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PERSISTENCE OF TICK-BORNE ENCEPHALITIS VIRUS IN MONKEYS. IV. VIRUS LOCALIZATION AFTER INTRACEREBRAL INOCULATION

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Summary. — Tick-borne encephalitis (TBE) virus was isolated from the brains and spinal cords, blood, livers, lymph nodes and kidneys from *Macaca rhesus* monkeys showing acute and subacute fatal encephalitis. In subacute encephalitis, virus titres in the CNS were lower than in acute disease (3.0—6.2 against 3.8—8.3 log LD_{50}/ml). TBE virus localization in chronic encephalitis was largely the same as in acute and subacute disease. In monkeys with a chronic course and stable paralysis of the upper extremity, infectious TBE virus was isolated on day 383 from subcortical ganglia and spinal cord. In lymph nodes and spleen, it could be detected only by a combination of methods (co-cultivation in association with fluorescent antibody technique and complement-fixation test, explantation of organ fragments) more sensitive than is the inoculation of mice with organ homogenates. TBE virus was detected by the same methods on day 90 in the CNS and internal organs of a monkey with chronic encephalitis in the stage of remission.

Key words: flavivirus; tick-borne encephalitis; monkeys; persistence

Introduction

Ilienko *et al.* (1974*a*) studied the pathogenicity for *Macaca rhesus* monkeys of 113 strains of tick-borne encephalitis (TBE) virus by intracerebral (i.c.) inoculation and selected those capable of producing a chronic disease. These authors, however, isolated the virus only by i.c. inoculation of mice with brain tissue homogenates without virological examination of the internal organs. Asher (1979) examined by the same method not only the brain and cerebellum but also the cerebrospinal fluid and spleen of *M. rhesus* monkeys inoculated with the Vasilchenko strain of TBE virus. He found no virus in the spleen and cerebrospinal fluid.

We examined both the CNS and internal organs of monkeys infected with (1) TBE virus strains capable of producing chronic disease in monkeys

Table 1. Virus localization in monkeys with acute and subacute fatal encephalitis

Organ	Titres (log LD_{50}/ml) at intervals* of								
	7 days	10—12 days				15—16 days			21 days
	41/65	41/65	Aina/ /1448	Pan-114 No. 1 M	Pan-114 No. 1 MAPH	Vasil-chenko	Pan-114 No. 1 MAPH	Pan-114 No. 1 M	Pan-114 No. 1 M
Brain:									
Cerebral cortex	6.5	5.5	5.5	4.5	7.8	4.3	6.2	3.5	5.2
Subcortical ganglia	7.0	5.3	6.2	4.3	6.3	0	4.5	5.0	0
Cerebellum	6.3	4.5	3.8	6.8	8.5	0	6.0	3.7	4.7
Stem	4.7	7.0	5.2	5.5	6.8	3.9	5.5	4.3	4.5
Spinal cord									
Cervical	4.4	5.1	5.5	0	6.1	4.2	4.3	4.3	0
Thoracic	4.8	7.0	6.5	4.3	5.7	3.0	5.3	0	0
Lumbar	4.7	5.3	4.2	5.0	7.1	4.1	5.8	0	3.7
Spleen	0	0	0	0	0	0	0	0	0
Liver	2.5	3.5	0	0	0	3.7	0	0	0
Lymph nodes	0	0	0	0	0	2.8	3.9	0	0
Kidney	0	3.0	0	0	0	3.0	0	0	0
Intestine	n. d.	n. d.	n. d.	3.7	0	n. d.	0	0	0
Blood	5.8	3.8	2.5	2.7	2.8	n. d.	2.5	3.5	0

* Intervals: 10—12 days — acute encephalitis; 15—21 days — subacute encephalitis.
n. d. — not done.

after i.c. inoculation or isolated from humans with chronic TBE and (2) mutants showing various degrees of attenuation. We used a combination of sensitive methods for the detection of persisting TBE virus with altered properties or of the virus-specific antigen at late post-inoculation (p.i.) intervals. These methods proved to be of value in detection of latent viruses (Shope *et al.*, 1972; Gavrilov *et al.*, 1974; Rajčáni *et al.*, 1977; Zuev, 1979).

