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POWASSAN VIRUS INFECTION IN SNOWSHOE HARES *(Lepus americanus)*

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Abstract: Sera from snowshoe hares *(Lepus americanus)* trapped near Rochester, Alberta, Canada were tested for Powassan virus antibody by the constant virus/serum dilution neutralization test. Of 1264 serum samples tested, 137 had an antibody titer of at least 1:4 for Powassan virus. Ten hares were inoculated with Powassan virus in the laboratory. Viremia lasted 4-5 days and ceased with the appearance of Powassan antibody in the serum. Neutralizing antibody reached a peak titer of 1:119 on day 15 post-inoculation and was still detectable 13 months post-inoculation.

INTRODUCTION

Powassan (POW) virus is a flavivirus closely related to the Russian spring-summer encephalitis virus complex, both immunologically and ecologically.[2,5] It was first isolated from the basal ganglia and cortex of the brain of a 5-year-old boy from Powassan, Ontario, Canada who died of encephalitis.[18] Several clinical cases have been reported since the original isolation, with sequelae ranging from mild to moderate.[9,25,27] A second human fatality involving an 82-year-old man was reported from New York state during 1975.[7] Serologic tests have detected human infection from Sonora, Mexico[1] to Ontario, Canada;[19] and from New York state[30,31] to British Columbia, Canada.[3,15]

POW is maintained primarily in small rodents,[17,23,29] especially members of the family Sciuridae. Ixodid ticks appear to be the principal vectors,[3,4,12,28] especially members of the genera *Ixodes* and *Dermacentor*. Several serologic surveys have documented infection in the snowshoe hare.[16,17,20,21] Reported antibody prevalence in hares ranged from 0% to 16%. The sample sizes in these studies were small and taken over a short period of time, usually a few days.

The main objective of the present study was to sample a sufficient number of hares to determine the age- and sex-specific antibody prevalence of POW infection in these animals in central Alberta during 1970-1974. In addition, hares were inoculated with POW in the laboratory. Antibody response of these hares was used to evaluate the immune status of wild-caught hares. Viremia results were used to assess the capacity of hares to serve as reservoirs of POW in nature.

MATERIALS AND METHODS

Study area. The study area for this project was near Rochester, Alberta, Canada (115° West longitude; 54° North latitude), an area situated on the southern fringe of the northern boreal forest biome, described previously.[26]

Hare sampling. Hares were trapped from April, 1970-July, 1974 using non-baited, single-door, 23 × 23 × 81 cm National live traps. [2] Greatest trapping effort each year was during May-August.

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[2] National Live Trap Co., Tomahawk, Wisconsin 54487, USA.

Trapping was not done from October, 1972-March, 1973 and October, 1973-December, 1973.

Hares were bled by cardiac puncture, using the Vacutainer system. [1] Serum was collected after centrifugation and stored at -20 C until tested. Blood was not collected from any hare more than once.

Cell line. An established, continuous line of medium M-199-adapted baby hamster kidney cells (BHK-21) was used throughout this study. [4] The passage level was not known.

Cell culture medium. Growth medium consisted of: medium M-199, [5] 10% heat-inactivated fetal calf serum, [5] 0.1% gentamicin sulfate, [6] 1.0% sterile tricine buffer, and 2.0% sterile sodium bicarbonate. Growth medium was also used as a diluent throughout this study.

Viruses. The POW used throughout this study was the L.B. strain isolated from the original human fatal case. [4] The virus was in 7th suckling mouse brain passage, after having been passaged twice in baby hamsters. The St. Louis Encephalitis virus (SLE) used as a flavivirus control was lot number CDC-904 obtained originally from the Center for Disease Control, Atlanta, Georgia. [4] It was originally isolated from a flicker *(Colaptes auratus)* in Kentucky during a 1955 outbreak of the disease. As used here, it was in 8th suckling mouse brain passage.

Hyperimmune mouse ascitic fluid. Hyperimmune mouse ascitic fluid (MAF) for use as positive control serum was prepared for both POW and SLE by the method of Feild and Kalter for epizootic hemorrhagic disease and Semliki Forest viruses.[8]

Serologic tests. Preliminary testing of the 1264 serum samples was done at a dilution of 1:4 in the constant virus/serum dilution neutralization test (NT).[24] Sera were inactivated at 56 C for 30 min. Virus test doses ranged from 50 to 250 tissue culture mean lethal doses ($TCLD_{50}$). The test was carried out in flat bottom 96-well tissue culture plates. [7] SLE was included to test for cross-reactive neutralization by the hare sera. A serum control was included for each sample. A serum sample was considered positive if cytopathic effect (CPE) was completely inhibited in either or both of the test wells.

