Antibody Responses Induced by Experimental West Nile Virus Infection with or without Previous Immunization with Inactivated Japanese Encephalitis Vaccine in Horses

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ABSTRACT. A group of horses immunized with inactivated Japanese encephalitis (JE) vaccine (JE-Immune Group) and a group of non-immunized horses (Non-Immune Group) were infected with West Nile virus (WNV). After WNV infection, neutralizing (Nt) antibody (Ab) titers to WNV were higher than those to JE virus (JEV) in the Non-Immune Group, but the NtAb titers to JEV were higher than those to WNV during most of the post-challenge observation period in the JE-Immune Group. Immunoglobulin M (IgM) Abs to WNV tested positive in the Non-Immune Group but negative in the JE-Immune Group, except for in one horse. These results suggest that diagnosis of WNV infection in JE-immunized horses requires serological tests for NtAb and IgM titers to both WNV and JEV.

KEY WORDS: antibody response, equine, Japanese encephalitis vaccine, serological tests, West Nile virus.

West Nile virus (WNV) is an emerging flavivirus that is transmitted predominantly by Culex mosquitoes [13]. WNV infection in horses is usually subclinical, but an increased incidence of neurological disease has been observed in recent outbreaks [3]. Affected horses display clinical signs such as weakness, ataxia and recumbency as a result of spinal cord nerve damage [3, 25]. Furthermore, abnormal behavior, dysmetria, teeth grinding, facial nerve paralysis and dysphagia have been observed as a result of brain damage due to WNV infection [3, 25]. Cases of equine infection have been reported during epidemics, or outbreaks, of WNV in many countries, including Italy [1], France [16], Canada [26] and the United States [17], and thus WNV infection has become a major concern of the horse industry.

In most of Asia, there has been no WNV detected thus far, but introduction of the virus is conceivable due to transportation and migration of birds from WNV-endemic areas. In addition, many potential WNV vector species inhabit Japan and nearby Asian countries [21, 22, 24], and our recent study indicated that jungle crows (Corvus macrorhynchos) could serve as amplifying hosts [18], which suggests that the virus could spread rapidly upon introduction into East Asia. Therefore, introduction of the virus is a serious concern for the horse industry. Instead of WNV, an antigenically related flavivirus, Japanese encephalitis (JE) virus (JEV), has been widely disseminated in Asia [13]. In Japan, which is part of a JEV-endemic area, horses have been mostly immunized with inactivated JE vaccine [4, 19]. Immunization with inactivated and live attenuated JE vaccines has been shown to induce cross-reactive immune responses to WNV in mice and hamsters, respectively [20, 23].

In the present study, we sought to evaluate antibody (Ab) responses to WNV and JEV in horses that were immunized with inactivated JE vaccine and subsequently infected with WNV. Our hope is that evaluation of Ab responses will provide useful information for diagnosis of WNV-infected horses in JEV-endemic areas in the case of WNV introduction into these areas.

