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MOLECULAR HYBRIDIZATION WITH CLONED FRAGMENTS OF TICK-BORNE ENCEPHALITIS (TBE) VIRUS cDNA IN ACUTE AND CHRONIC TBE INFECTION

V. V. POGODINA, T. V. FROLOVA, M. P. FROLOVA, S. G. SOBOLEV, V. A. SHAMANIN, A. G. PLETNEV

Institute of Poliomyelitis and Virus Encephalitides, Academy of Med. Sciences of U.S.S.R., Moscow, 142782; Institute of Bioorganic Chemistry, Siberian Branch of the Academy of Sciences of the U.S.S.R., Novosibirsk 630090, U.S.S.R.

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Summary. - Recombinant plasmid DNA was used as a probe to detect tick-borne encephalitis (TBE) virus RNA during incubation period, acute disease and persistent infection of syrian hamsters. Within the first three weeks post-infection the results of direct virus isolation and RNA detection in the brain agreed by a rate of 100 %, the virus titre ranging between $10^{1.9}$ to $10^{10.5}$ LD_{50}/ml and viral RNA concentration at 1-1000 pg. At the same time TBE virus RNA was detected in the spleen when the virus titre was $\geqq 10^{6.5}$ LD_{50}/ml. By 8 months post infection (p. i.) viral RNA was found in the brain, liver, and spleen in the absence of infectious TBE virus. No viral RNA was present in the thymus. In addition, electron microscopic findings in hamster brain confirmed the hypothesis that TBE virus persistence was accompanied by formation of virus-specific structures but impaired virion maturation.

Key words: tick-borne encephalitis virus; TBE virus RNA; cDNA/RNA hybridization; viral persistence

Introduction

It has been previously shown that TBE virus RNA can be detected using cloned DNAs as probes in biologic prepared samples from ticks, human blood, and animal organs (Pletnev *et al.*, 1985). In the present investigation we applied this method at early stages of infection and during viral persistence in Syrian hamsters, which were previously shown to represent a suitable model of nonacute TBE virus infection (Frolova *et al.*, 1982; Pogodina *et al.*, 1984; 1986).

Materials and Methods

Animals. Four to five-week-old Syrian hamsters were inoculated intracerebrally (0.03 ml) or subcutaneously (0.25 ml) with TBE virus in a dose of $10^{5.5}$ - 10^{6} LD_{50} to reproduce acute lethal, subacute, or chronic encephalitis, and/or asymptomatic infection; the latter animals were followed upto 8 months.

Virus. TBE virus strains belonging to two serotypes, the Eastern (Far Eastern) and Siberian (Aina/1448) were used. The strains were isolated from the brain of the dead patient (Sofjin), from a chronic TBE patient (No.41/65), from a patient with the mitigated form of disease (Vasilchenko), as well as from *Ixodes persulcatus* ticks (No.928). The properties of the strains and procedures of isolation have been described previously (Kraminskaya *et al.*, 1972; Malenko *et al.*, 1982; Pogodina *et al.*, 1986). The virus was maintained by intracerebral serial passages in white mice weighing 6-7 g.

Virus infectivity titration. The virus was titrated by inoculating 10-fold dilutions of virus-containing material into the brain of white mice (weighing 5-6 g) or in PEK cells. At the late stage the presence of viral antigens was determined by complement fixation and haemagglutination test (CFT, HAT) directly in the organ homogenates as well as in the culture fluid of tissue explants on days 10-15 in culture. To isolate the virus from explants the culture fluid was passaged in white suckling mice as described previously (Levina and Pogodina, 1981; Malenko *et al.*, 1982; Pogodina *et al.*, 1986).

Morphological examination. Hamster brain and spinal cord specimens were fixed in 10 % formalin, paraffin-embedded, cut and stained with haematoxylin and eosin or cresyl violet (Nissl). For each term and each strain the specimens were obtained from 3-5 animals. Electron microscopic examinations were carried out as reported previously (Roikhel *et al.*, 1983).

