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MAJOR ARTICLE

Search for Viral Infections in Cerebrospinal Fluid From Patients With Autoimmune Encephalitis

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Background. It has been reported that virus-mediated brain tissue damage can lead to autoimmune encephalitis (AE) characterized by the presence of antibodies against neuronal surface antigens. In the study, we investigate the presence of viruses in cerebrospinal fluid (CSF) from patients with AE using reverse transcription polymerase chain reaction (RT-PCR)/PCR and shotgun metagenomics.

Methods. CSF samples collected from 200 patients with encephalitis were tested for the presence of antibodies against antiglutamate receptor (NMDAR), contactin-associated protein 2 (CASPR2), glutamate receptors (type AMPA1/2), leucine-rich glioma-inactivated protein 1 (LGI1), dipeptidyl aminopeptidase-like protein 6 (DPPX), and GABA B receptor, and those found positive were further analyzed with real-time RT-PCR/PCR for common viral neuroinfections and shotgun DNA- and RNA-based metagenomics.

Results. Autoantibodies against neuronal cells were detected in CSF from 8 individuals (4% of all encephalitis patients): 7 (3.5%) had anti-NMDAR and 1 (0.5%) had anti-GABA B. RT-PCR/PCR identified human herpes virus type 1 (HSV-1; 300 copies/mL) and the representative of *Enterovirus* genus (550 copies/mL) in 1 patient each. Torque teno virus (TTV) was found in another patient using metagenomic analysis, and its presence was confirmed by specific PCR.

Conclusions. We detected the presence of HSV, TTV, and *Enterovirus* genus in CSF samples from 3 out of 8 AE patients. These findings support the concept of viral involvement in the pathogenesis of this disease.

Keywords. autoimmune encephalitis; anti-NMDAR; metagenomics; virus; NGS.

Autoimmune encephalitis (AE), which is characterized by the presence of antineuronal or antiglial antibodies, has been reported to constitute 4.2% and 7.9% of all encephalitis cases in the United States and the United Kingdom, respectively [1, 2]. However, some studies reported a markedly higher percentage [2, 3] among pediatric patients [4]. Autoantibodies in AE are classified into 2 main categories: antibodies against neuronal cell surface and against intracellular antigens [5]. While autoimmune reactions against intracellular antigens are linked with irreversible outcomes and limited response to therapy, patients with surface autoantibodies have a more favorable prognosis and respond well to immunosuppressive treatment [6, 7]. Intracellular antibodies are almost invariably part of paraneoplastic syndrome, but only a minority of patients with autoantibodies against surface antigens have concurrent tumors [8–10]. There is mounting evidence that in contrast to intracellular antibodies, autoantibodies against surface antigens such as anti-N-Methyl-d-aspartate receptor (anti-NMDAR) and anti-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (anti-AMPAR) are directly pathogenic [11–14].

It is currently unclear what causes activation and migration of autoantibody-producing B lymphocytes across the blood–brain barrier [15]. It has been proposed that virus-mediated brain tissue damage could lead to exposure of the normally sequestered neuronal cell antigens or that the cause of autoantibody production could be "molecular mimicry" of viral proteins [16–18]. The primary candidate is herpes simplex virus (HSV), which was reported to be capable of triggering autoimmunity to NMDAR, dopamine D2 receptor (D2R), and γ-aminobutyric acid type A receptor (GABA-AR) [18, 19]. However, a variety of other viral agents, including West Nile virus (WNV), varicella zoster virus (VZV), and even severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), have been associated with the development of AE [20–23].

In the present study, we used real-time reverse transcription polymerase chain reaction (RT-PCR)/PCR and next-generation sequencing (NGS) metagenomics to search for the presence of viruses in cerebrospinal fluid (CSF) from patients with AE.

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METHODS

Patients

The analysis was conducted on 200 consecutive patients who were part of a large ongoing single-center study on the etiology of encephalitis in Poland. Details of the study, including criteria, and data on the first 96 patients have been published [24]. CSF and serum were collected at the time of hospital admission. CSF was centrifuged at 1.200 rpm for 20 minutes at 4°C, and kept frozen at –80°C.