Eight 1- to 2-year-old horses of varying breeds were used in this study. Neutralizing Abs (NtAbs) to WNV and JEV were tested by using a 50% plaque-reduction neutralization test (PRNT50) [20], and all eight horses were confirmed to be negative for both viruses. The horses were moved to a biosafety level 3 facility on the day before either the WNV or placebo challenge. At the end of the experimental periods, all the horses were euthanized with an overdose injection of pentobarbital and ketamine hydrochloride administered intravenously. All experimental procedures and animal care was performed in compliance with the guidelines of the National Institute of Animal Health for the humane use of laboratory animals. The eight horses were randomly divided into three groups: the Non-Immune
Group (Horses 1–3) was challenged with WNV without prior immunization with JE vaccines; the JE-Immune Group (Horses 4–6) was immunized with an inactivated tissue culture JE vaccine (BM III strain; Nisseiken, Tokyo, Japan) and then subsequently challenged with WNV; and the Control Group (Horses 7–8) was immunized with the JE vaccine and challenged with a placebo (non-infected cell culture fluid). The JE-Immune and Control groups were immunized twice via an intramuscular injection in the neck over a 4-week period. Four weeks after the second immunization, the JE-Immune and Control groups were challenged with WNV and placebo, respectively. The JE-Immune and Non-Immune groups were inoculated subcutaneously in the neck with 10⁷ plaque-forming units (PFU) of the WNV NY99 strain (originally isolated from a horse, provided by the Diagnostic Virology Laboratory, National Veterinary Service Laboratories, U.S. Department of Agriculture) diluted in 1.0 ml of a maintenance medium [18]. Each horse was examined for signs associated with WNV infection, and rectal temperatures were recorded twice a day from the day of challenge. All the horses were euthanized and necropsied at 21 days post-challenge (DPC). Blood samples were collected at 0–10, 14 and 21 DPC for detection of WNV RNA and Abs in the sera. The blood samples were centrifuged at 800 g for 10 min, and the separated sera were then collected. At necropsy, samples for WNV RNA detection were collected from the cerebrum, cerebellum, midbrain, pons, medulla oblongata, spinal cord, heart, liver, kidney, lung, spleen, cerebrospinal fluid and peritoneal fluid. All the above samples were maintained at –80°C until they were tested. Tissues required for histopathology and immunohistochemical assay were collected from the same organs as the ones for WNV RNA detection, as well as from the pituitary gland, pancreas, trachea, adrenal gland, gonads, urinary bladder, skeletal muscle, skin, thymus, tonsil, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon and rectum. The tissues were fixed in 10% neutral buffered formalin.

After the challenge with WNV, ataxia was observed in two horses in the Non-Immune Group, Horses 1 (3 DPC) and 2 (3–11 DPC), and one horse in the JE-Immune Group, Horse 4 (4 DPC). The clinical signs were generally slight, but severe hindlimb ataxia was observed at 10 DPC in Horse 2. Horses 3 and 5–8 did not show any clinical signs during this study. No significant increases in rectal temperature were observed in any of the horses.

TaqMan reverse transcription polymerase chain reaction assay indicated the presence of WNV RNA in the serum samples collected from two horses in the Non-Immune Group, Horses 1 and 2 (0.3–2.2 and 0.2–3.3 PFU equivalents, respectively), at 3–7 DPC and from a horse in the JE-Immune Group, Horse 6 (0.7–2.0 PFU equivalents), at 1 and 3 DPC [10, 18]. All the other serum samples tested negative. In addition, all the tissue samples were negative for WNV RNA.

The results of PRNT₅₀ using the NY99 strain of WNV and JaGAr01 strain of JEV are shown in Fig. 1. In the Non-Immune Group, all of the horses developed NtAbs to WNV and JEV after the WNV challenge, and the titers to WNV and JEV were 1:640–1:1280 and 1:20–1:80, respectively (14 DPC). In the JE-Immune Group, immunization resulted in all of the horses developing NtAbs to JEV with titers of
1:20–1:320, and 2 of the horses also developed NtAbs to WNV with titers of 1:20 and 1:5 (Horses 4 and 6, respectively), while Horse 5 had no detectable NtAbs to WNV (0 DPC). After the challenge with WNV, NtAb titers to WNV and JEV were greatly increased in two horses of the JE-Immune Group (Horses 5 and 6). The NtAb titers to WNV at 14 DPC were 1:1280 in both Horses 5 and 6, and the titers to JEV were 1:2560 and 1:5120, respectively. Meanwhile, in Horse 4, the increases in NtAbs were less than those in Horses 5 and 6, and the titers to WNV and JEV were 1:40 and 1:640, respectively (14 DPC). In the Control Group, the NtAbs to JEV were 1:640 and 1:1280 in Horses 7 and 8, respectively, and the NtAbs to WNV were 1:10 in both horses at 0 DPC. The titers of NtAbs to each virus at 7, 14 and 21 DPC were less than or equal to those at 0 DPC, except that the NtAb titer to JEV at 21 DPC (1:1280) exceeded the titer at 0 DPC in Horse 7 (1:640).