TBE virus RNA was determined by hybridization on nitrocellulose filters (NCF). ^{32}P-cDNAs from recombinant plasmids pBR-TBEV1, pBR-TBEV7, pBR-TBEV10, pBR-TBEV15 were used as probes. These plasmids contained non-interlacing cDNA copies of TBE virus RNA portions which cover about 50 % of the genome. Restriction maps and nucleotide sequences of the DNA of these plasmids have been described previously (Yamschikov *et al.*, 1989). The cDNAs were isolated as described (Maniatis *et al.*, 1984) by centrifugation in CsCl and ethidium bromide gradients, labelled with α ^{32}P-TTP and α ^{32}P-dCTP (37 TBg/mmol) by nick-translation to a specific activity of 1 - 4 x 10^{8} cpm^{-1} μg^{-1} DNA (Maniatis *et al.*, 1984). *E. coli* DNA polymerase was obtained through the courtesy of Dr. A. G. Romaschenko (Institute of Cytology and Genetics, the Siberian Branch of the AS U.S.S.R.). Total RNA was phenol-extracted (60 ^{0}C, pH 5.2) from tissue samples in the presence of 0.5 % (mass/volume) sodium dodecylsulphate (Sherrer, 1972). Then the RNA was sedimented with ethanol, dissolved in distilled water and the nucleic acid concentration was measured (1 R. U. D_{260}^{1cm}= 40 μg RNA). The RNA dissolved in 10 x SSC was put on nitrocellulose filters (1 μg RNA/per point). To evaluate the sensitivity of the method and the level of the intensity of positive signal, the following standards were put on the filters: pBR-TBEV unlabelled DNA (1000, 100, 10, 1 pg) and the total RNA from uninfected hamster tissues. The NCF with specimens were dried under vacuum at 80 ^{0}C for 2 hr.

Hybridization procedure was carried out as described previously (Dobrikova *et al.*, 1986). The filters were preincubated at 65 ^{0}C for 1-2 hr in the hybridization mixture of following composition: 6 x SSC (1 x SSC = 0.15 mol/l NaCl - 0.015 mol/l Na-citrate, pH 7.0), 0.04 % (mass/volume) Ficoll 400, 0.04 % (mass-volume) polyvinylpyrrolidon, 0.04 % (mass/volume) bovine serum albumin, 0.5 % (mass/volume) DSC, 100 μg/ml poly C, 220 μg/ml yeast RNA. After prehybridization, ^{32}P-cDNA from pBR-TBEV (100 ng/ml) was added to the solution and incubated at 65 ^{0}C for 12 - 18 hr. Thereafter the filters were washed 3 times at 55 ^{0}C for 30 min in lx SSC solution, dried and exposed with X-ray film PM-1 for 4 - 48 hr.

The results were evaluated visually comparing the intensity of signals registered for the samples tested and for standards (known quantities of unlabelled DNA pBR-TBEV15 put on the NCF and hybridized with ^{32}P-cDNA pBR-TBEV15 under the same conditions). Hybridization was defined negative (-) when the intensity of signal was less than 1 pg DNA pBR-TBEV15; doubtful (±) when

it ranged from 1 to 10 pg DNA pBR-TBEV15; positive (+) when it was equal or exceeded 10 pg DNA pBR-TBEV15.

Results

Detection of TBE virus RNA in the course of infection

At early intervals (1 - 7, 14, and 21 days) and late stages (8 months) post-infection (p. i.) detection of infectious virus and viral antigen were carried out in 122 organ specimens and of viral RNA in 92 specimens (Table 1). Coincident results were obtained in the hamster brain independently of the TBE virus strain used and of the route of inoculation (intracerebral, subcutaneous). During the first 3 weeks the results of virus isolation and viral RNA detection coincided at 100 % rate (Table 2), similar correlation was noted between the virus titre in the brain tissue and the amount of viral RNA detected (Table 3). Eight months after subcutaneous infection with the Vasilchenko strain 6 hamsters were examined individually. All the animal exhibited a rather low titre of viral antigen: 1 : 8 - 1 : 16 in CFT, 1 : 20 - 1 : 160 in HAT. Five of 6 animals had viral RNA in their brain at a concentration of 1, 10 pg/1 μg total RNA (2 hamsters) and 10 pg (3 biospecimens). In none of the cases the infectious virus could be isolated from the brain. The negative results of infectious virus isolation were confirmed morphologically. The brain of suckling mice

