JOURNAL OF CLINICAL MICROBIOLOGY, May 1984, p. 664–667
0095-1137/84/050664-04$02.00/0

Vol. 19, No. 5

Detection of Specific Immunoglobulin M Antibody to Different Flaviviruses by Use of Enzyme-Labeled Antigens

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Received 7 November 1983/Accepted 17 January 1984

An enzyme immunoassay was developed for the detection of human immunoglobulin M (IgM) antibody to different flavivirus antigens. The IgM antibody of human sera was selectively bound to anti-IgM antibody-coated solid-phase plates. Flavivirus IgM antibodies were then detected by use of various enzyme-labeled antigens. The flavivirus antigens (dengue type 2 virus, West Nile virus, and tick-borne encephalitis virus) were produced in suckling mice. The antigens were labeled with horseradish peroxidase by adding the activated enzyme at alkaline pH to sucrose-acetone-treated antigens. Addition of unlabeled mouse brain suspension of uninfected animals to the diluted enzyme-labeled antigens effectively reduced nonspecific binding to the solid phase. In patients with acute flavivirus infections, viral IgM antibody could be demonstrated with high sensitivity. Furthermore, the enzyme-labeled antigen-IgM test showed greater specificity than the hemagglutination inhibition test.

Only a few publications have appeared on the use of specific immunoglobulin M (IgM) antibody detection for the diagnosis of acute flavivirus infections. In early reports, specific IgM antibody activity was demonstrated in the IgM fraction isolated from serum specimens by ultracentrifugation; alternatively, the titer reduction of a serum sample was measured after mercaptoethanol treatment (5, 10). Later, attempts were made to detect specific IgM antibody on plates coated with purified flavivirus antigens produced in tissue culture or in suckling mice (3, 7, 8, 11). In 1978 and 1979, publications first appeared on the use of anti-IgM-coated microtiter plates for the detection of IgM antibody to hepatitis A (4, 6) and rubella (9) viruses. With these new test systems, human IgM antibody can be selectively bound to the coated solid phase. A crude viral antigen is then applied, followed by an appropriate indicator system, such as radiolabeled antibody (4, 6) or erythrocytes, if a hemagglutinating antigen is involved (9). Recently, a similar test system was used for the detection of IgM antibodies to tick-borne encephalitis (TBE) virus (7) and Japanese B encaphalitis virus (1). The tests improved the diagnosis of these flavivirus infections considerably. These reverse tests were further simplified by using enzyme-labeled antigen (ELA). As shown with the detection of IgM and IgA antibodies to cytomegalovirus and Epstein-Barr virus (12, 14), the ELA-IgM method has the advantage of fewer incubation steps and can be carried out in a shorter period of time. Also, a labeled hyperimmune antiserum is not required, and interaction of rheumatoid factor IgM with labeled immunoglobulin cannot occur. We, therefore, have tried to extend the ELA-IgM method to detect IgM antibodies to certain flavivirus antigens.

MATERIALS AND METHODS

Labeling of flavivirus antigens. Dengue type 2 (New Guinea strain C) and type 3 (H87) viruses, West Nile virus, and TBE (strain Hyper) virus were propagated in suckling mice. Sucrose-acetone-extracted antigens were prepared according to Clarke and Casals (2). The antigens agglutinated goose erythrocytes to a titer of at least 1:1,280 at pH 6.2.

For antigen labeling, a mixture of 1 ml of antigen at alkaline pH (in 0.15 M NaCl–0.05 M carbonate buffer, pH 9) and an equal volume of activated peroxidase (16) was incubated at 4°C overnight (16 h). $NaBH_4$ (4 mg/ml; 1:30 [vol/vol] was then added for 30 min at 4°C. The ELA was immediately stored frozen at −20°C in the presence of 10% glycerol in 0.1-ml volumes. The diluent for the ELAs consisted of a mixture of 1 part normal mouse brain suspension, 1 part fetal calf serum, and 2 parts 0.3 M NaCl plus 1% Tween 20 (Serva, Heidelberg, Federal Republic of Germany).

