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Review of the doctoral dissertation submitted by Jana Elsterova entitled "Pathogenesis and clinical aspects of tick-borne encephalitis virus infection" prepared in the University of South Bohemia, Faculty of Science, Biology Centre of the Czech Academy of Sciences, Institute of Parasitology and Veterinary Research Institute, Ceske Budejovice under supervision of doc. RNDr. Daniel Ruzek, Ph.D.

Tick-borne encephalitis (TBE) represents one of the most important human viral infections of the central nervous system in Europe and Asia. The disease shows many forms, from an asymptomatic or mild flu-like illness to severe encephalitis, which can lead to paresis and even death. An individual's immune response and genetic background seem to play an important role in the development of tick-borne encephalitis, however some clinical aspects of TBE are still missing. Although the number of clinical TBE cases are highly increasing and the geographical ranges of the tick vector species are also expanding, no specific antiviral treatment is still available.

The doctoral thesis prepared by Jana Elsterova, which I have received for the review, fits well into this research topic as it is focused on the clinical impact of TBEV infection on a human organism. The topic taken up by the author is important and new. Better understanding of this aspect may help in the development of new therapies in future. It should be emphasized that the research constituting this thesis has been supervised by dr. Daniel Ruzek, an expert in the field of tick-borne encephalitis virus. Moreover, the interdisciplinary character of the research performed in cooperation with different scientific groups should be noticed.

The reviewed doctoral dissertation by J. Elsterova, prepared in the form of a book, comprises 168 pages in total and is based on seven original research articles and three reports presented in national and international scientific conferences. Six articles have been published in 2014-2017 in journals noted in Journal Citation Report (JCR): Journal of Infection (IF=5,099, Q1), Journal of General Virology (IF=2,089, Q2), Scientific Reports (IF=4,011, Q1), Journal of Medical Virology (IF=2,049, Q2), Virology (IF=2,657, Q2) and Ticks and Tick-borne Diseases (IF=3,055, Q1) J. Elsterova is the first author in one of these publications and in others she is the co-author. One additional manuscript, where J. Elsterova is also the first author, is in preparation. It should be emphasized that the exact role of J. Elsterova in all of these publications is clearly defined. Besides the copies of the papers, the extensive introduction section is included, which proves the great knowledge of the author in the field of research. Moreover, the summary of obtained results and conclusions as well as the description of Candidate scientific achievements were also provided.

The thesis begins with broad general introduction part, described on 34 pages, which is informative and well written. It contains concise description of the current knowledge about tick-borne encephalitis virus. In this section, the author characterizes among others: the classification and evolution of tick-borne encephalitis virus, the structure of TBEV and its replication cycle, vector-host interaction and virus transmission, TBEV epidemiology and pathogenesis, clinical course of infection as well as virus prevention, diagnosis and treatment. This part of the thesis is very solid and demonstrates the good knowledge of TBEV research based on the current literature. My remark is that Figure 4 is too small and therefore not legible. Additionally, in the “Reference” section, the DOI number of the article is sometimes included in the publication list, and sometimes not. Moreover, according to the general rule, “Manuscript submitted or in preparation” should not be cited in the reference section, but only in the text.

The main aim of the thesis is clearly defined. To achieve the aim of studying the clinical pathogenesis of TBEV infection in human patients, three research topics presented in 3 Chapters were specified. Firstly, it was intended to assess the clinical course of TBE and the potential reasons of TBEV neuropathogenesis in relation to the effect of a patient’s immune reaction. Secondly, the author aimed to reveal the detailed understanding of the neuroinvassion of the virus and the role of various cell types as compounds of CNS during an infection. Thirdly, the potential use of human intravenous immunoglobulins in treating severe cases of TBE was tested. After reading all of publications covering this Ph.D. thesis, I’m convinced that these goals have been fully accomplished. Moreover, the choice of appropriate methodology allowed the author to make important observations, which significantly broaden our knowledge about clinical aspects of TBE including pathogenesis and immune reaction to the virus infection.

In the Chapter I of the thesis, the results of three publications by J. Elsterova, which were focused on a deeper understanding of a patient’s immunity status and the conditions of the blood-brain barrier for determining the variable levels of TBEV neuropathogenicity, are presented. In the first presented publication

(Elsterova et al., 2020, in preparation) the role of the integrity of the blood-brain barrier (BBB) in pathogenesis during TBEV infection as well as the impact of age on severity of TBE was studied. This study was based on the analysis of samples collected from 56 patients diagnosed with acute TBE. The authors showed that the markers of blood-brain barrier disruption do not change with the severity of infection. It has been also demonstrated that age have a great impact on the permeability of BBB what correlate with the fact that more severe course of TBE are usually observed in elder patients. It has been also shown that high levels of non-specific intrathecal antibodies during TBE are observed predominantly in elder patients, however in the presented study no change in levels of specific serum antibodies with age has been observed although in previous studies it was noticed that severity of TBE corresponds clearly with lower levels of these antibodies.

The second publication (Palus et al., 2015), where J. Elsterova is one of the authors, was attempted to analyze the levels of 30 cytokines, chemokines, and growth factors in serum samples from 87 patients with clinically and serologically confirmed acute TBE in comparison with 32 control patients. These molecules have been never or rarely studied in TBE patients. Moreover, serum levels of serotonin, dopamine and noradrenaline were also measured using enzyme-linked immunosorbent assay. The obtained results clearly showed that the levels of pro-inflammatory cytokines interleukin IL-6, IL-8 and IL-12 are highly increased during TBEV infection, although no correlation between IL-6 levels and age has been observed. Interestingly, the levels of monoamine neurotransmitters were decreased in TBE patients. Moreover, the ratios of IL-12:IL-4 and IL-12:IL-10 were higher in TBE positive patients which points to global pro-inflammatory cytokine balance. Furthermore, the authors proved for the first time that the levels of hepatocyte growth factor and vascular endothelial growth factor were increased in TBE positive patients indicating their utility as biomarkers of TBE. The authors concluded that the knowledge of inflammatory mediators activated during TBE may be used to design new therapies against this important pathogen.

