

PERSISTENCE OF TICK-BORNE ENCEPHALITIS VIRUS IN MONKEYS

III. PHENOTYPES OF THE PERSISTING VIRUS

V. V. POGODINA, L. S. LEVINA, G. I. FOKINA, G. V. KORESHKOVA,
G. V. MALENKO, N. G. BOCHKOVA, O. E. RZHAKHOVA

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

Received November 5, 1980; revised June 24, 1981

Summary. — The properties of tick-borne encephalitis (TBE) virus persisting for 90—383 days after intracerebral and subcutaneous inoculation of *Macaca rhesus* monkeys were studied, namely (1) the type of infection produced directly in the tissues of the experimental monkeys; (2) the activating effect of co-cultivation and explantation procedures; and (3) the phenotype of the isolates by a set of markers. The virus was detected and analysed in 52 instances. Directly in monkey tissues the virus induced a productive infection rarely (5.8%) but more frequently (71.2%) an abortive infection detectable by immunofluorescence (presence of virus-specific antigen). In 23% of instances a non-productive infection was observed in monkey tissues. Like abortive infection it could be activated by the co-cultivation of cells and explantation procedures. The latter exerted a more marked activating effect than co-cultivation. The strains isolated from monkey tissues in productive infection or activated by explantation or co-cultivation were heterogeneous in their properties. The following virus phenotypes were found: virus highly virulent for mice, cytotoxic and antigenically complete; a cytotoxic virus of low virulence, possessing haemagglutinin; and a cytotoxic virus apathogenic for mice, devoid of haemagglutinin but synthesizing complement-fixing antigen and an antigen detectable by immunofluorescence.

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

Introduction

The information on the properties of tick-borne encephalitis (TBE) virus persisting in man and monkeys is very limited mostly because of the small number of agents which had been isolated and studied. The isolated strains proved to be virulent (Chumakov *et al.*, 1944; Kraminskaya *et al.*, 1972;

Iliencko *et al.*, 1974). At the same time failures to isolate persisting TBE virus were reported repeatedly. The available evidence gives no idea of the similarity or diversity of the properties of persisting virus. It is difficult to conclude whether the failures in virus isolation were due to its elimination from the host organism, loss of virulence, or the use of unsuitable isolation methods.

The present study demonstrated the heterogeneity of TBE virus persisting in monkeys after inoculation with Aina/1448, Vasilchenko, and 41/65 virus strains intracerebrally (chronic encephalitis) and subcutaneously (asymptomatic infection). We analysed 52 instances of virus isolation 90–383 days after inoculation of the monkeys.

Materials and Methods

For the TBE virus strains used, inoculation of monkeys and the methods employed for isolation and identification of persisting TBE virus see Pogodina *et al.* (1981a, b).

The TBE virus strains isolated from the infected monkeys were investigated for a set of markers. Because of the low activity of the strains isolated in persistent infection, they could not be assayed by routine methods. The quantitative evaluation of the properties of the strains and methods of their assay were modified for persisting virus strains (see Table 1). The virus-containing materials were examined in the form of 10% homogenates of monkey organs or culture fluid from explants and co-cultivated cells (in dilutions from 10^0 to 10^{-5}). The fluorescent antibody (FA) technique was employed to examine impression smears of organs and coverslip cultures of co-cultivated cells in the presence of 5-bromo-2-iododeoxyuridine (BUDR).

Results

Detection of persisting virus before activation

In the course of attempts at isolation of the persisting strains, the presence of virus was determined in tissues with or without a special treatment. The methods requiring no special treatment included examinations of tissue homogenates and detection of mouse-pathogenic virus as well as demonstration of virus-specific antigen in organ impression smears by the FA technique.

In 3 cases the tissues yielded a virulent or moderately virulent TBE virus which produced plaques in chick embryo cell (CEC) cultures and a cytopathic effect (CPE) in pig embryo kidney (SPEV) cells. The virus was isolated 102 days after inoculation (p.i.) from lymph nodes of a monkey inoculated subcutaneously (s.c.) with the Aina/1448 strain and from the spinal cord and subcortical ganglia at 383 days after intracerebral (i.c.) inoculation with the Vasilchenko strain. The virus titre was 10^3 – $10^{4.7}$ LD₅₀/ml. Virus-specific antigen was demonstrated in impression smears of the same tissues.

In 37 cases, brains and viscera of the monkeys were examined from 90 to 383 days after i.c. and s.c. inoculation with the TBE virus strains studied; no infectious virus was isolated but virus antigen was demonstrated in the tissues by the FA technique.