Materials and Methods

Twenty-six *M. rhesus* monkeys were inoculated with TBE virus strains Vasilchenko (6 monkeys), Aina/1448 (3), 41/65 (3), mutants Pan-114 No. 1 M APH (8), and Pan-114 No. 1 M (6); 1 ml of virus suspension (10^4—$10^{6.8}$ LD_{50}) was administered into the thalamus. The virus strains were described by Pogodina *et al.*, (1981*a*).

For virological examinations we collected brain (cerebral cortex: anterior central gyrus, subcortical ganglia, cerebellum; brain stem: mesencephalon and medulla oblongata), spinal cord, spleen, liver, kidneys, inguinal lymph nodes, small intestine, and blood. In the first 45 days, virus was isolated, in general, by i.c. inoculation of 8—10 random-bred mice aged 7—10 days with 10% organ homogenates. Titrations were performed in similar mice using 4 animals per each 10-fold dilution of the virus-containing material. At later intervals, virus was assayed by the same method, by the fluorescent antibody (FA) technique and co-cultivation of trypsinized organ cells with indicator pig embryo kidney (SPEV) cells in 4 modifications [without activators, with the addition of 3% dimethyl sulphoxide, DEAE-dextran, or 5-bromo-deoxyuridine (BUDR)], and the method of organ explants. The FA technique was used to examine both organ impression

Table 2. TBE virus localization in monkeys with chronic encephalitis

Monkey No., strain, time p. i.	Organs	Results of examinations			
		Homogenates	FA technique (impressions)	Co-cultivated cells	Explants
No. 9099	Cerebral cortex	0	0	n. d.	n. d.
Vasilchenko,	Subcortical ganglia	3.2	+	+	n. d.
383 days[1]-	Cerebellum	0	+	0	0
	Spinal cord	5.0	+	+	n. d.
	Spleen	0	+	+	+
	Liver	0	0	0	0
	Kidney	0	0	0	0
	Lymph node	0	+	+	0
No. 8910,	Cerebral cortex	0	+	+	n. d.
Vasilchenko,	Subcortical ganglia	0	+	+	+
90 days[2]-	Cerebellum	0	+	+	+
	Spinal cord	0	+	+	n. d.
	Spleen	0	+	n. d.	+
	Liver	0	+	n. d.	+
	Lymph node	0	+	n. d.	n. d.

1) At 9 days ataxia, at 22 days paralysis of the upper extremity, at 25 days hyperkinesia of masseter muscles, up to 383 days stable paralysis of the extremity.
2) Similar progressive development of the symptoms followed by recovery of motor functions. Histological examinations of the CNS revealed slowly progressing inflammatory process.
0 — negative results; + — positive results; n. d. — not done.

smears and co-cultivated organ-SPEV cells grown in the presence of BUDR. The above methods of virus assay and serological methods of TBE virus identification [complement-fixation, heamagglutination inhibition, diffusion and precipitation in agar (AGDP), neutralization test] have been already described (Pogodina *et al.*, 1981*b*).

Results

From 14 monkeys with acute fatal encephalitis, infectious TBE virus was isolated by i.c. inoculation of mice with homogenates of different parts of the brain and spinal cord in titres from $10^{4.3}$ — $10^{8.5}$ LD_{50}/ml, from the blood in titres from $10^{2.5}$ — $10^{5.8}$ LD_{50}/ml, in some cases from the liver and small intestine in titres from $10^{2.5}$ — $10^{3.5}$ LD_{50}/ml (Table 1).

