Virus Research xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

The nature of replication of tick-borne encephalitis virus strains isolated from residents of the Russian Far East with inapparent and clinical forms of infection

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ARTICLE INFO

Article history:
Received 29 November 2013
Received in revised form 3 April 2014
Accepted 6 April 2014
Available online xxx

Keywords:
TBEV Far East subtype
Molecular genetics
Virulence
Replication in cells
Hemagglutination

ABSTRACT

We describe the biological properties and molecular characteristics of complete genomes of 33 tick-borne encephalitis virus (TBEV) strains that induced different forms of infection, from inapparent to severe focal ones resulting in fatal outcome. Hemagglutinating activity of Oshima-like strains was higher at pH 5.8, while activity of Sofjin- and Senhzang-like strains were higher at pH 6.2 and 6.8, respectively. We determined susceptibility of porcine kidney (PK) cell cultures to these TBEV strains by cytopathic effect (CPE), plaque formation, and size of plaques. The clinical TBEV strains had higher virus titers both in tissue culture infectious dose 50(TCID50) and in plaque-forming unit (PFU) titers and larger plaques than the inapparent strains. A comparison of virus multiplication kinetics by PFU in culture fluid with kinetics of ELISA antigen and hemagglutinin accumulation suggested a different mechanism of interaction between these virus strains and PK cells at the initial stage of cell infection.

1. Introduction

Tick-borne encephalitis (TBE) is the most widespread neurovirus in Eurasia. Its niduses are found in many countries of Western, Central, Eastern, and, partly, Northern Europe, in many Russian regions, northern provinces of China and Mongolia, and on Hokkaido Island in Japan (Suss et al., 2004; Takashima et al., 1997; Zhang et al., 2012). TBEV is a member of the genus Flavivirus of family Flaviviridae and one of several members of the TBE group of flaviviruses. International Classification of flaviviruses divides TBEV into Far-Eastern, Siberian, and European subtypes (King et al., 2012). TBEVs in the Far East are believed to cause more severe forms of disease than those in other Eurasian territories (Borisov et al., 2000; Leonova, 2009). Predominant cases of TBE in the Far East were reported to be severe focal forms of the disease with high mortality. The Far-Eastern TBE is characterized by rapid symptomatology of overall lesions in the central nervous system (CNS) resulting in focal or diffuse meningoencephalitis involving the stem and spinal cord structures. Some researchers relate these manifestations of infection with a predominant circulation of the Far-Eastern TBEV subtype in the territory (Borisov et al., 2000; Zlobin et al., 2010). Until the 1990s, the mortality sometimes reached 30% (Leonova, 1997). However, since 1990, the ratio of non-focal TBE forms increased according to the reports of clinical manifestations of infection in the Far East (Leonova, 2009). For 1991–2000, focal forms (paralytic form with central nervous system involvement) of TBE averaged 44.1% and the non-focal (feverish) form 55.9%. The TBE mortality in the region in 2000 averaged 13.2%. The dead patients were not vaccinated against TBE. Clinical data indicates that the disease course of TBE in the Urals, Siberia, and parts of Europe part is milder than that in the Far East (Suss et al., 2004; Borisov et al., 2000; Iastrebov, 2007). At the same time, some researchers report that each TBEV subtype can cause the whole gamut of clinical manifestations of infection (Leonova, 2010; Pogodina et al., 1992). Additionally, each TBEV subtype is molecularly divided into genetically different lines or clusters reflecting heterogeneity of the virus population (Belikov et al., 2007; Ecker et al., 1999). Thus far, we have focused on the polymorphous nature of the TBE virus population, although nosology of TBE seems homogeneous (Belikov et al., 2007, 2010; Leonova,

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http://dx.doi.org/10.1016/j.virusres.2014.04.004
0168-1702/

Table 1
List of 40 primers used for amplification and sequencing of complete genomes.

