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**Title: Chemokines CXCL10 and CXCL11 in the cerebrospinal fluid of patients with
tick-borne encephalitis**

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Short title: CXCL10 and CXCL11 in tick-borne encephalitis

ABSTRACT

Objectives: The aim of our study was to determine whether cerebrospinal fluid (CSF) of patients with tick-borne encephalitis (TBE) contains CXCL10, CXCL11, p40 subunit of IL-12/IL-23, IL-18 and IL-15. We compared serum and CSF concentrations of CXCL10 and analysed the possible concentration gradient of this chemokine between the periphery and central nervous system (CNS).

Materials and methods: The study enrolled 19 TBE patients and 10 patients with non-inflammatory neurological diseases (NIND).

Results: CSF of TBE patients contained CXCL10 (median 217 pg/mL), CXCL11 (8.3 pg/mL), p40 subunit of IL-12/IL-23 (38.9 pg/mL), IL-18 (30.1 pg/mL) and IL-15 (5.9 pg/mL). CXCL10 in the CSF of TBE patients was higher compared with serum (median 62 pg/mL, $p < 0.001$).

Conclusion: CSF of TBE patients contains CXCL10, CXCL11, p40 subunit of IL-12/IL-23, IL-18 and IL-15. Increased CXCL10 CSF concentration suggests a role for this chemokine in the recruitment of CXCR3-expressing T-cells into the CSF of TBE patients.

INTRODUCTION

Tick-borne encephalitis is a viral infection of the central nervous system (CNS). The causative agent tick-borne encephalitis virus (TBEV) is transmitted to humans by infected *Ixodes ricinus* ticks (1,2). Infection with TBEV is characterized by a biphasic clinical course: early non-specific influenza-like symptoms and subsequent development of neurological symptoms. Permanent neurological sequelae (post-encephalitis TBE symptoms) that are observed in up to 46% of patients significantly alter their quality of life and, despite an efficient vaccine, TBE remains an important health problem.

Clinical presentation and laboratory findings in TBE have been well studied but the knowledge on the local immune responses in this disease is limited (3-7). This study focuses on the analysis of cytokines and lymphocyte subpopulations in the cerebrospinal fluid (CSF) of TBE patients.

CSF lymphocytes are responsible for the immunological surveillance of the brain in healthy persons and for the initiation of local immune response upon encounter with an antigen. The migration of lymphocytes from the blood into the CSF is mediated by chemotactic cytokines and their receptors. In response to the cytokine gradient between the periphery and CSF, lymphocytes that express a particularly cytokine receptor will migrate across the blood brain barrier (8).

The majority of experimental evidence on the role of chemokines and their receptors in the neurological diseases (multiple sclerosis, neuropsychiatric lupus, relapsing neuromyelitis optica, HIV-1 and lymphocytic choriomeningitis virus infections of the CNS, subacute sclerosing panencephalitis, neuroborreliosis etc.) focused on chemokine receptors CXCR3 and CCR5 and their soluble ligands (9-15). Two recent studies showed the presence

of CCL5 and CCL2 in the CSF of TBE patients but the role of other chemokines in this disease has not been previously investigated (16-17).

In this study, we investigated two non-ERL (lacking the Glu-Leu-Arg tripeptide motif) chemokines that are important for the recruitment of activated Th1 lymphocytes to sites of inflammation: CXCL10 (interferon-gamma inducible protein 10 kDa, IP-10) and CXCL11 (interferon-inducible T cell alpha chemoattractant, I-TAC). Both CXCL10 and CXCL11 bind to a chemokine receptor CXCR3 that is usually expressed on activated Th1 lymphocytes that participate in various inflammatory reactions (18). Our aim was to determine whether CSF of TBE patients contains CXCL10 and CXCL11 and whether CSF T-cells express CXCR3. In order to determine whether CXCL10 forms a concentration gradient between the periphery and CNS, we compared serum and CSF concentrations of this chemokine.

In an attempt to further characterize cytokine content of CSF from TBE patients, we quantified several cytokines responsible for the development, proliferation and activity of Th1 lymphocytes (IL-12, common p40 subunit of IL-12 and IL-23 and IL-18). Additionally, we analyzed CSF concentrations of IL-15 (IL-2-like cytokine) that has been shown to be a chemoattractant for human blood T-cells. To the best of our knowledge, this is the first experimental evidence on the CXCL10, CXCL11, IL-12, IL-15 and IL-18 in the CSF of TBE patients.

