

Immunoglobulin G Avidity in Differentiation between Early and Late Antibody Responses to West Nile Virus

Janet L. Fox,¹ Stuart L. Hazell,^{1*} Leslie H. Tobler,² and Michael P. Busch²

Panbio Ltd., Brisbane, Australia,¹ and Blood Systems Research Institute, San Francisco, California²

Received 15 April 2005/Accepted 24 October 2005

In 1999 West Nile virus (WNV) surfaced in the United States in the city of New York and spread over successive summers to most of the continental United States, Canada, and Mexico. Because WNV immunoglobulin M (IgM) antibodies have been shown to persist for up to 1 year, residents in areas of endemicity can have persistent WNV IgM antibodies that are unrelated to a current illness with which they present. We present data on the use of IgG avidity testing for the resolution of conflicting data arising from the testing of serum or plasma for antibodies to WNV. Thirteen seroconversion panels, each consisting of a minimum of four samples, were used. All samples were tested for the presence of WNV IgM and IgG antibodies, and the avidity index for the WNV IgG-positive samples was calculated. Panels that exhibited a rise in the WNV IgM level followed by a sequential rise in the WNV IgG level were designated “primary.” Panels that exhibited a marked rise in the WNV IgG level followed by a sequential weak WNV IgM response and that had serological evidence of a prior flavivirus infection were designated “secondary.” All samples from the “primary” panels exhibited low avidity indices (less than 40%) for the first 20 to 30 days after the recovery of the index sample (the sample found to be virus positive). All of the “secondary” samples had elevated WNV IgG levels with avidity indices of $\geq 55\%$, regardless of the number of days since the recovery of the index sample. These data demonstrate that it is possible to differentiate between recent and past exposure to WNV or another flavivirus through the measurement of WNV IgG avidity indices.

West Nile virus (WNV) is a member of the family Flaviviridae, which includes viruses that cause St. Louis encephalitis (SLE), yellow fever, Japanese encephalitis, and dengue fever. In 1999 West Nile virus surfaced in the United States in the city of New York (15). The virus has spread over successive summers to most of the continental United States and has entered both Canada and Mexico. Disease associated with WNV infection is commonly mild and self-limiting; however, more severe disease is observed in a subset of infected patients. The elderly are at the greatest risk, with mortality associated with severe neurological manifestations, including meningitis, encephalitis, and acute flaccid paralysis syndrome (15). Severe manifestations of infection in children may be associated with raised intracranial pressure and seizures.

The detection of immunoglobulin M (IgM) antibodies to WNV antigen is the most commonly used diagnostic method (3). While molecular biology techniques are available, serology remains important, for although the onset of symptoms is preceded by a period of viremia, this viremia persists for only a short period and disease usually manifests after the virus is no longer detectable. The classic primary immune response to an antigen results in an increased IgM titer followed by a rise in the IgG titer over the ensuing days to weeks. With WNV infection, however, WNV IgM levels have been shown to remain elevated for up to 1 year (3). Such persistent WNV IgM has also been reported in cerebrospinal fluid from WNV-infected patients presenting with central nervous system disease (12). While the absence of a WNV IgG response in the pres-

ence of WNV IgM is invariably an accurate marker of very recent WNV infection, the presence of specific WNV IgM and WNV IgG per se provides limited clinical information as to the time since infection. In this context there is a question about the utility of detectable WNV IgM in serum as a marker of primary infection due to the persistence of the WNV IgM response.

Avidity testing for the differentiation between a primary and an enduring antibody response to infectious disease was introduced in 1984 when a simple and reliable method was developed by using enzyme-linked immunosorbent assay (ELISA) and a mild protein-denaturing agent (10). The term avidity (or functional affinity) denotes the net antigen-binding force of populations of antibodies. Immunoglobulin G avidity is low after primary antigenic challenge but increases progressively during subsequent weeks and months due to affinity maturation and antigen-driven B-cell selection. Avidity assays have been used to differentiate between acute or primary infection and persistent infection, recurrent infection, or reactivated disease in a number of infections such as human immunodeficiency virus (17), parvovirus B19 (8), *Toxoplasma* (6), cytomegalovirus (1), measles virus (13), varicella-zoster virus (16), rubella virus (5), and tick-borne encephalitis virus (7) infections. Avidity assays have also been assessed for their utility in the analysis of vaccine failure (9, 14).

We present data on the use of IgG avidity testing to resolve conflicting data arising from the testing of serum or plasma for antibodies to WNV.

