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A RICKETTSIA-LIKE ORGANISM SHOWING POSITIVE IMMUNOFLUORESCENCE WITH ANTISERA TO *COXIELLA BURNETII* IN *HAEMAPHYSALIS INERMIS* TICKS

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Summary. — A rickettsia-like organism (RLO) was detected in the oocytes of *Haemaphysalis inermis* ticks. The RLOs were about 5 μm long bent rods with a definite inner structure. They were Gram-negative and could be visualized by Giemsa but not by Gimenez staining. Attempts to cultivate the RLO in chick embryo yolk sacs, various types of cell culture and tick body cavities were unsuccessful. The RLO displayed a bright immunofluorescence with antisera to *Coxiella burnetii*, but no immunofluorescence was obtained with antisera to representatives of typhus and spotted fever group rickettsiae, with the exception of very weak fluorescence with serum from a rabbit immunized with *Rickettsia akari* and one serum from *Apodemus flavicollis* immunized with *Rickettsia conorii*. These findings should be taken into consideration when studying the infestation of ticks with rickettsiae.

Key words: rickettsia-like organism; *Coxiella burnetii*; *Haemaphysalis inermis ticks*; *immunofluorescence*

Introduction

Examination by Gimenez staining and immunofluorescence of *Haemaphysalis inermis* females for the presence of rickettsia revealed organisms morphologically and antigenically similar to *Rickettsia slovaca*. Giemsa staining of parallel smears from ovaries of the same ticks disclosed rickettsia-like organisms (RLO) showing a distinct immunofluorescence with antisera to *Coxiella burnetii*. This observation was repeatedly confirmed in the course of a 5-year period (1976—1980). There arose the question as to whether these RLO represented *C. burnetii* undetectable in the tick ovaries by Gimenez staining or whether they represented RLOs sharing some antigenic determinants with *C. burnetii*. We are presenting the results of our studies aimed at the elucidation of this problem.

Materials and Methods

H. inermis ticks were collected in the Krupinská vrchovina hills (central Slovakia) and Pohronský Inovec mountains (West Slovakia). Our own laboratory breeds of *Dermacentor reticulatus*,

Table 1. Detection of RLOs in oocytes of H. inermis ticks by immunofluorescence with rabbit antirickettsial sera

Rabbit immunized with	Homologous antibody titre*	Fluorescence shown by RLO
C. burnetii (RSA)	512 (phase I)	bright
	4096 (phase II)	bright
C. burnetii (Florián)	256 (phase I)	bright
	4096 (phase II)	
R. akari (Kaplan)	16	very weak
R. sibirica (Netsvetaev)	128	none
R. slovaca (strain B)	64	none
R. typhi (Wilmington)	32	none
R. canada	32	none

*Titres to *C. burnetii* antigens were determined in the MA test, to antigens of other rickettsiae in the CF test.

D. marginatus, *Ixodes ricinus* and *Hyalomma dromedarii* ticks served as controls. Unengorged or halfengorged females fed on quinea pigs or rabbits were used throughout.

For *isolation experiments*, 20 % suspensions in phosphate buffered saline from ovaries of half-engorged females were inoculated in chick embryo yolk sacs (0.25 ml), monolayer cultures of primary chick embryo cells and L and HeLa cell lines, primary *H. dromedarii* tick cell cultures and organ cultures from *H. dromedarii*, *D. reticulatus* and *H.inermis* ticks (0.2 ml per culture) and into the body cavities of H. dromedarii and D. reticulatus (0.01—0.1 ml depending on the tick size). Chick embryo yolk sacs were harvested either from embryos killed between the 4th—10th day after infection (p.i.) or from the remaining live embryos on the 10th day p.i. Cell and organ cultures were observed daily up to the 14th day p.i. Inoculated ticks were examined by the haemocyte test 3 weeks p.i. The ovarial suspensions were checked for bacterial contamination on blood agar and in thioglycolate broth.

To visualize the RLOs, staining according to Gram, Giemsa and Gimenez was employed. Great attention was paid to the indirect immunofluorescence technique in order to determine antigenic relations of the RLOs to known rickettsial species. Sera from rabbits and different small rodents immunized with phase I *C. burnetii* strains RSA and Florián and representatives of typhus (strain Breinl of *R. prowazekii*, strain Wilmington of *R. typhi* and *R. canada*) and spotted fever group (strain Netsvetayev of *R. sibirica*, strain Simko of *R. conorii*, strains B and R 42 of *R. slovaca*, and strain Kaplan of *R. akari*) rickettsiae were employed. Their homologous antibody titres in the microagglutination (MA) test (*C. burnetii*) and in the complement-fixation (CF) test (other rickettsial species) varied from 256 to 4096 and from 32 to 512, respectively (see Tables 1 and 2). To avoid non specific fluorescence, the sera were absorbed with 20 % suspensions of *D. reticulatus* and *H. dromedarii* ticks.

