CLINICAL AND VACCINE IMMUNOLOGY, Sept. 2006, p. 1044–1051 Vol. 13, No. 9
1556-6811/06/$08.00+0 doi:10.1128/CVI.00105-06

Altered Enzyme-Linked Immunosorbent Assay Immunoglobulin M (IgM)/IgG Optical Density Ratios Can Correctly Classify All Primary or Secondary Dengue Virus Infections 1 Day after the Onset of Symptoms, when All of the Viruses Can Be Isolated

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Received 6 March 2006/Returned for modification 28 April 2006/Accepted 7 July 2006

We compared dengue virus (DV) isolation rates and tested whether acute primary (P) and acute/probable acute secondary (S/PS) DV infections could be correctly classified serologically when the patients' first serum (S1) samples were obtained 1 to 3 days after the onset of symptoms (AOS). DV envelope/membrane protein-specific immunoglobulin M (IgM) capture and IgG capture enzyme-linked immunosorbent assay (ELISA) titrations ($1/\log_{10}$ 1.7 to 1 $\log_{10}$ 6.6 dilutions) were performed on 100 paired S1 and S2 samples from suspected DV infections. The serologically confirmed S/PS infections were divided into six subgroups based on their different IgM and IgG responses. Because of their much greater dynamic ranges, IgG/IgM ELISA titer ratios were more accurate and reliable than IgM/IgG optical density (OD) ratios recorded at a single cutoff dilution for discriminating between P and S/PS infections. However, 62% of these patients' S1 samples were DV IgM and IgG titer negative ($<OD_{max}/2$ titer threshold), and in 35% of the S/PS infections, the patients' S1 and S2 samples were IgM titer negative. The IgM OD values were, however, much higher than those of IgG in the S1 samples of many of these, and the other, S/PS infections. This necessitated using higher ($\geq$2.60 and $<$2.60) discriminatory IgM/IgG OD (DOD) ratios on these S1 samples than those published previously to correctly classify the highest percentage of these P and S/PS infections. The DV isolation rate was highest (12/12; 100%) using IgG and IgM titer-negative S1 samples collected 1 day AOS, when 100% of them were correctly classified as P or S/PS infections using these higher DOD ratios.

The dengue viruses (DVs) are flaviviruses contained within their own antigenic complex of four serotypes defined by neutralization assays, and therefore, patients can encounter sequential (secondary) infections with different DV serotypes. The diagnosis of these viruses generally relies on virus isolation (cell culture) or the detection of viral RNA using reverse transcription-PCR and serological assays (20, 23, 24). DV surveillance programs also require virus isolates for subsequent comparisons with other DV strains (e.g., cDNA sequence determination and phylogenetic analyses). For this purpose, patients' sera must be obtained early in the acute phase of disease before the virus is neutralized by their rising titers of antibody. DV isolation is therefore usually unsuccessful using patients' sera obtained 6 or more days after the onset of symptoms (23). Thus, while DVs can be efficiently isolated from patients' sera collected early after the onset of symptoms, these sera are often DV-specific immunoglobulin M (IgM) and IgG titer negative in serological assays (10, 11, 14, 20, 21, 23). In addition to this first serum (S1) sample, a second serum (S2) sample, obtained 2 to 14 days afterwards, is therefore usually required to confirm a ≥4-fold increase in DV envelope/membrane (E/M) protein-specific titers of IgG or IgM and to classify infections as either acute primary (P) or secondary (S) flavivirus infections by IgG and IgM optical density (OD)/titer comparisons (24). IgM capture (MAC) and IgG capture (GAC) enzyme-linked immunosorbent assays (ELISAs) are now the favored serological assay for this purpose (24), and several different IgM/IgG antibody ratios have been described to discriminate between primary (nonanamnestic antibody responses) and secondary (anamnestic antibody responses) flavivirus infections (11, 14, 19). DV E/M protein-specific IgM/IgG ratios of ≥1.78 and <1.78 were the first to be derived to identify P and S infections, respectively (11). These ratios were, however, performed on IgM and IgG OD values after being converted to "units" that had been established using negative control sera at 1/100 dilutions (11, 21, 22). Subsequently, other workers classified acute primary and secondary flavivirus infections by applying different discriminatory values directly to the IgM/IgG OD ratios (i.e., without converting the OD values to units). In these studies, acute primary and secondary flavivirus infections were identified using DV E/M protein-specific discriminatory IgM/IgG OD ratios of either >1.4 and <1.4, respectively, using patients' sera at 1/20 dilutions (14), or ≥1.2 and <1.2, respectively, using patients' sera at 1/100 dilutions (18, 19, 20).

Since most dengue virus infections are presented at outpatient clinics in the Americas, these patients' infections need to be diagnosed using single serum samples (14), ideally as early

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as days 1 to 3 after the onset of symptoms. While these early acute-phase sera are often IgM and IgG titer negative (10, 11, 14, 23), we compared DV isolation rates for subsequent virological studies (e.g., cDNA sequence determination and phylogenetic comparisons) and the ability to still correctly classify acute primary and secondary flavivirus infections using these different discriminatory IgM/IgG ratios when these patients' S1 samples were obtained on days 1 to 3 after the onset of symptoms.

MATERIALS AND METHODS

Study area. Barranquilla, the capital city of the department (state) of Atlantico, lies at the mouth of the Magdelena River on the Caribbean coast and is the principal seaport of Colombia. Dengue fever (DF) is endemic throughout the year, but the circulating dengue virus serotypes and genotypes were unknown. When these patients' sera were collected (2000 to 2001), no yellow fever vaccination program had been established in the department of Atlantico and DF was the only human disease caused by a flavivirus known to occur in this department.

