Serological Differentiation of Infections with Dengue Virus Serotypes 1 to 4 by Using Recombinant Antigens

Diana Ludolfs, Stefan Schilling, Jan Altenschmidt, and Herbert Schmitz*

Bernhard-Nocht Institute for Tropical Medicine, Hamburg, Germany

Received 21 February 2002 /Returned for modification 5 May 2002 /Accepted 4 August 2002

The B domains of dengue virus serotypes 1 to 4 were expressed in *Escherichia coli*. The purified proteins were applied to immunoblot strips to detect serotype-specific antibodies in paired serum samples from 41 patients with primary and secondary dengue infections. A close correlation between the results obtained with the immunoblot strips and by type-specific reverse transcription-PCR (T. Laue, P. Emmerich, and H. Schmitz, *J. Clin. Microbiol.* 37:2543-2547, 1999) was observed.

Four virus serotypes can cause dengue fever, and secondary infections are frequently seen. The laboratory diagnosis of dengue can be carried out by detecting anti-dengue immunoglobulin M (IgM) antibodies, which are produced 3 to 5 days after the onset of the disease (2, 7).

Unfortunately, anti-dengue IgG and IgM antibodies in human sera cross-react with all four dengue virus serotypes (13) and even with other flaviviruses. Serotype differentiation has been possible only by testing immune responses by neutralization tests or type-specific reverse transcription (RT)-PCR (3, 9).

On glycoprotein E (10, 14), a highly dengue virus type-specific antigenic side, the B domain or domain III, has been characterized (17). The short sequence of about 100 amino acids was expressed in *Escherichia coli* for the Japanese encephalitis (JE) virus by Mason et al. (12) and for dengue viruses by Fonseca et al. (4). By use of these antigens, type-specific antibodies were found in mouse hyperimmune sera. The investigations were continued by Simmons et al. (16, 17), who applied a mixture of all four recombinant dengue virus B domains to establish a dengue virus-specific enzyme-linked immunosorbent assay.

Secondary dengue virus infections may be more serious than primary ones (6, 20). Therefore, after a primary dengue virus infection, tourists may want to know which serotype caused the disease. Moreover, a dengue virus serotype-specific antibody test could provide information on the serotypes prevailing in areas in which dengue is endemic. In vaccination studies, type-specific responses could be monitored. We therefore tested serum samples obtained from dengue fever patients and having known serotypes by using B domain antigens.

Samples were collected from European tourists and from patients during a dengue virus serotype 2 epidemic in Hue, Vietnam. Acute-phase serum samples (days 1 to 3 after onset) and follow-up serum samples taken 3 to 6 days after the first ones were obtained. IgM titers were determined for all patients by using a μ -capture enzyme-linked immunosorbent assay (Pan Bio, Brisbane, Australia). Anti-dengue virus IgG titers

were determined by an indirect immunofluorescence test. All patients had clinical signs of acute dengue virus infection. In all 41 patients (7 from Vietnam; 34 tourists), the serotype could be determined by using 5'-nuclease RT-PCR (9). Routine serum samples from an additional 142 dengue fever patients with specific anti-dengue virus IgM and IgG antibodies and samples from 85 healthy individuals without anti-dengue virus antibodies were used to calculate the sensitivity and specificity of the immunoblot assay. Serum samples obtained from four West Nile (WN) virus-infected individuals and containing anti-WN virus IgM antibodies (15) were also included (1).

In contrast to earlier expression strategies (4, 12, 16, 17), our antigens consisted of B domains with His tags for improved purification. For the amplification of the respective B domain coding regions, supernatants of dengue virus serotypes 1 to 4 (9), of WN virus (Wengler strain; SwissProt accession no. P06935), and of JE virus (Nakayama strain; SwissProt accession no. P14403) were available.

