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Enhancement of tick-borne encephalitis virus transmission by tick salivary gland extracts

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Abstract. To investigate the role of ticks in TBE virus transmission, salivary gland extract (SGE) was derived from partially fed female *Ixodes ricinus*, *Dermacentor reticulatus* and *Rhipicephalus appendiculatus* ticks. Guinea-pigs were infested with uninfected *R.appendiculatus* nymphs and inoculated with a mixture of TBE virus and SGE or with virus alone. The number of ticks which on average acquired virus from feeding on animals inoculated with TBE virus and SGE from partially fed ticks was 4-fold greater than the number that became infected by feeding on animals inoculated with virus alone or virus plus SGE from unfed *I.ricinus*. Viraemia was detected in 67% of guinea-pigs inoculated with virus plus SGE compared to 30% of guinea-pigs inoculated with virus alone. Virus titres in the blood were similar for both groups of animals [range 2.0–2.8 $\log_{10}$ plaque-forming units (PFU)/ml of blood]; however, the number of ticks that became infected was significantly higher on animals inoculated with virus plus SGE from partially fed ticks. No significant difference was observed with respect to the tick species used to derive SGE. The results indicate that TBE virus transmission is enhanced by factor(s) associated with the salivary glands of feeding ticks, and that these factor(s) may facilitate efficient transmission of TBE virus between infected and uninfected ticks even when they feed on hosts that have no detectable viraemia.

Key words. Tick-borne encephalitis virus, non-viraemic transmission, Acari, Ixodidae, ticks, salivary gland.

Introduction

Arthropod vectors of an animal virus become infected when they feed on the blood of a viraemic host (W.H.O., 1985). However, studies with Thogoto (THO) virus (family Orthomyxoviridae) and tick-borne encephalitis (TBE) virus (family Flaviviridae) have demonstrated efficient transmission from infected to uninfected ticks co-feeding on vertebrate hosts that develop sub-threshold levels of viraemia (Jones *et al.*, 1987; Alekseev & Chunikhin, 1990; Labuda *et al.*, 1993). Furthermore, investigations of the role of ticks in THO virus transmission indicate that a factor(s) associated with the salivary glands of ticks and secreted in tick saliva potentiates this novel mode of arbovirus transmission referred to as saliva activated transmission (SAT) (Jones *et al.*, 1987, 1992b).

Tick-borne encephalitis virus is endemic over a wide area covering Europe, northern Asia and China. Two types of TBE virus, Far eastern and European, have been distinguished (Gresikova & Calisher, 1988). These two viral subtypes cause encephalitis in humans with several thousand cases recorded annually (Monath, 1990). Their distribution corresponds to the geographical range of their primary tick vectors, *Ixodes persulcatus* Schulze (Far eastern subtype) and *I.ricinus* Linnaeus (European subtype).

Experimental studies have demonstrated that numerous tick species are competent vectors of TBE virus (European subtype); however, virus isolations from such ticks are rare. Consequently, they are either secondary vectors, e.g. *Dermacentor reticulatus* Fabricius and *D.marginatus* Sulzer, or not considered natural vectors as their distribution is not sympatric with that of the virus, e.g. *Rhipicephalus appendiculatus* Neumann is a competent vector of TBE

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virus but is confined to Africa, a continent in which TBE subgroup viruses have not been isolated (reviewed by Nuttall & Labuda, 1993). This study was undertaken to assess the potential of salivary gland extract (SGE) derived from *I.ricinus*, *D.reticulatus* and *R.appendiculatus* to enhance the transmission of TBE virus (European subtype) to uninfected ticks feeding on TBE virus-inoculated guinea-pigs.

Materials and Methods

Ticks. Laboratory colonies of *I.ricinus*, *D.reticulatus* and *R.appendiculatus* were maintained as previously described (Jones *et al.*, 1988). During the interval between feeding all ticks were held in perforated tubes at 21°C and 85% r.h.

Cells and virus. Pig stable (PS) kidney cells were propagated in modified Eagle's medium (EMEM) supplemented with 3% fetal bovine serum (FBS). The 198 isolate of TBE virus was used throughout these studies. The isolate was originally obtained from *I.ricinus* ticks collected in Czechoslovakia and subsequent virus stocks were derived by passage in sucking mouse brain (Labuda *et al.*, 1993).

