

Survival of rat cerebrocortical neurons after rickettsial infection

Monika Boháčsová^a, Peter Filipčík^b, Alena Opattová^b, Jana Valáriková^a, Marco Quevedo Diaz^a,
Ludovit Škultéty^a, Michal Novák^b, Zuzana Sekeyová^{a,*}

^a Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovakia

^b Institute of Neuroimmunology, Slovak Academy of Sciences, Bratislava, Slovakia

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Abstract

Neuroinvasive microorganisms are suspected to play an important role in the etiopathogenesis of neurological diseases. However, direct evidence for the pathogenic function is still missing. The main aim of this study was to investigate biochemical and morphological changes that may occur as a result of an *in vitro* infection of rat cerebrocortical neurons by selected members of the genus *Rickettsia*. Our results showed that survival of the neurons is significantly reduced after the infection. Intracellular level of ATP is gradually decreased and inversely correlates with the load of rickettsiae. Immunofluorescence revealed that rickettsiae can enter the neurons and are localized in perinuclear space and also in neuronal processes. Data obtained in this study correspond to the idea of possible involvement of rickettsiae in the etiopathogenesis of various neuropathies.

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1. Introduction

The central nervous system (CNS) is one of the targets for many *Rickettsia* species [3,7,12,25]. Rickettsiae primarily infect the microvascular endothelium leading to direct increase in microvascular permeability very early after invasion of the endothelium [32]. It has been demonstrated in mouse model that rickettsiae when injected intravenously, can cross the blood–brain barrier to reach brain parenchyma [22]. Moreover, rickettsial invasion of vascular endothelial cells into the brains was histologically confirmed also in humans [28,29].

Joshi and Kovács demonstrated that cerebellar granule neurons can be efficiently infected by *Rickettsia rickettsii*. Upon infection the neurons underwent apoptotic death, as detected by TUNEL assays [13].

Since rickettsiae may cause a whole spectrum of diseases with clear neurological manifestation, we employed an experimental model of rat embryonal and *in vitro* differentiated cerebrocortical neurons for investigation of direct effect of rickettsiae in the neurons, which may have implications for etiopathogenesis of serious neurological diseases [19]. We have provided first data on intraneuronal detection and neurotoxicity of selected rickettsial species, such as *Rickettsia helvetica*, *Rickettsia akari* and *Rickettsia slovaca* in rat cerebrocortical neurons.

2. Materials and methods

Prior to their purification by isopycnic density gradient centrifugation [2,31], rickettsiae were multiplied in Vero cell line (ATCC® CCL-81™) plated on 20 cell culture flasks with 175 cm² growth area. The purity of the rickettsial solution was checked by Gimenez staining [11]. *R. akari* strain MK (Kaplan), *R. slovaca* strain 13-B and *R. helvetica* strain C9P9

* Corresponding author. Department of Rickettsiology, Institute of Virology, Slovak Academy of Sciences, Dubravská cesta 9, 845 05 Bratislava, Slovakia.
E-mail address: viruseke@savba.sk (Z. Sekeyová).

were used for infection of differentiated rat neurons cultured *in vitro* for 8 days.

Rickettsiae were quantified by Real-Time qPCR using rickettsiae specific primers and Taqman probe designed in our laboratory, based on the gene sequence coding for 30S ribosomal protein S12 (*rpsL* gene) available in GenBank (NC009881). The sequence of forward and reverse primers are as follows: MQ25 5'-CCCGGTGAAAAGCATAGTGT-3' and MQ26 5'-ATTTCACACCCGGAAGATCA-3' respectively. The sequence of the probe is as follows: 5-FAM[®]-TTAGTAAGAGGCGGTCAAGTG-TAMRA[®]-3'. Based on the result of qPCR we have calculated amount of genomes used for infection of neurons. For infection of neuronal cultures we have used MOI (multiplicity of infection) 25 and 50 rickettsiae genomes per one neuron.

Cell cultures of rat cerebrocortical neurons were prepared as described previously [9]. Briefly, brains of anaesthetized rats (18-day-old embryos) were removed and cortices were dissected. After enzymatic treatment a cell suspension was prepared and cells were plated onto poly D-lysine-coated 96-well plates at densities of 70,000 and 25,000 cells/cm² for determination of cell survival and immunofluorescence, respectively. The neurobasal medium was supplemented with B27 (2%) and Pen/Strep, which were washed out 24 h before experiment. The neurons were maintained in a humidified atmosphere of 5% CO₂ at 37 °C for more than 8 days *in vitro* (DIV) and medium was replaced every 3–4 days.