Sample collection from suspected DF cases. Ethical approval to obtain the human S1 and S2 samples and these patients' clinical and personal details was provided by the Universidad del Norte Ethics Committee following the Colombian National Guidelines. Each patient, or the parents in the case of children or minors, was informed about the purpose of this study and signed a consent form before the blood samples and the patients' details (clinical and personal information, including addresses to acquire the S2 samples) were obtained. For this study, S1 and S2 samples were obtained from 100 patients with suspected DF from days 1 to 3 after the onset of symptoms at outpatient clinics throughout the city of Barranquilla and the adjoining district of Soledad and stored at −80°C.

Dengue virus growth. The growth of DVs in an insect cell line has been described previously (4, 16). To prepare test virus stocks for the immunofluorescence assays (IFAs) and ELISAs, 70% confluent *Aedes albopictus* (C6/36) cells, maintained in 80-cm^2 culture flasks (Costar) with Leibovitz (L-15) medium (cell culture medium) containing 10% (vol/vol) tryptose phosphate broth, 10% (vol/vol) fetal bovine serum, and antibiotics (Sigma), were infected with D-1V (Nauru Island), D-2V (New Guinea C), D-3V (PR-1340), and D-4V (Dominica).

DV isolations were attempted using the S1 samples from all patients with serologically confirmed acute/probable acute flavivirus infections. For this purpose, 100 μl of the patients' S1 samples was added to 2.5 ml of cell culture medium and incubated for 2 h at 28°C in 10-cm^2 flasks (Costar) containing 70% confluent C6/36 cell monolayers. This medium was subsequently collected, 7.5 ml of fresh media was added, and the detached C6/36 cells were reintroduced to the flask after centrifugation at 1,000 × *g* for 10 min and resuspension in 2.5 ml of fresh medium (total volume, 10 ml/flask). These flasks were then incubated for either 5 days (test virus stocks) or 7 days (patients' sera) at 28°C before collection and replacement with fresh media. The harvested media were centrifuged for 10 min at 1,000 × *g*, a 1/10 volume of 1 M Tris-HCl, pH 7.2, was added, and supernatant aliquots were stored at −80°C. The cell pellets were resuspended in a minimum volume of phosphate-buffered saline (PBS), and 10-μl volumes of media were added to each well of 12-well polytetrafluoroethylene-coated immunofluorescent slides (Hendley, United Kingdom). After being air dried, the cell pellets were fixed with cold (−20°C) acetone, air dried, stored at −20°C, and subsequently processed as described below. A second harvest of these cell culture supernatants was also performed on day 8 (test virus stocks) and day 12 (patients' sera).

Growth and purification of anti-DV monoclonal antibodies. The production of mouse monoclonal antibodies (MAbs) to live dengue type 2 (PR159) virus and the preparation of high titers of MAb ascitic fluid have been described previously (4). This work was performed under a personal animal procedures license (no. PIL 70/6903) issued by the Home Office of the United Kingdom.

The flavivirus complex-reactive mouse MAb 4G2 and the D-1V, D-2V, D-3V, and D-4V serotype-specific mouse MAbs 15F3, 3H5, 5D4, and 1H10, respectively, have been described previously (9) and were obtained from the American Type Culture Collection (ATCC).

MAbs 2F2.1 (flavivirus subgroup reactive) and 2C5.1 (flavivirus group reactive) (IgG2a subclass) (4, 5) were immunoaffinity purified using protein A-Sepharose CL-4B (P 3391, Sigma) and eluted using 0.1 M glycine-HCl, pH 3.0, and aliquots were immediately neutralized using a 1/10 volume of 1 M Tris-HCl, pH 7.6, as described previously (4). Antibody concentrations were determined using standard concentrations of purified mouse IgG (I 5381; Sigma) with a bicinchoninic acid protein assay (Pierce) in ELISA plates, and after a 60-min incubation at 37°C, the ODs were determined at 570 nm (MRX; Dynex).

IFAs. The processing of IFAs using flavivirus complex-reactive and DV serotype-specific MAbs was described previously (16). Dried IFA slides were rehydrated with 10 μl of PBS/well for 10 min at 25°C. For this assay, 1/500 dilutions of MAb 4G2 (flavivirus complex reactive), 15F3, 3H5, 5D4, or 1H10 (DV serotype specific) were prepared in PBS containing 2% (wt/vol) milk powder (PBS/M) (Marvel; Cadbury's, United Kingdom), and 10 μl/well was added and incubated for 1 h at 28°C. The slides were then washed with PBS/M, and 10 μl/well of a 1/500 dilution of fluorescein isothiocyanate-labeled goat anti-mouse IgG (115-095-062; Jackson ImmunoResearch) in PBS/M was added and incubated for 1 h at 28°C. The slides were then washed with PBS/M, briefly dipped in distilled water, gently blotted, mounted in 90% glycerol-PBS, pH 8.3, and viewed by immunofluorescence microscopy using the appropriate excitation and barrier filters.

MAC and GAC ELISAs. Positive and negative DF patients' sera and sera from patients with infections from other common pathogens (e.g., measles virus, influenza virus, and rubella virus) as well as *Leptospira* spp. and *Plasmodium vivax*, provided by the local health authorities (Oficinia Distrital de Salud Publica de Barranquilla), were used to optimize the MAC and GAC ELISAs. The reagent concentrations in each assay step were optimized using "checkerboard" titrations to give maximum OD (OD_{max}) signals. The average maximum OD "plateaus" (492 nm) for IgM and IgG were determined to identify the $OD_{max}/2$ titer threshold at which the reciprocal $\log_{10}$ 50% endpoint ($\log_{10}t_{50}$) IgM or IgG titer was identified. The panels of control sera ($\log_{10}t_{50}$ of negative sera plus 3 standard deviations) were used to establish the serum cutoff dilution (1/100) used in the IgM/IgG OD ratios (see below) and the final serum dilutions employed in the MAC and GAC ELISAs. IgM or IgG titers above and below the $OD_{max}/2$ titer threshold were identified as IgM or IgG titer positive and titer negative, respectively.

