0014-4754/93/090802-04$1.50 + 0.20 Birkhäuser Verlag Basel, 1993

Non-viraemic transmission of tick-borne encephalitis virus: a mechanism for arbovirus survival in nature

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Received 1 February 1993; accepted 3 June 1993

Abstract. The vectors of arthropod-borne viruses (arboviruses) become infected by feeding on the viraemic blood of an infected animal. This theory is based on transmission studies involving artificial infection of vertebrate hosts by syringe inoculation. To reproduce natural conditions of virus transmission, infected and uninfected vectors (ticks) of tick-borne encephalitis virus, the most important arbovirus in Europe, were allowed to feed together on uninfected wild vertebrate hosts. The greatest numbers of infected ticks were obtained from susceptible host species that had undetectable or very low levels of viraemia. The results suggest that 'nonviremic transmission' is an important mechanism for the survival of certain arboviruses in nature.
Key words. Arbovirus transmission; tick-borne encephalitis virus.

Over 500 viruses are listed in the International Catologue of Arboviruses[2], including the aetiological agents of important diseases such as yellow fever, dengue, and tick-borne encephalitis. The World Health Organization defines arboviruses as ones that '... multiply and produce viraemia in the vertebrates, multiply in the tissues of arthropods, and are passed on to new vertebrates by the bites of arthropods ...'[3]. This definition implies that only vertebrate species that develop detectable levels of virus circulating in the blood (viraemia) are significant in the epidemiology of arboviruses. However, we have demonstrated significant levels of 'nonviremic transmission' of two different tick-transmitted viruses when infected and uninfected ticks fed together on guinea pigs[4,5].

An important point raised by the phenomenon of nonviremic transmission is whether it occurs in nature. This question was addressed with tick-borne encephalitis (TBE), the most important arbovirus disease affecting humans in Europe[6]. A wide range of vertebrates, including at least 10 rodent species, are considered maintenance and reservoir hosts in the ecology of TBE virus[7]. In particular, field mice (*Apodemus flavicollis* and *A. sylvaticus*), and the bank vole (*Clethrionomys glareolus*), are implicated as major hosts because they are abundant in infection foci and they are readily infested with immature stages of *Ixodes ricinus*, the primary vector species of TBE virus in Europe. In general, the role of vertebrate species in maintaining and amplifying TBE virus has been extrapolated from their ability to produce a viraemia of sufficiently high titre for the infection of ticks feeding on them. The infection threshold of *I. ricinus* larvae fed on *A. flavicollis* mice was calculated as 2.0 $\log_{10}$ LD_{50}/0.02 ml blood[8]. However, as with other arboviruses, most studies have been based on infection of vertebrate host species by syringe inoculation of the virus[9].

To determine whether nonviraemic transmission plays a role in the circulation of TBE virus in nature, we examined the levels of viraemia and the extent of dissemination of TBE virus infection into target organs (lymphoid tissue and brain) of selected wild vertebrate species after exposure to infected ticks. Replication and release of TBE virus from lymphoid tissue is the source of viraemia[10], and infection of the brain is a measure of virus virulence. At the same time, the levels of virus transmission from infected to uninfected ticks were measured with respect to the vertebrate host species and the degree of contact between infected and uninfected ticks. The aims of the study were to determine the efficiency of tick to tick TBE virus transmission involving different natural host species of *I. ricinus*, and the contribution of viraemia developed by the host animals to virus transmission between cofeeding ticks. Adult vertebrates obtained from the wild were used because they represent the most common hosts of *I. ricinus* in nature, viz. animals that have been constantly exposed to ticks and therefore have had the opportunity to develop immunity to tick infestation.

Materials and methods

Cells and virus. Pig stable (PS) kidney cells were propagated in Earle's modification Eagle's medium (EMEM) supplemented with 3% foetal bovine serum (FBS). The 198 isolate of TBE virus, originally obtained from *I. ricinus* ticks collected in former Czechoslovakia, was used at the 24th mouse brain passage[5].