The following primers were used (restriction sites are underlined): 5'-ACGGGATCCGTAATGTGCACAGGGTCATTC-3' (dengue virus serotype 1 sense), 5'-ATGGAGCTCAC TGCTTCCCTTCTTGAACCA-3' (dengue virus serotype 1 antisense), 5'-ACGGGATCCCTCATCTCTATGTGCACAGGA-3' (dengue virus serotype 2 sense), 5'-ATGAAGCTTGC CGATAGAAGCTTCCTTCTT-3' (dengue virus serotype 2 antisense), 5'-ACGGGATCCATGAGCTATGCAATGTGCTTG-3' (dengue virus serotype 3 sense), 5'-ATGAAGCTTTT CCCAATCGAGCTTGGCTT-3' (dengue virus serotype 3 antisense), 5'-ACGGGATCCCTCATACAGATGTGCTCAGGA-3' (dengue virus serotype 4 sense), 5'-ATGAAGCTTAAT GGAGCTCCCTTCTCTGAA-3' (dengue virus serotype 4 antisense), 5'-CCCGAATTCGACAACATATGGA-3' (WN virus sense), 5'-CCCCTCGAGAGATTTGTGCCA-3' (WN virus antisense), 5'-ACGGGATCCATGTGTACAGAAAAA TTCTCGTTC-3' (JE virus sense), and 5'-ATGAAGCTTCC AATGGTGGTTGATCTGCTT-3' (JE virus antisense).

The amplified fragments were cut with restriction enzymes and ligated into plasmids pET22b and pQE30 (11). These recombinants were used to transform *E. coli* BL21(DE3)/pLysS and JM109 (both from Novagen, Madison, Wis.), respectively. Bacterial colonies were analyzed for the presence of the B domain gene fragment by restriction enzyme analysis.

* Corresponding author. Mailing address: Department of Virology, Bernhard Nocht Institute for Tropical Medicine, Bernhard Nocht Str. 74, D-20359 Hamburg, Germany. Phone: 49-40-42818-460. Fax: 49-40-42818-378. E-mail: schmitz@bni.uni-hamburg.de.

FIG. 1. Electrophoresis and immunoblotting results. (A) Silver-stained SDS-polyacrylamide gel showing the B domain antigen of dengue virus serotype 4 after purification by Ni-nitrilotriacetic acid affinity chromatography. Lane 1, 1 μ g of purified serotype 4 B domain antigen. Lane 2, molecular weight markers. The faintly visible dimeric band of the antigen (18) is marked with "D." (B) Reactivity of B domain antigens on immunoblot strips with various serum samples having known serotype specificities, as determined by 5'-nuclease RT-PCR. Den 1, dengue virus serotype 1 antigen; Den 2, dengue virus serotype 2 antigen; Den 3, dengue virus serotype 3 antigen; Den 4, dengue virus serotype 4 antigen; WNA, WN virus antigen (control); JE, JE virus antigen (control). Lanes 1, 2, 3, and 4, serum samples from patients with dengue virus serotype 1, 2, 3, and 4 infections, respectively; WN, WN virus antibody-positive serum; Neg., serum without flavivirus antibodies. For improved identification of the various protein bands, the upper rim of each band was marked with a pencil.

Proteins were purified by Ni-nitrilotriacetic acid affinity chromatography under denaturing conditions.

For immunoblotting, samples (200 μ g each) were run on sodium dodecyl sulfate (SDS)-15% polyacrylamide gels (8, 19). The monomer bands were excised and glued together with silicon paste, and the attached nitrocellulose sheet was cut into strips containing six recombinant antigens.

For antibody detection, the strips were stained according to routine procedures (11) by using peroxidase-labeled rabbit anti-human serum.

Upon SDS-polyacrylamide gel electrophoresis (Fig. 1A), a strong single band was visible. Its size varied, depending on the different B domains, from approximately 11 to 14 kDa, consistent with the expected sizes of proteins containing 110 to 120 amino acids and including the vector backbone and the six-His tag. Due to purification, contaminating proteins were only faintly visible in the gel (<1% of the purified protein). Bands consisting of dimeric (22 kDa; marked with "D" in Fig. 1A) and trimeric B domain proteins were observed. Upon blotting of the dengue virus serotype 2 B domain protein, these bands also reacted with type 2-specific anti-dengue virus monoclonal antibody 3H5-1 (ATCC HB-46).

