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Role of Maternal Antibody in Natural Infection of *Peromyscus maniculatus* with Sin Nombre Virus

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Data from naturally infected deer mice (*Peromyscus maniculatus*) were used to investigate vertical transmission of Sin Nombre virus (SNV) and SNV-specific antibody. The antibody prevalence in juvenile mice (14 g or less) was inversely proportional to the mass of the animal, with juvenile deer mice weighing less than 11 g most likely to be antibody positive (26.9%) and juvenile mice weighing between 13 and 14 g least likely to be antibody positive (12.9%). Although a significant sex bias in seropositivity was detected in adult deer mice, no significant sex bias in seropositivity was detected in juvenile animals. Ten juvenile deer mice were identified that had initially tested positive for SNV-specific immunoglobulin G (IgG) by enzyme-linked immunosorbent assay (ELISA) but had subsequently tested negative when recaptured as adults. SNV RNA was detected by reverse transcriptase PCR (RT-PCR) in the blood of ELISA-positive adult deer mice but not in the blood of ELISA-positive juveniles. One of the juvenile mice initially tested negative for SNV RNA but later tested positive when recaptured as an ELISA-positive adult. The RT-PCR results for that individual correlated with the disappearance and then reappearance of SNV-specific IgG, indicating that the presence of SNV RNA at later time points was due to infection with SNV via horizontal transmission. SNV-specific antibody present in both ELISA-positive juvenile and adult mice was capable of neutralizing SNV. Additionally, our data indicate that SNV is not transmitted vertically.

Interest in New World hantaviruses and their rodent hosts greatly increased following the first recognized outbreak of hantavirus pulmonary syndrome (HPS) in humans in 1993. The etiologic agent of HPS was found to be a previously unknown hantavirus, termed Sin Nombre virus (SNV) (3, 13, 23). This hantavirus was shown to primarily infect deer mice (*Peromyscus maniculatus*) (6). More than 200 HPS cases have occurred in the United States, with a mortality rate of about 40% (5). Since the 1993 outbreak, a number of new hantaviruses have been identified throughout North and South America, many of which have been associated with human disease (10, 16, 17, 20, 24, 27).

Hantaviruses are members of the family *Bunyaviridae*, genus *Hantavirus* (26). They contain a single-stranded, negative-sense, trisegmented RNA genome. The three segments of the genome are referred to as large (L), medium (M) and small (S). The S segment (1.7 to 2.0 kb) codes for the nucleocapsid protein, the M segment (3.6 kb) codes for a glycoprotein precursor which is cleaved to form two glycoproteins (G1 and G2), and the L segment (6.5 kb) codes for the virus transcriptase (8).

Hantaviruses are maintained in wild rodent populations, with each different hantavirus associated with a specific rodent reservoir (21). Unlike other members of the family *Bunyaviridae*, hantaviruses are not arthropod borne but are believed to be transmitted to humans from their rodent hosts by inhalation of aerosolized rodent excrement (15). The mode of SNV transmission between deer mice is unclear, but field studies have provided indirect evidence of horizontal transmission of SNV among rodents via biting and other aggressive behavioral interactions (2, 4, 18, 19).

Antibody specific for Old World hantavirus (Seoul virus) has been shown to be transferred from dam to offspring both in utero and via mammary secretions (7); however, the role of vertically transferred antibody is still unclear. To date, the evidence for maternal antibody in the SNV-deer mouse system is indirect and cross-sectional, and no evidence exists with regard to vertical transmission of actual virus. In deer mice, seroprevalence to SNV is high in the smallest deer mice, decreases slightly as mass increases, and then begins to increase with body size (18). This may indicate that offspring are obtaining passive immunity from infected mothers.

Although rodents infected with hantaviruses do mount a strong humoral immune response, and antibody is able to neutralize virus when assayed in vitro, data obtained from laboratory and field studies of both Old World and New World hantaviruses indicate that animals remain systemically and chronically infected (12, 15, 22, 28).

