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Tick-Borne Encephalitis Virus Transmission between Ticks Cofeeding

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To determine whether the portion of a vertebrate host population having specific immunity to tick-borne encephalitis (TBE) virus can participate in the TBE virus transmission cycle, natural hosts immunized against TBE virus were challenged with infected and uninfected ticks. Yellow-necked field mice (*Apodemus flavicollis*) and bank voles (*Clethrionomys glareolus*) were either immunized with TBE virus by subcutaneous inoculation of the virus, or they were exposed to virus-infected *Ixodes ricinus* ticks. One month later, when serum neutralizing antibody was detectable, the animals were infested with infected (donor) adult female ticks and uninfected (recipient) nymphal ticks; recipients were allowed to feed either in close contact (chamber 1) or physically separated (chamber 2) from the infected donor ticks. Following challenge with infected (and uninfected) ticks, viremia developed in all the control, nonimmune animals, whereas viremia was undetectable in all those animals naturally immunized by previous exposure to infected ticks. Despite the presence of neutralizing antibodies in all the immunized animals, 89% (24/27) immune animals supported virus transmission between infected and uninfected cofeeding ticks. Most transmission was localized, occurring within chamber 1; disseminated transmission from chamber 1 to chamber 2 was reduced. Immunization by tick bite was more effective than immunization by syringe inoculation in blocking cofeeding virus transmission. Nevertheless 75% (9/12) animals with "natural" immunity still supported transmission. The results demonstrate that natural hosts having neutralizing antibodies to TBE virus (and no detectable viremia) can still support virus transmission between infected and uninfected ticks feeding closely together on the same animal. These observations have important epidemiological implications relating to the survival of TBE virus in Nature.

INTRODUCTION

Tick-borne encephalitis (TBE) virus (genus *Flavivirus,* family *Flaviviridae*) is the etiological agent of the most important arbovirus infection affecting humans in Europe (World Health Organization, 1985). Two subtypes of TBE virus, Far eastern and European, have been distinguished; their distributions correspond to those of their primary tick vectors, *Ixodes persulcatus* and *I. ricinus,* respectively. A wide range of vertebrates are considered maintenance and reservoir hosts of TBE virus. Yellow-necked field mice (*Apodemus flavicollis*) and bank voles (*Clethrionomys glareolus*) are implicated as major hosts in Central Europe because they are abundant in foci of infection, they are readily infested with large numbers of *I. ricinus* ticks (reviewed by Nuttall and Labuda, 1994), and they support nonviremic transmission (Jones *et al.,* 1987) in which TBE virus is transmitted from infected to uninfected ticks as they feed together on a host that does not have a patent viremia (Labuda *et al.,* 1993, 1996). In the TBE foci of western Slovakia, yellow-necked field mice and bank voles comprise approximately 75% of the rodent population and about 15% of them have antibody to TBE virus (Kozuch *et al.,* 1990). Such immune animals are generally considered to be "dead-end" hosts; the infection is transmitted to them but is cleared by the anamnestic response, hence they do not participate in the virus transmission cycle. However, there is little evidence to support the assumption that immune hosts are indeed dead ends (Korenberg, 1974).

To address the important epidemiological question of whether immune hosts support the maintenance of TBE viral infections in wild rodent populations and thus contribute to the basic reproductive rate (R_0) (Anderson and May, 1991) of TBE viral infections, both field mice and bank voles were immunized with TBE virus; following the development of neutralizing antibodies, they were infested with infected donor and uninfected recipient *I. ricinus* ticks to determine their ability to support "cofeeding transmission" of the virus from infected to uninfected ticks feeding together on the same animal. Animals were immunized by either subcutaneous (s.c.) syringe inoculation of virus (the usual experimental approach) or by infective tick bite (the natural route of infection). Unlike laboratory strains of mice, field mice, and bank voles survive TBE virus infection.

