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Short Communication

Inflammatory response in human tick-borne encephalitis: analysis of postmortem brain tissue

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In Central European tick-borne encephalitis (TBE) mechanisms of tissue destruction are poorly understood. To evaluate the contribution of immunological mechanisms to tissue injury, the authors immunohistochemically analyzed paraffin-embedded autoptic brain tissue of 26 human TBE cases. In the parenchymal compartment, there was a predominance of macrophages/microglia and cytotoxic T cells. In addition, it was found that granzyme B–expressing lymphocytes were in close contact with TBE-expressing neurons up-regulating caspase-3. These findings indicate that cellular and humoral pathways of the immune system, especially granzyme B–releasing cytotoxic T cells and macrophages/microglia, mainly contribute to tissue destruction in TBE. *Journal of NeuroVirology* (2006) **12,** 322–327.

Keywords: cell death; cytotoxic T cells; immunohistochemistry; tick-borne encephalitis; virus

Introduction

Central European tick-borne encephalitis (TBE) is caused by a flavivirus and is transmitted by *Ixodes ricinus* tick bite. In humans, the infection may cause permanent disablement or even death by severe destruction of central nervous system (CNS) tissue (Haglund and Gunther, 2003; Gunther and Haglund, 2005). Flaviviral infections such as TBE, Japanese encephalitis, St. Louis encephalitis, and West Nile encephalitis share the lesioning pattern of gray matter, which consists of mononuclear inflammatory infiltrates, perivascular cuffing, microglial nodules, and neuronophagias affecting preferentially diencephalon, brainstem, and cerebellum (Johnson *et al*, 1985; Chambers and Diamond, 2003; Erman *et al*, 2001; Yasui, 2002; Sampson and Armbrustmacher, 2001; Budka, 1997).

We recently showed that TBE viral antigens are immunohistochemically detectable in large neurons of human brains of fatal cases with relatively short natural clinical course (Gelpi *et al*, 2005). We observed that areas with prominent inflammatory infiltrates and marked neuronal damage contain only few immunolabeled neuronal perikarya and/or processes. We hypothesized that immunological mechanisms might contribute to nerve cell destruction in human TBE virus infection. The inflammatory tissue response in human Central European TBE has not been studied in detail yet (Seitelberger and Jellinger, 1966; Osetowska and Wroblewska-Mularczyk, 1966; Környey, 1978; Gelpi *et al*, 2005). In addition, detailed descriptions of mechanisms leading to neuronal death based on human tissue are lacking for most of flaviviral diseases. Nevertheless, experimental studies have reported activation of apoptotic cascade and cytopathic effects of infection with TBE virus (TBEV) and West Nile virus (Shrestha *et al*, 2003; Isaeva *et al*, 1998). In this study, we characterized the inflammatory response in TBE. To this end, we immunohistochemically analyzed paraffin-embedded autoptic brain tissue of 26 human TBE cases.

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Table 1 Quantitative evaluation of inflammatory infiltrates and relative distribution of inflammatory cell subsets

