Acta virol. **25** : 337—343, 1981

PERSISTENCE OF TICK-BORNE ENCEPHALITIS VIRUS IN MONKEYS
I. FEATURES OF EXPERIMENTAL INFECTION

V. V. POGODINA, M. P. FROLOVA, G. V. MALENKO, G. I. FOKINA, L. S. LEVINA, L. L. MAMONENKO, G. V. KORESHKOVA, N. M. RALF

Institute of Poliomyelitis and Viral Encephalitides, U.S.S.R. Academy of Medical Sciences, 142782 Moscow, U.S.S.R.

Received November 5, 1980; revised June 24, 1981

Summary. — Sixty-seven *Macaca rhesus* monkeys were inoculated with 2 mutants and 3 virulent strains of tick-borne encephalitis (TBE) virus including strains isolated from patients with a chronic form of TBE. A model of the clinical course of acute, subacute, and chronic encephalitis was produced by intracerebral inoculation and that of asymptomatic infection was produced by subcutaneous inoculation [with or without administration of cyclophosphane (CP)]. Virus persistence developed after inoculation with all the strains, after non-fatal encephalitis and inapparent infection irrespective of CP administration. In monkeys recovering from encephalitis the virus persisted for at least 783 days. After asymptomatic infection, it persisted for 302 days; neither virus nor virus-specific antigen was detected at 356, 367, and 620 days.

Key words: Flavivirus; tick-borne encephalitis virus; persistence; monkeys

Introduction

Tick-borne encephalitis (TBE) belongs to neuroinfections which may run an asymptomatic or an overt course in acute, subacute and chronic forms (Panov, 1956; Shapoval, 1976). The clinical and morphological features of chronic TBE were reproduced by intracerebral (i.c.) inoculation of Macaca rhesus monkeys (Ilienko *et al.*, 1974*a*, *b*; Zlotnik *et al.*, 1976; Andzhaparidze *et al.*, 1978). The isolation of several virulent strains from patients (Chumakov *et al.*, 1944; Kraminskaya *et al.*, 1972) and i.c. inoculated monkeys in the chronic stage of the disease (Ilienko *et al.*, 1974*a*; Asher, 1979) suggest that pathogenesis of chronic TBE may be associated with persistence of TBE virus.

The development of chronic TBE is the most important medically but is not the only possible outcome of virus persistence. The features of TBE virus persistence in its hosts have been little studied, particularly with regard

to the conditions of development of this process, its frequency, duration, and mechanisms.

We studied the clinical, morphological, virological, pathogenetic and immunological aspects of TBE virus persistence in experimental monkeys. This paper deals with the dependence of virus persistence on strain properties, route of inoculation, the effect of an immunosuppressant, and type of the course of infection in its early stages.

Materials and Methods

Virus. The Vasilchenko strain was isolated in 1961 by Kvetkova *et* al. (1973) in Novosibirsk region from a patient with abortive TBE who showed no signs of chronic infection in subsequent follow-up for many years. The Aina/1448 strain was isolated by Kraminskaya *et al.* (1972) in Irkutsk region from the cerebrospinal fluid of a patient with chronic TBE (Kozhevnikov epilepsy syndrome) on the 108th day of the disease. The strain 41/65 was isolated by the same authors from the blood of a patient with chronic TBE 7 months after the onset of the disease which terminated fatally with progressive bulbar paralysis. The strains were passaged in 6—8 g random-bred white mice and reached titres of $10^{8.8}$—$10^{9.5}$ LD_{50}/ml after i.c. and $10^{7.1}$—$10^{7.6}$ LD_{50}/ml after subcutaneous (s.c.) inoculation. The Pan-114-IM and Pan-114-IM-APH mutants were derived by L. L. Mamonenko by treatment with the mutagen N-nitroso-N-ethylurea and plaque cloning of the virus progeny from the partially attenuated variant Pan-114, previously selected from the Pan strain population by Levkovich *et al.* (1971). These mutants had a mouse i.c. titre of $10^{8.5}$ LD_{50}/ml, markedly reduced virulence on s.c. inoculation ($10^{2.5}$ LD_{50}/ml), and poor invasiveness (invasiveness index 6 log.).

