

Extensive Recruitment of Plasma Blasts to the Cerebrospinal Fluid in Toscana Virus Encephalitis

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An unexpectedly extensive recruitment of B cells and plasma blasts to the cerebrospinal fluid (CSF) in a patient with Toscana virus (TOSV) encephalitis is described. Acute infection by TOSV was demonstrated by serological methods and by detection of TOSV-specific nucleic acid in the CSF by real-time polymerase chain reaction and sequencing.

Keywords. B cells; cerebrospinal fluid; encephalitis; plasma blasts; Toscana virus.

In the last years, Toscana virus (TOSV) infection has been identified as an important cause of meningoencephalitis in a subgroup of patients in certain areas of Mediterranean countries [1, 2]. Toscana virus belongs to the family of *Bunyaviridae*, genus *Phlebovirus*, with a trisegmented negative sense RNA genome [1–3]. In recent studies, TOSV infections in travelers from the Island of Elba, Italy, who developed meningoencephalitis in their home countries have been described [4, 5]. Although the seroprevalence of TOSV antibodies in selected Italian populations ranges from 22% to 77%, the reported number of cases with severe central nervous system (CNS) involvement is limited in those areas [6–9]. Serum anti-TOSV antibodies cross-react strongly with sandfly fever Naples virus (SFNV), and the

detection of viral RNA is often difficult due to the low concentration of TOSV-specific nucleic acid in cerebrospinal fluid (CSF).

We describe the case and extensive clinical work-up of a 73-year-old man with the clinical picture of encephalitis. Informed consent of the patient was obtained for this study. The patient's symptoms developed gradually over 3 days while he was returning to Germany from vacation on Elba in September 2014. Back in Germany, the patient was initially admitted to a local hospital with mild fever up to 38°C, headache, myalgia, and episodes of vomiting. As symptoms worsened, he was transferred to our university medical center, where he presented with signs of confusion, headache, nausea, and photophobia. The patient recovered gradually and had only minor episodes of headaches 13 days after disease onset.

Flow cytometry analysis identified a transient and remarkable recruitment of B cells with a large proportion of plasma blasts to the CSF compartment. Acute infection by TOSV was supported by anti-TOSV serum antibodies with significant titer changes during the course of disease. Central nervous system infection was demonstrated by TOSV-specific nested polymerase chain reaction (PCR) and sequencing of RNA extracted from the CSF of the patient.

MATERIALS AND METHODS

Serum and CSF samples were collected on day 5 and 13 after disease onset and immediately processed. A follow-up serum sample was obtained 27 days after disease onset. Basic CSF work-up consisted of microscopic cell counting and May-Grünwald cell stain; measurement of glucose/lactate levels and nephelometric protein analysis (albumin, immunoglobulin [Ig] G, IgA, IgM) were done in parallel.

For flow cytometry analysis, CSF samples were directly washed and processed. Ex vivo staining was performed with antibodies against CD45 (clone HI30; BD Biosciences [BD]), CD3 (clone SK7; BD), CD19 (clone J3.119; Beckman Coulter [BC]), CD138 (clones B-A38; BC), CD4 (clone SK3; BD), CD8 (clone SFC121Thy2D3; BC), CD14 (clone MfP9; BC), and CD56 (clone N901; BC). Stained cells were analyzed using an FACS cytometer (CYAN; BC) and FlowJo Software (Tree Star).

Two-fold dilutions of sera and CSF specimens were subjected to endpoint titration with starting dilutions of 1:2 (CSF) and 1:10 (sera), respectively, to determine IgM and IgG titers. Antibody detection was done by an indirect immunofluorescence assay (IFA) using a sandfly fever virus and a flavivirus mosaic (Euroimmun).