Case	*Disease duration (days)*	*Total inflammatory cells per mm²*	*CD3*	*CD4*	*CD8*	*CD20*	*CD79A*	*CD68*
1	4	1580	23%	12%	9%	0.1%	0.9%	55%
2	4	1606	20%	9%	12.7%	1.5%	1.8%	55%
3	4	1201	23%	5.8%	11%	1.6%	4.6%	54%
4	5	193	20%	3%	11%	0.5%	1.5%	64%
5	6	650	22%	7.8%	6%	0.8%	2.4%	61%
6	8	750	26%	8.5%	8%	0.5%	3.7%	53%
7	9	1637	26.7%	12.6%	10%	0.7%	8%	42%
8	10	1314	36%	4.8%	22.3%	0.3%	8.6%	28%
9	10	1150	24%	3.6%	11%	1.4%	14%	46%
10	11	894	32.2%	6.3%	13.2%	0.1%	4.2%	44%
11	13	721	12.3%	6.8%	2.7%	2.2%	22%	54%
12	13	2330	28.7%	3.4%	11.8%	1.6%	5.5%	49%
13	14	520	34.4%	8.8%	33.3%	1.2%	11%	11.3%
14	15	2221	19%	11.4%	11.3%	2.8%	3.5%	52%
15	16	2020	31.5%	7.9%	7.5%	2.5%	8.9%	41.7%
16	17	886	28%	20.2%	4.8%	5.3%	13.6%	28.1%
17	19	1722	40%	10.5%	20%	2.4%	5.1%	22%
18	20	558	22.5%	9.3%	11.3%	0	7.9%	49%
19	21	1227	31.7%	9%	7.3%	1.8%	3.6%	46.6%
20	23	2918	29.7%	14.8%	14.5%	3.7%	14%	23.3%
21	30	548	49%	14.8%	22.6%	0.5%	1.6%	11.5%
22	30	1251	14.5%	10.2%	9.8%	0.6%	12.9%	52%
23	35	967	25%	11.5%	10%	0.3%	6.5%	46.7%
24	60	1409	30.2%	15%	13.4%	5%	8.5%	27.9%
25	na	721	26.6%	7.2%	12.4%	1.4%	0.4%	52%
26	na	1158	29.8%	11.5%	2.9%	0.2%	0	55.6%

Results

Inflammation- and cell death–associated factors
Inflammatory infiltrates: Inflammatory infiltrates consisted predominantly of CD3-, CD4-, and CD8-positive T cells and macrophages/microglia (Table 1). B cells were more frequently observed in the perivascular compartment (Figure 1E, F). In 24/26 cases, <1% to 41% of cytotoxic T lymphocytes were in close contact with morphologically intact neurons, independently of disease duration (Figure 1C, *inset*). In 17 cases, <1% to 36% of parenchymal cytotoxic T lymphocytes expressed granzyme B (GrB).

Representative immunohistochemical results of inflammatory cell subsets are shown in Figure 1. Immunoreactivity for anti-β_2-microglobulin, used as a marker for major histocompatibility complex (MHC) class I molecules, was detected in cytoplasms of endothelial cells and lymphocytes. Cytoplasmic anti-β_2-microglobulin staining of neurons was only occasionally seen in 1/3 of the cases. Only single neurons showed selective membrane staining. A direct contact of β_2-microglobulin–labeled neurons with GrB-positive cytotoxic T cells was not observed using consecutive tissue sections.

Humoral factors: Analyzing humoral inflammatory factors, we found that antiimmunoglobulin G (IgG) stained diffusely the brain parenchyma in nearly all cases, whereas only the half of the cases showed this staining pattern for anti-IgM. In addition, anti-IgG– and anti–IgM positive neurons, glial, and hematological cells were observed. Furthermore, deposits of membrane attack complex (MAC, C5b9) were detected in the cytoplasm of single neurons.

Indicators of apoptosis: Immunohistochemical expression of Fas ligand was only found decorating the membrane of single neurons of anterior horns of spinal cord, in areas surrounded by inflammatory infiltrates. Most of the neurons showed no anti–Fas ligand immunoreactivity.

Caspase 3 was not prominently expressed and only few neurons surrounded by inflammatory infiltrates were labeled. On consecutive tissue sections, single anti-TBEV-positive neurons expressing caspase 3 were closely associated with GrB-expressing lymphocytes (Figure 2). Using the TUNEL method only some granule cells of cerebellar cortex in areas unaffected by inflammation and lymphocytes were stained.

Correlation with disease duration
The presence of T and B lymphocytes, macrophages/microglia, or GrB-expressing cells in both perivascular and parenchymal compartment did not significantly correlate with disease duration.