Table 1. Markers of persisting TBE virus strains and methods of their assay

Marker and test system	Quantitative characteristics
1. Virulence for random-bred white mice (5-7 g) inoculated i.c. with 0.03 ml volumes	(+) — capacity for passages with regular production of disease in 40% of 10-20 inoculated animals (±) disease of single mice in 1-2 passages (subsequently loss of ability to be passaged) (-) — no pathogenicity for mice or death of single mice out of 20-50 mice inoculated (without confirmation of the specificity of death) Additional characteristic sign: virus titre (LD ₅₀ /ml)
2. Plaque formation in CEC cultures by the method of Levkovich <i>et al.</i> (1971)	(+, -) — capacity of plaque formation present or absent Additional characteristics: plaque diameter, virus titre in PFU/ml
3. CPE in continuous SPEV and PS or primary SHK cell cultures	(+, -) — presence or absence of a CPE after inoculation of 4-8 cultures (±) — irregular CPE in passages Additional characteristics:
4. Haemagglutinin in culture fluid from infected SPEV or SHK cells at 37 °C; in brains from suckling and 6-8 g mice; and in fluids from explant cultures	time of CPE appearance; virus titre in TCD ₅₀ /ml (+, -) — presence or absence of haemagglutinin. Additional characteristic: haemagglutinin titre in a pH range from 6.2-6.4
5. Demonstration of precipitating antigen in infected SPEV cell culture fluid concentrated 500-fold with polyethylene glycol (mol. weight 6000-8000). Agar gel diffusion precipitation test performed as described by Bochkova and Pogodina (1980)	(+, -) — presence or absence of precipitation with TBE virus-specific immune sera. Additional characteristic: antigen titre
6. Demonstration of complement-fixing antigen in CEC cultures infected with specimens from mixed cultures as described previously (Pogodina <i>et al.</i> , 1981b)	(+, -) — presence or absence of antigen Additional characteristics: antigen titre; time of culture fluid sampling from co-cultivated cells
7. Demonstration of virus-specific antigen by the direct FA technique in organ impression smears or in cells co-cultivated on slides in the presence of BUDR as described previously (Pogodina <i>et al.</i> , 1981b)	(+, -) — presence or absence of antigen

In 12 cases, neither virus nor virus-specific antigen were found. After co-cultivation and explantation, however, the same tissues yielded agents identified as TBE virus. These results were obtained in examinations of the central nervous system (CNS) and viscera at 102-302 days after s.c. inoculations of the monkeys with all the strains studied.

Table 2. The activating effect of co-cultivation and explantation procedures in TBE virus persistence in monkey tissues

No. of observations	Results of tissue examination, virus properties							Activation procedure
	Before activation			After activation				
	Virus		Antigen	Virus		Antigen		
	Virulence for mice	FA	Virulence for mice	Plaques	CPE	HA	FA	
3 (5.8%)	+	+						
37 (71.2%)	-	-	(1) +	+	+	+	+	Explantation
			(2) -	+	±	-	+	Co-cultivation
			(3) -	-	-	-	+	Co-cultivation
			(4) -	-	-	-	-	Co-cultivation
12 (23%)	-	-	(1) +	+	+	+	+	Explantation
			(2) ±	+	+	+	+	Explantation
			(3) ±	+	+	-	+	Co-cultivation
			(4) -	-	-	-	+	Co-cultivation

HA = haemagglutinin; FA = antigen detectable by immunofluorescence.

+ and - : result positive and negative, respectively.

The effect of co-cultivation and explantation

The isolation of persisting TBE virus strains required also procedures which involved special treatment of tissues or special conditions of cultivation: explantation and co-cultivation of trypsinized organ cells with indicator SPEV cells in the absence or presence of DEAE-dextran, dimethylsulphoxide (DMSO) or BUDR. In cases in which before activation the tissues were only shown to contain virus-specific antigen these procedures gave 4 types of results (Table 2):

1. Isolation of a mouse-virulent or moderately virulent, cytopathic, plaque-producing virus possessing haemagglutinin and synthesizing an antigen detectable by the FA technique. These results were obtained by explantation of CNS and viscera from monkeys 90 and 302 days after i.c. and s.c. inoculation with the Vasilchenko and Aina/1448 strains.

2. After co-cultivation, isolation of a virus apathogenic for mice, producing plaques in CEC cultures and irregularly a CPE in SPEV cultures, synthesizing specific antigens (complement-fixing and detectable by immunofluorescence), and showing no haemagglutinating activity. This result was obtained with all strains persisting for 90-102 days in the brains, spinal cords and viscera.