The results of immunoglobulin M (IgM) Ab-capture enzyme-linked immunosorbent assay (MAC-ELISA) are shown in Fig. 2 [12, 14]. WNV-specific IgM Abs were detected in all the horses in the Non-Immune Group from 6 or 7 DPC to 21 DPC. The serum samples of Horse 5 at 9, 10 and 14 DPC were positive for IgM Abs to WNV (positive-to-negative (P/N) values were 2.2, 2.9 and 2.4, respectively), but all the other samples in the JE-Immune Group tested negative for the IgM Abs. IgM Abs to JEV tested positive in Horse 1 at 10 DPC (P/N value was 2.2), but all the other horses in the Non-Immune and JE-Immune groups tested negative.

Histopathology and immunohistochemical assays were performed as described previously [18], and no histological lesions were detected in any of the tissue samples collected from any of the groups. In addition, all the tissue samples tested negative for WNV antigens.

WNV has not been detected in most of Asia where JEV is endemic, and it has been unknown whether cross-reactive immune responses to WNV can be induced by using JE vaccine in horses. Several studies have evaluated the potential of JE vaccines to induce cross-reactive immune responses to WNV in vivo by using mice [20], hamsters [23] and monkeys [5] and in vitro by using human specimens [6, 27]; cross-reactive immune responses have been indicated in most of the studies. Such immune responses have not been evaluated in horses, and this has been a major concern for the horse industry in Asian countries, especially in Japan, where racehorses have some economic importance. Although the studies just described were performed mainly to determine whether JE vaccination has any protective effects against heterologous flavivirus infection, it is noteworthy that the authors of the study using hamsters suggested the difficulty in making a serologic diagnosis of WNV infection in animals [23]. This difficulty is also conceivable in horses, and thus, in the present study, we sought to evaluate Ab responses in horses by PRNT and MAC-ELISA, with the goal of providing useful information for making serologic diagnoses in horses. As described in the manuals for prevention of WNV infection created by the Ministry of Agriculture, Forestry and Fisheries of Japan [15], PRNT and MAC-ELISA can be used to make a WNV diagnosis in horses, but discrimination of WNV infection from other flavivirus infections and vaccinations is also required.

In the present study, specific antibody responses to WNV were observed in the Non-Immune Group, but they were not significantly observed in the JE-Immune Group. In the Non-Immune Group, all the horses developed NtAbs and IgM Abs to WNV, and the titers of the Abs to WNV were higher than those to JEV. In the JE-Immune Group, NtAb
responses after WNV infection were observed, but the NtAb titers to JEV were also increased after WNV infection, and the titers to JEV were higher than or equal to those to WNV at 0, 5–10, 14 and 21 DPC, except for in Horse 5 at 9 and 10 DPC. Similar results were observed in a study by Lim et al. [11], where mice were immunized with inactivated JE vaccine and subsequently infected with WNV. The mice developed significant NtAbs to JEV and WNV after WNV infection, and the titers to WNV and JEV were equal in some of the mice. In our study, IgM Abs to WNV all tested negative in the JE-Immune Group, except for in Horse 5 at 9, 10 and 14 DPC. Similar results were observed in a hamster model study; hamsters that were immunized with attenuated JE vaccine and subsequently infected with WNV developed minimal IgM Abs to WNV [23]. Our results suggested that the immune responses induced by the WNV challenge in the JE-Immune Group were not specific to WNV, but mainly boosted responses primed by the inactivated JE vaccine.