Six antigens were present on each immunoblot strip (Fig. 1B). The WN or JE virus antigen bands served as internal negative controls. With serum samples from patients with proven dengue virus serotype 1, 2, 3, or 4 infections, strong homologous reactions were observed. The WN virus antigen did not react with any samples from dengue patients but reacted with anti-WN virus sera and anti-WN virus monoclonal antibody 15R4.

In 33 patients with primary dengue infections, dengue virus RNA had been demonstrated in the early serum samples by RT-PCR. No IgG antibodies could be detected in these early

samples, but anti-dengue virus IgG and IgM antibodies appeared later in the consecutive samples. The immunoblot results obtained for the late samples are shown in Table 1. All samples from the 18 patients with dengue virus serotype 1 infections, as identified by RT-PCR, reacted with dengue virus serotype 1 antigen. Antibodies to dengue virus serotype 2 were found in 10 out of 11 confirmed dengue virus serotype 2 infections. We found only four individuals with proven dengue virus serotype 3 infections, and in the four late samples, antibodies to dengue virus serotype 3 antigen were present. Primary infections with dengue virus serotype 4 were not identified. A total of 85 serum samples from healthy individuals without antibodies to dengue virus were used as negative controls, including 27 serum samples from yellow fever vaccinees. Only 3 of these serum samples (one in the yellow fever group)

TABLE 1. Immunoblot reactivity of late serum samples from 33 patients with primary dengue virus infections^a

Serotype in 5'-nuclease PCR (no. of patients)	No. of patients with the following immunoblot reactivity:						Cross- reactive	Negative
	Positive for:							
	Den 1	Den 2	Den 3	Den 4	WN			
Den 1 (18)	18	5	1 ^b	1	0	5	0	
Den 2 (11)	1	10	2	0	0	3	1	
Den 3 (4)	1	1	4	0	0	2	0	
Den 4 (0)	0	0	0	0	0	0	0	
None (controls) (85)	0	3	0	0	0	0	82	

^a In the early samples, no IgG antibodies to dengue virus were found, but viral RNA was detected. Den 1, dengue virus serotype 1; Den 2, dengue virus serotype 2; Den 3, dengue virus serotype 3; Den 4, dengue virus serotype 4; WN, WN virus.

^b Strong heterologous reactivity with dengue virus serotype 3 antigen.

TABLE 2. Immunoblot reactivity of early and late serum samples from eight patients with secondary dengue virus infections^a

Patient (origin ^b)	Days of illness	Serotype in 5'-nuclease PCR	Immunoblot reactivity for:			
			Den 1	Den 2	Den 3	Den 4
1 (EU)	4	Den 4	—	—	+++	+++
	42		—	—	—	+++
2 (EU)	4	Den 4	—	—	+++	—
	42		—	—	++	+++
3 (EU)	2	Den 2	—	+	—	++
	36		—	++	—	—
4 (VN)	5	Den 2	—	++	—	+++
	8		—	+++	—	+
5 (VN)	4	Den 2	—	++	++	++
	15		—	+++	++	++
6 (VN)	5	Den 2	—	—	—	+++
	6		—	+	—	+++
7 (VN)	5	Den 2	—	—	+	+
	7		—	—	—	+
8 (VN)	3	Den 2	+	—	+	—
	8		+++	+	+	+

^a In the secondary infections, both dengue virus RNA and anti-dengue virus IgG antibodies were simultaneously present, while IgM antibodies were absent. +, weakly positive; ++, positive; +++, strongly positive; —, negative.

^b EU, European; VN, Vietnamese.

reacted with the dengue virus serotype 2 antigen, while the remaining 82 serum samples did not show any staining with the six antigens applied to the strips.

