at 4°C overnight, the plates were washed with PBS-T, and bound antigens were detected with anti-dengue virus mouse ascitic fluid prepared in our laboratory and diluted 1:10,000 in PBS-T-NDM, followed by the addition of conjugated goat anti-mouse IgG peroxidase (Sigma Laboratories) diluted 1:1,000 in

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TABLE 1. Comparison of the results of AAC-ELISA and MAC-ELISA for detection of dengue virus-positive reference sera

Days after the onset of DF	No. of serum specimens with the following result[a]:							% Agreement
	Total tested	IgA+, IgM+	IgA+, IgM−	IgA±, IgM+	IgA±, IgM−	IgA−, IgM+	IgA−, IgM−	
1–2	51	0	0	0	0	0	51	100
3	19	0	0	0	0	2	17	85
4	24	11	0	4	1	4	4	63
5	12	9	0	1	0	1	1	83
6–19	43	43	0	0	0	0	0	100
20–24	9	9	0	0	0	0	0	100
25–29	10	8	0	2	0	0	0	80
30–39	5	4	0	1	0	0	0	80
>40	5	1	0	0	0	3	1	40
Total	178	85	0	8	1	10	74	96

[a] +, positive; −, negative; ±, indeterminate.

PBS-T-NDM. Each of these two layers was subjected to incubation for 1 h at 37°C followed by washing. Tetramethylbenzidine was used as the substrate.

When positive reference sera appeared blue while the negative reference sera were still clear, 50 μl of 0.5 N sulfuric acid was added to each well to block the reaction. The optical density (OD) at 450 nm was read in an ELISA reader (model LP 300; Sanofi Diagnostics Pasteur, Marnes la Coquette, France).

For the AAC-ELISA, the mean ± standard deviation OD values for the negative controls were determined. A result was considered negative when the OD values were less than the mean value for the negative control plus 2 standard deviations, indeterminate when the OD values exceeded 2 standard deviations but scored less than 3 standard deviations, and positive when the OD values were above 3 standard deviations.

The results for the MAC-ELISA were calculated by dividing the absorbance of the antigen-containing well by the absorbance of the uninfected control well for each specimen, as described previously (3). A ratio of 3 or more was considered positive.

Statistical methods. To validate anti-dengue virus IgA detection as a predictor of acute DF, the sensitivity, specificity, positive and negative predictive values, and accuracy were calculated for reference sera and were compared with the results of MAC-ELISA.

RESULTS

Anti-dengue virus IgA titers were always increased when DF had been confirmed by virus detection and serology (Table 1). As seen with the sequential sera, however, the positivity for anti-dengue virus IgA often occurs 1 day later than that for IgM (Fig. 1). The mean delay for positivity for IgM was 3.8 days after the onset of DF, and the mean delay for positivity for IgA was 4.6 days after the onset of DF. The peak IgA titer occurs on day 8 after the onset of DF, and its decrease is more rapid than that of IgM; after 40 days, IgA is usually not found (Fig. 2). No difference in IgA titers was found between patients with secondary and primary infections or between patients with DF due to DEN-1, DEN-2, or DEN-4. The results of the AAC-ELISA were also positive for four DEN-3-positive serum samples collected on days 7 to 13 after the onset of illness.

The agreement between tests for IgA and IgM was 100% for reference sera taken before day 3 after the onset of the disease and for those collected between days 6 and 25 after the onset of disease. Discrepancies were seen between days 3 and 5 and after day 25 after the onset of disease (Table 1). Sera from most of the patients with infections other than DF and even those from patients recently infected with West Nile and yellow fever viruses gave negative results for dengue virus IgA (Table 2); two serum samples gave indeterminate results. In contrast, three patients with low HI titers were found to be positive for IgM, and for these patients there was no increase in the HI titer between the acute- and the recovery-phase sera. The positive predictive value and the specificity of the AAC-ELISA, determined with reference sera, were 100%, but its sensitivity and its negative predictive value were high only between days 6 and 25 after the onset of disease (Table 2). For sera taken between days 1 and 29 after the onset of disease, the MAC-ELISA was more sensitive and less specific than the AAC-ELISA, but the differences were not significant (Table 2).

For the sera sent to CNR for the diagnosis of DF, the discrepancies between the results of tests for IgM and IgA were greater (Table 3), with many serum samples being dengue virus IgM positive and dengue virus IgA negative. Most of these sera were taken between days 3 and 5 and after day 25 after the onset of fever; however, two serum samples collected between days 6 and 19 after the onset of fever remained IgA negative, whereas a significant rise in the anti-dengue virus HI titers was observed.

