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Authors: LEUNG, M. K., IVERSEN, J., McLINTOCK, J., and SAUNDERS, J. R.

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SUBCUTANEOUS EXPOSURE OF THE RICHARDSON'S GROUND SQUIRREL (*Spermophilus richardsonii* Sabine) TO WESTERN EQUINE ENCEPHALOMYELITIS VIRUS

M. K. LEUNG[1], J. IVERSEN[1], J. McLINTOCK[2] and J. R. SAUNDERS[1]

Abstract: Both adult and suckling Richardson's ground squirrels (*Spermophilus richardsonii*) were susceptible to subcutaneous exposure with western equine encephalomyelitis (WEE) virus, but the virus was more virulent for sucklings than adults. In sucklings, the incubation period was from 4 to 5 days, followed by apparent signs of central nervous system (CNS) involvement. Death occurred 10 to 13 days post-exposure. In adults, infections were inapparent or acute with typical signs of CNS involvement similar to those observed in sucklings. In both age groups, brain and lymph nodes were the most frequently involved tissues. The highest titres of virus were recovered from brain, sections of which also showed the most marked histological changes. Lesions in the brain included multifocal vasculitis, perivascular edema, perivascular cuffing, focal or diffuse gliosis, parenchymal hemorrhage, meningitis with infiltration of mononuclear cells, neuronal degeneration, and occasional demyelination. For both age groups, viremias were detected for 3 to 5 days with a maximum virus titre of 4 to 6 logs, a sufficient time and magnitude to infect numerous mosquitoes, further supporting the hypothesis that *S. richardsonii* may serve as an amplifying host of WEE virus in the prairie provinces.

INTRODUCTION

The Richardson's ground squirrel (*Spermophilus richardsonii*) is widely distributed and abundant throughout agricultural areas of the prairie provinces of Manitoba, Saskatchewan and Alberta.[8] Geographic distribution of this squirrel coincides with distribution of human and equine cases of western equine encephalomyelitis (WEE) and with the geographic distribution of *Culex tarsalis*, the vector of WEE virus.[21] During a long-term study (1964-1973), WEE seropositive squirrels were found each summer in the known enzootic regions of Saskatchewan.[16] Serologic data, coupled with isolations of WEE virus from Richardson's ground squirrels during the survey, led to the hypothesis that *S. richardsonii* could serve as early seasonal (*i.e.*, during June) amplifying hosts for the virus.[16]

To determine the dynamics of an infection that would closely resemble the natural infection in Richardson's ground squirrels, the present study was conducted utilizing subcutaneous exposure to obtain information by virologic and histopathologic methods on: 1) relative susceptibility of juvenile and adult squirrels to WEE virus infection; 2) duration and magnitude of WEE viremia; and 3) pattern of distribution of WEE virus in tissues of the squirrels.

MATERIALS AND METHODS

Virus

WEE virus strain WMIS 55-71 (originally isolated from *C. tarsalis* females collected in August, 1971, near Weyburn, Saskatchewan) was used as the inoculum. The virus used had undergone three successive passages in suckling mouse brains

[1] Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan.

[2] Canada Department of Agriculture, Research Station, University Campus, Saskatoon, Saskatchewan.

and had a titre of $10^{7.2}$ mouse $ICLD_{50}$/0.02 ml in 3-week-old Swiss white mice, $10^{8.5}$ mouse $ICLD_{50}$/0.02 ml in 2- to 3-day-old suckling mice, $10^{9.5}$ $TCID_{50}$/0.2 ml in BHK_{21} (baby hamster kidney line obtained from the Banting Institute, University of Toronto) cell culture, and $10^{10.8}$ plaque-forming units (P.F.U.)/ml in the same cell culture system.

Cell cultures

Titrations of the infectious WEE virus were made in continuous cultures of BHK_{21}. The growth medium was Medium 199 buffered with 0.22% sodium bicarbonate and containing 10% calf serum and antibiotics (100 units of penicillin and 100 mcg of streptomycin per ml). In flasks inoculated with viral suspensions, the maintenance medium was the same as the growth medium except that 5% agamma calf serum was used.

