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PERSISTENCE OF TICK-BORNE ENCEPHALITIS VIRUS IN MONKEYS. V. VIRUS LOCALIZATION AFTER SUBCUTANEOUS INOCULATION

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Summary. — In 28 *Macaca rhesus* monkeys inoculated subcutaneously (s.c.) with different strains and mutants of tick-borne encephalitis (TBE) virus and developing asymptomatic infection, TBE virus and the virus-specific antigen were found at different intervals up to 302 days post inoculation (p.i.) in the CNS and internal organs (liver, spleen, lymph nodes, kidneys). When cyclophosphane (CP) was used as an immunosuppressor, no significant changes in virus distribution were observed with the exception of more frequent isolations at early intervals from kidneys and at late intervals from spleen. With or without CP administration virus was found in the spinal cord at 3—6 days, in the cerebellum and subcortical ganglia at 11—14 days, in the cerebral cortex at 19 days p.i. Early after inoculation the virus was more frequently isolated from the CNS than from the internal organs; later (93—302 days p.i.) the persisting virus or virus-specific antigen were predominantly found in internal organs. The set of sensitive methods used facilitated to detect the persistence of TBE virus in noninfectious form in monkeys with asymptomatic infection.

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

Introduction

The problems of the pathogenesis and distribution of tick-borne encephalitis (TBE) virus in hosts with chronic and asymptomatic infection have been little studied. There are reports on the modeling of this process after s.c. inoculation in rodents (mice, hamsters), birds, and other animals (Vorobieva *et al.*, 1975; Kraminskaya and Kraminsky, 1975; Dremov *et al.*, 1979; Elečková *et al.*, 1976; Larina *et al.*, 1977). As for the chronic process in monkeys as a model most closely approaching man, such studies have been carried out mainly after intracerebral inoculation of the virus (Dubov *et al.*, 1969; Iliyenko *et al.*, 1974*a*, *b*; Zlotnik *et al.*, 1976; Asher, 1979). We studied virus distribution in monkeys inoculated subcutaneously with several strains of TBE virus.

Table 1. Isolation of infectious TBE virus from the organs of monkeys (with and without CP treatment) 3—45 days after s. c. inoculation

CP treatment	Days p. i.	Virus strain	Titre of isolated virus (mouse LD_{50}/ml)												
			Brain				Spinal cord								
			cortex	subcortical ganglia	cerebellum	stem	cervical	thoracic	lumbar	Spleen	Liver	Lymph nodes	Kidney	Intestine	Blood
	6	a	0	0	3.1	3.0	2.7	2.7	0	0	0	0	0	n. d.	0
	11	b	0	0	2.9	0	0	0	0	0	2.5	0	0	n. d.	0
	45	d	4.3	0	0	3.9	0	0	3.9	0	0	0	0	0	0
No	45	d	0	0	0	3.8	0	0	0	0	0	0	0	0	0
	45	d	0	0	0	0	0	0	0	0	0	0	0	0	3.0
	45	d	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	a	0	0	0	0	0	0	3.5	0	0	0	3.5	n. d.	n. d.
	5	c	0	0	0	0	0	2.8	0	0	0	n. d.	2.9	n. d.	n. d.
	10	c	0	0	0	0	0	0	0	0	0	3.0	2.8	n. d.	n. d.
	11	b	0	2.9	0	0	0	0	2.5	0	0	2.8	0	n. d.	n. d.
Yes	13	a	2.8	0	3.0	0	0	0	0	0	0	0	0	n. d.	n. d.
	14	c	0	0	2.5	0	0	0	2.5	0	0	0	0	n. d.	n. d.
	19	c	2.7	2.5	0	0	0	0	0	n. d.	0	n. d.	0	n. d.	0
	26	b	0	0	0	0	0	0	0	0	0	0	0	n. d.	n. d.

0 — negative result;
n. d. — not done.
a — Aina/1448; b — Vasilchenko; c — 41/65; d — Pan-114 No. 1 M.

