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Research Paper

Experimental Infection of Common Garter Snakes (*Thamnophis sirtalis*) with West Nile Virus

AMIR STEINMAN,[1] CAROLINE BANET-NOACH,[2] LUBOV SIMANOV,[2] NURIT GRINFELD,[1] ZACHI AIZENBERG,[1] OHAD LEVI,[1] DAN LAHAV,[3] MERTYN MALKINSON,[2] SHIMON PERK,[2] and NAHUM Y. SHPIGEL[1]

ABSTRACT

The role of various reptilian species in the infectious cycle of several arboviruses is documented, but their role in that of West Nile virus (WNV) is uncertain. Common garter snakes (*Thamnophis sirtalis*) were infected subcutaneously with 10^5 plaque forming units (PFU) WNV-Isr 98, five of nine snakes became viremic, and five exhibited persistent low levels of neutralizing antibodies. Four of the parentally infected snakes died and high titers of virus were found in multiple organ samples. In contrast, orally infected garter snakes did not become viremic, but viral RNA was detected in cloacal swabs. Since oral infection of predator birds by WNV is known, their ingestion of infected snakes may also result in their becoming infected. Key Words: West Nile virus—Common garter snake—*Thamnophis sirtalis*—Experimental infection—Viremia—Neutralizing antibodies—Reverse transcriptase–polymerase chain reaction. Vector-Borne Zoonotic Dis. 6, 361–368.

INTRODUCTION

THE ROLE OF VARIOUS reptilian species in the ecology of arboviruses, including Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV), western equine encephalitis virus (WEEV), eastern equine encephalitis virus (EEEV), and Venezuelan equine encephalitis virus (VEEV), has been documented (Hayes et al. 1964, Whitney et al. 1968, Shortridge et al. 1974, Bowen 1977, Thomas et al. 1980, Oya et al. 1983, Walder et al. 1984), but their role in that of West Nile virus (WNV) is uncertain.

WNV is a mosquito-borne flavivirus affecting mainly humans, horses, and birds. Birds are the natural reservoir hosts, and WNV is maintained in nature in a mosquito-bird-mosquito transmission cycle (Campbell et al. 2002). In addition to birds, reptiles have been proposed as possible reservoirs, and in 2001–2002, two epizootics of WNV infection occurred among captive alligators (*Alligator sp*.) on a South Georgia alligator farm, affecting hundreds of animals (Miller et al. 2003). This was one of the first reports of the virus in American reptiles and highlighted their possible role in its life cycle. WNV antibody in reptiles was first reported in Israel in 1965–1966, when 22 reptiles and 96 amphibians were tested by hemagglutination-inhibition against several arboviruses, including

[1]Koret School of Veterinary Medicine, Faculty of Agricultural, Food and Environmental Quality Sciences, Hebrew University of Jerusalem, Rehovot, Israel.
Departments of [2]Avian Diseases and [3]Pathology, Kimron Veterinary Institute, Beit Dagan, Israel.

WNV and one turtle (*Clemmys caspica*) was found seropositive for WNV (Nir et al. 1969). In 2002, we reported that, of 20 healthy farmed crocodiles (*Crocodylus niloticus*) examined serologically, 14 had neutralizing antibodies (Steinman et al. 2003). In experimental infections with WNV, low viremic levels were detected in green iguanas (*Iguana iguana*) (Klenk and Komar 2003), and high titered viremias were detected in American alligators (*Alligator mississippiensis*) (Klenk et al. 2004). Viremias sufficiently high to infect *Culex quinquefasciatus* mosquitoes were detected in naturally infected American alligators, reinforcing the possible role of alligators in the infectious cycle of WNV (Jacobson et al. 2005). Experimental infection of Florida garter snakes (*Thamnophis sirtalis sirtalis*) with 2,000 plaque forming units (PFU) of WNV did not cause viremia, but WNV was detected in spleens of three snakes killed 12 days post-inoculation (PI) (Klenk and Komar 2003).