Immunogenicity of RLOs was tested by determining the antibody response by immunofluorescence in white mice and *Apodemus flavicollis* immunized with ovarial suspensions of *H. inermis* ticks. The suspensions prepared from 100 females were injected three times at weekly intervals and the sera were harvested 7 days after the 3rd dose.

For preparation of ticks for electron microscopy see Řeháček *et al.* (1976*b*).

Results

During routine examination of ticks, besides rickettsiae similar to *R. slovaca*, a RLO was detected in *H. inermis* females collected in central and west Slovakia. It occurred in great amounts exclusively in the oocytes of each

Table 2. Detection of RLO in H. inermis oocytes by immunofluorescence with antirickettsial sera from small rodents

Rodent and antigen used for immunization		Homologous antibody titre*	Immunofluorescence shown by RLO
Mus musculus	*C. burnetii* (RSA)	1024	bright
White mouse	*C. burnetii* (RSA)	1024	bright
White mouse	*C. burnetii* (RSA)	256	bright
White mouse	*R. akari* (Kaplan)	64	none
Apodemus flavicollis	*R. akari* (Kaplan)	512	none
M. musculus	*R. akari* (Kaplan)	512	none
A. flavicollis	*R. slovaca* (R 42)	256	none
A. flavicollis	*R. sibirica* (Netsvetaev)	128	none
A. flavicollis	*R. conorii* (Simko)	128	wery weak
Clethrionomys glareolus	*R. conorii* (Simko)	128	none
A. flavicollis	*R. prowazekii* (Breinl)	256	none

*Titres to phase II *C. burnetii* antigen in the MA test; to antigens of other rickettsiae in the CF test.

tick examined (Fig. 1). The RLOs were stained intensively by Giemsa, but could not be visualized by Gimenez staining. They represented Gram-negative bent rods about 5 μm long, with ends gradually narrowed so that they appeared to possess a flagella.

Electron microscopy of *H. inermis* ovaries revealed numerous polymorphous RLOs in the oocyte cytoplasm. The RLOs had an inner cytoplasmic membrane and an outer limiting membrane. The former was 10-15 nm thick and consisted of two osmiophilic and one osmiophobic layer. The outer membrane in some places adhered close to the inner membrane or had a character of multilamellar membrane formed by several osmiophilic layers each 8-10 nm thick (Fig. 2). Similar membranous structures were described in the tick *Ornithodoros savignyi* (Roshdy, 1968). The RLOs possessed a definite inner structure formed by fine fibrillar accumulations of individual 10 nm thick fibrils and by granules similar to cytoplasmic ribosomes. The cytoplasm of oocytes contained abundant multilamellar bodies and showed a different degree of damage to mitochondria manifested by disarrangement of mitochondrial cristae or lamellar degeneration (Fig. 3).

Ovarial smears from *H. inermis* repeatedly showed bright immunofluorescence with sera from rabbits (Table 1) and small rodents (Table 2) immunized with phase I Florián or RSA strain of *C. burnetii*, irrespective of whether the sera were or were not absorbed with 20 % suspensions of *D. reticulatus* and *H. dromedarii* ticks. Immunofluorescence with rabbit and small rodent antisera to other rickettsial species was either negative or exceptionally very weak (one serum of a rabbit immunized with *R. akari* and one serum

of *A. flavicollis* immunized with *R. conorii*). In all cases, the ovarial smears contained similar great amounts of RLOs as evidenced by Giemsa staining.

The RLOs were detected only in *H. inermis* females. No such organisms were found in other tick species examined, namely *D. marginatus*, *D. reticulatus*, *I. ricinus* and *H. dromedarii*.

Attempts at cultivation or isolation of the RLOs in chick embryo yolk sacs, body cavities of tick, and various cell and organ cultures were unsuccessful. In all the substrates used no sign of multiplication of the RLOs was noted and they disappeared in successive blind passages, though the original inocula contained great amounts of the RLOs.