Materials and Methods

Virus, animals. — Twenty-eight *Macaca rhesus* monkeys weighing 2.2—3.2 kg were inoculated subcutaneously with TBE virus strains Vasilchenko (7 animals), Aina/1448 (8), and 41/65 (9) as well as with the mutant Pan-114 No. 1 M (4). The characteristics of the strains and the mutant were described by Pogodina *et al.* (1981*a*). Before inoculation the animals had no antibodies to viruses of the TBE complex. One ml of virus (10^6—$10^{8.2}$ LD_{50}) was inoculated in the skin of the back. The monkeys were divided into two groups; one group (CP^+) received the immunosuppressant cyclophosphane (CP) s.c. in a dose of 50—100 mg/kg body weight 1 day before and 1 day after infection while the other remained untreated.

At various intervals after inoculation (p.i.) from the 3rd to 620th day, specimens were collected from the animals for virological examinations including the brain (cerebral cortex: anterior central gyrus, subcortical ganglia, cerebellum; stem: mesencephalon and medulla oblongata), spinal cord, spleen, liver, kidneys, inguinal lymph nodes, small intestine and blood. In the first 45 days p.i. the virus was isolated as a rule by intracerebral inoculation of 10% organ suspensions in 8—10 random-bred mice aged 7—10 days. The virus was titrated in similar animals, using 4 mice for each 10-fold dilution of the virus-containing material. At later intervals, attempt at virus detection were done by mice inoculation, the fluorescent antibody (FA) technique, 4 modifications of the method of co-cultivation of trypsinized organ cells with indicator SPEV cells [without activators, with 3% dimethyl sulphoxide, DEAE-dextran, or bromodeoxyuridine (BUDR)], as well as by the method of organ explants. These methods of virus assay and procedures of serological identification of TBE virus have been described by Pogodina *et al.* (1981*b*).

Table 2. Detection of persisting TBE virus in organs of monkeys untreated or treated with CP at 92—620 days p. i.

CP treatment	Days p. i.	Virus strain	No. of isolates*)	Localization of isolates								
				Brain				Spinal cord	Spleen	Liver	Lymph nodes	Kidney
				Cortex	Subcortical ganglia	Cerebellum	Stem					
	102	a	4	0	0	0	0	+	0	+	+	+
	176	b	2	+	0	0	0	+	0	0	0	0
No	271	a	6	+	0	+	0	+	0	+	+	+
	292	a	7	+	+	+	0	0	+	+	+	+
	620	b	0	0	0	0	0	0	0	0	0	0
	92	c	4	0	0	0	0	0	+	+	+	+
	111	b	1	0	0	0	0	0	0	+	0	0
	111	c	3	+	0	0	+	0	0	0	0	0
	176	a	0	0	0	0	0	0	0	0	0	0
Yes	236	c	3	0	0	0	0	0	+	0	+	+
	279	a	1	0	0	0	0	0	0	+	0	0
	292	b	5	0	+	+	0	0	+	+	+	0
	302	a	4	+	0	+	n. d.	n. d.	+	n. d.	0	+
	356	c	0	0	0	0	0	0	0	0	0	0
	367	c	0	0	0	0	0	0	0	0	0	0

*) The isolates were not pathogenic for mice with the exception of one isolated at 102 days from a lymph node.

TBE virus strains: a — Aina/1448; b — Vasilchenko; c — 41/65; n. d. — not done

Results

S.c. inoculation of CP treated monkeys and untreated ones resulted in asymptomatic infection and virus penetration into the brain and spinal cord (Table 1). In untreated monkeys, infectious virus was isolated from the cerebellum, brain stem, cerebral cortex and some parts of the spinal cord at 6—45 days p.i., once from the liver at 11 days, and blood at 45 days p. i. It should be noted that infectious virus was irregularly detected in the CNS 45 days p.i. No TBE virus was isolated from the spleen, kidneys, or lymph nodes.

In CP treated monkeys, infectious virus could be detected in the spinal cord at 3—5 days p.i., in the brain from 11th day on. After 19 days p.i. no virus could be isolated from the CNS. The virus could be regularly found earlier in kidneys (3, 5, 10 days p.i.) and later in lymph nodes (10—11 days p.i). No virus could be isolated from tissues of the spleen and liver.