Discussion

The exact mechanism of neuronal death and tissue destruction in most human viral CNS infections

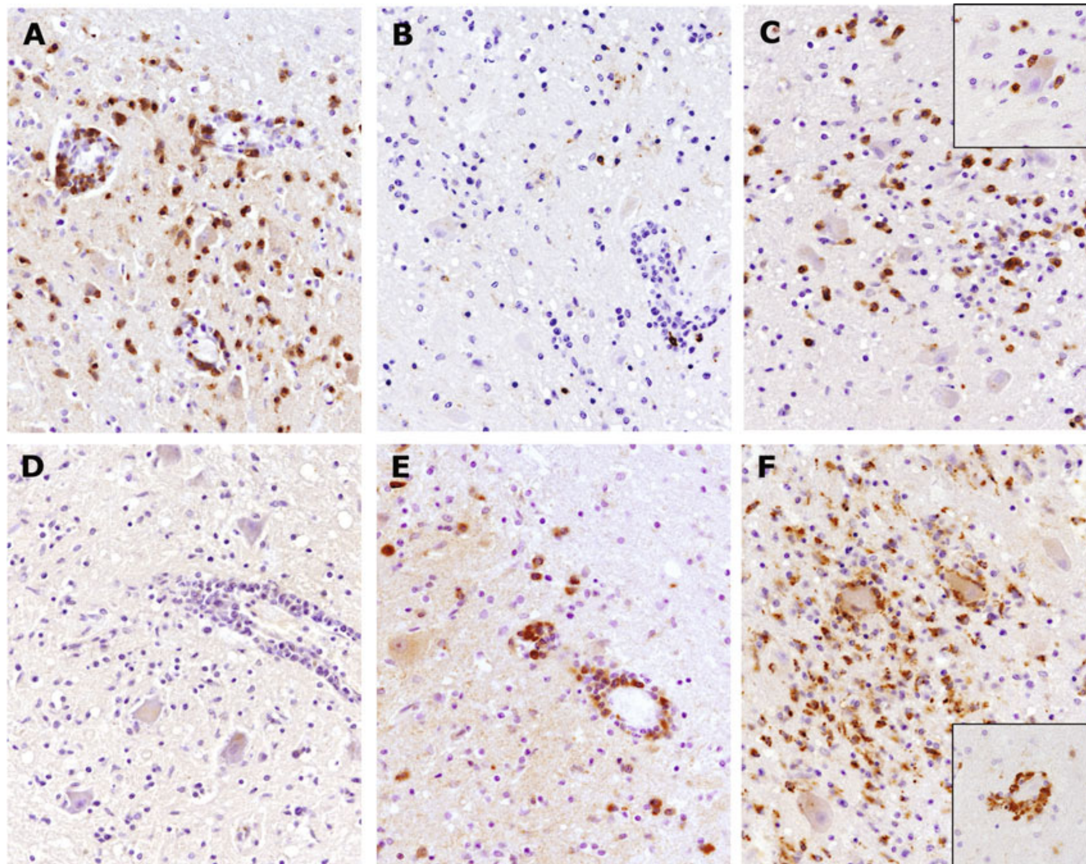

Figure 1 Immunohistochemical characterization of inflammatory cell subsets and inflammation-associated factors. T lymphocytes were detected in the parenchymal and perivascular compartment (**A:** anti-CD3, ×400; **B:** anti-CD4, ×400; **C:** anti-CD8, ×400). Among these, CD8-positive cytotoxic lymphocytes predominated in the parenchymal compartment (**C**). Up to 41% were in close contact to morphologically intact neurons (**C**, *inset*). In contrast, B lymphocytes were mainly found in the perivascular compartment (**D:** anti-CD20, ×400; **E:** anti-CD79A, ×400). In addition, numerous CD68-positive cells (**F**, ×400) were detected in both compartments. In addition, numerous neuronophagias were observed (**F**, *inset*; ×600).

including TBE is still unclear (Chambers and Diamond, 2003; Hunsperger and Roehrig, 2005). Three possible mechanisms of tissue destruction have been postulated: that the virus itself causes direct neuronal damage (lytic infection) and that the virus induces an inflammatory response, which finally leads to neuronal death, or a combination of both.

