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PECULARITIES OF TICK-BORNE ENCEPHALITIS VIRUS REPRODUCTION IN *HAEMAPHYSALIS INERMIS* TICKS AND THEIR EXPLANTS

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Summary. — Virological, electron microscopic and immunomorphological investigation on the reproduction of tick-borne encephalitis (TBE) virus in *Haemaphysalis inermis* ticks and in tissue explants from nymphs during their metamorphosis revealed that the virus reproduced in the cells of different tissues and organs of ticks which were in different phases and stages of life cycle. During the eclipse phase of TBE virus persistence in ticks, when no virus could be demonstrated by infectivity assay, viral particles were detected by electron microscopy.

Key words: *tick-borne encephalitis virus*; *Haemaphysalis inermis ticks*; *tick tissue explants*; *eclipse phase*; *tick hypodermis cells*; *salivary and dermal glands*; *nephrocytes*

Introduction

There is only one reference in the literature on TBE virus reproduction in *H. inermis* ticks (Nosek *et al.*, 1972) and no report, to our knowledge on tissue explants from this tick species. Meanwhile, the peculiarities of TBE virus reproduction, as determined by embryogenesis, physiology and morphology of the vector, can be fully investigated by parallel use of ticks as well as of their tissue explants. There has been found (Chunikhin *et al.*, 1981; 1984) that explants preserve the histological specificity and characteristics of tick tissues which makes it possible to study varying susceptibility of different tick cells, tissues and organs during virus reproduction. In experiments with arboviruses the use of tissue explants was found preferable in comparison with ticks, because of the possibility of long-term (more than 7 months) investigation of the same specimen (Chunikhin *et al.*, 1984).

One of the least studied peculiarities of arbovirus reproduction in ixodid ticks consists in the presence of the so called "eclipse" phase of infection, when no virus can be demonstrated by infectivity assay. The eclipse phase of virus persistence in ixodid ticks is of different duration obviously depending

on the physiology of ticks (Nosek *et al.*, 1984). Absence of detailed information on hormonal regulation of the moulting process in ixodid ticks does not allow at present to reveal association between the character of morphological, physiological and functional transformation of the arthropod organism in a given phase of metamorphosis and the infectivity of arboviruses which they transfer. At the same time, the presence of "latent" periods during arbovirus infection of ticks may lead in several cases to the false conclusion on "getting the vector free of" the virus and deforming the idea on the character of vector agent relationships.

In this paper the results are presented of virological, electron microscopic and immunomorphological studies on TBE virus reproduction in *H. inermis* ticks and tissue explants of nymphal phase of their metamorphosis.

Materials and Methods

Ticks. Larvae, nymphs and adults of *H. inermis* ticks of the first laboratory generation originating from ticks collected in West Slovakia in November 1982 were employed.

Explants cultures. Explants of nymphal tissues were prepared according to the method of Yunker and Cory (1967) with a modification described by Chunikhin *et al.* (1981). Tick larvae at final stage of their preparation for moulting to nymphs were used for culture preparation. The larvae were disinfected for 10 min by 70% ethanol and then washed many times with sterile physiological saline. Afterwards , the ticks were immersed by ventral side into wax, covered by a drop of sterile medium and dissected. The nymphal tissues devoid of larval cuticula were transferred on the cover glass, put into tubes and allowed for one hr to fix the explants. Then, 1 ml of medium was added into each tube and the tubes with explants were incubated in the thermostat at 27 °C.

Medium. For cultivation of the explants, medium based on Hank's solution supplemented with 0.5% lactalbumin hydrolysate, 20% foetal calf serum inactivated for one hr at 56 °C, penicillin (100 units/ml) and streptomycin (100 μg/ml) was used. The fresh medium was added when taking samples for demonstration of virus presence.

Virus. Experiments were carried out with strain 198 of TBE virus isolated in Czechoslovakia in 1981. The virus strain was in the 6th brain passage on newborn white mice (NWM). *H. inermis* larvae were infected with TBE virus by feeding on NWM 2 days after their intracerebral (i.c.) inoculation with the virus. Tissue explants as well as the phases of metamorphosis (nymphs, imagoes) following the larval phase were not further infected.

Virus titration. The virus was bioassayed on NWM which were inoculated i.c. with 0.01 ml of ten-fold dilutions of virus-containing material. Culture fluids from the tissue explants were examined for virus presence on days 2, 7, 14 and 21 after their preparation. The virus titre was calculated according to Reed and Muench.