For these assays, high-binding ELISA plates (Immulon 4; Dynatech, United Kingdom) were loaded at 10 to 20 μg/ml (50 μl/well) with affinity-purified goat anti-human IgM or IgG (109-005-129 and 109-005-098; Jackson ImmunoResearch) in 1.6%/2.93% (wt/vol) sodium carbonate-bicarbonate (pH 9.8) overnight at 4°C. After being washed with PBS, the plates were blocked using 1% (wt/vol) gelatin (G 6650; Sigma) in PBS at 25°C for 2 h. Serial four- or fivefold dilutions from 1/50 ($1/\log_{10}$ 1.7) of these patients' S1 and S2 samples were prepared in PBS containing 0.02% (vol/vol) Tween 20 (P 1379; Sigma) (PBS/T) with 0.25% (wt/vol) gelatin (PBS/T/G) in low-adsorption ELISA plates (240 μl/well) (Immulon 1; Dynatech, United Kingdom). After the blocked plates were washed with PBS/T, 50-μl volumes of the diluted sera were transferred to the IgM and IgG capture plates (n.b., 50 μl × 4 < 240 μl) working up the dilution gradients. After incubation for 2 h at 25°C, the plates were washed with PBS/T, a 1/2.5 dilution of the pooled dengue type 1 to type 4 virus C6/36 culture supernatants in PBS/T/G was added to each well, and the plates were incubated for 2 h at 25°C. After the plates were washed with PBS/T, 50 μl of 1 μg/ml of affinity-purified anti-dengue virus MAb 2F2.1 and/or 2C5.1 (4, 5) in PBS/T/G was added to each well and the plates were incubated overnight at 4°C. After the plates were washed with PBS/T, 50 μl/well of a 1/2,000 dilution of peroxidase-labeled goat anti-mouse IgG (115-035-062; Jackson ImmunoResearch) in PBS/T/G was added to each well and the plates were incubated for 2 h at 25°C. After being washed with PBS/T, the bound antibodies were detected by the addition of 0.012% (wt/vol) *o*-phenylenediamine dihydrochloride (P 1526; Sigma) with 0.09% (vol/vol) H_2O_2 in citrate-phosphate buffer (pH 5.0). After incubation for 10 min at 25°C, 0.2 M H_2SO_4 was added and the average OD value for each serum sample dilution was determined at a dual wavelength of 490 nm and 630 nm (MRX; Dynex).

An acute flavivirus infection was confirmed when there was a ≥4-fold ($\log_{10}$ 0.60) increase in their IgM or IgG (50% endpoint) titers, with an S1-to-S2 interval of 2 to 14 days (24). The patients' S1 and/or S2 samples that had moderate to high IgM and/or IgG titers but showed less than a fourfold increase in IgM or IgG titers, with S1-to-S2 intervals of 2 to 14 days, were classified as recent flavivirus infections. When there was a ≥4-fold increase in IgG or IgM (50% endpoint) titers but the S1-to-S2 interval was more than 14 days, they were classified as probable acute flavivirus infections. These classifications were, however, also dependent upon the known durations of the IgM and IgG titers during DV infections (10, 11, 19, 20). The patients' S1 and S2 samples which contained only low to medium-low IgG titers, with similar or reduced IgG titers between their S1 and S2 samples, were classified as previous flavivirus infections.

The IgM/IgG OD ratio for each S1 and S2 sample was recorded at a 1/100 dilution. These OD ratios were then directly analyzed using three different discriminatory IgM/IgG OD ratios (11, 14, 19) to compare their concordances in the accurate classification of the acute primary and acute/probable acute sec-

TABLE 1. Classification of 37 confirmed flavivirus infections[a]

Infection type	No. of patients	S1-S2 interval (days)	IgM/IgG OD ratio range (S1 sample)	S2 sample	
				IgM/IgG OD ratio range	IgG titer ($\log_{10}t_{50}$) range
Classical					
Acute primary infection	2	8	3.17–3.67	2.21–2.30	<2.00
Acute secondary infection	16	6–14	0.31–1.00	0.27–1.03	5.18–6.85
Low S2 IgG acute secondary infection	3	9–10	0.40–0.80	0.59–0.69	3.73–4.21
Probable acute secondary infection	6	18–52	0.63–1.15	0.24–0.88	5.44–6.65
Nonclassical					
High S1 IgG acute primary infection	1	7	1.20	1.88	<2.00
High S1 IgM acute secondary infection	5	7–13	1.39–1.75	0.80–0.99	5.13–6.27
High S1 IgM and low S2 IgG acute secondary infection	2	9–13	2.04–2.55	0.77–1.41	4.18–4.38
High S1 IgM probable acute secondary infection	2	27–28	2.33–2.52	0.98–1.15	4.93–5.70

[a] Twenty-seven serologically classical and 10 serologically nonclassical infections.

ondary flavivirus infections. The ≥1.78 and <1.78 discriminatory IgM/IgG OD ratios described previously (11) were, however, directly used on these IgM/IgG OD ratios without conversions to antibody units, and the >1.4 and <1.4 discriminatory IgM/IgG OD ratios described by Kuno et al. (14) were used as ≥1.40 and <1.40.

IgG/IgM titer ratios were also used to classify acute primary and acute/probable acute secondary infections. For this method, the IgM titers ($1/\log_{10}t_{50}$) were subtracted from the IgG titers to give $\log_{10}$ IgG/IgM titer ratios, which were then converted to real number IgG/IgM titer ratios. When either IgM or IgG was titer negative, a titer of $<\log_{10}$ 2.0 (1/100 titer threshold cutoff) was assigned to them and the results were expressed as more than or less than the IgG/IgM titer ratio. Because of the greater dynamic ranges of these $\log_{10}t_{50}$ IgM and IgG titers ($\log_{10}t_{50}$ IgM titers, <2.00 to 4.10 = $>\log_{10}$ 2.10 = >125.89 [five-digit range]; $\log_{10}t_{50}$ IgG titers, <2.00 to 6.85 = $>\log_{10}$ 4.85 = >70,794.58 [seven-digit range]), the accuracy and reliability of the IgG/IgM titer ratios were compared to those of the three-digit IgM/IgG OD ratios from these patients' sera.