MATERIALS AND METHODS

Animals

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 labo-

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ratory generation ticks fed on out-bred guinea pigs were used; none of the animals on which these ticks were maintained developed TBE virus-neutralizing antibodies, indicating that none of the ticks was infected with TBE virus prior to experimentation. Yellow-necked field mice and bank voles were live-trapped in areas of western Slovakia known to be free of TBE virus. Only adult animals that had no neutralizing antibodies to TBE virus were selected.

Infection and transmission experiments

Ticks and rodents were infected experimentally with the 198 isolate of TBE virus, originally obtained from *I. ricinus* ticks collected in Slovakia and used at the 26th mouse brain passage. All the female *I. ricinus* used in the immunization and in the transmission experiments were infected with TBE virus by parenteral inoculation (mean titer, 3.0 $\log_{10}$ PFU/tick). Infected ticks were maintained at ambient temperature for at least 14 days prior to use; all the infected ticks fed successfully on their experimental hosts.

Field mice and bank voles were immunized either by sc inoculation of 4.0 $\log_{10}$ PFU in 0.5 ml of appropriately diluted mouse brain virus stock, or by infestation with two infected *I. ricinus* female ticks that were retained in a transparent plastic chamber attached to the back of each animal and allowed to feed to repletion. Subsequent transmission experiments were carried out 37–38 days after sc virus inoculation or commencement of infected tick feeding; nonimmune animals of each species were included in the experiments as controls. Feeding ticks were retained on each individual animal within two transparent chambers as previously described (Labuda *et al.,* 1993). Chamber 1 contained two TBE virus-infected female ticks (donors of infection), two uninfected males, and 20 uninfected nymphs (recipients); chamber 2 contained 20 uninfected recipient nymphs. Nymphs were allowed to feed for 3 days and then the animals on which they were feeding were killed humanely and the ticks collected (these conditions gave the highest yield of infected ticks). The ticks, together with blood, brain, spleen, and lymph nodes from their hosts, were stored at −70° prior to infectivity assays. For virus assays, nymphal and adult ticks, and vertebrate tissues, were homogenized individually in 1 ml of EMEM containing 10% fetal bovine serum (FBS) and antibiotics appropriate to inhibit bacterial growth. Plaque titrations of clarified tick and tissue homogenates were performed using the continuous line of porcine stable (PS) kidney cells propagated in Earle's modification of Eagle's medium (EMEM) supplemented with 3% FBS and antibiotics and incubated at 36° for 4 days prior to fixation and staining. Virus titers were expressed as $\log_{10}$ PFU/sample. When blood samples (0.1 ml of 1:10 dilution in EMEM) were negative by plaque assay (less than 2 $\log_{10}$ PFU/ml), the parallel sample of whole blood in heparin (1:1) was titrated by intracranial (ic) inoculation of 1- to 2-day-old mice, as previously described (Labuda *et al.,* 1996). The efficiency of virus transmission was determined by the number of recipent nymphs that became infected (Tables 1 and 2). Serum samples, collected prior to termination of the tick cofeeding, were assayed for antibodies using the plaque reduction neutralization test (DeMadrid and Porterfield, 1969); neutralizing antibody titers were expressed as the serum dilution which resulted in a 50% reduction in plaque number compared to the controls (Tables 1 and 2).

Statistical analyses

Analyses of the results centered around two models considering the two different methods of immunization (immunity as a factor with three levels) and the two species as explanatory variables. For the first model, the proportion of ticks infected within chamber 1 was taken as the response variable, and in the second model, the difference in proportions between chambers 1 and 2 was taken as the response variable. Proportions were arcsin transformed before analysis with normal errors. The design involves a mixture of fixed and random effects; the variation between animals within species/immunity treatment combination was taken as the error term. In all cases model checking procedures were employed on the final models.