Neuron survival was determined by measurement of intracellular ATP using CellTiter-Glo[®] assay (Promega), which quantifies ATP level in viable cells and toxicity was determined by measurement of adenylate kinase activity in culture medium. The “ATP assay” was designed for quantitative analysis of viability of rat primary cerebrocortical neurons and was used according to manufacturer's protocol. Medium from cell cultures was mixed with adenylate kinase substrate ADP (AbD Serotec) and subsequently the released adenylate kinase (AK) from damaged cells was measured using a Fluoroscan Ascent FL luminometer (MTX Lab Systems, Inc).

A triple immunofluorescent staining technique (IFA) was used to detect and localize rickettsiae in the infected neuronal cells [27]: rabbit anti-*Rickettsia* primary antibody was overlaid by Alexa Fluor[®]-488 Goat anti-rabbit secondary antibody to visualize rickettsiae. To label neuronal neurites, tau-specific monoclonal antibody DC-25 (Axon-Neurosciences) and Alexa Fluor[®]-546 anti-mouse polyclonal antibody were applied. Cells were counterstained with DAPI to selectively colour DNA. We have used confocal microscope Zeiss LSM 510 META for co-localization analysis of the fluorescent probes. The resulting signal was analysed by image analysis software from Zeiss.

One experimental group was represented by data from at least six wells of a 96-well plate. The experimental data were repeatedly confirmed and survival was determined after several different time points. Statistical differences were evaluated by one way ANOVA. Differences were considered to be statistically significant when $p < 0.05$.

3. Results

Rat cerebrocortical neurons cultured *in vitro* for eight days (8 DIV) were infected with three different species of the genus *Rickettsia*. We observed considerable phenotypic disparities between neurons after rickettsial infection when compared to rickettsia-free neuronal cultures by a light microscope. Control cells were pictured with a large round soma and well developed branches of neuronal neurites. The neuronal cultures infected with *R. akari*, *R. slovaca* and *R. helvetica* showed the signs of subtotal neuron destruction, i.e. neurite fragmentation, loss of shape and cell fragmentation (Fig. 1).

The presence of rickettsiae inside the neurons was confirmed by indirect immunofluorescence. Co-localization of anti-*Rickettsia* primary antibodies and antibodies specifically labelling intraneuronal microtubule associated protein tau in neurons demonstrated that all three rickettsiae species tested are able to enter into neurons (Fig. 2). We assume that they can propagate intraneuronally forming large aggregates.

To further investigate the effect of rickettsia infection directly on the neurons we measured intracellular concentration of ATP and the level of adenylate kinase (AK) secreted to the culture medium. The neurons were inoculated with three different *Rickettsia* species at an MOI of 50 rickettsiae per cell, full load (L1), and 25 half load (L1/2), for time period of 24 and 48 h. While there is no difference in concentration of AK in the culture medium between infected and non-infected neurons (data not shown), we found a profound differences in the level of intracellular ATP. The rickettsia induced a strong and statistically significant decrease in viability of the neurons, which can be observed as soon as 24 h after the infection (not shown). After 48 h post infection resulted in the loss of 45.1% of ATP in case of *R. helvetica* and 31.5% in case of *R. akari* when compared to ATP level in mock treated control cultures (Fig. 3). Interestingly we observed no compromised viability of neurons when infected with *R. slovaca*. These data indicate significant differences in neurotoxicity of different species of *Rickettsia*, which can be relevant to their potential role in etiopathogenesis of various neuropathies.

4. Discussion

Rickettsioses are a group of arthropod-borne diseases caused by bacteria of the genus *Rickettsia* and *Orientia*, which belong to the *Rickettsiaceae* family. Clinical manifestations of these diseases affecting various organs, including the CNS, have been assigned within the differential diagnoses [1,5].