Inoculation and examinations of monkeys. Sixty-seven *M. rhesus* monkeys weighing 2.2 to 3.2 kg, without antibodies to viruses of the TBE complex before inoculation, were used. Twenty-eight monkeys were inoculated into the thalamus with 1 ml of inoculum (10^4—$10^{6.8}$ LD_{50}/ml of virus) and 39 monkeys were inoculated with virus doses of 10^6—$10^{8.2}$ LD_{50}/ml into the thigh; of the latter, 30 animals were given 50—100 mg/kg body-weight cyclophosphane (CP) one day before and one day after inoculation (p.i.). The animals were observed for up to 3 years and some of them were examined virologically for up to the 783rd day.

For *virological examinations*, brains (cerebral cortex, subcortical ganglia, cerebellum, brain stem), spinal cords (pooled cervical, thoracic, and lumbar areas), livers, spleens, kidneys, inguinal lymph nodes and small intestines were collected. The same organs were collected from 4 control monkeys given no virus or treated with CP alone.

Detection of persistent virus (starting from the 26th day p.i.) was attempted by a set of methods, including i.c. inoculation of 6—8 g mice with organ homogenates, co-cultivation by the method of Shope *et al.* (1972) of trypsinized organ cells with indicator continuous pig embryo kidney (SPEV) cells, explantation method (Rajčáni *et al.*, 1977), and direct immunofluorescence of impression smears of organs and co-cultivated cells (organ + SPEV) grown in the presence of 5-bromo-2-iododeoxyuridine.

The isolated viruses were identified as TBE virus by immunofluorescence, and neutralization, complement-fixation (CP), haemagglutination inhibition (HI) and agar gel diffusion and precipitation tests with immune rabbit and rat sera to TBE virus and a set of control sera (normal sera and immune sera to Japanese encephalitis and West Nile flaviviruses).

Results

The clinical course of infection

Of the 67 monkeys, 20 were infected with the Vasilchenko strain, 14 with the Aina/1448, 15 with the 41/65 strain, 10 with the Pan-114-IM mutant and 8 with the Pan-114-IM-APH mutant. The monkeys inoculated i.c. (28 animals) were observed for 783 days. All animals became ill. Against the background of general infection and meningeal symptoms, there developed

Table 1. Course of experimental TBE in monkeys

Route of inoculation	Course of the disease	Outcome	Total observation period (days)	No. of monkeys (n = 67)	TBE virus strains, mutants
Intracerebral	Acute encephalitis	Fatal	12	14	Aina/1448, 41/65 Pan-114-IM, Pan-114-IM-APH
		Recovery	783	2	Vasilchenko
	Subacute encephalitis	Fatal	24	9	Vasilchenko Pan-114-IM, Pan-114-IM-APH
	Chronic encephalitis [1]	Paralyses	383	2	Vasilchenko, Pan-114-IM
		Recovery of motor functions	90	1	Vasilchenko
Subacutaneous	Asymptomatic infection		620	9	Vasilchenko, Aina/1448, Pan-114-IM
Subcutaneous + CP + CP	Asymptomatic infection		1112	30	Vasilchenko, Aina/1448, 41/65

[1]) Histological examinations revealed a slowly progressive inflammatory process in the CNS of the monkeys.

signs of impaired coordination of movements, marked ataxia, epileptic attacks, tonic and clonic convulsions. Some animals showed paresis and paralysis of the upper and lower extremities, more marked in proximal parts, as well as hyperkinesia. All strains produced similar symptoms. At the same time, the severity and outcome of the disease varied. In i.c. inoculated monkeys, the following variants of the course of experimental infection were observed (Table 1):

1. Acute encephalitis: a short incubation period (4—6 days) and a short course (2—7 days). Depending on the outcome, we distinguished: (*a*) acute fatal encephalitis in which the animals died within 6—12 days p.i. or were killed at the same intervals in agony with rectal temperature declining to 33—32 °C; and (*b*) acute non-fatal encephalitis in which the condition improved on the 5th—6th day, the symptoms regressed and the animals remained normal for over 2 years of observations.