Designation[a]	Sequence (5′ → 3′)	Position[b]
1F	AGATTTTCTTGCACGTGCGTGCG	1–23
500F	CCGTGTTGAAGTCTTTCTGGAA	301–322
500R	GTGAGTCATCACACCATGATCC	615–594
1000F	CTGGCTCCGGTTTATGCCTCAC	954–975
1000R	ACGCATCCTCCCAGTTCCAG	1063–1044
1500F	CCTGTGTCAAGGCGTCTTGTG	1315–1335
1500R	ACTCCAGTCTGGTCTCCAAGG	1759–1739
2000F	GCGTTCTCTGGGACCAAACCC	1962–1983
2000R	TTGGGTGTTATCAACATGGCCAC	2052–2031
2500F	ATGAGGAATCCGACCATGTCC	2382–2402
2500R	AGTTCCCTCCTCGAAGGTCTC	2621–2601
3000F	GGACTTCAGACAGGAATCAAC	2966–2986
3000R	GGTCTGTGTGGACTGCCATGCC	3051–3030
3500F	TGAAAGGACCATGGAAGTACTC	3253–3274
3500R	TTCCAGTGGCTGGCCTCCTC	3612–3593
4000F	GGTGTTGGACTTCTGCTCATGG	3969–3990
4000R	CAACATGAGGCCACGCCC	4063–4046
4500F	CATTCAGTGAACCACTGACTG	4207–4227
4500R	CGTCCACAATCCCATCACAC	4568–4549
5000F	GAACTGCTCCTGGACACAGGT	4947–4967
5000R	ACCACTCCCTGGGAGTTGAGG	5044–5024
6000F	CACAGGGACCAGACGTGTGAC	5936–5956
6000R	TCATCATCACACTGTCCTGAG	6046–6026
6500F	CGTGTCTAGTGTGACAAGTCG	6263–6283
6500R	CTGCCAGGCTCCTCATGCATC	6571–6551
7000F	CGGTGTTGTGGAGTGAACATG	6952–6972
7000R	TGGTGTGAACAGGGAGACCAC	7067–7047
7500F	AAGTCTTCTTCTCGGCAATGG	7321–7341
7500R	CACAGGCCACTGGCATCGTC	7578–7559
8000F	ATGTGGAAGAGGCGGCTGGTC	7907–7927
8000R	GCTCTGAACTTGATCAGGTTC	8047–8027
8500F	GCGGAGGACTGGTGAGGAC	8275–8294
8500R	AGTACTGCCAGGTCCTGTACG	8583–8563
9000F	TGAGCAGAACAGGTGGTCAAG	8909–8929
9000R	GTACCATATGGCCCGGCTTCC	9095–9075
9500F	AGGGTGAACACAAGCAACTGG	9340–9361
9500R	TCCGAGACGTTCTTCTCCATG	9615–9635
10000F	TCCGTGAAACTGCCTGCCTTTC	9910–9930
10000R	GTCCTCTGTGGTCATCCAGGC	10,091–10,071
11000R	GCGGGTGTTTTTCCGAGTCAC	10,893–10,873

[a] F indicate a viral-sense orientation, with R indicate a reverse orientation.
[b] Genome positions are given according to the published sequence of strain Sofjin-HO.

2010). To date, there have been no comparative studies on biological and molecular genetic characterizations of a large group of strains inducing inapparent to severe focal TBE forms. In this regard, based on the comparative studies of biological and molecular genetic properties of TBEV strains inducing different infectious courses, from asymptomatic to fatal outcome, in patients living in the Far East, we aimed to identify the differences in replication characteristics of these strains.

2. Materials and methods

2.1. Materials

We studied the properties of 33 TBEV strains that caused severe focal (Group 1, $n = 10$), febrile (Group 2, $n = 4$), and inapparent (Group 3, $n = 19$) forms of the infection (Table 1).