MATERIALS AND METHODS

Plasma panels. Thirteen seroconversion panels were obtained from the Blood Systems Research Institute, San Francisco, Calif. (2). Each panel consisted of between four and seven plasma samples that had been serially collected from

* Corresponding author. Mailing address: Panbio Limited, 532 Seventeen Mile Rocks Road, Sinnamon Park, Queensland 4073, Australia. Phone: 617 3363 7100. Fax: 617 3363 7199. E-mail: stuart_hazell@panbio.com.

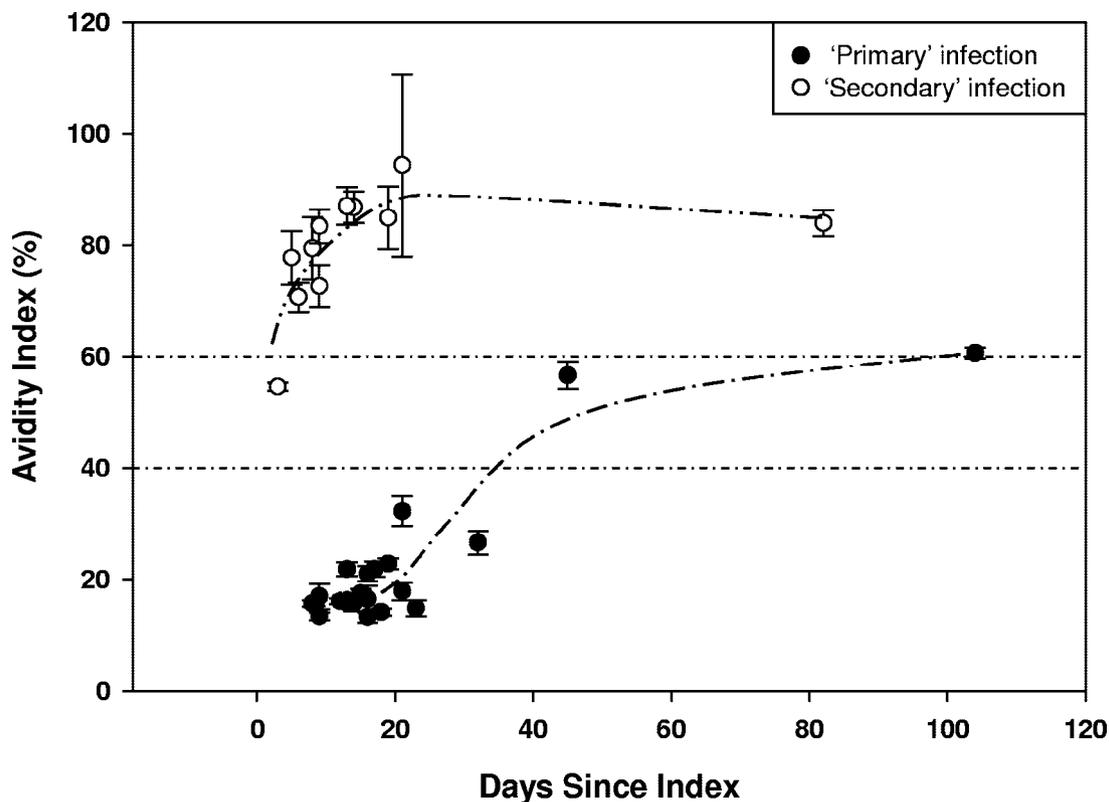


FIG. 1. Plots of the avidity test results for plasma samples exhibiting “primary” IgG profiles and “secondary” IgG profiles from samples where there is evidence of an anamnestic response. Plots show the mean \pm 1 standard deviation for each sample tested.

U.S. blood donors. The index sample (day 0) was viremia positive by a WNV transcription-mediated amplification (TMA) assay and antibody nonreactive when it was tested for WNV IgM. The next sample was drawn less than 10 days after the index sample was obtained. Each panel also contained at least two other follow-up specimens taken at various intervals ranging from day 10 to day 208 after collection of the index sample. All samples were independently tested by the Centers for Disease Control and Prevention (CDC) by a plaque reduction neutralization test (PRNT) that targeted a range of flaviviruses.

