Use of Immunoglobulin G Avidity Assays for Differentiation of Primary from Previous Infections with West Nile Virus

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Since its introduction in 1999, West Nile virus (WNV) infections have spread rapidly across the North American continent. Diagnosis of acute WNV infection by detection of WNV-specific immunoglobulin M (IgM) is complicated by the persistence of detectable IgM for more than 1 year in some patients. IgG antibody avidity testing was assessed as a supplemental assay in the diagnosis of current infections. Three groups of serum samples were assayed in parallel by two different IgG avidity test systems (indirect immunofluorescence test [IIFT] and prototype enzyme-linked immunosorbent assay [ELISA]; EUROIMMUN, Luebeck, Germany). Group I (40 sera taken between 2 and 9 days after the onset of influenza-like symptoms) and group II (40 sera taken between 10 and 43 days after onset) were acute and convalescent specimens from patients with a positive anti-WNV IgM test (ELISA; Focus Diagnostics, Cypress, CA). Group III consisted of 43 patient sera collected between 6 and 12 months after infection. IgG antibodies specific for WNV were detected in 38% (ELISA) and 50% (IIFT) of group I sera, in 90% (ELISA and IIFT) of group II sera, and in 100% (ELISA and IIFT) of group III sera. Low-avidity IgG antibodies were demonstrated in 86% (ELISA) and 95% (IIFT) of IgG-positive patient samples taken between 2 and 43 days after the onset of symptoms (groups I and II). High-avidity IgG antibodies were detected in 100% of group III sera obtained 6 months or more after the onset of symptoms (ELISA and IIFT). IgG avidity tests for WNV infections are rapid and simple to perform. The determination of IgG avidity provides additional diagnostic certainty in differentiating between recently acquired and previous infections with WNV.

West Nile virus (WNV) has spread rapidly across North America since it was first detected in New York in 1999 (6, 8, 12, 14, 15). Although WNV was first detected in Canada in 2001, the first cases of human illness were not identified until 2002 (5). In 2003, large outbreaks of WNV-associated illness occurred in the Midwestern United States (2) and the prairie provinces of Canada (http://www.phac-aspc.gc.ca/wnv-vwn/pdf _sr-rs_2004/surveillance_table_042904_hm.pdf). As a result, a significant proportion of the population of Saskatchewan seroconverted during the 2003 mosquito season. In the southwestern region of the province, approximately 10% of the population were seropositive when surveyed in the spring of 2004 (7), most of whom had no history of WNV infection.

The diagnosis of WNV infection has relied heavily upon the detection of immunoglobulin M (IgM) antibodies in blood (or in the cerebrospinal fluid in cases of neurological infection). Unfortunately, IgM antibodies are persistent for more than 1 year in some patients (17). This finding has significant implications for the differentiation of recent or current infection with WNV from persistent seropositivity derived from the previous WNV season. The ability to identify immune responses persisting from previous years may be important for focusing public health efforts to control acute outbreaks. It is often difficult to obtain convalescent samples from patients after mild illness; thus, it may not be possible to confirm recent

infection by detection of seroconversion using a hemagglutination (HA) inhibition (HI) assay and a plaque reduction neutralization test (PRNT). We investigated the use of IgG avidity to exclude historical infection as a cause of IgM reactivity.

MATERIALS AND METHODS

Serum specimens. Three groups of specimens, representing recent and previous infections, were studied. These included 80 sera from patients whose symptoms were consistent with a diagnosis of acute WNV infection, comprised of group I (40 sera taken between 2 and 9 days after the onset of influenza-like symptoms) and group II (40 sera taken between 10 and 43 days after onset). Group III contained 43 sera collected 6 to 12 months after exposure from patients known to have been infected with WNV in the summer of 2003.

WNV IgM ELISA. IgM antibodies against WNV were detected using an IgM capture enzyme-linked immunosorbent assay (ELISA) (Focus Diagnostics, Cypress, CA), as described by the manufacturer. Reactive sera were retested following a background subtraction procedure (9) to eliminate false-positive IgM results.

WNV IgG ELISA and HI assay. Serosurvey samples were screened for the presence of flavivirus IgG antibody using a monoclonal antibody-based capture enzyme-linked immunosorbent assay as previously described (10). The HI assay was performed on selected serum samples essentially as described previously (3). In brief, dilutions of acetone-treated serum were mixed with 8 HA units of suckling mouse WNV antigen and incubated at 4°C overnight. Goose erythrocytes were then added to the mixture, and the solution was incubated for an additional hour at room temperature. The HI titer was determined as the highest dilution of serum that caused complete inhibition of erythrocyte agglutination by 8 HA units of viral antigen.

