Human Pegivirus in Patients with Encephalitis of Unclear Etiology, Poland

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Human pegivirus (HPgV), previously called hepatitis G virus or GB virus C, is a lymphotropic virus with undefined pathology. Because many viruses from the family Flaviviridae, to which HPgV belongs, are neurotropic, we studied whether HPgV could infect the central nervous system. We tested serum and cerebrospinal fluid samples from 96 patients with a diagnosis of encephalitis for a variety of HPgV sequences.
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Human pegivirus (HPgV) was originally described as a hepatitis virus by 2 independent groups of researchers and called GB virus C and hepatitis G virus (1,2). Whereas the infection was found to be common in patients with forms of chronic hepatitis, and particularly prevalent in patients with chronic hepatitis C infection, it is not associated with liver injury in the absence of concomitant infection with hepatitis C virus (HCV) or hepatitis B virus (HBV). Furthermore, the liver is not the primary replication site for this virus (3,4). This virus was recently renamed as pegivirus and assigned to a new genus (Pegivirus) within the family Flaviviridae (5).

Infection with HPgV is common worldwide; ≈5% of healthy blood donors in industrialized countries are viremic, whereas in some developing countries the prevalence of viremia among blood donors is ≈20% (6). There is evidence that HPgV is transmitted parenterally, sexually, and also vertically from mother to child (7). However, the high proportion of HPgV infection in apparently healthy blood donors and the general population suggests existence of nonparenteral routes. The reasons for the high prevalence of infection in developing countries are not entirely clear but could be related to overall poor hygienic conditions, as well as to the time of exposure. In sub-Saharan Africa, where HPgV is particularly common, this virus is transmitted mainly during childhood, which may facilitate the establishment of chronic infection (7). Because no association between HPgV and disease has been consistently identified, blood donors are not routinely screened for the virus.

Interest in the HPgV infection was revived when several studies identified its beneficial effect on the survival of HIV-infected persons (8,9); anti-HIV replication effects of HPgV were confirmed in vitro (8). Several in vivo and in vitro studies suggest that HPgV may directly interfere with HIV replication and affect host cell factors necessary for the HIV life cycle; specific mechanisms include modulation of cytokine and chemokine release and receptor expressions and lowering of T-cell activation and proliferation (9). However, infection with HPgV may not be totally benign; some studies found an association between infection and non-Hodgkin lymphoma (10,11), which could be the result of lowered immune activation.

Many viruses from the family Flaviviridae, most prominently arthropodborne viruses (arboviruses) such as West Nile virus (WNV) and tick-borne encephalitis virus (TBEV), are neurotropic and a prominent cause of encephalitis in Europe and North America (12). These factors raise the question whether HPgV could be neurotropic and whether it could be an etiologic agent in neuroinfections. Of note, despite substantial progress in diagnostics, the etiology of encephalitis remains unclear in 40%–80% of patients (13,14). A plethora of pathogens may cause encephalitis; many of these pathogens are rare and thus testing is not performed to identify them, and others have not yet been identified.

Three recent case reports described HPgV RNA in the human central nervous system (CNS), demonstrating that the virus can be present in the brain under certain circumstances (15–17). In the first study, viral sequences were detected postmortem in brain tissue from a patient with multiple sclerosis, not encephalitis (15). In the second study, the presence of HPgV might have been related to a severely compromised blood–brain barrier; the patient was HIV-positive and had cerebral toxoplasmosis and fungal encephalitis (16). Although the full-length virus was recovered from the patient’s brain tissue, it is unclear which cells harbored the virus and it was possible that the actual source was blood. Furthermore, the association of HPgV with multiple sclerosis could not be established because the study was limited to a single case. In the third study, HPgV was detected in serum and CSF of a patient with a severe form of encephalitis of unclear origin (17). Of these 3 studies, none included comparison of serum- and CNS-derived virus. We conducted a study of 96 consecutive patients with diagnosis of encephalitis (18) in Poland during 2012–2015 to determine whether HPgV could be found in the CNS.