RESULTS

Host infection and immunity

The neutralizing antibody titers of field mice and bank voles 6 weeks after immunization by either virus syringe inoculation or exposure to two infected ticks were similar (1:16 or 1:32), with two exceptions (field mouse No. 7 and bank vole No. 11; Tables 1 and 2, respectively). Viremia was not detected in the 12 animals immunized by tick bite but was detected in all of the 10 control, nonimmune animals, and in 5/8 field mice and 2/7 bank voles immunized by syringe inoculation. The incidence of viremia was not significantly different between the two species, but was different between control animals, those immunized by syringe inoculation, and those immunized by tick bite (proportion of animals exhibiting viremia analyzed with binomial errors, $\chi^2 = 30.3$, $P < 0.001$), immunization via tick bite resulting in no cases of viremia. In addition to viremia, field mouse No. 5 was the only immune animal with virus infection observed in the spleen and lymph nodes. All the other spleen and lymph node tissues from immune animals and all brain tissues were negative by plaque assay. By contrast, spleen and lymph nodes of control, nonimmune animals were all infected, with one exception (lymph node tissues from field mouse No. 16).

TABLE 1

Transmission of Tick-Borne Encephalitis Virus by Cofeeding of *Ixodes ricinus* Ticks on *Apodemus flavicollis* Hosts Immunized by either (a) Subcutaneous Inoculation of TBE Virus or (b) Infected *I. ricinus* Tick Bite

Animal no.	Anti-TBE antibody[a]	Viremia[b]	Percentage (No. nymphs infected/fed) Chamber 1		Chamber 2	
(a) Immunized by sc inoculation of TBE virus						
1	1:32	<1	65%	(13/20)	0%	(0/2)
2	1:32	<1	21%	(3/14)	0%	(0/15)
3	1:32	1.5	67%	(8/12)	0%	(0/10)
4	1:16	1.8	83%	(10/12)	67%	(4/6)
5	1:16	1.8	78%	(11/14)	21%	(3/14)
6	1:16	1.5	58%	(11/19)	0%	(0/8)
7	1:4	1.8	80%	(4/5)	60%	(6/10)
8	1:16	<1	37%	(7/19)	0%	(0/19)
Percentage mean (totals):			58%	(67/115)	15%	(13/84)
(b) Immunized by a bite of infected tick						
9	1:32	<1	19%	(3/16)	0%	(0/10)
10	1:32	<1	20%	(2/10)	0%	(0/8)
11	1:16	<1	25%	(2/8)	14%	(2/14)
12	1:32	<1	0%	(0/6)	0%	(0/6)
13	1:16	<1	50%	(8/16)	0%	(0/17)
14	1:16	<1	8%	(1/12)	0%	(0/6)
Percentage mean (totals):			24%	(16/68)	3%	(2/61)
(c) Nonimmune control animals						
15	<1:2	1.3	83%	(10/12)	35%	(6/17)
16	<1:2	1.0	60%	(9/15)	50%	(8/16)
17	<1:2	1.5	71%	(10/14)	35%	(6/17)
18	<1:2	1.3	75%	(15/20)	33%	(6/18)
Percentage mean (totals):			72%	(44/61)	38%	(26/68)

[a] Reciprocal serum dilution resulting in a 50% reduction in TBE viral plaque number compared to the controls.
[b] Virus titer expressed as $\log_{10}$ ic mouse LD_{50}/0.02 ml blood; <1, <1 LD_{50}.

Virus transmission between cofeeding ticks: Field mice compared with bank voles

A greater number of ticks became infected on field mice compared with bank voles (Tables 1 and 2). The significantly greater efficiency of field mice in supporting TBE virus transmission between cofeeding ticks ($F_{1,31} = 12.0$, $P < 0.01$) has been reported previously (Labuda *et al.,* 1993). The difference may relate to the observation that bank voles, but not field mice, develop an immunologically based resistance response to tick feeding that may impede virus transmission (Dizij and Kurtenbach, 1995). All the animals used in the experiments were captured in the field and consequently they would have been exposed to ticks in Nature. There was no difference in the mean virus titer of nymphs fed on field mice ($\log_{10}$ 1.9 PFU/tick) compared with bank voles ($\log_{10}$ 1.6 PFU/tick).

