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EXPERIMENTAL MODEL OF TRANSOVARIAL TRANSMISSION OF ŤAHYŇA VIRUS IN *AEDES AEGYPTI* MOSQUITOES

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Summary. — The progeny of 31 viruliferous *Aedes aegypti* females infected with Ťahyňa virus by sucking on viraemic newborn mice was investigated for virus presence. Out of 1587 individuals of the F_1 generation, 16 suspensions representing the progeny of 7 females were positive in 146 trials. Individuals of the F_1 generation failed to transfer the virus by sucking. Electron microscopy revealed the presence of Ťahyňa virus particles in the cytoplasm of maturing oocytes inevitably confirming the transovarial transfer of the virus by germinal cells of *Aedes aegypti* mosquitoes.

Key words: transovarial transmission; Ťahyňa virus; Aedes aegypti mosquitoes; electron microscopy

Introduction

Since the isolation of La Crosse virus in 1972 from *Aedes triseratus* larvae collected in nature (Pantuvatana *et al.*, 1974) and from the first description of transovarial transmission of this virus under laboratory conditions (Watts *el al.*, 1973), the vertical transfer of arboviruses in mosuqitoes has been repeatedly confirmed. Arboviruses were isolated from immature stages of mosquitoes collected in nature (Berry *et al.*, 1974, 1977; Le Duc *et al.*, 1975; McLintock *et al.*, 1976; Crane *el al.*, 1977) and their vertical transfer to the progeny was proved under laboratory conditions (Miller *et al.*, 1977; Rosen *et al.*, 1978; Christensen *et al.*, 1978; Beaty *et al.*, 1980; Tesh, 1980; Tesh and Shroger, 1980; Tesh and Cornet, 1981). Vertical transfer of Ťahyňa virus was confirmed by isolation of the virus from *Culiseta annulata* larvae collected in nature (Bárdoš *et al.*, 1976) and by laboratory experiments with *Aedes vexans* mosquitoes (Danielová and Ryba, 1979).

Our work was aimed at elucidating the extent of Ťahyňa virus transmission to the progeny of infected *Aedes aegypti* mosquitoes including changes in the ratio of infectious progeny from one gonotrophic cycle to another. We were also interested in ascertaining the localization of the virus within the oocytes of mother generation to gain additional support for the concept of transovarial transmission.

Materials and Methods

Virus and mosquitoes. Aedes aegypti mosquitoes, kept in our laboratory breed since 1981, were obtained from School of Hygiene and Tropical Medicine, London. The mosquitoes used in experiments, were fed on 2- to 4-day-old viraemic white mice, which had been inoculated intracerebrally (i.c.) 24 hr earlier with 0.01 ml of virus containing 10% mouse brain suspension. This suspension titered $10^{7.5}$ mouse i.c. LD_{50}/0.01 ml of the M2 strain of Ťahyňa virus, which was isolated from 20 *Aedes vexans* mosquitoes collected in September 1981 in the suburbs of Bratislava (Labuda and Kožuch, 1982). The strain was used in its third i.c. passage in newborn white mice. After 24 hr, the viraemia in i.c. inoculated mice titered $10^{2.5}$ mouse i.c. LD_{50}/0.01 ml.

Transmission and vertical transmission experiments. Selected optimum engorged mosquitoes were kept in plastic containers 9 cm in diameter and 10 cm high. The mosquitoes were fed with 10% glucose solution soaked into cotton swabs. They were allowed to lay eggs onto a wet filter paper. By interval of 7 days, an 1- to 3-day-old mouse was put into contact with each mosquitoe to allow its feeding and virus transfer. Four subsequent series of virus transmissions were made. Each batch of eggs was counted and stored at room temperature till further work.

About 8—10 weeks later, the eggs were separately immersed into water according to individual batches and the metamorphosis was allowed to proceed at temperature of 27 °C and relative air humidity of 75—80%. Larvae of the 4th instar and pupae were sampled for virological examination: the imagoes were examined 15—20 days after hatching. Transfer experiments were performed with the females of F_1 generation from each batch of eggs amass using 5 newborn mice. The females were always investigated at 2 intervals, namely from 7 to 12 days and from 14 to 18 days after hatching.