Materials and Methods

Patients and Routine Diagnostics

We prospectively enrolled patients with encephalitis at the Warsaw Hospital for Infectious Diseases (Warsaw, Poland) from June 2012 through July 2015. The details of this study were published previously (18). We defined encephalitis as an acute-onset illness with altered mental status, decreased level of consciousness, seizures, or focal neurologic signs, together with ≥1 abnormality of the CSF (leukocyte count >4 cells/mm³ or protein level >40 mg/dL). We obtained written informed consent from all patients or from close relatives of patients unable to give...
consent due to their condition. The Internal Review Board of the Medical University of Warsaw approved the study.

We collected CSF and serum samples from patients at admission (5–7 days after symptom onset) and kept them frozen at -80°C until analysis. We tested the samples from all 96 patients for the presence of 5' untranslated region (UTR) HPgV RNA. We performed real-time quantitative PCR (qPCR) or real-time quantitative reverse transcription PCR (qRT-PCR) to detect human herpesvirus (HHV) 1 and 2, varicella zoster virus (VZV), cytomegalovirus (CMV), HHV-6, enteroviruses (coxackievirus A9, A16, B2, B3, B4, B5; echovirus 5, 6, 9, 11, 18, 30; and enterovirus 71), TBEV, WNV, and human adenovirus (HAdV) in CSF samples. We used commercial serologic tests to test CSF and paired serum samples for HHV-1, HHV-2, VZV, TBEV, and WNV as described (18). We detected autoantibodies against neuronal surface antigens using the Autoimmune Encephalitis Mosaic 6 assay (Euroimmun AG, Luebeck, Germany).

**HPgV 5’ UTR and E2 Amplification**

We extracted total RNA with TRIzol LS (ThermoFisher Scientific, Waltham, MA, USA) from 400 µL of CSF or serum and suspended RNA in 20 µL of water, 5 µL of which was subsequently used for each amplification reaction. We amplified the HPgV 5’ UTR by nested reverse transcription PCR (RT-PCR) as described previously (19), resulting in 421 bp-length product; we amplified the E2 region following the RT-PCR protocol published by Smith et al. (20). The final product was 422 bp in length.

**Single-Strand Conformation Polymorphism**

We subjected the amplified PCR products to single-strand conformational polymorphism analysis, as described previously (21). In brief, we purified HPgV 5’ UTR and HPgV-E2 PCR products by using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA). We then subjected the products to thermal denaturation, ran them on nondenaturing 1% polyacrylamide gels at 400V in 25°C, fixed them with acetic acid, and stained them with silver stain.

**Next-Generation Sequencing**

We reamplified RT-PCR products with primers specifically designed for the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Each primer contained the following: sequences complementary to the adapters on a flow cell; an 8-nt index sequence; sequences corresponding to the Illumina sequencing primers; and sequence-specific nested primers for the 5’ UTR and E2 region. Amplification of the 5’ UTR region included initial denaturation at 94°C for 5 min, 20 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and final elongation at 72°C for 10 min. HPgV E2 amplification included denaturation at 94°C for 5 min followed by 20 cycles of 94°C for 18 s, 55°C for 20 s, 72°C for 90 s, and 1 cycle at 72°C for 10 min. We trimmed the libraries by using the LabChip XT apparatus (PerkinElmer, Waltham, Massachusetts, USA) with the DNA 300 Assay Kit (PerkinElmer); the range of fraction collection was 370–430 bp for 5’ UTR and 460–530 bp for E2.

We assessed the quality and average length of next-generation sequencing libraries by using Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). We equimolarly pooled the indexed samples and sequenced them on Illumina MiSeq with 301 bp-end reads according to the manufacturer’s protocol.

