Interferon-γ and Cortisol Levels in Cerebrospinal Fluid and its Relationship to the Etiology of Aseptic Meningoencephalitis

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Abstract: The aim of the study was to analyze the concentrations of Th1/Th2 cytokines and cortisol in the cerebrospinal fluid (CSF) from patients with aseptic meningoencephalitis (AM). The study enrolled 37 patients with AM and 11 control subjects. CSF concentrations of IL-2, IL-4, IL-5, IL-10, IFN-γ, and TNF-a were analyzed using cytokine bead array and flow cytometry; CSF cortisol concentrations were measured by a RIA method. Cortisol was detected in 37 CSF samples (100%) from patients with AM, and it was significantly elevated in comparison to control subjects. IFN-γ was detected in 32 CSF samples (86.5%) and IL-10 was detectable in 9 CSF samples (24.3%). The CSF cortisol levels correlated negatively with the duration of AM. The intrathecal concentration of IFN-γ correlated positively with CSF numbers of leukocytes and lymphocytes, and negatively with the duration of AM. The etiology of AM influenced the CSF cortisol concentration, which was significantly higher in patients with tick-borne encephalitis when compared to persons with AM of unknown origin and control subjects. The results indicate that the prevailing intrathecal immune reaction during AM is shifted to a Th1-like response, whereas anti-inflammatory response in the brain is executed by the effect of cortisol.

Introduction

Aseptic meningoencephalitis (AM) represents an important cause of morbidity and mortality worldwide. In the Czech Republic, 1266 cases of AM were reported in 2004. The most frequently diagnosed etiology of AM in the Czech Republic is the western subtype of the tick-borne encephalitis (TBE) virus. Increasingly, the incidence of AM has been associated with enteroviruses in 2005 [1]. In addition, herpesviruses appear to play a significant role in AM, especially with regard to a severe course of central nervous system (CNS) infections due to Herpes simplex virus (HSV)-1 in immunocompetent adults and HSV-2 in neonates [2]. AM may also develop as a result of a *Borrelia burgdorferi* infection, which can invade the CNS during the early stages of the infection [3].

Intrathecally, phagocytosis and intracellular killing by glial cells is essential for elimination of infectious agents [4]. The innate immune response to intracellular pathogens also involves activation of NK cells, NK-T cells, monocytes, and mast cells. However, under physiologic conditions, there is a lower number of these cells in the CNS than during an infection [4]. Efficient clearance of an intracellular infection is additionally provided by cytotoxic T-cells, which may either kill intracellular pathogens together with infected cells, or suppress the infection via non-cytolytic mechanisms. It has been suggested that a non-cytolytic mechanism employs interferon (IFN)-γ mainly produced by Th1 ($CD4^+$) cells, which is the predominant cell subset in the cerebrospinal fluid (CSF) during TBE and neuroborreliosis [5]. Activation of Th1 cells can be inhibited by anti-inflammatory mediators such as interleukin (IL)-10 and cortisol providing negative feed-back mechanisms of CNS inflammation. However, there is a lack of data about these mediators in the CSF during the course of AM.

The aim of our study was to analyze the predominant type of intrathecal immune response – Th1 or Th2 – in patients with AM, based on the detection of cytokines in the CSF. We also determined CSF cortisol levels and assessed its relationship to the intrathecal cytokine concentrations. Additionally, we evaluated the correlation of CSF cortisol and cytokines levels with laboratory parameters routinely determined in CSF as well as the etiology and duration of AM.