The purpose of the present study was to determine whether the common garter snake (*Thamnophis sirtalis*) is susceptible to WNV when infected with high doses of WNV. We determined the duration of viremia following subcutaneous and oral inoculation, the distribution and titers of virus in their tissues, and the formation of neutralizing antibodies. Oral inoculation study was conducted in order to determine to what extent this route of infection is important, despite the fact that garter snakes very unlikely to be exposed to WNV via diet.

METHODS

Animals

Eighteen common garter snakes were acquired from a commercial source, provided with food and water *ad libitum*, and maintained at constant laboratory conditions (28°C) under a regime of 12-h light/dark cycles (Vagvolgyi and Halpern 1983). Snakes were fed frozen whole fish, slices of fillets, and occasionally goldfish. The snakes were given an acclimatization period of 2 weeks before the start of the experiment. The study was approved by the animal experimentation committee of the Kimron Veterinary Institute, Beit Dagan, Israel.

Experimental design

In order to determine the infectious dose, initially two groups of three garter snakes each, were inoculated subcutaneously with either 10^3 (Table 1; snakes no. 1–3) or 10^5 (snakes no. 4–6)

Table 1. Isolation of West Nile Virus (WNV) from the Serum and Presence of WNV Neutralizing Antibody of Infected Garter Snakes (Days Post-Infection)

Snake no.	*Dose and route of infection*	*Viremia*[a]	*Neutralizing antibody*[a]
1	10^3 PFU/SC	Negative	>17 days
2	10^3 PFU/SC	Negative	Negative
3	10^3 PFU/SC	Negative	Dead (day 9)
4	10^5 PFU/SC	Day 9	>17 days
5	10^5 PFU/SC	Days 2–13	Dead (day 13)
6	10^5 PFU/SC	Negative	>17 days
7	10^5 PFU/SC	Negative	Dead (day 3)
8	10^5 PFU/SC	Day 2	>16 days
9	10^5 PFU/SC	Negative	>18 days
10	10^5 PFU/SC	Negative	Dead (day 2)
11	10^5 PFU/SC	Days 2–4	Dead (day 24)
12	10^5 PFU/SC	Days 2, 3	>21 days
13	10^5 PFU/PO	Negative	Negative
14	10^5 PFU/PO	Negative	Dead (day 13)
15	10^5 PFU/PO	Negative	>28 days
16	10^5 PFU/PO	Negative	Negative
17	10^5 PFU/PO	Negative	Negative
18	10^5 PFU/PO	Negative	Negative

[a]Day PI positive.
SC, subcutaneous; PO, *per os*; PFU, plaque-forming units.

PFU of a WNV isolated from a goose (WN-Isr98) in 1 mL of Eagle's medium (Malkinson et al. 2002). Blood samples were collected from each snake before infection for serological and virological examination, and daily, PI for seven consecutive days, and then every two to three days for 3 weeks. The snakes were bled by puncture of the ventral tail vein, and one drop of blood was collected on a filter paper disk. Snakes were observed daily for clinical signs for one month PI. Dead snakes were necropsied, and all organs removed aseptically and kept frozen at −70°C until further examination.

In a second study, six garter snakes were inoculated orally using a stomach tube (Table 1; snakes no. 13–18), and six subcutaneously (snakes no. 7–12) with 10^5 PFU WNV in 1 mL of Eagle's medium. Blood samples were collected as before, the last one at 7 weeks PI. Cloacal swabs were obtained concurrently using cotton-tipped applicators, and placed in 0.15 mL of phosphate buffered saline (PBS) to resuspend the sample. The snakes were inspected daily for clinical signs for 2 months. Dead snakes were necropsied, and all organs were removed aseptically and kept frozen at −70°C until further examination.

Serology

Serum was eluted from the filter paper disks by addition of 50 μL of PBS per disk, the sample was then vortexed and incubated at room temperature for one hour. Prior to testing the sample was centrifuged for 1 min at 6000*g*. Thirty microliters of the sample was immediately extracted for RNA, and the remainder was stored at −70°C until examination by virus neutralization as previously described (Banet-Noach et al. 2003). For the latter, doubling dilutions of the eluate from 1/10 to 1/1280 were made in 96-well plates in 50 μL Eagle's medium containing 5×10^5 PFU/mL WNV. The plates were allowed to stand for 1 h at room temperature. Confluent Vero cells were trypsinized 1:4 in Eagle's medium containing with 8% fetal calf sera (FCS), and 50 μL of cells were added to each well, the plates were placed at 37°C in 5% CO_2/95% air for 4 days, and the cells inspected daily for cytopathic effect (CPE). The neutralizing antibody titer was calculated as the highest dilution at which complete neutralization of CPE was observed. The results are presented in terms of dilution rather than conventional units because of the very small amounts of serum which were collected.