Coating of plates. Round-bottomed polystyrene microtiter plates were exposed to gamma-irradiation (2 mrads) before use, to improve the protein binding capacity. They were coated by adding 100 μl per well of anti-human IgM (goat anti-human μ-chain antibody, affinity chromatography purified; Medac, Hamburg, Federal Republic of Germany) at a dilution of 1:500 in phosphate-buffered saline, pH 7.5. The plates were stored at 4°C in a moist chamber and could be used after 2 days adsorption time. The plates could be lyophilized after deep-freezing (−20°C) and could be stored at room temperature after vacuum sealing in aluminum-coated plastic bags.

ELA-IgM test procedure. The plates were washed with buffer (0.15 M NaCl, 0.1 Tris [pH 7.5], 0.05% Tween 20, 0.1 mg of NaN_3 per ml) as previously described (13). Then 50 μl of the serum specimens diluted in twofold steps from 1:100 through 1:12,800 in phosphate-buffered saline were incubated in eight wells for 2 h at room temperature. Also, two positive and three negative control sera diluted 1:100 were included in each assay. After another washing, ELA (50 μl per well) was applied for a further 2 h at 4°C. After a final washing, the substrate (1 mg of *o*-phenylenediamine per ml in 0.1 M phosphate buffer [pH 6] plus 0.1% H_2O_2) was added, and the reaction was stopped with 2 M H_2SO_4 after 10 min. The test was read with a spectrophotometer at 495 nm.

Separation of IgM by ultracentrifugation in sucrose gradients. The sera (0.1 ml) were ultracentrifuged in an SW 41 rotor for 20 h at 180,000 × *g*. Before ultracentrifugation, the sera were stained by addition of fluorescein-isothiocyanate to permit direct visualization of the IgM band (13).

HI test. The hemagglutination inhibition (HI) test was performed by standard procedures (15). The sera were freed

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TABLE 1. HI and ELA-IgM antibody titers to dengue type 2 antigen in serum specimens and in corresponding IgG and IgM fractions of two dengue fever patients; the results obtained with the SPIT technique are included for comparison

Serum	Titer for:		
	HI	SPIT	ELA-IgM
Total serum LI II	5,120	3,200	12,800
IgG fraction	2,560	Negative (<50)	Negative (<50)
IgM fraction	320	3,200	25,600
Total serum WE III	160	Negative	100
IgG fraction	160	Negative	Negative
IgM fraction	Negative (<10)	Negative	100

from inhibitors by repeated acetone treatment. Sera were tested at serial dilutions from 1:10 against 8 U of antigen.

Detection of neutralizing antibodies to dengue viruses. The dengue serotypes 1 through 4 (1, Hawaii; 2, New Guinea C; 3, H87; and 4, H241) were passaged in our laboratory in suckling mice. The method for detecting neutralizing antibodies in human sera followed that described by Shope and Sather (15). Briefly, mixtures of virus suspensions (containing 10 and 100 50% lethal doses) and human sera (diluted 1:5) were incubated for 2 h at 37°C. For each dilution of virus and for each serotype, six suckling mice were inoculated. The suckling mice were observed daily, and 14 days after inoculation the neutralization index was determined.

SPIT. As an alternative for specific IgM antibody detection, the solid-phase immunosorbent technique (SPIT) of Krech and Wilhelm (9) was investigated. The initial steps of this method were identical to those of the ELA-IgM procedure (see above). Again the sera were incubated on the anti-IgM-coated microtiter plates at dilutions of 1:100 up to 1:6,400 for 2 h. After washing, 2 U of hemagglutinating flavivirus antigen was applied overnight at 4°C, and after another washing a suspension of 0.1% goose erythrocytes was added in a mixture of 1 part of 0.4% bovine-albumin-borate-saline (pH 9) and 1 part of the appropriate 0.2 M phosphate buffer (15).

