Acta virol. **31** : 243—248, 1987

BIOLOGICAL PROPERTIES OF ESTERO REAL VIRUS

D. MÁLKOVÁ, J. HOLUBOVÁ, Z. MARHOUL, K. BLAŽEK

Institute of Parasitology, Czechoslovak Academy of Sciences,
370 05 České Budějovice, Czechoslovakia

Received June 6, 1986

Summary. — The pathogenicity for animals, multiplication characteristics in animals, chick embryos and cells, and antibody formation to the Estero Real (ER) virus are reported.

Key words: Estero Real virus; pathogenicity; multiplication; antibody formation

Introduction

Virological investigation of argasid ticks in Cuba in 1980 resulted in isolation of 8 strains of an antigenically identical virus, called Estero Real (ER) virus (Málková *et al.*, 1985*a*). Serological identification attempts of ER virus showed that, at present, it belonged to antigenically ungrouped viruses and should be considered for a new virus (Málková *et al.*, 1985*b*). Here we report

Table 1. Pathogenicity of Estero Real virus (strain K 329) for experimental animal

Animal	Age (weight)	Inoculation	Course of infection	Virological findings
Mouse	newborn (2 days)	i.c.	death	5.5—7.0*
		i.p.	death	2.5
		s.c.	survival	
	wenaling (8—10 g)	i.c.		
		i.p.	survival	antibodies, viraemia
		s.c.		
	adult (20—22 g)	i.c.		
		i.p.	survival	antibodies
		s.c.		
Golden hamster	newborn	i.c.		
		i.p.	survival	antibodies
		s.c.		
Chicken	2 days	i.c.		
		i.p.	survival	antibodies
		s.c.		

* mouse log LD_{50}/0.01 ml

Table 2. Multiplication of Estero Real virus (strain K 329) in chick embryos

Inoculation (Age of CE)	Inoculum (log LD_{50})	Course of infection	Virus detection	
			organ	virus titre***
Chorioallantoic membrane (10 days)	5.24	survival*	membrane	0.78
			embryo	4.35
Amniotic fluid (10 days)	5.24	survival*	fluid	1.75
			embryo	5.00
Yolk sac (5 days)	5.65	death**	sac	4.83
			embryo	4.77

* Virus control 7th day p.i.
** death on days 3 to 4 p.i.
*** mouse log LD_{50}/0.01 ml

the biological properties of ER virus such as experimental pathogenicity for animals, multiplication in mice, chick embryos and in cell lines and antibody induction.

Materials and Methods

Virus. The K 329 strain of the virus was used in the form of fresh or lyophilized supernatant of mouse brain suspension after centrifugation at 2000 rev/min for 10 min. For virus detection 2—3-day-old mice were infected intracerebrally (i.c.) and observed for 3 weeks; titres were calculated according to Reed and Muench.

Experimental pathogenicity. Susceptibility of mice, golden hamsters and chickens was studied (Table 1). The virus distribution was investigated in 12—15g mice infected into footpad and in 48 hr old chickens infected subcutaneously (s.c.) into occipital area of the head with about 10,000 mouse LD_{50}. The course of infection was followed from 24 hr to 10 days p.i. For each time interval 5 animals were used. In mice the virus was tested in regional lymph nodes, in blood and spleen; in chickens in blood and spleen. For virus detection 10% organ suspensions centrifuged at 2000 rev/min for 10 min were prepared and the blood was mixed with 50 units of heparin per ml. The samples were inoculated into 2-day-old mice intracerebrally (i.c.).

Multiplication in chick embryos (CE) was investigated after inoculation on chorioallantoic membrane, into amnion or into the yolk sac (Lennette and Schmidt, 1969).

Cultivation on cell cultures. The following cell cultures were tested: PS, Cl_4, CV-1, VERO BHK-21, LLC, MK_2, LB, HeLa, XTC, CE cells. The CPE, plaque formation and detection of antigen by immunofluorescence (IF) (Holubová, 1980) were investigated. Cell monolayers infected by adsorption at 37 °C for 60 min were observed for 10 days p.i. For virus titration plaque micromethod on plates was used (De Madrid and Porterfield, 1969; Marhoul, 1980).

Serological methods. Hyperimmune sera were prepared in mice after application of 5 immune doses (first intraperitoneal, then subcutaneous) of mouse brain suspension containing cca. 100,000 LD_{50} of virus in a single dose. In the experiments studying dynamics of antibody formation the blood samples from 5 mice after each immune dose were collected on the 10th and 28th days p.i. Virus-neutralization (VN) and immunofluorescent (IF) antibodies were investigated.

