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EXPERIMENTAL DOUBLE INFECTION WITH *COXIELLA BURNETII* AND TICK-BORNE ENCEPHALITIS VIRUS IN *DERMACENTOR RETICULATUS* TICKS

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Summary. — Experimental parenteral inoculation of *Dermacentor reticulatus* ticks with the rickettsia *Coxiella burnetii* (*C.b.*) and tick-borne encephalitis (TBE) virus resulted in a generalized rickettsial and viral infection, irrespective of whether the agents were given simultaneously or by a 7 days interval apart. Both agents multiplied intensively in ticks, *C.b.* being detectable predominantly in cytoplasmic vacuoles and TBE virus mostly in the endoplasmic reticulum.

Key words: Dermacentor reticulatus ticks; mixed infection; Coxiella burnetii; tick-borne encephalitis virus

Introduction

During feeding on their hosts tick may naturally receive microorganisms from the blood. These can be inactivated in the gastrointestinal tract of the arthropod, may persist there passively being excreted into the environment during further feeding or may penetrate through the gastrointestinal tract wall into the body where they multiply and eventually undergo further development, depending on the relationship between the tick and the microorganism developed by evolution.

Relatively frequently ticks use to be infected simultaneously with several microbial species, most often with different bacterial species. Very nearly all tick species become the hosts of rickettsia-like organisms, which occur almost exclusively in the ovaries and Malpighian tubes. Comparatively frequent are mixed infections of ticks with rickettsiae, namely with *Rickettsia sibirica* and *C.b.* (Daiter, 1979). Mixed microbial tick infections represent an interesting biological phenomenon from the standpoint of mutual interactions of these microorganisms in the arthropods, including the possible inhibition of transmission of individual pathogens on the vertebrate host.

In our search for rickettsiae in the ticks we suceeded in detection of dual infections of *Dermacentor marginatus* ticks with *Rickettsia slovaca* and bacillary rickettsia-like organisms (Řeháček *et al.*, 1976) or with *R. slovaca* and

a virus probably from family Bunyaviridae (Diehl *et al.*, 1980) as well as of *Ixodes ricinus* ticks with *R. slovaca* and a microsporidian designated *Nosema slovaca* (Weisser and Řeháček, 1975). These findings led us to investigate mixed infection of the ticks with *C.b.* and TBE virus trying to solve the principal question whether these two agents can simultaneously multiply in their vector both at the organ and cellular levels.

Materials and Methods

Ticks and microorganisms. Experiments were carried out on *D. reticulus* females collected in the vicinity of Bratislava. The ticks were examined by the haemocyte test for the presence of rickettsiae and rickettsia-like organisms to exclude positive individuals from further experiments. The ticks after feeding for 5 days on healthy guinea pigs were inoculated intracoelomally with *C.b.* and TBE virus either simultaneously or apart in a 7 days interval. For infection we used phase I *C.b.* strain Nine Mile (with 3 egg passages) and the Skalica strain of TBE virus (in the 4th mouse brain passage; Grešíková *et al.*, 1976); 0.01 ml volumes of brain-heart infusion (BHI) contained 10^4 EID_{50} of *C.b.* and 10^4 mouse LD_{50} of TBE virus, respectively.

Detection of C.b. and TBE virus in the ticks. To detect the agents under study in the tick bod y the haemolymph, organs and whole-tick suspensions always of 3 ticks were examined in each group at 2—6 day intervals on days 7—20 post-infection (p.i.) starting from the second inoculation day. The amount of TBE virus and *C.b.* in the tick haemolymph and individual organs was estimated by immunofluorescence technique (IFT) and expressed in 4 degrees based on the number of fluoresceing cells and intensity of fluorescence; in the case of *C.b.* Gimenez staining was used for evaluation of the amount of rickettsiae in the field of view as described by Kazár *et al.* (1973). The yields of TBE virus and *C.b.* from whole-tick suspensions were assayed in suckling white mice by determining the LD_{50}/0.01 ml values of TBE virus after intracerebral inoculation and the ID_{50}/0.01 ml of *C.b.* after combined intracerebral and intraperitoneal inoculations.