Serum specimens. Twenty sera were obtained from nine adult German tourists just returned from Southeast Asia and the Maldive Islands. In all these patients dengue-like clinical symptoms (high fever, rash, and arthralgia) were observed. High HI antibody titers (>640) to dengue antigen were detected in all patients. A fourfold rise in antibody titer to dengue type 2 antigen was demonstrable in three patients (CA, HE, and SCH; see Table 2), when early acute-phase sera could be obtained. In two others (WE and SM; see Table 2), a significant (fourfold) decrease in titer was detected, and in two a twofold decrease in titer could be demonstrated between early and late convalescent-phase sera. With the HI test, all dengue sera showed marked cross-reactivity to West Nile antigen.

To confirm the diagnosis of dengue fever in the patients, neutralizing antibodies to different dengue serotypes were looked for in their sera. In all dengue patients, neutralizing antibodies could be detected. The sera of six of our patients (WE, SM, LI, PL, SC, and KO) neutralized dengue type 1 virus. The sera of three patients (CA, HE, and SCH) neutralized dengue type 2 virus (neutralization index > 2).

Furthermore, 11 sera of four German patients with TBE were included in our investigations. All patients had developed a meningitis or meningoencephalitis 11 to 14 days after exposure to tick bites. As all patients were infected in southern Germany, early sera were available and a fourfold increase in HI titer to TBE virus could be demonstrated in all of them.

Moreover, control sera were examined from 100 healthy adult Germans and from 58 healthy adult refugees from Laos, who had arrived in the Federal Republic of Germany 2 months previously.

RESULTS

As a baseline control, we began IgM antibody detection of dengue type 2 antigen by testing isolated IgM fractions after ultracentrifugation in the HI test. Dengue IgM antibody could be demonstrated in a serum sample taken early (LI II; Table 1) after acute dengue fever, whereas in the IgM fraction of a late serum sample (WE III), specific IgM antibody was no longer detectable. Similar results were obtained when the IgM fractions were tested by SPIT (Table 1). Moreover, by using SPIT, unfractionated serum specimens could be used for specific IgM detection, because human IgG was effectively eliminated by the first washing step (see above). This is documented with the IgG antibody

TABLE 2. IgG and IgM antibody titers to dengue antigen in serial serum specimens of patients with clinical evidence of dengue[a]

Patient code	Days after onset	Titer for:			
		HI			ELA-IgM
		Total serum	IgG	IgM	
WE					
I	13	640	640	160	6,400
II	18	1,280	1,280	80	1,600
III	68	160	160	Negative (<50)	100
IV	270	160	160	Negative	Negative
SM					
I	22	>5,120	5,120	160	1,600
II	42	>5,120	5,120	40	800
III	67	1,280	640	<10	200
CA					
I	4	320	n.t.	n.t.	3,200
II	120	1,280	1,280	n.t.	<50
HE					
I	4	20	20	20	400
II	14	320	320	80	800
SCH					
I	4	160	320	40	400
II	11	2,560	2,560	160	3,200
III	51	2,560	2,560	80	800
IV	117	5,120	5,120	<10	<50
LI					
I	7	5,120	2,560	80	12,800
II	12	5,120	2,560	320	25,600
PL	16	640	320	80	3,200
SC	7	2,560	2,560	40	3,200
KO	5	5,120	2,560	320	1,600

[a] Dengue antibodies were detected by using the HI- and ELA-IgM assays. n.t., Not tested; IgM and IgG, isolated IgM and IgG fractions.

fraction of the serum LI II (Table 1), which was negative both in the SPIT and in the ELA-IgM test.

Compared with SPIT and HI, the ELA-IgM method appeared to detect lower amounts of specific IgM, as shown with serum sample WE III (Table 1) taken on day 68; traces of antidengue IgM in the late-convalescent-phase serum were detected by the ELA-IgM test only. Finally, 270 days after onset of the disease (WE IV; Table 2), specific IgM was no longer demonstrable.

All dengue and TBE sera (listed in Tables 2 and 3) were tested not only by the ELA-IgM test but also by SPIT. With both methods, the lowest serum dilution tested was 1:50 which gave complete saturation of the IgM binding capacity of the plates. However, titers in the ELA-IgM test were higher (about fourfold) as compared with SPIT. Sera with low antibody titers (<200) were not detected by SPIT. Because the ELA-IgM test was more sensitive and more reproducible than SPIT, only ELA-IgM results are shown in the tables.

