Efficient Transmission of Tick-Borne Encephalitis Virus Between Cofeeding Ticks

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ABSTRACT Most of the data on oral infections of ticks with tick-borne encephalitis virus have been derived from experiments using animals infected by syringe inoculation. To mimic the natural conditions of virus transmission, tick-borne encephalitis virus-infected *Ixodes ricinus* (Linnaeus) or *Rhipicephalus appendiculatus* Neumann adults (donors) were cofed with uninfected nymphs (recipients) of either tick species on uninfected guinea pigs. Two tick-retaining cells were attached to each guinea pig: cell 1 contained uninfected nymphs and virus-infected adults, and cell 2 contained uninfected nymphs. Following engorgement, 55% of *I. ricinus* nymphs and 65% of *R. appendiculatus* nymphs were shown to have acquired the virus while cofeeding with *I. ricinus* donor ticks. Similarly, 66% of *R. appendiculatus* recipient nymphs that cofed with *R. appendiculatus* virus-infected adults were infected. Some of the guinea pigs on which the ticks cofed were apparently nonviremic. The results indicate that efficient transmission of tick-borne encephalitis virus can occur between cofeeding ticks even when the host on which they feed does not develop a detectable viremia.

KEY WORDS tick-borne encephalitis virus, virus transmission, ticks

Tick-borne encephalitis virus (family *Flaviviridae,* genus *Flavivirus*) is endemic over a wide area covering Europe, northern Asia, and China. Several thousand cases are recorded annually, with considerable variation from year to year (Monath 1990). Two subtypes of tick-borne encephalitis virus, Far eastern and European, have been distinguished (Mandl et al. 1989, Pletnev et al. 1990). Their distributions correspond to those of their primary tick vectors, *Ixodes persulcatus* Schulze (Far eastern subtype) and *I. ricinus* (Linnaeus) (European subtype). Experimental studies have demonstrated that numerous other tick species are competent vectors. For example, *Rhipicephalus appendiculatus* Neumann is a competent vector of tick-borne encephalitis serogroup virus under laboratory conditions (Alexander & Neitz 1933), but this tick is confined to Africa, a continent in which tick-borne encephalitis viruses have not been recorded.

For arthropod-borne viruses it is considered axiomatic that a virus must induce a viremia of sufficiently high titer in the vertebrate host to overcome the threshold of infection of the feeding vector (Hardy et al. 1983, WHO 1985). However, studies with Thogoto (THO) virus (a tick-borne member of the family *Orthomyxoviridae*) have challenged this dogma by demonstrating tick-borne virus transmission involving a "nonviremic" vertebrate host (Jones et al. 1987). This phenomenon was shown to be mediated by a protein(s) found in salivary gland extract derived from feeding ticks and secreted in tick saliva (Jones et al. 1989, 1992). The term *saliva-activated transmission* was coined to describe this novel mode of virus transmission.

In nature, abundant rodent and insectivorous species are regarded as maintenance hosts of tick-borne encephalitis virus because these species develop levels of viremia that exceed the threshold of infection (Grešíkova & Calisher 1988). By contrast, vertebrate hosts that develop sub-threshold levels of viremia are not considered to play a role in the maintenance cycle of tick-borne encephalitis virus, although they may support the tick vector population. In view of the reported "nonviremic transmission" of THO virus *(vide supra),* the role of such vertebrate hosts was readdressed using an experimental model. The efficiency of tick-borne encephalitis (European subtype) virus transmission was determined when infected and uninfected ticks were allowed to feed together on guinea pigs. In addition to *I. ricinus,* we also tested *R. appendiculatus* because the efficiency of this tick species in saliva-activated transmission of THO virus has been determined (Jones et al. 1990).

Materials and Methods

Cells and Virus. Porcine stable kidney cells were propagated in modified Minimum Essential Medium with Earle's salts (EMEM) supplemented with 3% fetal bovine serum. The 9001 and 198 isolates of tick-borne encephalitis virus were originally obtained from *I. ricinus* ticks collected in Czechoslovakia (Danielová 1979, Kožuch et al. 1982). Virus stocks of both isolates were derived by passage in suckling mouse brain; isolate 9001 was used in its second passage and isolate 198 in its 24th passage.

Ticks. *Ixodes ricinus* nymphs and adults were collected by flagging the vegetation in selected areas of Western Slovakia and Central Bohemia, Czechoslovakia, areas where tick-borne encephalitis virus has not been detected. A laboratory colony of *R. appendiculatus* was maintained by feeding all three stages of *R. appendiculatus* on outbred Dunkin Hartley guinea pigs (Jones et al. 1988). During the interval between feeding, both tick species were held in perforated tubes inside a dessicator at a temperature of 21°C and 85% RH.