West Nile virus IgM capture ELISA and IgG ELISA. The samples were run on a Panbio WNV IgM capture ELISA (catalog no. E-WNV02M) and a Panbio WNV IgG ELISA (catalog no. E-WNV01G), according to the manufacturer’s instructions. The WNV IgM assay required the predilution of an antibody-antigen horseradish peroxidase (HRP) tracer before commencement of the assay. The WNV IgG assay contained ready-made HRP-conjugated anti-human IgG antibody. For both assays, 100 μ l of test samples, controls, and calibrator sera diluted 1:100 was added to the individual wells of microtiter plates. The plates were incubated at 37°C for 1 h for the WNV IgM assay and for 30 min for the WNV IgG assay and then washed six times with a phosphate-buffered saline (PBS)-Tween wash buffer provided with the assay kits. One hundred microliters of the appropriate preprepared antibody-antigen tracer (IgM assay) or HRP-conjugated anti-human IgG antibody (IgG assay) was added to each well, and the plates were incubated and washed as described above. The plates were developed by the addition of 100 μ l of tetramethylbenzidine to each well, and the color was allowed to develop for 10 min before the reaction was stopped by the addition of 100 μ l of a stop solution. The plates were read immediately at a wavelength of 450 nm with a reference filter of 600 to 650 nm. The samples tested for IgM were run in duplicate, and the samples tested for IgG were run in triplicate. All equivocal samples were retested.

West Nile Virus avidity IgG ELISA. The avidity assay was carried out by the WNV IgG ELISA procedure, except for the following steps. After incubation with diluted patient plasma, the avidity plate was washed twice with the PBS-Tween wash buffer. One hundred microliters of Buffered Avidity Reagent (catalog no. O-AVR02G; Panbio) was then added to each well and the plate was incubated for 5 min at room temperature. The plate was then washed a further six times, as described for the standard ELISA, before the HRP-conjugated

anti-human IgG was added to the plate. The rest of the assay was carried out as described above for the WNV IgG ELISA. Samples tested by the avidity assay were run in triplicate.

Calculation of results. The absorbance obtained for each test plasma sample by the WNV IgG ELISA was designated absorbance A. The absorbance obtained for each test plasma by the WNV avidity IgG ELISA was designated absorbance B. The avidity index (in percent) was calculated by using the following formula: (absorbance B/absorbance A) \times 100.

An avidity index was calculated only for samples that tested positive by the standard IgG ELISA (absorbance A). The mean and standard deviation of the triplicates were calculated.

Avidity assay precision. Interassay variation was determined by repeat testing of 13 samples from the original plasma panels, chosen on the basis of their positivity by the WNV IgG assay. Three different operators, who used the same kit lot number, ran each sample on three consecutive days, and the coefficient of variation was calculated.

Ethics. All individuals involved in this study gave informed consent, and the Ethics Committee of the Blood Systems Research Institute approved the protocol for sample collection and analysis.

RESULTS

The WNV IgG and IgM profiles for each panel from individuals were reviewed. Panels that exhibited a rise in the IgM titer followed by a sequential rise in the IgG titer were designated “primary” to indicate a classical primary response to infection. Panels that exhibited IgG in the index RNA-positive specimen or a marked rise in IgG reactivity followed by a sequential weak IgM response were designated “secondary”; this is consistent with an anamnestic response associated with a previous WNV infection or an infection with another flavi-

TABLE 1. Interassay variation data for 13 WNV IgG-positive samples

Parameter	Sample no.												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Range of avidity indices	52–66	15–22	18–24	11–18	13–18	18–26	48–59	58–66	59–69	59–75	58–72	60–78	62–74
Mean avidity index	59	18	21	14	16	21	55	62	64	68	66	69	68
Standard deviation	4.25	2.25	1.72	2.07	1.67	3.05	4.02	2.89	3.67	4.55	4.47	6.12	3.94
Coefficient of variation (%)	7	12	8	15	11	14	7	5	6	7	7	9	6

virus. This classification was confirmed by the PRNT data from CDC.

Nine of the panels exhibited antibody profiles that led to the designation of a “primary” response. Those samples with a primary response were PRNT positive for WNV (data not shown). Three of the panels exhibited an antibody profile indicative of a “secondary” response. The PRNT data from CDC showed that these specimens had high titers for SLE virus, despite the detection of WNV in the index sample (data not shown). This is consistent with an anamnestic response resulting from prior exposure to SLE virus. One panel exhibited an early IgG response that was determined to be nonspecific, and this panel was eliminated from further analysis.

When the IgG avidity profiles were plotted for WNV IgG-positive samples from the panels of individuals with a “primary” response, all samples from the first 20 to 30 days after recovery of the index sample (TMA positive and IgM negative on primary donation) exhibited low avidity indices (less than 40%). For these panels, ~40 days after recovery of the index sample, the avidity index rose to over 40% (Fig. 1). For the “secondary” samples (WNV positive by TMA and SLE virus positive by PRNT), all samples positive for IgG had avidity indices of $\geq 55\%$, regardless of the number of days since the index sample donation (Fig. 1).