In 10 of the 33 primary dengue virus infections (Table 1), some cross-reactivity with heterologous dengue virus antigens was observed (30%). However, with only one exception (one patient with dengue virus serotype 1 infection), the cross-reactive band was always weaker than the homologous one. Thus, the correct serotype would have been identified by means of the immunoblot assay for 31 of 33 dengue cases (94%).

In the early samples from eight patients with secondary dengue infections (Table 2), both anti-dengue virus IgG antibodies (titer, ≥ 40) and virus RNA were present simultaneously, while anti-dengue virus IgM antibodies were absent. In addition to the early samples, late samples also were available. Three of the eight patients (patients 1, 2, and 3) were frequent travelers, and five were Vietnamese. Compared to the primary dengue infections, the secondary dengue infections showed clearly broader reactivity; however, some unexpected changes in the heterologous reactions were observed.

To obtain information on the sensitivity of the immunoblot assay for detecting antibodies irrespective of the dengue virus serotype, we tested serum samples from 142 patients with acute dengue fever. With these serum samples, the sensitivity of the immunoblot assay was 89.4%. The sensitivities for detecting serotype-specific antibodies, as calculated from the 41 late samples from the serotyped patients, were 94% for dengue virus serotype 1 (18 patients), 94% for dengue virus serotype 2 (17 patients), and 100% for both dengue virus serotype 3 (only 4 patients) and dengue virus serotype 4 (only 2 patients) antigens.

A total of 82 of 85 samples from healthy individuals without antibodies to dengue virus were negative in the immunoblot assay (96.5% specificity). For obvious reasons, the specificity of the immunoblot assay for detecting individual serotypes was

confined to the primary dengue cases. The specificity of dengue virus serotype 3 antigen detection was 80% (four of five samples correctly detected). In all other serotype reactions, the specificity was 100%, indicating that weak cross-reactions were not interpreted as false-positive results.

The four serotypes of dengue viruses can be identified in early serum samples containing virus RNA. The immunoblot assay may provide a simpler alternative to PCR typing and, in contrast to PCR, can be applied to healthy seropositive individuals. The blots can be stored at 4°C for several weeks, probably because the B domain proteins are very stable (21).

In only one case could the type-specific (dengue virus serotype 1) reaction not be differentiated from the heterologous one (dengue virus serotype 3). This result might have been caused by the high degree of homology (70%) among the B domains of dengue virus serotypes 1 and 3 (4). The cross-reactivity in a primary infection must be differentiated from the multiple reactivity in secondary infections. This multiple reactivity is most likely due to consecutive immunizations with several dengue virus serotypes.

A serotype-specific immunoblot assay could be used to determine the distributions of different dengue virus serotypes in areas of endemicity. Our RT-PCR data on secondary dengue fever patients in Hue, Vietnam, support a major role of dengue virus serotype 2 in this region and are in agreement with recent data on dengue virus serotype 2 isolation in Vietnam (5).

We thank H. G. Zeller (Institut Pasteur, Paris, France) for providing WN-containing serum samples; Le Van An for cooperation at Hue, Vietnam; Thomas Laue and Marcus Panning for testing sera by 5'-nuclease RT-PCR; and Gabriele Rietdorf for excellent technical assistance.

