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Original article

Different tick-borne encephalitis virus (TBEV) prevalences in unfed versus partially engorged ixodid ticks – Evidence of virus replication and changes in tick behavior

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ABSTRACT

There is some evidence that tick-borne encephalitis virus (TBEV) prevalence in ticks, removed from humans, is higher than that in field-collected ticks from the same area. There are two possible explanations: (i) Infected ticks are more active and aggressive and can be found on humans more often. (ii) Some questing ticks are infected with TBEV in a low, undetectable concentration; during tick feeding, virus replicates and reaches the titers that can be detected. The aim of our work was to evaluate both hypotheses. Using unfed adult *Ixodes ricinus*, we compared three methods of tick infection with TBEV: (i) injection of the virus under the tick's 4th coxa (percoxal method), (ii) injection through anus (rectal method), and (iii) immersion of ticks in virus-containing medium. The percoxal method showed the best results and was used in further experiments. We compared the dynamics of virus reproduction in ticks that remain unfed after inoculation and in partially engorged ticks fed on mice. When ticks fed for 15 h, the titer of the virus increased in 3 $\log_{10}$PFU/tick since inoculation, while in unfed ticks it did not change. We also studied the reaction on the repellent DEET of uninfected versus TBEV-infected *Ixodes ricinus* ticks of the physiological age levels III and IV. We investigated ticks movements upwards in the direction of the bait on the cotton tape, impregnated with an increasing concentration of DEET. Obtained data showed that infected ticks were more active and tolerant to DEET. About 70% of the non-infected ticks and only 13% of the infected ticks did not get over the lowest concentration of the repellent (0.1%). Only infected ticks (5.6%) got over 1% concentration of DEET. Ticks of the physiological age level IV from both infected and uninfected groups were the most active and tolerant to the repellent. Both above-mentioned hypotheses were approved and can be used to explain higher virus prevalences in partially engorged ticks than in field-collected ticks.

Introduction

There is some evidence that tick-borne encephalitis virus (TBEV) prevalence in ixodid ticks, removed from humans, is higher than that in field-collected ticks from the same area. In the 1990s in Irkutsk region and in the Republic of Buryatia (Siberia, Russia), higher TBEV prevalences were observed in unfed and partially engorged *I. persulcatus* ticks, removed from humans and animals, than in questing ones, collected from the vegetation in nature (Melnikova et al., 1997). In Germany, it was noted that virus prevalence in *I. ricinus* nymphs, removed from humans, was 18 times higher than in questing ticks, collected in nature during the same period. In partially engorged females, this parameter was 9 times higher (Süss et al., 2004). In Tomsk city (Russia), TBEV infection rates of *I. persulcatus* and *I. pavlovskyi* were also higher in fed ticks (48.8% and 35%, respectively) than in ones without signs of preceding feeding (9.4% and 3.7%, respectively) (Romanenko and Kondrat'eva, 2011). In the Leningrad region, adult *I. persulcatus* ticks found on human body and clothes were 7 times more often infected with TBEV than unfed questing ticks from the same area, and this ratio was even higher in Latvia (A.N. Alekseev, pers. communication).

One of the possible explanations of these facts is that infected ticks under TBEV influence became more active and aggressive so that they can be found more often on humans and animals. Another possible explanation is the replication of the virus in ticks during the blood meal. Perhaps in nature, some questing ticks contain TBEV at an undetectable level, and during tick feeding, virus replicates and reaches detectable titers.

There are published data that partially confirm both theories. It was shown on 50 *I. persulcatus* females that TBEV infection substantially changes their behavior: ticks became more active and

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traversed a longer path with higher speed toward the bait and against a humidity gradient than uninfected ones (Alekseev et al., 1988a). Moreover, it was shown the TBEV quantity in the saliva, discharged by *I. persulcatus* females and measured at different periods of bloodsucking (at least during the first 3 days), was by 10–100 times higher in comparison with unfed ticks (Alekseev and Chunikhin, 1990). Also, depending on the TBEV strain, the virus titer in 6 infected *I. ricinus* ticks had increased by 100–1000 times after 3 days of feeding (Khasnatinov et al., 2009).

The aim of our work was to evaluate carefully both hypotheses explaining the phenomenon of higher TBEV prevalences in ticks, removed from humans and animals, when compared with questing ticks field-collected from the vegetation.

Materials and methods

Cells and viruses

A pig embryo kidney (PEK) cell line was maintained at 37 °C in medium 199 (PIPVE, Russia), supplemented with 5% bovine serum (Furo, Russia).

In our work, we used TBEV strain Absettarov of European subtype (GenBank: AF091005.1; Kozlovskaya et al., 2010). Ticks were inoculated with the culture supernatant from TBEV-infected PEK cells with a virus concentration of 6.7–7.2 logarithm of plaque forming units per ml ($\log_{10}$PFU/ml). Virus production and cell culture maintenance were described earlier (Romanova et al., 2007).

