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DUAL INFECTION OF *IXODES RICINUS* TICKS WITH TWO VIRUSES OF THE TICK-BORNE ENCEPHALITIS COMPLEX

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Summary. — *Ixodes ricinus* female ticks were inoculated with Skalica (SK) virus (non-pathogenic for adult albino mice by subcutaneous route) and 14 days later they were challenged with strain 198 of Tick-borne Encephalitis (TBE) virus (highly pathogenic for adult albino mice by subcutaneous route). After additional 14 days of incubation, 42.9 to 65.0% of the adult (10—12 g) albino mice infested with these double infected ticks developed antibodies to TBE without signs of sickness (transmission of SK virus), while paralysis or death was registered in 35.0 to 57.1% of infested mice (transmission of strain 198) depending on the concentration of strain 198 used for inoculation of ticks. However, a low degree of interference to superinfection with strain 198 was observed, when the dissected tick salivary glands were examined by subcutaneous inoculation of adult albino mice (more than 90% of examined salivary glands contained strain 198 virus).

Key words: *dual infection*; *interference*; *Ixodes ricinus ticks*

Introduction

Dual infections of vectors with various arboviruses became evident with the evidence of mixed natural foci of arbovirus infections. The mixed foci of different tick-borne virus combinations [e.g. tick-borne encephalitis and Uukuniemi viruses; or tick-borne encephalitis (TBE) and Tribeč virus] were investigated in Slovakia (Nosek *et al.*, 1980; Kožuch *et al.*, 1982; 1987).

The relationship of viruses in double infected vectors and/or vector tissues was studied by many investigators. In cell or organ cultures from ticks no interference of any kind was observed after subsequent infection with the unrelated viruses (e.g. Libíková *et al.*, 1974; Řeháček and Libíková, 1979; Chunikhin *et al.*, 1981; Řeháček, 1987). Similar results have been reported in mosquitoes infected with alphaviruses and flaviviruses (Chamberlain and Sudia, 1957; Lam and Marshall, 1968).

Inhibition of the replication of alternate California serogroup bunyaviruses in *Aedes triseriatus* mosquitoes has been observed for mosquitoes previously

infected with the La Crosse virus. By contrast, prior to infection of mosquitoes with La Crosse virus it did not interfere significantly with the subsequent infection and replication of Guaroa bunyavirus (Bunyamwera serogroup), or heterologous viruses such as West Nile flavivirus, or vesicular stomatitis rhabdovirus (Beaty *et al.*, 1983). The onset of interference was time dependent. The majority of mosquitoes infected with the temperature-sensitive mutant of La Crosse virus were resistant to superinfection with wild type at 72 hr post-infection (p.i.) but not within 24 hr p.i. (Sundin and Beaty, 1988).

The importance of the study of dual infection of ticks with the closely related viruses of TBE complex raised again after isolation of peripherally non-pathogenic Skalica (SK) virus belonging to this complex (Grešíková *et al.*, 1976) in the area where natural foci of TBE virus of western type (-Central European Encephalitis-CEE) are present (Kožuch *et al.*, 1983).

Materials and Methods

Viruses. Two viruses of the tick-borne encephalitis complex differing in their neurovirulence for adult albino mice after peripheral inoculation were used throughout the study. Skalica (SK) virus isolated from the blood of bank-vole (*Clethrionomys glareolus*) caught in the locality Radimov near Skalica town in western Slovakia (Grešíková *et al.*, 1976) was used in its fourth mouse intracranial (i.c.) passage; this virus is non-pathogenic for adult albino mice (SPF, farm Černý Vůl) following subcutaneous (s.c.) inoculation (Grešíková and Sekeyová, 1980; Rajčáni and Grešíková, 1982). The strain 198 isolated from *Ixodes ricinus* nymphs in the locality Gbelce in southern Slovakia (Kožuch *et al.*, 1982) highly pathogenic for adult albino mice following the s.c. inoculation was used in its twenty second mouse i.c. passage.

