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Environmental Modification of Western Equine Encephalomyelitis Infection in the Snowshoe Hare (*Lepus americanus*)

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The snowshoe hare (*Lepus americanus*) could be infected with western equine encephalomyelitis (WEE) virus and produce a viremia. Furthermore, viremia in hares exposed to variable climatic conditions differed significantly from viremias seen in the control animals held at constant temperatures. The viremia duration and titer were increased in animals subjected to fluctuating temperature and humidity. The time of onset of viremia was accelerated. Antibody response also increased in animals exposed to varying temperatures when compared with controls held at constant temperature and humidity. Snowshoe hares were studied at two distinct seasonal periods: winter, before reproductive activity; and summer, during reproductive midseason. Winter animals experienced greater viremia than did summer hares when exposed to fluctuating temperatures, suggesting a seasonality in the hare's susceptibility to host modification by environmental influences. These findings implicate the snowshoe hare as a possible mammalian amplifying host for WEE virus in the boreal forest.

Western equine encephalomyelitis (WEE) virus is an agent of significant public health importance, the epizootiology of which is not yet completely understood. Throughout most of its range, the virus is believed to be maintained in a sylvan cycle between mosquitoes and birds (15). In the boreal forest, however, there is no known vector for WEE virus and the role that small mammals play in the ecology of this virus is unstudied.

It was previously believed that small mammals were not important in the epizootiology of WEE (11, 15). However, during the course of a long-term population study in the boreal forest near Rochester, Alberta, Canada, neutralizing antibody to WEE virus has been detected on several occasions in the snowshoe hare (7). On two occasions, epizootics of WEE swept through that hare population several months prior to epidemics in human beings and horses in the transitional aspen parkland area 100 miles south of Rochester (8, 22). Serological studies indicate that snowshoe hares in Alberta have been infected in the field (23). Demonstration of the presence of neutralizing antibodies, however, does not prove that this species responds to infection by the development of viremia adequate for the infection of arthropod vectors.

Vertebrate host response to viral infection has been studied with both domestic and captive wild animals by artificially infecting them in the laboratory and monitoring viremia and antibody production. These are traditional procedures for establishing epidemiological relationships between host and parasite. It has been assumed that such relationships are not significantly altered by the laboratory milieu, and that the course of infection under experimental and natural conditions is essentially the same. Consequently, relatively little consideration has been given to the role the environment plays in modifying the response of a vertebrate host to a replicating virus. In these studies, the effect of environmental modification of a naturally occurring host-virus relationship was investigated.

This investigation employed the snowshoe hare to determine whether it could develop a viremia great enough to serve as a source of infectious blood meals for susceptible arthropods. Since changes in viremia levels would alter the ability of an infected animal to serve as a potential amplifying host for the acquisition of virus for transmission by mosquito vectors, we also examined the effect temperature-humidity fluctuations, acting as environmental stressors, had on the levels of circulating virus and antibody in WEE virus-infected snowshoe hares.

This communication reports the results of our experiments.

MATERIALS AND METHODS

Experimental animals. Snowshoe hares from the area of Rochester, Alberta (54° north latitude), were trapped, tagged, shipped to Madison, and held in a

quarantine facility at Charmany Farm. Hares were housed individually in cages (84 by 38 by 61 cm), fed a specially made rabbit ration (University of Wisconsin feedmills), and watered ad libitum. Animals were bled for serological studies from the central ear artery using 5-ml plastic syringes equipped with 23-gauge needles. Serum was aseptically collected and stored in 1-dram vials at −25 C. The BHK-21 microneutralization test (13) was employed for antibody assay.

Cell cultures and media. BHK-21 cells (clone 13) adapted to fetal bovine serum, originally obtained from the Department of Virology, U.S. Army Medical Component, SEATO, Bangkok, Thailand, were grown in 32-oz (about 0.946 liters) glass prescription bottles in 30 ml of medium. Growth medium for cell cultures consisted of medium 199 in Hanks balanced salt solution without sodium bicarbonate (GIBCO, Grand Island, N.Y.), 5% fetal bovine serum heated at 56 C for 30 min, and antibiotics giving a final concentration of 200 U of potassium penicillin G per ml and 200 μg of streptomycin sulfate per ml, adjusted to a pH of 7.2. Outgrowth medium for use in the microneutralization test was similar to the above except that 10% fetal bovine serum was used and the final pH was adjusted to 7.5. All pH adjustments were accomplished using either 0.1 N HCl or 7.5% $NaHCO_3$.