In these studies, data collected during 4 years of field sampling in the Walker River Basin of Nevada and California were used to investigate vertical transmission of SNV and SNV-specific antibody in deer mice. Juvenile deer mice (14 g and under) were identified that had initially tested positive for SNV-specific immunoglobulin G (IgG) but had subsequently tested negative at later time points. The presence of SNV RNA was assayed for in the blood of these rodents by reverse transcriptase PCR (RT-PCR) using primers specific for the SNV M segment. Studies have shown that maternal antibodies to Seoul virus prevent infection of juvenile rats that are challenged with Seoul virus (7). For this reason, the ability of antibodies involved in passive and active immunity to neutralize SNV was also investigated.

MATERIALS AND METHODS

Study area. Samples were collected at 150 independent field sites in the Walker River Basin of Nevada and California. Walker River Basin is ecologically

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diverse and is more fully described in reference 2. At least seven human cases of HPS have occurred in or near Walker River Basin since 1993. Mark and recapture (longitudinal) data were collected at 15 of these 150 sites that were sampled repeatedly at monthly intervals for periods ranging from 7 months to 4 years. All site locations were chosen randomly by a computer algorithm that used vegetation maps and other data derived from satellite imagery to select a sample set representing maximal ecological diversity (2). Mark and recapture sites were selected based on the presence of substantial deer mouse populations (allowing a maximum number of individual mice to be monitored).

Trapping and blood collection. Deer mice were live trapped in 1995 to 1998 according to our standard protocol (2). Briefly, blood was collected from each deer mouse by retro-orbital puncture with a heparinized capillary tube or Pasteur pipette. Blood samples were placed on dry ice until they could be returned to the laboratory for analysis. Animals were also weighed using Pensola scales (which are accurate only to the nearest gram) and visually identified as to sex and maturity. On longitudinal sites, each animal was tagged on the ear with an individually numbered tag, allowing identification of the animal on subsequent recaptures.

ELISA. Enzyme-linked immunosorbent assays (ELISAs) tests were conducted on all blood samples to detect antibody to hantavirus. The wells of polyvinyl chloride microtiter plates (Dynatech Laboratories, Chantilly, Va.) were coated with recombinant nucleocapsid antigen (SNV recombinant antigen) (9) diluted 1:2,000 in phosphate-buffered saline, pH 7.4 (PBS), and incubated overnight at 4°C. As a control, a negative recombinant antigen was also coated to plates. After incubation, unbound antigen was removed from the plates by washing three times with wash buffer (PBS, 0.5% Tween 20). Heat-inactivated mouse sera were diluted at 1:50 in serum dilution buffer (PBS, 0.5% Tween 20, 5% skim milk), added in duplicate to both the positive and negative antigen-coated wells, and incubated at 37°C for 1 h. After incubation with serum, the wells were washed three times with wash buffer and incubated with secondary antibody (horseradish peroxidase-labeled goat anti-*Peromyscus leucopus*) at 37°C for 1 h. The plates were washed three times with wash buffer and incubated at 37°C for 30 min with 100 μl of ABTS Microwell peroxidase substrate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). The absorbance (A_{405}) was recorded with a Ceres 900 enzyme immunosorbent assay workstation (Bio-Tek Instruments, Inc., Winooski, Vt.). For each sample, values greater than 3 standard deviations of the two negative control wells were considered positive. Additionally, scalar scores were assigned to each sample based on the mean plate reader value for the three positive wells minus 3 standard deviations from the negative control. Absorbance values ranging from 0 to 1 were given a score of 1; values ranging from >1 to 2 were given a score of 2; values ranging from >2 to 3 were given a score of 3; values ranging from >3 to 4 were given a score of 4; values of >4 were scored as 5.

RNA extraction, RT-PCR amplification, and sequencing. To avoid RNA template or PCR cross-contamination, RNA was extracted and first-round RT-PCRs were prepared in a laminar flow biological safety cabinet in a biosafety level 3 (BSL3) facility. Second-round PCRs were prepared in a laminar flow biological safety cabinet in a separate laboratory. Total RNA was extracted from 10 μl of blood clot, using an RNaid Plus kit (Bio101, La Jolla, Calif.) according to the manufacturer's instructions. SNV RNA was amplified by nested RT-PCR using hantavirus-specific primers as described previously (25). Products were analyzed by gel electrophoresis and direct sequencing.