3. Detection in co-cultivated cells of specific fluorescence while tests for virulence in mice, cytopathic activity and plaque formation were negative.

Table 3. Isolation of TBE virus by co-cultivation and explantation procedures from subeortical ganglia of a monkey 90 days after i.e. inoculation with the Vas/ichenko strain

Methods of detection and test system	Results
Tissue homogenate (mice)	—
Tissue impression smears (FA technique)	+
Co-cultivation:	
without activator (mice, CPE, plaques)	—
with DMSO (mice, CPE, plaques)	—
with BUDR (mice, CPE, plaques)	—
with DEAE-dextran	—
	CPE in SPEV cell culture at 5 days → plaques in CEC culture at 4 days → demonstration of CF antigen (titre 16) → identification in CF test with immune serum to TBE virus; 2nd passage in SPEV cells: CPE at 7 days → death of individual mice in passages (6/50).
Tissue explant	Mice: irregular deaths (4/20); positive CPE in SPEV cell cultures (3 passages); haemagglutinin present in a titre of 64; identification in the HI test with immune serum to TBE virus

The immunofluorescence was observed at 90 and 383 days in organs of monkeys inoculated with different strains.

4. The lack of virus and virus-specific antigen after co-cultivation of the cells in the presence of BUDR. This finding applied to livers, brains and spinal cords of monkeys inoculated with the Aina/1448 strain at 102 and 302 days p.i.

In 12 cases neither virus nor virus-specific antigen was found directly in the tissues, but active virus could be demonstrated after co-cultivation of the cells or explantation:

1. A virus highly virulent for mice, cytopathic, producing plaques, possessing haemagglutinin and precipitating antigen was isolated by the explantation method.

2. A cytotoxic virus of low mouse virulence, exhibiting haemagglutinating activity and positive immunofluorescence was isolated after explantation of the cerebellum from a monkey 302 days after s.c. inoculation with the Aina/1448 strain.

3. A low-virulent, cytopathic and plaque-forming virus, synthesizing complement-fixing and fluorescent antigens but possessing no haemagglutinin or precipitating antigen was isolated after co-cultivation of cells in the absence or presence of DMSO and BUDR at intervals of 102 and 176 days.

4. Specific fluorescence was demonstrated in cells co-cultivated in the presence of BUDR, while the results of infectious virus isolation were negative. These results were obtained with all the strains studied when monkey CNS were examined at 202, 292, and 302 days p.i.

Table 4. Isolation of TBE virus by the co-cultivation procedure from the cerebral cortex of a monkey 176 days after s.c. inoculation with the Vasilchenko strain

Methods of detection and test systems	Result
Tissue homogenate (mice)	—
Tissue impression smears (FA technique)	—
Co-cultivation:	
with DMSO (mice, plaque, CPE)	—
with BUDR (FA technique)	+
without activator	Out of 20 mice one sick at 13 days → 3 suckling mice sick at 2 days → storage for 37 days at -20 °C → CPE in SHK cells at 6 days → plaques in CEC cultures at 6 days → CPE in SPEV cell culture at 4 days → plaques in CEC at 4 days

Properties of the isolates

The results of virus isolation after co-cultivation and tissue explantation are presented in Table 3. Virus-specific antigen was detected by the FA technique in impression smears of subcortical ganglia of a monkey 90 days after i.c. inoculation with the Vasilchenko strain, no infectious virus having been detected in the tissue. Co-cultivation in the absence or presence of DMSO or BUDR had no activating effect. Co-cultivation in the presence of DEAE-dextran yielded a virus forming plaques in CEC culture, synthesizing a specific complement-fixing antigen in these cells (titre of 16), irregularly producing a CPE in SPEV cells and poorly pathogenic for mice. An explant of the same tissue yielded a TBE virus regularly producing a CPE in SPEV cell cultures and possessing haemagglutinin (in a titre of 64), but poorly pathogenic for mice.