Virus Assay. Nymph and adult ticks were homogenized individually in a microtissue grinder in 1 ml of EMEM containing 10% newborn bovine serum and appropriate antibiotics to inhibit bacterial growth. Larvae were treated similarly but in groups of ten. Blood samples were obtained from anesthetized guinea pigs or mice. Prior studies showed that in the few guinea pigs that developed a viremia following subcutaneous inoculation of tick-borne encephalitis virus, the virus was detectable in blood 3–5 d after infection. Hence, blood samples were obtained routinely 4 d after infestation of guinea pigs with ticks. Plaque titration of blood and tick material was done using porcine stable cells incubated at 36°C for 4 d, before fixation and staining.

Per Os Infection of Ticks. *Ixodes ricinus* nymphs were allowed to feed on adult albino mice (obtained from Velaz Cerny Vul, Czechoslovakia) inoculated intracerebrally with 6.2 $\log_{10}$ plaque forming units tick-borne encephalitis virus (isolate 9001). Maximum viremic titers of 2.7–3.5 $\log_{10}$ plaque forming units per milliliter of blood were obtained 3 d after inoculation. Thus, mice were infested with ticks at the time of inoculation as they take 4–6 d to complete engorgement. For per os infection of *R. appendiculatus* larvae, four guinea pigs were inoculated with a mixture of 6.0 $\log_{10}$ plaque forming units of tick-borne encephalitis virus (isolate 198) and 40 µg salivary gland extract derived from *R. appendiculatus* female ticks that had fed for a period of 6 d, using the method previously described (Jones et al. 1989). These infected ticks were used at the adult stage in cofeeding experiments.

Infection of Ticks by Cofeeding on Guinea Pigs. Thirteen uninfected guinea pigs were each infested with either virus-infected adult *I. ricinus* or *R. appendiculatus* ticks (donors; equal sex ratio). In the same retaining cell as the virus-infected adult ticks, a further 50 uninfected *I. ricinus* or *R. appendiculatus* nymphs were added. An additional 50 uninfected *I. ricinus* or *R. appendiculatus* nymphs were placed in a separate retaining cell.

Statistical Analysis. The results were analyzed using a GLIM program that fits a series of generalized linear statistical models to the data (Baker & Nedler 1978). Results (which were binomially distributed) were not transformed, because the GLIM program adjusts each model to fit the given data. Binomial errors were specified with the y variate representing the number of ticks that became infected per replicate animal. Initially a Null model was fitted to give total model deviance (total variation in data). The reduction in model deviance caused by each parameter was then determined. The significance of each reduction in deviance was assessed by construction of the appropriate F table using values generated by the GLIM model. The GLIM program expressed the results of binomially distributed data as a logit function of a linear model (x), which could be back transformed to the natural state (r) using the equation $r = 1/(1 + e^{-x})$. Mean transmission rates and their nonsymmetrical standard errors resulting from the binomial nature of the data distribution were calculated using this formula.

Results

Eight guinea pigs were infested with *I. ricinus* adult ticks: females (donors; Table 1, cell 1) that had fed on tick-borne encephalitis virus-infected mice at the preceding nymphal stage, and uninfected males (cell 1). Guinea pigs 1–4 were also infested with uninfected *I. ricinus* nymphs (recipients in cells 1 and 2); guinea pigs 5–8 were treated in a similar manner, but *R. appendiculatus* nymphs were used as recipient ticks. Following engorgement, virus was not detected in donor female ticks that had fed on guinea pig 7 and guinea pig 8; recipient ticks from these animals were excluded from all subsequent analyses. Similar experiments were undertaken using *R. appendiculatus* female ticks as donors and *R. appendiculatus* nymphs as recipients (guinea pigs 9–13; Table 1). Female ticks that fed on guinea pig 13 were negative when assayed for virus; recipient nymphs from this animal were excluded from all subsequent analysis.

No significant difference in susceptibility to tick-borne encephalitis virus infection was observed between recipient tick species that cofed with *I. ricinus* donor ticks (guinea pigs 1–6). Following engorgement, 55.4 ± 51.8–58.9%

Table 1. Per os infection of *I. ricinus* and *R. appendiculatus* nymphs feeding with tick-borne encephalitis virus-infected adults

Guinea pig	Tick retaining cell	Viremia $\log_{10}$ PFU/ml blood[a]	Unfed ticks			Ticks after feeding		
			Tick species[b]	Tick nos.[c]	Virus[d]	No. infected	No. tested	% Infected
1	1	<2.0						
	2	2.6						
	2	<2.0						
	2 / 1	3.0						
	2	<2.0						
	2	3.1						
	2 / 1	2.8						
	2 / 1	<2.0			− / + / −			
	2 / 1	2.6			− / + / −			
	2 / 1	3.3			− / + / −			
	2							

[a] PFU, plaque forming units.
[b] *I. ric*, *I. ricinus*; *R. app*, *R. appendiculatus*.
[c] Numbers of adult females (F) and nymphs (N) added to retaining cell.
[d] +, infected ticks; −, uninfected nymphs.