RESULTS

Patients. The 100 suspected DF patients, whose ages ranged from 1.5 to 84 years (average, 13.4 years old), presented with fever at neighborhood outpatient clinics throughout Barranquilla and the neighboring town of Soledad when the S1 samples were obtained. None of these patients were subsequently hospitalized.

Optimization of the MAC and GAC ELISAs. Positive and negative DF patients' sera and sera from patients with acute infections caused by other locally common pathogens as well as *Leptospira* spp. and *Plasmodium vivax* were used to optimize the MAC and GAC ELISAs. Since the average maximum OD "plateaus" (492 nm) for these patients' IgM and IgG antibodies were approximately 1.44, the $\log_{10}t_{50}$ titers were determined at an OD of 0.72 ($OD_{max}/2$) and the panel of positive and negative control sera (highest OD of the negative control sera plus 3 standard deviations) was used to establish the 1/100 IgM cutoff dilution and the final serum dilution of $1/\log_{10}$ 6.6 used in the GAC ELISA.

Initial serological studies. MAC ELISA and GAC ELISA titrations from $1/\log_{10}$ 1.7 to $1/\log_{10}$ 6.6 dilutions were performed on the S1 and S2 samples from all of the 100 suspected DF patients. Most of the samples (60/100) had S1-to-S2 intervals of 2 to 14 days, while the others ranged from 17 to 69 days. A high proportion (38/100) of the patients' S1 and S2 samples were anti-dengue virus IgM and IgG titer negative. Based on the WHO classification (24), 25/100 of the other patient samples were classified as (i) recent primary ($n = 2$), (ii) recent secondary ($n = 5$), (iii) probable recent primary ($n = 6$), (iv) probable recent secondary ($n = 8$), or (v) previous ($n = 4$) flavivirus infections.

Division of confirmed acute or probable acute flavivirus infections into serologically classical or nonclassical flavivirus infections. Another 37/100 patients were found to have acute flavivirus (S1-to-S2 interval, ≤14 days) or probable acute flavivirus (S1-to-S2 interval, >14 days) infections by observing ≥4-fold increases in the IgM or IgG titers between their S1 and S2 samples (Table 1). The S1 samples from most of these patients (23/37; 62%) were IgM and IgG titer negative (<0.72 OD titer threshold). In the majority of these cases (27/37; 73%), all three of the discriminatory IgM/IgG OD ratios (≥1.78, 1.40, and 1.20 and <1.78, 1.40, and 1.20) previously described (11, 14, 19) identified each of them as acute primary, acute secondary, which included an additional group who generated low S2 sera IgG titers (IgG $\log_{10}t_{50}$ <4.4), or probable acute secondary flavivirus infections when they were applied to these patients' S1 and S2 IgM/IgG OD ratios (Table 1). These cases were, therefore, defined as serologically classical flavivirus infections.

In another 10/37 (27%), defined as serologically nonclassical flavivirus infections, there were either conflicting or erroneous classifications resulting from the application of these three IgM/IgG discriminatory OD ratios to all of their S1, and one of their S2, IgM/IgG OD ratios (Table 1). All three of these discriminatory IgM/IgG OD ratios (11, 14, 19), however, correctly classified 9/10 (90%) of these infections as acute primary, acute secondary, low S2 IgG titer acute secondary, or probable acute secondary flavivirus infections when they were applied to these patients' S2 IgM/IgG OD ratios (see below). These patients, therefore, had either an unusually low S1 IgM/IgG OD ratio for an acute primary flavivirus infection ($n = 1$) or an unusually high S1 IgM/IgG OD ratio for acute secondary ($n = 5$), low S2 IgG acute secondary ($n = 2$), or probable acute secondary ($n = 2$) flavivirus infection.

Full analysis of 27 serologically classical acute primary/secondary and probable acute secondary flavivirus infections. Twenty-seven of the patient samples were classified as serologically classical acute primary, acute secondary, low S2 IgG acute secondary, or probable acute secondary infections, since

TABLE 2. Details of 27 serologically classical acute primary/secondary and probable acute secondary flavivirus infections when S1 samples were collected on days 1 to 3 after the onset of symptoms