Those sera which had given a positive reaction in the preliminary test were then retested by NT at dilutions of 1:4, 1:16, 1:64, and 1:256. A 0.025 ml microdiluter appartus [8] was used to make the dilutions.

A POW titration, cell controls, and a known positive serum (anti-POW MAF) were included with each group of sera tested.

Snowshoe hare inoculations. In a preliminary experiment, two snowshoe hares (1 male, 1 female) without detectable POW antibody were inoculated with 0.5 ml of 10^3 $TCLD_{50}$/0.5 ml of POW by both the intramuscular (IM) and subcutaneous (SC) routes. These hares had been held in captivity for more than 2 years. The hares were bled from the marginal ear vein or central ear artery. Blood was drawn daily for 9 days, every second day for 8 days, every third day for

[1] Becton, Dickinson and Co., Rutherford, New Jersey 07070, USA.

[4] Supplied by B.D. Nassif, Department of Veterinary Science, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA.

[5] International Scientific Industries, Inc., Cary, Illinois 60013, USA.

[6] Schering Corp., Kenilworth, New Jersey, 07033, USA.

[7] Linbro Scientific, New Haven, Connecticut 06510, USA.

[8] Cooke Laboratory Products, Alexandria, Virginia 22313, USA.

9 days, and monthly thereafter. One ml aliquots of each of these blood samples were defibrinated by shaking in a sterile vial with sterile glass beads. This sample was stored in a mechanical freezer at -65 C for 1 month or less, and then 0.03 ml aliquots of ten-fold serial dilutions were inoculated intracerebrally (IC) into 7 to 10 albino Swiss mice 1-2 days of age,[9] to quantify viremias. Another 1.0 ml aliquot of the hare blood samples was allowed to clot. Serum was collected after centrifugation and stored at -20 C until it was tested for POW antibody by NT.

To confirm results obtained from these preliminary tests, identical inoculations were performed with eight additional hares (4 male, 4 female) from the same captive group.

RESULTS

The majority of sera was obtained during 1970 and 1971 at a time of peak hare population densities. The smaller number of samples for 1973 and 1974 reflects a declining population, as does the small number of juvenile sera obtained during these 2 years. Of the 1264 sera tested, 137 (11%) were positive, i.e. inhibited CPE caused by POW. One serum inhibited both POW and SLE and none of the sera neutralized only SLE. In the serum titration NT, POW was neutralized by 121 sera at a dilution of 1:4, 15 at a dilution of 1:16, and 1 at a dilution of 1:64. Yearly antibody prevalences are presented in Table 1. Prevalences for 1972 and 1973 are significantly different from all other years, and 1970, 1971, and 1974 were similar ($P<0.001$ by the chi square test). There were no significant differences in antibody prevalence between the various sex and age classes.

The magnitude and duration of viremia in the experimentally POW-infected hares are presented in Figure 1. One of the hares in the second group was very weak prior to inoculation. It struggled little when removed from its cage for bleeding. It neither ate nor drank quantities equivalent to the other nine hares. It died on day 45 PI. Because the duration and magnitude of the viremia in this hare were quite different from the other nine, results were not included in calculations represented in Figure 1. On day 1 PI, mean viremia was 1.49 logs of virus per 0.03 ml of blood with a range of 0.29 to 2.29. On day 2 PI, mean viremia was 2.29 logs with a range of 0.00 to 4.40. On day 3 PI, mean viremia was 1.63 logs with a range of 1.00 to 2.83. On day 4 PI, mean viremia was 0.12 logs with a range of nil to 0.38. Mean duration of viremia was 3.33 days with a range of 3 to 4 days. On day 1 PI, viremia in seven of nine hares exceeded the level necessary to infect *Dermacentor andersoni*.[3] On day 2 PI, viremia exceeded this level in eight of nine hares. On day 3 PI, viremia exceeded this level in all 9 hares. On day 4 PI, viremia did not exceed this level in any of the hares. Viremia for the weak hare peaked on day 4 PI at 4.5 logs of virus per 0.03 ml, which is later and higher than any of the other hares. The production of POW antibody by the hares is represented in Figure 2. Antibody response of the weak hare was similar to the other hares but was not included in calculations represented in Figure 2. Two hares survived beyond 1 year post-infection (PI).