**Data Analysis**

We trimmed raw reads using cutadapt version 1.2.1 (https://github.com/marcelm/cutadapt/); (22), then used FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html) for additional processing. We removed all Phred quality score reads <20 using FASTQ/A Artifacts Filter and preprocessed the remaining reads (grouping, counting, and frequency arrangement) using R scripts (23). To diminish the contribution of false positive variants to genetic diversity, we applied the experimentally established sequencing error cutoff of 1.22%. Finally, we aligned remaining sequences and generated phylogenetic trees with ClustalX version 2.0 (http://www.clustal.org/clustal2/) (24). We assessed nucleotide diversity per site and the number of substitutions with respect to the dominant serum sequence in each patient using DnaSP version 6.11.06 (25). We predicted the RNA secondary structures of the 5' UTR using Mfold version 3.2 (http://unafold.rna.albany.edu/?q=mfold) (26), and searched for putative B cell epitopes within the E2 region using BepiPred-2.0 (http://www.cbs.dtu.dk/services/BepiPred/) (27).

**Results**

Four patients were positive for 5' UTR HPgV RNA in serum, and 3 of these patients were also positive in CSF. We analyzed the samples from those 3 patients; their samples were collected at admission, which was 5–7 days after symptom onset. We diagnosed encephalitis of unclear origin for all 3 patients because they were negative for all the pathogens tested (Table 1). The small number of HPgV-infected patients did not allow for statistical analysis, but these patients were not strikingly different from other encephalitis patients. We initially suspected 1 patient, who had a severe illness with prolonged hospitalization, of having HHV infection, but tests did not confirm HHV. All 3 patients recovered without any neurologic sequelae.

We compared serum- and CSF-derived 5' UTR and E2 amplicons from the 3 patients by single-strand conformational polymorphism analysis (Figure 1). Because this
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analysis suggested the presence of differences between the serum- and CSF-derived viral sequences in individual patients, we subjected all amplicons to next-generation sequencing. After filtering, the mean number of reads per sample was 70,759 (range 2,706–183,046) (Table 2).

When we compared 5′ UTR and E2 sequences phylogenetically, we found that serum- and CSF-derived sequences clustered together in individual patients; no sequence was found in multiple patients (Figure 2). Of note, we found several variants to be unique in the CSF compartment. These sequences comprised 2.28%–29.32% of all variants for 5′ UTR and 0%–41.78% of all variants for E2 (Table 2; Figure 3). Unique CSF-derived sequences were also present when we analyzed the E2 region on the amino acid level (Figure 4). The changes were serine to phenylalanine at aa position 508 in patient 2 and proline to leucine at position 572 in patient 3. Both changes were within the predicted B cell epitopes (aa 506–522 and 559–572).

We analyzed all 5′ UTR sequence variants to determine the predicted stability of their secondary RNA structure. We deemed the effect of variations minor because most were localized in the nonbasepaired parts (data not shown), and free energies of the hypothetical secondary structures were only occasionally and mildly affected (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/10/18-0161-Techapp1.pdf) (28).

Discussion

We detected HPgV sequences in CSF from 3/96 patients with encephalitis. We classified all 3 cases as encephalitis.

Table 1. Comparison of clinical characteristics of 3 encephalitis patients infected with human pegivirus compared with patients with other forms of encephalitis, Poland, 2012–2015*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Encephalitis patients infected with pegivirus</th>
<th>Other encephalitis patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient 1</td>
<td>Patient 2</td>
</tr>
<tr>
<td>Male sex</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Median age, y (range)</td>
<td>55</td>
<td>28</td>
</tr>
<tr>
<td>Pharmacological immunosuppression present</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HIV positive</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cancer</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Median length of hospital stay, d (range)</td>
<td>41</td>
<td>30</td>
</tr>
<tr>
<td>Fever &gt;38°C</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Headache</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Altered mental status</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Focal neurologic signs</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Seizures</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Stiff neck</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CSF analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median leukocyte count, cells/mm² (range)</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Median protein level, g/L (range)</td>
<td>0.68</td>
<td>0.26</td>
</tr>
<tr>
<td>Death</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Values are no. (%) unless otherwise indicated. CSF, cerebrospinal fluid
†Identified infections were human herpesvirus 1 (n = 22), enterovirus (n = 6), varicella zoster virus (n = 5), tick-borne encephalitis virus (n = 6), and cytomegalovirus (n = 2).
‡All markers of viral infection were negative, but 5 patients had antibodies against N-methyl-D-aspartate receptor and a diagnosis of autoimmune encephalitis.