Patients and methods

Patients

The study enrolled 37 patients hospitalized in the Department of Infectious Diseases, Teaching Hospital Na Bulovce – Prague, Czech Republic, from October 2002 through December 2003. The group of patients consisted of 15 females and 22 males with a mean age 37.5 ± 18.1 years (range 9–67 years). Patients' demographic, clinical and laboratory data are presented in Table 1. The control group comprised 11 persons (6 females and 5 males, mean age of 35.8 ± 17.3 years and range 21–69 years). These control subjects had meningeal irritation with normal CSF cytology (i.e. white blood cells $< 5/mm^3$ of CSF) and negative serology of TBE, neuroborreliosis and enteroviruses. Polymerase chain reaction (PCR) of CSF of those persons was also negative for *B. burgdorferi*, TBE virus, enteroviruses and herpesviruses. The demographic and laboratory data of control subjects are presented in Table 2. The study was approved by the local ethics committee and it was performed in accordance with the Helsinki Declaration as revised in 1996. For the analyses, we used only CSF leftovers after routine laboratory tests. These specimens were stored at –80°C until further analyses.

Routine CSF examinations

Leukocytes in CSF were enumerated using a Fuchs-Rosenthal counting chamber (Fein Optik, Jena, Germany) after staining with crystal violet (0.2%) and lysis of erythrocytes with 4% acetic acid. Using the counting chamber the absolute number of mononuclear cells was determined as well. CSF glucose and protein levels were defined calorimetrically with an automated clinical chemistry analyzer Vitron™ (Ortho Clinical Diagnostics, Inc., Rochester, NY, USA).

Serology for TBE, Lyme borreliosis and enteroviruses

TBE was diagnosed serologically by the detection of IgM antibodies against TBE virus using a commercial capture enzyme immunoassay (EIA) – EIA TBEV IgM™ – obtained from Test Line Ltd. (Brno, Czech Republic), which provides sufficient specificity [6]. Diagnosis of neuroborreliosis was based on the detection of specific IgM or IgG antibodies against *Borrelia burgdorferi* in CSF by ELISA and/or western blotting [7]. Neuroinfection caused by enteroviruses was diagnosed by a commercial ELISA kit for the detection of specific IgM in serum (Virotech, Russelsheim, Germany).

Table 1 – Demographic, clinical and laboratory characteristics of patients with aseptic meningoencephalitis

		Cerebrospinal fluid								
Age (years)	Sex	Protein (g/L)	Glucose (mmol/L)	WBC* (cell/μL)	Lympho (cell/μL)	IFN-γ (pg/mL)	IL-10 (pg/mL)	Cortisol (nmol/L)	Duration# (days)	Etiology
27	M	0.7	3.4	77	39	172	N/D	30.2	3	TBE
32	M	1.39	2.6	515	395	535	N/D	24.1	3	TBE
33	M	1.13	3.20	256	52	112	N/D	36.5	3	TBE
48	M	0.96	2.9	70	63	421	N/D	21.1	5	TBE
58	M	0.76	5.9	41	11	98	71	34.3	4	TBE
29	F	0.83	2.9	119	22	105	N/D	18.6	7	TBE
35	F	0.72	2.9	32	24	102	N/D	14.6	12	TBE
61	F	1.47	3.7	90	67	267	N/D	35.9	3	TBE
67	F	0.50	3.0	170	2	N/D	N/D	30.3	1	TBE
9	M	1.02	2.9	296	296	122	113	19.4	4	NB
49	M	1.57	2.7	280	255	86	N/D	11.5	10	NB
56	M	1.22	3.9	25	21	58	66	13.7	35	NB
56	M	0.43	3.2	2	2	N/D	N/D	12.6	110	NB
21	F	0.98	2.8	299	291	105	57	15.2	6	NB
64	F	0.74	4.1	99	88	N/D	N/D	15.5	6	NB
24	M	1.21	2.9	201	124	111	N/D	15.4	4	TBE+NB
18	M	0.57	3.5	587	587	127	N/D	12.3	3	EM
23	M	0.81	3.1	1232	248	523	124	28.8	2	EM
25	F	1.36	2.3	172	167	N/D	N/D	32.0	8	EM
33	F	0.72	3.6	154	144	197	78	16.8	2	EM
35	M	0.74	2.9	317	307	76	N/D	12.9	4	HSV-1
67	M	0.85	4.9	251	235	140	N/D	5.0	10	HSV-1/EBV
16	F	1.49	3.1	432	405	1793	N/D	14.9	2	HSV-1/HHV-7
50	M	1.74	2.9	387	323	127	N/D	21.6	12	CMV
66	M	1.78	6.2	78	76	48	N/D	16.9	17	VZV
56	F	0.61	2.9	61	53	91	N/D	14.0	8	VZV
10	M	0.24	3.7	103	97	96	N/D	7.4	4	AM UO
15	M	0.55	3.1	47	43	112	N/D	6.3	6	AM UO
18	M	0.57	3.6	133	23	51	N/D	25.6	3	AM UO
20	M	0.55	2.8	312	208	153	62	24.5	7	AM UO
28	M	0.76	3.8	149	123	239	134	28.6	4	AM UO
41	M	0.61	3.2	25	21	79	N/D	14.2	7	AM UO
13	F	0.54	3.3	216	213	130	N/D	5.6	1	AM UO
28	F	0.34	3.1	35	27	N/D	N/D	12.3	10	AM UO
52	F	0.68	3.1	213	113	147	N/D	22.1	6	AM UO
52	F	1.91	2.2	528	427	51	N/D	5.0	13	AM UO
52	F	0.87	2.4	58	55	110	39	13.2	2	AM UO