Focus reduction neutralization assay. Neutralization assays were performed on confluent Vero-E6 cell monolayers grown in 12-well plates (Costar, Corning, N.Y.) using SNV strain CC107. All work with live virus was done in a class II biological safety cabinet in a BSL3 laboratory. *P. maniculatus* serum samples were diluted (1:100 and 1:1,000) in Iscove's tissue culture medium, combined with an equal volume of virus containing approximately 100 PFU/0.2 ml, and incubated at 37°C for 1 h. SNV not incubated with immune serum served as a positive control. Serum from an ELISA-negative *P. maniculatus* was used for a negative control. Each serum-virus mix was added to the monolayer of cells; after a 1-h absorption period at 37°C, the unabsorbed serum-virus inoculum was removed. Each mixture was assayed in duplicate. Following absorption, the cells were overlaid with 0.3% agarose in Iscove's medium supplemented with 2% fetal bovine serum. Plates were incubated at 37°C in a 5% CO_2 incubator for 10 days and then fixed at room temperature with methanol-acetone (3:1) for 10 min. The cells were washed three times at room temperature with PBS containing 0.2% Tween 20, followed by incubation at 37°C with convalescent human serum diluted 1:300 in PBS containing 0.2% Tween 20. Cells were rinsed as described previously and incubated with alkaline phosphatase anti-human IgG (Vector Laboratories, Burlingame, Calif.) diluted at 1:100 in PBS containing 0.2% Tween 20 at 37°C. Cells were again rinsed as described above, and an alkaline phosphatase substrate kit (Vector Laboratories) was used to detect foci as described by the manufacturer. An 80% or greater reduction in plaque counts compared to the positive control was considered positive for neutralization (11).

Statistical analysis. Deer mice from all 150 sample sites were placed into different weight categories (based on criteria established by Mills et al. [18]) representing different degrees of maturity. Chi-square tests (SAS version 6.12 software; FREQ procedure) were used to test for differences in antibody prevalence among different weight and/or sex categories, using a Bonferroni-adjusted significance level of 0.0071 (i.e., 0.05 divided by 7 posthoc comparisons).

TABLE 1. Antibody prevalence

Group	Wt (g)	No. of antibody-positive rodents/no. tested (%)		
		Male	Female	All
1	<11	24/88 (27.3)	32/120 (26.6)	56/208 (26.9)
2	11–12	25/143 (17.5)	39/183 (21.3)	65/329 (19.8)
3	13–14	39/280 (13.9)	36/300 (12.0)	75/581 (12.9)
4	15–18	198/812 (24.4)	79/526 (15.0)	277/1,351 (20.5)
5	≥19	191/491 (38.9)	154/643 (24.0)	347/1,145 (30.3)
All combined		407/1,611 (25.3)	293/1,534 (19.1)	703/3,166 (22.2)

RESULTS

To investigate the incidence of SNV infection in various populations of deer mice in the Walker River Basin of Nevada and California, blood samples were obtained from 3,166 deer mice captured as part of an ongoing longitudinal study. For the demographic analysis, seroprevalence data (as determined by ELISA assay for SNV-specific IgG antibody) were categorized according to the weight and sex of the animal (Table 1), using the categories defined by Mills et al. (18). The overall antibody prevalence was 22.2% (703/3,166). Male deer mice weighing 19 g or more were most likely to be antibody positive (38.9%), and female mice weighing between 13 and 14 g were the least likely to be antibody positive (12.0%). The antibody prevalence in juvenile mice (14 g or less) was inversely proportional to the mass of the animal. Within the juvenile group of mice, mice weighing less than 11 g were most likely to be antibody positive (26.9%), and juvenile mice weighing between 13 and 14 g were least likely to be antibody positive (12.9%). Comparison of group 1 (the smallest deer mice) with group 3 (the heaviest juvenile deer mice) showed that seroprevalence was significantly lower for group 3 ($P < 0.0001$). Seroprevalence was also significantly lower in group 3 than in group 5 (the heaviest adult deer mice) ($P < 0.0001$).