MATERIALS AND METHODS

Patients and study design:

This cross-sectional study was carried out at the University Hospital for Infectious Diseases, Zagreb and General Hospital „Dr. Tomislav Bardek“, Koprivnica, Croatia between 2004 and 2005.

The study included 19 patients with biphasic course of TBE (8 females, 11 males, median age 38 years, range 21-47 years). The patients were at the second stage of disease that presented in the form of aseptic meningitis, meningoencephalitis or encephalomyelitis. TBE patients were diagnosed based on anamnestic and epidemiological data (a history of recent tick-bite in an endemic area), clinical presentation, CSF analysis (pleocytosis) as well as IgM antibodies against the TBE virus in the serum and CSF and/or seroconversion or significant increase of specific IgG antibodies in acute and convalescent sera.

Additionally, we included 10 patients with non-inflammatory neurological diseases (NIND, 7 females, 3 males, median age 29 years, range 24-41 years). NIND patients were tested and found negative for infection with a group of neurotropic viruses (described in section Serological testing).

Informed consent was obtained from all patients. Ethics committee of the Hospital approved this study.

Samples:

CSF samples were collected from TBE patients and patients with NIND who underwent a diagnostic lumbar puncture on day 0 or 1 of admission. Sample volumes ranged between 1 mL and 2.5 mL. The number of cells per μL in CSFs was determined by counting in a Fuchs-Rosenthal chamber. The samples were stored at -80°C until testing. Concentrations

of CXCL10, CXCL11, IL-12, common p40 subunit of IL-12/IL-23, IL-15 and IL-18 and percentages of selected lymphocyte subpopulations were determined in the CSF of all TBE patients. Expression of CXCR3 on CSF T-cells was determined in 8 TBE patients.

Serum samples from TBE patients (for the quantification of CXCL10 only) and NIND patients were collected on the day of lumbar puncture and stored at -80° C until testing.

Serological testing

Specific IgM and IgG antibodies were tested for tick-borne disease virus (TBD) by enzyme-linked immunosorbent assay (Genzyme Virotech GmbH, Russelheim, Germany) in paired sera and CSF of all patients. Serologically confirmed acute infections showed positive specific IgM and/or seroconversion or significant increase of specific IgG antibodies in acute and convalescent sera. Patients with NIND were tested and found negative for infection with neurotropic viruses. Specific IgM and IgG antibodies were tested for herpes virus type 1 and 2 (HSV-1, HSV-2), cytomegalovirus (CMV) (DiaSorin, Saluggia, Italy), tick-borne disease virus (TBD) (Genzyme Virotech GmbH, Russelheim, Germany), *Borrelia burgdorferi* (Biomedica, Wien, Austria). Specific IgM, IgG and IgA specific antibodies for varicella zoster virus (VZV), enterovirus, influenza A and influenza B virus (Genzyme Virotech GmbH, Russelheim, Germany), *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *C.psittaci* (Savyon Diagnostics LTD, Israel) were tested in paired sera and CSF in all patients with non-inflammatory CNS diseases. In some patients (based on clinical signs), antibodies IgM and IgG for adenovirus, morbilli, mumps (Genzyme Virotech GmbH, Russelheim, Germany), and antibodies IgM, IgG and IgA for parainfluenza (Genzyme Virotech GmbH, Russelheim, Germany), and toxoplasma (DiaSorin, Saluggia, Italy) were also tested. All tests were used and interpreted according to the guidelines of the manufacturer.

Cytokines immunoassays

Concentrations of CXCL10 and CXCL11 in the CSF and serum of TBE and NIND patients were determined by Quantikine Human IP-10/CXCL10 Immunoassay and Quantikine Human I-TAC/CXCL11 Immunoassay (R&D Systems, Inc., USA). Concentrations of IL-12, p40 subunit of IL-12 and IL-15 were determined by Quantikine human IL-12 Immunoassay, Quantikine human IL-12p40 Immunoassay and Quantikine human IL-15 Immunoassay, respectively. IL-18 was quantified by Human IL-18 ELISA Kit (MBL, Nagoya, Japan). The minimum detectable doses of commercially available EIAs for IL-12, p40 of IL-12, IL-15 and IL-18 were 5 pg/mL, 15 pg/mL, 2 pg/mL and 12.5 pg/mL, respectively. All enzyme-immunoassays were performed according to manufacturer's instructions.