Day and infection type[a]	Dengue virus serotype[b]	S1 sample			S2 sample		
		Titer (OD at 1/100)[c]		IgM/IgG OD ratio (P or S)[d]	Titer (OD at 1/100)[c]		IgM/IgG OD ratio (P or S)[d]
		IgM	IgG		IgM	IgG	
1							
S		— (0.41)	5.28 (1.34)	0.31 (S)	— (0.50)	5.97 (1.44)	0.35 (S)
S	4	— (0.22)	— (0.45)	0.49 (S)	— (0.53)	5.75 (1.45)	0.37 (S)
S	4	— (0.28)	— (0.53)	0.53 (S)	2.88 (0.97)	5.28 (1.33)	0.73 (S)
S	4	— (0.33)	— (0.42)	0.78 (S)	— (0.38)	5.82 (1.42)	0.27 (S)
S	2	— (0.47)	— (0.60)	0.78 (S)	3.50 (1.32)	5.88 (1.28)	1.03 (S)
S	4	— (0.40)	— (0.50)	0.80 (S)	2.73 (0.90)	5.61 (1.35)	0.67 (S)
S	4	2.23 (0.75)	4.66 (0.94)	0.80 (S)	3.26 (1.24)	6.22 (1.35)	0.92 (S)
S	4	— (0.29)	— (0.29)	1.00 (S)	— (0.64)	5.83 (1.47)	0.43 (S)
LS		— (0.29)	2.63 (0.73)	0.40 (S)	— (0.44)	3.73 (0.75)	0.59 (S)
PS	1	— (0.55)	— (0.50)	1.10 (S)	3.04 (1.03)	6.16 (1.34)	0.77 (S)
2							
P		— (0.57)	— (0.18)	3.17 (P)	3.05 (1.46)	— (0.66)	2.21 (P)
S		— (0.45)	4.25 (0.90)	0.50 (S)	— (0.69)	5.18 (1.25)	0.55 (S)
S		— (0.61)	3.62 (0.78)	0.78 (S)	3.07 (1.06)	6.23 (1.45)	0.73 (S)
S	1	— (0.36)	— (0.37)	0.97 (S)	2.33 (0.74)	5.82 (1.50)	0.49 (S)
LS		— (0.50)	2.38 (0.73)	0.68 (S)	— (0.54)	4.21 (0.78)	0.69 (S)
PS		— (0.63)	3.40 (0.75)	0.84 (S)	3.18 (1.17)	6.43 (1.33)	0.88 (S)
PS	4	— (0.55)	— (0.48)	1.15 (S)	— (0.66)	5.44 (1.25)	0.53 (S)
3							
P		— (0.55)	— (0.15)	3.67 (P)	2.88 (1.08)	— (0.47)	2.30 (P)
S		— (0.38)	4.61 (1.06)	0.36 (S)	— (0.52)	5.76 (1.39)	0.38 (S)
S	2	2.21 (0.76)	4.90 (1.16)	0.66 (S)	3.97 (1.34)	6.47 (1.34)	1.00 (S)
S		2.43 (0.86)	4.42 (1.04)	0.83 (S)	2.92 (1.09)	5.73 (1.38)	0.79 (S)
S	4	2.83 (1.18)	5.18 (1.36)	0.87 (S)	3.60 (1.41)	6.85 (1.45)	0.97 (S)
S	4	— (0.61)	— (0.64)	0.95 (S)	3.23 (1.23)	5.65 (1.37)	0.90 (S)
LS		— (0.48)	— (0.60)	0.80 (S)	— (0.48)	4.00 (0.75)	0.64 (S)
PS		2.72 (0.84)	6.02 (1.34)	0.63 (S)	2.97 (1.01)	6.65 (1.36)	0.74 (S)
PS	4	— (0.21)	— (0.28)	0.75 (S)	— (0.34)	5.73 (1.41)	0.24 (S)
PS	2	2.70 (0.81)	4.97 (1.07)	0.76 (S)	3.28 (1.12)	6.01 (1.30)	0.86 (S)

[a] Infection type classification as acute primary (P), acute secondary (S), low S2 IgG acute secondary (LS), or probable acute secondary (PS) flavivirus infection.
[b] Dengue type 1 (1), type 2 (2), or type 4 (4) viruses isolated.
[c] $\log_{10}t_{50}$ IgM and IgG titers (— indicates <0.72 OD titer threshold) and IgM and IgG ODs at a serum dilution of 1/100 (in parentheses).
[d] IgM/IgG OD ratios and classifications of the S1 and S2 samples as primary (P) or secondary (S) flavivirus infection by concordance of all three discriminatory IgM/IgG OD ratios (≥1.78, 1,40, and 1.20 or <1.78, 1.40, and 1.20) (in parentheses).

they could all be correctly classified using the three discriminatory IgM/IgG OD ratios on their S1 and S2 IgM/IgG OD ratios. The IgM and IgG titers and dengue virus isolation rates were then compared when these patients' S1 samples were obtained on days 1, 2, and 3 after the onset of symptoms (Table 2).

The majority (7/10; 70%) of the patients' S1 samples obtained on day 1 after the onset of symptoms were both IgM and IgG titer negative (<0.72 $OD_{max}/2$ titer threshold) (Table 2). Dengue viruses of three different serotypes (dengue type 1, 2, and 4 viruses) were isolated from each of these patients' S1 samples and from one acute secondary infection that had high S1 IgM and IgG titers, but not from the other two patients' S1 samples which had moderately high (low S2 IgG acute secondary infection) or high (acute secondary infection) IgG titers.

In contrast, only three of seven (43%) of the patients' S1 samples obtained on day 2 after the onset of symptoms were both IgM and IgG titer negative (Table 2). Only two dengue viruses (dengue type 1 and type 4 viruses) were isolated from these IgM and IgG titer-negative patients' S1 samples that had either an acute secondary or a probable acute secondary (PS) infection.

Only 4/10 (40%) of the patients' S1 samples obtained on day 3 after the onset of symptoms were both IgM and IgG titer negative (Table 2). Only two dengue viruses could be isolated from them (acute secondary infection, dengue type 4 virus; probable acute secondary infection, dengue type 4 virus), but three other dengue viruses were also isolated from these patients' S1 samples which had high IgM and IgG titers (two acute secondary infections, dengue type 2 and type 4 viruses; one probable acute secondary infection, dengue type 2 virus).

Because of their greater dynamic range, the IgG/IgM titer ratios gave greater accuracy when used on the IgM or IgG titer-positive S1 samples (IgG/IgM titer range, $\log_{10}t_{50}$ 4.42 − $\log_{10}t_{50}$ 2.43 = $\log_{10}$ 1.99 = 97.72 [acute secondary infection] [four-digit range] to $\log_{10}t_{50}$ 5.28 − $<\log_{10}t_{50}$ 2.00 = $>\log_{10}$ 3.28 = >1,905.46 [acute secondary infection] [six-digit range]) and S2 samples (IgG/IgM titer range, $<\log_{10}t_{50}$ 2.00 − $\log_{10}t_{50}$ 3.05 = $<\log_{10}t_{50}$ −1.05 = <0.09 [acute primary infection] [three-digit range] to $\log_{10}t_{50}$ 5.97 − $<\log_{10}t_{50}$ 2.00 =

TABLE 3. Details of 10 serologically nonclassical acute primary/secondary and probable acute secondary flavivirus infections when the S1 samples were collected on days 1 to 3 after the onset of symptoms