2. Subacute encephalitis: prolonged incubation period (8—11 days), a longer course, death after 15—24 days.

3. Chronic encephalitis: prolonged incubation period, progressive development of neurological symptoms for 1 month, followed by persistent paralysis of the extremities or partial recovery of motor functions. Histological examinations revealed a slowly progressive inflammatory process in the

Table 2. Development of persistent TBE virus infection

	TBE virus strains				Total
	Vasilchenko	Aina/1448	41/65	Pan-114-IM	
Condition of infection					
Intracerebral (acute nonfatal encephalitis; recovery; chronic encephalitis)	3/3	nd	nd	1/1	4/4
Subcutaneous (asymptomatic infection)	1/2	3/3	nd	2/4	6/9
Subcutaneous + CP (asymptomatic infection)	2/3	2/3	3/5	nd	7/1
Total	6/8	5/6	3/5	3/5	17/24
Virus and virus-specific antigen at the indicated day p.i.					
found	90, 176, 202, 292, 383, 783,	102, 271, 279, 292, 302	92, 111, 236	45	
not found	26, 620	176	356, 367	45	
No. of instances in which TBE virus and virus-specific antigen was detected	20	22	10	8	60

Every agent detected after 26 days and identified in serological test as TBE virus was taken into account. Several positive results could be obtained in examinations of a single monkey (different parts of the CNS, viscera). If in the materials examined (e.g., spleen) the virus was identified simultaneously by several methods, the positive result was recorded only once. Numerator — No. of monkeys with established TBE virus persistence; denominator — No. of inoculated monkeys. nd = not done.

central nervous system (CNS) of monkeys. The pathomorphology of the experimental infection will be described elsewhere.

The course of the disease showed no dose-response relationship within the range of 4—6.8 log LD_{50}/ml).

The 8 monkeys inoculated i.c. with the Vasilchenko strain showed a different course of infection: 2 had acute non-fatal encephalitis and 4 subacute encephalitis. One monkey had progressively developing symptoms: at 9 days cerebellar ataxia, at 22 days paralysis of an upper extremity and at 25 days hyperkinesis of masseter muscles; paralysis of the extremity persisted for up to 383 days when the animal was killed. In the 8th monkey, the symptoms developed similarly, but then the motor functions recovered; this animal was killed on the 90th day.

Among the 8 monkeys inoculated with the Pan-114-IM-APH mutant, 6 developed acute fatal encephalitis and 2 subacute encephalitis. Of the 6 monkeys inoculated i.c. with the Pan-114-IM mutant, 2 had acute encepha-

litis, 3 subacute fatal encephalitis and 1 had chronic encephalitis. Three monkeys inoculated with the Aina/1448 strain and 3 with the 41/65 strain developed acute fatal encephalitis.

The group of monkeys inoculated s.c. without the administration of CP consisted of 9 animals, of which 4 were infected with the Pan-114-IM mutant, 3 with the Vasilchenko strain, and 2 with the Aina/1448 strain. During the observation period of 620 days, no clinical signs of acute, subacute or chronic encephalitis were noted. A group of monkeys inoculated s.c. and given CP included 30 animals of which 12 were infected with the 41/65 strain, 9 with the Aina/1448 and 9 with the Vasilchenko strain. The observation period lasted 1112 days. No clinical symptoms were observed in any animal.

Detection of TBE virus persistence

To detect virus persistence at intervals from 26 to 783 day p.i., 24 monkeys were examined. They were divided into 5 groups depending on the inoculation route and clinical manifestations of infection: 1 — chronic encephalitis with steady paralysis of the extremities (2 monkeys examined at 45 and 383 days); 2 — chronic encephalitis, stage of recovery of motor functions (1 monkey, 90 days); 3 — acute non-fatal encephalitis, recovery (1 monkey, 783 days); 4 — asymptomatic infection after s.c. inoculation without CP (9 monkeys examined at 45, 102, 176, 271, 292, and 620 days); 5 — asymptomatic infection after s.c. inoculation and CP administration (11 monkeys examined at 26, 92, 111, 176. 202, 236, 279, 292, 302, 356, and 367 days).