Flow cytometry

Lymphocyte subpopulations in the CSF were analyzed by flow cytometry as previously described by Kivisäkk et al (6). CSF cells were collected by centrifugation (600 g, 5 min.) at room temperature. The pellet was resuspended in 200 Izoton II Azide-free balanced electrolyte solution (Beckman Coulter, Inc., Fullerton, CA, USA). Non-specific binding to Fc receptors was blocked with 0.2 mg/ml of IgG1 (clone MCG1, IQ Products, Groningen, Netherlands).

Percentages of CD4⁺ T-cells and memory CD45RO⁺CD4⁺ T-cells in the CSF of TBE patients were determined by staining with antibodies specific for CD3 (clone UCHT1), CD4 (clone MT310), and CD45RO (clone UCHL1, DakoCytomation A/S, Glostrup, Denmark, 3-color flow cytometry).

The number of CXCR3 molecules on PB and CSF T-cells was determined by using CELLQUANT Calibrator kit for leukocyte surface antigen quantitation by flow cytometry (Biocytes, Marseille, France) as recommended by the manufacturer. Indirect immunostaining

of CSF cells was performed by using a monoclonal antibody specific for CXCR3 (unconjugated, clone 49801, R&D Systems, Minneapolis, MN, USA) and polyclonal antibody anti mouse IgG-FITC (provided by the manufacturer of the kit). Counter-staining used to gate and analyze CD4⁺ T-cells was CD4-PE (Biotex, Marseille, France). PB samples were prepared by using Whole blood erythrocyte lysing kit (R&D Systems, Minneapolis, MN, USA). The samples were analyzed on Epics XL-MCL Flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA).

Statistical analysis

Statistical analysis was performed by using Statistica for Windows (Version 6.1, StatSoft Inc.). Comparison between two independent groups was done with Mann-Whitney U test and Wilcoxon test **was used for paired data**. Statistical significance was determined at the 0.05 level.

RESULTS

Cytological and biochemical findings

Seventy-five percents of patients had a positive epidemiological anamnesis for a tick-bite in endemic area. The most frequent clinical signs and symptoms in TBE patients were moderate and included headache, fever, vomiting, dizziness, arthralgia and abdominal pain. None of the patients had received TBE vaccine. Median number of CSF cells in TBE patients was 738 cells/ μ L [interquartile range (IQR) 116-1569 cells/ μ L]. Median CSF/blood glucose ratio was 0.55 (IQR 0.45-0.80). Median protein concentration in the CSF was 725 mg/mL (IQR 520-1485 mg/mL). A group of patients with non-inflammatory neurological diseases (NIND) included persons with headache (n=5), back pain (n=3) and cerebrovascular disease (n=2). All NIND patients had less than 10 CSF cells per μ L (median 2.5 cells/ μ L). Additionally, all NIND patients had normal concentrations of glucose in the blood (normal values 4.2-6.4 mol/L) and CSF (reference values 2.25-4 mol/L). Concentration of proteins in the CSF of NIND patients was also within normal ranges (150-450 mg/L).

CSF cytokines

CSF of TBE patients contained CXCL10, CXCL11, p40 subunit of IL-12/23, IL-15 and IL-18 (Table 1).

Median CSF concentration of CXCL10 in TBE patients was 217 pg/mL, IQR 63-1065 pg/mL. CXCL10 concentration in the CSF of NIND patients (median 9, IQR 7.2-12.9 pg/mL) was significantly lower compared with TBE patients ($p < 0.001$).

We compared CXCL10 concentration in CSF and serum (taken on the same day) of TBE and NIND patients. Concentration of CXCL10 in CSF samples was higher than in serum samples in all TBE patients. Statistical analysis showed that CXCL10 concentrations in the

CSF and serum (median 62, IQR 51-73 pg/mL) of TBE patients were significantly different ($p < 0.001$) (Figure 1). Contrary to this finding, CXCL10 concentration in the CSF of NIND patients were lower compared with serum in all patients. CXCL10 concentrations in the serum (median 98, IQR 75-117 pg/mL) and CSF of NIND patients were significantly different as well ($p < 0.001$).

CSF of TBE patients also contained CXCL11 (median 8.3 pg/ml; range 1.6-145 pg/mL). Concentration of CXCL11 in the serum (median 20.5 pg/mL, range 15.7-131 pg/mL) and CSF of TBE patients were significantly different ($p < 0.001$).