DISCUSSION

Because sera from 137 of 1264 hares tested had POW NT antibody, it appears that POW infection was present in the snowshoe hare population near Rochester during 1970-1974. The overall prevalence of 11% in the present study

[9] From Charmany Farms colony, Dept. of Veterinary Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA.

TABLE 1. Powassan virus neutralizing antibody prevalence,[1] by sex, age and year of capture, for snowshoe hares captured near Rochester, Alberta, Canada.

Age and Sex	1970	1971	1972	1973	1974	Accumulated Total	
						No.	% Prev.
Adult Male	12/92	17/129	2/99	10/19	7/80	48/419	12
Adult Female	13/82	18/134	3/59	5/19	5/50	44/344	13
Juvenile Male	11/123	12/75	1/36	0/1	1/5	24/240	10
Juvenile Female	8/130	13/88	0/44	0/0	0/3	21/261	8
Total	44/427	60/426	6/234	15/39	12/138	137/1264	11
% Prevalence	10	14	3	39	9		

[1]Expressed as number positive/number tested. Sera tested by constant virus-serum dilution neutralization test. Sera considered positive if neutralization of cytopathic effect occurred at serum dilution of 1:4 or greater.

(Table 1) compared favorably with the composite prevalence of 9% (5 of 57) for hares in three studies conducted by McLean *et al.* in the neighboring province of British Columbia.[13,15,19] The observed prevalence does not approach the 42-45% rate found in three major studies of groundhog *(Marmota monax)* populations,[14,16,22] however.

A relatively small proportion of the hares had POW antibody. Possible explanations for this include: (1) POW transmission was common among the fauna of this area, but was only infrequently transmitted to hares (perhaps as a result of transmission by a vector which only rarely feeds on hares); (2) POW transmission was infrequent among all mammals of the area; (3) POW antibody titers declined rapidly in wild hares; or (4) most hares which were infected with POW in the wild died as a result of the infection. Results of our laboratory inoculations discount the validity of hypotheses 3 and 4. In subsequent serological tests with sera from several mammalian species captured near Fort MacKay, Alberta (500 km NNE of Rochester), we found no evidence of POW activity.[33] This would lend support to hypothesis 2. An index to the abundance of *Haemaphysalis leporis-palustris* found on hares near Rochester was calculated. This index was used to determine if environmental conditions may have affected the abundance of all tick species (presumably including those which transmit POW) during 1970-1974. No significant differences in yearly tick indices were detected. Therefore, changes in tick abundance do not appear to explain the differences in yearly POW antibody prevalences. Nor can changes in recruitment of juvenile hares (presumably susceptible to POW infection) to the population be used to explain these differences in yearly prevalences.

Only one serum of the 1264 tested neutralized SLE, the only other flavivirus previously known to be pre-

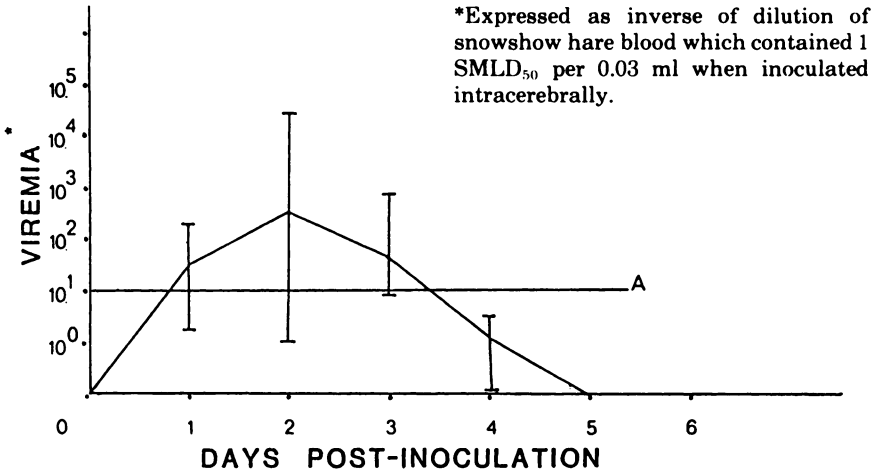

FIGURE 1. Powassan viremia (geometric mean titers with range) in 9 snowshoe hares following inoculation 0.5 ml of 10^4 $TCID_{50}$/0.5 ml by both subcutaneous and intramuscular routes. Line A represents the titer of Powassan virus necessary to infect *Dermacentor andersoni* ticks.

sent in western Canada. This lends credence to the belief that it was POW specific antibody which was being measured. A subsequent study in northeast Alberta has resulted in isolation of a varient of Modoc virus from the blood of *Peromyscus maniculatus*.[34] Antigenically, this virus is not closely related to POW. The possibility of an additional undescribed, closely related flavivirus circulating in the hare population near Rochester cannot be discounted, however.

