Culex tarsalis Coquillett as a Vector of an Attenuated Strain of Western Equine Encephalomyelitis Virus.* (30790)

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An attenuated variant of western equine encephalomyelitis (WEE) virus with low neuropathogenicity for young adult mice(1,2) has been tested as a live virus vaccine for immunization of horses(3). Although 5 horses inoculated subcutaneously with vaccine failed to circulate virus, one horse injected intramuscularly developed a low level of viremia. This evidence of viremia in a vaccinated horse prompted inquiry into the infectivity of the attenuated virus for arthropod vectors. Preliminary studies showed that *Culex tarsalis*, the primary arthropod vector of WEE virus in the western United States(4) became infected after ingesting the attenuated virus. Further studies were undertaken to determine whether *C. tarsalis* could transmit the virus and whether the virus would retain its attenuated properties after multiplication in vertebrate hosts and mosquitoes. This paper reports the results of these studies.

Materials and methods. Dr. H. N. Johnson of the Rockefeller Foundation provided stock suspensions of the non-neuroadapted strain (B628, hereafter referred to as HK3 virus) of WEE virus in its third passage in hamster kidney cell cultures and the attenuated variant (Clone 15) derived from this strain. The histories of these viruses have been described in detail(1,2).

The mosquitoes, obtained from a laboratory colony of *C. tarsalis* maintained at Bakersfield, Calif., were 5 to 7 days old when fed on infected hosts. Fed mosquitoes, held in screened cages or pint ice cream cartons covered with nylon netting, were incubated at 78 to 85°F in the insectary. Cotton pads soaked in sugar solution on top of the containers provided food between blood meals and moist towels placed over the containers provided humidity.

Viremic chicks were the source of virus to infect mosquitoes. White leghorn chicks (7 days of age) were inoculated subcutaneously with $10^{1.5}$ to $10^{5.5}$ suckling mouse (SM) intracranial (IC) LD_{50} of HK3 virus or with $10^{1.8}$ to $10^{5.8}$ SM IC LD_{50} of Clone 15 virus. Groups of 25-50 mosquitoes were allowed to feed for 30 minutes upon each chick at 24-hour intervals for 4 days after the chicks were inoculated. Immediately after exposure to mosquitoes, a blood sample for viral titration was taken from each chick, ampouled and stored at −70°C. The mosquitoes that fed on each chick were kept in separate containers, except in one experiment when mosquitoes were pooled that had fed on 5 chicks infected with Clone 15 virus. Viral transmission was attempted 14 to 21 days after mosquitoes had fed on viremic chicks by allowing infected mosquitoes to bite normal chicks. All mosquitoes that re-fed on each chick were pooled, sealed in glass ampoules, and stored at −70°C. Mosquitoes that did not re-feed were ampouled in pools of 2-10 mosquitoes. Each host chick was bled 36-40 hours after mosquitoes had fed and the blood was ampouled and stored at −70°C. The recovery of virus from re-fed mosquitoes and from the blood sample taken from the host chick after mosquitoes had re-fed was accepted as proof of transmission.

Just prior to assaying for virus, clotted bloods were thawed, homogenized in an equal volume of 10% normal rabbit serum (NRS) in Hanks' balanced salt solution (HBSS) with antibiotics, and decimally diluted in phosphate-buffered saline containing 4% normal calf serum. Mosquito pools were triturated in 2 ml of 33% NRS in HBSS plus antibiotics including mycostatin and centrifuged at 400 *g* for 10 minutes.

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TABLE I. Viremias in Chicks Infected Subcutaneously with Varying Concentrations of a Non-Neuroadapted Strain (HK3) of WEE Virus and Its Attenuated Variant (Clone 15).

Virus strain	Log_{10} SM IC LD_{50} of virus inoculated into chicks	Log_{10} SM IC LD_{50} of virus per .01 ml of blood by hours post-inoculation			
		24 hr	48 hr	72 hr	96 hr
HK3	1.5	5.0	5.4	2.7	.8
		3.5	6.3	3.9	NT
	3.5	6.7	5.4	1.7	<.3
		5.2	4.5	2.1	NT
	5.5	7.3	6.6	2.1	<.3
		7.0	4.4	1.0	"
Clone 15	1.8	3.7	1.3	<.3	NT
		2.5	4.4	1.5	<.3
	3.8	4.1	3.1	<.3	"
		3.7	1.5	.3	"
		3.3	4.4	1.5	NT
		"	3.7	.3	"
	5.8	4.1	.8	<.3	<.3
		3.1	2.4	NT	NT

NT = not tested.