TBE, tick-borne encephalitis; NB, neuroborreliosis; EM, enteroviral meningoencephalitis; HSV, Herpes simplex virus; EBV, Epstein-Barr virus; HHV, Human herpes virus; CMV, Cytomegalovirus; VZV, Varicella-zoster virus; AM UO, aseptic meningoencephalitis of unknown origin

*WBC, white blood cells; # duration of symptoms from onset to lumbar puncture; N/D, not detectable

PCR for TBE, enteroviruses and herpesviruses

The DNA and RNA were co-purified from 140 ml of CSF using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Positive and negative controls were extracted with every batch of samples. Enterovirus was detected using a semi-nested PCR strategy in the real-time format utilizing a TaqMan hydrolysation probe. A sequence conserved among enterovirus serotypes was amplified in one-step real-time RT-PCR with the forward primer 5'-CCCTGAATGCGGCTAATCC-3' and the reverse primer 5'-ATTGTCACCATAAGCAGCCA-3' followed by the second round of amplification using two forward primers, 5'-CGTAACGCGCAAGTCTGTGG-3' and 5'-GTCGTAATGGGYAACTCYGCAGCG-3' together with the reverse primer identical to the first round and a TaqMan probe 5'-FAM-AACCGACTACTTTGGGTGTCCGTGTTTC-TAMRA-3' [8]. The quantitative standards were derived from a dilution series of RNA extracted from supernatants of cell cultures infected by five different enterovirus strains. The TBE virus was detected using a real-time RT-PCR assay described by Schwaiger quantitative standards obtained from the inactivated vaccine strain of the virus [9]. The CSF samples were also tested for herpesviruses: HSV, EBV (Epstein-Barr virus), CMV (Cytomegalovirus), VZV (Varicella-zoster virus), HHV (Human herpes virus)-6 and HHV-7 using previously published real-time PCR methods, with some of the primers repositioned for better performance; the sequences are available from the authors of this article. All reactions were performed using the Qiagen chemistry and run on an ABI 7700 machine with the Sequence Detector Software 1.9.