Autoantibody Detection

The presence of antibodies against NMDAR, contactin-associated protein 2 (CASPR2), AMPAR1/2, leucine-rich glioma-inactivated protein 1 (LGI1), dipeptidyl aminopeptidase-like protein 6 (DPPX), and GABA B receptor was evaluated using a commercially available indirect immunofluorescence test (Autoimmune Encephalitis Mosaic 6, Euroimmune, Lübeck, Germany). Fluorescence was read with a Nikon Eclipse 80i (Nikon, Tokio, Japan) microscope at ×20 and ×40 magnifications. CSF samples from all patients were tested regardless of whether any etiological factor of encephalitis was identified. All positive results were confirmed in an independent run and read by a technician experienced in routine diagnostics of autoimmune encephalitis antibodies.

Virus-Specific RT-PCR/PCR

CSF samples were analyzed with in-house quantitative real-time RT-PCR/PCR assays, as previously described [25–28], using RNA and DNA extracted from 200 µL of CSF by Trizol LS (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the NucleoSpin Plasma XS kit (Macherey Nagel, Düren, Germany), respectively. The following pathogens were searched for: herpes simplex viruses type 1/2 (HSV-1/2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus type 6 (HHV-6), human adenoviruses (HAdVs), and enteroviruses (EVs: Coxsackie A9, A16, B2, B3, B4, B5; ECHO 5, 6, 9, 11, 18, 30; and enterovirus 71). Limits of detection (LODs) for quantitative PCRs were as follows: for HSV-1 – 253 viral copies/mL, HSV-2 – 369 viral copies/mL, VZV – 150 viral copies/mL, CMV – 403 viral copies/mL, EBV – 226 viral copies/mL, HHV-6 – 111 viral copies/mL, HHV-7 – 153 viral copies/mL, HAdV – 102 viral copies/mL, and EV – 240 viral copies/mL.

Serological Testing

Routine serological testing included anti-HSV-1/2 IgG/IgM, anti-VZV IgG/IgM, and anti-TBEV IgG/IgM (Institut Virion/Serion GmbH, Würzburg, Germany). Tests were performed and interpreted following the manufacturer's recommendations.

Viral Metagenomics

Two hundred twenty-five microliters of each CSF was filtrated using the Millex-HV Syringe Filter Unit (Merck KgaA, Darmstadt, Germany) with a pore size of 0.45 µm and digested with 2U of TURBO DNase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 30 minutes. Next, filtrated and digested CSF samples were subjected to RNA extraction with TRIzol LS (Thermo Fisher Scientific, Waltham, Massachusetts, USA) or DNA extraction using the NucleoSpin Plasma XS kit (Macherey-Nagel, Düren, Germany). RNA and DNA were eluted in 5 µL and 12 µL of water, respectively. Due to the typically low yield of DNA/RNA extraction from CSF, a preamplification step was introduced to generate NGS libraries for sequencing. In short, 5 µL of RNA was first reverse-transcribed for 5 minutes at 65°C and preamplified by a single-primer isothermal amplification (Ribo-SPIA) using the Ovation RNA-Seq V2 system (NuGEN, San Carlos, San Francisco, USA). For DNA, the whole amount was preamplified with the SeqPlex Enhanced DNA Amplification kit (Sigma-Aldrich, Saint Louis, Missouri, USA). Preamplified cDNA/DNA was purified using a 0.8 ratio of Agencourt AMPure XP beads (Beckman Coulter, Brea, California, USA) to reaction mixture and eluted in 30 µL of water. Libraries for sequencing were prepared using the Nextera XT Kit (Illumina, San Diego, California, USA) with 1 ng of preamplified cDNA/DNA and following the manufacturer's protocol with small modifications: Amplification was performed with 14 cycles instead of 12, and the ratio of Agencourt AMPure XP beads (Beckman Coulter, Brea, California, USA) added to the reaction mixture was 0.6. The quality and average length of NGS libraries were assessed using Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) and the DNA HS kit (Agilent Technologies, Santa Clara, California, USA). Finally, the samples were indexed, pooled, and sequenced on Illumina HiSeq (101 nt, paired-end reads).