Richardson's ground squirrels

Adult Richardson's ground squirrels were caught by live-traps in the agricultural areas of Saskatchewan during the summer of 1973. They were maintained individually in cages in the laboratory at 20 C for 2 to 3 months prior to experimentation, at which time they weighed 350 to 500 g. Suckling squirrels of litters born in captivity were also used as host animals. Sucklings were 22- to 28-days-old and weighed 85 to 150 g at exposure. Prior to exposure, all squirrels were bled by puncturing the retro-orbital venous plexus. During experimentation, all squirrels were maintained at 20 C.

Experimental inoculations

Experiment 1

Adult Richardson's ground squirrels were inoculated subcutaneously with ten-fold dilutions of the stock virus. Inoculated squirrels were bled retro-orbitally daily for 7 days to determine titres and duration of viremias. Squirrels were observed for clinical signs for at least 3 weeks. After 3 weeks, survivors of the titration were bled for serology.

Experiment 2

Adult Richardson's ground squirrels were killed sequentially after receiving 3.3 $\log_{10}$ suckling mouse $ICLD_{50}$ of WEE virus subcutaneously. On the designated day, squirrels were anesthetized in a CO_2 chamber, bled to death by cardiac puncture, and their tissues taken for histopathology and viral assay.

Experiment 3

Sucklings were inoculated subcutaneously with 1.8, 2.8 and 3.8 logs of WEE virus for a titration of infectivity. For each virus dose, four sucklings were inoculated with virus, and four were used as uninoculated controls. Both the inoculated and control sucklings were kept in the same cage with their mothers. Inoculated sucklings were allowed to develop disease and die naturally. At 29 days post-exposure all survivors, including the controls and their mothers, were bled for determination of serum neutralizing antibodies in response to infection and possible contact transmission.

Experiment 4

Six sucklings were each inoculated with 2.8 logs of WEE viral suspension. One suckling was used as a control. Inoculated sucklings were killed on a daily schedule. Tissues were collected aseptically for viral assay and histopathology.

Viral assay

Each tissue was removed with sterile instruments, washed with phosphate buffered saline (PBS) with a pH 7.4 at 0-4 C), and divided into two parts. One part was weighed and triturated with diluent to make a 20% suspension. The tissue homogenate was then centrifuged at 1020 x g for 10 min at 4 C, and the supernatant collected for viral assay. Titres of virus in blood or tissue homogenates were established by inoculating ten-fold dilutions of each suspension onto BHK_{21} cell culture monolayers. The inoculum was allowed to adsorb for one hour at 37 C and the maintenance medium was added. Cultures were observed 5 to 7 days for cytopathic effect and the

$TCID_{50}$ calculated by the Karber method.[15] The virus recovered from Richardson's ground squirrel tissues was confirmed as WEE virus by neutralization with hyperimmune rabbit WEE antiserum in BHK_{21} cell cultures.

Serology

The presence of antibodies to WEE virus in squirrel sera was determined by the plaque reduction neutralization method.[15] Serum-virus mixture was allowed to adsorb onto BHK_{21} monolayers for 1 hr at 37 C before agar overlay was added.

Histopathology

Tissue sections of 5 to 6 μm were processed by the Carnoy-chloroform-paraffin technique.[3] Sections were stained with hematoxylin and eosin.

RESULTS

Pre-exposure serology

No adult or suckling Richardson's ground squirrels had detectable WEE virus neutralizing antibody prior to the experiment.

Experiment 1

Clinical signs and mortality:

Clinical illness was observed in 5 of 9 inoculated adult squirrels. The onset of disease was from 6 to 8 days, and the signs were central nervous system (CNS) disturbance with depression, excitability, circling, paralysis of hind limbs and marked involuntary salivation. During the first through third day post-exposure, there was a 1 to 2 C elevation in rectal temperature. Three of nine inoculated squirrels died, one on day seven (Richardson's ground squirrel 104) and two on day eight (Richardson's ground squirrels 102 and 105). Titres of virus in their brains were 4.7, 5.2 and 4.7 logs, respectively.

Viremia:

Subcutaneous exposure produced infection in all inoculated adult squirrels with one exception (Table 1). In squirrels receiving 2.5 logs or more of virus, the viremia lasted 3 to 5 days with peak titres varying from 2.2 to 6.5 logs per ml of blood. The peak of the febrile responses tended to coincide with onset of viremia. There was a tendency for earlier viremias in squirrels receiving higher doses of virus.