Virus assay. Ticks were homogenized individually in a microtissue grinder in 1 ml of EMEM containing 10% newborn bovine serum (NBS) and appropriate antibiotics to inhibit bacterial growth. Blood samples were obtained from anaesthetized guinea-pigs on day 4 post-infestation (Labuda *et al.*, 1993). Titration of blood and tick derived material was performed in PS cells incubated at 36°C for 4 days, prior to fixation and staining.

Salivary gland extracts. Salivary glands were dissected from uninfected adult female *R.appendiculatus* and *D.reticulatus* ticks which had fed for a period of 6 days, and from *I.ricinus* female ticks which were either unfed or had fed for a period of 4 or 5 days. The dissected salivary glands were placed in phosphate-buffered saline (PBS) pH 7.2, extracted by homogenization and low-speed centrifugation, and frozen at −20°C. Experiments were standardized by infesting guinea-pigs (a minimum of two per salivary gland sample) with approximately 50 uninfected *R.appendiculatus* nymphs. Each guinea-pig was inoculated subcutaneously with 6.0 $\log_{10}$ plaque-forming units (PFU) TBE virus mixed with salivary gland extract (SGE, 40 µg protein per animal); control guinea-pigs were inoculated with virus alone. Virus transmission was measured by the number of uninfected ticks that acquired virus; ticks were assayed for virus on day 0 post-engorgement to estimate the amount of virus taken up during feeding. Results were analysed as previously described using the 'GLIM' program (Baker & Nedler, 1978; Labuda *et al.*, 1993).

Results

The effect of tick SGE derived from either unfed *I.ricinus*, or from partially fed *I.ricinus*, *D.reticulatus* or *R.appendiculatus*, on the acquisition of TBE virus by uninfected ticks, is shown in Table 1 (GP 1–13); control guinea-pigs were inoculated with virus alone (GP 14–23). Enhancement of virus transmission was observed with salivary glands of all the above partially fed tick species, i.e. a 3–5-fold increase in the number of recipient ticks that became infected (mean range 28.6–51.7%), compared with the control ticks which fed on guinea-pigs inoculated with virus alone (9.8%). In contrast, salivary glands from unfed *I.ricinus* did not result in an increase in the percentage of infected ticks (8.6%) compared with the controls. No significant difference was observed with respect to the tick species used to derive SGE (Table 1); however, a significant reduction in the number of recipient ticks that acquired virus was observed with day 4 SGE compared to day 5 SGE derived from *I.ricinus* ticks (mean 28.6% and 51.7%, respectively). Of the animals tested, 6/9 guinea-pigs inoculated with virus plus SGE developed viraemia (mean 2.6 $\log_{10}$ PFU/ml blood, range 2.0–2.8) compared to 3/10 animals inoculated with virus alone (mean 2.6 $\log_{10}$ PFU/ml blood, range 2.5–2.6). Neither of the two animals inoculated with unfed *I.ricinus* salivary glands had a detectable viraemia. Although there was considerable variation in virus titres in ticks (maximum titre 4.0 $\log_{10}$ PFU/tick), there were no significant differences in virus titres between the groups of ticks tested.

Discussion

Efficient transmission of tick-borne encephalitis (TBE) virus can occur between co-feeding ticks even when the vertebrate host on which they feed does not develop a detectable viraemia (Labuda *et al.*, 1993). A similar phenomenon demonstrated for THO virus is associated with a SAT factor synthesized in tick salivary glands during feeding and secreted, in tick saliva, into the feeding site (Jones *et al.*, 1992b). The results reported herein indicate that a comparable mechanism of SAT operates in the transmission of TBE virus. Thus, the number of infected ticks from guinea-pigs inoculated with TBE virus plus SGE from partially fed ticks was approximately 4-fold greater than the numbers of infected ticks from guinea-pigs inoculated with either virus plus SGE from unfed *I.ricinus* or virus alone (Table 1).