For example, in Herpes simplex virus encephalitis a host cell lysis accompanying productive infection has been described (Kent *et al*, 2004). Also for

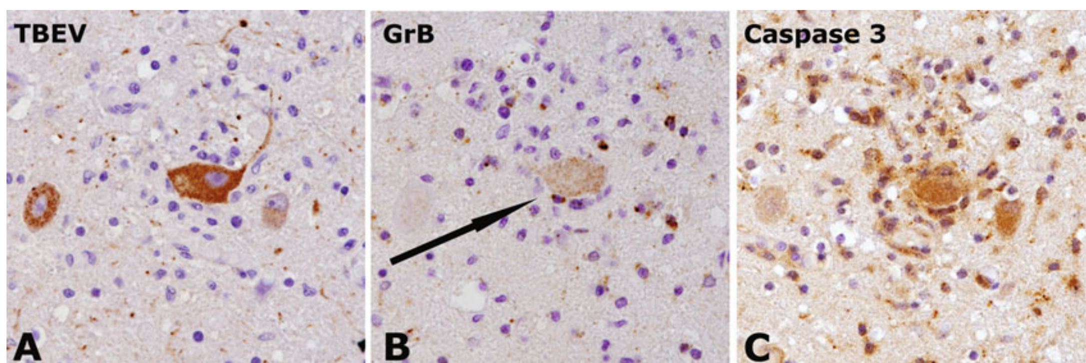

Figure 2 Consecutive tissue sections. Using consecutive tissue sections granzyme B–expressing lymphocytes (**B**, ×600, arrow) were found in close contact with TBEV-expressing neurons (**A**, ×600), up-regulating caspase 3 (**C**, ×600).

poliovirus a cytolytic effect after neuronal infection has been recognized. In contrast, in flaviviral infections divergent observations have been reported. In Japanese encephalitis a noncytopathic infection of specific neuronal population in mosquitos has been reported by one group (Leake and Johnson 1987), whereas more recently other studies observed a cytopathic effect in cell culture (Raung *et al*, 2005). For West Nile virus infection, experimental studies on its pathogenesis performed in murine neural cell cultures and human glioblastoma cells have shown that the selective infection of these cells has a cytopathic effect (Ceccaldi *et al*, 2004; Koh and Ng, 2005). In contrast, in our study we observed numerous TBEV-infected neurons that appeared morphologically intact, which might suggest that direct neuronotoxicity by viral proteins is not prominent.

Activation of apoptotic pathways as a mechanism of neuronal death has been described for several flaviviral infections such as Japanese Encephalitis and West Nile virus infections in experimental animal models and tissue cultures (Yasui, 2002; Shresta *et al*, 2003). In contrast, Lee *et al* have observed that flaviviral infection of cell cultures blocks caspase-dependent apopototic cell death at the early stage of virus infection (Lee *et al*, 2005). Furthermore, on an ultrastructural level, a dysfunction of neuron rather than a destruction of neurons was previously postulated as a mechanism of neuronal damage (Hase *et al*, 1990).

Accordingly, in our study we did not observe prominent signs of neuronal apoptosis by anti–caspase 3 immunohistochemistry, TUNEL method, or morphology. These results indicate that immediate activation of the apoptotic cascade is not a predominant mechanism of neuronal cell death in human cases of fatal TBE.