The interassay variation data are presented in Table 1. With three different operators who used the same kit lot number and who ran each of the test samples on three consecutive days, the coefficient of variation ranged from 5% to 15%.

DISCUSSION

The primary immune response to an antigen classically results in an increase in the IgM titer followed by a rise in the IgG titer. Once antibody has been produced, primed B memory cells remain in circulation. These cells are responsible for the anamnestic or memory response to the same antigen due to subsequent infection. This secondary response is generally characterized by a predominantly IgG response. Avidity testing of positive IgG samples, where a subject presents with a positive IgM response and a positive IgG response, has been established to be of utility in the differentiation of recent primary infection from the presence of residual or persistent antibody or antibody to the antigen from a secondary infection (4–11).

Avidity assays have been used for the serodiagnosis of a number of viruses that are members of the Flaviviridae, including hepatitis C virus (HCV) and dengue fever virus. In patients with primary HCV infection, low-avidity antibody was detected within 50 days of seroconversion, whereas in patients

with long-term HCV infection (300 days after seroconversion), the mean avidity was significantly higher (19). A recent study confirmed those earlier data, with a significant difference in avidity being found between patients with primary HCV infection ($7.7\% \pm 6.8\%$) and those with either chronic infections ($77.0\% \pm 21.8\%$) or past infections ($44.5\% \pm 12.6\%$) (11). A similar result was seen with dengue patients, with the mean avidity index for the group with secondary infection ($50\% \pm 18.0\%$) being significantly higher than that for the group with primary infection ($12\% \pm 4.6\%$) (4).

The cutoff for the definition of a recent infection based on a low avidity index varies depending on the infectious agent, the disease state, the formulation of the avidity reagent, and the ELISA used. Narita et al. (13, 14) suggests a low-avidity cutoff of $<30\%$ for both measles and mumps patients when 8 M urea is used. This shows alignment with the results shown for HCV (7.7 to 24%) (11, 18) and dengue fever ($12\% \pm 4.6\%$) (5). Based on the data presented here, a WNV IgG avidity index of $\leq 40\%$ for samples positive for WNV IgG and IgM supports a presumption of recent primary infection with WNV (<20 days). Samples with an intermediate IgG avidity index (between 40 and 60%) may indicate primary infection with WNV within a period of from ~30 to 60 days earlier. A high avidity index ($>60\%$) suggests infection on the order of ~40 days or greater in a patient with a primary WNV infection or an anamnestic response resulting from a previous flavivirus infection. These time periods are indexed against the time of donations for the detection of viremia and would presumably be 5 to 10 days after the infectious mosquito bite and 3 to 5 days prior to IgM seroconversion.

Although more data are needed to refine the time periods over which the WNV IgG response changes from low avidity to high avidity, it is apparent that a WNV avidity assay will prove to have utility both for the detection of WNV as the etiologic agent in clinical cases and in seroepidemiologic studies in settings where prior WNV or cross-reactive flavivirus infections confound the interpretation of the results of standard IgM and IgG serological assays.

REFERENCES

- Baccard-Longere, M., F. Freymuth, D. Cointe, J. M. Seigneurin, and L. Grangeot-Keros. 2001. Multicentre evaluation of a rapid and convenient method for determination of cytomegalovirus immunoglobulin G avidity. *Clin. Diagn. Lab. Immunol.* 8:429–431.
- Busch, M. P., S. Caglioti, E. F. Robertson, J. D. McAuley, L. H. Tobler, H. Kamel, J. M. Linnen, V. Shyamala, P. Tomasulo, and S. H. Kleinman. 2005. Screening the blood supply for West Nile virus RNA by nucleic acid amplification testing. *N. Engl. J. Med.* 353:460–467.
- Centers for Disease Control and Prevention. 29 September 2004. Information and guidance for clinicians. West Nile virus: clinical description. [Online.] <http://www.cdc.gov/ncidod/dvbid/westnile/clinicaldescription>.
- De Souza, V. A. U. F., S. Fernandes, E. S. Araujo, A. F. Tateno, O. M. N. P. F. Oliveria, R. D. R. Oliveira, and C. S. Pannuti. 2004. Use of an immuno-

