DENGUE VIRUS TRANSMISSION BY MOSQUITOES INCUBATED AT LOW TEMPERATURES[1]

D. M. MCLEAN, M. A. MILLER AND P. N. GRASS

Division of Medical Microbiology, University of British Columbia, 2075 Wesbrook Place, Vancouver, B.C., V6T 1W5, Canada

ABSTRACT. Dengue-2 virus from the Southwest Pacific region has been transmitted to mice by bites of *Aedes aegypti* mosquitoes 32 days after they inbibed 3 mouse LD_{50} of virus in its first mosquito passage, following incubation at 13° C. A Caribbean strain of dengue-2 virus, both in zero passage and after attenuation through 10 passages in primary grivet monkey kidney tissue cultures, replicated in *A. aegypti* following feeding and intrathoracic injection, after incubation both at 13° C and 21° C. The attenuated strain was transmitted after 13 and 20 days incubation at 21° C following feeding and after 20 and 27 days incubation at 21° C following intrathoracic injection. Multiplication of all three dengue-2 strains was demonstrated in wild-caught *A. communis* and/or *A. hexodontus* from the Yukon Territory following intrathoracic injection and incubation for 1 to 5 weeks at 13° C and 1 to 3 weeks at 21° C.

INTRODUCTION. Transmission of zero-passage dengue-2 virus (NC-6) contained in the blood of a human resident of a Southwest Pacific island who developed clinical dengue was demonstrated by *Aedes aegypti* mosquitoes following 6 and 10 days incubation at 32° C and 13 days at 27° C, after imbibing an infective blood meal (McLean et al. 1974b). Comparable results were obtained with another Pacific dengue-2 isolate following one intrathoracic passage in *A. albopictus* mosquitoes. After intrathoracic injection of *A. aegypti,* mosquitoes transmitted the NC-6 strain after 6 days incubation at 32° C, 6 to 27 days at 24° C and 13 to 20 days at 13° C. The present paper reports transmission of the NC-6 isolate by *A. aegypti* after incubation at 13° C following ingestion of an infective blood meal, and its multiplication in wild-caught Yukon mosquitoes after incubation at 0, 13 and 21° C following intrathoracic injection.

A Caribbean isolate of dengue-2 virus in zero-passage (PR-159) was transmitted by *A. aegypti* after 6 days incubation at 35° C and 10 days at 25° C following intrathoracic injection (McLean et al. 1974b). Development of an attenuated clone (S-1 P-19a) of the PR-159 strain following 10 tissue culture passages, stimulated attempts to determine whether this agent would replicate in and be transmitted by *A. aegypti,* and Yukon mosquitoes which are known vectors of the snowshoe hare subtype of California encephalitis (CE) virus (McLean et al. 1974a, Ritter and Feltz 1974).

METHODS. The Caribbean strain of dengue-2 virus, PR-159 was supplied by Col. P. K. Russell, Walter Reed Army Institute of Research (WRAIR), Washington, D.C. in lyophilized serum from a patient who contracted dengue fever in 1969. After 6 passages in primary grivet monkey kidney monolayer tissue cultures at WRAIR (GM pass 6), the virus pool induced both small and large plaques on grivet monkey kidney monolayers. After 2 plaque purification steps, giving a cumulative total of 10 passages in grivet kidney monolayers, the S-1 P-19a clone was derived which induced small plaques exclusively. This clone showed reduced virulence for rhesus monkeys. It was studied at WRAIR as a candidate strain for production of attenuated dengue-2 vaccine (P. K. Russell, personal communication).

The Pacific strain of dengue-2 virus, NC-6 was supplied by Dr. Leon Rosen,

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Pacific Research Section, N.I.A.I.D., Honolulu, Hawaii in lyophilized serum from a resident of an island in the Southwest Pacific Ocean who developed dengue fever during the 1971–72 epidemic.

All infectivity titrations were conducted by intracerebral injection of Webster Swiss mice less than 24 hours old, as described previously (McLean et al. 1974b).

Aedes aegypti mosquitoes were supplied from the British Columbia Research Council mosquito colony. They were infected either by feeding on newborn mice rendered viremic by intracerebral injection with dengue virus immediately beforehand, or by intrathoracic injection of 0.001 ml amounts of virus diluted in sterile water (McLean et al. 1974b). After incubation at the stated temperatures, mosquitoes were placed individually on newborn mice in attempts to induce feeding. The virus content of salivary glands and thoraces of mosquitoes was assayed by inoculation of newborn mice and titers were expressed as $\log_{10}$ mouse LD_{50} per mosquito part (McLean et al. 1974b). The minimum infectivity titer detected was 1.5 log mouse LD_{50}. After mosquito bites, suckling mice were returned to their mothers, and 7 days later their brains were removed and assayed for virus content (Coleman and McLean 1973). The agent transmitted to mice by mosquito bite was confirmed as dengue-2 virus by neutralization with dengue-2 hyperimmune mouse ascitic fluid prepared by WRAIR.