Plaque reduction neutralization test. ELISA/HI-reactive samples were confirmed for the presence of WNV-specific antibody by carrying out a PRNT. The standard PRNT was performed essentially as described previously (1). In brief, mixtures of virus (200 PFU) and dilutions of human sera were incubated at 37°C

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TABLE 1. Detection of IgG antibodies and IgG avidity in patients with recent or previous W NV infection

Patient group (no. of samples)	Days after onset of symptoms	No. of IgG-positive samples (%)		No. of low-avidity samples (%)[a]	
		IIFT	ELISA	IIFT	ELISA
Recent infection					
Acute (40)	2–9	20 (50)	15 (38)	18 (90)	13 (87)
Convalescent (40)	10–43	36 (90)	36 (90)	35 (97)	31 (87)
Previous infection (43)	180–360	43 (100)	43 (100)	0	0

[a] Percentage of IgG-positive samples.

for 1 h and added to six-well plates containing Vero cell monolayers. After a 37°C incubation of 1 h, a nutrient agar overlay was added, and the plates were placed in a CO_2 incubator for approximately 3 days, after which a second overlay containing neutral red as a vital stain was added. Plates were then checked periodically over the next few days for plaque formation. The highest serum dilution with a plaque reduction of at least 90% was defined as the titration end point.

IgG avidity IIFT. Sera were tested using an indirect immunofluorescence test (IIFT) (Euroimmun AG, Luebeck, Germany). Briefly, 25 μl of serum diluted 1:10 in buffer was added to each of two reaction fields on a BIOCHIP slide. Each field contained two BIOCHIPS, one with WNV-infected cells and the second with uninfected cells. After incubation at room temperature for 30 min, cells were exposed to either 6 M urea solution or to phosphate-buffered saline–Tween for 10 min. After washing with phosphate-buffered saline–Tween, cells were incubated with fluorescein-labeled anti-human globulin for 30 min and then viewed at ×20 magnification using a 488-nm excitation filter and a 520-nm blocking filter. In a specific positive reaction, the cytoplasm of infected cells demonstrated fine to coarse granular fluorescence and inclusion bodies in some cells. The presence of low-avidity IgG antibodies was inferred by the absence or significant reduction of fluorescence of cells treated with urea compared with the buffer-treated controls.

IgG avidity ELISA. Sera were tested using a prototype ELISA (Euroimmun AG, Luebeck, Germany). Briefly, 100 μl of serum diluted 1:100 in buffer was added to each of two wells coated with WNV. After incubation for 60 min at 37°C, wells were exposed to either 6 M urea solution or to phosphate buffer for 10 min. After washing, wells were incubated with peroxidase-labeled anti-human IgG for 60 min at 37°C, substrate was added, and the reactions were stopped after 15 min by the addition of 100 μl stop solution per well. The reactions were read immediately at a wavelength of 450 nm using a reference wavelength of 650 nm. A relative avidity index (RAI) was calculated for each specimen and was expressed as the percentage of reactivity remaining in the urea-treated well. Samples with RAIs of <40% were regarded as having low avidity, and those with RAIs of >60% were regarded as having high avidity.

RESULTS

IgM antibodies were detected in all 80 specimens from acutely ill patients with WNV infection, and the 43 patients with previous infections were diagnosed by detection of IgM

and IgG antibodies and of specific neutralizing antibodies by PRNT. IgG antibodies were detected by IIFT in 20 samples (50%) from acutely ill patients, rising to 90% of convalescent samples (Table 1). The IgG ELISA was slightly less sensitive than the IIFT, and only 15 (38%) of the acute specimens were IgG positive by ELISA. In the convalescent samples, both methods detected IgG antibodies in 36 patients (90%). In the specimens collected 6 months or more after the onset of symptoms, IgG seroprevalence was 100% using both IIFT and ELISA methods.

Among the IgG-positive acute and convalescent samples, the great majority were of low avidity (Table 1). The number of low-avidity samples was highest in the convalescent samples, reflecting the higher prevalence of IgG in this group. In addition, the number of low-avidity samples was greater using the IIFT than the ELISA because of the higher sensitivity of the IIFT.

When tested using the IgG ELISA, there was very little difference in the proportion of IgG-positive samples showing low avidity, but the IgG ELISA appeared to be less sensitive than the IIFT for detection of IgG (Table 1). No samples from previously infected subjects showed low avidity (Fig. 1), although a few specimens from recently infected patients exhibited high avidity indices.