Histology. Brain, spinal cord, lungs, heart, liver, spleen, intestine were investigated. The organs were fixed in 10% formalin and embedded into parafin. The sections were stained with haematoxylin and eosin and according to Giemsa.

Table 3. Susceptibility of cell cultures to Estero Real virus strain K 329

Cell lines	CPE		Plaques	Immunofluorescence		
	Character-ization	Titre $TCID_{50}$/ /0.2 ml	Character-ization	Titre PFU/0.2 ml	Time p.i.	Fluorescent cells (%)
PS-Cl_4	0*		0*		hr 72 day 6	50 80
CV-1	since hr 48^{+++} p.i.	$10^{5.3}$	clear-cut ⌀ 1—2 mm	2.5×10^6	hr 46 hr 72	20—30 90
VERO	since hr 48^{++} p.i.	$10^{4.6}$	very small, difficult to count	$\geqslant 10^5$		ND
LLC-MK_2	since hr 72^{+} p.i.	$10^{3.2}$	very small, turbid	impossible to count	hr 46 day 5	50 90
BHK 21	0		0		hr 72	90—100
LB	0		0		hr 72	<10
HeLa	0		0		hr 72 day 7	0* 0
XTC	since hr 72^{++} p.i.	$10^{5.2}$	very small difficult to count	$\geqslant 10^5$	hr 72	60
primoculture CEC	0 0		0 0		hr 72 day 8	0 0

0* — no CPE, plaques or fluorescence demonstrated.
ND — not done.
0* — no CPE, plaques or fluorescence demonstrated.
$^{+}$, $^{++}$, $^{+++}$ intensity of CPE

Table 4. Multiplication dynamics of Estero Real virus (strain K 329) in CV-1 cells

Time (hours p.i.)	Immuno-fluorescence (% of fluorescent cells)	Virus titre**	
		in cells	in medium
3	neg	ND	ND
6	neg	ND	ND
8	neg	ND	ND
16	0.98	ND	ND
24	4.04	ND	ND
48	26.1	3.28	2.68
72	90*	4.35	3.89
96	100	2.5	3.0

* beginning of CPE
** mouse log LD_{50}/0.01 ml

Results

Experimental pathogenicity and virus multiplication in animals

As can be seen from Table 1, the virus killed suckling mice after i.c. infection in high, after i.p. infection in low dilutions; however, mice infected by s.c. route survived. Most susceptible were 2—3—days-old mice. They died 7—8 days after i.c. infection (reaching 6.5 g of weight by 9—10 days age), mice weighing 8 g (cca. 18-day-old) already survived i.c. infection. Golden hamsters and chickens did not die following any route of infection.

Investigating virus distribution in weaning mice infected by s.c. route, the virus was found in regional lymph nodes from 24 to 120 hr p.i. in titres ≥ 1.5 log, in blood from 48 hr to day 7 in titres ≤ 1.0 log and in the spleen from

Table 5. Neutralizing and immunofluorescence antibodies to Estero Real virus (strain K 329)

Method	Immunization (doses)									
	1		2		3		4		5	
	Antibody control on days p.i.									
	10	28	10	28	10	28	10	28	10	28
NT (log)	0.39	neg	0.26	0.14	1.2	0.56	1.31	1.06	1.38	2.19
PRNT	8	8	8	16	64	64	64	64	128	64
IF	32	16	64	64	256	256	512	512	2048	512

NT = neutralization test; PRNT = plaque reduction neutralization test; IF = indirect immunofluorescence

72 to 120 hr p.i. in titres $\leq$ 1.0 log LD_{50}/0.01 ml. In 48 hr old chicken the virus was detected neither in blood nor in spleen, where the antibodies were found only.

Histological changes were found in brain of mice showing signs of encephalitis. Activation of glial elements often with degenerative changes in nucleus, dystrophy of ganglion cells, vascular mononuclear infiltrates were seen. In two out of five cases myocarditis was observed. No pathological changes were found on other organs.

Multiplication in chick embryo

Embryos survived infection of chorioallantoic membrane and inoculation into amnion up to observation day 7. Virus titres in the suspension prepared from chorioallantoic membranes or in amniotic fluid were low, in the suspension prepared from the embryos during the same experiment they were significantly higher. After infection into the yolk sac, the embryos died on days 3—4 p.i., the virus titres were high in the yolk sac as well as in the embryo (Table 2).