Virus transmission between cofeeding ticks: Chamber 1 vs chamber 2

The efficiency of virus transmission on both immune and nonimmune hosts depended on the location of recipient nymphs compared with infected donor ticks. Localized transmission in chamber 1 yielded a higher proportion of infected nymphs than disseminated transmission from infected donors feeding in chamber 1 to uninfected recipient nymphs feeding in chamber 2 (Tables 1 and 2). This was illustrated by analyzing the difference in proportions of nymphs infected in chambers 1 and 2: the difference was significantly greater than zero ($t_s = 11.8$, $df = 35$, $P < 0.001$). No other terms were found to be significant, indicating that the effect of distance between donor and recipient ticks was the same for both species, and for both methods of inducing immunity to THO virus. Chamber 1 represents the natural situation in which feeding ticks aggregate together in localized sites such as on the ears of mammals or around the beaks of birds (Randolph *et al.,* 1996).

Virus transmission between cofeeding ticks: Natural and artificial immunity vs nonimmune hosts

For both species, tick bite (the natural mode of immunization) was found to be more effective than immunization

TABLE 2

Transmission of Tick-Borne Encephalitis Virus by Cofeeding of *Ixodes ricinus* Ticks on *Clethrionomys glareolus* Hosts Immunized by either (a) Subcutaneous Inoculation of TBE Virus or (b) Infected *I. ricinus* Tick Bite

Animal no.	Anti-TBE antibody[a]	Viremia[b]	Percentage (No. nymphs infected/fed) Chamber 1		Chamber 2	
(a) Immunized by sc inoculation of TBE virus						
1	1:32	1.5	29%	(4/14)	0%	(0/3)
2	1:32	1.3	25%	(3/12)	0%	(0/4)
3	1:32	<1	20%	(1/5)	0%	(0/3)
4	1:32	<1	27%	(3/11)	0%	(0/5)
5	1:32	<1	22%	(2/9)	0%	(0/4)
6	1:32	<1	50%	(1/2)	0%	(0/3)
7	1:16	<1	20%	(4/20)	—	(0/0)
Percentage mean (totals:)			25%	(18/73)	0%	(0/22)
(b) Immunized by a bite of infected tick						
8	1:32	<1	11%	(1/9)	0%	(0/8)
9	1:32	<1	0%	(0/5)	0%	(0/7)
10	1:32	<1	0%	(0/3)	0%	(0/5)
11	1:8	<1	78%	(7/9)	0%	(0/4)
12	1:32	<1	25%	(2/8)	0%	(0/8)
13	1:32	<1	27%	(4/15)	9%	(1/11)
Percentage mean (totals):			29%	(14/49)	2%	(1/43)
(c) Nonimmune control animals						
14	<1:2	1.8	55%	(5/9)	50%	(2/4)
15	<1:2	2.8	50%	(6/12)	20%	(1/5)
16	<1:2	1.8	14%	(2/14)	0%	(0/9)
17	<1:2	2.7	27%	(3/11)	20%	(2/10)
18	<1:2	1.8	56%	(5/9)	50%	(2/4)
19	<1:2	2.8	58%	(7/12)	20%	(1/5)
Percentage mean (totals):			42%	(28/67)	22%	(8/37)

[a] Reciprocal serum dilution resulting in a 50% reduction in TBE viral plaque number compared to the controls.
[b] Virus titer expressed as $\log_{10}$ ic mouse LD_{50}/0.02 ml blood; <1, <1 LD_{50}.