Day and infection type[a]	Dengue virus serotype[b]	S1 sample			S2 sample		
		Titer (OD at 1/100)[c]		IgM/IgG OD ratio (P and/or S)[d]	Titer (OD at 1/100)[c]		IgM/IgG OD ratio (P and/or S)[d]
		IgM	IgG		IgM	IgG	
1							
*S	2	— (0.25)	— (0.18)	1.39 (S/S/S/P)	3.20 (1.16)	6.01 (1.40)	0.83 (S)
*S	4	— (0.35)	— (0.25)	1.40 (S/S/P/P)	3.51 (1.42)	5.92 (1.44)	0.99 (S)
*S	2	— (0.40)	— (0.25)	1.60 (S/S/P/P)	3.83 (1.37)	6.27 (1.43)	0.96 (S)
*S	1	— (0.49)	— (0.28)	1.75 (S/S/P/P)	2.96 (1.10)	5.78 (1.38)	0.80 (S)
*LS	1	— (0.28)	— (0.11)	2.55 (S/P/P/P)	4.10 (1.44)	4.38 (1.02)	1.41 (S/P/P)
2							
*LS		— (0.57)	— (0.28)	2.04 (S/P/P/P)	— (0.61)	4.18 (0.79)	0.77 (S)
*PS	1	— (0.68)	— (0.27)	2.52 (S/P/P/P)	3.97 (1.40)	4.93 (1.22)	1.15 (S)
3							
*P		— (0.48)	— (0.40)	1.20 (S/S/S/P)	2.78 (0.90)	— (0.48)	1.88 (P)
*S		— (0.55)	— (0.35)	1.57 (S/S/P/P)	3.24 (1.23)	5.13 (1.32)	0.93 (S)
*PS	2	2.70 (0.91)	— (0.39)	2.33 (S/P/P/P)	3.63 (1.42)	5.70 (1.45)	0.98 (S)

[a] Infection type classification as high S1 IgG acute primary (*P), high S1 IgM acute secondary (*S), high S1 IgM and low S2 IgG acute secondary (*LS), or high S1 IgM probable acute secondary (*PS) flavivirus infection.
[b] Dengue type 1 (1), type 2 (2), or type 4 (4) viruses isolated.
[c] $\log_{10}t_{50}$ IgM and IgG titers (— indicates <0.72 OD titer threshold) and IgM and IgG ODs of S1 and S2 sera at a 1/100 dilution (in parentheses).
[d] IgM/IgG OD ratios and classifications of the S1 and S2 samples as acute primary (P) and/or secondary (S) flavivirus infection using the ≥2.60, 1.78, 1.40, and 1.20 or <2.60, 1.78, 1.40, and 1.20 discriminatory IgM/IgG OD ratios on the patients' S1 samples (in parentheses) and the ≥1.78, 1.40, and 1.20 or <1.78, 1.40, and 1.20 discriminatory IgM/IgG OD ratios on the patients' S2 samples (in parentheses). A single P or S for the patients' S2 samples indicates concordance between the three discriminatory IgM/IgG OD ratios.

$>\log_{10}$ 3.97 = >93,332.54 [acute secondary infection] [seven-digit range]) from these patients than the three-digit IgM/IgG OD ratios.

Full analysis of 10 serologically nonclassical acute primary/secondary and probable secondary flavivirus infections. The three discriminatory IgM/IgG OD ratios (11, 14, 19) correctly classified 9/10 (90%) of the serologically nonclassical acute primary and acute/probable acute secondary flavivirus infections when applied to their S2 IgM/IgG OD ratios (Table 3). The other patient, who had an S2 IgM/IgG OD ratio of 1.41, could be correctly classified as having high S1 lgM and low S2 lgG acute secondary flavivirus infection using only the highest of the three discriminatory IgM/IgG OD ratios (<1.78) (Table 3) (11). In this case, the S2 IgM OD (1.44) was higher than the IgG OD (1.02) value, while the IgG titer ($\log_{10}t_{50}$ 4.38) was nearly two times higher (IgG/IgM titer ratio, 1.91) than the IgM titer ($\log_{10}t_{50}$ 4.10). Similarly, in another patient with a high S1 IgM probable acute secondary infection, the S2 IgM OD (1.40) was higher than the IgG OD (1.22), but the IgG titer ($\log_{10}t_{50}$ 4.93) was nine times higher (IgG/IgM titer ratio, 9.12) than the IgM titer ($\log_{10}t_{50}$ 3.97) (Table 3). The IgG/IgM ELISA titer ratios were, therefore, more accurate and reliable than the three-digit IgM/IgG OD ratios performed at the single 1/100 cutoff dilution for classifying acute primary and acute/probable acute secondary flavivirus infections using all of the S2 samples from these serologically nonclassical flavivirus infections (IgG/IgM ELISA titer ratio ranges, $<\log_{10}$ 2.00 − $\log_{10}$ 2.78 = $<\log_{10}$ −0.78 = <0.17 [high S1 IgG acute primary infection] [three-digit range] to $\log_{10}$ 5.78 − $\log_{10}$ 2.96 = $\log_{10}$ 2.82 = 660.69 [high S1 IgM acute secondary infection] [five-digit range]), as was found using the S2 samples from the serologically classical flavivirus infections (see above).

Nearly all (9/10; 90%) of the S1 samples from these patients with serologically nonclassical flavivirus infections were, however, IgM and IgG titer negative, but their S1 IgM/IgG OD ratios showed that these patients had high S1 IgG acute primary, high S1 IgM acute secondary, high S1 IgM and low S2 IgG acute secondary, or high S1 IgM probable acute secondary flavivirus infections (Table 3). This led to conflicting or erroneous classifications using the three different discriminatory IgM/IgG OD ratios (≥1.78, 1.40, and 1.20 or <1.78, 1.40, and 1.20) (11, 14, 19) on these patients' S1 IgM/IgG OD ratios. Because none of the three discriminatory IgM/IgG OD ratios could correctly classify each of these infection types when applied to the patients' S1 IgM/IgG OD ratios, an "adjusted" discriminatory IgM/IgG OD ratio of ≥2.60 and <2.60 was employed to correctly classify the highest number of them (9/10; 90%). The IgM and IgG titers and dengue virus isolation rates were then compared when these patients' S1 samples were obtained on days 1, 2, and 3 after the onset of symptoms.