DISCUSSION

IgM antibodies to WNV have been shown to persist for at least a year and for up to 500 days in some patients (17). It is not known whether persistence for longer periods occurs and, if so, if it occurs in sufficient proportions of cases and at titers high enough to be clinically confusing. Since IgM persistence
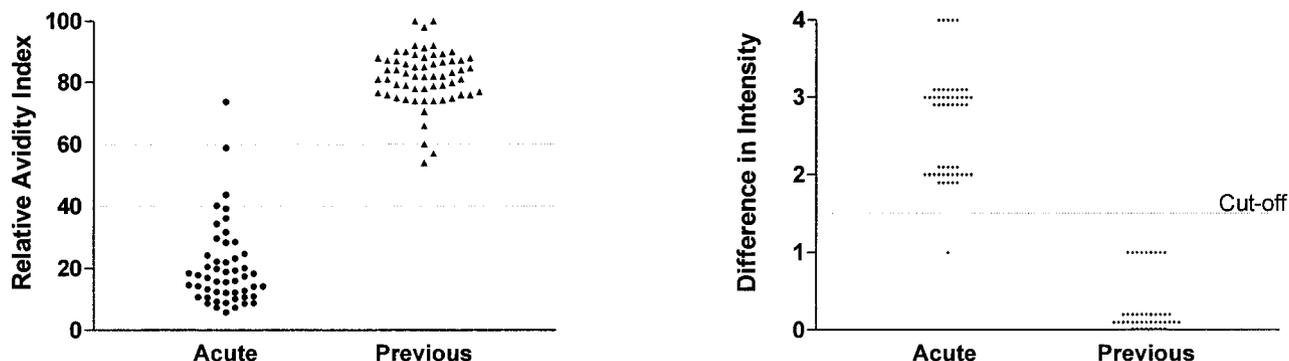

FIG. 1. Difference in relative avidity index between recent and previous infection detected by ELISA (left) and difference in intensity of fluorescence in IIFT (reduction of fluorescence of cells treated with urea compared with the buffer-treated cells) (right).

may be problematic when current cases of WNV infection are identified using serological assays such as the IgM ELISA, other approaches may need to be considered. Documentation of seroconversion using acute- and convalescent-phase sera has been the "gold standard" for demonstrating a current infection; however, convalescent samples are not always available, and rises in antibody titer may be missed if acute samples are collected too late after exposure. Several approaches have been used to differentiate recent from previous flavivirus infection. Detection of IgA antibodies in patients with recent, but not previous, WNV infection has also been reported (16); further studies are needed to determine the duration of IgA persistence and its diagnostic value in helping to characterize the time frame of exposure.

IgG avidity has been employed to distinguish primary and secondary dengue virus infections (4, 13) with sensitivity similar to those of the assays described in this study. In the early stages of the immune response, IgG antibodies are produced against a wide range of antigenic epitopes, producing an IgG response of relatively low specificity, which results in low IgG avidity. Thus, the detection of low-avidity IgG after a recent febrile illness, in conjunction with a positive IgM ELISA, is presumptive evidence of an acute or recent WNV infection.

In contrast, as the immune response matures and B-cell clonal selection occurs, the resulting IgG antibodies are more specific, and the IgG avidity increases. In this study, all sera tested 6 months or more after exposure to WNV contained high-avidity IgG antibodies. Thus, our results indicate that individuals exposed to WNV during the preceding mosquito season will in almost all cases demonstrate high-avidity IgG antibodies if tested the following spring.

Antibodies to flaviviruses are cross-reactive, but cross-reactivity is somewhat less pronounced in immunofluorescence assays than in ELISA formats (11). Moreover, in many cases, differentiation is possible using a flavivirus BIOCHIP mosaic (dengue, St. Louis encephalitis, tick-borne encephalitis, and yellow fever viruses) with the determination of antibody profiles. A very small proportion of specimens from recent infections showed high IgG avidity (Fig. 1). It is not known whether cross-reacting flavivirus antibodies will result in a high-avidity result, leading to a recent WNV infection being misclassified as a previous infection, but this possibility requires further study.

The Euroimmun prototype IgG ELISA appeared to be less sensitive than the IIFT for detection of IgG early after the onset of symptoms. However, there was minimal difference between ELISA and IIFT methods for avidity determination. An ELISA using a more purified antigen, in order to increase the sensitivity of IgG detection and reduce cross-reactivity, is in development.

The value of avidity determination will be great in years following large outbreaks of WNV infection, as occurred in Saskatchewan in 2003. Weather conditions in Western Canada during the summer of 2004 limited vector development and activity, resulting in very few cases of WNV infection. Thus, most of the positive IgM tests recorded in 2004 were reflective of previous infections. Epidemiological investigation of these cases consumed much time and effort, which might have been saved if the capacity to test for the presence of high-avidity IgG antibodies had existed.

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