Table 1. Comparative results of TBEV indication and viral RNA detection in the course of the infectious process

Organ	Time points and results of studies*						
	days 1 - 7		days 14 - 21		8 months		
	virus	RNA	virus	RNA	virus	antigen**	RNA
Brain	25/25	25/25	8/8	8/8	0/6	6/6	5/6
Spleen	24/25	6/25	4/6	1/6	0/6	3/6	2/6
Thymus	12/30	n.t.***	0/2	0/2	0/6	0/6	0/6
Liver	n.t.	n.t.	2/2	2/2	0/6	4/5	5/6

* the numerator designates the number of positive results, the denominator - the total number of samples tested;

** at 8 months the viral antigen was detected directly on organ homogenates and in the culture fluid of explants by CFT, HAT, and immunofluorescence assay (Frolova *et al.*, 1982);

*** not tested

Table 2. TBE virus isolation and detection of viral RNA in the brain of infected hamsters

Route of inoculation, TBE virus strains*	Time after inoculation, days	Results **		
		TBE virus isolation	TBE virus RNA detection	RNA concentration
Intracerebral	1	3/3	3/3	±, +
Sofjin strain	2	3/3	3/3	+
No. 41/65	3	3/3	3/3	+
No. 928	4	3/3	3/3	+
	5	3/3	3/3	+
	6	4/4	4/4	+, ++
	7	3/3	3/3	++
Subcutaneous	7	3/3	3/3	+, ++
Sofjin strain	14	3/3	3/3	±, +
No. 41/65	21	3/3	3/3	+
No. 928				
Subcutaneous				
Vasilchenko strain	14	2/3	2/2	+
	8 months	0/6	5/6	±, +

* At each interval (1–5, 7, 14, 21 days p. i.) brain samples from 18 hamsters collected in 3 specimens were examined (each of them included 6 brain samples from hamsters infected with No. 41/65, 928, and Sofjin strains). At 6 day p. i. 24 brain samples were examined (4 biospecimens were prepared: one for each of 41/65 and 928 strains, and 2 for Sofjin strain; each of them had brain samples derived from 6 hamsters).

** The numerator designates the number of positive specimens exibiting the presence of viral RNA. The denominator – the total number of specimens examined. TBE virus RNA concentration is shown according to the conventional system: (–) for the intensity of signal lower than 1 pg of pBR-TBE virus 15 DNA; (±) for the intensity of signal ranging from 1 to 10 pg pBR-TBE virus 15 DNA; (+, ++) for the intensity of signal equal or higher than 10 pg pBR-TBE virus 15 DNA. See also Fig. 1.

used for blind passages of samples from the culture fluid of explants revealed no pathological changes typical for TBE.

At early stages of infection the virus was constantly isolated from hamster spleens (24 out of 25 samples) in a titre of $10^{3.5}$ – $10^{7.3}$ LD_{50}/ml. Viral RNA was detected in 6 out of 25 specimens. On days 14 and 21 viral RNA was detected in 1 out of 6 spleen samples taken for each term. As a rule, viral RNA could be detected if the TBE virus titre in the spleen was $10^{6.5}$ LD_{50}/ml or higher. TBEV RNA concentration in these samples was not over 10 pg. Eight months after infection viral antigens were detected in 3 out of 6, and viral RNA in 2 of 6 spleen samples, the infectious virus was isolated in none of the cases. The concentration of viral RNA in the spleen at this term ranged from 10 to 100 pg/