The enhancing potential of SGE derived from the prostrate tick species *I.ricinus* was similar to that shown by the metastriate tick species *D.reticulatus* and *R.appendiculatus*. In contrast, SAT factor activity for THO virus was only detected in the salivary glands of metastriate ticks and not in the salivary glands of prostriate ticks (Jones *et al.*, 1992a). However, *I.ricinus* is the primary vector of TBE virus, and is not a competent vector of THO virus. These observation are consistent with the previous report of an apparent correlation between vector competence and the ability of different tick species to mediate SAT (Jones *et al.*, 1992a).

More guinea-pigs developed a detectable viraemia following inoculation of virus plus SGE from partially fed ticks compared with inoculation of virus plus SGE from

Table 1. The effect of SGE derived from ixodid tick species on the ability of *R.appendiculatus* nymphs to acquire TBE virus.

Guinea-pig	Viraemia log_{10}PFU/ml	SGE*	No. infected/ no tested†	% infected	Mean titre log_{10}PFU/tick	x % infected (+95% CL)
GP1	2.8	*I.ric.* D5	25/48	52	1.65	51.7
GP2	2.5	*I.ric.* D5	5/10	50	2.06	(38.8–64.4)
GP3	2.8	*I.ric.* D4	15/35	43	1.79	28.6
GP4	<2.0	*I.ric.* D4	5/35	14	1.95	(15.9–45.7)
GP5	<2.0	*I.ric.* D0	3/30	10	2.71	8.6
GP6	<2.0	*I.ric.* D0	3/40	7	1.97	(3.8–18.0)
GP7	NT	*D.ret.* D6	12/48	25	2.03	35.4
GP8	NT	*D.ret.* D6	22/48	46	2.96	(20.6–51.9)
GP9	2.7	*R.ap.* D6	8/28	29	1.83	35.3
GP10	2.5	*R.ap.* D6	27/51	53	1.78	(25.6–46.4)
GP11	2.0	*R.ap.* D6	12/29	38	1.76	
GP12	<2.0	*R.ap.* D6	12/35	34	1.62	
GP13	<2.0	*R.ap.* D6	19/72	26	2.40	
GP14	2.6	Control	4/36	11	3.53	9.8
GP15	2.6	Control	2/35	6	1.95	(6.1–16.0)
GP16	2.5	Control	9/40	22	1.75	
GP17	<2.0	Control	3/19	16	1.10	
GP18	<2.0	Control	5/32	16	1.06	
GP19	<2.0	Control	1/7	14	4.00	
GP20	<2.0	Control	3/33	9	1.42	
GP21	<2.0	Control	3/48	6	2.10	
GP22	<2.0	Control	0/7	0	–	
GP23	<2.0	Control	0/48	0	–	

* Salivary gland extract (SGE) from either *I.ricinus* (*I.ric.*), *D.reticulatus* (*D.ret.*) or *R.appendiculatus* (*R.ap.*). Salivary glands were collected from unfed ticks (D0), or from partially fed ticks on days 4–6 (D4, D5 or D6) of engorgement. Control guinea-pigs were inoculated with virus alone.

† Individual ticks were assayed for virus on day 0 post-engorgement.

NT, not tested.

unfed ticks or virus alone. In a previous study of TBE virus transmission between co-feeding infected and uninfected ticks, more ticks became infected on guinea-pigs that developed viraemia (Labuda *et al.*, 1993). However, on viraemic guinea-pigs, the number of ticks that acquired an infection was greater among the cohorts that fed in the same feeding chamber as the infected ticks compared with the cohorts that fed on the same animal but in a separate feeding chamber. Furthermore, co-feeding ticks became infected when feeding on guinea-pigs that had no detectable viraemia. These observations are inconsistent with the hypothesis that viraemia (i.e. virus circulating in the blood) is the principal source of TBE virus infection of ticks feeding on guinea-pigs. The results obtained by inoculation of virus ±SGE suggest that the SAT factor enhances the level of infection in the host (reflected by a greater probability of developing viraemia) and that the nature of the enhanced infection, rather than the level of viraemia *per se*, determines the number of ticks that become infected. We are currently testing this hypothesis with natural hosts of TBE virus.

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