Serology:

With the exception of one inoculated adult squirrel (Richardson's ground squirrel 109), all survivors produced titres of neutralizing antibodies of 2.3-3.0 or more logs (Table 1).

Experiment 2

Viral assay of tissues:

With the exception of the lymph nodes and brain, very few tissues of adult squirrels contained WEE virus. For the first 2 days post-exposure, all tissues tested were negative for WEE virus with the exception of blood. Virus was detected in kidney on the third day post-exposure and did not reach lymph nodes and brain until the fourth and sixth days respectively. In brain and lymph nodes, WEE virus persisted for as long as 5 and 9 days, respectively. Highest titres of the virus were in brain (Table 2).

Histopathology:

Consistent significant lesions were observed in the brain of adult squirrels and affected primarily the cerebral cortex, cortex, brain stem, cerebellum and the overlying leptomeninges, whereas the olfactory lobes were not affected. Changes observed in the involved areas consisted of multifocal vasculitis and perivascular cuffing. Affected vessels had rounded, swollen endothelial cells that reduced the vascular lumen. Frequently, the vessels were surrounded by proteinaceous fluid. Focal and diffuse gliosis accompanied by neuronophagy and parenchymal hemorrhage persisted throughout the interval when virus was detected in the brain. The non-suppurative leptomeningitis overlying affected areas of brain and adjacent to inflamed congested meningeal vessels was a

TABLE 1. Viremias of WEE virus and post-exposure WEE antibodies in Richardson's ground squirrel adults — Experiment 1.[a]

Richardson's ground squirrel No.	Virus dose [b]	Titres of Viremia by Days Post-exposure [c]							Antibody titre [d]
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	
101	7.5	3.2	4.2	3.7	—	—	—	—	>3.0
102	7.2	4.2	3.2	2.2	—	—	—	ND*	ND
103	6.5	3.5	4.2	3.7	4.5	—	—	—	2.3
104	4.5	—	3.9	6.5	4.2	<0.5	—	—	ND
105	4.3	1.9	<0.5	—	2.2	—	—	ND	ND
106	3.5	2.2	4.5	5.2	4.2	—	—	—	>3.0
107	2.5	—	2.7	6.0	6.2	3.0	—	—	>3.0
108	1.5	—	—	—	1.7	—	—	—	—
109	0.5	—	—	—	—	—	—	—	—

[a] Each adult squirrel received 0.1 ml of viral suspensions subcutaneously.
[b] Log_{10} suckling mouse $ICLD_{50}$.
[c] Titre = log_{10} TCD_{50} (BHK_{21} cells) per ml of blood.
[d] Log_{10} TCD_{50} neutralized by serum 20 days after exposure.
* ND = not tested.

TABLE 2. Recovery of WEE virus from tissues of killed Richardson's ground squirrel adults — Experiment 2.[a]

Richardson's ground squirrel No.	Day	Titres of WEE virus in adults killed post-exposure										
		Blood	Brain	Lymph node	Liver	Spleen	Bone marrow	Adrenal	Kidney	Lung	Skeletal muscle	Brown fat
110	1	—	—	—	—	—	—	—	—	—	—	—
111	2	2.2[b]	—	—	—	—	—	—	—	—	ND[c]	—
112	3	—	—	—	—	—	—	—	2.5	—	—	2.2
113	4	1.9	—	4.2	—	—	—	—	—	—	—	3.7
114	5	—	—	—	—	—	<0.5	—	ND	—	—	—
115	6	—	6.2	3.2	—	—	—	—	—	—	—	—
116	7	—	6.2	2.2	—	—	—	—	—	—	—	—
117	12	—	3.2	2.2	—	3.2	2.2	—	—	1.7	—	—
118	15	—	—	—	—	—	—	—	—	—	—	—
119	20	—	—	—	—	—	—	—	—	—	—	—
Mean titre of positive tissues		2.1	5.2	3.0	0	3.2	1.4	0	2.5	1.7	0	1.4

[a] Each adult squirrel received 3.3 $\log_{10}$ suckling mouse $ICLD_{50}$ of WEE virus subcutaneously.
[b] Titre = $\log_{10}$ TCD_{50} (BHK_{21} cells) per ml of blood or per gram wet weight of tissue.
[c] ND = not tested.

common occurrence. Involvement of the cerebellum was minimal with some degeneration of Purkinje cells and focal hemorrhage in the granular and intermolecular layer.