Table 4 demonstrates an instance of TBE virus isolation after co-cultivation from a tissue in which originally neither virus nor virus-specific antigen was found. Cerebral cortex was examined 176 days p.i. with the Vasilchenko strain. Specific fluorescence appeared in the cells co-cultivated in the presence of BUDR. A specimen from the culture grown without a chemical activator produced disease in one out of 20 mice. This mouse yielded an agent producing a CPE in SPEV cell cultures and plaques in CEC cultures. From individual vials with a CPE and individual plaques, 11 lines of this agent were isolated. Ten lines were cloned once or twice in CEC cultures. The clones underwent 7–10 passages in SPEV and Syrian hamster kidney (SHK) cell cultures, producing a CPE in 6 and later in 4 days; the virus had a titre of $10^{4.6}$ – $10^{5.1}$ TCD₅₀/ml. These clones were apathogenic for 6–8 g mice but caused death in some newborn mice. Plaques 1.2 mm in diameter appeared in CEC cultures in 3–4 days; the virus titre was 10^3 PFU/ml. One of the lines produced no plaques. Twelve attempts at detecting haemagglutinin in the brains of infected mice and SPEV and SHK cell cultures with a marked CPE gave negative results. No precipitating antigen was found. The isolate was identified as TBE virus by the FA technique and by the plaque reduction neutralization test with immune serum to TBE virus.

Table 5. Isolation of TBE virus by explantation procedure from the liver of monkey 202 days after s.c. inoculation with the Vasilechenko strain

Methods of detection and test systems	Result
Tissue homogenate (mice)	—
Tissue impression smears (FA technique)	—
Co-cultivation with SPEV cells without an activator or with the addition of DMSO or BUDR	—
Detection in mice, CEC (plaques), SPEV cells (CPE)	—
Tissue explant, detection in mice	+
PS cells (CPE)	+
CEC (plaques)	+

Properties of the isolate: can be passaged in mice and cell cultures. Virus titres: 10^{10} i.c. mouse LD₅₀/ml, $10^{8.3}$ s.c. mouse LD₅₀/ml, and $10^{7.5}$ TCD₅₀/ml in PS cell cultures. Titre of haemagglutinating antigen in the brain 2560.

Identification:

HI test positive with immune serum to TBE virus 1 : 640, with serum to Japanese encephalitis virus 1 : 10.

Neutralization test: virus titre with normal serum 6×10^9 PFU/ml, with immune TBE serum 7.5×10^5 PFU/ml.

Agar precipitation test with immune serum to the isolate: antigen of TBE virus — 1 : 32, antigen of West Nile virus — no reaction.

Virus isolation by the explantation procedure from the liver of a monkey 202 days after s.c. inoculation with the Vasilchenko strain is illustrated in Table 5. Examinations of the organ homogenates by inoculation of mice and of liver impression smears by the FA technique were negative. None of the co-cultivation variants produced any activation effect. The explant yielded a virus highly virulent for mice (titre 10^{10} i.c. LD₅₀/ml, $10^{8.3}$ s.c. LD₅₀/ml), cytopathic and plaque producing (titres of $10^{7.5}$ TCD₅₀/ml and 10^9 PFU/ml), and showing haemagglutinating activity (titre of 2560). The activated virus was definitely identified as TBE virus by neutralization, HI and agar gel diffusion precipitation tests with a set of immune and control sera.

Examinations of organs from 4 uninfected monkeys by co-cultivation and explantation demonstrated no activation of spontaneous viruses.

Discussion

The present studies demonstrated a heterogeneity of persisting TBE virus which produced different types of infection in the infected tissue, showed a different capacity for activation under the influence of co-cultivation and explantation, and exhibited various degrees of antigenicity and infectivity after activation.

Virulent, antigenically complete virus could be found directly in the tissue examined, without any special treatment, extremely rarely (5.8%) (productive type of infection). A more typical feature of the persisting TBE virus was an abortive infection detectable by the FA technique (presence of

virus-specific antigen). By co-cultivation and explantation, the abortive infection could be changed into a productive one in which either a virulent, cytotoxic virus possessing all antigens or a virus lacking haemagglutinin and apathogenic for mice was produced. In a number of cases the FA technique again revealed in co-cultivated cells an abortive infection, or virus-specific antigens could not be detected by any of the tests used. The latter results suggest that the persisting virus is capable of producing abortive infection only in monkey tissues and is lacking the capacity to infect other cells (e.g., indicator SPEV cells).

In 23% of cases, the presence of virus-specific structures in monkey tissues could not be demonstrated by the markers of virulence for mice and antigenicity; it was only by the use of activation methods that the presence in tissues of virus-specific material could be confirmed. The level of activation in such cases varied: activation could be manifested by the expression of virus-specific antigen demonstrable by the FA technique; production of a cytotoxic virus of low virulence, lacking haemagglutinin; production of virus of low virulence possessing haemagglutinin; or, finally, synthesis of highly virulent, invasive and antigenically complete virus. The latter maximal level of activation indicates that the repression of functions in persisting TBE virus is not due to defects in the genetic material (at least, to deletions of genes associated with the markers of virulence, plaque formation, cytopathic activity, and synthesis of haemagglutinin, precipitating and complement-fixing antigens and the antigen detectable by the FA technique).