Ticks. Ixodes ricinus nymphs and adults were collected by flagging the vegetation in selected areas of western Slovakia where TBE virus has not been detected. First

laboratory generation ticks fed on out-bred guinea pigs and rabbits were used; none of the animals on which these ticks were maintained developed TBE virus-neutralizing antibodies. All the female *I. ricinus* used in the experiments were infected with TBE virus by parenteral inoculation [mean titre, 3.0 $\log_{10}$ plaque forming units (PFU)/tick], and subsequently they all fed successfully on their experimental hosts.

Experimental animals. Mammalian species were live-trapped from May to June, 1992, in areas known to be free of TBE virus: *A. flavicollis*, *C. glareolus* and *Erinaceus europaeus* in western Slovakia, and *A. agrarius* and *Pitymys subterraneus* in northern Moravia. Pheasants were obtained from the Malacky farm near Bratislava. Captured animals were held for at least 14 days in the laboratory prior to experimentation. Only adult animals that had no neutralizing antibodies to TBE virus were selected.

Virus assay. Nymphal and adult ticks, and vertebrate tissues, were homogenized individually in 1 ml of EMEM containing 10% newborn bovine serum and antibiotics appropriate to inhibit bacterial growth. Plaque titrations were performed in PS cells incubated at 35 °C for 4 d prior to fixation and staining, and virus titres expressed as $\log_{10}$ plaque-forming units (PFU)/tick or organ. Blood samples were assayed in 2 day-old mice (as this method is approximately 10 times more sensitive than plaque assay) and the titre expressed as the 50% lethal dose (LD_{50})/0.01 ml blood.

Statistical analysis. Data were arcsin transformed and subject to ANOVA with viraemia and number of ticks feeding as co-variates within a GLIM program[11,5]. Host species, location of ticks, and level of viraemia all had significant effects on the proportion of ticks becoming infected; there were no significant interactions.

Results and discussion

Nymphs showed variable feeding success, with 77% (216/280) engorging on field mice but only 44% (130/280) on bank voles and 14% (17/120) on pine voles (table 1). On two hedgehogs, 60% (48/80) nymphs fed successfully, and five pheasants yielded 32% (97/300) engorged nymphs. The differences in feeding success, particularly between field mice and blank voles, may reflect the relative abilities of the host species to develop immune-mediate resistance to tick feeding[12]. Indeed, laboratory-bred mice and to a lesser extent bank voles develop resistance to repeated infestations with *Ixodes* ticks but wood mice (*Apodemus sylvaticus*) do not[13].

The six wild vertebrate species examined showed a remarkable diversity in their response to cofeeding of infected and uninfected ticks (tables 1 and 2). At one end of the spectrum, pheasants and hedgehogs were largely resistant to virus infection and did not support TBE virus transmission between cofeeding ticks, although at least one third of nymphs fed successfully. In

Table 1. Infection of *Ixodes ricinus* nymphs by cofeeding with infected ticks on wild hosts

Vertebrate host species /animal no.§	Nymphs in cell 1†		Nymphs in cell 2	
	no. infected /no. fed	% infected	no. infected /no. fed	% infected
A. flavicollis				
1	8/13	62%	11/17	65%
2	9/16	56%	4/17	24%
3	10/11	91%	16/20	80%
4	15/18	83%	*	
5	16/18	89%	5/20	25%
6	18/19	95%	10/16	62%
Totals/means:	76/95	80%	46/90	51%
A. agrarius				
1	11/12	92%	7/7	100%
2	7/12	58%	0/1*	
Totals/means:	18/24	75%	7/8	88%
C. glareolus				
	2/5	40%	1/16	6%
2	3/15	20%	1/5	20%
3	4/7	57%	4/6	67%
4	1/6	17%	3/14	21%
5	3/11	27%	0/8	0%
6	*	*	9/14	64%
7	2/2	100%	2/6	33%
8	*	*	1/15	7%
Totals/means:	15/46	33%	21/84	25%
P. subterraneus				
1	3/3	100%	1/2	50%
2	6/6	100%	0/2	0%
3	2/3	67%	0/1	0%
Totals/means:	11/12	92%	1/5	20%
E. europaeus				
1	1/11	10%	0/16	0%
2	1/10	10%	0/11	0%
Totals/means:	2/21	10%	0/27	0%
Ph. colchicus				
1	0/8	0%	0/30	0%
2	0/17	0%	0/16	0%
3	0/5	0%	0/2	0%
4	0/3	0%	0/6	0%
5	0/5	0%	0/5	0%
Totals/means:	0/38	0%	0/59	0%

§Yellow-necked field mouse, *Apodemus flavicollis*; striped field mouse, *A. agrarius*; bank vole, *Clethrionomys glareolus*; pine vole, *Pitymys subterraneus*; hedgehog, *Erinaceus europaeus*; pheasant, *Phaseanus colchicus*.