Table 2 – Demographic and laboratory data of control subjects

		Cerebrospinal fluid						
Age (years)	Sex	Protein (g/L)	Glucose (mmol/L)	WBC* (cell/μL)	Lympho (cell/μL)	IFN-γ (pg/mL)	IL-10 (pg/mL)	Cortisol (nmol/L)
23	M	0.55	3.3	2	1	N/D	N/D	8.3
26	M	0.40	3.5	5	5	N/D	N/D	7.8
31	M	0.35	3.0	1	1	N/D	N/D	10.7
39	M	0.29	4.0	2	2	N/D	N/D	20.2
61	M	0.61	3.7	0	0	N/D	N/D	15.2
21	F	0.33	3.4	1	1	N/D	N/D	11.1
22	F	0.25	4.0	1	1	N/D	N/D	9.9
23	F	0.66	2.7	3	2	N/D	N/D	13.3
26	F	0.17	3.3	0	0	N/D	N/D	5.9
53	F	0.48	3.1	1	1	N/D	N/D	8.0
69	F	0.85	3.0	1	1	N/D	N/D	10.1

*WBC, white blood cells; N/D, not detectable

Cytokine analysis in CSF
Cytokine concentrations in CSF were analyzed with a CBA™ kit (BD™ Cytometric Bead Array – Human Th1/Th2 cytokine kit) and three-color flow cytometer FACScalibur™ (Becton-Dickinson, San Jose, CA, USA). The detection limit of the cytokines was 20 pg/mL.

Cortisol analysis in CSF
The concentration of cortisol in CSF was determined by a radioimmunoassay (RIA) using commercial DSL-2000 kit (Diagnostic Systems Laboratories, Webster, TX, USA). The detection limit of cortisol was 5 nmol/L.

Statistical analysis
Statistical analyses were performed using the SigmaStat® 3.0 software (Jandel Scientific, Chicago, IL, USA). Statistical differences between laboratory parameters of AM patients and control subjects were tested with Student's *t* test. Spearman's correlation test was employed to determine a correlation between variables. The influence of the etiology of AM on the analyzed parameters was tested using One-way ANOVA test with Dunn's method. The analyses consisted of two-tailed tests with α level < 0.05. Data are presented as mean $\pm$ standard deviation.

Results
The total number of leukocytes in CSF from the patients with AM was significantly higher than that of the control subjects (217.9 $\pm$ 229.5 vs. 1.5 $\pm$ 1.4 cells/mm^3; $p < 0.01$). Also, the total number of lymphocytes was significantly elevated in comparison to the control group (152.6 $\pm$ 145.8 vs. 1.4 $\pm$ 1.4 cells/mm^3; $p < 0.01$). Similarly, the mean CSF protein concentration of the patients with AM was significantly elevated when compared to control subjects (0.92 $\pm$ 0.43 vs. 0.40 $\pm$ 0.18 g/L; $p < 0.01$). The mean glucose level in CSF of the AM patients was not significantly different from the controls (3.32 $\pm$ 0.84 vs. 3.36 $\pm$ 0.42 mmol/L; normal range 2.8–3.9 mmol/L). There was no difference in the routine CSF parameters due to the etiology of AM.

Serum IgM antibodies against TBE virus were detected in 9 of 37 patients (24.3% of AM patients); six patients demonstrated intrathecal synthesis of antiborrelial antibodies (13.5% of AM patients); IgM antibodies against enteroviruses were detected in sera from two patients. In addition, one patient demonstrated the signs of dual infection – positive serology for neuroborreliosis and TBE concomitantly.

In six of 37 CSF samples (16.2% of AM patients), viral DNA or RNA of one virus was detected: PCR for VZV and enterovirus was positive in two samples; HSV or CMV DNA was detected in one sample. In two CSF samples (5.4% of AM patients), herpesviruses were presented simultaneously: HSV-1/HHV-7

and HSV-1/EBV. TBE RNA was not detected in any of the CSF samples from patients with serologically diagnosed TBE.

