Antibody Responses to Asian and American Genotypes of Dengue 2 Virus in Immunized Mice

Lidice Bernardo, Adriana Yndart, Susana Vázquez, Luis Morier, and María G. Guzmán*

Virology Department, PAHO/WHO Collaborating Center for Viral Diseases, “Pedro Kouri” Tropical Medicine Institute, Autopista Novia del Mediodía, Havana, Cuba

Received 28 June 2004/Returned for modification 21 September 2004/Accepted 17 November 2004

The possibility of a correlation between dengue virus genotype groups and disease severity is currently under discussion. The objective of this investigation was to identify any immunogenic difference between the American and Asian dengue 2 virus genotypes through the study of antibody development (virus-binding immunoglobulin G and neutralizing antibodies) in mice. Differences in the neutralization pattern between the strains studied were observed, suggesting the presence of slight antigenic variations among them. The lack of recognition of one of the Asian genotype strains was remarkable.

Currently, dengue viruses (Den1 to Den4) are considered the most serious arboviruses pathogens causing morbidity and mortality. The development of a vaccine against these pathogens has been hampered by the need to protect against the four serotypes in order to prevent the risk of the severe form of the disease, dengue hemorrhagic fever (DHF) (13).

Dengue virus strains of a common serotype have been classified into genotypes. In the particular case of Den2 viruses, both the American and Asian genotypes have been associated with distinct clinical presentations in humans. The first, isolated in 1953 in Trinidad and Tobago, has not been associated with DHF epidemics; in contrast, both branches of the Asian genotype have been related to the main DHF epidemics that have occurred in the American regions since 1981 (8, 11).

The relevance of genotypic and antigenic differences among dengue virus strains to disease severity and vaccine efficacy remains unclear. At present several vaccine candidates based on attenuated live or genetically engineered viruses have been developed and tested in human trials; however, the cross-neutralization patterns among dengue virus strains of different serotypes have not been very well established (4). Studies on Japanese encephalitis virus (JEV), a related flavivirus, have also evidenced differences among strains. These previous results suggest that JEV strains differ in their ability to be neutralized by vaccine-induced immunity. Recently the abilities of human, monkey, and rabbit anti-JEV sera to neutralize different wild-type strains of JEV were studied, and large differences in the abilities of these sera to neutralize the panel of wild-type JEV strains were shown (9).

Considering that the presence of specifically neutralizing dengue virus antibodies is one of the most important markers of protection against dengue virus, we proposed to identify any immunogenic difference between the American and Asian Den2 genotypes by studying the antibody patterns (virus-binding immunoglobulin G [IgG] and neutralizing antibodies) in the hyperimmune sera and ascitic fluids obtained from immunized mice.

Three Den2 strains previously isolated from the sera of dengue fever patients were employed in the study: the Cuban strain A15 (1981 epidemic), the Cuban strain 58/97 (1997 epidemic), and strain I348600 (Colombia, 1986). The first two strains have been classified as belonging to the Asian genotype and were isolated during DHF epidemics. The third strain belongs to the American genotype and was associated with mild disease (2, 11, 12). Viruses were grown in C6/36 HT cell cultures, and supernatants were concentrated by 3 h of ultracentrifugation at 80,000 × g and 4°C on a 30% sucrose cushion prepared in phosphate-buffered saline.

Groups of 10 6-week-old female BALB/c mice were immunized by the intraperitoneal route with a single immunization dose of live virus (10⁶ PFU) and an equivalent antigen titer from each strain. Animals were bled retro-orbitally 7 days post-immunization. Twenty-three days after immunization, mice were inoculated with 0.5 ml of a suspension of Sarcoma 180 tumor cells. Hyperimmune mouse ascitic fluid (HMAF) was collected 10 days later. Both sera and HMAF were tested individually.

The levels of IgG binding antibodies were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described (5). Neutralizing antibody titers were determined by a plaque reduction neutralization technique (PRNT) on BHK21 cells as previously described by Morens et al. (10). End point titration was calculated by using probit analysis. The serum dilution resulting in 50% plaque reduction was considered the end point titer. In both assays, ELISA and PRNT, culture supernatants of the three Den2 strains were used as antigens. Geometric mean titers (GMT) of antibodies were calculated, and data were analyzed by Fisher’s test using Epi Info, version 6.04a (text, databases, and statistical process for public health; Centers for Disease Control and Prevention, Atlanta, Ga.).

The reciprocals of GMT of antibodies determined by ELISA both in serum samples and in HMAF are shown in Table 1. Serum samples obtained from mice immunized with Den2 strain A15 showed similar GMT of antibodies to all of the strains studied. In contrast, serum samples obtained from animals immunized with strain 58/97 or I348600 showed the highest titers to the homologous strain. In both groups of serum
samples, the difference among titers of antibodies to the three Den2 strains tested was statistically significant ($P < 0.05$). Titers observed in HMAF from 58/97-immunized mice were highest; however, the antibodies induced were cross-reactive to the heterologous Den2 strains.

In spite of the antibody titers detected by ELISA, no neutralizing antibody was detected at a 1/10 serum dilution. This phenomenon could be related to the time required for antibody maturation (6).

Table 2 shows the reciprocals of GMT in HMAF as detected by PRNT. Low titers of neutralizing antibodies were observed. In order to improve the specificity of the antibody response, only one immunization dose was used for each animal. This could be the cause of the low neutralizing-antibody titers obtained. Two patterns of neutralization were observed: a specific antibody response to the homologous virus was observed in mice immunized with strain A15, and a heterologous antibody response was observed in mice immunized with either strain 58/97 or strain I348600. In Den2 58/97 immunized mice, the differences among titers of antibodies to the three Den2 strains tested were statistically significant ($P < 0.001$).