via syringe inoculation in suppressing virus transmission (Fig. 1). Both immunization treatments resulted in significantly fewer infected ticks when compared with the controls (Tables 1 and 2; Fig. 1). Although analysis of the proportion of nymphs infected in chamber 1 showed that both immunity and species were significant ($F_{2,31} = 7.74$, $P < 0.01$ and $F_{1,31} = 12.0$, $P < 0.01$, respectively), there was no evidence of an interaction between species and immunity ($F_{2,31} = 2.89$, $P > 0.05$), indicating that field mice and bank voles responded similarly to the two methods of immunization. The apparent difference in the "transmission blocking" capacity of tick bite versus syringe inoculation suggests that either tick-borne virus transmission induces a stronger immune response (possibly by stimulating cell-mediated immunity) or that the response to tick feeding per se in hosts previously exposed to tick infestation can inhibit virus transmission. Evidence supporting the latter hypothesis has been observed for the tick-borne transmission of the Lyme disease spirochaete, *Borrelia burgdorferi,* and has been named "saliva-inhibited transmission" or SIT (Kurtenbach, 1996).

Despite the presence of neutralizing antibodies, 89% (24/27) immune animals supported virus transmission between infected and uninfected ticks. Furthermore, there was a suprisingly high degree of localized virus transmission from infected donor ticks to uninfected recipient ticks feeding together in chamber 1 of immune animals (Fig. 1).

DISCUSSION

The basic reproductive rate, R_0, of a virus is defined as the average number of secondary infections produced when one infected individual is introduced into a host population where every individual is susceptible (Anderson and May, 1991). This concept is central to the population biology of parasites including viruses and is used to design control stategies; e.g., in predicting the level of herd immunity required to eliminate a particular infectious disease. For a virus infection to survive in a population, $R_0 > 1$ (i.e., one infected host must give rise to at least one new infected host). Vertebrate hosts immune to

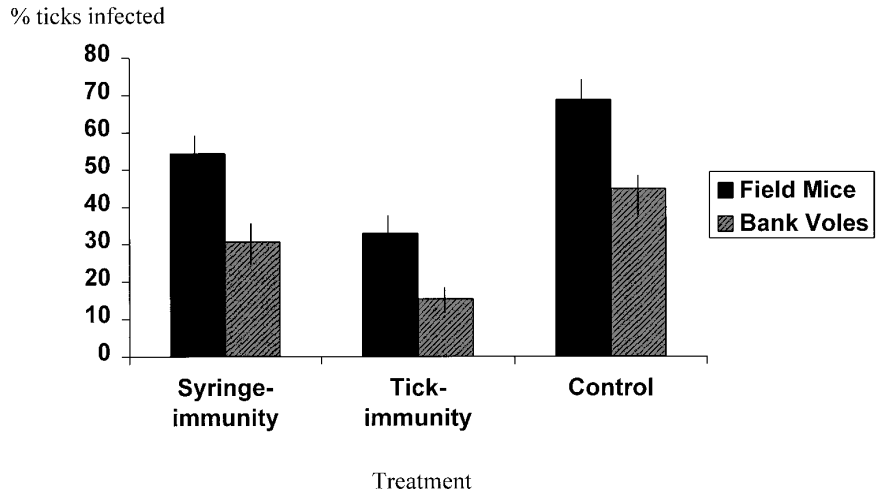

FIG. 1. The percentage of nymphal ticks feeding in chamber 1 that became infected during cofeeding with TBE virus-infected ticks on natural hosts that were either immune or nonimmune to the virus. "Syringe-immunity" represents animals immunized by syringe inoculation of mouse brain-derived TBE virus; "tick-immunity" represents animals immunized by infestation with TBE virus-infected ticks. The fitted values were derived from the statistical model for three treatments and two species (see Materials and Methods) using the data presented in Tables 1 and 2. Bar, Standard error of the difference between the two species (field mice and bank voles).

virus infection are generally considered dead-end hosts. Thus, immunity has a negative effect on R_0 at the population level because susceptible hosts are removed from the host population supporting the infection (Anderson and May, 1991).