In untreated monkeys, virus titres in the CNS did not exceed $10^{4.3}$ LD_{50}/ml, in the internal organs $10^{2.6}$ LD_{50}/ml. In CP treated animals, virus titres in the CNS and internal organs were lower than $10^{3.5}$ LD_{50}/ml. At later intervals no infectious virus could be detected in either group. On one occasion, 102 days after inoculation of an untreated monkey with the Aina 1448 strain,

Table 3. Isolation of TBE virus from lymph nodes of a monkey 102 days p. i. with the Aina/1448 strain

Methods of virus detection	Pathogenicity for mice Titre in LD_{50}/ml	Plaque production in CEC culture Titre in PFU/ml	CF test	FA technique
1. Tissue homogenate	10^3	8×10^4	n. d.	n. d.
2. Co-cultivation of tissue with SPEV cells*				
a) without activator	+	1.3×10^3	1 : 32	n. d.
b) with dimethyl-sulphoxide	+	2×10^3	1 : 8	n. d.
c) with BUDR	+	1.5×10^3	n. d.	+
3. Organ impressions				+

* Cellular virus harvested at 10 days was examined.
+ = positive result, no titration done.
This set of methods yielded a total of 4 lines of persisting TBE.

TBE virus of moderate virulence was isolated from lymph nodes (Tables 2 and 3).

The use of a set methods for the detection of persisting virus and viral antigen (FA technique, explantation, co-cultivation of trypsinized cells and indicator SPEV cells in the absence of activators, in the presence of dimethylsulphoxide, DEAE-dextran, or BUDR) showed that the persisting virus could be detected in both the CNS and internal organs of s.c. inoculated monkeys. Virus persisted in monkeys treated or untreated with cyclophosphane for at least 302 days.

In untreated monkeys the persisting virus was more frequently located in the cerebral cortex, cerebellum, and spinal cord as well as in lymph nodes, liver, and kidneys, less frequently in subcortical ganglia and spleen (Table 2), whereas in CP^+ animals the persisting virus was more frequently found in internal organs (6 monkeys) and less frequently in the CNS (3 monkeys). It was mainly located in the spleen, liver, lymph nodes, kidneys. Persistent virus was found twice in the cerebral cortex and subcortical ganglia and once in the spinal cord. No virus was detected in the brain stem at intervals of 90 to 302 days p.i. neither in CP^+ nor in the untreated groups.

No virus persistence was detected at 256, 367 (CP^+ group) or 620 days (untreated group) p.i., respectively.

Tables 3 and 4 demonstrate the isolation of the persisting virus from lymph nodes and spleens of subcutaneously inoculated monkeys in course of infection (102 and 236 days p.i.). The highest functional activity was shown by the persisting virus (agent A-102) isolated from a lymph node of a monkey 102 days p. i. with the Aina/1448 strain (Table 3). Four lines of this agent were obtained in parallel by inoculation of mice with a 10% tissue homogenate as well as by 3 modifications of the co-cultivation method. Virus-specific antigen was demonstrated both in organ impression smears and in co-cultivated cells. The agent was identified by the FA technique and CF tests. Agent 1-102 was moderately virulent for mice which was sufficient for

Table 4. Isolation of TBE virus from the spleen of a CP+ treated monkey 236 days p. i. with the 41/65 strain

Methods of virus detection	Pathogenicity for mice	CPE in SPEV cells		FA technique
		primary	in passages	
1. Tissue homogenate, mice	—			
2. Co-cultivation with SPEV cells in the presence of*				
a) no activator	—	—	—	±
b) dimethylsulphoxide	2/20**	+	±	+
c) BUDR	—	+	n. d.	+
3. Organ impressions				+

* Culture fluid harvested at 13 days was tested.
** Numerator — number of sick mice, denominator — number of inoculated mice.
+ = positive result, ± = irregular detection, — = negative result.
n. d. — not done.

primary isolation but not for regular passaging in these animal species. The agent produced small plaques (1.8—2.5 mm) in CEC culture, no distinct cytopathic effect in SPEV cultures, and CF antigen after inoculation of susceptible cells.