Leyssen and coworkers observed in a mouse model of flaviviral Modoc virus encephalitis that in addition to direct virus-induced damage to neurons, immunological factors markedly contribute to progression of Modoc virus encephalitis (Leyssen *et al*, 2003). Some groups have also demonstrated that lack of the cytotoxic effector function (Fas ligand and perforin) in mice protected the animals from flavivirus-mediated encephalitis, thus indicating that the immune response is one major cause of tissue damage (Licon Luna *et al*, 2002). Similarly, in early experiments on herpes simplex infection in mice, depletion of lymphocytes at an early stage prior to invasion of the CNS increases the incidence of CNS infection and death. In contrast, if the same immunosuppression is induced after virus has invaded the CNS, the time of death is delayed. Thus, cell-mediated immunity protects against CNS invasion, but once CNS invasion occurs, the cytotoxic actions accelerate the disease (Nahmias *et al*, 1969). CD8+ T cell–mediated immunity is essential for recovery from many primary viral infections, but may contribute to tissue damage beyond the mere clearing of virus (Dorries, 2001). Cytotoxic CD8-expressing T cells may destroy infected cells that present viral antigens using MHC class I. Cytotoxic T cells interact with MHC class I complex and release granzyme B, a serine protease that causes target cell death by induction of apoptosis and/or necrosis (Budihardjo *et al*, 1999). Our data show that CD8/GrB-positive cytotoxic T cells contribute significantly to neuronal damage in human TBE. These findings are in concordance with previous studies on human flavivirus encephalitides such as Japanese virus encephalitis and fatal human and animal West Nile virus infection (Johnson *et al*, 1985; Sampson and Armbrustmacher, 2001; Wang *et al*, 2003; Liu *et al*, 1989). We additionally detected several CD8-positive and GrB-expressing cytotoxic T cells that were in close contact with TBEV-infected neurons, some of these up-regulating activated caspase 3. Moreover, some noninfected neurons were also in close contact with GrB-releasing cytotoxic T lymphocytes. A similar phenomenon has been postulated by King *et al* for West Nile virus infection *in vitro*, as result of a recruitment of low-affinity self-reactive T-cells by excessive up-regulation of MHC class I molecules and costimulatory molecules. This, together with activation of microglia, was associated with severe tissue lesioning (King and Kesson, 2003).

Macrophages release many molecules that create an unfavorable microenvironment for viral replication and spread but can also be detrimental to the host. We detected large amounts of CD68 and HLA-DR immunoreactive cells in early and late disease stages. In addition, neuronophagias were a frequent finding. These findings show that macrophages/microglia play an important role in tissue destruction in human TBE (Johnson, 1998; Rock *et al*, 2004), as was reported for other flaviviral infections including Japanese encephalitis (Johnson *et al*, 1985).

In addition to T cell– and macrophage-mediated tissue damage, humoral responses may also contribute to tissue inflammatory response in viral encephalitides (Johnson, 1998). We found prominent presence of B cells, mainly in the perivascular compartment. This indicates that inflammatory response in CNS of human TBE includes antibody-mediated tissue damage. We found diffuse tissue as well as cytoplasmic neuronal and glial anti-IgG labeling in all cases. Similar results were found for anti-IgM in a fraction of cases. Cell-associated deposition of membrane attack complex was observed in few neurons. However, the interpretation of these findings is impeded by the possibility of tissue imbibition of serum proteins due to disruption of the blood-brain barrier.

Interpreting these data, one could postulate that neuronal infection by TBEV triggers rapid microglial/macrophage activation, liberation of soluble inflammatory mediators, overexpression of MHC class I molecules, and recruitment of cytotoxic T cells. Both the host innate and adaptative immune response aiming at the abrogation of the CNS infection

might cause bystander effects detrimental to the host tissue. That TBEV itself causes a cytopathic effect of neurons or induces apoptotic neuronal death in human fatal cases is not supported but cannot be excluded by our observations in human postmortem autoptic brain tissue.

In sum, our findings indicate that human TBE is a complex inflammatory process that involves multiple, closely interrelated elements of the cellular and humoral immune system. Nevertheless, granzyme B–releasing cytotoxic T cells and macrophages/microglia mainly contribute to tissue destruction in TBE.

Materials and methods

Patients

A total of 26 human autopsy cases of tick-borne encephalitis were included in this study. Clinical data of these cases have been detailed in a previous study (Gelpi *et al*, 2005). All cases had TBE confirmed by serology and/or immunohistochemical detection of viral antigens in autoptic brain tissue. Patients' age ranged from 13 to 76 years (median 55 years) and disease duration from 4 to 60 days (median 13.5 days). In each case, one block of CNS tissue showing prominent inflammatory infiltrates was selected for this study. Brain areas included cerebral and cerebellar cortex, nucleus basalis Meynert, thalamus, substantia nigra, pons, medulla oblongata, and spinal cord.