Organ sample preparation

Organs were extracted by passage through a 2.5-mL syringe and a 19-gauge needle. The extracts were suspended 1:4 in PBS, homogenized, vortexed vigourously for 1 min, and clarified by centrifugation at 1000*g* for 5 min. The supernatant was filtered through a 0.22-μm filter, and the filtrate was either inoculated on Vero cell monolayers (100 μL) or treated for RNA extraction (200 μL). The remaining extracts were stored at −70°C.

RNA extraction and direct reverse transcriptase–polymerase chain reaction

RNA extraction from the sera was performed with RNEasy kit (Qiagen Gmbh, Hilden, Germany) according to the manufacturer's protocol and resuspended in 30 μL of RNAse-free water. Cloacal swabs in PBS were clarified by centrifugation for 2 min at 6000*g* prior to removing 100 μL of the supernatant for RNA extraction. Trizol (Invitrogen, Jerusalem, Israel) was used to extract RNA from the cloacal swabs and the organ extracts according to the manufacturer's protocol, the RNA was resuspended in 30 μL of RNAse-free water. RT-PCR was performed as described previously (Banet-Noach et al. 2003).

Virus isolation

Vero cell cultures in 12-well plates were inoculated with organ filtrates. 90% confluent Vero cell monolayers were gently washed with PBS. 100 μL of filtrate was added to each well and held for 1 h at room temperature. Maintenance medium (Eagle's medium + 5% FCS without antibiotics) was then added, and the culture maintained for 3 days at 37°C in 5% CO_2/95% air, then inspected daily for 4 additional days for CPE. The expected CPE for WN is rounding up and lifting of the cells from the plastic surface, at it appears usually after more than 4 days post-infection (Banet-

Noach et al. 2003). Still samples were considered positive only on the basis of the described CPE appearance and it was not confirmed by other tests. Organs were considered negative after two further passages. Titration of virus in the positive organs was performed by diluting the original filtrate twofold ranging from 1/10 to 1/20,480 in maintenance media in 96-well plates similarly as for virus isolation. Briefly, Vero cells were plated in 96-well plates, and inoculated the following day with 50 μL of the diluted sample, allowed to stand for 1 h at room temperature, and then 50 μL of fresh media was added. Plates were observed daily for 1 week or until the appearance of CPE.

To detect viremia, 96-well plates, each well containing 100 μL of Vero cells suspension were inoculated with 10 μL of eluted sera in the first well and then with doubling dilution of the sera (1/20 to 1/1280). A preliminary experiment using experimentally infect geese was perform in order to test the validity of the method, and showed no significant reduction of the viral titer in the eluted sera (data not shown). Dilutions, rather than other methods, were used because of the small amounts of sera which were available. Plates were placed at 37°C in 5% CO_2/95% air for 3 days, and inspected daily for 4 additional days for CPE. Medium was removed from the wells where CPE was observed. After 7 days, the plates were frozen and two further passages were made on the supernatant of the first well into 12-well plates similarly to the virus isolation procedure for the organ extract.

Histology and immunohistochemistry

Only samples from one snake that was inoculated with 10^5 PFU and died on day 13 PI were examined histologically and by immunohistochemistry (IHC). The samples were fixed in 10% neutral-buffered formalin for histopathologic examination. Formalin-fixed tissues were trimmed, embedded in paraffin, sectioned at 5 μm and processed for microscopic examination. IHC for WNV was performed on selected organs following a standard peroxidase-anti-peroxidase method with amplification by the EnVision+™ system (Dako Corp., Carpinteria, CA), using a mouse monoclonal anti-WNV antibody (mice 8907; Pasteur Institute, Paris, France) (Perl et al. 2002).