NGS reads were evaluated for quality using FastQC software [29], and those with a Phred quality score >30 were trimmed with Trimmomatic [30] and mapped to the human genome using Stampy [31]. Next, all nonhuman reads were aligned by Bowtie2 [32] to a database containing complete viral genomes, obtained from the NCBI Reference Sequence Database (RefSeq). Reads matching viral genomes were sorted and counted with SAMtools [33] and the phyloseq package in R [34]. Visualization of alignments and coverage were analyzed using CLC Genomics Workbench (Qiagen, Hilden, USA).

The following criteria were applied for positive virus detection by metagenomic analysis: (i) a minimum of 3 reads specific to a particular viral species had to be present; (ii) specific reads had to be distributed over the genome. Similar criteria were previously used by other groups for viral identification by NGS [35, 36].

Patient Consent Statement

All patients gave written informed consent, and all research was performed in accordance with the relevant guidelines and regulations. The study was approved by the Internal Review Board of the Medical University of Warsaw, Poland.

RESULTS

Autoantibodies against neuronal cells were detected in 8 individuals (4% of all encephalitis patients): 7 (3.5%) had anti-NMDAR and 1 (0.5%) had anti-GABA B. Only 1 of these patients had anti-NMDAR in serum (Table 1). All these samples were tested at serial dilutions of 10^1–10^4 and deemed to be low positive (1:10). None of the patients had teratoma or any other type of cancer.

The age of autoantibody-positive patients ranged from 20 to 62 years, and the majority were female (62.5%). Seizures were the most prominent clinical manifestation, as they were present in almost every patient, and 4 patients had psychotic symptoms. Some demographic and clinical data on these patients are presented in Table 1.

All patients positive for autoantibodies were further analyzed for the presence of common neurotropic viruses in CSF using quantitative in-house RT-PCR/PCR and for the presence of specific antiviral antibodies using commercial assays (Table 1). One patient was found to be infected with HSV-1 (viral load, 300 copies/mL). Another patient was found to be infected by an enterovirus (our quantitative RT-PCR would detect several enteroviruses but did not discriminate between species), and the estimated viral load was 550 copies/mL.

Detailed data on metagenomic analysis and corresponding PCR and serological test results are shown in Table 1. Our metagenomic analysis consisted of a 2-pronged approach based on separate RNA and DNA preamplification. The numbers of raw reads per sample for DNA and RNA analysis were 12 893 313 and 14 469 597, respectively. After quality checking and trimming, the average number of filtered reads for RNA and DNA analysis was 12 653 056 and 14 137 536, respectively. The number of reads aligning to viral genomes ranged from 1015 to 132 884 (median, 6645) and was similar for RNA and DNA analysis (median, 6493 vs 6645) Only 1 eukaryotic virus—Torque teno virus (TTV)—was identified (Table 1). The presence of TTV-DNA was confirmed by a specific PCR detecting viral noncoding region using primer NG133 (91–115 nucleotide position based on GenBank AB017610.1) and NG147 (position 211–233) and following the procedure described by Okamoto et al. [37].

Although metagenomics identified eukaryotic viral agent in only 1 CSF sample, viral reads were detected in all. The vast majority of these viruses were bacteriophages, whereas the remaining viral reads either did not fulfill the criteria for positivity or were classified as contaminants or artifacts (not shown).