Figure 1. Single-strand conformation polymorphism analysis of 5′ UTR and E2 region human pegivirus amplicons from 3 patients with encephalitis of unclear origin, Poland, 2012–2015. CSF, cerebrospinal fluid; S, serum; UTR, untranslated region.
of unknown origin because they were negative for serologic and molecular markers of common CNS pathogens. Furthermore, we demonstrated that these viral sequences differed from those circulating in serum. The presence of viral RNA in CSF could be due to a compromised blood–brain barrier, which was possible in these patients with encephalitis. However, the presence of differences in circulating and CSF-derived sequences is more compatible with the existence of separate viral compartments and thus independent replication. Similar compartmentalization in which distinct blood and CNS viral populations indicate separately evolving populations has been described for other viruses, most prominently for HIV (29) but also for HCV (30,31) and human BK polyomavirus (32).

### Table 2. 5 Human pegivirus variants in serum and cerebrospinal fluid in 3 patients with encephalitis of unknown origin, Poland, 2012–2015

<table>
<thead>
<tr>
<th>RNA region</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>CSF</td>
<td>Serum</td>
</tr>
<tr>
<td>5′ untranslated region</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. reads before error cutoff</td>
<td>124,987</td>
<td>110,331</td>
<td>108,002</td>
</tr>
<tr>
<td>No. reads after error cutoff</td>
<td>61,783</td>
<td>57,668</td>
<td>49,075</td>
</tr>
<tr>
<td>No. nucleotide variants†</td>
<td>3</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>No. unique nucleotide variants in CSF†</td>
<td>5 (29.32)</td>
<td>–</td>
<td>1 (2.28)</td>
</tr>
<tr>
<td>No. nucleotide substitutions</td>
<td>2</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Nucleotide diversity per site</td>
<td>0.004</td>
<td>0.007</td>
<td>0.004</td>
</tr>
</tbody>
</table>

| E2 region | | | | | | |
| No. reads before error cutoff | 70,460 | 38,025 | 77,656 | 76,918 | 2,706 | 82,738 |
| No. reads after error cutoff | 26,720 | 20,619 | 26,558 | 42,609 | 453 | 34,887 |
| No. nucleotide variants† | 8 | 4 | 8 | 9 | 4 | 7 |
| No. unique nucleotide variants in CSF† | – | 0 | – | 5 (41.78) | – | 3 (27.28) |
| No. nucleotide substitutions | 4 | 2 | 5 | 5 | 2 | 3 |
| Nucleotide diversity per site | 0.007 | 0.004 | 0.006 | 0.007 | 0.004 | 0.005 |
| No. amino acid variants† | 2 | 2 | 2 | 5 | 2 | 4 |
| No. unique amino acid variants in CSF† | – | 0 | – | 3 (27.28) | – | 2 (27.28) |

*Numbers in parentheses are percentages. CSF, cerebrospinal fluid
†After applying the 1.22% sequencing error cutoff.