Virus. WEE virus employed in experimental inoculation was a lyophilized stock of the Fleming isolate from a fatal human case in California. Seed virus was supplied by the Center for Disease Control with a recorded history of ten passages in mice and two passages in eggs. The wet-stock virus used in the microneutralization test was of the same origin with a history of 11 mouse passages and was stored as a 10% solution of growth medium containing 10% fetal bovine serum in a mechanical freezer at −65 C.

Host modification experiments. Two experiments were performed at two distinct periods of the year. Snowshoe hares inoculated in February 1973 (winter study) were reproductively inactive. Those inoculated in June 1973 (summer study) were reproductively active. Prior to each experiment, hares were segregated into two groups, experimental and control. Animals were evenly distributed among groups by age and sex. All experimental group animals were exposed to fluctuating temperature (range, −10 to 25 C) and humidity (range, 70 to 80% relative humidity). Control animals for these studies were held at 10 C and 50% relative humidity. The fluctuating-temperature program was based on actual weather data gathered at the station in Athabasca, Alberta, near the Rochester research site.

All host modification experiments were performed in the University of Wisconsin Biotron, a controlled-environment facility. Experimental and control snowshoe hares were individually caged in two separate but identical rooms (2.6 by 3.7 m), where temperature, humidity, and photoperiod were controlled within narrow limits by a central computer. Prior to inoculation, all animals were acclimated for 6 days to a cyclical daily high (15.5 C) and then a low (4.5 C) temperature at a constant 50% relative humidity. Within 12 h after the termination of the acclimation sequence and the beginning of the experimental environmental temperature regimen, snowshoe hares were inoculated subcutaneously with a 2-ml suspension of virus described below for each experiment.

All animals were bled from the central ear artery for 6 days at 24-h intervals for titration of antibody and circulating virus. For viremia titrations, 1 ml of fresh whole blood was placed in a 1-dram vial, capped, sealed with Parafilm (American Can Co., Neenah, Wis.), and quick frozen in an alcohol/dry-ice bath. The remaining blood was allowed to clot for serum extraction. After the initial 6-day period, blood was taken for serum samples only on the following schedule: every other day for week 2, every third day for week 3, once in week 4, and once in the following month. Viremia was quantified by intracerebral inoculation in 3- to 4-day-old suckling mice, using the Reed and Muench end-point determination method (14). Unless otherwise stated, all viremia titers were expressed as $\log_{10}$ of the virus dilution. Antibody titration was performed on serial twofold dilutions of sera made from the initial 1:2 dilution and tested against 100 mean tissue culture lethal doses of virus. End points for serum titration were read microscopically as the highest serum dilution which prevented cytopathic effect.

Statistical analysis. Differences in viremia amplitude and duration and antibody response were tested for significance by the simple, unpaired, one-tailed Student's t test. Although animals were evenly distributed as to age and sex, this was not a pairing. The one-tailed test of significance was used since a one-directional deviation could be reasonably expected from the literature. Differences in the time of onset of viremia between groups were tested for significance in a contingency table (2 by 2) (18). Significance of this test was computed with one-tailed z values at the 0.05 level.

Antibody analysis was based on geometric mean $\log_2$ titers.

RESULTS

Effect of temperature-humidity fluctuation on viremia. (i) Winter study. A total of 14 snowshoe hares was inoculated with 7.0×10^4 mean suckling mouse lethal doses of WEE virus. All seven experimental hares and six of seven controls developed a demonstrable viremia. The mean viremia titers of experimental hares on days 1, 2, and 3 (2.54, 4.65, 3.17) were significantly higher ($P < 0.05$) than those of controls (1.40, 2.93, 2.53) (Table 1). Control hare 64 was not included in these calculations because it developed no demonstrable viremia during the 5-day period of observation. Day 4 viremias were found not to be significantly different because of the small number of animals having a persistent viremia.

The differences in viremia duration and time of onset between the two groups were also tested for significance. Duration was calculated as the total days of viremia in each group divided by the number of animals in that group which developed a demonstrable viremia during the 5-day observation period. The difference, 3.57

days (experimental) compared with 2.51 days (control), was significant at $P < 0.01$. Time of viremia onset was compared in a contingency table (2 by 2) as described. All of the experimental hares developed detectable viremia on day 1, whereas only half (three of six) of the controls did so. This difference was significant at $P < 0.05$.