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Table 1. Protein and Leukocyte Analysis in the CSF at Day 5 and 13 After Disease Onset

| | CSF/Serum Albumin Ratio ($\times 10^{-3}$) | Intrathecal IgM Synthesis (mg/L) | Leukocyte Count ($\times 10^9/L$) | CD3 ⁺ CD4 ⁺ T Cells | CD3 ⁺ CD8 ⁺ T Cells | CD14 ⁺ Monocytes | CD56 ⁺ NK Cells | CD19 ⁺ CD138 ⁻ B Cells | CD19 ^{low} CD138 ⁺ Plasma Blasts |
|--------|--|--|---|--|--|--------------------------------|-------------------------------|---|---|
| Day 5 | 22.4 | 136.0 (91%) | 264 | 50.5 | 6.3 | 2.1 | 4.9 | 9 | 20.2 |
| Day 13 | 12.5 | 24.2 (55%) | 25 | 55 | 27.6 | 1 | 6.5 | 4.4 | 0.6 |

Abbreviations: CSF, cerebrospinal fluid; NK, natural killer.

Immune cell subset frequencies given in % of all CSF leukocytes; reference values for CD19⁺CD138⁻ B cells and CD19^{low}CD138⁺ plasma blasts in non-inflammatory neurological patients: 0.6% (B cells) and 0.1% (plasma blasts) [13].

Viral RNA was extracted from CSF obtained at day 5 using QIAamp viral RNA Mini Kit (Qiagen) and tested for West Nile virus (WNV) by artus WNV LC real-time reverse-transcription (RT)-PCR Kit (Qiagen) according to the manufacturer's instructions. Toscana virus-specific RT-PCR was carried out according to a protocol previously described [10]. A partial sequence of the S-segment was obtained by nested PCR. In brief, a 918 base pair (bp) fragment was amplified from the extracted nucleic acid using the primers TOS 873f 5'-ACTgCTCTTTCCACCTTTTgg-3' and TOS 1791r 5'-ACA TTgCTCTTgCTTTTCTTgATg-3'. The obtained PCR product was subjected to nested PCR with the primers TOS 960f 5'-AgAgTgACAAAgTggCTgCCTAg-3' and TOS 5'-1741r CAA TgCATgggTgAATgAgTTTg-3'. All protocols were carried out using a superscript III high fidelity kit (Life Technologies). The obtained 782 bp fragment was analyzed by gel electrophoresis and Sanger sequencing (GATC Biotech). Phylogenetic tree analysis of a 663 nucleotide (nt) partial small (S) segment sequence was performed using the maximum likelihood method based on the Tamura-Nei model and 1000 bootstrap replicates with Mega 5.0 [11,12]. Identical clusters and highly similar confidence values were obtained by maximum parsimony method and neighbour-joining method.

RESULTS

Cerebrospinal fluid taken 5 days after disease onset revealed moderate pleocytosis (264 white cells/ μ L) with normal levels for glucose and lactate (Table 1). Protein analysis revealed blood-CSF barrier dysfunction and evidence for a strong intrathecal IgM (but not IgG or IgA) synthesis of 91% (Table 1). Cerebrospinal fluid smears showed a dominance of plasmacytoid lymphocytes (Figure 1A). Flow cytometry analysis demonstrated increased CD19⁺CD138⁻ B cell numbers (9% of leukocytes; reference value in noninflammatory neurological patients: 0.6%) with an extensive CD19^{low}CD138⁺ plasma blast recruitment to the CSF of 20.2% (reference value: 0.1%; Figure 1B, Table 1) [13].

Thirteen days after disease onset, CSF analysis revealed decreased cell numbers (25 cells/ μ L), normalization of blood-

CSF barrier and reduced intrathecal IgM synthesis of 55% (Table 1). This was paralleled by decreased CD19⁺CD138⁻ B-cell counts (4.4% of leukocytes) with a remarkable drop of CD19^{low}CD138⁺ plasma blasts to 0.6% in the CSF approaching normal levels (Figure 1B, Table 1).

In contrast to B cells and plasma blasts, intrathecal CD8⁺ and CD4⁺ T cells increased between day 5 and 13, whereas CD14⁺ monocyte frequencies decreased over time; CD56⁺ natural killer cells remained largely stable (Table 1).

Neuroborreliosis, neurosyphilis, and tickborne encephalitis as well as infections due to herpes simplex virus, enteroviruses, cytomegalovirus, and Epstein-Barr virus could be ruled out by serological and molecular methods. Bacteriological work-up including testing for meningococcal infection was negative. Thus, antibiotic and antiviral treatment with acyclovir was stopped. Brain magnetic resonance image scanning did not reveal any pathological changes or evidence for meningeal enhancement.