Virus titrations. The mosquitoes of mother generation were examined for the virus presence. Each mosquito was suspended in 0.5 ml of basal Eagle's medium (BEM) containing 10% calf serum. Larvae, pupae and imagoes of F_1 generation were sampled by groups of 10, rarely separately. Suspensions were made in 1 ml of BEM. All suspensions were inoculated into 1-to 3-day-old mice by i.c. route in a dose of 0.01 ml. The isolated virus was identified by hyperimmune mouse serum against Ťahyňa virus. The neutralizing index of the serum was 10^6 as tested in i.c. inoculated suckling mice.

Electron microscopy. The ovaries of infected females were removed between 7—19 days post infection. The ovaries were fixed in 2.5% glutaraldehyde in 0.2 mol/l sodium cacodylate buffer at pH 7.2 for 30 min at 4 °C. After washing in the same buffer, the ovaries were post-fixed for 1 hr in 1% OsO_4 (dissolved in the same buffer) at room temperature. Fixed samples were dehydrated in increasing acetone concentrations and embedded into Araldite C_y 212 resin. Ultrathin sections were prepared on Ultratome III LKB, stained with 2% uranylacetate, contrasted with lead citrate and examined in Philips EM 300 microscope at 80 kV.

Results

Demonstration of Ťahyňa virus in mosquitoes and in their progeny

Out of 50 selected mosquitoes (numbered 1—50) engorged on viraemic newborn mice 31 became infectious (infectivity rate 62%). Of them, at least a single transmission appeared in 27 cases (87%). As detected in 4 independent subsequent experiments, mosquitoes No. 11, 14. 17, 18, 26, 35 and 42 transferred the virus 3 times, the mosquito No. 22 four times. In the first gonotrophic cycle established after the mosquito sucking on infected mice, 41 egg batches originating from the 50 females investigated; 27 batches were found coming from 31 viruliferous mosquitoes. After the first trial to transfer the virus to newborn mice, 25 egg batches were counted from 39 females, from which 13 were posited by 24 viruliferous mosquitoes. In the second trial (the 3rd gonotrophic cycle) 21 batches of eggs were obtained from 32 females out of which 11 came from 19 viruliferous mosquitoes. In the third trial, 11 egg batches were gained from 24 mosquitoes; of these 6 were from 15 viruliferous individuals. Finally, in the last trial the 5th gonotrophic

Table 1. Survey of F_1 generation progeny from 31 viruliferous mosquitoes

Gonotrophic cycle	Number of suspensions	Number of progeny	MFIR
1	0/79*	871	—
2	0/24	228	1 : 28.5
3	5/28	302	1 : 60.4
4	1/10	124	1 : 124.0
5	1/5	62	1 : 31.0
Total	16/146	1587	1 : 99.1

* Positive out of examined.

cycle) 5 egg batches were obtained from 7 viruliferous mosquitoes left (some mosquitoes not viruliferous but still alive were succumbed at the 3rd trial for infectivity control). Together out of 103 batches of eggs 62 originated from viruliferous females. Of the latter, 1587 mosquitoes of F_1 generation were examined in 146 isolation experiments. Out of these, 16 were positive, namely 8 in 2nd, 5 in 3rd, 1 in the 4th and 2 in the 5th gonotrophic cycles (Table 1).

The progeny nursed from eggs of the first cycle was negative. The lowest minimal filial infection rate (MFIR), i.e. the lowest rate between the number of positive suspensions to the total number of progeny tested, reaching the value of 1 : 28.5 was found in the 2nd gonotrophic cycle (Table 1). The 16 positive suspensions coming from 7 females out of 31 viruliferous mosquitoes (22.6%) of these, mosquito No. 33 yielded infected progeny twice, namely in the 2nd and 3rd cycles. Four females had infected progeny in the 2nd, two in the 3rd, one in the 4th and one in the 5th cycle (Table 2). The MFIR ranged from 1 : 12.2 to 1 : 77.

All 24 trials on the virus transfer by females of F_1 generation were negative, although some females were positive in infectivity titrations later performed.