Rhe RLOs were apathogenic for white mice and *A. flavicollis*, because the rodents developed no sign of disease following repeated inoculation of suspensions of *H. inermis* ovaries. Sera from *A. flavicollis* inoculated with ovarial suspensions reacted with the RLOs in a dilution of 1 : 100 in the immunofluorescence test, but they did not react with *C. burnetii* antigens in the MA test.

Discussion

Along with rickettsiae also RLOs are commonly seen in different tick species. As follows from the results of many authors (Roshdy, 1961; Balashov and Daiter, 1976; and others), the RLOs occur almost in every tick, being localized usually in the Malpighian tubes and ovaries. They have always been found in the cell cytoplasm, but never in the nuclei (Balashov and Daiter, 1976). Such organisms were also detected in ovaries and Malpighian tubes of *H. inermis* ticks (Sixl-Voigt *et al.*, 1977), but these authors took them for Wohlbachiae-like organisms based on the results of electron microscopic observations. In fact, their morphology differed from that of the RLOs observed in our study, like the structure observed in the oocytes of other tick species (Hecker, 1970; Burgdorfer *et al.*, 1973).

Burgdorfer (1970) found in the haemocytes of *Dermacentor andersoni* ticks a RLO showing immunofluorescence with antisera to spotted fever group rickettsiae. He suggested that they were non-pathogenic rickettsiae which had developed from originally pathogenic rickettsial organisms. Rickettsiae displaying a bright immunofluorescence with antiserum to *R. slovaca* were frequently seen in the present (Fig. 5) as well as previous study (Řeháček *et al.*, 1976*a*). But the RLOs occurring in *H. inermis* oocytes gave only very weak immunofluorescence with two antisera against representatives of spotted fever group rickettsiae. This immunofluorescence can be considered as non specific, because it was not observed repeatedly and no immunofluorescence was found with other sera from rabbits and small rodents immunized with typhus and spotted fever group rickettsiae. On the other hand, the immunofluorescence with antisera to *C. burnetii* was bright in all cases encountered during a 5-year period. Our observation, i. e. positive immunofluorescence of the RLOs in smears from *H. inermis* ovaries with antisera to *C. burnetii* and no immunofluorescence with antisera to typhus and spotted fever group rickettsiae was confirmed by the microimmunofluorescence

technique by Dr. R. N. Philip (Rocky Mountain Laboratory, Hamilton, Montana, U.S.A.), but he did not consider the positive immunofluorescence as specific.

So far, all types of antibody to *C. burnetii* have appeared to be highly specific and no cross-reactivity with other microorganisms was demonstrated. Our finding of RLOs showing positive immunofluorescence with antisera to *C. burnetii* indicates the possibility of such a cross-reactivity, which is of both practical and theoretical importance. From the practical point of view, the possibility of imitation of *C. burnetii* by RLOs in smears from *H. inermis* ticks must be taken into account. Theoretical aspects consist in the fact that the positive immunofluorescence indicates some antigenic similarity or even relatedness of the RLOs with *C. burnetii*. We consider the RLO as a highly specified symbiote of *H. inermis* tick, from which *C. burnetii* could have developed evolutionarily, also because *H. inermis* belongs to the most primitive representatives of ixodid ticks. This assumption is not contradictory to a theory according to which rickettsiae had originated from obligatory symbiotic bacteria of arthropods (Krieg, 1963).

Our finding of a novel type of RLOs might be also of help for tick taxonomy. Differences in morphology and structure of RLOs found in different ticks could serve for distinguishing between tick species. But this requires accumulation of new data on RLOs and stresses the need for their further investigation.

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Explanation of Micrographs (Plates XLIII-XLV):

Fig. 1. Smears from *H. inermis* ovaries, showing numerous RLOs. Giemsa stain.

Fig. 2. Cytoplasm of an oocyte from *H. inermis* with abundant RLOs. Inner cytoplasmic membrane of the RLO (im), outer limiting membrane (om), and multilamellar character of outer limiting membrane (arrow). × 15 000.

Fig. 3. Longitudinal sections of RLOs in an *H. inermis* oocyte. Inner cytoplasmic structure of the RLO with fibrillar accumulations (asterisk), multilamellar cytoplasmic body (arrow), lamellar degeneration of mitochondiral cristae (m). × 15 000.

Fig. 4. Smears from *H. inermis* ovaries. A bright fluorescence of RLOs as shown by the indirect technique with rabbit antiserum to phase I *C. burnetii* strain Florián.

Fig. 5. Smear from *H. inermis* haemolymph. Bright fluorescence of RLOs with rabbit antiserum to *R. slovaca*.