DISCUSSION

In our study, the results of the AAC-ELISA were always positive when DF was confirmed, but they were positive about 1 day later than those of MAC-ELISA. The finding that IgA did not appear in two patients presenting with seroconversion to flaviviruses but in whom no virus could be isolated or detected by reverse transcription-PCR did not appear to be due

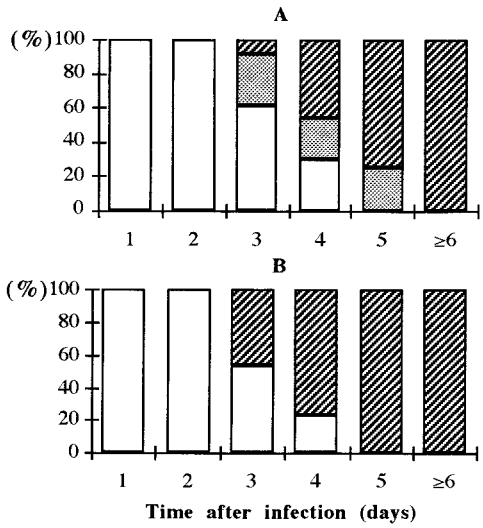

FIG. 1. Percentages of positive (▨), indeterminate (▤), and negative (□) results for AAC-ELISA (A) and MAC-ELISA (B) for 14 patients from whom sequential sera were available.

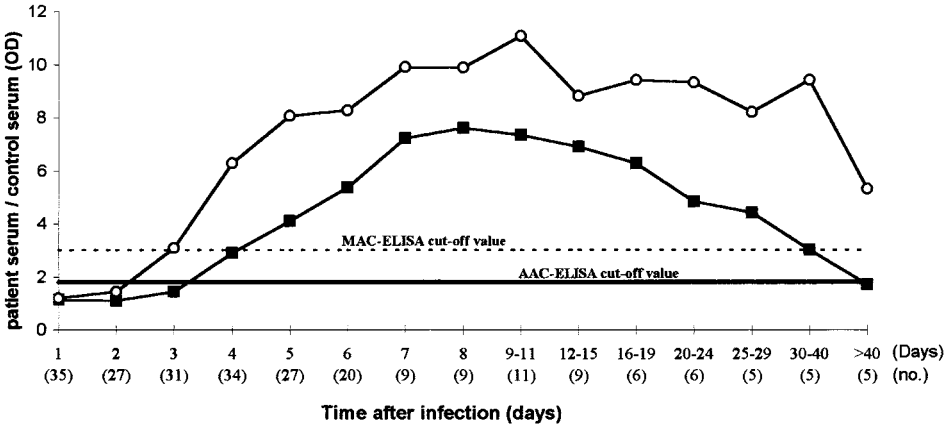

FIG. 2. Evolution of the mean ratios of the absorbance for the well containing the serum specimen divided by the mean of the absorbance values for the negative wells for AAC-ELISA (■) and by the absorbance for the uninfected control well for MAC-ELISA (○) after the onset of DF. The means were determined for 178 reference serum samples and 61 sequential serum samples. Numbers in parentheses represent the numbers of serum samples tested.

to IgA deficiency since the levels of IgA in serum were normal. Therefore, either the seroconversion was due to another flavivirus circulating in the Amazonian forest complex (9) or dengue virus IgA is sometimes not present during DF. Since similar levels of dengue virus IgA were detected in patients with DF due to DEN-1, DEN-2, DEN-3, and DEN-4, the lack of increase in the IgA titer does not seem to be the result of an infection with a particular serotype of dengue virus. Further studies, especially in countries where dengue viruses are the only flaviviruses present, are necessary to answer to this question.