All five of the patients' S1 samples collected on day 1 after the onset of symptoms were IgM and IgG titer negative (Table 3). Each of them could, however, be correctly classified as high S1 IgM acute secondary or high S1 IgM with low S2 IgG acute secondary flavivirus infection using the "adjusted" discriminatory IgM/IgG OD ratio (≥2.60 and <2.60), while only four of five, one of five, and zero of five were correctly classified using the ≥1.78 and <1.78 (11), 1.40 (14), and 1.20 (19) discriminatory IgM/IgG ratios, respectively. Dengue viruses of three serotypes (dengue type 1, type 2, and type 4 viruses) were isolated from each of these five patients' S1 samples.

Both patients' S1 samples collected on day 2 after the onset of symptoms were IgM and IgG titer negative but were correctly classified as high S1 IgM and low S2 IgG acute secondary or high S1 IgM probable acute secondary flavivirus infections using the "adjusted" discriminatory IgM/IgG OD ratio (<2.60)

(Table 3). One dengue virus (dengue type 1 virus) was isolated from a patient's S1 sample with the high IgM probable acute secondary flavivirus infection.

Two patients' S1 samples collected on day 3 after the onset of symptoms were both IgM and IgG titer negative (Table 3). One of these patients, who had an S1 IgM/IgG OD ratio of 1.20, could be correctly classified as having a high S1 IgG acute primary infection using only the ≥1.20 (19) discriminatory IgM/IgG OD ratio. The other patient, with an S1 sample IgM/IgG OD ratio of 1.57, could be correctly classified as having a high IgM acute secondary infection using only the "adjusted" (<2.60) and the highest of the other three (<1.78) (11) discriminatory IgM/IgG OD ratios. One other patient, with an S1 IgM/IgG OD ratio of 2.33, could be correctly classified as having a high IgM probable acute secondary infection using only the "adjusted" (<2.60) discriminatory IgM/IgG OD ratio. Only one dengue virus (dengue type 2 virus) was isolated from these three patients' S1 samples.

Overall, therefore, dengue virus isolation rates of 100%, 50%, and 33% were attained from the patients' S1 samples collected on days 1, 2 and 3 after the onset of symptoms, respectively, and the ≥2.60 and <2.60, 1.78 (11), 1.40 (14), and 1.20 (19) discriminatory IgM/IgG OD ratios correctly classified these serologically nonclassical flavivirus infections in 9/10, 5/10, 1/10, and 1/10 of these cases, respectively.

Highest overall dengue virus isolation rates from patients' S1 samples collected on days 1, 2, and 3 after the onset of symptoms. Dengue virus isolation rates were highest from the patients' S1 samples collected on day 1 after the onset of symptoms, which were IgM and IgG titer negative (12/12; 100%) (total for all S1 samples, 13/15 [87%]), when 100% of them could be correctly classified as acute secondary, low S2 IgG acute secondary, probable acute secondary, high S1 IgG acute primary, or high S1 IgM and low S2 IgG acute secondary flavivirus infections using the "adjusted" (≥2.60 and <2.60) discriminatory IgM/IgG OD ratios (Tables 2 and 3).

DISCUSSION

The MAC and GAC ELISAs have surpassed the use of the hemagglutination assay because of their simplicity and sensitivity, without the need to pretreat patients' sera to remove nonspecific hemagglutination inhibitors and agglutinins (11, 14, 20, 23, 24). For these assays, most laboratories use a patient's S1 and S2 samples at single cutoff dilutions of either 1/20 (14) or 1/100 (11, 19, 20, 21). A 50% endpoint titer cutoff dilution of 1/100 was also identified in our study. Some of these other MAC and GAC ELISAs described previously, however, had low maximum OD values (11, 14), and in one of these assays, the maximum signal was deliberately reduced to an OD of 0.4 (11). We, instead, opted to maximize the sensitivity of our assays to increase the ability to more accurately measure the IgM/IgG OD ratios of these very early acute-phase S1 samples. We also opted to perform full IgM and IgG ELISA titrations in these patients' sera and to maintain accuracy at each serum dilution by preparing the serial four- or fivefold dilutions of the patients' sera at 240-μl volumes in other 96-well plates before transferring 50-μl volumes of each dilution (working up the concentration gradient) to the paired MAC and GAC ELISA plates. Although not suitable for high-throughput patient screening, the greater dynamic ranges of these IgG/IgM ELISA titer ratios could be used to more accurately and reliably classify and subgroup the acute primary and acute/probable acute secondary infections using patients' S2 samples and 14/37 (38%) of the S1 samples which were IgM or IgG titer positive. We therefore wish to compare the discriminatory IgG/IgM ELISA titer ratios from this panel of DF patients with those from other countries where dengue hemorrhagic fever and dengue shock syndrome are endemic.

While other studies also used acute-phase sera from DF patients in the Americas to identify acute primary and acute/probable acute secondary flavivirus infections (14), they did not test whether the discriminatory IgM/IgG OD ratios from IgM and IgG titer-negative sera could still be used to correctly classify these acute primary and secondary flavivirus infections. Our ability to correctly classify 36/37 (97%) of the acute primary and acute/probable acute secondary infections in these early S1 samples from DF patients using the "adjusted" discriminatory IgM/IgG OD ratios, and 100% (37/37) of them using the S2 samples with the ≥1.78 and <1.78 discriminatory IgM/IgG OD ratios (11) or IgG/IgM ELISA titer ratios, compared favorably with those described previously (11, 14, 19).