REFERENCES

- Berthet, F. X., H. G. Zeller, M. T. Drouet, J. Rauzier, J. P. Digoutte, and V. Deubel. 1997. Extensive nucleotide changes and deletions within the envelope glycoprotein gene of Euro-African West Nile viruses. *J. Gen. Virol.* 78:2293–2297.
- Bundo, K., and A. Igarashi. 1985. Antibody-capture ELISA for detection of immunoglobulin M antibodies in sera from Japanese encephalitis and dengue hemorrhagic fever patients. *J. Virol. Methods* 11:15–22.
- Deubel, V., M. Laille, J. P. Hugnot, E. Chungue, J. L. Guesdon, M. T. Drouet, S. Bassot, and D. Chevrier. 1990. Identification of dengue sequences by genomic amplification: rapid diagnosis of dengue virus serotypes in peripheral blood. *J. Virol. Methods* 30:41–54.
- Fonseca, B. A., K. Khoshnood, R. E. Shope, and P. W. Mason. 1991. Flavivirus type-specific antigens produced from fusions of a portion of the E protein gene with the *Escherichia coli* trpE gene. *Am. J. Trop. Med. Hyg.* 44:500–508.
- Ha, D. Q., N. T. Tien, V. T. Huong, H. T. Loan, and C. M. Thang. 2000. Dengue epidemic in southern Vietnam, 1998. *Emerg. Infect. Dis.* 6:422–425.
- Halstead, S. B. 1988. Pathogenesis of dengue: challenges to molecular biology. *Science* 239:476–481.
- Innis, B. L., A. Nisalak, S. Nimmannitya, S. Kusalerdchariya, V. Chongswasdi, S. Suntayakorn, P. Puttisri, and C. H. Hoke. 1989. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *Am. J. Trop. Med. Hyg.* 40:418–427.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Laue, T., P. Emmerich, and H. Schmitz. 1999. Detection of dengue virus RNA in patients after primary or secondary dengue infection by using the TaqMan automated amplification system. *J. Clin. Microbiol.* 37:2543–2547.
- Mandl, C. W., F. Guirakhoo, H. Holzmann, F. X. Heinz, and C. Kunz. 1989. Antigenic structure of the flavivirus envelope protein E at the molecular level, using tick-borne encephalitis virus as a model. *J. Virol.* 63:564–571.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Mason, P. W., P. C. McAda, J. M. Dalrymple, M. J. Fournier, and T. L. Mason. 1987. Expression of Japanese encephalitis virus antigens in *Escherichia coli*. *Virology* 158:361–372.

13. Nawa, M., K. I. Yamada, T. Takasaki, T. Akatsuka, and I. Kurane. 2000. Serotype-cross-reactive immunoglobulin M responses in dengue virus infections determined by enzyme-linked immunosorbent assay. *Clin. Diagn. Lab. Immunol.* **7**:774–777.
14. Nowak, T., and G. Wengler. 1987. Analysis of disulfides present in the membrane proteins of the West Nile flavivirus. *Virology* **156**:127–137.
15. Schmitz, H., and P. Emmerich. 1984. Detection of specific immunoglobulin M antibody to different flaviviruses by use of enzyme-labeled antigens. *J. Clin. Microbiol.* **19**:664–667.
16. Simmons, M., G. S. Murphy, and C. G. Hayes. 2001. Antibody responses of mice immunized with a tetravalent dengue recombinant protein subunit vaccine. *Am. J. Trop. Med. Hyg.* **65**:159–161.
17. Simmons, M., K. R. Porter, J. Escamilla, R. Graham, D. M. Watts, K. H. Eckels, and C. G. Hayes. 1998. Evaluation of recombinant dengue viral envelope B domain protein antigens for the detection of dengue complex-specific antibodies. *Am. J. Trop. Med. Hyg.* **58**:144–151.
18. Stiasny, K., S. L. Allison, A. Marchler-Bauer, C. Kunz, and F. X. Heinz. 1996. Structural requirements for low-pH-induced rearrangements in the envelope glycoprotein of tick-borne encephalitis virus. *J. Virol.* **70**:8142–8147.
19. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
20. Vaughn, D. W., S. Green, S. Kalayanarooj, B. L. Innis, S. Nimmannitya, S. Suntayakorn, T. P. Endy, B. Raengsakulrach, A. L. Rothman, F. A. Ennis, and A. Nisalak. 2000. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *J. Infect. Dis.* **181**:2–9.
21. Winkler, G., F. X. Heinz, and C. Kunz. 1987. Characterization of a disulphide bridge-stabilized antigenic domain of tick-borne encephalitis virus structural glycoprotein. *J. Gen. Virol.* **68**:2239–2244.