We have investigated what can be the role of rickettsiae in the brain, specifically whether or not the rickettsiae can enter the neurons, how they can change cell morphology and affect neuron survival. For this purpose we have performed an *in vitro* infection of rat cerebrocortical neurons with three different species of spotted fever group rickettsiae; *R. helvetica*, *R. akari* and *R. slovaca*, which represent three genetically different clusters of rickettsiae.

Dermacentor marginatus, the major vector of *R. slovaca*, while feeding blood is known to prefer a neck or head area on

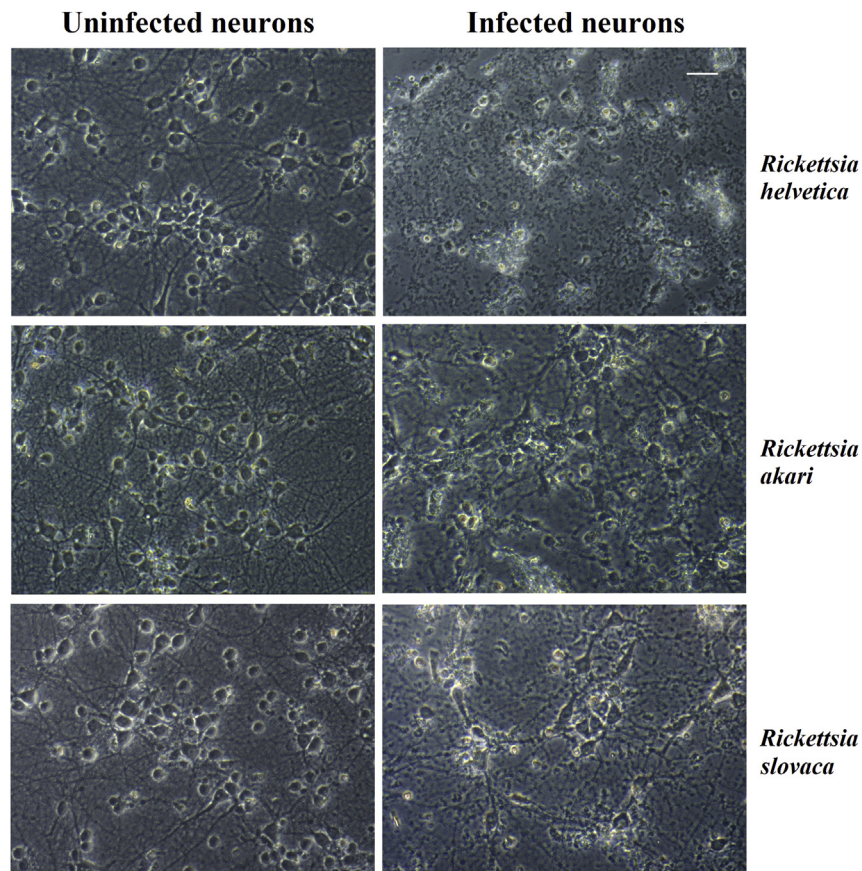


Fig. 1. Rat cerebrocortical neurons infected *in vitro* with *R. helvetica*, *R. akari* and *R. slovaca*. Two days post infection we observed extensive damage of neurons induced by rickettsiae, which was manifested by fragmented neurites and obvious destruction of neuronal morphology as revealed by light microscopy. Scale bar represents 40 μ m.