IFN-γ was detected in 32 of 37 CSF samples (86.5% of AM patients) with the mean concentration of 180.6 ± 298.7 pg/mL. IL-10 was detected in nine samples (24.3% of AM patients); the mean IL-10 concentration was 35.2 ± 31.4 pg/mL. IL-2 and IL-5 were detected only in one CSF sample. No CSF sample was positive for IL-4 and TNF-a . There was no significant difference in CSF cytokines due to the etiology of AM. The control subjects had undetectable CSF levels of all investigated cytokines. The CSF IFN-γ concentration positively correlated with the total numbers of leukocytes ($r = 0.40$; $p = 0.015$) and lymphocytes ($r = 0.43$; $p < 0.01$). Additionally, CSF levels of IFN-γ correlated negatively with the duration of AM, which was considered as the interval between the onset of clinical symptoms and diagnostic lumbar puncture ($r = –0.514$; $p = 0.01$).

Cortisol was detected in all CSF samples from patients with AM or control subjects. The mean CSF cortisol concentration was significantly elevated when compared to the control subjects (18.6 ± 8.9 vs. 11.0 ± 4.0 nmol/L; $p < 0.01$). The CSF cortisol concentration correlated negatively with the interval between the onset of the disease and diagnostic lumbar puncture ($r = –0.37$; $p = 0.025$). As shown in Figure 1, the CSF cortisol level in the patients with TBE was significantly higher than that of the patients with AM of unknown etiology (27.3 ± 8.0 nmol/L vs. 15.0 ± 8.8 nmol/L; $p < 0.05$). TBE patients also demonstrated significantly elevated CSF cortisol levels in comparison to control group ($p < 0.05$).

Discussion

AM is usually a moderate to severe disease, which can be responsible for acute neurological damage as well as for long-term sequel as was shown in patients with TBE [10]. The pathogenesis of AM is strongly associated with the inflammation

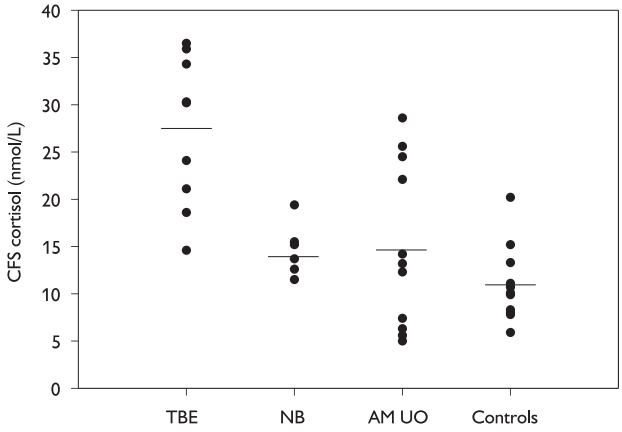

Figure 1 – Comparison of CSF cortisol levels in tick-borne encephalitis (TBE), neuroborreliosis (NB), aseptic meningoencephalitis of unknown origin (AM UO) and control subjects.

within the brain parenchyma elicited by both – brain resident immune cells and cells migrating to the CNS [11]. Because the damage to resident CNS parenchymal cells may also be caused by a cytopathic effect of pathogens, suppression of the infection of neurons as well as astrocytes is necessary [12]. However, intrathecal immunity needs to be carefully regulated to prevent inflammatory brain injury caused by cytotoxic immunity.

It is suggested that elimination of viruses as well as intracellular bacteria is executed within the CNS by non-cytolytic mechanisms, similar to immune responses that can control replication of viruses during viral hepatitis. Suri et al. [13] have reported that replication of hepatitis B virus in hepatocytes is suppressed by IFN-γ, which enhances intracellular killing without the necessity of cytotoxicity. Similarly, we detected elevated CSF levels of IFN-γ in the majority of AM patients. We posit that this strong proinflammatory cytokine has been mainly produced by Th1 cells, because $CD4^+$ T-cells represent the prevailing cell subset in the CSF during neuroinfection as well as in other inflammatory brain diseases [4, 5]. However, the increased CSF IFN-γ concentration observed in our study may also reflect migration and activation of IFN-γ producing $CD8^+$ T-cells in the brain during CNS infection. These immune cells demonstrated protective immunity in mice with an experimental CNS infection caused by the parasite *Toxoplasma gondii* [14]. Possible role of IFN-γ producing T-lymphocytes during AM is supported by the correlation between the number of lymphocytes and IFN-γ concentration in CSF observed in our study. It appears that the IFN-γ response declines over time, since the intrathecal concentrations of IFN-γ were higher in the patients with shorter duration of symptoms.