The laboratory inoculation of hares with POW demonstrates that these animals can be infected and circulate virus. The mean viremias for days 1-3 PI are adequate to infect *Dermacentor andersoni* (Figure 1, line "A") a natural vector of POW.[3] These results are further evidence for the feasibility of natural infection of hares. These hares also developed high neutralizing antibody titers, which declined to a low value (less than or equal to 1:4) within 8 months PI but remained above 1:2 beyond 1 year PI (Figure 2).

The antibody response of the experimentally infected hares was used in the interpretation of results with sera from wild-caught hares. Antibody titer of the majority of seropositive wild-caught hares was 1:4, indicating that hares in this category were either sampled within 9 days PI (an unlikely occurrence), or were convalescent from an infection acquired a minimum of 120 days prior to the time of capture. Based on the results of the experimental infections, hares captured between these two times (9 days PI to 120 days PI) would be expected to have higher antibody titers. In addition, hares with titers of at least 1:16 (indicating recent infection) were detected in all years except 1973. This indicates that POW transmission occurred in at least 4 of the 5 years of this study.

McLean *et al.* suggested that POW transmission is at its peak in the spring of the year.[15] In the present study, 11 of the 16 hares with titers of at least 1:16 were captured during May-July, supporting this hypothesis. However, five hares were found to have titers of at least

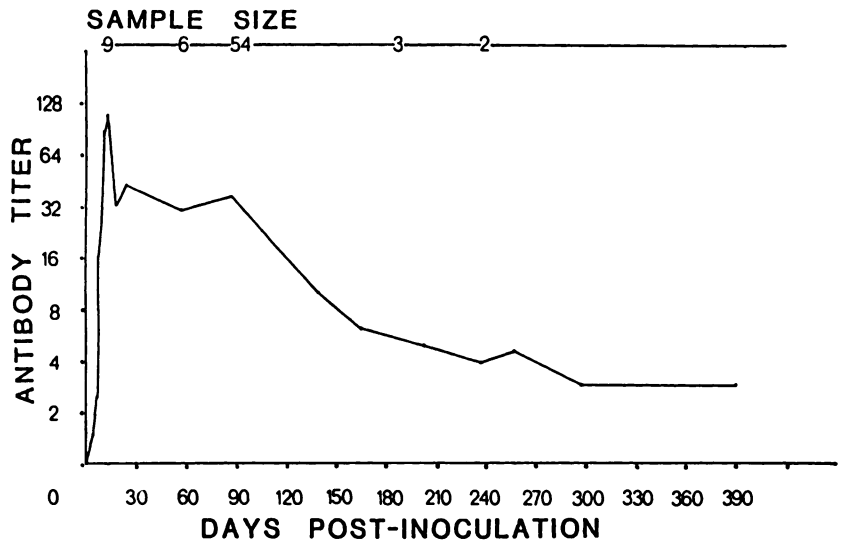

FIGURE 2. Powassan geometric mean antibody titer (expressed as inverse of dilution which neturalized 100 $TCID_{50}$ of Powassan virus) in 9 snowshoe hares following inoculation of 0.5 ml of 10^3 $TCID_{50}$/0.5 ml by both the subcutaneous and intramuscular routes.

1:16 during March and April. This would indicate that transmission occurred during the winter months as well, or that a few individuals maintained high antibody titers for periods longer than those observed for the experimentally infected hares. Both *Ixodes marxi* and *I. cookei* are active and feeding principally in the spring and summer months. However, collections of both species have been made from wild mammals in December.[6] Transmission of POW at this time could have resulted in the 1:16 titers observed during March and April.

The mode of transmission of POW on the study area was not obvious. *D. andersoni* was not observed on any vertebrates, and *Ixodes* spp. were rare (L.B. Keith, Department of Wildlife Ecology, University of Wisconsin, pers. commun.). Although many *H. leporis-palustris* ticks from Rochester area hares have been tested for viruses, POW has never been isolated.[10]

Viremias of experimentally infected hares and antibody levels of wild-caught hares indicate that snowshoe hares could serve as wildlife reservoirs of POW. However, POW has never been isolated from a hare, although virus isolation has been attempted many times.[10,11,32] There has never been a human case of POW encephalitis reported from western Canada. Apparently, the hare poses no serious public health threat as a carrier of POW.

Aknowledgements

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