Subclinical Tick-Borne Encephalitis Virus in Experimentally Infected *Apodemus agrarius*

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Key Words

Tick-borne encephalitis virus · *Apodemus agrarius* · Experimental infection · Subclinical encephalitis

Abstract

Objectives: In this study, we investigated the dose dependence of tick-borne encephalitis virus (TBEV) infection in one of the reservoirs, i.e. *Apodemus agrarius*, a small rodent species. **Methods:** The animals were challenged with TBEV per os and intramuscularly with infectious doses ranging from 1 to 1,500 plaque-forming units (pfu). Clinical signs were recorded and clinical and pathological features were evaluated by histological, immunohistochemical, and serological methods. **Results:** High perorally administered infectious doses resulted in virus replication in the brain, which is the first sign of subclinical viral encephalitis in the *Apodemus* genus. The animals seroconverted at infectious doses greater than 100 pfu, and all animals remained asymptomatic. **Conclusion:** Our work shows the first evidence that subclinical TBEV encephalitis may occur in *Apodemus* species, depending on the virus load of the inoculum. The antiviral response of the local innate immune system may influence the resis-

tance of *Apodemus* individuals to lower infectious doses. Per oral/nasal infection seems to be more dangerous for the host than other routes of infection.

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Introduction

Tick-borne encephalitis (TBE) is a well-known vector-borne flaviviral disease and one of the most important zoonoses in Europe, with increasing numbers of annual human cases [1]. Humans react to TBE virus (TBEV) infection with a wide range of symptoms. In the majority of human cases, the infection is unapparent [2]. However, some people overcome the disease after a short period of headache and fever; 10–20% of patients are hospitalized with clinical encephalitis [3], while 0.1–0.2% of cases result in chronic paralysis (usually of the arms) or death [4].

The following 3 patterns of pathogenesis were delineated on the basis of peripheral experimental West Nile virus (a flavivirus closely related to TBEV) infections in laboratory mice: (1) fatal encephalitis, preceded by early viremia and invasion of the central nervous system (CNS);

(2) unapparent infection, with no detectable viremia and no evidence of CNS invasion, and (3) subclinical encephalitis, usually preceded by trace viremia with minimal transient levels of virus in the brain. In the latter type of subclinical infection with a potentially lethal virus, the immune response probably plays an important role in recovery [5].

The role of wild rodents in the spread and maintenance of natural TBEV foci is known but not completely understood. Histological studies on free-ranging small mammals are limited; experimental infection of the wood mouse (*Apodemus sylvaticus*) did not lead to detectable virus in the CNS [6], although virus-infected neurons have been demonstrated in the CNS of bank voles, i.e. *Myodes glareolus* [7].

A. agrarius (striped field mouse) is a species of the *Apodemus* genus that is widespread from Central-Eastern Europe through Southern Siberia to China, Korea, and Taiwan. Its habitat almost completely overlaps with endemic TBE virus areas. As one of the main reservoir species for TBEV, the striped field mouse was found to be susceptible to TBEV infection, as the virus was isolated from [8] and seroconversion was proven [9] in individuals captured in nature.

Classical histological stains for the detection of tissue lesions caused by TBEV have been used for decades in TBEV research. A variety of chronic and acute pathological changes in the CNS have been documented in several vertebrate hosts [10, 11].

Immunohistochemical (IHC) techniques are generally used as an additional diagnostic tool to prove the presence of viral antigen in TBE cases [12, 13]. The only comprehensive study utilizing IHC examined the distribution of viral antigens in the CNS in 28 fatal human TBEV cases [14]. That study demonstrated that immunoreactivity was most prominent in Purkinje cells and large neurons of the dentate nucleus, inferior olives, and anterior horns and, to a lesser extent, in neurons of the other brainstem nuclei, isocortex, and basal ganglia. Signs of a severe inflammatory response (triggered by cytotoxic T cells) were detected, indicating the role of antiviral immune functions in neuron damage in TBEV encephalomyelitis.

Serological tests are widely used in TBEV diagnosis, although the serum neutralization test appears to be the most specific method despite the requirements for live virus, biological containment facilities, cell culture facilities, and time [15].

The aim of the present study was to test the sensitivity of a free-ranging murine species to a wide range of TBEV infectious doses.

Material and Methods

Wild-caught striped field mice were challenged perorally ($n = 10$) and intramuscularly ($n = 10$) with 5 infectious doses of $1, 10^1, 10^2, 10^3$, and 1,500 plaque-forming units (pfu) in a 100- μ l inoculation volume (thus, there were 2 mice per infectious dose). Inoculation through the intramuscular route was administered via the musculus semimembranosus, 2–3 mm below the skin, while oral inoculation was undertaken using a syringe with a blunt-end needle. Clinical signs were recorded daily, and 2 animals were included as mock-infected controls. The animals were killed humanely at 7 days postinfection, following blood sampling for serological analysis.

Permission for this animal study was granted by the National Food Chain Safety Office (permission No. PEI/001/1792-4/2014), and this study was undertaken under the supervision of the local animal protection committee at the Institute for Veterinary Medical Research.