Electron microscopy of germinal cells

The ovaries of viruliferous females were examined after oviposition, 24 hr after sucking, then 7 and 19 days post infection. The cytoplasm of oocytes

Table 2. Survey of the progeny of F_1 generation from viruliferous "families"

Parent mosquito No.	Gonotrophic cycle	No. of suspensions	No. of progeny tested	MFIR
15	2	1/3*	28	1 : 28
23	2	1/4	41	1 : 41
26	2	2/3	27	1 : 13.5
33	2	4/6	65	1 : 16.2
20	3	4/6	49	1 : 12.2
33	3	1/4	77	1 : 77
18	4	1/1	16	1 : 16
22	5	2/2	40	1 : 20

* Positive out of examined.

repeatedly contained round shaped virus particles 100—110 nm in diameter. Ultrastructural changes characteristic for bunyavirus replication were present. The smooth endoplasmic reticulum and the Golgi complex formed numerous proliferating vesicles. The particles were situated either in cytoplasm rich of free ribosomes or in the smooth membrane-bound vacuoles. The particles in cytoplasm were mostly electron translucent; occasionally more electrondense particles were present in different proportion. The electron translucent cytoplasmic particles had a denser limiting membrane 5 nm thick and were covered with 10—11 nm long more electron-translucent spikes (Fig. 2). The particles within the smooth membrane-bound vacuoles had a more dense core than those found in cytoplasm. These particles were budding on the vesicle wall (Fig. 1) inside the smooth cytoplasmic membranes.

Virions in the oocyte cytoplasm were mostly free or they were seen less frequently in the granular matrix inside vacuoles (Fig. 3). In the course of yolk accumulation, virions were seen in the vicinity of membrane bound vesicles not only in cytoplasm of oocytes but also in that of follicular epithelium cells (Fig. 4).

Discussion

Negative results on the progeny of the first gonotrophic cycle are in accord with the suggestion that Tahyňa virus did not enter the already maturing oocytes in engorged mosquitoes fed on the viraemic host. The virus does not appear in oocytes sooner than by the second gonotrophic cycle. Similar results were found after oral infection of *Aedes trivittatus* with the La Crosse virus (Miller *et al.*, 1979). The highest number of positive isolates and the lowest MFIR were observed just in the second cycle progeny. In forthcoming cycles, a decreased infectivity of the progeny was no so clear in our experiments as those with *Aedes aegypti* mosquitoes infected with yellow fever virus (Beaty *et al.*, 1980). The latter authors argued that the mechanism of transovarial transmission of flaviviruses and bunyaviruses might be different.

There was no direct correlation between the transovarial transmission and the virus transfer by sucking. For example, the mosquito No. 33, in which the transovarial transmission had been confirmed in 2 subsequent gonotrophic cycles, did not transfer the virus to newborn white mice during feeding.

The attempts to transmit virus by the females of F_1 generation failed despite the presence of infectious virus as proved by isolation experiments. Though we have not enough data for final conclusions; it seems probable that successful virus transfer by infected progeny could depend on the optimal number of trials. It might also reflect, however, the different distribution of virus in the organs and tissues following transovarial infection. This should be taken into account when evaluating the role of transovarial transmission in the virus circulation in nature.

Electron microscopic examination confirmed the ovarial transmission of Ťahyňa virus and its replication in the maturing oocytes. In the oocytes cytoplasm morphological changes typical for virus presence were found. During the second gonotrophic cycle, in which involvement of oocytes was apparent and associated with the active virus replication, a great many of viruliferous females (11 out of 24) did not participate in oviposition. Further experiments are needed to show whether this could be caused by virus induced damage to the ovarial tissue.

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Explanation of Electron Micrographs (Plates XXVIII–XXX):

Fig. 1. Cytoplasm of the oocyte from a female 7 days post infection and 1 day after feeding. Proliferation of smooth endoplasmic reticulum and vesicles of the Golgi complex. Electron-translucent virus particles in cytoplasm (wide arrow) and electrondense ones in membrane bound vesicles (long thick arrow). Budding of Ťahyňa virus particles on the membranes of vacuoles (thin arrow). × 32,000.

Fig. 2. Detail of the oocyte cytoplasm from a female 19 days post infection. Free ribosomes and Ťahyňa virus particles in the cytoplasm. Fine spikes 10–11 nm long on the surface of virions (arrow). × 52,800.

Fig. 3. Oocyte from Fig. 2 showing numerous virus particles in the cytoplasm (arrows). × 9000.

Fig. 4. Oocyte from Fig. 1 with yolk accumulation (Y). Virus particles visible in the oocyte (○) as well as in the follicular epithelium cells (FE). × 11 200.