†Feeding ticks were retained on each individual animal within two neoprene cells as previously described[5]. Cell 1 contained two TBE virus-infected female ticks, two uninfected males, and 20 uninfected nymphs; cell 2 contained 20 uninfected nymphs. Pheasants received 30 uninfected nymphs instead of 20. Nymphs were allowed to feed to repletion (4 days). The animals were then humanely killed and ticks, blood, brain, spleen and lymph nodes collected, and then frozen and assayed for virus (see table 2). The titres of virus in ticks from field mice and bank voles were very similar ($\log_{10}$ mean: 1.80 and 1.87 PFU/tick, respectively) but ticks from pine voles had significantly higher titres ($\log_{10}$ mean 2.8 PFU/tick, 95% CL: 3.3–2.3). However, the titres reflect the level of virus in engorged ticks and do not necessarily relate to the titres of infective ticks after the nymphs have moulted to adults. *ticks eaten by host.

Table 2. TBE virus titres in the organs of wildlife hosts after tick feeding

Species/No.§	Viraemia†	Spleen	Lymph nodes*						
					ax	ms	prih	in	po
A. flavicollis									
1	<1	<1	<1	<1	<1	<1	<1		<1
2	<1	<1	<1	<1	<1	<1	<1	1.0	-
3	1.3	<1	-	<1	<1		<1	<1	<1
4	<1	<1		<1	<1	<1	<1	<1	<1
5	1.3	2.5	<1	1.0	<1	<1	<1	<1	<1
6	1.5	<1	<1	<1	<1	<1	<1	<1	<1
No. infected/total:	3/6	1/6	0/4	1/6	0/6	0/5	0/6	1/5	0/5
A. agrarius									
	2.0	<1	<1	<1	<1	<1		-	
2	1.5	<1	<1	<1	<1	<1	<1	<1	1.5
No. infected/total:	2/2	0/2	0/2	0/2	0/2	0/2	0/1	0/1	1/1
C. glareolus									
	1.3	1.5	.3	<1	<1	1.5	1.5	1.0	<1
2	2.0	2.6	<1	<1	-	2.4	1.5	<1	<1
3	2.5	<1	<1		<1	<1		-	-
4	1.5	1.8	<1	<1	<1	2.1	1.5	<1	<1
5	1.5	<1	<1	<1	.6	1.3	1.3	-	<1
6	3.5	3.7						-	
7	3.3	3.2		<1	<1	<1	1.5	2.4	
8	3.2	4.0	2.6	3.5		2.3	2.2	<1	<1
No. infected/total:	8/8	6/8	2/6	1/6	1/5	5/7	6/6	2/5	0/5
P. subterraneus									
1	3.7	4.7	1.6	<1	<1	4.7	4.7	-	<1
2	4.8	4.0	4.6	1.9	2.1	4.8	-	<1	-
3	3.9	3.0	1.0		<1	1.7	<1		<1
No. infected/total:	3/3	3/3	3/3	1/2	1/3	3/3	1/2	0/1	0/2

§Animals and treatments are as shown in table 1.
In summations of data, titres <1.0 are considered uninfected. Pine voles were the only animals that showed clinical signs of infection and had virus infection of the brain (up to 2.4 $\log_{10}$ PFU); moreover, 3/6 of the experimental animals died before the ticks completed engorgement. The level of viraemia was significantly lower in field mice (mean 1.5 $\log_{10}$ LD_{50}/ml, 95% CL: 1.6–1.4) compared to bank voles (2.6 $\log_{10}$ LD_{50}/ml blood, 2.8–2.3) ($F_{1,180} = 60.13$, $p = <0.001$). Infectious TBE virus was not detected in the blood or target organs of hedgehogs, and target organs of pheasants; blood of pheasants contained trace levels of virus (≤ 1.0 LD_{50}/0.01 ml blood). *s + m, submental and mandibular; md, mediastinal; ax, axillary; ms, mesenteric; prih, pancreactic, renal, iliac and hypogastric; in, inguinal; po, popliteal.