Viral titrations were performed by IC inoculation of SM and occasionally by plaquing on 48-hour monolayer cultures of duck embryonic cells (DECC). Each sample was inoculated into 8 SM (0.01 ml/mouse, 1-4 days old). Mice were observed for deaths daily for 14 days and the LD_{50} calculated (5). Cell cultures were grown in 5% calf serum, 0.25% lactalbumin hydrolysate, 0.1% yeastolate, and 1.0% vitamin stock (Microbiological Associates, MEM vitamin mixture, 100× concentration) in HBSS plus antibiotics. Two replicate cultures were each inoculated with 0.1 ml of test material and incubated for 1-1.5 hours at 37°C before overlaying with a medium consisting of 1.2% agar (Difco, purified), 2% calf serum, and 0.002% neutral red in 199 medium (modified to include 0.02 M sodium phosphite and 0.038 g of $NaHCO_3$/100 ml). Plaques were counted after 2-4 days' incubation at 36°C and viral titers were expressed as plaque forming units (PFU)/0.1 ml.

The attenuated character of Clone 15 virus after multiplication in chicks and mosquitoes was determined by comparing (1) average survival time (AST) of SM, (2) morbidity and mortality rates of weanling mice, and (3) plaque size of Clone 15 stock virus with 2 viral isolates from infected chick bloods and 2 isolates from pools of infected mosquitoes. Similar specimens from chicks and mosquitoes infected with the HK3 virus were tested concurrently. Five weanling mice (21-28 days old) were inoculated IC with 0.03 ml of test material and observed for 14 days for morbidity and death.

Results. Chicks were inoculated subcutaneously with varying concentrations of HK3 virus and Clone 15 virus to establish comparative viremia profiles. HK3 virus reached higher titers in blood and persisted slightly longer than did Clone 15 virus regardless of the infecting dose (Table I). The 6 chicks inoculated with HK3 virus had maximum viremia titers of $10^{5.2}$ to $10^{7.3}$ LD_{50} 24 and 48 hours after infection and viremias were present in 6/6 and 1/4 chicks at 72 and 96 hours respectively. In contrast, 8 chicks inoculated with Clone 15 virus developed peak viremia titers of only $10^{3.1}$ to $10^{4.4}$ LD_{50} 24 and 48 hours after infection and virus was detectable in bloods of 4/7 and 0/4 chicks at 72 and 96 hours, respectively.

Culex tarsalis became infected after ingesting HK3 virus or Clone 15 virus from viremic chicks (Table II). All pools of mosquitoes were infected that had fed on chicks circulating from $10^{4.5}$ to $10^{7.3}$ LD_{50} of HK3 virus. Clone 15 virus was not recovered from 94 mosquitoes, tested as 18 pools, that had fed on chicks having titers ranging from $10^{0.3}$ to $10^{2.5}$ LD_{50}. However, Clone 15 virus was

TABLE II. Infection of *Culex tarsalis* After Feeding on Chicks Infected with HK3 and Clone 15 Strains of WEE Virus.

Virus strain	Log_{10} SM IC LD_{50} of virus in chick bloods when mosquitoes fed	Mosquito infection*		
		No. mosquitoes tested	No. pools tested	No. pools infected
HK3	4.5–7.3	35	9	9
Clone 15	.3– .8	39	7	0
	1.2–1.5	30	6	0
	2.4–2.5	25	5	0
	3.1–3.7	47	10	9
	4.1–4.4	22	5	5

* Mosquitoes were tested 21 days after feeding on viremic chicks.

recovered from 14/15 pools of mosquitoes that had fed on chicks with viremia titers above $10^{3.0}$ LD_{50}.

No attempt was made to determine whether HK3 and Clone 15 viruses multiplied at the same rates in mosquitoes. However, pools of *C. tarsalis* infected with either virus contained essentially the same amount of virus after 21 days of extrinsic incubation. For example, pools of 2, 3, and 4 *C. tarsalis* infected with HK3 virus had titers of $10^{4.3}$, $10^{4.0}$, and $10^{5.0}$ PFU/0.1 ml, respectively; and pools of 2, 4, and 5 *C. tarsalis* infected with Clone 15 virus had titers of $10^{4.6}$, $10^{5.2}$, and $10^{4.5}$ PFU/0.1 ml, respectively.

Three mosquitoes infected with HK3 virus re-fed on susceptible chicks and transmitted virus. Clone 15 virus was transmitted on 3 of 7 occasions when 1 to 5 infected mosquitoes re-fed on susceptible chicks.

The attenuated character of Clone 15 virus did not change after passage through chicks and mosquitoes. This was established by comparing the mouse pathogenicity and plaque characteristics of the stock Clone 15 virus with the viruses recovered from: 2 chick bloods and 2 pools of mosquitoes infected with Clone 15 virus, and samples of HK3 virus with similar passage histories (Table III).