Electronmicroscopic examination. The samples of haemolymph and of the most frequently infected organs (ovaries, hypodermis and salivary glands) were examined further electronmicroscopically to localize more precisely the site of multiplication of either agent and to determine whether both agents multiply in the same cell. The tissues were fixed by 2.5 % glutaraldehyde in 0.2 mol/l sodium cacodylate buffer, pH 7.2, for 60 min at 4 °C, washed in the same buffer for 20 hr and postfixed for 60 min in 1 % OsO_4 in this buffer at room temperature. Fixed material was dehydrated in increasing concentrations of acetone and embedded into Araldit. Ultrathin sections were stained by uranyl acetate and lead citrate, and examined in the electron microscope Philips EM300 at 80 kV. Because of detection of the agents by this method is laborious and time consuming, the samples of tick tissues were examined electronmicroscopically only on days 7 and 20 p.i. with the second inoculated agent.

Results

The results of three experiments are summarized in Tables 1—3. As to the *C.b.* detection, in Tables are given only the results obtained by Gimenez staining which was found more suitable than IFT for quantitative determination of namely lower amounts of this rickettsia. Nevertheless, each finding was verified by IFT, the number of fluoresceing particles and intensity of fluorescence roughly corresponding to the number of rickettsiae as determined by Gimenez staining.

In the experiment in which primary *C.b.* infection were followed 7 days later with TBE virus inoculation, a rapid multiplication of the former agent was observed in all tick organs except of the intestine. In the haemolymph starting from the 4 th day after second inoculation and in organs from 6—13

Table 1. Mixed C. burnetii and TBE virus infection in D. reticulatus ticks inoculated 7 days earlier with the former agent

Tick inoculated with	Tick organs	Days p.i. 2	4	6—7	13	18—20	Examination**
C. burnetii + TBE virus	haemolymph	2/—	3*/—	4*/1	4*/1	4*/2	Gimenez staining/ IFT
	hypodermis	2/1	1 /—	4*/1	4*/1	4*/2	
	Malpighian tubes	—/—	—/1	2/—	1/—	2*/—	
	ovaries	1/—	—/—	3*/—	4*/—	4*/—	
	intestine	—/—	—/—	1/—	1/—	1/—	
	salivary glands	—/—	2/1	4*/—	4*/1	4*/1	
	neural ganglion	1/—	—/—	3*/—	4*/1	3*/—	
	Gene's organ	1/—	—/—	1/—	2/—	3*/—	
	whole tick suspension (in log EID_{50}/ /LD_{50} units)	6.3/1.3	> 7.0/2.7	> 7.0/2.3	> 7.0/2.3	> 7.0/3.5	
C. burnetii + BHI	haemolymph	2	3*	4*	4*	4*	Gimenez staining
	hypodermis	1	1	4*	4*	4*	
	Malpighian tubes	—	1	1	3*	4*	
	ovaries	1	1	3*	3*	4*	
	intestine	1	—	3*	2	3*	
	salivary glands	—	1	4*	3*	4*	
	neural ganglion	—	—	2*	3*	4*	
	Gene's organ	—	—	2*	3*	4*	
	whole tick suspension (in log EID_{50} units)	6.0	7.0	6.7	> 7.0	> 7.0	
BHI + TBE virus	haemolymph	1	1	1	1	2	IFT
	hypodermis	—	—	1	1	2	
	Malpighian tubes	—	—	—	—	1	
	ovaries	—	1	—	—	1	
	intestines	—	—	—	—	1	
	salivary glands	—	1	1	1	1	
	neural ganglion	—	—	1	1	1	
	Gene's organ	—	—	—	—	1	
	whole tick suspension (in log LD_{50} units)	2.0	2.5	3.0	2.7	2.7	

* Foci of C. burnetii

** Scoring of Gimenez staining and immunofluorescence: (4) — uncountable number, (3) — — hundreds, (2) — tens, (1) — single, (—) — none rickettsiae in the field of view.