Nine monkeys showing subacute fatal encephalitis with prolonged incubation period and protracted course yielded infectious virus from the brain and spinal cord, although the virus spread in the CNS was less diffuse and the titres were slightly lower than in acute encephalitis (10^3—$10^{6.2}$ LD_{50}/ml). Infectious virus was also detected in the blood, liver, kidney, and lymph nodes in titres from $10^{2.5}$—$10^{3.9}$ LD_{50}/ml.

In chronic encephalitis, infectious virus was isolated on day 45 from the blood, spleen, liver, kidneys and inguinal lymph nodes (in titres from $10^{3.7}$ to $10^{5.5}$ LD_{50}/ml) of one monkey and on day 383 from subcortical ganglia of the brain and from the spinal cord (in titres from $10^{3.2}$ — 10^5 LD_{50}/ml) of another one with signs of residual paralysis of the upper extremity. The same

Table 3. Detection and identification of persisting TBE virus in the spinal cord of monkey No. 9099, 383 days after i.c. inoculation with the Vasilchenko strain

Mouse passage No.	Virulence for mice			Plaque formation PFU/ml	CPE in SPEV culture, log TCD_{50}/ml	Identification
	Morbidity[1])	Incubation period, days	Virus titre			
0	11/20	4	i. c., 5	n. d.	n. d.	FA technique
1	15/20	8	n. d.	n. d.	n. d.	FA technique
2	17/21	5	i. c., 8.9 s. c., 6.6 i. p., 6.6	n. d.	n. d.	FA technique
3	39/50	4—5	i. c., 9.2	CEC: 2.7×10^9 SPEV: 1.5×10^{10}	5.6	Neutralization test in mice, log NI 3.6 Neutralization test in CEC culture, plaque reduction, log NI 5

[1]r) Numerator: No. of sick and dying animals; denominator: No. of inoculated animals
[2]) Route of inoculation (i. c. — intracerebral; s. c. — subsutaneous; i. p. — intraperitoneal) and titre in log LD_{50}/ml values.
n. d. — Not done.

parts of the CNS showed specific fluorescence and the virus was isolated by the co-cultivation method. By the same methods the virus was detected in lymph nodes and the spleen. From the latter, the virus was recovered also by the explanation method. Virus-specific antigen was demonstrated in the cerebellum by the FA technique (Table 2). The properties of the virus isolated from the spinal cord of this monkey at 383 days were studied by inoculation of one-week-old mice with a brain tissue homogenate. The virus was highly virulent for mice by all the inoculation routes, showed marked cytopathic activity in SPEV cell culture and produced plaques 2.5—3 mm in

Table 4. Isolation of TBE virus from the liver of monkey No. 8910 90 days after i. c. inoculation with the Vasilchenko strain

Methods of detection (test systems)	Result
Tissue homogenate (mice)	0
Organ impressions (FA technique)	+
Cocultivation with SPEV cells without activators, with dimethylsulphoxide, BUDR (mice, plaques), CPE	n. d.
Tissue explant	Mice: no deaths (0/20) SPEV/CPE: + in 3 passages for 38 days. Virus titre $10^{4.7}$ TCD_{50}/ml. Haemagglutinin: +, titre 1 : 32. Identification by neutralization test in PS culture and AGDP test with immune serum to the Sofin strain of TBE virus

diameter in chick embryo cell (CEC) and SPEV cell cultures (Table 3). The isolate was serologically identified as TBE virus by the FA technique and two variants of the neutralization test.

In a monkey with chronic encephalitis examined at 90 days p.i. in the stage of remission, no infectious virus was found either in the CNS or in internal organs (Table 2). Virus-specific antigen, however, was demonstrated by the FA technique in all the examined parts of the brain and spinal cord, in the spleen, liver, kidneys, and lymph nodes (Figs. 1—3). The presence of virus was also established by co-cultivation of trypsinized cells of organs with indicator SPEV cells in the cerebral cortex, subcortical ganglia, cerebellum and spinal cord, as well as by the explant technique (subcortical ganglia, cerebellum spleen, liver) (Tables 2 and 4).