DISCUSSION

In the current study, antineuronal autoantibodies were detected in 8 (4%) out of 200 patients with a clinical diagnosis of encephalitis, which is similar to the proportion reported for the United States (4.2%; based on anti-NMDAR) [38] and only slightly lower than that reported for Hungary (5.8%; based on anti-NMDAR, anti-LGI1, anti-Caspr2, anti-GABABR, anti-AMPAR1, and anti-AMPAR2) [39] and the United Kingdom (7.9%; based on anti-VGKC and anti-NMDAR) [40]. In our study, the vast majority of detected autoantibodies were anti-NMDR (87.5%), which is compatible with other reports; for example, in the study by Saraya et al. [41], anti-NMDAR antibodies were present in 81% of AE cases with detectable neuronal surface antibodies.

However, our study could have underestimated the prevalence of AE, as patients were required to have increased CSF pleocytosis and/or elevated protein to be diagnosed with encephalitis, and some AE cases have been observed without these findings [42]. The International Encephalitis Consortium has published guidelines for the identification of encephalitis that might decrease this bias using a combination approach specifically designed to include autoimmune causes [43], and some recent studies using these less strict criteria reported an AE proportion as high as 26% among all encephalitis cases [4].

The etiology of AE is often unclear, but many cases have been associated with pseudoneural expression in teratomas [44], and the results of several studies suggest that viral infections, and particularly HSV, are implicated in its pathogenesis [45, 46]. In the current study, using quantitative RT-PCR/PCR and RNA- and DNA-based metagenomics, we detected viral sequences in CSF from 3 out of 8 patients with AE. To our knowledge, this is the first study employing shotgun metagenomics for the analysis of CSF from patients with AE. The role of antecedent viral infection among our AE patients could have been overestimated due to the aforementioned inclusion criteria, as higher pleocytosis and protein values are typically associated with viral etiology.

HSV-1 infection has previously been associated with AE and particularly with anti-NMDAR receptor encephalitis [45, 47, 48]. A close association between HSV and AE was also suggested by the results of a retrospective study that found anti-NMDAR antibodies in 13 (30%) out of 44 patients with HSV-1 encephalitis [49]. Similarly, Salovin et al. found that markers of past HSV-1 infection are more common in anti-NMDAR encephalitis than in age-matched controls with other neuroinflammatory disorders [50]. While the exact mechanisms are still speculative, the most widely accepted hypothesis is molecular mimicry between virus-associated antigens and the NMDA receptor [17, 48]. A similar mechanism was also postulated to be behind the association between HSV infection and such autoimmune diseases as stromal keratitis [51] and myasthenia gravis [52].

Table 1. Demographic, Clinical, Laboratory, and NGS-Based Metagenomic Data in Patients With Autoimmune Encephalitis

Pt.	Age Gender	AE-ab in CSF	Clinical Manifestation	GCS	CSF Cytosis/μL lymph. No. (%)[b]	PCR in CSF	NGS Workflow	Reads After Trimming	Viral Reads	% of Viral Reads	Phage/All Viral Species, %	Eukaryotic Viruses in NGS
S1	59 M	NMDAR+	Headache, fever, seizures, meningeal signs, VII nerve palsy, hearing impairment	15	24 (80)	Neg	DNA	15 234 632	38 360	0.252	71.67	Neg
							RNA	15 369 597	4316	0.028	9.52	Neg
S2	22 F	NMDAR+	Headache, fever, seizures, psychosis, myoclonus, peripheral nerve palsy	6	208 (10)	TTV	DNA	11 359 791	8728	0.077	64.71	TTV
							RNA	17 488 272	8555	0.049	10.0	Neg
S3	21 M	NMDAR+[a]	Headache, fever, seizures	13	20 (99)	EV[c] (550 c/mL)	No metagenomic analysis					
S4	55 F	NMDAR+	Headache, memory disorders	14	76 (95)	Neg	DNA	12 276 106	10 959	0.089	75.72	Neg
							RNA	14 894 031	46 880	0.315	16.67	Neg
S5	62 F	GABA+	Seizures, memory disoders	8	72 (95)	Neg	DNA	13 558 199	1805	0.013	75.92	Neg
							RNA	17 233 575	16 954	0.098	22.73	Neg
S6	20 F	NMDAR+	Seizures, psychosis	13	23 (51)	Neg	DNA	12 950 423	4259	0.033	75.61	Neg
							RNA	13 407 464	4735	0.035	60.71	Neg
S7	27 F	NMDAR+	Seizures, psychosis, memory disorders, peripheral nerve palsy	10	2 (81)	Neg	DNA	12 931 073	1101	0.009	44.44	Neg
							RNA	10 793 065	4310	0.040	5.56	Neg
S8	20 F	NMDAR+	Fever, seizures, psychosis, myoclonus	10	2 (63)	HSV-1 (300 c/mL)	DNA	12 243 743	132 884	1.085	72.41	Neg
							RNA	13 783 361	9658	0.070	9.52	Neg