Ticks. For the experimental purposes *Ixodes ricinus* adults were collected by flagging the vegetation in the locality Rusovce near Bratislava, where CEE virus was never isolated. The collected ticks were kept in the test tubes at 4 °C until used (up to 14 days). Infected tick females were kept in the test tubes at the ambient temperature. A few leaves of the fresh grass were added to the each tube and were changed in a few days intervals to keep the humid environment inside the test tubes.

Infection of ticks and transmission experiments. Tick females were infected parenterally into the membrane between the coxa and trochanter of the second left leg by the means of the microcapilary. All ticks (with the exception of control groups) were inoculated with 0.2 µl of 20,000 suckling mouse (SM) i.c. LD_{50} of SK virus. After 14 days incubation at ambient temperature the ticks were challenged with 0.2 µl of 2, 20, and 200 SMic LD_{50} of strain 198. One control group of ticks was inoculated with 0.2 µl of PBS only and after 14 days of incubation was infected with 2, 20, and 200 SM i. c. LD_{50} of 198 virus. The other control group of ticks was inoculated with 20,000 LD $_{50}$ of SK virus and then with PBS only. After additional 14 days of incubation infected ticks were allowed to feed on adult (10—12 g) albino mice. Infected females were accompanied with uninfected males and each pair was placed individually into a transparent capsule fixed on the back of the mouse (Nosek, 1965). Tick females were allowed to feed to repletion. The mice exposed to the tick bite were observed for the symptoms of illness or death and after 21 and 28 days two samples of the blood were taken from sinus orbitalis of surviving mice for the detection of neutralizing (N) antibodies to CEE virus.

Sera from the blood of mice were assayed against 100 CPD_{50} of CEE virus in the cloned PS porcine epithelial cells, in which this virus exert a cytopathic effect (Kožuch and Mayer, 1975).

Tick salivary glands. The salivary glands of engorged and by mice not removed ticks in the second of two performed experiments were dissected and processed for virus isolation. Suspensions of salivary glands of individual ticks were prepared in 0.5 ml of MEM supplemented with 10% heat-inactivated calf serum and antibiotics. With 0.01 ml of 10-fold diluted suspension 1—3 days old albino mice were i.c. inoculated and 0.1 ml of the same diluted suspension was paralelly s.c. inoculated into 10—12 g albino mice. The suckling and adult mice were observed for the symptoms of illness for 14 and 21 days, respectively.

Table 1. Transmission of virus to adult albino mice by Ixodes ricinus females inoculated with (a) 20,000 suckling mouse (SM) i.c. LD_{50} of Skalica virus (non-pathogenic for adult mice inoculated peripherally), (b) 20,000 SMic LD_{50} of Skalica virus and, subsequently, 2, 20, or 200 SMic LD_{50} of the pathogenic strain 198, (c) 2 or 20 SMic LD_{50} of strain 198

		Apparent/inapparent infections	Per cent transmission	
			Skalica	198
(a)		0/17	100	—
(b)	2	7/13	65.0	35.0[a]
	20	9/12	57.1	42.9[a]
	200	8/6	42.9	57.1[b]
(c)	2	10/0	—	100
	20	10/0	—	100

[a] Two ticks from this portion of the study did not transmit virus, based on absence of neutralizing antibody in mice on which these ticks were fed.

[b] One tick from this portion of the study did not transmit virus, based on absence of neutralizing antibody in the mouse on which this tick was fed.

Results

Transmission experiments

Out of 17 ticks inoculated with SK virus only, all transmitted the virus to mice in two feeding experiments as confirmed by N-antibodies. No mouse died, neither developed symptoms of illness.