The isolates obtained directly from monkey tissues or activated by co-cultivation and explanation methods had different phenotypes. The differences concerned the level of virulence, the presence or absence of haemagglutinin, complement-fixing and precipitating antigens and the degree of cytopathic and plaque-forming activity. No relationship between the phenotype of the persisting virus and conditions of infection of the monkey (virus strain, inoculation route — i.c. or s.c.) or the type of tissue from which the virus was isolated has been established.

Our experiments clearly showed that explantation exerted a more marked activating effect than co-cultivation. The maximum level of activation (production of a virulent virus possessing haemagglutinin) was achieved by explantation. Co-cultivation stimulated some patterns of infectivity of the virus, mostly its cytopathic activity, as well as the synthesis of antigens detectable by the FA technique and complement fixation. Explantation and co-cultivation may contribute to activation owing to the elimination of immunological factors or due to the effect of trypsin, DEAE-dextran, DMSO, or BUDR on the permeability of membranes and cell metabolism; they can enhance the adsorption processes or alter virus properties (Ochuchi and Homma, 1976; Schumacher and Albrecht, 1970).

Activation of persisting TBE virus observed in experiments on co-cultivation and explantation, may apparently also occur directly in the host owing to the effect of various exogenous and endogenous factors. The level of activation achieved (restoration of the antigenic functions or virulence)

may be one of the mechanisms affecting the course, outcome and sequelae of persistent infection (stimulation of immunity, development of a pathological process).

The different levels of repression of the functions of the persisting virus as well as different levels of activation observed in our experiments with all TBE virus strains examined thus represent typical features of persistent infection produced by TBE virus in primates. These features are an explanation of the fact that isolations of virulent strains have been rare and that it is necessary to use a set of various methods and tests for the detection of persisting TBE virus.

References

- Bochkova, N. G., and Pogodina, V. V. (1980): Immune typing of Japanese encephalitis virus strains (in Russian). *Vop. Virus.* **25**, 318—322.
- Chumakov, M. P., Vorobieva, N. N., and Belyaeva, A. P. (1944): Study of ultraviral encephalitides. Communication III. Kozhevnikov's epilepsy and tick-borne encephalitis (in Russian). *Nevrol. i Psichiatr.* **13**(2), 65—68.
- Ilienکو, V. I., Komandenko, N. I., Platonov, V. G., Prozorova, I. N., and Panov, A. G. (1974): Pathogenic study on chronic forms of tick-borne encephalitis. *Acta virol.* **18**, 341—346.
- Kraminskaya, N. N., Zhivolyapina, R. R., and Meierova, R. A. (1972): Attempts at virological studies on hyperkinetic forms of tick-borne encephalitis with a proгредиент course (in Russian), pp. 224—225. In M. P. Chumakov (Ed.): *Aktualnye Problemy Virusologii i Profilaktiki virusnykh Zabolevaniy, Tezisy XVII Nauchnoi Sessii Inst. Poliomyel. i Virusnykh Entsefal.*, Moskva.
- Levkovich, E. N., Karpovich, L. G., and Zasukhina, G. D. (1971): *Genetika i Evolutsiya Arbovirusov*, Meditsina, Moskva.
- Ochuchi, M., and Homma, M. (1976): Trypsin action on the growth of Sendai virus in tissue culture cells. IV. Evidence for activation of Sendai virus by cleavage of glycoprotein. *J. Virol.* **18**, 1147.
- Pogodina, V. V., Frolova, M. P., Malenko, G. V., Fokina, G. I., Levina, L. S., Mamonenko, L. L., Koreshkova, G. V., and Ralph, N. M. (1981a): Persistence of tick-borne encephalitis virus in monkeys. I. Features of experimental infection. *Acta virol.* **25**, 337—343.
- Pogodina, V. V., Malenko, G. V., Fokina, G. I., Levina, L. S., Koreshkova, G. V., Rzhakhova, O. E., Bochkova, N. G., and Mamonenko, L. L. (1981b): Persistence of tick-borne encephalitis virus in monkeys. II. Effectiveness of methods used for virus detection. *Acta virol.* **25**, 344 to 351.
- Schumacher, H., and Albrecht, P. (1970): Optimal conditions for isolation of neurotropic measles virus from brain tissue. *Proc. Soc. exp. Biol. Med.* **134**, 396—402.