No significant sex bias in seropositivity was detected in juvenile animals within any weight class ($P > 0.38$ for groups 1 to 3) (Table 1). However, for the last two groups (adult mice), there was a significantly higher prevalence of antibody in male mice ($P < 0.0001$ for both groups), a finding confirmed in several other studies (1, 2, 4, 14, 18).

For longitudinal analysis, 10 juvenile deer mice were identified that were initially positive by ELISA for SNV-specific IgG but negative at a later time point(s) (Table 2). RNA was extracted from each blood clot sample, and RT-PCR was used to amplify SNV RNA. RT-PCR products were sequenced to ensure that the product was not due to laboratory contamination. Nine of the ten rodents tested negative for SNV RNA at all time points. One of the rodents (rodent 7) initially tested negative for SNV RNA but then tested positive at later time points (Table 2). However, the RT-PCR results of that individual correlated with the disappearance and then reappearance of SNV-specific IgG. In other words, as the rodent became seropositive as an adult, SNV RNA was detected at most of the subsequent time points. Since viral RNA is not always detectable in the blood of hantavirus-infected, ELISA-positive rodents (12), the absence of detectable SNV RNA at two of the time points was not unexpected. This indicates that the presence of SNV RNA at later time points was due to infection with SNV via horizontal transmission.

Sera from five deer mice were assayed for the ability to neutralize SNV virus (Table 3). Two of the mice (rodents 4 and 7) were seropositive and SNV RNA negative when first captured as juveniles and subsequently became seronegative, al-

TABLE 2. Data from longitudinal study: juvenile rodents suspected of having maternal antibody (as determined from ELISA and RT-PCR results)

Capture date (yr/mo/day)	Rodent	Sex[a]	Age[b]	Wt (g)	ELISA[c]	RT-PCR[d]
97/07/12	1	F	J	10	4	−
97/08/09	1	F	A	25	0	−
97/08/31	1	F	A	27	0	−
97/06/17	2	F	J	12	3	−
97/07/12	2	F	A	14	0	−
98/06/07	3	M	J	8	5	−
98/06/28	3	M	J	12	2	−
98/06/07	4	M	J	8	5	−
98/06/28	4	M	J	14	2	−
98/07/22	4	M	A	14	1	−
98/08/15	4	M	A	14	0	−
97/08/27	5	F	J	9	5	−
97/09/21	5	F	A	20	0	−
97/10/13	5	F	A	18	0	−
97/08/27	6	M	J	8	5	−
97/09/21	6	M	A	13	1	−
97/08/27	7	F	J	8	5	−
97/09/21	7	F	A	11	1	−
97/10/13	7	F	A	14	0	−
97/11/04	7	F	A	14	0	−
98/06/07	7	F	A	19	5	+
98/06/28	7	F	A	19	4	−
98/07/22	7	F	A	23	4	+
98/08/15	7	F	A	22	3	+
98/09/09	7	F	A	26	5	+
98/10/14	7	F	A	19	4	−
98/07/22	8	F	J	10	4	−
98/08/15	8	F	A	13	3	−
98/09/09	8	F	A	14	0	−
98/10/14	8	F	A	13	0	−
98/08/15	9	M	A	13	1	−
98/09/09	9	M	A	14	0	−
98/10/14	9	M	A	14	0	−
98/09/09	10	F	J	12	3	−
98/10/14	10	F	A	16	0	−

[a] F, female; M, male.
[b] Juvenile (J) or adult (A), estimated by pelage and weight of animal.
[c] Scored from 0 (negative) to 5 (strong positive).
[d] RT-PCR products were sequenced to confirm authenticity.

though one (rodent 7) became seropositive and positive for SNV RNA at a later time point. Two of the mice (rodents 11 and 12) were seropositive adults when first captured and were SNV RNA positive at at least one time point. One rodent (rodent 14) was a seronegative, SNV RNA-negative adult that served as a negative control.