Generally, in the ELA-IgM test the P/N ratio (ratio of the optical density of a positive standard serum to that of a negative control pool) is mainly influenced by background reactions of the ELAs with the coated solid phase (12, 14). To reduce this background, concentrated control antigen (normal mouse brain extract) was added to the diluted ELAs. This mixing of ELA and control antigen (see above) proved to be highly effective in reducing nonspecific reactions. For example, by addition of control antigen the P/N ratio in the dengue ELA-IgM test was increased from 2.5 to 8. Also, the presence of 1% Tween 20 in the ELA diluent improved the P/N ratio. For each ELA, the optimum test dilution had to be determined (11, 14). As it happened, with all three antigens used maximum P/N ratios were obtained at a dilution of 1:100.

To define the cutoff level between negative and positive results, 100 sera of adult European patients without a history of recent flavivirus infection were examined with the ELA-IgM tests. The arithmetic mean (m) of the optical densities of these control sera plus three standard deviations (m + 3s) was 0.14 for the two dengue ELAs, 0.12 for the West Nile ELA, and 0.12 for the TBE ELA. Moreover, we included the sera of 58 healthy refugees from Laos, 24 (41%) of which had dengue antibodies in the HI test. All sera were completely negative (cutoff levels < 0.13) in the dengue type 2, type 3, and West Nile ELA-IgM tests. Serum dilutions with optical densities exceeding the corresponding cutoff level were considered to be positive (12). For the determination of the titration endpoint, twofold serum dilutions were applied in the ELA-IgM test. The highest dilution with an optical density still exceeding the cutoff level was defined as the IgM antibody titer (14). The interassay variation was rather low in the ELA-IgM tests (variation coefficient < 36%; serum sample LI I; eight independent determinations with the dengue type 2, type 3, and West Nile ELAs).

TABLE 3. IgM and IgG antibody titers to TBE antigen in sera and in IgG and IgM fractions of four patients with acute TBE infections

Patient code	Days after onset	Titer for: HI[a] Total serum	HI[a] IgM	ELA-IgM
DO I	4	160	320	25,600
DO II	14	640	80	6,400
DO III	22	640	20	800
HN I	2	40	20	400
HN II	4	80	40	800
HN III	7	640	80	1,600
SO I	3	160	n.t.	800
SO II	8	640	n.t.	1,600
SO III	12	640	n.t.	3,200
SH I	5	640	80	6,400
SH II	16	2,560	20	800

[a] IgG was not tested by HI.

TABLE 4. Cross-reactions of IgM antibody titers and HI titers to three different flaviviruses[a]

Patient	Titer for flaviviruses: Antidengue ELA-IgM	Antidengue HI	Anti-West Nile ELA-IgM	Anti-West Nile HI	Anti-TBE ELA-IgM	Anti-TBE HI
Dengue						
WE I	6,400	640	6,400	1,280	Negative (<50)	80
SM I	1,600	>5,120	Negative	>5,120	Negative	1,280
CA I	3,200	1,280	3,200	1,280	Negative	160
HE I	400	20	100	40	Negative	10
SCH I	400	160	400	n.t.	Negative	n.t.[b]
PL I	3,200	640	3,200	n.t.	Negative	n.t.
SC I	3,200	2,560	1,600	n.t.	Negative	n.t.
LI I	12,800	5,120	12,800	5,120	Negative	1,280
KO	12,800	5,120	800	5,120	Negative	640
TBE						
DO I	Negative	Negative	Negative	10	25,600	160
HN I	Negative	Negative	Negative	Negative	400	40
SO I	Negative	10	Negative	20	800	160
SH I	Negative	Negative	Negative	Negative	6,400	640

[a] The results obtained with early serum specimens of nine patients with evidence of dengue fever and of four patients with TBE are shown.

[b] n.t., Not tested.