A. agrarius were live-trapped in Southwestern Hungary and transferred to an animal accommodation where they were assessed for seronegativity prior to virus inoculation. The individuals were mature adults (presumably 2–8 months old). Pairs of animals were kept in isolated plastic cages. The infectious virus was a Hungarian TBEV isolate from sampled *Ixodes ricinus* larvae (2011) propagated and titrated on an N2A mouse neuroblastoma cell line.

For histological studies, the classical hematoxylin-eosin (HE) staining and monoclonal antibody-based IHC were applied. Tissue samples were collected from the brains (longitudinal sections). Formalin-fixed and paraffin-embedded tissue sections (4- μ m thickness) were prepared and stained with HE. Serial tissue sections were deparaffinized, treated in citrate buffer (pH 6.0) in a microwave oven at 750 W for 20 min, and incubated at room temperature in 3% H_2O_2 solution for 10 min and then with a 2% solution of skimmed-milk powder for 20 min. The sections were incubated at 4°C overnight with TBEV-specific mouse antibody 19/1,367 (IgG2b) [16]. Antibody binding was detected using an ARK Animal Research Kit (HRP; DAKO Denmark A/S, Glostrup, Denmark) according to manufacturer's instructions. The sections were counterstained with Mayer's hematoxylin and covered with glycerol gelatin. All sections were examined at magnifications of $\times 100$ to $\times 400$.

A virus neutralization assay was used to assess serum samples for seroconversion. Serum taken from an additional mouse experimentally infected 2 weeks previously was used as a positive control. Sera were not titrated; 10 μ l of undiluted serum samples were mixed with 100 pfu of TBEV virus in 2 wells of a 96-well tissue culture plate (Greiner Bio-One, Kremsmünster, Austria) and incubated at 37°C for 1 h, time point at which 10^4 N2A cells were added to the wells in 200 μ l of 4.5% high glucose DMEM with 10% fetal calf serum and antibiotics. The cells were cultured for 5–7 days before monitoring of the cultures for a cytopathic effect.

Results

All *A. agrarius* individuals were free of any clinical signs.

Histological examinations revealed no TBEV antigens or histological alterations in intramuscularly infected mice or any of the controls.

Color version available online

Fig. 1. *A. agrarius* (per os). Several cells are immunostained for TBEV in the bulbous olfactorius.

Color version available online

Fig. 2. *A. agrarius* (per os) brain. Few cells are immunostained for TBEV in the caudate putamen.

TBEV antigens were found in the brains of the per os infected (1,500 pfu) striped field mice. Few infected neurons and glial cells in the olfactory bulb and in the anterior olfactory nucleus and the presence of mild lymphohistiocytic vasculitis restricted to this area were evident (fig. 1). Few infected neurons and glial cells were observed in the caudate putamen, which was accompanied by mild lymphohistiocytic vasculitis in the same area (fig. 2). Necrosis of few neurons and glial nodules was also present in the brainstem.

Detectable levels of TBEV-specific antibodies were found in *A. agrarius* individuals (inoculated by both routes) given an infectious dose of 100 pfu, but not in individuals inoculated with lower doses of TBEV.

Discussion

Previous studies have been unable to detect TBEV in the *Apodemus* CNS [6]. However, we have demonstrated that TBEV virions can infiltrate and replicate within cells of the CNS in a free-ranging wild murine *Apodemus* species, without induction of clinical signs, even if a relatively high infectious dose is administered. These results prove that under certain circumstances a wild murine species can also be sensitive to subclinical TBE, as previously shown in bank voles [7]. However, these are preliminary data only, as the small test group size ($n = 2$) implies that the statistical significance of the analyses would be difficult to interpret.

Subclinical, recovered encephalitis induced by high levels of infectious virions, along with serological results (detectable virus-specific antibodies following inoculation of >100 pfu of virus), suggests involvement of the immune system, including the innate immune system (local antiviral response in the skin), in resistance. This is in accordance with previous studies in which TBEV proteins were detected by IHC on local dendritic cells of murine skin in the early phase of infection [17]. Inoculating virus doses <100 pfu are most likely eliminated by local immune cells at the site of inoculation, and therefore limited virions are carried by antigen-presenting cells to lymph nodes, which are not sufficient to trigger a serological response.

The recovered subclinical encephalitis suggests that effective antiviral functions exist within the CNS; either CNS cells (astrocytes and glial cells) or circulating immune cells enter the CNS from the periphery to eliminate the virus.

We observed, that subclinical encephalitis occurred only in per os infected *A. agrarius*. As oral/nasal cavities and the pharynx are practically confluent spaces, perorally administered inoculum could easily reach nasal epithelium and olfactory nerve endings, which explains the heavy involvement of olfactory bulbs in the infection. These are slight indications that per os administration could be a more efficient route of infection, but the small sample size did not enable statistical analysis.

Our work shows the first evidence that subclinical TBEV encephalitis might occur also in *Apodemus* species,

depending on the infectious dose. The effectiveness of the local innate antiviral immune response might provide an explanation for the resistance of *Apodemus* individuals to lower infectious doses, while the per os route may be a more efficient route of infection.

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