Table 2. Mixed C. burnetii and TBE virus infection in D. reticulatus ticks inoculated 7 days earlier with the latter agent

Ticks inoculated with	Tick organs	Days p.i. 2	4	6—7	13	18—20	Examination**
TBE virus + C. burnetii	haemolymph	—/2	—/3	2/3*	1/4*	1/4*	IFT and Gimenez staining
	hypodermis	—/—	—/1	1/3*	1/4*	1/4*	
	Malpighian tubes	1/1	—/1	1/2	—1/4*	1/4*	
	ovaries	—/—	1/1	1/2	1/3*	2/4*	
	intestine	1/—	—/—	1/2*	—/2	1/3*	
	salivary glands	—/—	—/2	—/3	—/4*	1/3*	
	neural ganglion	—/—	—/—	—/3*	—/2*	1/4*	
	Gene's organ	—/—	—/—	—/1	—/4*	1/4*	
	whole tick suspension in log EID_{50}/LD_{50} units)	6.5/2.0	> 7.0/2.7	6.7/3.5	> 7.0/4.0	> 7.0/3.5	
TBE virus + BHI	haemolymph	1	—	2	2	3	IFT
	hypodermis	1	1	2	1	2	
	Malpighian tubes	1	—	1	—	1	
	ovaries	1	—	2	1	1	
	intestine	—	—	1	—	—	
	salivary glands	1	—	1	—	2	
	neural ganglion	—	—	—	—	2	
	Gene's organ	—	—	1	—	2	
	whole tick suspension (in log EID_{50} units)	2.7	3.5	4.5	4.5	4.5	
BHI + C. burnetii	haemolymph	2	3	3*	3*	4*	Gimenez staining
	hypodermis	1	1	3*	3*	4*	
	Malpighian tubes	1	1	1	2	4*	
	ovaries	1	1	1	4*	4*	
	intestines	—	—	1	2*	4*	
	salivary glands	1	1	2*	4*	4*	
	neural ganglion	1	1	2	3*	4*	
	Gene's organ	1	1	2	3*	4*	
	whole tick suspension (in log LD_{50} units)	6.7	6.7	6.5	> 7.0	> 7.0	

* and ** — see explanations in Table 1.

to 20 days, massive (4 crosses) multiplication of *C.b.* was accompanied by the formation of foci of rickettsia (Figs. 1 and 2). Almost similar situation occurred in control ticks which were inoculated with BHI 7 days after the primary *C.b.* infection. Gradual multiplication of TBE virus in double-infected ticks was detected namely in hypodermis, salivary glands and sporadically in

Table 3. Mixed C. burnetii and TBE virus infection in D. reticulatus ticks inoculated simultaneously with both agents

Tick inoculated with	Tick organs	Days p.i. 2	4	6—7	13	18—20	Examination**
C. burnetii + TBE virus	haemolymph	1/2	1/2	3*/2	3*/1	4*/2	Gimenez staining and IFT
	hypodermis	1/1	1/2	3*/2	3*/1	4*/2	
	Malpighian tubes	—/1	1/2	2/2	2/—	3/2	
	ovaries	—/1	1/1	2*/1	3*/1	4*/2	
	intestine	—/—	—/2	1/2	—/—	2/1	
	salivary glands	—/1	1/2	2*/2	3*/—	4*/2	
	neural gnaglion	—/—	—/—	1*/—	3*/—	4*/1	
	Gene's organ	—/—	—/—	1/—	3/1	2*/—	
	whole tick suspension (in log EID/LD_{50} units)	6.5/1.5	>7.0/2.0	>7.0/2.5	>7.0/2.5	>7.0/2.5	
C. burnetii + BHI	haemolymph	1	2	3	3*	4*	Gimenez staining
	hypodermis	1	1	2	3*	4*	
	Malpighian tubes	—	1	—	1	2	
	ovaries	—	1	2	2	2*	
	intestine	—	—	1	—	1	
	salivary glands	—	1	1	3	4*	
	neural ganglion	—	1	—	2	2*	
	Gene's organ	1	1	—	1	2*	
	whole tick suspension (in log EID_{50} units)	6.3	7.0	>7.0	>7.0	>7.0	
TBE virus + BHI	haemolymph	1	2	1	1	1	IFT
	hypodermis	1	2	1	1	2	
	Malpighian tubes	—	2	—	1	2	
	ovaries	—	2	1	1	1	
	intestine	—	1	2	1	2	
	salivary glands	1	2	1	1	1	
	neural ganglion	—	—	—	1	1	
	Gene's organ	—	—	—	—	—	
	whole tick suspension (in log LD_{50} units)	1.5	1.5	2.5	2.7	3.0	

* and ** — see explanations in Table 1.