Antibodies present in the sera of the juvenile mice neutralized SNV at both dilutions tested (Table 3). However, once these mice became seronegative adults, the sera no longer neutralized virus. Rodent 7 regained seropositivity at later time points, and sera from these time points were able to neutralize virus. Antibodies present in the sera of the three seropositive, SNV RNA-positive adult mice were also able to neutralize SNV. Sera from the seronegative, SNV RNA-negative control mouse was not able to neutralize virus.

TABLE 3. Focus reduction neutralization assay results

Rodent	Capture date (yr/mo/day)	Age[a]	ELISA[b]	RT-PCR	Neutralization[c]
4	98/06/07	J	5	−	+
	98/08/15	A	0	−	−
7	97/08/27	J	5	−	+
	97/11/04	A	0	−	−
	98/06/07	A	5	+	+
	98/09/09	A	5	+	+
11	97/08/31	A	4	+	+
	97/10/06	A	4	+	+
12	98/06/07	A	4	+	+
	98/06/28	A	2	+	+
14	97/10/06	A	0	−	−

[a] Juvenile (J) or adult (A), estimated by pelage and weight of animal.
[b] Scored from 0 (negative) to 5 (strong positive).
[c] Positive control had an average of 100 PFU/0.2 ml; neutralization test was considered positive if there was an 80% or greater reduction in plaques compared to the positive control.

DISCUSSION

Our findings provide the first longitudinal evidence of maternal SNV-specific antibody transmission for deer mice. Data from several experiments support the hypothesis that antibody detected in small juvenile deer mice is maternally transmitted. These data include the following. (i) Although there is an overall trend for seroprevalence to increase with body mass, the smallest group of juvenile mice were more likely to be seropositive than the two groups of larger juveniles. (ii) There is a significant sex bias in seropositivity in each group of adult mice; however, there is no significant sex bias in mice weighing 14 g or less. (iii) ELISA-positive juvenile rodents that later became seronegative did not have any detectable SNV RNA in their blood, while ELISA-positive adults commonly do (2). (iv) The antibody was capable of neutralizing SNV when tested in vitro. Although data taken from one experiment alone would not provide conclusive evidence, taken together these data are much more indicative of maternal antibody transmission.

Many of our findings require captive animal studies to be clearly confirmed. For example, while it is interesting that the maternal antibody was able to neutralize SNV in vitro, antibody present in persistently infected adult mice is also capable of in vitro neutralization. Due to the fact that research on SNV infection in *P. maniculatus* requires a BSL4 facility, many questions regarding SNV transmission among rodents have yet to be addressed in captive animal studies.

Our data suggest that the virus itself is not transmitted maternally, although conclusive evidence is not available. If SNV is transmitted vertically, one would expect the smallest group of juvenile mice to have SNV-specific antibody, remain seropositive, and have detectable SNV RNA at at least one time point. However, if the presence of maternal antibody prevents infection, one would expect the smallest group of juvenile mice to be ELISA positive initially, lose seropositivity upon early adulthood, and have no detectable SNV RNA during the initial period of seropositivity. Data from all nine of the juvenile mice with adequately extensive time lines support the hypothesis that the presence of maternal antibody protects neonates and young juvenile mice from infection.

Hantavirus infection persists in deer mice even in the presence of antibodies that are capable of neutralizing virus when

tested in vitro (12, 22). However, since juvenile mice with maternal antibody do not have detectable SNV RNA in their blood even after being reared by an infected mother, it is likely that maternal antibodies are capable of neutralizing SNV both in vitro and in vivo. The apparent discrepancy between in vivo neutralizing capabilities of maternal and nonmaternal antibodies may be due to temporal effects of the infection. For example, when adult rodents are infected, the virus is able to replicate to a high enough titer to prevent complete viral clearance once antibodies are generated. However, since juveniles acquire maternal antibody in utero, maternal antibodies may be able to neutralize virus before an infection is established. It is also possible that SNV persists intracellularly in seropositive adult mice and while extracellular virus is neutralized by circulating antibody, the infection is chronic due to the presence of intracellular virus.

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