Discussion

We showed that after i.c. inoculation of monkeys with TBE virus strains isolated from patients with chronic or subclinical forms of encephalitis and with mutants of various degrees of attenuation, TBE virus spread early after inoculation (6—24 days) both in the CNS and to internal organs irrespective of the strain and clinical manifestations of the disease. In subacute fatal encephalitis we found only slightly lower virus titres than in the acute disease. From the point of view of the development of clinical forms, the Vasilchenko strain proved to be most interesting, in agreement with the data reported by Ilienko *et al.* (1974*b*) and Asher (1979). Subacute diseases with protracted course and chronic forms developed after inoculation with this strain more frequently than with other strains.

The monkeys developing chronic encephalitis were subjected to detailed virological examinations using a number of sensitive methods allowing the detection of persistent virus or virus-specific antigen. The methods were described previously (Pogodina *et al.*, 1981*a*, *b*). In parallel, infectious virus was isolated by i.c. inoculation of mice with homogenates of organs from the infected mice. The isolation at 383 days p.i. of infectious virus from subcortical ganglia and the spinal cord of a monkey with residual paralysis is in agreement with the report by Asher (1979) on virus isolation from the brain in the presence of neurological signs. Unlike Asher, we were able to detect persisting TBE virus and the virus-specific antigen in the CNS and internal organs both in case of a chronic disease with residual neurological symptoms and in case of recovery. One out of 6 monkeys infected with an attenuated mutant (Pan-114 No. 1 M) also developed a chronic form of TBE with ataxia and paralysis of the lower extremity. It was killed at 45 days, and infectious virus was isolated from its blood, spleen, and other internal organs.

As distinct from other authors working on chronic forms of TBE in monkeys (Ilienko *et al.*, 1974*a*, *b*; Zlotnik *et al.*, 1976; Asher, 1979) we examined virologically the internal organs of monkeys and obtained positive results. Persisting virus was regularly detected at remote intervals after inoculation in the spleen, whereas in the first 24 days it was not found there. Infectious virus in the spleen was first found in a rather high titre (5.5 log LD_{50}/ml) at 45 days in a chronic disease. Subsequently, at 90 and 383 days, the FA

technique revealed noninfectious virus capable of activation by co-cultivation of the test specimen with sensitive cells in the presence of a stimulator (BUDR) of viral activity as well as by explant cultivation. Some workers studying chronic forms of TBE in rodents observed the presence of virus-specific antigen in the spleen detectable by the FA technique at remote intervals: by 55 days in mice (Vorobieva *et al.*, 1975) or by 121 days in hamsters (Dremov *et al.*, 1979).

In our studies, although the virus appeared early in other organs (7 to 12 days, in lymph nodes from the 15th day), there was also a trend for more frequent isolation of virus in cases of persistence. Capability of TBE virus to multiply in organs of the reticuloendothelial system (RES) in acute infection was observed by Málková *et al.* (1961), Nan Shi-gie and Pogodina (1964), Mayer (1972) and Dubov *et al.* (1975). At the same time, most latent and persisting viruses (some herpesviruses and adenoviruses of man, etc.) are known to be capable of replicating in immunocompetent cells damaging the immune system which, in turn, is conducive to establishment of persistence (Mims, 1978). In this respect TBE virus may be similar in its properties to known persisting and latent viruses.

Using more sensitive methods for virus isolation from organs of the infected monkeys we showed that the localization of virus in chronic forms of infection was essentially similar to that in acute infection (CNS and internal organs, lymphoid organs).

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Explanation of Micrographs (Plate XXL):

Specific fluorescence in impression smears of the spleen (Fig. 1) and lymph node (Fig. 3) from monkey No. 8910 (90 days after i.c. inoculation of the Vasilchenko strain); ×200.

Fig. 1. Specific fluorescence in the spleen impression smear.

Fig. 2. Spleen impression smear stained with heterologous serum (no fluorescence).

Fig. 3. Specific fluorescence in a lymph node smear.