Previously, hosts immune to TBE virus were considered dead-end hosts. Studies on a Far eastern subtype of TBE virus (Dumina, 1958) and louping ill virus (Alexander and Neitz, 1935) demonstrated that recipient ticks did not acquire virus when cofeeding with infected donor ticks on immune hosts. Similarly, in studies on cofeeding transmission of Thogoto virus on virus-immune guinea-pigs, disseminated transmission was almost completely inhibited (Jones and Nuttall, 1989). These published results are comparable to those reported here on the transmission of TBE virus from infected donor ticks feeding in chamber 1 to uninfected recipient ticks feeding in chamber 2. However, in all previously reported experiments, the capacity of immune hosts to support localized cofeeding transmission (represented here by chamber 1 data) was not tested, even though localized cofeeding represents the aggregated feeding distribution of ticks on their hosts in Nature (e.g., Fig. 4 of Randolph *et al.,* 1996).

The clustered distribution of feeding ticks is one of several epidemiologically important natural features of tick–host interactions (Randolph *et al.,* 1996). It results from the fact that, at any one time, some individual animals have many ticks feeding upon them, whereas others of the same species have none or only a few ticks (an overdispersed distribution that is typical for parasites on their hosts). Another important feature of tick–host interactions is that tick saliva induces immunomodulation of the host at the skin site of tick feeding (Wikel *et al.,* 1994). These features greatly facilitate cofeeding pathogen transmission in Nature (Randolph *et al.,* 1996). Indeed, comparison of the relative index of R_0 for viremic and nonviremic transmission pathways shows that survival of TBE virus in Nature depends more on cofeeding transmission between ticks feeding on nonviremic hosts ($R_0 = 1.65$) than on classical viremic transmission ($R_0 = 0.98$). The relative R_0 values of TBE virus are low, particularly when compared with equivalent values (10 to 60 times higher) for the Lyme disease spirochete, *Borrelia burgdorferi* sensu lato, which is maintained by the same vector and host species but is much more widespread and of greater prevalence than TBE virus.

Longitudinal studies (1981–1986) of small mammals collected in Western Slovakia revealed neutralizing antibody to TBE virus in 14.6% (426/2922). The antibody prevalence varied seasonally and according to species, e.g., in March–May, 25% subadult and in June–August, 25% adult *A. flavicollis* were positive, whereas in September–November, 18% subadult and 11% adult were positive (Kozuch *et al.,* 1990). At these levels of herd immunity, it is unlikely that TBE virus would survive in Nature, given its low relative R_0 values (see above), if immune hosts are dead-end hosts. The results presented here show that immune hosts can indeed contribute to the susceptible host population and therefore effective host density is much higher than previously thought. Even relatively low transmission efficiencies, in which only 25% of recipient ticks acquire infection, could be critical for the survival of TBE virus in a natural focus of infection, especially if these immune hosts repeatedly support virus transmission and thereby contribute significantly to the numbers of newly infected ticks.

The mechanism by which virus passes from infected to uninfected ticks feeding together on the same host is unknown. However, recent data suggest that TBE viral infection of Langerhans cells at the skin site of infected

tick feeding, combined with cellular infiltration and migration at the feeding sites of infected and uninfected ticks, play key roles in cofeeding transmission (Labuda *et al.,* 1996). In addition, the ability of tick saliva components to potentiate arbovirus transmission (reviewed by Nuttall *et al.,* 1994), possibly by suppressing natural killer cell anti-viral activity together with other immunosuppressive effects (Kubeš *et al.,* 1995, Wikel *et al.,* 1995), help explain why arbovirus transmission between cofeeding ticks is independent of the development of a patent viremia by the tick-infested host. The observation that 17/20 of the immunized animals that had no detectable viremia (Tables 1 and 2) were still able to support virus transmission between cofeeding ticks supports the concept that viremia is a product, rather than a prequisite, of tick-borne virus transmission.

Besides the epidemiological significance, virus transmission involving immune hosts has important implications for virus evolution. Neutralizing antibodies exert a strong selective pressure on viral phenotypes. The effect of such evolutionary pressure could be tested by comparing the phenotype (e.g., reactivity with monoclonal antibodies) of TBE virus transmitted by donor ticks with that of the virus acquired by recipient ticks cofeeding on specific immune natural hosts.

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