RESULTS

Clinical observations

Following subcutaneous inoculation of three snakes with 10^3 PFU of WNV, one died (no. 3) on day 9 PI without noticeable signs. Following subcutaneous inoculation with 10^5 PFU, four (no. 5, 7, 10, 11) of nine snakes died; two (no. 7, 10) on days two and three PI without clinical signs, and two (no. 5, 11) died later with clinical signs. Of these two snakes, snake no. 5 died on day 13 PI after showing unusual behavior: aggression and immobility of the caudal part of the body. The second snake (snake no. 11) died on day 24 after showing weakness and cachexia, which might be attributed to inappetance. One snake (no. 14) of the group of six inoculated *per os* with 10^5 PFU died on day 13 PI without showing clinical signs. All the other snakes remained clinically normal during the experimental period.

Serology

None of the snakes had neutralizing antibodies during the first two weeks after inoculation. One (no. 1) of the two surviving snakes following inoculation with 10^3 PFU of virus developed low level antibodies, first detected on day 17 PI (Table 1). Five of the six snakes inoculated subcutaneously with 10^5 PFU, and were alive three weeks PI, exhibited persistent low levels of neutralizing antibodies (Table 1). Only one snake (no. 15) of the group inoculated *per os* had low levels of neutralizing antibodies from day 28 PI onwards (Table 1).

Viremia

None of the snakes inoculated subcutaneously with 10^3 PFU or *per os* with 10^5 PFU became viremic. Following subcutaneous inoculation with 10^5 PFU, five (no. 4, 5, 8, 11, 12) of nine snakes (no. 4–12) developed detectable viremia, which persisted 1–11 days (Table 1).

TABLE 2. WEST NILE VIRUS (WNV) TITERS IN TISSUES OF FOUR GARTER SNAKES INOCULATED SUBCUTANEOUSLY WITH 10^5 PFU WNV

	Tissue									
Snake no.	*Liver*	*Kidney*	*Heart*	*Intestine*	*Skin*	*Muscle*	*Spleen*	*Brain*	*Lung*	*Trachea*
5	$>10^4$	$>10^4$	$>10^4$	$>10^4$	$>10^4$	$>10^4$	NT	NT	$>10^4$	NT
7	Neg	Neg	Neg	320	$>10^4$	NT	NT	160	1280	160
10	Neg	Neg	10	Neg	Neg	NT	NT	NT	5120	320
11	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NT	Neg	NT

NT, not determined; neg, negative (<1/10); PFU, plaque-forming units.

Virus isolation from the organs of dead snakes

WNV was not detected in the organs of the snake that was inoculated subcutaneously with 10^3 PFU, nor from the snake that died following inoculation *per os*. In contrast, WNV was detected in many of the organs of three (no. 5, 7, 10) of the four snakes that died following subcutaneous inoculation with 10^5 PFU (Table 2). Virus isolation was positive up to dilution of 1/20480 in some of the tissues.

Histology and immunohistochemistry

Only samples from snake number 5 (Table 1) were submitted for IHC. The lobular pattern of hepatic cords was distorted, and there was ballooning, degeneration and multifocal to coalescent areas of coagulative necrosis accompanied by a very mild inflammatory response consisting mainly of histiocytes. The cytoplasm of the hepatic histiocytes stained positively for WNV. The kidney revealed a small area of necrosis with the presence of a mild histiocytic infiltration. Extensive IHC staining was seen in the endothelial cells and cytoplasm of the histiocytes (Fig. 1). IHC staining was visible in a few histiocytic cells of the spleen.

Viral RNA in cloacal swabs and sera

Cloacal swabs were assayed only by RT-PCR, and therefore can only indicate the presence of viral RNA. Viral RNA was found intermittently between 1 and 28 days PI, in cloacal swabs from all snakes inoculated *per os*, and in cloacal swabs from four snakes (no. 7, 8, 11, 12) inoculated subcutaneously with 10^5 PFU.