Experiment 3

Clinical signs:

In suckling squirrels, signs of disease indicating involvement of the CNS appeared after an incubation period of 4 to 5 days. Affected squirrels were depressed or excitable, with frequent squealing, ataxia, incoordination of movement, arching of the back accompanied with ruffling of fur, and involuntary salivation. In the terminal stages, when the squirrels were comatose and laying on their sides, intermittent convulsions, stretching of hind limbs and tremors were observed.

Mortality pattern:

Only four of 12 inoculated sucklings survived (Table 3). Although clinical signs appeared at 4 to 5 days post-exposure, mortality was delayed until 10 to 13 days. None of the 12 contact controls showed signs of disease or developed WEE virus neutralizing antibodies.

TABLE 3. Mortality ratios in Richardson's ground squirrel sucklings by dose of WEE virus — Experiment 3.[a]

Virus dose [b]	No. infected [c] No. exposed	No. mortality/ No. exposed	Days to death (post-exposure)
1.8	2/4	2/4	13
2.8	3/4	3/4	11
3.8	4/4	3/4	10 - 11

[a] Each squirrel received subcutaneously 0.1 ml of WEE viral suspension.

[b] Log_{10} suckling mouse $ICLD_{50}$ per 0.02 ml.

[c] Infected = dead or seropositive.

Experiment 4

Viremia:

Virus titres in the blood occurred from day one through day five with a peak on the second day, and they varied from 1.0 to 4.2 logs (Table 4). The average titre was 2.7 logs for the entire viremia. An elevation in rectal temperature of 1 to 2 C was assciated with the viremia.

Viral assay of tissues:

In addition to the CNS, all of the extraneural tissues tested were positive for WEE virus at one time or another (Table 4). Lymph nodes and brain were the most frequently positive tissues. The pattern of involvement was sequential for the various tissues. Virus was found in the lymph nodes throughout the experiment. From the second day onward, the brain was involved. The number of positive tissues was maximal on the third and fourth days. As viremia declined, the number of positive tissues decreased so that on the sixth day only the brain, lymph nodes, spleen and adrenals remained positive. The highest average virus titers were from the brain.

Histopathology:

Lesions in the brain occurred in the cerebral cortex, brain stem, cerebellum and the meninges overlying these structures. Squirrels killed at 2 and 3 days post-exposure had no vascular endothelial changes although the virus titre was 1 to 2 logs in the brain. On the fourth day and thereafter, widespread perivascular

TABLE 4. Recovery of WEE virus from tissues of killed Richardson's ground squirrel sucklings — Experiment 4.[a]

RGS No.	Day	Titres of WEE virus in sucklings killed post-exposure										
		Blood	Brain	Lymph node	Liver	Spleen	Bone marrow	Adrenal	Kidney	Lung	Skeletal muscle	Brown fat
120	1	3.2[b]	—	2.2	—	—	—	—	—	—	—	—
121	2	4.2	2.2	3.7	—	—	—	—	—	—	—	—
122	3	3.0	1.7	3.7	3.2	3.7	1.7	1.7	1.7	—	3.2	1.7
123	4	2.0	7.2	4.7	—	4.2	3.2	1.7	—	1.7	ND[c]	1.7
124	5	1.0	8.2	3.2	—	3.2	1.7	—	1.7	—	ND	1.7
125	6	—	7.7	3.7	—	2.7	—	—	—	—	1.7	—
126 (control)	7	—	—	—	—	—	—	—	—	—	—	—
Mean titre of positive tissues		2.7	5.4	3.5	3.2	3.5	2.2	1.7	1.7	1.7	2.5	1.7

[a] Each suckling squirrel received 2.8 $\log_{10}$ suckling mouse $ICLD_{50}$ of WEE virus subcutaneously.