In the third publication (Palus et al., 2014), were J. Elsterova is also a co-author, the function of blood-brain barrier in neuropathogenesis of tick-borne encephalitis was studied for the first time by measuring the levels of matrix metalloproteinase-9 (MMP-9) and its tissue inhibitor (TIMP-1) in serum samples from 147 TBE patients. The presented results clearly showed that the level of MMP-9 is increased as well as the MMP-9/TIMP-1 ratio is higher in positive TBE patients and this metalloproteinase can be used as a marker of inflammatory damage to the brain during TBE. These results are in agreement with previous data that the TBEV infection is associated with higher levels of IL-6 and IL-8, as they act as stimulators of MMPs. Also, in this study, no correlation between age of the patient and MMP-9 activity was observed.

Chapter II of this Ph.D. thesis, which enclosed three publications, where J. Elsterova is one of the authors, was concentrated on the mechanisms involved in crossing of the virus through the intact blood-brain barrier using constructed BBB *in vitro* model. It is well known that neurons act as primary targets for TBEV infection. In the first publication included in this chapter (Palus et al., 2014), published in the Journal of General Virology, in which Jana Elsterova is one of the co-authors, the infection of non-neuronal CNS cells

such as primary human astrocytes have been tested. It should be emphasized that the conducted research showed for the first time that TBEV is capable to infect astrocytes and that the infection is associated with the production of various pro-inflammatory cytokines and chemokines among others IL-1β, IL-6, IL-8, IFN-α. Also, the high levels of MMP-9 were observed in TBEV-infected astrocytes. It may be associated with TBEV-induced neurotoxicity and blood-brain barrier breakdown during infection. Moreover, using three-dimensional electron tomography, several novel ultrastructural changes including formation of tubule-like structures in the rough endoplasmic reticulum of TBEV-infected cells were observed. Different types of nanotubes are the potential virus transfer pathways between the cells protecting viral particles against the immune system which is of great importance for the development of the infection. To conclude, the publication was the first to show that not only neurons, but also astrocytes, are sensitive to TBEV infection what should be taken into account during designing of new antiviral therapies.

In the next publication published in Virology in 2017 (Palus et al., 2017) J. Elsterova together with her colleagues investigated the possibility of TBEV infection of primary human brain microvascular endothelial cells (HBMECs), which are the main component of BBB. The results confirmed the TBEV infection of HBMECs without the cytopathic effect. Although the number of infected cells was very low, it was sufficient for virus transmission through BBB and initiation of transfection in the brain. Also, the ability of TBEV to migrate across BBB was demonstrated. All together the data demonstrated that TBEV invades the central nervous system by HBMECs infection without altering BBB integrity.

In the last publication of this chapter (Bily et al., 2015) published in Scientific Reports, where J. Elsterova is one of the co-authors, the electron tomography analysis of tick-borne encephalitis virus infection in human neurons was performed. As mentioned above, neurons are primary target for TBEV infection in the central nervous system. The presented work was the first to visualize the architecture of cellular components involved in tick-borne encephalitis virus replication and transport in neurons. Moreover, the major ultrastructural changes that occur in response to the infection were also identified. The authors have gained the confidence that in TBEV-infected neurons, at early time points after infection, the virus is present mainly in the whole neuron body, while at later points it accumulates mainly in rough endoplasmic reticulum (RER). The morphology and 3D organization of TBEV-induced, tubule-like structures located in the RER of infected human neurons was described. Furthermore, the analysis lead to important conclusion that two sizes of tubule-like structures are present in the RER of single infected cell with the presence of E protein. Since the function of these structures in TBEV replication is still not known, are further experiments planned by the author in future? The authors also showed 3D topographical organization of membranous whorls and autophagic vacuoles in infected neurons.

The third, last Chapter of the thesis was focused on the useless of human-pooled high dose intravenous immunoglobulin (IVIG) as potential antivirals against TBE. Since IVIGs are used to treat many diseases, the idea to use it for TBEV treatment was interesting, as there is no approved therapeutic agent for TBE treatment. The paper published in 2017 in Ticks and Tick-borne Diseases, where J. Elsterova is the first

author (Elsterova et al., 2017), investigated the application of two, commercially available IVIG lots, which were found to vary in the presence of anti-TBEV specific antibodies, as a potential treatment for TBE in a mouse model. It was observed that only IVIG containing anti-TBEV antibodies possess efficient TBEV-neutralizing capacity. In my opinion, this observation is very important, because it shows that only IVIG with high TBE antibody titers might represent a potential post-exposure prophylaxis for patients suffering from severe TBE.

In summary, the doctoral thesis by Jana Elsterova deserves a very high mark. A huge amount of data presented in J. Elsterova papers contributed to the understanding of clinical aspect of TBE including pathogenesis and immune reaction to the virus infection. In her dissertation, J. Elsterova demonstrated the ability to plan, perform and interpret experiments, as each time the obtained results were objectively discussed in the relevant discussion section. After reading the thesis, I am fully convinced that the author is a mature investigator, able not only to perform the experiments but also capable of proper interpretation of the results against current state of knowledge in the field.

In a final conclusion, in my opinion the dissertation presented by Jana Elsterova fulfills all the requirements for a Ph.D. thesis. I propose to accept it and to proceed with further steps of the doctoral procedure. Taking into account the high scientific value of the thesis, I recommend awarding the thesis with an appropriate prize.