The rapid decrease in the dengue virus IgA titer (about 40 days) is very interesting for countries where many serotypes of dengue viruses cocirculate. In French Guiana in 1996 and 1997, dengue viruses were very active, with an epidemic due to DEN-1 and DEN-2 occurring from September 1996 to April 1997 (13). Individuals could therefore be infected with both viruses within a short period of time. All DF-like syndromes are not necessarily due to dengue viruses, however, and the presence of dengue virus IgM after a febrile syndrome is not always proof of recent infection but may be due to DF that occurred some months previously. For example, two of the three patients positive for dengue virus IgM but for whom a diagnosis of acute DF could be eliminated were known to have had DF five and seven months earlier, respectively. These patients illustrate the limitation of MAC-ELISA alone for diagnosing DF in countries where it is endemic. An indicator of a very recent infection, such as the presence of IgA, is therefore useful. The limit of positivity was chosen in order to eliminate false positivity. By using this cutoff value, the positive predictive value of the AAC-ELISA is excellent, and positivity by this test is proof of recent DF. Although the sensitivity of AAC-ELISA is not very good, it is almost the same as that of MAC-ELISA. The absence of IgA coupled with the presence of IgM is usually due to a delay after onset of DF that is too short. Indeed, in our study IgM antibodies were detected about 1 day sooner than IgA antibodies when sequential sera were tested, even though many trials were performed to improve the sensitivity of the AAC-ELISA. However, a positive MAC-ELISA result in association with a negative AAC-ELISA result at the beginning of a febrile illness can also be due to an older infection. This has been confirmed at least three times with sera from patients with other febrile illnesses. It was also probably the case for sera sent to CNR for the diagnosis of DF, but this is difficult to prove since a recovery-phase sample was not available. Knowledge of the date of the disease's onset and the availability of a recovery-phase serum sample are important in drawing a conclusion. However, when the dengue virus IgA is absent from a blood sample obtained between days 6 and 25 after the onset of the disease, a diagnosis of DF is highly improbable, since the negative predictive value is 100% during this period (Table 2). The presence of IgM coupled with the absence of dengue virus IgA during this period probably indicates an old infection. Indeterminate AAC-ELISA results in association with positive MAC-ELISA results usually reflect DF at a very early or late phase (Fig. 1; Table 1). Assays of

TABLE 2. Results of AAC-ELISA and MAC-ELISA with reference sera and sera from patients with other febrile illnesses

Days after onset	Capture ELISA	No. of samples with the following result:					% Sensitivity (95% CI)[a]	% Specificity (95% CI)	% Positive predictive value (95% CI)	% Negative predictive value (95% CI)	% Accuracy (95% CI)
		Total	True positive	False positive	True negative	False negative					
1–29	IgA	272	80	0	104	88	48 (40–55)	100 (97–100)	100 (96–100)	54 (47–61)	68 (62–73)
	IgM	272	94	3	101	74	56 (48–63)	97 (93–99)	97 (92–99)	58 (50–65)	72 (66–77)
6–25	IgA	132	52	0	80	0	100 (94–100)	100 (96–100)	100 (94–100)	100 (96–100)	100 (97–100)
	IgM	132	52	3	77	0	100 (94–100)	96 (90–99)	94 (82–97)	100 (96–100)	98 (94–99)
All	IgA	290	85	0	112	93	48 (40–55)	100 (97–100)	100 (96–100)	55 (48–61)	68 (63–73)
	IgM	290	103	3	109	75	58 (51–65)	97 (93–99)	97 (93–99)	59 (52–66)	73 (68–78)

[a] 95% CI, 95% confidence interval.

TABLE 3. Results of AAC-ELISA and MAC-ELISA with sera submitted to CNR for diagnosis of DF

Days after the onset of DF	No. of samples with the following result[a]:							% Agreement
	Total tested	IgA+, IgM+	IgA+, IgM−	IgA±, IgM+	IgA±, IgM−	IgA−, IgM+	IgA−, IgM−	
1–2	75	0	0	0	0	5	70	93
3	33	3	0	2	2	3	23	85
4	56	28	0	4	0	5	19	84
5	32	20	0	4	0	4	4	75
6–19	83	70	0	2	0	3	8	94
20–24	29	4	0	2	0	0	23	93
25–29	9	4	0	4	0	1	0	44
30–39	3	2	0	0	0	1	0	67
>40	9	0	0	8	0	0	1	11
Unknown	113	12	0	2	0	11	88	88
Total	442	143	0	28	2	33	236	86

[a] +, positive; −, negative; ±, indeterminate.

sequential sera from 14 patients indicated that at the beginning of DF, when the results of MAC-ELISA are positive, the results of AAC-ELISA are usually positive or indeterminate (Fig. 1).

In conclusion, the presence of dengue virus IgA is a better indicator of dengue virus infection than the presence of IgM on days 6 to 25 after the onset of infection. Since the date of onset is not always known, however, MAC-ELISA remains the best tool for the serological diagnosis of DF. Use of the two assays in conjunction provides useful information for interpreting the results of dengue virus serology. The experimental procedures for AAC-ELISA and MAC-ELISA are quite similar, and the only limiting factor is the doubling of the cost of dengue virus serology.

IgA detection may also be useful for determining the rates of infection during a DF epidemic. Since the IgA titer decreases rapidly, it gives an indication only of the individuals infected during the past month; thus, successive testing can provide an indication of the progression of the epidemic. Dengue virus IgA could be determined in saliva since this immunoglobulin is excreted in large quantities. If reliable, testing for IgA in saliva would be useful in large-scale epidemiological studies and for the diagnosis of DF in babies and children, eliminating the need for venipuncture.

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