Virus persistence was established in 17 out of 24 monkeys, including all animals surviving after actue non-fatal and chronic encephalitis, in 6 out of 9 monkeys inoculated s.c. without CP administration, and in 7 out of 11 monkeys infected s.c. and given CP (Table 2).

Virus was isolated from different parts of the brains and spinal cords, spleens, livers and lymph nodes from 6 out of 8 monkeys infected with the Vasilchenko strain; 20 isolates were identified as TBE virus. Two monkeys had chronic encephalitis (examined at 90 and 383 days), 1 had acute non-fatal encephalitis (783 days), 3 had asymptomatic infections (examined at 176, 202, and 292 days). No virus was isolated from 2 monkeys at 26 and 620 days after s.c. inoculation (with and without CP administration).

TBE virus was isolated from 5 out of 6 monkeys inoculated s.c. with the Aina/1448 strain; 22 isolates were obtained from the cerebral cortex, cerebellum, spinal cord, spleen, kidneys, liver, and lymph nodes. Three monkeys given no and 2 given CP were examined at 102, 271, 292, and 302 days. No virus persistence was demonstrated in one monkey 176 days after s.c. inoculation and CP administration.

TBE virus was recovered from 3 out of 5 monkeys inoculated s.c. with the 41/65 strain and given CP, at 92, 111, and 236 days p.i. Ten isolates from the cerebral cortex, subcortical ganglia, spinal cord and viscera were identified. Two monkeys examined at 356 and 367 days were negative.

Virus was isolated from 3 out of 5 monkeys 45 days p.i. with the Pan-114-IM mutant: one had a chronic disease and two asymptomatic infections. Eight isolates were identified. Two monkeys inoculated s.c. were negative.

Attempts at virus isolation from 4 monkeys of the control group were negative.

Discussion

Models of different manifestations of the infection: acute fatal and non-fatal encephalitis, subacute fatal encephalitis, chronic course as well as asymptomatic infection were reproduced in inoculated monkeys, these features being in several similar to diverse forms of TBE manifestation in man (Panov, 1956; Shapoval, 1976) and monkeys (Ilienko *et al.*, 1974*b*).

According to Ilienko *et al.* (1974*b*), TBE virus strain vary in their capacity to produce chronic TBE forms in monkeys. Our observations with the Vasilchenko strain resembled the results of Ilienko *et al.* (1974*a*, *b*) and Asher (1979), since they confirmed the tendency of this strain to produce chronic disease in monkeys. Subacute and chronic forms of the disease were also produced by mutants of the Pan strain, which is important for characterization of attenuated TBE virus strains. The Aina/1448 and 41/65 strains, isolated from patients with chronic TBE, induced acute fatal encephalitis in i.c. inoculated monkeys in our experiments. Apparently the capacity of a strain to induce a chronic course should be regarded not as an inherent property, but rather as a more or less manifest tendency against a background of multiple clinical manifestations.

At the same time we found no variations among the strains in their capacity to persist in monkeys. This capacity was observed both in highly virulent strains with marked invasiveness (Vasilchenko, Aina/1448, 41/65) and in the induced mutants of the Pan strain showing a poor invasiveness.

We showed that persistence in monkeys developed regularly: (*a*) after inoculation with all the strains tested (*b*) after i.c. and s.c. inoculation; (*c*) after non-fatal encephalitis and asymptomatic infection; and (*d*) with or without administration of CP.

In the animals surviving encephalitis with steady paralysis of the extremities, the virus was not eliminated for 383 days. In contrast to Asher (1979), we demonstrated that the disappearance of motor disorders in monkeys was not accompanied by virus elimination. Moreover, after complete clinical recovery, the virus persisted in the animals for more than 2 years. Long-term TBE virus persistence was also observed in asymptomatic infection induced by s.c. inoculation with or without administration of CP.