Abbreviations: AE-ab, autoimmune encephalitis autoantibodies; CSF, cerebrospinal fluid; EV, enterovirus; GCS, Glasgow Coma Scale; HSV-1, human herpes virus type 1; lymph., lymphocytes; nd, not done; neg, negative; NGS, next-generation sequencing; NMDAR, N-Methyl-d-aspartate receptor; PCR, polymerase chain reaction; TTV, Torque teno virus.

[a]Anti-NMDAR were detected in CSF and serum.

[b]Counted in 1 mL of CSF.

[c]EV: assay detected Coxsackie A9, A16, B2, B3, B4, B5; ECHO 5, 6, 9, 11, 18, 30; and enterovirus 71.

Importantly, the presence of anti-NMDAR has been described in patients with a relapsing form of HSV encephalitis in which neurological symptoms develop a few weeks or even months after the initial infection, and this disease is at times indistinguishable from AE [53]. Furthermore, anti-NMDAR is also found in the course of uncomplicated HSV encephalitis in as many as 30% of all patients [49], pointing to the potential for autoimmunity inherent in this infection. It is plausible that similar effects could be induced by other viral pathogens and could at least occasionally lead to full-blown AE. However, in many patients anti-NMDAR could represent nothing more than a transient epiphenomenon related to viral neuroinfection.

Another viral pathogen identified in 1 of our patients belonged to the *Enterovirus* genus. Enteroviral infection is a common cause of encephalitis, and in the California Encephalitis Project this particular etiology constituted 25% of all viral encephalitis cases [38]. Although an association between enteroviral infection and AE has not been previously reported, Nakajima et al. described a case of a female patient with chronic progressive enteroviral limbic encephalitis who developed anti-NMDA epsilon2 receptor antibodies [54].

Metagenomic analysis detected TTV infection in 1 of our AE patients, and this finding was confirmed by specific PCR. TTV is highly prevalent in the general population and is considered to be an orphan virus. However, recent NGS-based studies reported on the presence of TTV in the CSF of patients with encephalitis/meningitis [55–57]. Interestingly, TTV has previously been associated with such human autoimmune conditions as bullous pemphigoid and lupus erythematosus [58, 59].

While metagenomics allowed for the unexpected detection of TTV, it did not detect HSV sequences, which were found by our routine amplification assays. This inconsistency could be due to general lower sensitivity of metagenomic workflows compared with real-time RT-PCR/PCR assays. Using serial dilutions of HIV- and HSV-positive sera in negative CSF, we previously established that the limit of detection for RNA- and DNA-based metagenomics was no better than 10^2 and 10^3 viral copies/reaction, respectively [60].

While the presence of anti-NMDAR in CSF in association with clinical symptoms is considered specific in the diagnosis of NMDA receptor AE [61], most of our patients did not fit the typical characteristics of AE, as they were older and had a low mononuclear cell CSF count, and only 1 was serum-positive. Serum-negative AE represented 15% of all anti-NMDAR encephalitis cases, and such patients were reported to be older, have milder neurologic symptoms, and have a lower frequency of tumors, similar to our AE population [62].

In conclusion, we detected the presence of HSV, TTV, and *Enterovirus* genus in CSF samples from AE patients. These findings support the concept of viral involvement in the pathogenesis of this disease.

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Author contributions. All authors have seen and approved the manuscript and contributed significantly to the work.

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