Malpighian tubes and neural ganglion. In control ticks, i.e. in those infected with TBE virus 7 days after BHI inoculation, sporadic detection of the virus in the haemolymph and some organs was followed by a distinct viral multiplication in the haemolymph and all organs tested on days 18—20 p.i. No

significant differences in the yields of both *C.b.* and TBE virus were noticed irrespective of whether the whole-tick suspensions were titrated from the groups of double- or single-infected ticks (Table 1).

The primary tick inoculation with TBE virus followed 7 days later with *C.b.* inoculation resulted in a chronologically increasing massive infection by either agent of the haemolymph and all organs tested. The virus multiplication in dually infected ticks did not differ substantially from control ticks, i.e. those which were inoculated first with TBE virus and then with BHI. A similar result was obtained with *C.b.* which multiplied to approximately the same extent in dually infected ticks and control ticks infected by *C.b.* only. Again, no marked quantitative differences in the titres of TBE virus as well as of *C.b.* were found when titrating the suspensions of whole-ticks double infected or single-infected with one agent during the interval of 2—20 days after second inoculation.

Simultaneous inoculation of ticks with both agents led to a massive multiplication of *C.b.* from the 6th day p.i. in the haemolymph and all organs except of the intestine and Malpighian tubes, in which no focus formation was observed. TBE virus was detected in the haemolymph and gradually in all tick organs except the Gene's organ. Differences were found neither qualitative, i.e. in distribution of *C.b.* and TBE virus in the organs tested, nor quantitative, i.e. in the yield of either agent from the whole-tick suspensions, between the groups of double-infected ticks as compared to those infected with one agent only.

Electronmicroscopic examination of samples from all groups of infected ticks under study revealed the presence of either agent both in the haemolymph and other organs tested, the agents being the most frequently found in the haemolymph and hypodermis. The results obtained demonstrated the multiplication and accumulation of *C.b.* and TBE virus directly in the same individual cells, but interestingly enough in different cell areas. TBE virus multiplied on the ribosomes of rough endoplasmic reticulum, the mature virus particles accumulating in the vacuoles of smooth endoplasmic reticulum. The cisternae of rough endoplasmic reticulum were dilated (Fig. 3). *C.b.* multiplied exclusively in the cytoplasmic vacuoles of infected cells (Fig. 4). In some cells also giant vacuoles containing *C.b.* and an invaginated part of cytoplasm with replicating TBE virus were observed (Fig. 5). The vacuolized cytoplasm of infected cells contained moreover the vacuoles with *C.b.* corpuscles and mature TBE virus particles without any signs of further multiplication, which were probably prepared for the release from the double-infected cell (Fig. 6).

It can be concluded that results of all the three experiments showed that during dual infection with *C.b.* and TBE virus of *D. reticulatus* ticks no substantial inhibition of multiplication of one agent by another occurred. Either agent multiplied chronologically in the haemolymph and in the organs tested, regardless of the fact which agent had been introduced first, and the whole event manifested finally in a form of generalized infection of the tick host by both pathogens.

Discussion

Double infections in ticks are investigated mainly from the view of possible interference between the pathogens. Comparatively great attention was paid to double infection of ticks with *Rickettsia sibirica* and *C.b.*, with rickettsiae and TBE virus or *Borrelia recurrentis*. In these studies *C.b.* always appeared as the most and *B. recurrentis* as the least active pathogen. The results of such studies depended on the tick species, its developmental stage and physiological state during the experiment, the interval between inoculation of the pathogens, on their properties, namely their virulence, and naturally on the amounts applied (reviewed by Daiter, 1979).