In the present study, two horses in the Non-Immune Group and one horse in the JE-Immune Group showed ataxia. One of these horses, Horse 2 in the Non-Immune Group, showed ataxia for 9 days and developed severe hind-limb ataxia at 10 DPC. In contrast, Horse 4 in the JE-Immune Group showed ataxia for only 1 day. The time during which WNV RNA was detected in the sera was relatively shorter in the JE-Immune Group than in the Non-Immune Group; WNV RNA was detected for 5 days in two of the three horses in the Non-Immune Group and was detected for 2 days in one of the three horses in the JE-Immune Group. These results suggest that viremia and illness caused by WNV infection might be milder in horses previously immunized with inactivated JE vaccine than in horses not immunized with the vaccine; however, our data is not adequate to indicate cross-protection because of the small group size. It should be noted that WNV infection in horses often results in a subclinical infection [3] and that only one horse showed clinical signs in experimental WNV infection of twelve horses [2]. Also, viremia levels in WNV-infected horses are often low and sometimes undetectable, as seen previously in an experimentally infected horse [2] that was similar to Horse 3 in the Non-Immune Group in the present study. Further experiments with larger group sizes will be required to determine whether cross-protection occurs in horses. In addition, we should mention that Horse 4 exhibited ataxia at 4 DPC without detectable WNV RNA in the serum in the present study. This horse had cross-reactive NtAb to WNV (1:20) at 0 DPC, and the Ab might have prevented viremia, but the horse exhibited clinical signs. We cannot explain the reason for this phenomenon in the present study, and further investigation will be needed.

Histological lesions, WNV RNA and WNV antigen were not detected in any of the infected horses at necropsy. Histological lesions and WNV were detected in the central nervous systems of horses that showed polioencephalomyelitis and other neurological disorders due to WNV infection [3]; however, only transient ataxia was observed in horses in the present study, and thus, the histological lesions and WNV dissemination in these horses might have been limited or undetectable at 21 DPC.

Diagnosis of WNV infection would be possible in some horses that have previously been immunized with JE vaccine and recently infected with WNV. High NtAb titers to JEV and WNV would be observed if JEV-immunized horses are naturally infected with WNV. In this case, testing for NtAbs and IgM Abs would be important for discrimination between WNV and JEV infections. If a horse tests positive for NtAbs to both viruses, infection with both viruses should be suspected, while the presence or absence of IgM Abs to the viruses in this case could indicate whether the horse has recently been infected with either virus. If a horse tests positive for IgM Abs to either virus, the result would indicate recent infection of the virus, like Horse 5 in the present study, which tested positive for IgM Abs to WNV at 9, 10 and 14 DPC. In addition, infection with JEV or WNV may induce IgM Abs, which react to both of the viruses in some JEV-immunized horses. In such horses, it would be important when making a diagnosis to draw a comparison between the P/N values of the IgM Abs to both viruses. Thus, testing NtAbs and IgM Abs to JEV and WNV in parallel would be essential for a WNV diagnosis in horses immunized with JE vaccine.

Combined with clinical observations, pathological tests and epidemiological considerations, serological tests could confirm WNV infection; however, some horses may not develop IgM Abs to WNV despite infection, like Horses 4 and 6 in the present study. For WNV diagnosis in such cases, new serological diagnostic methods with higher specificity, such as epitope-blocking ELISA and complement-dependent cytotoxicity assay [7, 8], need to be developed.

In the present study, the small group size and individual differences in Ab responses prevented evaluation of the statistical significance of the differences noted between the non-immunized and JE-immunized horses, though our results do suggest that making serological diagnoses would be possible in some cases, such as in the case of Horse 5 in this study, but would be impossible in other cases, such as in the case of Horses 4 and 6.

Although the present study results suggested that immunization with a primary series of JE vaccine can induce cross-reactive Ab against WNV infection, two things should be considered in relation to Japan; many horses over 1 year age have been immunized with inactivated JE vaccine annually prior to the epidemic season of JEV, and natural JEV infection has been occurring among horses [9]. Thus, it is conceivable that many horses in Japan have higher cross-immunity against WNV than the horses in the present study, and horses in other countries in JEV-endemic areas might also have higher cross-immunity than our study horses. Further experiments will be required to evaluate cross-reactive immunity by using horses that have been immunized annually with JE vaccine and kept in a JEV-endemic area for several years. Such studies would provide valuable information.
for not only serological diagnosis but also for prevention of WNV infection in horses in the case of WNV introduction into a JEV-endemic area.

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