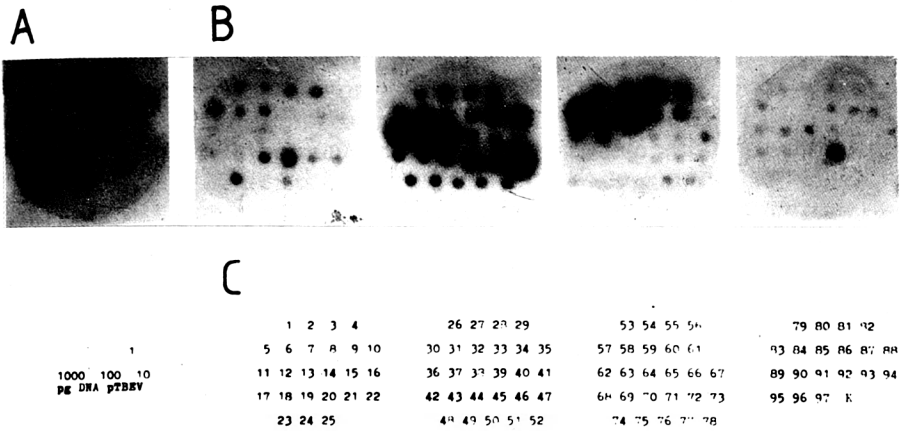

Fig. 1

TBE virus RNA detection by hybridization in the tissues of hamsters infected by i. c. and s. c. routes

As standards, unlabelled DNA of pBR-TBEV in the amount of 1000, 100, 10, and 1 pg was dotted on a filter "A". For negative control, RNA derived from uninfected hamsters spleen (point 97) was dotted on filter "B". Samples: hamster tissue extracts, Vasilchenko strain, subcutaneous inoculation: liver - 8 months (points 1-6), 14 days (point 7); thymus - 8 months (points 8-13); 14 days (point 14); spleen - 8 months (points 15-20), 14 days (points 21,22); brain - 8 months (points 23-28), 14 days (points 29,30).

Hamster brain extracts on days 1-7 after i. c. inoculation with Sofjin strain: points 31-37; with No. 41/65 strain: points 41-47; with No. 928 strain: points 51-58. Hamster brain extracts on days 7, 14, 21 after s. c. inoculation with Sofjin strain: points 38-40; with No. 41/65 strain: points 48-50: with No. 928 strain: points 59-61.

Hamster spleen, on days 1-7 after i. c. inoculation with Sofjin strain: points 64-70; with No. 41-65 strain: points 74-80; with No. 928 strain: points 84-91. Hamster spleen, on days 7, 14, 21 after s.c. inoculation with Sofjin strain: points 71-73; with No. 41/65 strain: points 81-83; with No. 928 strain: points 92-94. Points 62, 63, 95, 96 K are unrelated to the given experiment.

1μg of cellular RNA, i. e. it was higher than in the spleen at early stages of infection and in the brain by 8 months later.

TBE virus isolation was not always successful from the thymus of i. c. infected hamsters at early stages of infection (12 out of 30 samples); on days 3 - 7 the virus titre was $10^{4.1}$ - $10^{6.1}$ CPE_{50}/ml. On days 14 and 21 after i. c. inoculation with Sofjin strains No. 41/65 and No. 928, and also on day 14 and 8 months after s. c. inoculation with Vasilchenko strain none of thymic tissue specimens revealed infectious virus, viral antigen, or viral RNA (Table 1). Liver specimens of the same hamsters contained viral RNA (the number of positive samples on day 14 was 2 out of 2, by 8 months it was 5 out of 6). In half of the cases viral RNA concentration was higher than 10 pg. At the stage of virus persistence (8

months) viral RNA and viral antigens were found in the liver, brain, and spleen, whereas no infectious virus was isolated. Viral RNA was detected in individual organs at the 8 month pattern, i. e. in some animals in brain and liver samples while in others in brain, liver, and spleen.

The course of viral RNA accumulation in organs depends on the properties of the TBE virus strain. Daily comparative examination of hamsters for 7 days after i.c. inoculation showed that the highest rate of accumulation had the RNA of Sofjin strain. After infection with this strain the concentration of viral RNA in the brain on days 4 - 5 exceeded 10 pg (Fig. 1, points No. 34, 35), on day 6 it reached 100 pg (point No. 36), on day 7 1 ng (point No. 37) for 1μg of total cellular RNA. After inoculation of No. 928 strain in the same infectious dose, viral RNA accumulated in the indicated amount about 2 days later (points No. 55, 56) and its highest concentration of 1 ng was not reached during the observation period. Spleen samples from Sofjin strain-infected hamsters revealed viral RNA on days 3 - 4 p. i., whereas in the case of No. 928 strain - on days 6 - 7. These data correlate with a different course of viral reproduction in brain and spleen samples, the rates being higher for Sofjin strain as compared to No. 41/65 and for No. 928 strains. The accumulation of Sofjin strain in the thymus was at most on days 3 - 5, of No. 41/65 and No. 928 strains on days 5 - 6. Viraemia following infection with Sofjin and No. 928 strains lasted for 7 days reaching the peak on days 2 - 5 (the virus titre $10^{3.8}$ - $10^{7.0}$ LD_{50}/ml). Strain No. 41/65 induced 2-wave viraemia, its first peak being on day 2 and the second one on day 6 p. i.