Discussion. This study demonstrated that *C. tarsalis* could become infected with and transmit an attenuated strain of WEE virus when mosquitoes fed upon chicks circulating greater than $10^{3.0}$ SM IC LD_{50} of virus. This infective threshold value for *C. tarsalis* corresponds closely with values reported for other strains of WEE virus(6,7,8). Thus, the decrease in neurotropism for mice that was achieved by cloning the virus on chicken embryonic cells(1,2) was not accompanied by a corresponding decrease in infectivity to mosquitoes.

The pathogenicity for mice and plaque size of the attenuated virus did not change after a single passage in chicks and mosquitoes. Similarly Whitman(9) found that 17D yellow fever virus did not alter in its pathogenicity for monkeys after multiplication in larval *Aedes aegypti*; and Mussgay and Suarez(10) were unable to demonstrate a reversion of attenuated Venezuelan equine encephalitis virus to the pathogenic form after multiplication in *A. aegypti*. No attempt was made to determine whether the attenuated properties of Clone 15 virus would remain stable after further passage in mosquitoes. However, other studies(11) have shown that Clone 15 virus remained unchanged in its pathogenicity for weanling

TABLE III. Mouse Pathogenicity and Plaque Size of HK3 and Clone 15 Strains of WEE Virus Before and After Passage in Chicks and *Culex tarsalis*.

Virus strain	Source of virus	Results in mice					
		Suckling mice		Weanling mice			Diameter of plaques on DECC (mm)‡
		Log_{10} SM IC LD_{50} of virus inoc (.01 ml)	AST (days)	Log_{10} SM IC LD_{50} of virus inoc (.03 ml)	Morbidity*	Mortality†	
HK3	Stock virus	3.0	2.1	5.8	5/5	5/5	7–9
	Chick blood	2.7; 2.8	2.4; 2.1	5.0; 5.9	" ; 5/5	4/5; 5/5	6–9
	C. tarsalis	2.8; 2.9	2.0; 2.0	3.3; 3.4	" ; "	" ; 3/5	6–8
Clone 15	Stock virus	2.9	5.3	5.4	0/5	0/5	3–4
	Chick blood	2.7; 3.0	4.3; 4.1	4.2; 4.9	" ; 0/5	" ; 0/5	2–4
	C. tarsalis	2.4; "	5.8; 5.3	2.9; 3.5	" ; "	" ; "	"

* No. sick/No. inoculated. † No. dying/No. inoculated. ‡ Measured on day 2, post-inoc.

mice after 8 to 25 passages in either chicken embryos, chicken embryonic tissue cultures, or suckling hamsters.

Viremic chicks rather than viremic horses were used in this study as a source of virus to infect mosquitoes. However, the possibility that *C. tarsalis* could become infected by feeding on the blood of a viremic horse vaccinated with Clone 15 WEE virus seems remote. Kemp and Johnson(3) reported that even those horses injected intramuscularly with large doses of the attenuated WEE virus circulated less than $10^{1.0}$ LD_{50} of virus/0.01 ml of blood, and this low level of viremia would not be expected to infect *C. tarsalis*.

Summary. *Culex tarsalis* can be infected with a non-neuroadapted strain of western equine encephalomyelitis virus and an attenuated variant with low neuropathogenicity derived from this strain. The infective threshold of the attenuated variant for *C. tarsalis* was greater than $10^{3.0}$ suckling mouse intracranial LD_{50} of virus/0.01 ml of blood. The original and attenuated strains multiplied equally in mosquitoes and both were transmitted to chicks by mosquito bite. The mouse pathogenicity or plaque size of the attenuated virus was not altered after a single passage in chicks and mosquitoes. The attenuated variant developed lower levels of viremias in chicks than the original strain.

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Immunofluorescence of Human Adenovirus Type 12 in Various Cell Types.* (30791)

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The discovery in 1962 of the oncogenic properties of human adenovirus type 12 (A-12) in hamsters(1,2,3) and the fact that other human adenoviruses and other heterologous species share this property(4,5,6) has brought new facets to the subject of cell-virus relations resulting in cancer. Some animals with primary tumors develop neutralizing antibodies(7), and animals with primary or grafted A-12 tumors develop complement-fixing antibodies to the type specific C antigen (8). Antibodies detectable by complement fixation, gel diffusion(9) and fluorescent antibody(10) to early antigens associated with the adenovirus multiplication cycle are also made. These findings make a study of the antigens detectable by fluorescent antibody in infected and transformed cells of considerable interest. It has been shown by other workers that a nearly parasynchronous single cycle of viral replication can be initiated in susceptible

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