- globulin G avidity test to discriminate between primary and secondary dengue virus infections. *J. Clin. Microbiol.* **42**:1782–1784.
5. **Enders, G., and F. Knotek.** 1989. Rubella IgG total antibody avidity and IgG subclass-specific antibody avidity assay and their role in the differentiation between primary rubella and rubella reinfection. *Infection* **17**:218–226.
 6. **Flori, P., L. Tardy, H. Patural, B. Belleste, M. Varlet, J. Hafid, H. Raberin, and R. T. M. Sung.** 2004. Reliability of immunoglobulin G antitoxoplasma avidity test and effects of treatment on avidity indices of infants and pregnant women. *Clin. Diagn. Lab. Immunol.* **11**:669–674.
 7. **Gassmann, C., and G. Bauer.** 1997. Avidity determination of IgG directed against tick-borne encephalitis improved detection of current infection. *J. Med. Virol.* **51**:242–251.
 8. **Gray, J. J., B. J. Cohen, and U. Desselberger.** 1993. Detection of human parvovirus B19-specific IgM and IgG antibodies using a recombinant viral VP1 antigen expressed in insect cells and estimation of time of infection by testing for antibody avidity. *J. Virol. Methods* **44**:11–24.
 9. **Hidaka, Y., T. Aoki, H. Akeda, C. Miyazaki, and K. Ueda.** 1994. Serological and clinical characteristics of measles vaccine failure in Japan. *Scand. J. Infect. Dis.* **26**:725–730.
 10. **Inouye, S., A. Hasegawa, S. Matsuno, and S. Katow.** 1984. Changes in antibody avidity after virus infections: detection by an immunosorbent assay in which a mild protein-denaturing agent is employed. *J. Clin. Microbiol.* **20**:525–529.
 11. **Kanno, A., and Y. Kazuyama.** 2002. Immunoglobulin G antibody avidity assay for serodiagnosis of hepatitis C virus infection. *J. Med. Virol.* **68**:229–233.
 12. **Kapoor, H., K. Signs, P. Somsel, F. P. Downes, P. A. Clark, and J. P. Massey.** 2004. Persistence of West Nile virus (WNV) IgM antibodies in cerebrospinal fluid from patients with CNS disease. *J. Clin. Virol.* **31**:289–291.
 13. **Narita, M., S. Yamada, Y. Matsuzono, O. Itakaura, T. Togashi, and H. Kikuta.** 1996. Immunoglobulin G avidity testing in serum and cerebrospinal fluid for analysis of measles virus infection. *Clin. Diagn. Lab. Immunol.* **3**:211–215.
 14. **Narita, M., Y. Matsuzono, Y. Takekoshi, S. Yamada, O. Itakaura, M. Kubota, H. Kikuta, and T. Togashi.** 1998. Analysis of mumps vaccine failure by means of avidity testing for mumps virus-specific immunoglobulin G. *Clin. Diagn. Lab. Immunol.* **5**:799–803.
 15. **Nash, D., F. Mostashari, A. Fine, J. Miller, D. O'Leary, K. Murray, A. Huang, A. Rosenberg, A. Greenberg, M. Sherman, S. Wong, and M. Layton.** 2001. The outbreak of West Nile virus in the New York City area in 1999. *N. Engl. J. Med.* **344**:1807–1814.
 16. **Ono, E., M. M. Lafer, L. Y. Weckx, C. Granato, and M. I. de Moraes-Pinto.** 2004. A simple and cheaper in house varicella zoster virus antibody indirect ELISA. *Rev. Inst. Med. Trop. Sao Paulo* **46**:165–168.
 17. **Parekh, B. S., M. S. Kennedy, T. Dobbs, C.-P. Pau, R. Byers, T. Green, D. J. Hu, S. Vanichseni, N. L. Young, K. Choopanya, T. D. Mastro, and J. S. McDougal.** 2002. Quantitative detection of increasing HIV type 1 antibodies after seroconversion: a simple assay for detecting recent HIV infection and estimating incidence. *AIDS Res. Hum. Retrovir.* **18**:295–307.
 18. **Prince, H. E., and A. L. Leber.** 2002. Validation of an in-house assay for cytomegalovirus immunoglobulin G (CMV IgG) avidity and relationship of avidity to CMV IgM levels. *Clin. Diagn. Lab. Immunol.* **9**:824–827.
 19. **Ward, K. N., W. Dhaliwal, K. L. Ashworth, E. J. Clutterbuck, and C. G. Teo.** 1994. Measurement of antibody avidity for hepatitis C virus distinguishes primary antibody responses from passively acquired antibody. *J. Med. Virol.* **43**:367–372.