The wide range of DF patients' IgM and IgG antibody responses identified in this study prompted us to divide these acute primary and acute/probable acute secondary flavivirus infections into eight subgroups. While variations in patients' immune responses during DV infections have thoroughly been described (2, 10, 17, 18, 19, 20, 21, 23), subgroup classifications of these patients' antibody responses have not been established. In these previous studies, DV E/M protein-specific IgG antibodies were shown to rise before, or at the same time as, IgM antibodies during acute secondary flavivirus infections, while the IgM titers were usually lower than those generated during acute primary flavivirus infections (2, 10, 11, 19, 23). In a small number of secondary DV infections, however, the rate of IgM synthesis was found to be higher than that of IgG (14), which may therefore account for our findings. We found IgM/IgG OD ratios greater than or equal to 1.20, 1.40, and 1.78 in patients' S1 samples of 9/34 (26%), 8/34 (24%), and 4/34 (12%) of these acute/probable acute flavivirus infections, respectively. Since all of the patients' S1 samples from these serologically nonclassical flavivirus infections were IgM and IgG titer negative, the IgG titers may be more accurately assessed using an "indirect" dengue virus ELISA as previously described (3, 7, 10, 15, 20, 23).

MAC and GAC ELISAs, optimized using different components, are likely to result in some variations, thereby necessitating an appropriate cutoff dilution to be determined for each assay using appropriate panels of positive and negative patients' sera (23, 24). The antiflavivirus subgroup (MAb 2F2.1) and group-reactive (MAb 2C5.1) anti-envelope protein MAbs used in our assays weakly cross-reacted with the DV premembrane (5) and nonstructural 1 (NS1) (6) proteins. This weak cross-reaction of MAb 2C5.1 with the high levels of the uncleaved premembrane protein present on DV liberated from infected insect cells (12), however, would have been expected to increased the patients' IgM and IgG detection sensitivities. In addition, since anti-NS1 IgM antibodies are generated later than 9 days after the onset of symptoms (19) and dengue virus-infected insect cells do not secrete the dengue virus NS1

protein, the weak cross-reaction of MAb 2F2.1 with this protein could also not have accounted for these higher S1 IgM OD values. Other ELISA reagents (e.g., the anti-human IgM and IgG polyclonal antibodies) may possibly have inadvertently led to a bias towards obtaining high discriminatory IgM/IgG OD ratios in these S1 samples from 9/34 (27%) of the acute/probable acute secondary flaivivirus infections. These possibilities may, however, be resolved by comparing the IgG OD results obtained using these patients' S1 samples in the GAC ELISA with those obtained using an "indirect" ELISA (3, 15). IgM titers were found in the S2 samples of patients with acute/probable acute secondary infections that were similar to, and in some cases higher than, those from patients with acute primary infections in this study. These results, therefore, confirmed that high-titer anti-DV IgM responses can be generated by some patients during acute secondary/probable secondary flavivirus infections. This observation was further complicated by finding that some (5/34; 15%) of the patients with acute/probable acute secondary flavivirus infections generated only low S2 DV E/M protein-specific IgG titers, as reported previously (11, 23). A previous vaccination (or natural infection) with a live heterologous flavivirus, such as yellow fever virus, resulted in unusually high IgM responses when these patients were subsequently infected with dengue virus (11). None of the patients in our study, however, had received a flavivirus vaccine, nor were any other human diseases caused by flaviviruses known to occur in the department (state) of Atlantico in Colombia. To account for these findings, we, therefore, wish to perform NS1 protein serotype-specific IgG capture ELISAs on some of these patients' S1 samples to identify the DV serotypes previously encountered by these patients, as described previously (19), using our dengue complex-reactive anti-NS1 mouse MAbs (6).

We also found that the percentage of patients with acute/probable acute secondary infections whose S1 and S2 samples were IgM titer negative (35%) was slightly higher than that reported in some other studies (22% and 28%) (2, 17), confirming the need for diagnostic laboratories to quantify patients' IgM and IgG antibodies in the serological diagnosis of DV infections, preferably using paired S1 and S2 samples (7, 10, 11, 14, 23, 24). In our study, only 1/15 (7%) of these patients' S1 samples was IgM titer positive on day 1 after the onset of symptoms, while 6/13 (46%) were IgM titer positive on day 3 after the onset of symptoms, consistent with previous reports (10, 23, 24).

While we showed similar abilities to correctly classify the acute primary and acute/probable acute secondary infections using S1 samples obtained on day 1 to day 3 after the onset of symptoms, we could efficiently isolate DVs using the S1 samples collected only on day 1 after the onset of symptoms, which were IgM and IgG titer negative. High (98%) DV isolation rates from a panel of patients' S1 samples collected over a 72-hour (1- to 3-day) period after the onset of symptoms was however reported in a study conducted in Thailand (21). The lower viremias probably generated in the patients from our study site in South America, where only DF has been reported, may have accounted for these differences. This city, however, has sea, air, and road connections to countries in the Americas where dengue hemorrhagic fever is endemic, and virulent (Southeast Asian) strains of each dengue virus serotype now circulate in many countries in the Americas (8).

We believe that the ability to correctly classify all acute primary and acute secondary flavivirus infections using these "adjusted" discriminatory IgM/IgG OD ratios on patients' IgM and IgG titer-negative S1 samples collected 1 day after the onset of symptoms, when all of these viruses can be efficiently identified using reverse transcription-PCR (20), a DV protein-specific (e.g., the nonstructural 1 glycoprotein) detection assay (1, 13), or IFA after cell culture, may therefore negate the time-consuming need to collect these patients' S2 samples. For our dengue virus surveillance and control program in Barranquilla, the cDNA sequence determination of these viruses will be used to identify their geographical origins, study their distribution and movement within this city, and subsequently identify the importation of new dengue type 1, 2, or 4 virus strains or dengue type 3 virus.

ACKNOWLEDGMENTS

We thank Duane J. Gubler for kindly supplying the dengue type 3 (PR-1340) virus strain used in this study.

This investigation received financial support from the Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnologia "Francisco Jose de Caldas" (COLCIENCIAS) (no. 1215-04-14364) and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) (no. 990892).

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