Of interest was the finding of the workers of Rocky Mountain Laboratory in Hamilton (Montana, U.S.A.), who succeeded in the discovery of a non-pathogenic rickettsia-like organism named "East side agent" in *Dermacentor andersoni* ticks which were collected in eastern side of the Bitterroot Valley. The organism undistinguishable morphologically and serologically from the pathogenic *Rickettsia rickettsii* is nonpathogenic for small rodents and guinea pigs. However, by occupation of the cells and tissues of some inner tick organs it prevents an invasion of pathogenic rickettsiae into the ovaries, resulting in inability of the tick to transmit pathogenic rickettsiae transovarially (Burgdorfer *et al.*, 1980).

Electron microscopic examination of the haemolymph and organs of ticks double-infected with *C.b.* and TBE virus revealed that both agents multiplied and accumulated in cells and tissues of *D. reticulatus* tick. Similar observation of mixed infection by TBE and Powassan viruses in the explants of *Hyalomma anatolicum* and *H. dromedarii* ticks was reported by Chunikhin *et al.* (1984).

As control of double infection in our experiments served inoculation by BHI before or after single administration of the another infectious agent to exclude the possibility of (re)activation of rickettsia-like organisms in the organs of their arthropod host caused by BHI or by the injection only. Even though such organisms are distinguishable morphologically or by IFT from pathogenic rickettsiae, they could have led to misinterpretation of the results of electron microscopic examination. Nevertheless, no invasion of these organisms into the haemolymph and tick organs was observed.

We used an artificial (by injection) and not a natural (by blood-sucking) way of tick infection. This way was chosen for sake of simplicity of our investigation, based on the observation of relatively frequent mixed occurrence of different infectious agents in the haemolymph and organs of ticks collected in the field, which always disseminated only following the alimentary infection after overwhelming the gastrointestinal tract barrier. The method of parenteral infection of half-engorged ticks with viruses and rickettsiae we successfully employed already earlier (Řeháček, 1966; Řeháček *et al.*, 1971) and in our presented experiments it has been demonstrated to be useful again. The suitability of the method used was confirmed also by separate titrations of TBE virus from several individual tick females (infected with TBE virus only), which in most cases were found identical.

We are aware of the fact that determination of the yield of *C.b.* and TBE virus from all tick organs would be more exact than subjective visual evaluation of their infestation with either agent based on the IFT and Gimenez stainings, respectively. This requirement, however, because of the extent of such experiments was hardly possible, so that the whole-tick suspensions were employed to determine the titres of the agents in infected ticks.

Even though the amounts of *C.b.* and TBE virus used for double or single tick infection were the same, i.e. 10^4 EID_{50} and $LD_{50}/0.01$ ml, respectively, in all cases a massive multiplication of *C.b.* but markedly less replication of TBE virus was observed apparently due to the properties of the infectious agents and their relationship to the tick species itself. Although *D. reticulatus* tick does not belong to the important vectors of TBE virus and *C.b.*, it was proved to be susceptible to both pathogens.

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Legend to Figures (Plates XVIII—XXIII):

Fig. 1 and *2*. Hypodermal cells containing foci of *C. b.* on day 13 p.i. with this agent only (1200×).

Fig. 3. Portion of the cytoplasm of hypodermal cell 7 days after simultaneous infection with *C.b.* and TBE virus. Dilated cisternae of rough endoplasmic reticulum (ER) and maturing particles of TBE virus on the ribosomes (arrows). Cisterna of smooth ER with TBE virus particles (81,000×)

Fig. 4. Portion of tick haemocyte cytoplasm first infected with TBE virus and 7 days later with *C.b.*: multiple vacuoles containing *C.b.* 7 days after inoculation with the latter agent (16,000×).

Fig. 5. Cytoplasmic vacuole in tick haemocyte first infected with TBE virus and 7 days later with *C.b.* Invaginated cytoplasm with multiplying and mature particles of TBE virus and *C.b.* cells 7 days after inoculation with the latter (50,000×).

Fig. 6. Cytoplasmic vacuole in tick haemocyte infected first with TBE virus and 7 days later with *C.b.* Mature particles of TBE virus and *C.b.* cells 20 days after inoculation with the latter (31,250×).