[b] Titre = $\log_{10}$ TCD_{50} (BHK_{21} cells) per ml of blood or per gram wet weight of tissue.

[c] ND = not tested.

cuffing, vasculitis, neuronophagy, neuronal vacuolation and necrosis, and microglial infiltration were abundant. Focal and diffuse parenchymal hemorrhage, eosinophilic bodies, demyelination and spongy degeneration were also frequently observed in the cerebral cortex, the brain stem and the cerebellum. On occasion, mineralized neurons were found in the cortex.

DISCUSSION

Although both adult and suckling Richardson's ground squirrels were susceptible to subcutaneous exposure, WEE virus was more virulent for sucklings than adults. At a relatively low dose of 1.8 logs of virus (a quantity equivalent to that delivered by a mosquito), mortality occurred in sucklings, whereas a comparable dose in adult squirrels produced no mortality. Age-dependent susceptibility to encephalitis has been recognized as a common feature in many experimental arbovirus infections.[7,11,12,13,14,19,20,23]

Albrecht[1] emphasized the importance of the extraneural phase as a determinant in the invasion of the CNS. The present study is consistent with this latter point of view since comparable doses of virus in adult and suckling squirrels produced a marked difference in the outcome of the infection, *viz.*, dissemination of WEE virus was much less widespread and somewhat delayed in the adults. On occasion, the virus probably bypassed the peripheral resistance in adults, it spread to the CNS by replication via the cerebrovascular endothelial cells, and encephalitis occurred as the neural tissues remained susceptible.

The experimental subcutaneous infections provide some clues as to the impact of WEE virus on squirrel populations. Isolations of virus from brains of apparently healthy adult squirrels and detection of many seropositive adult animals during the previously reported long-term field survey in Saskatchewan,[16] coupled with non-fatal experimental infections following subcutaneous exposure of adult squirrels to WEE virus, suggest the virus would have little effect on the adult segment of the population. On the other hand, widespread WEE virus infection in juvenile squirrels could be expected to have a pronounced impact on the Richardson's ground squirrel population. Results of the present study are consistent with this hypothesis since the virus was markedly virulent for suckling squirrels. Since contact transmission among sucklings did not occur in this study, it must be assumed the transmission among young Richardson's ground squirrels involves mosquitoes. Richardson's ground squirrels are born in the first week of May, however young do not emerge from their burrows of natality until 3 or 4 weeks old,[18] the age of the sucklings used in this study. Emergence of the young coincides with the emergence of the spring aedine mosquitoes.[17] Whereas adult Richardson's ground squirrels hibernate during July, young of the year remain active above ground during August, the peak of WEE virus infection in mosquitoes in the prairies.[17,18] Therefore, young of the year remain exposed throughout the summer cycling of the virus.

In the Canadian prairies, evidence is accumulating that an early seasonal amplification of WEE virus occurs in wild mammal populations. Early seasonal amplification of the virus was reported in the snowshoe hare (*Lepus americanus*) in Alberta during 1963[24] and 1965,[10] epidemic years of WEE in that province. During the long-term survey of WEE virus in Richardson's ground squirrels in Saskatchewan, all isolations of WEE virus and the maximum rate of seropositive squirrels occurred during June, a time 1 to 2 months in advance of the peak of the *C. tarsalis*-bird cycle.[16,17] Early seasonal amplification in the Richardson's ground squirrel could feasibly serve as a source for the epidemic cycle of late summer in the prairies. Previous experimental studies on mosquito transmission utilizing avian hosts indicated a titre of 1.0 to 3.2 logs mouse LD_{50} was sufficient to infect 20 to 50% of *C. tarsalis* and a somewhat lower threshold (1.5 to 2.5 logs) for 30 to 100% *Aedes* mosquitoes.[4,5,6,8,9,22]

In Richardson's ground squirrels, a viremia lasting 3 to 5 days with a maximum virus titre of 4 to 6 logs in adults or sucklings would be sufficient in time and magnitude to infect numerous mosquitoes. During the early summer, the potential mosquito vectors amongst squirrels would include the aedines (*viz., Aedes flavescens, A. spencerii, A. vexans, A. campestris* and *A. dorsalis*), all known mosquito hosts of WEE virus in Saskatchewan.[17]

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