(mean ± SEM) of *I. ricinus* nymphs and 65.3 ± 71.4–58.6% (mean ± SEM) of *R. appendiculatus* nymphs became infected. Similarly, there was no apparent difference in vector efficiency of donor *I. ricinus* (guinea pigs 5 and 6) and *R. appendiculatus* (guinea pigs 9–12) when *R. appendiculatus* nymphs acted as recipients (change in model deviance 0.92, df = 2; 65.3 ± 71.4–58.6% [mean ± SEM] and 66.3 ± 70.5–61.7% [mean ± SEM], respectively, of *R. appendiculatus* nymphs acquired virus).

A considerable amount of variation was observed in the percentage of recipient ticks that acquired virus from different guinea pigs. Nevertheless, a significantly higher proportion of cofeeding recipient ticks acquired virus on animals that developed a detectable viremia; i.e., >2.0 $\log_{10}$ plaque forming units per milliliter of blood (Table 2). The mean percentage of infected nymphs from viremic guinea pigs was 72.2% (95%; CL = 68.0–76.0) compared with 41.1% (33.7–48.0) that fed on nonviremic animals.

The infection prevalence of recipient nymphs that fed in either the same cell as donor ticks (cell 1), or in a separate cell (cell 2), was compared (Table 2). When guinea pigs had a detectable viremia, the percentage of recipient ticks that became infected when feeding in close contact with donor ticks (84.4 ± 90.9–74.6%) was significantly greater than when recipient ticks fed in a separate cell (60.7 ± 72.7–48.2%). In contrast, no significant difference in infection prevalence was observed in recipient ticks that fed on apparently nonviremic animals (cell 1, 45.2 ± 56.2–34.8%; cell 2, 36.9 ± 47.9–27.1%). A similar analysis of the virus titers of donor and

Table 2. GLIM model (with bionomial distribution specified) for the number of nymphs that became infected when cofeeding with infected adult ticks

Factor investigated	Change in model deviance	Change in df	χ^2 significance
Guinea pig	32.79	5	P = 0.001
Viremia	45.88	1	P = 0.001
Location (cell 1 or cell 2)	20.73	1	P = 0.001
No. of donor ticks	0.61	1	NS
Tick species (donor/recipient)	0.92	2	
Residual deviance	27.67	9	
Total	128.60	19	

recipient ticks showed no correlation with the factors listed in Table 2, with the exception of tick species. The virus titer of *R. appendiculatus* (maximum titer, 4.0 $\log_{10}$ plaque forming units per nymph) was significantly greater than that of *I. ricinus* (maximum titer, 2.0 $\log_{10}$ plaque forming units per nymph), reflecting the larger blood meal and longer feeding period of the former species.

Discussion

In nature, uninfected and infected vectors often feed together on the same vertebrate host. These conditions were mimicked in the laboratory to investigate tick-borne transmission of tick-borne encephalitis virus. In cofeeding studies using adult ticks as donors, 4 of 10 guinea pigs had no detectable viremia. Of the ticks feeding on these animals, 37 of 82 (45%) *I. ricinus* and 32 of 75 (43%) *R. appendiculatus* nymphs became infected. By contrast, only 16 of 111 (14%) *R. appendiculatus* nymphs were infected after feeding on three viremic guinea pigs infected with tick-borne encephalitis virus by syringe inoculation (viremic titer 2.5–2.6 $\log_{10}$ plaque forming units per milliliter of blood, 4 d after inoculation). The results demonstrate that virus transmission by ticks is more efficient than transmission resulting from syringe inoculation of the vertebrate host. Furthermore, the data indicate that vertebrate hosts can play a significant role in tick-borne encephalitis virus transmission even when they do not develop a detectable viremia (as determined by plaque assay).

The infection prevalence was significantly higher for recipient ticks that fed on guinea pigs with a detectable viremia (72%) compared with apparently nonviremic animals (44%). However, when feeding on a viremic guinea pig, more ticks became infected when they fed together with the infected donor ticks (in cell 1) compared with separately (in cell 2). This result suggests that, even on viremic animals, the proportion of ticks that become infected is not directly related to the level of virus circulating in the blood.

There was no significant difference in infection prevalence of recipient ticks that fed in cell 1 or cell 2 on apparently nonviremic animals. Similar observations have been reported for THO virus (Jones et al. 1989). Tick-borne encephalitis virus infection of *Dermacentor* ticks was observed only if recipients fed in close contact with donor *Dermacentor* ticks on nonviremic guinea pigs (Alekseev et al. 1991). The authors postulated that this was caused by passive transfer of the virus via the saliva (Alekseev & Chunikhin 1990). When *R. appendiculatus* recipients fed in close contact with virus-infected adults on Belgian hares, they did not acquire tick-borne encephalitis virus (unpublished data). This result, and results previously obtained with

THO virus (Jones et al. 1987, 1992), indicate that virus is not transferred passively between cofeeding ticks. Indeed, the considerable variation between guinea pigs in the proportion of recipient ticks that became infected (Table 1) suggests that the vertebrate host plays an active role in virus transmission. The significance of efficient virus transmission between cofeeding ticks should be explored with regard to the ecology of tick-borne encephalitis virus involving various tick and vertebrate species involved in the natural cycle.

Acknowledgment

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