Ticks

In all experiments, we used a 2-year laboratory *I. ricinus* tick culture, which was obtained from a female, collected in the TBE-free Kaluga region (Vysokinichi Village, Russia). Living ticks were kept in glass tubes with a humidity gradient. Glass tubes were filled to 1/3 with distilled water. Tight cotton swab 2–3 cm in length was inserted so that it almost entirely ended up in the water. Filter paper was placed on the top of the cotton swab, corresponding to the diameter of the tube. Also, a strip of filter paper was inserted into each tube to allow ticks migrate in the tubes according to their preferred humidity. All tubes were tightly closed with cotton-gauze cap.

Four main physiological age levels of unfed ixodid ticks were introduced by Balashov (1962) using a histological method. We estimated the physiological age of ticks according to Razumova's method (2001). This method allowed to determine the physiological age of unfed females using anatomic and morphologic age-specific characteristics of ticks such as body thickness, wrinkles and color of the cuticle and transcuticular visibility of the viscera. According to this method, ticks of the physiological age level I had convex, roundish body with surface wrinkles and unobservable internal organs; ticks of the age level II had a weakly convex body with surface wrinkles and barely visible organs; ticks of the age level III had a flat or slightly flattened body with wrinkled cuticle and visible viscera; and ticks of the age level IV had a strongly flattened, deeply wrinkled body and clearly visible viscera (especially intestine diverticula, Malpighian tubules, tracheae). Ticks used in our experiments with DEET belonged to the physiological age levels III and IV. The ratios of the different levels of physiological age was as follows: in the infected group, 37 and 15 ticks had physiological age level III or IV, respectively; in the intact group, 57 and 20 ticks had physiological age level III or IV, respectively; in the control group, 20 and 7 ticks had physiological age level III or IV, respectively.

Methods of tick infection with TBEV

Percoxal infection

The infection was carried out according to a modified Alekseev's method (Alekseev, 1965; Alekseev and Chunikhin, 1987). Instead of glass microneedles we used microsyringes (volume 25 μl) and special needles with tip No. 33 (length 7 mm) and element No. 25 connected to the syringe (length 8 mm) (Exmire, Ito Corp., Japan). The device for tick infection consisted of a binocular microscope and a vacuum holder that was connected to the vacuum pump. Questing ticks were immobilized by fixing their ventral surface up to the vacuum holder and under the binocular, virus suspension was injected in the joint of the tick coxa and trochanter of the 4th pair of legs. The infectious dose for *I. ricinus* females was 1 μl of virus (4.2 $\log_{10}$PFU), for males 0.5 μl (3.9 $\log_{10}$PFU). The same volume of culture supernatant of uninfected PEK cells was inoculated into ticks from the control group. All manipulations were carried out at room temperature (23–25 °C). A total of 160 female and 30 male ticks was infected by the percoxal method.

Rectal infection

Rectal infection was carried out according to percoxal infection, with one difference: TBEV was inoculated in ticks through the anus in the same volume and dose. A total of 25 female and 10 male ticks was infected by the rectal method.

Infection of ticks by immersion in virus suspension

Immersion of ticks in virus suspension was carried out according to the modified method, described for ixodid tick larvae (Policastro and Schwan, 2003). Two to 3 adult ticks were placed in a 1.7-ml test tube (Corning) with 0.5 ml of culture supernatant from TBEV-infected PEK cells (virus concentration 7.2 $\log_{10}$PFU/ml) and were incubated at 34 °C for 45 min. Every 5–10 min, tubes with ticks were shaken. After incubation, tubes were kept on ice for 2–3 min and were subsequently centrifuged on 200 × *g* for 30 s. Thereafter, ticks were removed from the virus-containing medium and washed with saline solution. After 2 washes, ticks were dried on filter paper in a Petri dish and finally irradiated with UV for 1 min to inactivate virus on the tick's surface. With the ticks from the control group, we carried out the same manipulations using the culture supernatant of uninfected PEK cells instead of a virus-containing suspension. A total of 22 female and 10 male ticks was infected by immersion.

After infection, all ticks were kept in groups in glass tubes with gradient humidity at room temperature.

Tick feeding

Outbred mice were used for tick feeding. One female and one male of *I. ricinus* were placed on each mouse. Males were used only for mating with females (for proper process of females feeding) and were not used in the experiment. Before feeding ticks, we cut off the hair in the region of the bladebones of mice in the form of a square (size 1.5 cm^2). Then we pasted some gauze of the same size to this area. Ticks were put under the gauze for feeding after the glue had dried (1–2 h).

TBEV replication in ticks during feeding

Ten males and 44 females of *I. ricinus* were infected with TBEV by the percoxal method. Virus dosage per female was 3.7 $\log_{10}$PFU, per male 3.4 $\log_{10}$PFU. One day post infection, 50% of the infected ticks were put on mice and the other 50% were left in glass tubes with gradient humidity. At different times after the beginning of tick feeding (8 h, 15 h, 38 h, 96 h), 5–6 ticks were removed from mice and the same number of ticks was taken from glass tubes. Ticks