Histological and electron microscopic examinations

Early stages of CNS infection can be characterized as acute meningoencephalomyelitis with inflammatory degenerativge changes manifested to a different degree. On days 50 - 70 in apparently healthy animals inoculated s. c. with Vasilchenko strain the examination of CNS showed dystrophic changes of neurons, occasional foci of neuron destruction, and astrocyte proliferation. Later on some animal showed residual lesions such as foci of neuron destruc-

Table 3. Correlation between the value of TBE virus infectious titre and the amount of viral RNA detected in hamster brain (on days 1 - 21 of infection)

The amount of the TBE virus RNA, pg	Number of experiments	TBE virus titre (log LD_{50}/ml)	
		variations	M ± m, p= 95 %
> 1 < 10 (±)	2	3.75; 5.02	4.38 ± 4.82
> 10 < 100 (+)	22	1.85 - 10.02	6.44 ± 0.98
> 100 < 1000 (++)	7	8.02 - 10.52	9.13 ± 0.9

tion, astrocytic glial proliferation in g. hippocampi, cerebellum and anterior horns of spinal cord.

Electron microscopy revealed morphologic changes in the CNS and of TBE virus virions characteristic for persistent infection (Figs. 2 - 3). In contrast, acute periods of infection, on days 50 - 90 mature virus particles were detected extremely infrequently. Forty-two specimens of cerebral cortex, cerebellum, subcortical ganglia, medulla oblongata, i. e. of those parts of CNS where persisting TBE virus is usually present (Malenko *et al.*, 1982; Fokina *et al.*, 1982) were examined. Typical virions resembling mature particles could be seen only in 1 case in the channels of granular endoplasmic reticulum (ER) of a neuron in the brain stem examined on day 70 p. i. with the Vasilchenko strain.

In other cases particles of 50 - 60 nm in size resembling virus particles were found, however, their bounderies were illegible, and the majority of them had a ring-like form of varying diameter (30 - 80 nm). Ultrastructural changes in cells related to virion formation were detected such as aggregation of „transformed" ER membranes free of ribosomes situated in parallel, smooth membrane-bound vesicles in the lumens of granular ER, aggregation of smooth-contoured membranes resembling protein crystals in the cytoplasm; associated ribosomes located in the hyaloplasm or on membranes of the channels of ER were larger in size (35 - 40 nm) and more electron-dense. All these changes could be considered for morphological manifestations of the production of virus-coded components required for virion formation.

Discussion

Plasmid cDNA probes were used to demonstrate the TBE virus RNA in organs of infected hamsters at late stages post-infection, in agreement with our previous conclusion on the regular development of persistent TBE infection (Pogodina *et al.*, 1986). The localization of viral RNA varied: in some cases the viral genome was present in the brain and liver, in the others in brain, spleen, and other organs except of thymus. These data confirm the neuro-and lymphotropism of the persisting TBE virus. However, TBE virus has been reported to persist for 46 days also in the thymus of BALB/c mice (Vargin and Semenov, 1985). In our hamsters neither the virus, nor the viral RNA or viral antigens were detected in thymic tissues during the 8-months persistence.