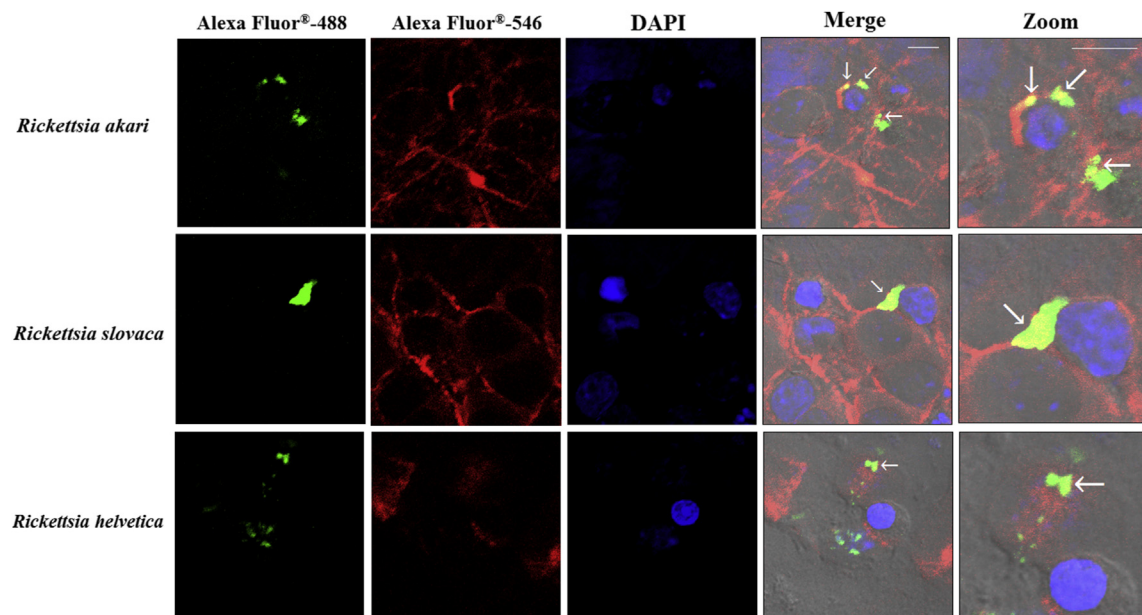


Fig. 2. Indirect immunofluorescence of cerebrocortical neurons infected *in vitro* with *R. akari*, *R. slovaca* and *R. helvetica*. Anti-*Rickettsia* rabbit serum (primary antibody) and goat anti-rabbit Alexa Fluor®-488 secondary antibody (green signal) were applied in order to visualize rickettsiae. To label intraneuronal antigen, tau-specific primary monoclonal DC-25 and Alexa Fluor®-546 anti-mouse secondary antibody (red signal) were employed. Cell nuclei were stained by DAPI (blue signal). Confocal microscopy revealed co-localization of signals from rickettsiae and tau protein which indicates intracellular localization of bacteria (Merged figure). Scale bar represents 10 μ m.