It is noteworthy that no neutralizing antibodies to strain 58/97 were detected in any of the HMAF studied. Cecilia and Gould reported the existence of JEV mutant strains that could not be recognized by homologous or heterologous neutralizing antibodies (1). The possibility that strain 58/97 could represent an escape mutant virus deserves further study, considering the hypothesis that argues the rise of natural escape mutants during dengue epidemics in order to explain the rapid increase in several severity markers were noted (3).

Kochel et al. first reported a one-way cross-strain neutralization pattern in the American and Asian Den2 genotypes. Seventeen serum samples, from individuals probably infected with the American Den2 genotype, neutralized four Den2 strains of both genotypes with variable titers (7). The cross-strain neutralization patterns in serum samples from individuals first infected by a Den2 Asian genotype (strain A15) are being studied; preliminary results suggest a higher specificity for the infecting virus (M. G. Guzman et al., unpublished data). These observations are in agreement with the neutralization pattern observed in our study in serum samples from mice inoculated with strain A15.

The differences observed by our approach could be related to specific antigenic differences among Den2 strains. Recently, Leit Meyer et al. (8) reported some important amino acid differences in the membrane and envelope proteins of Den2 strains of the two genotypes. Neutralizing epitopes are expected to occur on both structural proteins. The Cuban strains employed in this study contain the amino acid changes proposed by Leit Meyer et al. to be important for pathogenicity (Guzman et al., unpublished).

In conclusion, the differences observed in the neutralization pattern suggest the presence of slight antigenic differences among the strains studied; the lack of recognition of the Den2 strain 58/97 is remarkable. These differences could be important for vaccine development. Studies using sera from Den2-immunized monkeys and immune individuals to evaluate the humoral response to different Den2 genotype are under way.

### REFERENCES

1. Cecilia, D., and E. Gould. 1991. Nucleotide changes responsible for loss of neuroinvasiveness in Japanese encephalitis virus neutralization-resistant mutants. Virology 181:70–77.

2. Guzman, M. G., V. Deubel, J. L. Pelegri, D. Rosario, M. Marrero, C. Saroli, and G. Kouri. 1995. Partial nucleotide and amino acid sequences of the envelope and nonstructural protein-1 gene junction of four dengue 2 virus strains isolated during the 1981 Cuban epidemic. Am. J. Trop. Med. Hyg. 52:241–246.

3. Guzman, M. G., G. Kouri, and S. B. Halstead. 2000. Do escape mutants explain rapid increases in dengue case fatality rates within epidemics? Lancet 355:1902–1903.

4. Halstead, S. B., and J. Deen. 2002. The future of dengue vaccines. Lancet 360:1243–1245.

5. Hermita, C., M. Pupo, M. G. Guzman, G. Gonzalez, and R. Marcer. 1992. Empleo de un anticuerpo monoclonal anticomplejo dengue en la purificación viral. Rev. Cub. Med. Trop. 44:171–176.

6. Janeway, C. A., and P. Travers. 1994. Host defense against infection, p. 931–942. In M. Robertson, R. Ward, E. Lawrence, et al. (ed.), Immunobiology: the immune system in health and disease, 1st ed. Blackwell Scientific Publications, London, United Kingdom.

7. Kochel, T. J., D. M. Watts, S. B. Halstead, C. G. Hayes, A. Espinoza, V. Felices, R. Caceda, C. T. Bautista, Y. Montoya, S. Douglas, and K. L. Russell. 2002. Effect of dengue-1 antibodies on American dengue-2 viral infection and dengue haemorrhagic fever. Lancet 360:310–312.

8. Leit Meyer, K. C., D. W. Vaughn, D. M. Watts, R. Salas, I. Villalobos, C. Ramos, and R. Rico-Hesse. 1999. Dengue virus structural differences that correlate with pathogenesis. J. Virol. 73:4738–4747.

9. Mohanty, T., I. Levenbook, K. Sulke, Z. X. Zhang, M. Ratterree, K. Draper, A. D. T. Barret, R. Nichols, R. Weltzin, J. Arroyo, and F. Guirakhoo. 2000. Chimeric yellow fever virus 17D-Japanese encephalitis virus vaccine: dose-response effectiveness and extended safety testing in thersus monkeys. J. Virol. 74:1742–1751.

10. Morens, D. M., S. B. Halstead, P. M. Repik, R. Putvata, and N. Raybourne. 1985. Simplified plaque reduction neutralization assay for dengue viruses by semimicro method in BHK-21 cells: comparison of the BHK suspension test with the standard plaque reduction neutralization. J. Clin. Microbiol. 22:250–254.

11. Rico-Hesse, R., M. L. Harrison, R. A. Salas, D. Tovar, A. Nisalak, C. Ramos, J. Boshell, M. T. R. Mesa, M. R. Nogueira, and A. T. D. Rosa. 1997. Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 230:244–251.

12. Rosario, D., M. Alvarez, S. Vazquez, N. Amin, R. Rodriguez, K. Valdez, and M. G. Guzman. 2001. Application of molecular methods to the diagnosis and characterization of a dengue outbreak in Cuba. Bioterrorism. Apr 18:201–203.

13. Stapoli, L. M. P. Frenkiele, F. Megret, and V. Deubel. 1997. Affinity-purified dengue 2 virus envelope glycoprotein induces neutralizing antibodies and protective immunity in mice. Vaccine 15:1946–1954.