(ii) Summer study. All six experimental and six control animals inoculated with 3×10^5 mean suckling mouse lethal doses of WEE virus responded with viremia. Mean viremia titers for experimental and control hares for days 1, 2, and 3 post-inoculation were 1.8, 3.2, 2.7 and 1.0, 3.1, 1.5. These differences were not significant at $P < 0.05$ (Table 2). Viremia duration and onset were compared as previously described. Viremia lasted 3.2 days in experimental hares and 2.2 days in controls. This difference of 1.0 days was highly significant at $P < 0.001$. On day 1 post-inoculation, four of five experimental hares developed detectable viremia, whereas only two of five controls did. These differences were not significant at $P < 0.05$.

Effect of temperature-humidity fluctuation on antibody. (i) Winter study. All experimental and six of seven control hares responded with at least a fourfold increase in antibody titer against WEE virus. Above this level, responses were highly varied and were reflected in a large standard error (Table 3). Comparison of $\log_2$ geometric mean titers showed experimental hares had significantly higher antibody titers than did controls.

(ii) Summer study. All surviving experimental hares (three of five) and four of five controls developed fourfold increases in antibody titer. Antibody to WEE virus appeared on day 4 and rose slightly on days 5 and 6. On day 6, this study was terminated and all surviving hares were removed to the animal holding facilities at Charmany Farm. On day 14, these animals were bled. Comparison of $\log_2$ geometric titers showed no difference between experimental and control hares (Table 4).

TABLE 1. *Mean viremia titer[a] of snowshoe hares inoculated with 7×10^4 mean suckling mouse lethal doses of WEE virus at fluctuating low mean or constant low temperatures (winter study)*

Days post-inoculation	Temperature group[a]		Level of significance[b]
	Low fluctuating	Low constant	
1	2.54 ± 0.37	1.40 ± 1.54	0.05
2	4.65 ± 0.83	2.93 ± 1.81	0.05
3	3.17 ± 0.48	2.53 ± 0.35	0.05
4	1.00 ± 1.25	0.36 ± 0.89	NS

[a] Titer expressed as the $\log_{10}$ per milliliter ± the standard error.

[b] Calculated as one-tailed Student's *t* test. NS, Not significant ($P < 0.05$).

TABLE 2. *Mean viremia titer[a] of snowshoe hares inoculated with 3×10^5 mean suckling mouse lethal doses of WEE virus at fluctuating low mean or constant low temperatures (summer study)*

Days post-inoculation	Temperature group		Level of significance[b]
	Low fluctuating	Low constant	
1	1.8 ± 1.0	1.0 ± 1.3	NS
2	3.2 ± 0.9	3.1 ± 0.7	NS
3	2.7 ± 0.3	1.5 ± 1.4	NS
4	0.9 ± 1.3	0.4 ± 0.9	NS

[a] Titer expressed as the $\log_{10}$ per milliliter ± standard error.

[b] Calculated as one-tailed Student's *t* test. NS, Not significant ($P > 0.05$).

DISCUSSION

Our investigation showed that snowshoe hares could be infected with WEE virus, developed transient viremias, and produced neutralizing antibodies. This is the first report of WEE virus infection with viremia in the snowshoe hare. In addition, the results indicate that the responses of the snowshoe hares to WEE virus infection are not static with respect to either season or environmental conditions. When exposed to fluctuating low mean temperatures, these animals exhibited changes in viremia and antibody compared with controls held at constant temperature. To our knowledge, this is the first report of environmental modification of the response of a non-hibernating wild mammalian host after infection by a togavirus.

We did not investigate the possible mechanisms underlying the observed viremia and antibody changes. Perhaps the most likely explanation is the concept of stress response of mammals (16) and the effects that stress and corticoids have been shown to have as immunosuppressants (1, 5, 6, 17, 20) and on nonimmune (nonspecific) defense systems (10), including reduction of interferon production (3, 4, 9).