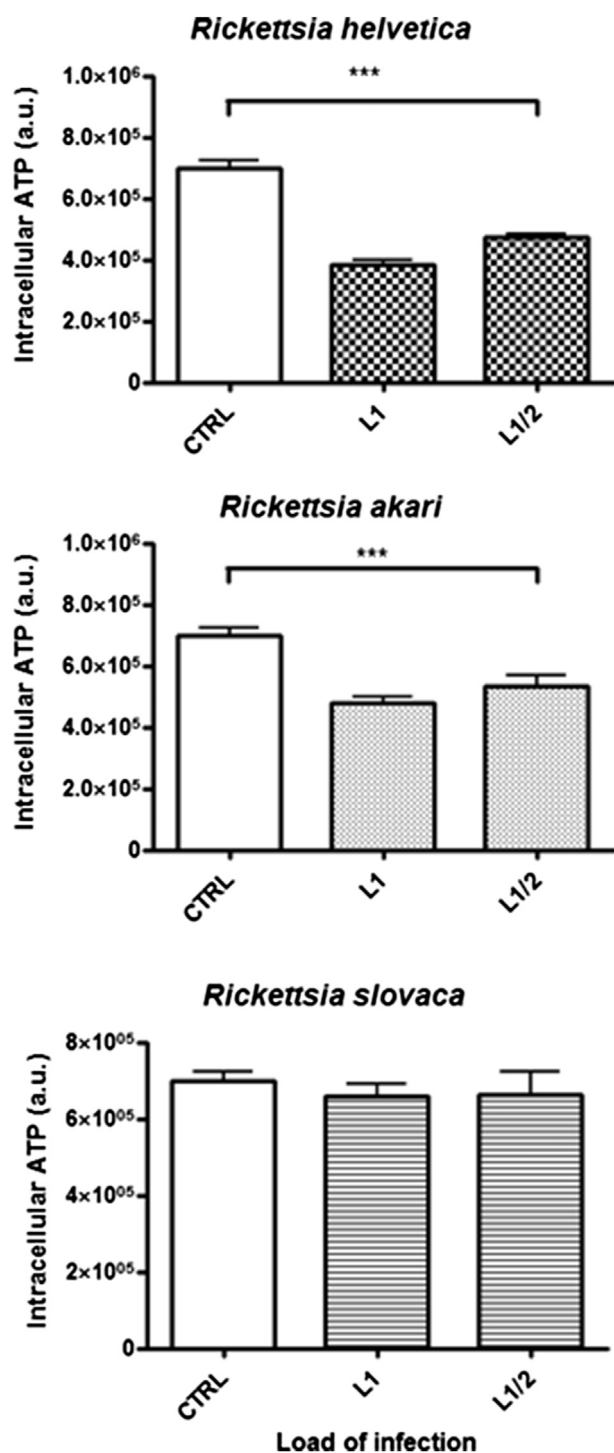


Fig. 3. The effect of rickettsial infection on the level of intracellular ATP. Cerebrocortical neurons were treated for 48 hrs using the same load of three different *Rickettsia* species. Both *R. helvetica* and *R. akari* caused significant and dose dependent decrease in viability of the neurons, which corresponds to the loss of 45.1% in case of *R. helvetica* and 31.5% loss of ATP in case of *R. akari*. These two rickettsia species strongly compromised neuron survival. Infection with *R. slovaca* did not lead to significant decrease of ATP content. Control neurons were treated with the same preparation just omitting rickettsiae (***) indicates $P < 0.001$; $n = 6$). L1 stands for full load (MOI 50), L1/2 stands for half load (MOI 25).

a prey organism [15,24,26]. The clinical features of patients with tick borne lymphadenopathy (TIBOLA) are fever, headache, an inoculation eschar surrounded by a circular erythema and painful regional lymphadenopathies [21]. Symptoms suggestive of acute encephalitis with febrile relapses have also been reported [16,17].

Another vector, *Ixodes ricinus*, is a leading tick path of *R. helvetica* [23]. The infection due to *R. helvetica* is presented with a low grade fever, headache and myalgia [10]. However, a more severe clinical diseases have been demonstrated and linked to serious infections of CNS [18].

The mite-transmitted *R. akari*, the agent of rickettsialpox, is also known to be prevalent in Europe [20]. Rickettsialpox is characterized by fever, headache, papulovesicular rash over the trunk and extremities, and in 80% of cases appearance of an eschar was described [4]. No case of CNS involvement has been reported so far.

In this study we have showed similarly to an earlier report of Joshi and Kovács, who used in their experiments cerebellar granule neurons (Joshi et al., 2007), that rickettsial pathogens are able to enter and very likely also propagate in cerebrocortical neurons causing severe damage to them. By the means of indirect immunofluorescence using anti-tau monoclonal antibody we showed, that each of tested rickettsiae was able to infect the neurons and were present cell bodies and also in the neuronal processes.

To determine how rickettsial infection impacts neuron survival, the level of intracellular ATP and adenylate kinase activity in culture medium was measured as an indicator of *Rickettsia*-induced cytotoxicity.

Rickettsiae are strict intracellular bacteria requiring a host cell to replicate [4]. They have developed transport systems specific for many of the compounds found in eukaryotic cytoplasm [8]. Although rickettsiae are not strict energy parasites, it is presumed that they transport ATP from the host cytoplasm using the ATP/ADP translocases during the early phase of the infection across a membrane in an exchange with ADP. When the host cytosol ATP is exhausted, they can synthesize their own ATP through tricarboxylic acid cycle and oxidative phosphorylation [14,30]. The remarkable drop in ATP level of neurons, which we observed shortly after the infection, can be explained by consumption of neuronal ATP by rickettsiae. This leads to profound impairment in energetic metabolism of the neurons.

Rickettsia slovaca induced a development of neuronal damage and changed morphology of neurons *in vitro* observably, but caused modest decrease in ATP level. Further investigation is needed to understand the behaviour of this agent.

Based on our results, the intracellular parasites such as rickettsiae can be regarded as an important risk factor for development of neurological diseases, specifically in context with the pathogenic role of other agents particularly those, which are able to disrupt energetic metabolism of neurons [6].

5. Conclusion

Here we report that rickettsiae can infect rat cerebrocortical neurons *in vitro*. Obvious cytopathic effect of *Rickettsia* spp.

was clearly reflected by profound morphological changes of neurons and significant drop of intracellular level of ATP. Triple immunofluorescence has proved the intracellular localization and indicates a multiplication of bacteria within the neuronal cells.

Conflict of interest

The authors have no conflict of interest.

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