CNS, liver and spleen of hamsters were shown to synthesize viral antigens, viral RNA was detected but no infectious virus was found even when highly sensitive isolation procedures (tissue explantation) were used. The concentra-

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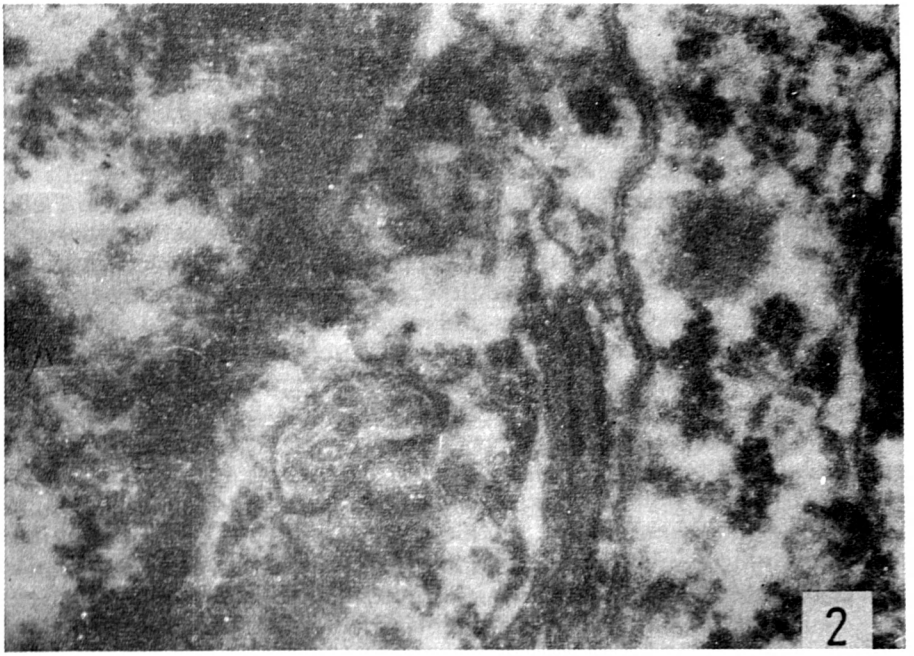
Fig. 2. Hamster cerebellum 50 days after s. c. inoculation with TBE virus. Transformed ribosome-free membranes of GEN in the cellgranule, magn. x 54 000.

Fig. 3. Hamster brain stem 7 days after s. c. inoculation with TBE virus. The aggregation of smooth contour membrane formations in the neuron cytoplasm, magn. x 36 000.



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tion of TBE virus RNA reached 10 pg by 8 months (about 2 x 10^6 TBE virus RNA molecules/1 μg cellular RNA), and in some cases its level was significantly higher in the spleen. These data agree with the results of electron microscopic examination and on the whole they indicate that in the course of persistence virus-specific components necessary for the formation of mature virions are produced in the brain and internal organs, however, the maturation of viral particles is hampered. A similar picture of ultrastructural changes was discovered during chronic TBE in monkeys (Tulakina and Erman, 1983). The data obtained on the production of virus-specific components shed more light on the relative readiness of persisting TBE virus activation under the influence, for example, of some antibiotics or stress-inducing factors (Frolova *et al.*, 1982; Frolova and Pogodina, 1984; Malenko *et al.*, 1984). The activation occurs, as a rule, in the background of immunosuppression (Pogodina *et al.*, 1986). Apparently, there is an immunologically-mediated mechanism of eliminating the inhibition of virion maturation.

At early stages of infection (the first 3 weeks) viral RNA was constantly detected in cerebral tissues, but not regularly in the spleen despite the presence of the infectious virus. The possible reason for discordant results of virus isolation and viral RNA detection in the spleen are as follows: a) the lower concentration of TBE virus RNA as compared to the brain; b) the higher level of RNase. Model experiment (their results are not reported here) showed that after TBE RNA extraction from the spleen (as well as from blood or liver) TBE virus DNA may be deteriorated and at subsequent hybridization the signal becomes significantly weakened. Apparently, it is necessary to modify the extraction procedure to preserve the RNA. For this it appears worthwhile to apply guanidine chloride (Cheley and Anderson, 1984) or guanidine isotiocyanate (Maniatis *et al.*, 1984). It is likely that because of abundant RNases the samples should be stored shortly (in our experiments the samples were stored at - 70 ^{0}C for 6 months).

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