Electron microscopy. For electron microscopy we used nymphs infected in the larval stage (taken on day 12 after moulting) and imagos (males and females) taken on day 18 after moulting. Besides that we investigated nymphs and adult males which had been in diapause for about 8 months. The ticks were washed with 0.1 mol/l cacodylate buffer, pH 7.2, fixed with 2% glutaraldehyde solution prepared in the same buffer and post-fixed with 1% OsO_4 solution. After dehydration in increasing concentrations of alcohol, the material was embeded into Epon-Araldit (Mollenhauer, 1964). Ultrathin sections prepared on the OM-3 Reichert ultramicrotome were contrasted with uranyl acetate and lead citrate. The preparations obtained were examined in the JEM-100B electron microscope at 80 kV.

Immunofluorescece. The ticks with incised culticula were fixed in 10% formalin in phosphate buffer solution (PBS) and embedded into paraffin. Deparaffinized sections after transition through a series of decreasing concentration of alcohols were washed with distilled water for 5 min and then incubated for 2 hr at 37 °C in 0.25% standard trypsin solution in PBS (pH 7.2—7.4) containing 0.02% $CaCl_2$. Then the preparations were washed 3 times with PBS and after washing in distilled water they were stained by indirect immunofluorescence using hyperimmune

guinea pig serum to the PK-49 strain of TBE virus and anti-guinea pig fluoresceine-labelled serum prepared in the Gemaleaya Institute of Epidemiology and Microbiology, U.S.S.R. Academy of Medical Sciences, Moscow. After examination in fluorescent microscope, the individual preparations were stained with haematoxylin and eosin and examined in the light microscope.

Results

H. inermis larvae were infected with TBE virus on June 12, 1983. At the beginning of July, when ready for moulting, they contained from 2.0 to 4.0 log LD_{50}/0.01 ml of virus. The tissue explant from infected larvae were prepared on July 5—6. We investigated altogether 29 explants, which, based on the degree of formation of tissues of the nymphal stage of metamorphosis were divided into 3 groups: 1. explant with "young" tissues up to formation of nymphal cuticula (14 specimens); 2. explants with the elements of cuticula (5 specimens); 3. explants with cuticula, on which the pigment areas appeared (10 specimens). Pools of the culture fluid for virus demonstration and titration were collected on days 2, 7, 14 and 21 after the "seeding" of explants. Virus titre in the pools varied from 0 to 1.4 log LD_{50}/0.01 ml. In parallel, we also assayed the suspensions of nymphs, which developed from the group of larvae serving for explantation. The latter yielded 4.4—5.5 log LD_{50}/0.01 ml of TBE virus (with exception of one negative result). Virus reproduction was found only in the explants with unformed tissues. Based on this information, it has been suggested that the eclipse phase of persistent TBE virus infection in ticks started from the moment of cuticula formation in the consecutive phase of tick metamorphosis covered by previous phase. Since in the nymphs the eclipse phase ended by the 6th day, but in explants it was preserved up to the 21st observation day, it was suggested that due to the interruption of hormonal influence on the process of tissue formation, the virus in explants had remained arrested in eclipse phase. As many as 376 days from the beginning of the experiment, the eclipse phase was found in the nymphs undergoing a long-term (more than 240 days) diapause in engorged state. However, at the moment of moulting (nymphs into imagos), TBE virus was demonstrated by electron microscopy, although the tissue when investigating the same specimens was not infectious for NWM.

The nymphs were engorged on non-infected white mice on July 26, 1983. On August 27—30, one part of them moulted into imagos, while another underwent diapause which lasted for more than 8 months (the last nymphs were investigated on April 29, 1984). In the period from September 7 to October 12, 1983 (i. e. between 7 and 52 days after moulting), TBE virus was detected in 16 out of 18 virologically examined imagos. The virus titre which was 4.5—6.0 log LD_{50}/0.01 ml in the interval from 7 to 15 days after moulting decreased to 1.5—2.0 log LD_{50}/0.01 on day 52 after moulting. Of the last 4 imagos examined on day 240 after moulting, the virus was found in only one with a titre of 0.1 log LD_{50}/0.01 ml. In the nymphs in diapause on day 240 from its beginning, the virus titre was 1.0—2.5 log LD_{50}/0.01 ml (with one negative result).