Viral RNA was not found in any of the serum samples collected from snakes inoculated subcutaneously with 10^3 PFU. Viral RNA was found intermittently in samples collected from seven (no. 4–8, 11, 12) of nine snakes inoculated subcutaneously with 10^5 PFU. Five (no. 4, 5, 8, 11, 12) of these snakes had detectable levels of viremia, and WNV was detected in some of the tissues of the sixth (no. 7) that died on day 3 PI. Viral RNA was found only once on day 5 in snake no. 18 (Table 1) of six snakes inoculated *per os* with 10^5 PFU.

DISCUSSION

The role of reptiles in the ecology of several arboviruses, led to the suggestion that they too, may have a role in the ecology of WNV (Dauphin et al. 2004, Zeller and Schuffenecker

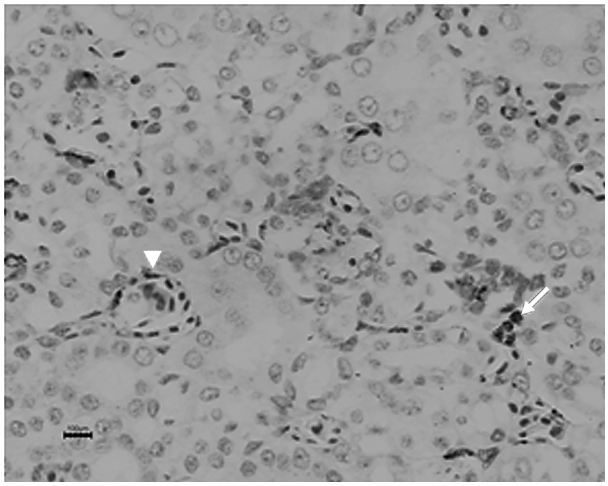

FIG. 1. Immunohistochemical staining of a kidney from a West Nile virus–infected garter snake with endothelial cells (arrowhead) and histiocytes (arrows) staining positive for West Nile virus. Hematoxylin counterstaining. Scale bar = 100 μm.

2004). The present study showed that common garter snakes are susceptible to WNV, can develop viremia, and the virus is found in many of their tissues following subcutaneous inoculation. Thus they might infect predator birds.

In the present study, subcutaneous inoculation with 10^3 PFU of WNV did not result in viremia, moreover WNV was not detected in the organs of snake no. 3 that died on day 9 PI (Table 1). However, low but persistent levels of neutralizing antibody were detected from day 17 PI in snake no. 1 of the two surviving snakes. These results are supported by another study, in which subcutaneous inoculation with 2000 PFU did not cause viremia in a group of 19 infected Florida garter snakes. However, three snakes that were euthanized on day 12 PI had WNV-positive spleens (Klenk and Komar 2003).

Since inoculation of 10^3 PFU of WNV did not result in viremia in the preliminary experiment, we increased the dose to 10^5 PFU, and 4 out of nine snakes inoculated subcutaneously died within three weeks, Of these, 2 demonstrated clinical signs, which might be attributed to the infection. Poor to moderate bodily condition with no ingesta in the gastrointestinal tract was reported in one WNV positive alligator (Miller et al. 2003). Thiamine (vitamine B_1) deficiency cannot be ruled out as the cause of the neurological signs of snake no.5, as was previously reported in garter snakes (Lawton 1992). However, this snake was persistently viremic from day 2 PI, until its death on day 13 PI. High levels of WNV were recovered from many of its organs. WNV was also detected in many of the tissues of two of the three other dead snakes, suggesting that they too may have died from WNV infection.

Snake no.5 that died on day 13 PI with clinical signs had IHC-positively stained liver, kidney and spleen, similar to those found in human cases of WNF (Guarner et al. 2004). IHC staining in many tissues, including the spleen, liver and kidney has been reported in several avian species (Jozan et al. 2003) and in American alligators (Jacobson et al. 2005). IHC staining was seen in renal tubular epithelial cells in a WNV-infected patient with AIDS (Guarner et al. 2004), in a WNV-infected dog (Lichtensteiger et al. 2003), and in all three infected Eastern fox squirrels (*Sciurus niger*) (Kiupel et al. 2003). In most incidental hosts WNV has a definitive neurotropism, however, and is rarely found in non-CNS tissues (Lichtensteiger et al. 2003, Guarner et al. 2004).