contrast, pine voles were highly susceptible to the virus but comparatively few nymphs completed engorgement. The most striking results were obtained with field mice and bank voles. Four times as many nymphs became infected on field mice as on bank voles even though the level of virus infection in bank voles was significantly greater than in field mice. This difference was due to greater feeding success and higher transmission frequencies in field mice. A significantly higher proportion of ticks became infected when cofeeding on *Apodemus* species (mean: 67.7%, 95% CL: 73.7–61.1) compared with bank voles (27.7%, 38.4–19.0). Examination of the differences in proportion of ticks infected between cells in *A. flavicollis* and *C. glareolus* showed that, although the trend was the same in both species (i.e. ticks in cell 1 had a higher probability of infection than ticks in cell 2), the significance of this effect was greater on *A. flavicollis* ($X^2 = 17.2$, $df = 1$, $p < 0.001$). The infection results for nymphs fed on field mice strongly suggest that viraemia is not the source of infection of ticks. If it was, we would expect to find similar proportions of infected ticks in cell 1 and cell 2, and a greater proportion of infected nymphs from bank voles compared to field mice.

If *Apodemus* species do not mount an immune response to *I. ricinus* (see above) they have no means of controlling their exposure to TBE virus-infected ticks. For TBE virus and *Apodemus*, evolutionary pressures appear to have reached an equilibrium in which selection for virulence of the virus (particularly high levels of viraemia) has been reduced by an efficient mechanism of nonviraemic transmission. The advantages for the survival of TBE virus infections in nature are two-fold. First, high virulence may result in mortality before ticks have completed engorgement and become infected. This was the apparent situation with pine voles. Thus low virulence for the host ensures that tick vectors complete their relatively long feeding period. Second, if uninfected feeding ticks only become infected when the host develops threshold levels of virus circulating in the

blood, as convention dictates, many ticks will complete engorgement before the viraemia develops. In contrast, nonviraemic transmission is not dependent on initial infection of target organs (as observed with field mice) and consequently maximizes the chances of transmission between cofeeding ticks.

The advantages of nonviraemic transmission appear to be related to the long feeding period of ixodid ticks. Nonviraemic transmission does not obviously provide similar benefits for arboviruses transmitted by the more rapidly feeding insect vectors, such as mosquitoes and sandflies. However, parallel observations have been made with sandflies (*Lutzomyia* and *Phlebotomus* spp., vectors of *Leishmania*) and ticks. A peptide secreted in sandfly saliva supresses the host immune response thereby promoting infection with *Leishmania* species[14–16]. Similarly, a protein secreted in tick saliva enhances the transmission of Thogoto virus between cofeeding ticks, facilitating transmission in the absence of a detectable viraemia[17], and a comparable phenomenon has been demonstrated recently in the transmission of TBE virus[18]. Collectively, these factors secreted by feeding arthroped vectors can be termed saliva-activated transmission (SAT) factors. The SAT factors act by inducing modifications in the skin site of feeding (presumably to facilitate vector blood-feeding) which are exploited by the vector-transmitted pathogen. SAT factors are believed to mediate nonviraemic transmission of arboviruses[19]. Given that SAT factor activity has been detected in the salivary glands of numerous ixodid tick species[18,20] and at least one group of insects (sandflies[16]), their general role in transmission and epidemiology needs to be explored, particularly as such factors offer a novel target for the control of arthropod-borne diseases.

Acknowledgments. We thank Dr S. E. Randolph and R. D. J. Rogers for their helpful comments on the manuscript, and Dr J. Chmela for field assistance. Dr M. Labuda was supported by a Wellcome Trust Research Fellowship.

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