Multiplication in cell cultures

CPE and plaque formation were observed in CV-1, VERO, BHK-21, LLC, MK2 and XTC cells. In all of these viral antigen was demonstrated by IF (Table 3). Most susceptible were the CV-1 cells. CPE on these cells started early; it was distinct, the plaques were clear-cut and the antigen was well demonstrable by IF. The multiplication dynamics studied on CV-1 cells (Table 4) infected with cca. 10,000 mouse LD_{50} showed that the CPE started from 48—72 hr p.i. with the highest titre at 72 hr p.i. As for plaque counting, the 7th day p.i. was the optimal period. Using the above mentioned virus doses viral antigen was found by IF first at 16 hr p.i. in single cells. After 24 hr fluorescence was observed in groups of cells ("nests") and at 72 hr p.i. the majority of monolayer cells showed positive fluorescence. Positive fluorescence was demonstrated in perinuclear cytoplasm first, later on in cytoplasm as a whole (Fig. 1).

Antibody formation

The hyperimmune sera prepared in mice were used. Neutralizing, complement fixing (CF) and IF antibodies were demonstrated. Neutralization indices (NI) in different mouse sera after 5 immunization doses were 2.2—4.0 log. Dynamics of antibody formation in mice as detected by neutralization test, plaque reduction neutralization test and IF method is summarized in Table 5. The most distinct results were found by IF.

Discussion

The presented biological studies with ER virus enabled to characterize some important virus properties which can be well utilized in laboratory work. The experiments showed that the virus multiplied in mice, chick

embryos as well as in different mammalian cells. In contrast, it did not multiply in chickens and chick embryo cell cultures.

The 2—3-day-old suckling mice were found the most susceptible and suitable for virus isolation. Signs of encephalitis were seen by histological examination. The susceptibility of mice decreased depending on the age of animals; mice weighing 8 g did not die after i.c. inoculation. Nevertheless, the virus was found after s.c. infection of weaning mice in regional lymph nodes, blood and spleen. However, the virus titres demonstrated in these organs were low. Virus multiplication reached quite high titres in chick embryos inoculated into the yolk sac. When we followed virus multiplication in cell cultures, the best results were obtained with CV-1 cells. In these the virus induced distinct CPE, clear-cut plaques and virus antigen was well visible by IF in the cytoplasm of cells. We recommend these cells for titration as well as for serological investigations.

Neutralizing, complement fixing (Málková *et al.*, 1985*b*) and IF antibodies were demonstrated in mice. Corresponding serological methods gave reliable results. Following the process of antibody formation, IF antibodies increased quicker and reached higher titres than neutralizing antibodies. The prolonged time interval (28 days) did not play any role in the antibody level in comparison with the antibody level found on day 10 after immunization.

References

De Madrid, A. T., and Porterfield, J. S. (1969): A simple microculture method for the study of group B arboviruses. *Bull. Wld Hlth Org.* **40**, 113—121.

Holubová, J. (1980): Experiences with an indirect immunofluorescence method for identification and serological investigations of certain arboviruses, pp. 527—533. In M. Labuda and C. H. Calisher (Eds): Internat. Symposium *New Aspects in Ecology of Arboviruses,* Smolenice n Bratislava, Slovak Acad. Sci., Bratislava, 1979.

Lennette, E. H., and Schmidt, N. J. (1969): *Diagnostic Procedures for Viral and Rickettsial Infections,* 4th ed., Amer. Publ. Health Assoc. Inc., New York.

Málková, D., Holubová, J., Marhoul, Z., Černý, V., Daniel, M., Fernández, A., De La Cruz, J., and Herrera, M. (1985*a*): Virological investigation of soft ticks in Cuba. *Folia Parasitol.* (Praha) **32**, 95—96.

Málková, D., Holubová, J., Černý, V., Daniel, M., Fernandez, A., De La Cruz, J., Herrera, M., and Calisher, C. H. (1985*b*): Estero Real virus: A new virus isolated from argasid ticks *Ornithodoros tadaridae* in Cuba. *Acta virol.* **29**, 247—250.

Marhoul, Z. (1980): Use of cell cultures for identification of certain less known arboviruses, pp. 521—525. In M. Labuda and C. H. Calisher (Eds): Internat. Symposium *New Aspects in Ecology of Arboviruses,* Smolenice n. Bratislava, Slovak Acad. Sci., Bratislava 1979.

Explanation to Figures (Plate XLIII):

Fig. 1. Immunofluorescence findings in CV-1 cells infected with ER virus strain K 329. Magn × 120
1-I 16 hr p.i.
1-II 72 hr p.i.