Antibody titers of winter study experimental hares were observed to be significantly higher than those of controls. There are three possible interpretations of this observation: (i) fluctuating temperature stress induced enhancement of the immune response in experimental hares; (ii) exposure to constant temperature caused depression of the immune response in control hares; (iii) the different temperature regimen had little effect on the immune response, and differences observed in antibody titers reflected

TABLE 3. *Comparison of mean antibody titers[a] of snowshoe hares inoculated with 7 × 10⁴ mean suckling mouse lethal doses of WEE virus at fluctuating and constant low mean temperatures (winter study)*

Days post-inoculation	Temperature group[a]		Level of significance[b]
	Low fluctuating	Low constant	
4	1.58 ± 1.42	1.21 ± 1.39	NS
6	6.90 ± 2.10	2.70 ± 1.61	0.05
8	12.95 ± 2.85	2.92 ± 1.49	0.01
10	17.60 ± 4.70	3.86 ± 1.66	NS
14	49.80 ± 5.62	7.25 ± 2.16	0.05
17	300.00 ± 7.06	13.24 ± 2.27	0.01
21	300.00 ± 8.18	13.24 ± 3.71	0.02
28	259.00 ± 4.56	17.7 ± 4.50	0.02
41	310.00 ± 2.22	11.4 ± 1.92	0.001

[a] Reciprocal of twofold dilution end point (± standard error) in microneutralization test.

[b] Calculated using Student's one-tailed *t* test and geometric mean titers. NS, Not significant ($P > 0.05$).

TABLE 4. *Comparison of mean antibody titers[a] of snowshoe hares inoculated with 3.0 × 10⁵ mean suckling mouse lethal doses of WEE virus at fluctuating low and constant temperatures (summer study).*

Days post-inoculation	Temperature group		Level of significance[b]
	Low fluctuating	Low constant	
5	4.0 ± 2.6	2.8 ± 1.4	NS
6	5.3 ± 2.4	5.3 ± 2.8	NS
14	465 ± 10.0	64 ± 5.9	NS

[a] Reciprocal of twofold dilution end point (± standard error) in microneutralization test.

[b] Calculated using Student's one-tailed *t* test and geometric mean titers. NS, Not significant ($P > 0.05$).

the levels of viremia which were in turn indicative of antigenic mass.

Although stress-induced enhancement of antibody production has been postulated, such a phenomenon has been largely discredited. Neither appearance nor titer of antibody elicited in stressed or control mice infected with vesicular stomatitis virus differed (21). Our observation of increased viremia under conditions of fluctuating temperatures does not appear to be consistent with the hypothesis that these same conditions enhance the immune response. Nor does the hypothesis that constant temperatures suppress the immune response seem likely. Exposures to constant cold temperature did not alter the immune response of mice (2) or rabbits (12).

The most likely interpretation of the antibody data presented here is that fluctuating temperature stress has little effect on the antibody-producing system of snowshoe hares infected with WEE virus. In our study, the significantly greater antibody titers in experimental hares closely paralleled the significantly greater viremia in this group compared to controls. In summer study hares, no difference between experimental and control group antibody titers was associated with no difference between group viremia titers.

A comparison of winter and summer study experimental group viremia data suggests that animals were more susceptible to stress plus infection earlier in the year than later. This difference in host modification by environmental influences cannot be explained by season alone, since winter and summer studies showed similar viremia response under constant temperatures. Similar seasonal phenomena have been observed in laboratory hamsters (19). These animals showed a greater susceptibility to stress plus infection with poliomyelitis virus in February than in either May or September. These differences appeared to be correlated with adrenal cortical activity, although further investigation in this area is needed.

In this study, the more natural condition of variable climatic conditions must be considered more stressful than the artificial laboratory or control situation of static temperature and humidity. One implication of these findings is that in laboratory studies designed to test host-pathogen relationships among wild species, erroneous conclusions may be reached if the results of such studies are extrapolated to the field condition without considering environmental effects.

That the snowshoe hare may be a host of WEE virus in the wild is consistent with two epidemiological studies made during the last decade. Between the period April to June 1963, and again in 1965, epizootics of WEE swept through the hare population in the boreal forest around Rochester, Alberta (23). Within these 3-month periods, neutralizing antibody prevalence in captured hares rose from less than 10% to greater than 90% for WEE virus. After June, the prevalence declined and then leveled off. On both occasions, the epizootics in hares were followed 2 to 3 months later by epidemics of WEE in human beings and epizootics in horses in the transitional aspen parkland to the south. The present report supports the earlier suggestion by Yuill and Hanson (22) that the snowshoe hare may have acted as a virus amplifier on both occasions.