Pro-inflammatory responses are strongly associated with anti-inflammatory mechanisms, a process crucial for the prevention of tissue injury. With regard to anti-inflammatory responses, many studies have indicated an important role for cortisol and IL-10 [15]. These anti-inflammatory mediators were detectable in the CSF samples from AM patients. This finding is not surprising for IL-10, which can be produced by many cell types within the brain including microglia, astrocytes, perivascular macrophages and T cells [16]. Significantly elevated CSF cortisol levels observed in our study indicate an important role of this hormone during AM. Cortisol is a small lipid-soluble molecule produced in adrenal glands after stress stimulation such as infection, trauma or major surgery. Cortisol may easily cross the blood brain barrier (BBB). Schwarz et al. [17] demonstrated that cortisol transport through BBB is not increased by its leakage and is rather dependent on serum concentrations. It has been suggested that intrathecal concentrations of steroid hormones are maintained by their active transport out of the brain [18]. Also, elevated intrathecal levels of IFN-γ and increased CSF cortisol concentrations that we observed in our patients with AM may reflect stimulation of the hypothalamo-pituitary-adrenal (HPA) axis. IFN-γ can stimulate HPA axis indirectly by activation of microglia, which can produce IL-1β [19].

Interesting differences were observed in cortisol levels according to the etiology of AM, in that the highest cortisol levels in CSF were observed in the TBE patients. TBE causes significant morbidity and mortality, which in the majority of patients is caused by paralytic disease and permanent CNS dysfunction. Moreover, it was reported that the development of paretic complications during the course of TBE is negatively influenced by the treatment with corticosteroids administered for the prolonged period of time [20]. It seems to be possible that elevated intrathecal cortisol levels can exacerbate neuronal injury. Such mechanism has been proposed in experimental study in rabbits, in that corticosteroids induced apoptosis of neuronal cells in the hippocampus [21]. Corticosteroids may also shift immune response from Th1 to Th2 type, which is characterized by pronounced antibody production. Interestingly, TBE patients with a severe course of disease demonstrated intense intrathecal synthesis of specific antibodies [22]. Also, an increased concentration of intrathecal antibodies can exacerbate activation of microglia causing release of reactive oxygen and nitrogen species.

PCR analyses of CSF performed in our patients revealed also some interesting findings. First, two herpesviruses were detected in CSF simultaneously in two of six patients with a herpetic neuroinfection. This finding could reflect either co-infection with two herpesviruses or their reactivation as was already reported in the previous study [23]. Herpesviruses usually display synergism, which may influence significant pathology especially associated with necrotizing encephalitis caused by HSV-1 and more rarely HSV-2 [24]. Secondly, TBE patients had no detectable viral RNA in CSF. This observation is in accordance with previous studies of CSF from patients with TBE [9, 25]. Whether this may represent spread of the TBE virus from CSF into the brain parenchyma or clearance of the viral infection is not known. It also raises an important question about the disease pathophysiology, which may involve mechanisms similar to those causing autoimmune disease rather than reflect the cytopathic effect of TBE virus.

Conclusion

Our results indicate important role of IFN-γ in the pathogenesis of AM. Increased intrathecal cortisol concentrations observed during the course of AM suggest induction of the HPA axis response and/or alterations in the cortisol transport mechanisms through BBB. Whether these changes of cortisol homeostasis in the CNS reflect suppression of intrathecal inflammatory response or may be related to development of the brain tissue injury, as it seems possible during the course of TBE, should be further elucidated.

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