RESULTS. Infectivity was demonstrated in salivary glands and thoraces of *A. aegypti* mosquitoes after 42 and 91 days incubation at 13° C, and after 46 and 80 days incubation at 21° C, following ingestion of 3 mouse LD_{50} of the NC-6 strain in zero passage. Virus transmission was attained after 46 days incubation at 21° C (Table 1). Following ingestion of the same amount of the NC-6 strain which received one intrathoracic passage in *A. aegypti*, virus transmission was demonstrated after 32 days incubation both at 13° C and 21° C. Infectivity titers in

Table 1. Transmission of Pacific dengue-2 virus strain NC-6 by *Aedes aegypti* mosquitoes after imbibing infective blood meals.

Passage level	Mosq. part	Days of extrinsic incubation at stated temperatures						
		13° C				21° C		
		32	42	74	91	32	46	80
Zero	SG		1/1		1/1 (3.5)		1*/1	1/1
	TH		2/2		1/1 (3.5)		1/1	1/1
	TR						1/1	
First	SG	1*/1 (3.0)*		1/1		2*/2 (3.5)		
	TH	1/1 (4.0)		1/1		2/2 (3.5)		
	TR	1/1				2/2		

SG: salivary glands; TH: thorax.
Proportions indicate number positive/number tested.
Infectivity titers (in parentheses) are expressed as log mouse LD_{50} per mosquito part.
TR: transmission ratio (number of mosquitoes transmitting virus/number tested).
* Virus transmission demonstrated.

salivary glands were 3.0 and 3.5 log respectively.

After ingestion of 3 mouse LD_{50} of zero passage PR-159 strain in blood of suckling mice rendered viremic immediately beforehand by intracerebral injection, dengue-2 virus titers attained 4.0 log in salivary glands of *Aedes aegypti* mosquitoes following incubation for 42 and 80 days at 13° C and viral replication was detected following 42 days incubation at 21° C (Table 2). Comparable infectivity titers were attained in salivary glands 13 days after ingestion of 3 mouse LD_{50} of GM pass 6 stock following incubation both at 13° C and 21° C, and titers remained undiminished after 20 days incubation at 21° C. Following ingestion of 3 mouse LD_{50} of S-1 P-19a clone, infectivity titers in salivary glands attained 5.0 log after 13 days incubation at 13° C and 4.0 log after 20 days incubation at 21° C. Mosquitoes transmitted virus by biting suckling mice after 13 and 20 days incubation at 21° C. Differences between infectivity titers of salivary glands and thoraces did not exceed 1.0 log.

After intrathoracic injection of *A. aegypti* mosquitoes with 3 mouse LD_{50} of zero passage PR-159 strain, infectivity titers in salivary glands attained 4.0 log after 17 days incubation at 13° C and after 10 days incubation at 21° C. The latter group of mosquitoes transmitted virus (Table 2). Replication of the GM pass 6 stock was demonstrated as early as 7 days following incubation at both 13° C and 21° C. Maximum infectivity in salivary glands was also detected after injection with the S-1 P-19a clone following incubation for 7 and 13 days at 13° C. Following incubation at 21° C, virus transmission was effected at 20 and 27 days.

Mosquitoes collected in the Yukon Territory during June and July 1974 were injected intrathoracically with 3 mouse LD_{50} of the NC-6 and PR-159 strains in zero passage and the S-1 P-19a derivative of the PR-159 strain in its tenth tissue culture passage. Multiplication of the NC-6 strain was detected in *A. communis* after incubation for 7 to 29 days at 13° C and 6 to 22 days at 21° C, with maximum infectivity titers of 3.0 log mouse LD_{50} for salivary glands after 22 and 29 days incubation at 13° C. This strain also replicated in salivary glands of *A. hexodontus* after 27 and 53 days incubation at 0° C, and after 12 to 34 days incubation at 13° C (Table 3). The PR-159 strain multiplied in salivary glands both of *A. communis* and *A. hexodontus* after incubation at 0, 13 and 21° C, and both species transmitted virus by biting suckling mice during the fifth week of extrinsic incubation at 13° C. The S-1 P-19a strain multiplied in *A. communis* and attained titers of 4.0 log mouse LD_{50} per salivary gland after 22 days incubation at 13° C. Viral replication was detected in *A. hexodontus* after incubation at 21° C for 6 and 22 days.

DISCUSSION. Transmission of a Pacific strain of dengue-2 virus after one mosquito passage by bites of *A. aegypti* following 32 days incubation at 13° C after imbibing infective blood, demonstrated that reduction of environmental temperature to the minimum compatible with longevity of the Vancouver strain of *A. aegypti* did not prevent this mosquito species from serving as a dengue vector in the laboratory. Viral replication and transmission of this strain in zero passage, by *A. aegypti,* was demonstrated after 46 days incubation at 21° C, 13 days at 28° C and 6 and 10 days at 32° C (McLean et al. 1974b). These results compare with accelerated rates of multiplication of dengue viruses in *A. albopictus* (Rosen and Gubler 1974) and eastern equine encephalitis virus in *A. triseriatus* (Chamberlain and Sudia 1955) with increase of environmental temperature from the standard 28° C to 32° C.