Based on the recent travel history to Tuscany with reported insect bites, a diagnostic work-up for sandfly infection was carried out. By IFA, high serum antibody titers against TOSV (IgM: 1:1280; IgG: 1:2560) and SFNV (IgM: 1:1280; IgG: 1:2560) were observed on day 5. Serum anti-TOSV antibodies temporarily increased (IgM: 1:2560; IgG: 1:10240) on day 13 and dropped on day 27 after disease onset (IgM: 1:1280; IgG: 1:5120). In contrast, antibody titers in the CSF against TOSV and SFNV dropped significantly from 1:512 (IgM) and 1:256 (IgG) on day 5 to 1:32 (IgM and IgG) on day 13. It is noteworthy that negative molecular and serologic test results were obtained for acute WNV infection in CSF and sera, respectively.

Toscana virus-specific RNA could be detected by real-time RT-PCR from the CSF, and a 782 bp fragment of TOSV was successfully amplified by means of nested PCR. Based on the obtained sequence data, the TOSV could be grouped in genotype A (Tos A) clustering most closely with another Italian strain (gb|KM275771; Figure 1C) [11].

DISCUSSION

Meningoencephalitis caused by TOSV infection is an important differential diagnosis in patients from endemic regions of

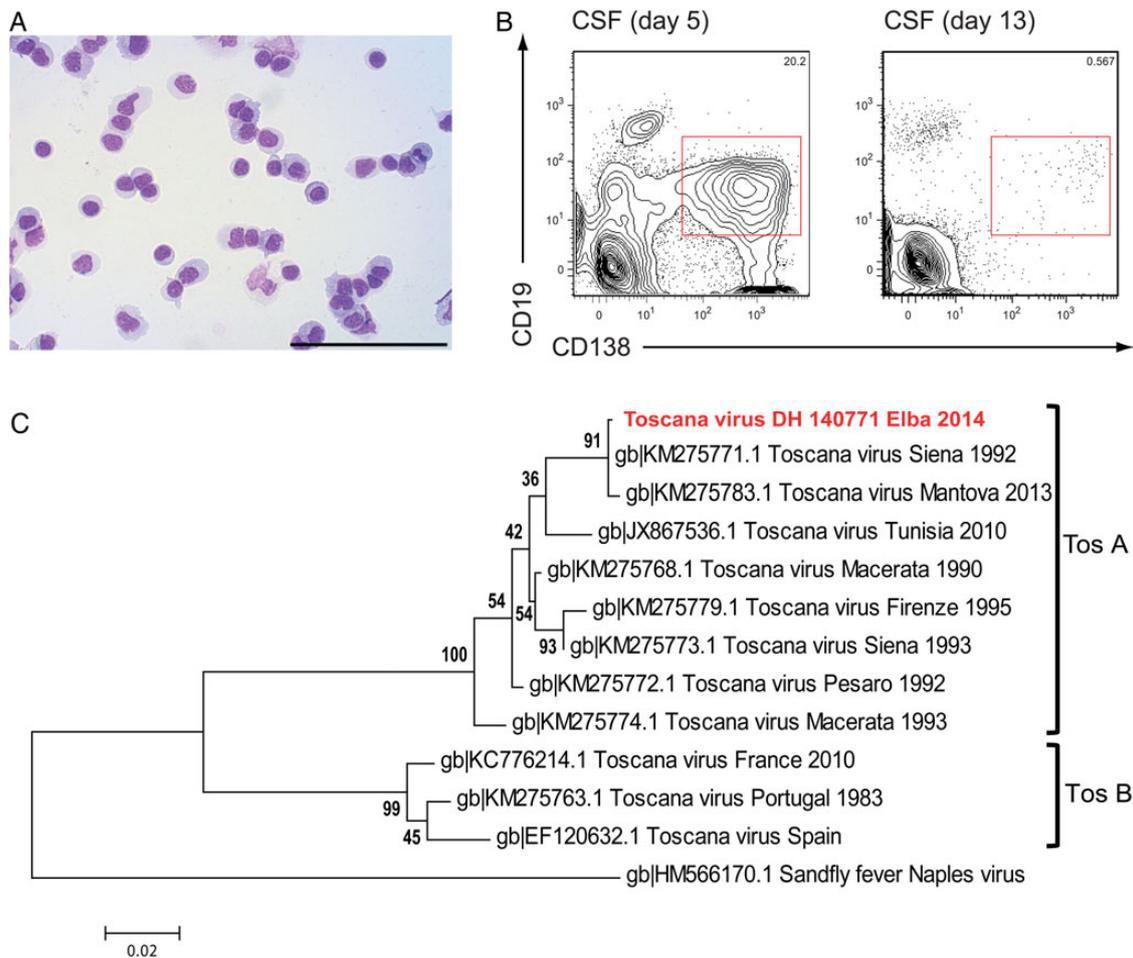


Figure 1. (A) May-Grünwald cell smear stain of cerebrospinal fluid (CSF) obtained 5 days after disease onset shows dominance of plasmacytoid lymphocytes and plasma blasts (scale bar: 50 μ m). (B) Flow cytometry analysis of sequential CSF reveals extensive recruitment of CD19^{low}CD138⁺ plasma blasts at day 5 after disease onset with dramatic decline during clinical recovery at day 13 (see framed gates for CD19^{low}CD138⁺ cells; frequencies in % of all CSF leukocytes). (C) Phylogenetic tree analysis of a 663 nucleotide (nt) partial S segment sequence by maximum likelihood method based on the Tamura-Nei model and 1000 bootstrap replicates with Mega 5.0; our herein described case *Toscana virus DH 140771 Elba 2014* is highlighted in red bold letters (scale bar indicates number of nt substitutions per site).

Phlebotomus spp sandflies and needs to be considered in travelers presenting with symptoms of viral meningoencephalitis. Due to climate changes, there is a risk that the vector will move farther North and establish itself in regions of central Europe, which consecutively may lead to the emergence of sandfly fever virus infections [14]. It is likely that the majority of TOSV infections in travelers returning from endemic regions remain undetected because only a limited number of specialized laboratories are able to carry out the required diagnostics. Patients with TOSV meningitis usually show a mild to moderate clinical course and resolve without any sequelae. However, a few severe cases including encephalitis have been described in the literature [7, 9, 15, 16].