Another persisting agent (41/65-236) was isolated from the spleen of a CP+ monkey subcutaneously inoculated with the 41/65 strain of TBE virus. The persisting virus was detected 236 days p.i. by several methods in parallel: by the FA technique in organ impression smears and by co-cultivation in the presence of dimethylsulphoxide and BUDR (Table 4). Attempts at virus isolation by inoculation of mice with organ homogenates and by co-cultivation of trypsinized cells without activator failed. The isolate produced a CPE in SPEV all cultures, induced disease in some of the inoculated mice after an incubation period of 7 and 11 days, but had no plaque-forming capacity in CEC cultures. The agent 41/65—236 had undergone 6 passages in SPEV cell cultures with irregular CPE; the final material was identified as TBE virus by the FA technique. The isolate could not be passaged in mice by the intracerebral route. It produced no CPE in Syrian hamster kidney cell culture. No haemagglutinin could be detected in the culture fluid of infected SPEV cultures (4 tests).

Discussion

Our studies showed that in asymptomatic infection of *M. rhesus* monkeys after subcutaneous inoculation, TBE virus was distributed largely in the same organs as in the acute infection. At early intervals (3—19 days) infectious TBE virus was isolated largely from the CNS (cerebral cortex, subcortical ganglia, cerebellum, brain stem, spinal cord). At 45 days p.i. the rate of infectious virus isolation decreased. At later intervals, infectious TBE virus could not be detected in the CNS by inoculation of mice with organ homogenate. The observations were continued for up to 620 days p.i. In previous papers (Pogodina *et al.*, 1981*a*, *b*) we described a set of sensitive methods facilitating the detection of persistent TBE virus. Persistence of TBE virus

or virus-specific antigen was observed for 302 days p.i. but infectious virus was isolated only in one case at 102 days from the lymph node of a monkey inoculated with the Aina/1448 strain. Virus was isolated also by means of 3 modifications of the co-cultivation method of trypsinized lymph node cells with sensitive SPEV cells: without activator, with DMSO and with BUDR, and identified in CF tests and by the FA technique as well as by FA examination of impression smears. The technique is known to allow the detection of virus-specific antigen of TBE virus in absence of pathomorphologic lesions in cells and organs and when infectious virus is undetectable by other methods (Han Shi-gie and Pogodina, 1964; Zlotnik *et al.*, 1971). A combination of immunofluorescence, co-cultivation of trypsinized organ cells with sensitive cells in the presence of BUDR and explantation methods allowed the detection of persisting TBE virus, which had lost its infectious and some other properties, at late intervals p.i. (92 to 302 days). The virus was isolated both from internal organs (spleen, liver, lymph nodes, kidneys) and from the CNS (cerebral cortex and spinal cord, subcortical ganglia, cerebellum). However the persisting virus and virus-specific antigen was predominantly located in the internal organs.

Our experiments on CP as immunosuppressor did not confirm the observations by other authors such as a more active virus penetration into the CNS, increased virus titres, exacerbation of the process or prolongation of the incubation period, and longer virus persistence (Nathanson and Cole, 1970; Zlotnik *et al.*, 1971, 1972; Mayer, 1972; Vargin and Khozinsky, 1974; Barinsky *et al.*, 1975; Larina *et al.*, 1977). These discrepancies might have been due to the experimental models used, conditions of CP administration, and methods employed for the detection of persisting virus. It cannot be excluded that the lack of a CP effect may have been associated with a comparatively low dose of the drug used. In our experiments both with and without CP, TBE virus penetrated into the CNS after similar intervals. At early intervals (3—19 days p.i.), against the background of immunosupression, TBE virus was less frequently isolated from the CNS and more so from internal organs than after conventional inoculation of the virus. Rather frequent isolation of virus from the kidneys early after inoculation is noteworthy. Longer persistence could not be observed either: virulent virus could not be isolated after 19 days, persisting virus after 302 days. At later intervals, persisting virus was also predominantly isolated from internal organs. Noteworthy is the more frequent TBE virus detection, against the background of immunosuppression, in the spleen. This is likely to be associated with the damaging effect of CP on lymphocytes which, in turn, may be conducive to the establishment of virus persistence. Zlotnik *et al.* (1971) also observed in guinea pigs treated with CP later and longer isolation of louping-ill virus from the spleen than in the animals given no CP.

No persistent virus could be detected at 356, 367, and 620 days p.i. It is possible that by this time it was eliminated from the body. Our study thus showed that *M. rhesus* monkeys inoculated subcutaneously with TBE virus develop symptomatic infection in which the virus penetrates into the CNS and persists for long periods both in the CNS and, mostly, in internal organs.