Following subcutaneous inoculation with 10^5 PFU, 5 of the nine garter snakes developed viremia, which lasted up to 11 days. The persistent viremia is longer than that reported in most species, and therefore increases the probability of infecting mosquitoes and predators when compared with 2-3 days of viremia in chickens (Langevin et al. 2001), 1-4 days in dogs and cats (Austgen et al. 2004), and up to 3 days in horses (Bunning et al. 2002). Similar, short time intervals were reported in American alligators (Klenk et al. 2004) and in several species of birds (Komar et al. 2003), including young geese (Banet-Noach et al. 2003). In contrast, a prolonged viremia was also reported in garter snakes (Thomas et al. 1958) and Texas tortoises (*Gopherus berlandieri*) following inoculation with WEEV (Bowen 1977), while an intermittent viremia was reported in other species (Bunning et al. 2002, Banet-Noach et al. 2003, Austgen et al. 2004). Only small amounts of blood were collected in this study, and viremic titers could not be determined, therefore we cannot determine whether mosquitoes could become infected, and further studies are required.

Viral RNA was found in cloacal swabs of all snakes inoculated *per os*, which might be expected because of the inoculation route; however, since none became viremic, the existence of infectious virus in the lower gastrointestinal tract is open to conjecture. In contrast, viral RNA was also found in cloacal swabs of four snakes inoculated subcutaneously with 10^5 PFU, which were also viremic. In another study, WNV was isolated from oral and cloacal swabs of green iguanas, but not from swabs of garter snakes (Klenk and Komar 2003). In this study, viral RNA was detected in the serum samples of 3 snakes although they were not viremic. This could be explained by the presence of low levels of WNV below that detectable on Vero cells, or that only RNA was present and was not infectious virus.

WNV was found in many organs of 3 of the four dead garter snakes following subcuta-

neous inoculation with 10^5 PFU, with levels up to 1/20480 in some of the tissues. In another study, WNV was found in spleens of garter snakes killed on day 12 PI (Klenk and Komar 2003), but none of the other organs contained WNV, however. In one out of three green iguanas WNV was detected in the spleen and small intestine (Klenk and Komar 2003), while high viral loads were detected in many tissues of three naturally infected American alligators (Jacobson et al. 2005). Predators of snakes include raptors, such as hawks, kestrels, and harriers, and the persistence of WNV in many of their organs up to 15 days PI would contribute to predator infection via oral transmission. Since oral infection occurs in many species of birds (Komar et al. 2003), direct transmission of WNV from snakes to birds is feasible.

Larger reptiles, including many snakes, alligators and crocodiles, feed on birds which might be infected with WNV. In this study, none of the snakes inoculated *per os* with 10^5 PFU WNV developed a detectable viremia, whereas in one snake (no. 15) low levels of neutralizing antibody were detected. Oral infection of several avian species (Komar et al. 2003), cats (Austgen et al. 2004), and experimental infection of alligators was recently reported (Klenk et al. 2004). In this study failure to demonstrate oral infection might be attributed to the dose of inoculum.

Garter snakes developed detectable antibodies only on day 16 PI, which is later than in warm-blooded species, and is possibly related to a slower reptilian humoral response time. In another study, green iguanas developed neutralizing antibodies at 28 days PI (Klenk and Komar 2003). In many avian and mammalian species, neutralizing antibodies were developed within two weeks PI (Langevin et al. 2001, Bunning et al. 2002, Banet-Noach et al. 2003, Austgen et al. 2004).

In conclusion, following experimental infection of garter snakes with WNV, a relatively persistent viremia and neutralizing antibodies were detected, and, most importantly, high titers of virus were found in multiple organ samples. These findings suggest that infection of predator birds is feasible following ingestion of infected snakes.

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Address reprint requests to:
Dr. Nahum Y. Shpigel
Hebrew University of Jerusalem
P.O. Box 12
Rehovot 76100, Israel

E-mail: shpigeln@agri.huji.ac.il