In this case, it was possible to demonstrate a moderate pleocytosis with an extensive appearance of B cells and plasma blasts in the CSF during the peak of clinical symptoms by microscopic

analysis and flow cytometry. We recently reported increased B cell and plasma blast counts in the CSF of patients with neuroinfectious diseases compared with multiple sclerosis and other CNS diseases [13, 17]. Accordingly, elevated B cell and plasma blast numbers have been described in the CSF of patients with viral meningitis, human immunodeficiency virus infection, and neuroborreliosis, however not at the high levels observed in this TOSV patient [13]. Given that a dramatic decline in CSF plasma blast numbers during the clinical recovery of the patient was noted, it can be assumed that the detected CD19^{low}CD138⁺ cells belong to a group of short-lived plasma cells that have the ability to strongly proliferate upon initial antigen contact with an intense antibody synthesis [17, 18].

In recent studies of West Nile encephalitis, a strong plasma cell pleocytosis in the CSF has been reported by cytological smear analysis [19]. It is interesting to note that both TOSV

and WNV are arthropod-borne and enveloped RNA viruses that can cause meningoencephalitis in overlapping areas of Mediterranean countries [14, 20].

CONCLUSIONS

In summary, we present a case of meningoencephalitic TOSV infection acquired on Elba, Italy. Direct and indirect proof of the infection was achieved by an extensive diagnostic work-up. For the first time, it was possible to conduct an immune cell subtyping in the CSF in TOSV encephalitis showing an exceptionally strong intrathecal recruitment of B cells and plasma blasts. Because this has not been observed in other common viral meningitis patients (eg, due to herpes or enterovirus infection), it seems possible that arthropod-borne RNA viruses such as WNV and TOSV lead to a unique recruitment of B cells to the CNS. Because these observations are only based on single cases, further investigations are necessary to study immune cell subsets in WNV and TOSV encephalitis and the mechanism that leads to the specific B-cell recruitment.

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