References

Asher, D. M. (1979): Persistent tick-borne encephaeitis 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.

Barinsky, I. F., Shubladze, A. K., Tolmacheva, V. D., and Vanag, K. A. (1975): Effect of cyclophosphane on the course of chronic neuroinfections (in Russian), pp. 129—132. In *Arbovirusy*, Moskva.

Dremov, D. P., Yerofeev, V. S., and Miryutova, T. L. (1979): Latent infection of syrian hamsters with attenuated tick-borne encephaeitis virus (in Russian). *Vop. Virus.* **24**, 70—74.

Dubov, A. V., Koshukov, S. D., Grigorieva, L. V., and Kostylev, S. G. (1969): Morphologic changes in CNS of monkeys after intracerebral inoculation of tick-borne encephalitis virus (Yelantsev strain) (in Russian). *Tr. Tyumensk. NII krayev. Patologii* **3**, 63—74.

Elečková, F., Rajčáni, J., and Grešíková, M. (1976): An attempt to detect persistent infection with tick-borne encephalitis virus in dogs. *Acta virol.* **20**, 89.

Han Shi-gie, and Pogodina, V. V. (1964): Use of fluorescent antibody technique in studies on Far East tick-borne encephalitis in mice, hamsters and pigs. *Acta virol.* **8**, 22—29.

Ilienko, V. I., Komandenko, N. I., Platonov, V. G., Prozorova, I. N., and Panov, A. G. (1974*a*): Pathogenetic 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 from different foci (in Russian). *Vop. Virus.* **19**, 414—418.

Kraminskaya, N. N., and Kraminsky, V. A. (1975): Studies on experimental tick-borne encephalitis virus infection in birds (in Russian). In G. P. Somov, V. D. Kostin (Eds): *Virusologicheskie Issledovania na Dalnem Vostoke*, 2, vol. **28**, 37—41.

Larina, G. I., Karpovich, L. G., Levkovich, E. N., and Ugbaeva, T. D. (1977): Study of the ability to persistence in vivo of the viruses of tick-borne encephalitis complex with different degree of neurovirulence. Sn *Abstr. Int. Symp. on Chronic Virus Infections*, Smolenice, 1977, Czechoslovakia.

Mayer, V. (1972): Viral infection and resistance in immunosuppressed host. I. Activation patterns of silent arbovirus infections. *Acta virol.* **16**, 323—335.

Nathanson, N., and Cole, J. A. (1970): Immunosuppression and experimental virus infection of the nervous system. *Advanc. Virus Res.* **16**, 397—448.

Pogodina, V. V., Frolova, M. P., Malenko, G. V., Fokina, G. I., Levina, L. S. Mamonenko, L. L., Koreshkova, G. V., and Ralf, N. M. (1981*a*): 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., Kereshkova, G. V., Rzhakhova, O. E., Bochkova, N. G., and Mamonenko, L. L. (1981*b*): Persistence of tick- or-e encephalitis virus in monkeys. II. Effectiveness of methods used for virus detection. *Acta virol.* **25**, 344—351.

Vargin, V. V., and Khozinsky, V. V. (1974): Effect of CPA on the course of experimental arbovirus infections in mice. (in Russian), pp. 138—143. In *Arbovirusy* **1**, Moskva.

Vorobyova, M. S., Gavrilov, V. I., Dzagurov, S. G., Barsukh, E. A., et al. (1975): Modeling of chronic infection established with an attenuated tick-borne encephalitis virus strain in outbred white mice and syrian hamsters (in Russian), pp. 58—59. *Vop. med. Virus. Abstr. Conf.*, Moskva.

Zlotnik, J., Carter, G. B., and Grant, D. P. (1971): The persistence of Louping ill virus in immunosuppressed guinea pigs. *Brit. J. exp. Path.* **52**, 395—407.

Explanations of Micrographs (Plate XLI)

Fig. 1. Specific fluorescence in spleen impression from monkey No. 8481 (292 days after subcutaneous inoculation with the Aina strain). ×200

Fig. 2. Specific fluorescence in cerebral cortex cells co-cultivated with SPEV cells (monkey No. 8484 autopsied 175 days after subcutaneous inoculation with the Vasilchenko strain)

Fig. 3. Lack of fluorescence in the control SPEV cell culture