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LITERATURE CITED

1. Brown, G. L., M. V. Rothlauf, R. H. Heinzerling, and T. S. Myers. 1971. Antibody formation in rabbits adapted and nonadapted to high altitude. Infect. Immun. **3**:358-360.
2. Chaffee, R. R. J., and C. M. Martin. 1962. The immune response in cold acclimated mice. Proc. Soc. Exp. Biol. Med. **111**:375-377.
3. Chang, S-S, and A. F. Rasmussen, Jr. 1965. Stress-induced suppression of interferon products in virus infected mice. Nature (London) **205**:623-624.
4. DeMaeyer, E., and J. DeMaeyer. 1963. Two-sided effect of steroids on interferon in tissue culture. Nature (London) **197**:724-725.
5. Elliot, E. V., and N. R. St. C. Sinclair. 1968. Effect of cortisone acetate on 19S and 7S haemolysin antibody. A time course study. Immunology **15**:643-652.
6. Gisler, R. H., and L. Schenkel-Hulliger. 1971. Hormonal regulation of the immune response. II. Influence of pituitary and adrenal activity on immune responsiveness *in vitro*. Cell. Immunol. **2**:646-657.
7. Hoff, G. L., T. M. Yuill, J. O. Iversen, and R. P. Hanson. 1970. Selected microbial agents in snowshoe hares and other vertebrates of Alberta. J. Wildl. Dis. **6**:472-478.
8. Iversen, J. O., G. L. Seawright, and R. P. Hanson. 1971. Serologic survey for arboviruses in Central Alberta. Can J. Public Health **62**:125-132.
9. Jensen, M. M. 1973. Possible mechanisms of impaired interferon production in stressed mice. Proc. Soc. Exp. Biol. Med. **142**:820-823.
10. Kass, E. H., and M. Finland. 1953. Adrenocortical hormones in infection and immunity. Annu. Rev. Microbiol. **7**:361-388.
11. Miles, J. A. R. 1960. Epidemiology of arthropod-borne encephalitides. Bull. W. H. O. **22**:339-371.
12. Northey, W. T. 1964. Studies on the interrelationships of cold environment, immunity and resistance to infection. I. Qualitative and quantitative studies on the immune response. J. Immunol. **94**:649-657.
13. Pantuwatana, S., W. H. Thompson, D. M. Watts, and R. P. Hanson. 1972. Experimental infection of chipmunks and squirrels with LaCrosse and Trivittatus viruses and biological transmission of LaCrosse virus by *Aedes triseriatus*. Am. J. Trop. Med. Hyg. **21**:476-491.
14. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27**:493-497.
15. Reeves, W. C., and W. McD. Hammon. 1962. Epidemiology of the arthropod-borne encephalitides in Kern County, California, 1943-1952. Univ. Calif. Berkeley Publ. Public Health. **4**:1-257.
16. Selye, H. 1950. The physiology and pathology of exposure to stress. Acta Inc. Med. Publ., Montreal.
17. Solomon, G. F. 1969. Stress and antibody response in rats. Int. Arch. Allergy **35**:97-104.
18. Steele, R. G. D., and J. Torrie. 1960. Principles and procedures of statistics, p. 372. McGraw-Hill, New York.
19. Teodoru, C. V., and G. Schwartzman. 1956. Endocrine factors in pathogenesis of experimental poliomyelitis in hamsters. Role of inoculatory and environmental stress. Proc. Soc. Exp. Biol. Med. **91**:181-187.
20. Vessey, S. H. 1964. Effect of grouping on levels of circulating antibodies. Proc. Soc. Exp. Biol. Med. **115**:252-255.
21. Yamada, A., M. M. Jensen, and A. F. Rasmussen, Jr. 1964. Stress and susceptibility to viral infection. III. Antibody response and viral retention during avoidance-learning stress. Proc. Soc. Exp. Biol. Med. **116**:677-680.
22. Yuill, T. M., and R. P. Hanson. 1964. Serologic evidence of California encephalitis virus and western equine encephalomyelitis in snowshoe hares. Zoo. Res. **3**:153-164.
23. Yuill, T. M., J. O. Iversen, and R. P. Hanson. 1969. Evidence for arbovirus infections in a population of snowshoe hares. A possible mortality factor. Bull. Wildl. Dis. **5**:248-253.