CSF of TBE patients also contained p40 subunit of IL-12/IL-23 (median 38.9 pg/mL, range 15-204 pg/mL), IL-15 (median 5.9 pg/mL, range 3.1-18.9 pg/mL) and IL-18 (median 30.1%, range 14.2-157 pg/mL) (Table 1). Concentration of IL-12 in the CSF was below the level of detection of the commercially available ELISA (< 5 pg/mL) used in this study.

Lymphocyte subpopulations and CXCR3 expression in the CSF

Percentages of CD4⁺ T-cells in the CSF of TBE patients (median 71%, IQR 65-83%) were significantly increased compared with the blood (median 55%, IQR 38-65%). The majority of CSF CD4⁺ T-cells ($> 85\%$) were of memory phenotype (CD45RO⁺). CD4⁺ T-cells in the PB and CSF of TBE patients expressed CXCR3. The majority of CSF CD4⁺ T-cells expressed CXCR3 (median 3304 molecules/cell, IQR 2689-3830 molecules/cell). The number of CSF cells in the NIND group (< 10 cells/ μ L) was too small to allow the analysis of chemokine receptor expression.

DISCUSSION

This study showed that CSF of patients with TBE contains chemokines (CXCL10 and CXCL11) and interleukins (common p40 subunit of IL-12/IL-23, IL-15 and IL-18). Furthermore, we demonstrated a serum-CSF concentration gradient of CXCL10 in TBE. Based on these results we propose that CXCL10 plays an important role in the recruitment of CXCR3-expressing T-cells into the CSF in TBE patients.

Literature data on the local and systemic immune response to TBE are rather limited. Quantification of various cytokines in the serum of TBE patients showed increased levels of tumor necrosis factor-alpha (TNF- α), IL-1 α , IL-6, IL-10 and their corresponding soluble receptors during the first week of hospitalization (6). Another study suggested a possible positive correlation between the disease severity and serum concentrations of IL-6 in TBE (7). More recent studies showed increased concentrations of CCL2 and CCL5 in the CSF of TBE patients (at the second stage of disease) compared with controls (neuroinfection excluded) (16,17). Our study showed, for the first time, the presence of CXCL10 and CXCL11 in the CSF of TBE patients. CXCL10 and/or CXCL11 have also been previously shown in the CSF of patients with neuroborreliosis, HIV-1 infection, neuropsychiatric lupus patients lymphocytic choriomeningitis virus-infected central nervous system subacute sclerosing panencephalitis (11-15).

We reported significantly higher concentrations of CXCL10 and CXCL11 in the CSF of neuroborreliosis patients compared with the corresponding serum samples (15). We concluded that CXCL10 and CXCL11 create a chemokine gradient between the CSF and serum and recruit CXCR3-expressing memory CD4⁺ T-cells into the CSF of neuroborreliosis patients. The present study also demonstrated a concentration (CSF-serum) gradient of

CXCL10 in TBE and suggested the common mechanism of T-cell recruitment into the CSF in both TBE and neuroborreliosis.

IL-18 has been previously demonstrated in the CSF of patients with multiple sclerosis, HIV-infection of the CNS, neuroborreliosis and Creutzfeldt-Jakob disease (20-23). Our study also showed the presence of IL-18 in the CSF of TBE patients. IL-12 is the principal regulator of the development, proliferation and activities of Th1 lymphocytes and the presence of this cytokine in the CSF of our patients suggests the importance of Th1 type of response in the local immune reaction to TBEV.

CSF of patients with neuroborreliosis, echovirus meningitis and Creutzfeldt-Jakob disease contains IL-12 (22, 24,25). IL-12 is a heterodimeric cytokine composed of a 40 kDa (p40) subunit and a 35 kDa (p35) subunit. We failed to detect IL-12 in the CSF of TBE patients. However, CSF of our patients contained p40 subunit of IL-12 that is shared with a novel cytokine IL-23. Although the p40 and p35 subunits themselves do not have IL-12 activity, the homodimer of p40 has been shown to bind the IL-12 receptor and is an IL-12 antagonist. Therefore, despite the absence of fully functional IL-12 in the CSF of our patients, the presence of p40 subunit of IL-12 might suggest an important role of IL-12 and possibly IL-23 in the local immune response to TBEV.