The development of persistence should, therefore, be regarded as a universal property of different strains and a typical pattern of TBE virus interaction with primate hosts.

It must be noted, however, that no virus could be demonstrated in 7 out of 24 monkeys examined at 26, 45, 176, 356, 367, and 620 days p.i. The negative results were obtained in s.c. inoculated monkeys given or not given CP. This could mean that the potential of a strain for persistence is not realized in every host or that the process of persistence is limited in time. Our experimental results suggest that, in asymptomatic infection, the virus is eliminated from the host approximately within a year. In cases of recovery from encephalitis, the duration of persistence exceeds two years and its limits are yet to be determined.

References

Andzhaparidze, O. G., Rozina, E. E., Bogomolova, N. N., and Boriskin, Yu. S. (1978): Morphological characteristics of the infection of animals with tick-borne encephalitis virus persisting for a long time in cell cultures. *Acta virol.* **22**, 218—224.

Asher, D. M. (1979): Persistent tick-borne encephalitis infection in man and monkeys: relation to chronic neurologic disease, pp. 179—195. In *Arctic and tropical arboviruses, Proc. 2nd Intern. Symp. on Arctic Arboviruses, Mont Gabriel, Canada.* Academic Press, New York.

Chumakov, M. P., Vorobieva, N. I., and Belyaeva, A. P. (1944): Investigation of ultravirus encephalitides. Communication III. Kozhevnikov's epilepsy and tick-borne encephalitis (in Russian). *Nevropat. i Psichiat.* **13**(2), 65—68.

Ilienko, V. I., Komandenko, N. I., Platonov, V. G., Prozorova, I. N., and Panov, A. G. (1974*a*): Pathogenesis study on chronic forms of tick-borne encephalitis. *Acta virol.* **18**, 341—346.

Ilienko, V. I., Platonov, V. G., and Smorodintsev, A. A. (1974*b*): Biological variants of tick-borne encephalitis virus isolated in different foci of the disease (in Russian). *Vop. Virus.* **19**, 414—418.

Kraminskaya, N. N., Zhivolyapina, R. R., and Meierova, R. A. (1972): Attempts at virological investigations of hyperkinetic forms of tick-borne encephalitis with a progredient course (in Russian), pp. 224—225. In M. P. Chumakov (Ed.): *Aktualnye Problemy Virusologii i Profilaktiki virusnykh Zabolevanii*, Tezisy XVII Nauchnoi Sessii Inst. Poliomielita i Virusnykh Entsefalitov, Moskva.

Kvetkova, E. A., Perekhodova, S. K., and Kalinina, E. E. (1973): Genetic markers of "wild" tick-borne encephalitis virus strains with reference to problems of pathogenesis (in Russian), pp. 144—149. In V. L. Kvetkov (Ed.): *Voprosy infektsionnoi Patologii*, Nauchn. Trudy Omskogo Medinstituta, Omsk.

Levkovich, E. N., Karpovich, L. G., and Zasukhina, G. D. (1971): *Genetika i Evolutsiya Arbovirusov, Meditsina, Moscow.*

Panov, A. G. (1956): *Kleshchevoi Entsefalit*, Medgiz, Leningrad.

Rajčáni, J., Čiampor, F., Sabó, A., Libíková, H., and Rosenbergová, M. (1977): Activation of latent Herpesvirus hominis in explants of rabbit trigeminal ganglia: The influence of immune serum. *Arch. Virol.* **53**, 55—69.

Shapoval, A. N. (1976): *Khronicheskie Formy kleshchevogo Entsefalita*, Meditsina, Leningrad.

Shope, Th. C., Klein-Robbenhaar, J., and Miller, H. (1972): Fatal encephalitis due to herpesvirus hominis. Use of intact brain cells for isolation of virus. *J. inf. Dis.* **125**, 542—544.

Zlotnik, I., Grant, D. P., and Carter, G. B. (1976): Experimental infection of monkeys with viruses of tick-borne encephalitis complex. Degenerative cerebellar lesions following inapparent forms of the disease or recovery from clinical encephalitis. *Brit. J. exp. Path.* **57**, 200—210.