2.2. Virology methods

All these strains were isolated by intracerebral injection in 2-day old inbred mice. To isolate the strains, we used autopsy material (brain) of dead patients. Brain particles were homogenized in 10% suspension in medium-199 ("Biolot", St. Petersburg, Russia) that contained 100 μg/ml gentamicin, centrifuged at 4000 rpm for 15 min. The blood of sick people came to the laboratory of the hospital. To isolate the virus from infected patients after being bitten by ticks, we also used fresh blood from a vein. We made daily observations of clinical symptoms of disease in animals for 14 days after inoculation. If the mice showed clinical symptoms (tremor, convulsions, paralysis), the brain of mice was included in the passage. In this study, we took the strains of passages III–IX in mice. To determine the titer of TBE virus strains were prepared 10% of infected mice brain suspension in medium-199 and centrifuged it at 4000 rpm for 20 min. Then 10-fold dilutions of suspensions were prepared from 10^{-1} to 10^{-10} in medium 199, and 0.03 ml of each dilution were infected in the brain of white mice (4 animals 3–4 weeks of age). Observation of the animals was carried out for 3 weeks. Titer was considered that the dilution of virus that causes 50% mortality in infected mice (lg LD50). Titer calculation performed by Reed and Muench (1938).

Mouse experiments were conducted in a vivarium at the Research Institute of Epidemiology and Microbiology according to the USSR Ministry of Public Health Regulation No. 1189 of 10.10.1983. Experiments were performed according to the USSR Ministry of Public Health Regulations No. 755 of 12.09.1977 and No. 701 of 27.07.1978 for the humane treatment of animals.

We studied hemagglutinating activity (HA) of TBEV strains in a hemagglutination-inhibition test (HIT) at different pH values (5.4, 6.2, and 6.8) of buffer solutions (Clarke and Casals, 1958). For this purpose, we prepared antigens from 10% brain suspension of infected mice in borate buffer, followed by purification with protamine sulfate (Serva). The protamine sulfate solution was prepared at the concentration of 25 mg/ml in saline. The 1/10 volume of the protamine solution was added to the antigen. It was periodically shaken for 20 min. Then, the samples were centrifuged at 4000 *g* for 15 min. HIT was performed using a micro-method using 0.4% goose erythrocyte suspension, prepared in a phosphate buffer pH 5.4, 6.2, 6.8. Goose erythrocyte suspension with pH 6.2. used for study the HA in cell culture fluid PK, infected different strains of TBEV.

We determined the susceptibility of porcine kidney (PK) cell cultures to the regional TBEV strains based on CPE, PFU, and size of plaques. An overnight monolayer of PK cells grown on 24-well plates was injected with tenfold dilutions of the virus. After 1 h incubation at 37 °C, the inoculated monolayer was washed with the culture medium-199 followed by addition of cell-maintenance medium-199 with gentamicin and 1% fetal bovine serum (FBS) and then incubated. The cytopathic effect of the strains was observed for 5–7 days.

To study the virus multiplication in PK cell culture as measured by PFU and plaque size, we used 0.6% carboxymethyl cellulose solution (ICN Biomedicals Inc.) in medium-199 supplemented with 1% FBS as the cell maintenance medium. On days 5–6 of incubation, after staining with Amido black 10B, counting of the plaques (number and plaque size) was carried out. All titers of TBEV strains are shown in Table 2 at the rate of 1 mL.

We studied the virus growth of different TBEV strains in PK cell culture. The overnight cell monolayers grown in vitro were injected with each strain; the TBEV infecting dose was 2–3 log of tissue cytopathic dose ($TCID_{50}$). Experimental samples were collected after 1, 3, 6, 12, and 24 h and on days 2, 3, 4, 5, and 6. Afterwards, we carried out simultaneous studies of samples with culture fluid on the virus growth by titration of the virus according to PFU, HA, and viral coefficient (K-factor) reflecting the accumulation of specific antigen.

We determined TBEV antigen in ELISA using the immune-enzyme test system "Vecto-TBE-antigen-strip" (JSC Vector-Best, Novosibirsk, Russia). The results were recorded by optical density (OD) in the spectrophotometer at 450 nm. To interpret the results, according to the manufacturer's instructions, we introduced a threshold value (a critical OD value–$OD_{critical}$): the mean OD

Please cite this article in press as: Leonova, G.N., et al., The nature of replication of tick-borne encephalitis virus strains isolated from residents of the Russian Far East with inapparent and clinical forms of infection. Virus Res. (2014), http://dx.doi.org/10.1016/j.virusres.2014.04.004