A recent study by Rentzos et al (2006) showed significantly higher concentration of IL-15 in the CSF of multiple sclerosis patients compared with patients with other inflammatory or non-inflammatory CNS diseases (26). No significant correlations between serum and CSF levels of IL-15 with clinical parameters important for multiple sclerosis were found. Our study showed IL-15 in the CSF of TBE patients. This finding suggests a possible role for the cytokines in the immunopathogenesis of TBE.

In conclusion, CSF of TBE patients contains CXCL10, CXCL11, p40 subunit of IL-12/IL-23, IL-18 and IL-15. Increased CXCL10 CSF concentrations (compared with serum)

suggest a possible role for this chemokine in the recruitment of CXCR3-expressing T-cells into the CSF of TBE patients. Our future research will focus on the comparison of CSF cytokines in patients with severe and moderate clinical course.

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Figure 1. CXCL10 in the cerebrospinal fluid (CSF) and serum of patients with tick-borne encephalitis (TBE) and non-inflammatory neurological diseases (NIND)

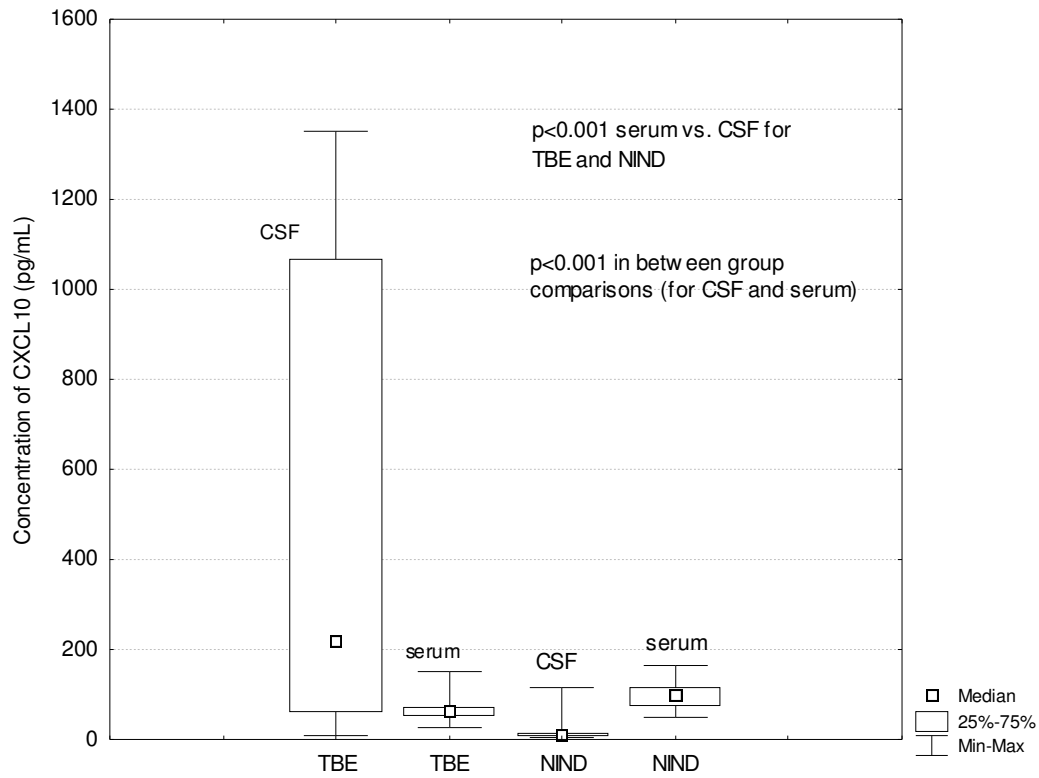


Table 1. Concentration of cytokines in the cerebrospinal fluid (CSF) of patients with tick-borne encephalitis (TBE)

Cytokine in the CSF (concentration in pg/mL)	TBE patients	NIND patients	p
CXCL10	217 (63-1065)	9 (7.2-12.9)	< 0.001
CXCL11	8.3 (1.6-145)	Not determined	
IL-12	not detectable	Not determined	
p40 subunit of IL-12	38.9 (15-204)	Not determined	
IL-18	30.1 (14.2-157)	Not determined	
IL-15	5.9 (3.1-18.9)	Not determined	

- values expressed as median, interquartile range

IL=interleukin