Three different virus concentrations were used to inoculate the ticks with strain 198 only. All 10 mice biten by ticks inoculated with 20 SMic LD_{50} died 10 to 15 days from the beginning of the tick feeding. Out of 11 mice biten by ticks inoculated with 2 SMic LD_{50} died 9, 1 developed paralysis of the hind legs but survived and the serum of the last one was negative in VN test.

The third group consisted of ticks inoculated with SK virus and subsequently challenged with the 198 strain. Together 60 *Ixodes ricinus* females inoculated with 20,000 SMic LD_{50} of SK virus and with three different concentrations of 198 strain were used in the two transmission experiments. Out of 15 mice fed upon by ticks inoculated with 200 SMic LD_{50} of 198 strain three died, 5 became paralysed, 6 developed N-antibodies and 1 was negative. Out of 23 mice on which ticks inoculated with 20 SMic LD_{50} of 198 strain were fed, 6 died, 3 became paralysed, 12 developed antibodies, and 2 were negative. Out of 22 mice in contact with ticks inoculated with 2 SMic LD_{50} of 198 strain died 3, 4 were paralysed, 13 developed antibodies and two were negative.

Control mice in contact with ticks inoculated with SK virus developed N-antibodies only. The other control group of mice in contact with ticks inoculated with 198 strain only, died or were paralysed in 100%. Mice infested with double infected ticks developed antibodies without signs of illness

in 42.9 to 65.0%. Paralysis or death was registered in 35.0 to 57.1% accordin to the concentration of 198 strain used for inoculation of ticks. Out of 2 apparently infected mice with ticks dually inoculated 12 (50%) survived Out of 20 apparently infected mice with ticks inoculated only with 19 strain 1 mouse (5.0%) survived.

Table 1 summarizes the results of both transmission experiments accordin to the apparent and inapparent infections of mice and mice with negativ N-antibodies, which was regarded as negative transmission.

Examination of tick salivary glands

Out of 6 tested salivary gland suspensions of double infected ticks whic transmitted virus to the mice developing subsequent inapparent infectio (SK virus transmission), all were virus-positive as expected (all i.c. inoculate suckling mice died). Out of these however, 3 were negative when tested b s.c. inoculation of adult albino mice (only SK virus was present); 2 sample came from ticks challenged with 2 SMic LD_{50}, and 1 from a tick challenge with 200 SMic LD_{50} of 198 strain. In the other salivary gland suspensions 19 strain was present, as confirmed by the death of adult mice inoculated by s.c route. Even when present, 198 strain was not transmitted by these tick

Discussion

Interference between mosquito-borne arboviruses is a well documented but incompletely understood phenomenon. Infection of mosquitoes with mutant Sindbis virus has been shown to inhibit replication of a superinfectin wild-type Sindbis virus (Peleg, 1975). Prior to infection of *Culex annulirostri* mosquitoes with a *ts* mutant of Semliki Forest virus also completely sup pressed subsequent infection by wild-type parental virus (Davey *et al.*, 1979) It has also been shown that infection of mosquitoes with mutant flaviviruse interfered with superinfection by wild-type strains of flaviviruses (Altman 1963; Rozemboom and Kassira, 1969).

Our results demonstrated that *Ixodes ricinus* ticks infected parenteral with peripheraly non-pathogenic SK virus are less susceptible to infectio with, and are less capable of transmitting the highly pathogenic 198 TBI virus strain.

The reduction in the transmission rate of the subsequently inoculated viru was high (42.9 to 65.0%), but the reduction in the infection rate of salivar glands by the second virus was low (less than 10%).

The role of observed interference in ticks remains to be demonstrated afte oral infection by feeding on viremic hosts in the stage of larva and nymph or nymph and imago. The molecular basis of such kind of interference i unknown. In view of the specificity of the interference it is possible that th initial infection induce defective interfering particles (Huang *et al.*, 1980) Alternate explanations could include alteration of viral receptors on targe cells or induction of antiviral activity in the infected ticks, or mosquitoe respectively (Riedel and Brown, 1979).

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