In our nine patients with acute dengue fever (Table 2) and in the four patients with TBE (Table 3), specific IgM antibody was found in sera taken early after onset of the disease. IgM antibody titers ranged from 1:400 to 1:25,600 in sera of patients. A maximum P/N ratio of 15, corresponding to a titer of 25,600, was obtained with serum LI II (Table 2). Specific IgM antibody seemed to disappear several months after onset of the disease, whereas IgG titers persisted considerably longer. All ELA-IgM tests for IgM antibodies to dengue virus were carried out with two different dengue ELAs (prepared from dengue type 2 and type 3 virus antigens). However, significant differences in the antidengue IgM titers were not observed in the ELA-IgM test when the dengue type 2 ELA was substituted by the dengue type 3 ELA.

Table 4 reflects the cross-reactivity of early dengue and TBE sera, using different flavivirus antigens in the HI and ELA-IgM tests. In the dengue group, the HI antibody titers to the closely related West Nile antigen were almost identical to the dengue HI titers, whereas antibody titers to the TBE antigen were considerably lower (usually one-fourth of the dengue HI titers). Most dengue patients had not only HI but also high IgM antibody titers to West Nile antigen, although exceptions were seen in three cases (SM, HE, and KO; Table 4). Here the much higher titers to dengue antigen seemed to support the suspected diagnosis of dengue fever. In contrast to the results of the HI test, in the ELA-IgM test the dengue sera did not show any cross-reactivity to TBE antigen.

On the other hand, the sera of the TBE patients (Table 4; TBE patients) had only very low HI antibody titers (<40) to the heterologous dengue and West Nile antigens, and in the ELA-IgM test cross-reactions were completely absent.

DISCUSSION

The strong cross-reactivity of HI antibodies of patients with flavivirus infections is well known (15), and from the HI test data it would be impossible to differentiate between acute dengue and West Nile infections. Similar cross-reactions were also observed with flavivirus IgM antibodies in the ELA-IgM test. But in contrast to the HI antibodies, the IgM antibodies of three patients showed an increased specificity for the dengue antigen, and cross-reactions with the heterologous TBE antigen were not observed. Thus the specificity of the ELA-IgM test seems to be better than that of the HI test. To confirm the diagnosis of dengue fever in the patients studied here, we looked for neutralizing antibodies to the different dengue virus serotypes, but it should be kept in mind that the diagnosis of an acute flavivirus infection is helpful to the clinician even when a type-specific virus diagnosis is not obtained.

As a simple alternative for specific IgM antibody detection to flaviviruses, SPIT was used. However, SPIT had a lower sensitivity as compared with the ELA-IgM test. Moreover, with SPIT the exact amount of hemagglutinating virus seemed to be very critical to obtain reproducible results. On the other hand, most of the acute dengue and TBE cases would have been diagnosed by SPIT, which might be helpful when more sophisticated methods are not available.

As previously described (11, 13), peroxidase-labeled antigens can be used with high efficiency for routine IgM antibody detection. An alternative method with unlabeled antigen and labeled antibody was described earlier (1, 3, 4, 6, 7). With this latter method, relatively unpurified antigens can be used for IgM antibody detection, if the specific antigen is in turn detected by a labeled antibody of high specificity. Such highly specific antibodies might be obtained by hybridoma technology. On the other hand, the production of viral proteins by genetic engineering seems to become increasingly popular, which might favor the ELA production. Furthermore, the ELA-IgM test is less complicated and can be carried out more rapidly. Also, the ELA-IgM test is not influenced by the presence of rheumatoid factor, and it is less likely that rheumatoid factor will bind to a labeled antigen than to a labeled antibody or antibody fragment (12). We were able to show here that extensive purification of the ELAs is not required, if an excess of unlabeled control antigen competes with the labeled host material in the ELA and unwanted anticellular reactivity is suppressed. By this approach further ELAs and ELA-IgM tests may easily be developed. In contrast to our TBE studies, our dengue tests were confined to patients from nonendemic areas. Therefore, the significance of the dengue or West Nile ELA-IgM tests for the diagnosis of acute flavivirus infections in endemic areas needs to be further investigated.

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