In ticks (nymphs, male and female adults) which did not undergo diapause,

virus particle were electron microscopically detected in the cells of different tissues and organs during different stages of post-moulting development. The virion were most frequently found in highest amounts in the cells of hypodermis, salivary (granules secreting alveoles and alveoles of the 1st type) and dermal glands as well as in non-differentiated epithelial cells of intestine. A distinct specific fluorescence was observed in these cells (Figs. 1 and 2) as an evidence for active virus reproduction. Less amounts of viral particles were found in the muscle cells surrouding the intestine, in the cells of striated musculature, in the epithelial cells of the cervical part of vagina and in the cells of Malpighian tubes. In the latter cells occurred the so-called "symbiotic" rickettsiae, whose presence is typical of all species of ixodid ticks (Balashov and Daiter, 1973). Massive accumulation of extracellular virions was demonstrated practically in every place, including the area of tracheal complex and around the nephrocytes.

Intracellular localization of viral particles was specific as known for flaviviruses. The viral particles were detected first of all in the lumens of rough endoplasmic reticulum, in cisterns and vacuoles of Golgi complex and occasionally in perinuclear areas. Proliferation of smooth and granular membranes typical of flavivirus infection predominantly occurred in hypodermis cells and in the non-differentiated intestine epithelium (Fig. 3).

Diffrent were the results of electron microscopic analysis of engorged nymphs which underwent diapause and were investigated 8 months after feeding. In such case, TBE virions were found almost exclusively in extracellular spaces, a great number of viral particles being observed along the basal membrane of nephrocytes. A part of virions was seen inside of tubular invaginations, in the basis of which desmosome-like structures were present (Fig. 4). Data on the amount of viral particles detected in ultrathin sections well correlated with the results of infectious virus titration. The number of virions as well as the virus titres decreased in following order: nymphs in the state of diapause, nymphs to the end of diapause and in the premoulting stage, nymphs at the beginning of moulting into imagos.

Discussion

Electron microscopic and immunomorphological investigations made it possible to obtain visual evidence for an effective TBE virus reproduction in cells of different tissues and organs of *H. inermis* ticks which were in diffrent phases stages of their life cycle. Judging according to the results obtained, the virus reproduced most actively in the cells of ectodermal origin as previously demonstrated by immunofluorescence (Rajčáni *et al.*, 1976). The virions were detected almost constantly in the hypodermis, salivary and dermal glands. At the same time, it was shown that in virus reproduction were involved not only the granules secreting-alveoles of salivary glands a noticed before (Nosek *et al.*, 1972), but likewise the 1st type alveolar cells responsible for the regulation of watersalt balance in tick body. It should be mentioned that the muscle cells also participated in virus reproduction,

namely those of the intestine. In this connection it is convenient to remind the paper of Tikhomirova and Karpovich (1966) and Tikhomirova *et al.* (1967) according to which muscle cells support TBE virus multiplication in the chick embryo cell culture.

Of particular interest is the finding of viral particles under the basal membrane of nephrocytes. It is known, that a basic function of these cells similarly to nephrocytes of other arthropod species is pinocytosis of haemolymph proteins and other high molecular substances (Balashov, 1979). Regular decrease in the number of virions detectable under the basal membrane of nephrocytes and in their invaginations is apparently caused, on one hand, by an increased activity of these cells during the preparation to moulting and, on other hand, by a decreased metabolic processes in the cells of other organs which may be manifested by their ability to reproduce the virus.

In any case, the observed pattern of active phagocytosis of virions by nephrocytes and their ultrastructural peculiarities testify for an intensification of lysosomal processess, pointing to the important role which they play in engulfment and further processing of TBE virus particles.

Indirect evidence obtained favours the assumption that eclipse phase of persistent TBE virus infection in ixodid ticks is connected with morphological, physiological and functional reconstruction of the arthropod host. The beginning of this phase coincides with the period of cuticula formation following the stage of tick development under the covers of previous stages. In vitro (in tick tissue explants) the eclipse phase was not completed; this may serve as evidence for hormonal regulation, which in ticks would accomplish this phase. During the eclipse phase electron microscopy demonstrated TBE virus particles although the same tissue was not infectious for NWM.

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Explanation to Figures (Plates XLVI–XLVII):

Fig. 1. Specific fluorescence in hypodermis and in the cells of dermal gland ducts (I); the same area conterstained with haematoxylin and eosin (II) (magn. × 460).
Fig. 2. Specific fluorescence in salivary gland granulosecretory alveolar cells (I); the same area conterstained with haematoxylin and eosin (II) (magn. × 460).
Fig. 3. TBE virus particles in the cisternae of dilated endoplasmic reticulum of gut epithelium cell of adult tick infected in larval stage. Arrows indicate the smooth membrane-bound vesicular structures (magn. × 50 000).
Fig. 4. Nymph nephrocyte during diapause which lasted for 8 months. Extracellular TBE particles are situated between the cell membrane and basement membrane as well as in the lumen of invaginations (arrows). Magn. × 60 000.