Histology and immunohistochemistry

Brain tissues were routinely formalin fixed and paraffin embedded. Of each block 3 to 5 μm thick sections were obtained. Hematoxylin-eosin (H&E) and Klüver-Barrera stainings were performed for standard histopathological evaluation.

For characterization of inflammatory infiltrates, the following monoclonal (mc) and polyclonal (pc) antibodies were used: anti-LCA (mc; 1:1000; DAKO, Glostrup, Denmark), anti-CD3 (mc; 1:500; DAKO), anti-CD4 (mc; 1:50; DAKO), anti-CD8 (mc; 1:100; DAKO), anti-CD20 (mc; 1:200; DAKO), anti-CD79A (mc; 1:50; DAKO), anti-CD68 (mc; 1:100; DAKO), anti-HLA-DR (mc; 1:100; DAKO), anti-C5b9 (mc; 1:500; Calbiochem, La Jolla, CA, USA), anti-granzyme B (mc; 1:50; LabVision, Fremont, CA, USA), anti–caspase 3 (mc; 1:100; Cell Signaling Technology, MA, USA), anti-β_2-microglobulin (pc; 1:200, DAKO), anti-IgG (mc; 1:50, DAKO), anti-IgM (mc; 1:10; Cymbus Biotechnology, Chandlers Ford, UK), anti-Fas Ligand (pc; 1:100; Abcam, Cambridge, UK), and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling method (TUNEL; In Situ Cell Death Detection Kit; Roche Molecular Biochemicals, Mannheim, Germany). For immunohistochemical detection of TBEV antigens, a noncommercial rabbit polyclonal hyperimmune serum (Institute of Virology, Medical University of Vienna, Austria; dilution 1:1000) was used (for details see Gelpi *et al*, 2005).

In addition, in three representative cases with a clinical duration of 4, 16, and 21 days, we performed immunohistochemical stains in consecutive tissue sections with the following antibodies: anti-CD8, anti–granzyme B, anti-TBEV, anti–caspase 3, TUNEL assay, anti-C5b9, anti-IgG, anti-β_2- microglobulin, and anti–Fas ligand.

Tissue section pretreatment for antigen retrieval was performed by boiling sections in 10 mM citrate buffer at pH 6 for 10 min or by protease XXIV 0.03%, digestion at 37°C.

Detection of immunostaining was performed using the Envision kit, and diaminobenzidine was used as chromogen.

Quantitative and semiquantitative evaluation of immunolabeling

The total amount of inflammatory cells positive for anti-CD3, anti-CD4, anti-CD8, anti-CD20, anti-CD79A, anti-CD68, and anti-GrB was counted in all 26 cases in the areas of maximal inflammatory infiltrates, in an area of 1 mm^2, using an eye grid at 400-fold magnification. The parenchymal and the perivascular compartment were counted separately. In addition, the number of cells positive for anti-GrB and anti-CD8 and in close contact to neuronal surface was evaluated in each case. Furthermore, the presence of structures labeled for anti-IgG, anti-IgM, anti-β_2-microglobulin, anti-C5b9, anti-Fas ligand, and anti–caspase 3, including neurons, glial cells, hematogeneous cells, and/or brain parenchyma, was evaluated separately for each structure as negative, moderate, or abundant. In adjacent tissue sections, we analyzed concomitant immunolabeling of anti-GrB-positive T cells, and neurons positive for TUNEL, anti-TBEV, anti–caspase 3, anti-C5b9, IgG, anti-β_2-microglobulin, and anti–Fas ligand.

Statistical analysis

Correlation of inflammatory cell subsets with disease duration was performed using Pearson correlation coefficient. The SPSS statistical software system (SPSS, Chicago, IL, USA) was used for statistical calculations.

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