Replication of all three strains of dengue-2 virus after intrathoracic injection of one or both species of wild-caught Yukon mosquitoes at temperatures ranging from 0 to 21° C paralleled the demonstration of multiplication of CE virus following intrathoracic injection of *A. communis* and other Yukon mosquito species after

Table 2. Transmission of Caribbean dengue-2 virus strain PR–159 by *Aedes aegypti* mosquitoes after feeding or intrathoracic injection.

Passage level	Mosq. part	Days of extrinsic incubation at stated temperatures																	
		Fed								Injected									
		13° C				21° C				13° C				21° C					
		7	13	42	80	7	13	20	42	7	13	17	20	3	7	10	20	27	
Zero	SG			1/1 (4.0)	1/1 (4.0)				1/1			1/1 (4.0)		0/1		3*/4 (4.0)			
	TH			1/1 (3.5)	1/1 (4.0)				1/1			1/1 (4.0)		0/1		3/3 (3.0)			
GM pass 6	SG	1/1 (3.0)	1/1 (4.0)			1/1	1/1 (4.0)	1/1 (4.0)		1/1 (3.5)					1/1		1/1		
	TH	1/1 (3.0)	1/1			1/1	1/1	1/1 (4.0)		1/1 (3.5)					1/1		1/1		
S–1 P–19a	SG	1/1	1/1 (5.0)				2*/2	1*/1 (4.0)		1/1 (4.0)	1/1 (4.0)		1/1		1/1		3*/3 (4.0)	1*/1 (4.0)	
	TH	1/1 (4.0)	1/1 (4.5)				2/2	1/1 (5.0)		1/1 (4.0)	2/2 (4.0)		1/1		1/1		3/3 (3.5)	1/1 (4.0)	
	TR						+	1/1									2/3	2/2	

* Virus transmission demonstrated.

Table 3. Propagation of zero-passage dengue-2 virus strains in Yukon mosquitoes after intrathoracic injection.

Passage level	Mosquito species	Mosq. part	Days of extrinsic incubation at stated temperatures												
			0° C				13° C						21° C		
			27	33	41	53	7	12	22	29	32	34	6	12	12
NC–6	*A. communis*	SG					1/1	2/2	1/1 (3.0)	1/1 (3.0)			1/1	1/1	1/1
		TH						2/2	1/1 (2.5)	1/1 (3.0)			1/1	1/1	1/1
	A. hexodontus	SG	1/1			2/2		1/1	1/1			1/1 (2.5)			
		TH										1/1 (3.0)			
PR–159 zero	*A. communis*	SG		1/1					1/1	1/1	*		1/1 (2.0)	1/1	
		TH							1/1	1/1	1/1		2/2		
	A. hexodontus	SG			1/1		1/1					1/1* (3.5)			1/1
		TH			1/1							1/1 (3.0)			1/1
PR–159 S–1 P–19a	*A. communis*	SG					1/1		1/1 (4.0)						
		TH					1/1		1/1 (3.0)						
		TH											1/1		1/1
		TH											1/1		1/1

* Virus transmission demonstrated.

incubation at 4 to 21° C (McLean et al. 1974a, 1975). These results also extend the range of *Aedes* mosquito species susceptible to dengue-2 virus by intrathoracic inoculation beyond the *Stegomyia* subgenus.

Passage of dengue-2 virus in tissue culture, both before and after selection of a clone with reduced monkey virulence, left unchanged the ability of these derived strains to replicate in *A. aegypti* following feeding or intrathoracic injection, which parallel the findings of Rosen and Gubler (1974). These results extend our previous findings that *A. aegypti* supported multiplication of high mouse-passage stocks of prototype dengue-1 and dengue-4 viruses, following incubation at temperatures from 13 to 35° C (Coleman and McLean 1973). Although the attenuated strain of dengue-2 virus was transmitted by *A. aegypti,* both after imbibing infective blood and after intrathoracic injection, it seems unlikely that this agent would spread extensively in nature, by analogy with the lack of transmission of the attenuated TC-83 strain of Venezuelan equine encephalitis by mosquitoes to horses (Taylor and Buff 1972).

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INFLATION

The book edited by K. G. V. Smith, "Insects and Other Arthropods of Medical Importance," which was reviewed by Harry D. Pratt in the March number of *Mosquito News* sells for 15 British pounds, not 6.5. This is ca. $37.00. We are indebted to Dr. John Lane for this information. The British Museum (Natural History) is the source of the error.