Figure 2. Phylogenetic analysis of A) 5′ UTR and B) E2 region sequences of human pegivirus from 3 patients with encephalitis of unclear origin, Poland, 2012–2015. Phylogenetic trees were generated using ClustalX version 2.0 (http://www.clustal.org/clustal2/). Viral variant frequencies follow haplotype number. Red indicates patient 1; blue, patient 2; green, patient 3. Scale bars indicate number of nucleotide substitutions per site. C, cerebrospinal fluid; S, serum; UTR, untranslated region.
Figure 3. Comparison of E2 region human pegivirus sequences amplified from serum and cerebrospinal fluid from 3 patients with encephalitis of unclear origin, Poland, 2012–2015. Numbers in parentheses represent the number of reads representing a given sequence. Shading indicates sequences unique to cerebrospinal fluid. Nucleotide numbering follows the reference strain published by Linnen et al (2) (GenBank accession no. NC_001710.1). C, cerebrospinal fluid; S, serum.
It is unclear how HPgV could access the CNS. Although initially HPgV was thought to be a hepatotropic virus, the viral negative strand, which is the putative replicative intermediate of the virus, was not detected in liver tissue (3); however, it was found in bone marrow and spleen (33,34). The virus is now considered lymphotropic because it can be detected at a low level in multiple lineages of peripheral leukocytes (35,36), and it has been speculated that the primary target could be a progenitor hematopoietic stem cell. The route for CNS access could be through infected leukocytes; all basic groups (T cells, B cells, macrophage/monocytes, and NK cells) have the ability to enter the brain under certain conditions (37). Certain monocyte family members are constantly replaced as part of normal physiology (38,39), while the entry of T cells and B cells depends largely on their activation state (40,41). Such a phenomenon related to trafficking of infected leukocytes through the blood–brain barrier has been long postulated for HIV-1 neuroinfection (42).

Whether HPgV was the causative factor of encephalitis in the patients we describe is not clear. Viral pathogens are typically present only transiently in CSF and this window could be easily missed, particularly when the spinal tap is done too late in the course of illness (43). If HPgV encephalitis exists, it could be a rare phenomenon. In a previous study of 17 encephalitis and aseptic meningitis cases of unknown cause, no CSF sample was positive (44). However, even infections with well-known neurotropic agents from the Flaviviridae family, such as WNV or TBEV, are usually subclinical or asymptomatic; clinical signs and symptoms develop only in 5%–30% of cases (45,46). Of note, in 2 of the cases we describe, the encephalitis was mild, and all 3 patients recovered without any neurologic sequelae. Obviously, the mere presence of a pathogen in the CNS in patients with encephalitis does not prove causality; for example, HCV sequences are commonly detected in brain and CSF of infected patients without any accompanying evidence of encephalitis (29). In our study, we detected HPgV sequences in CSF only in patients without an obvious cause of encephalitis and in none of the patients in whom a known pathogen was identified.
The identified 5′ UTR and E2 region sequence differences between CSF and serum compartments could have biological meaning. The 5′ UTR contains an internal ribosomal entry site that allows cap-independent viral translation (28). Such structures were identified within the 5′ UTR of the picornaviruses and were shown to interact with cellular proteins, thus affecting the host range of individual viruses (47). Research has also shown that, for HCV, translation efficiencies of brain-derived internal ribosomal entry site variants are generally lower than those found in serum, which could be a viral strategy favoring latency in the CNS (31). Taking this into consideration, we speculated that at least some of the 5′ UTR changes in the patients we report represent tissue-specific adaptation. Viral adaptive changes could be relatively small and yet make a huge difference; for example, it has been demonstrated for lymphocytic choriomeningitis virus in mice that variants differing by a single amino acid substitution are competitively selected either by the liver and spleen or by neurons (48).

On the amino acid level, we saw 2 unique E2 region changes in CSF variants compared with serum: in patient 2, serine was changed to phenylalanine at aa position 508, and in patient 3, proline was changed to leucine at position 572. Both were within regions predicted to contain B-cell epitopes, thus suggesting that they were the effect of immune pressure. Furthermore, the change in patient 3 was located in the region of E2 that was experimentally shown to contain a strong antigenic site and likely to be involved in cell binding or fusion (49).

RNA viruses in particular are characterized by a high degree of genetic heterogeneity; probably because the lack of proofreading 3′ 5′ exonuclease activity in viral RNA polymerases causes low fidelity. As a result, viruses circulate in the infected host as a population of closely related but nonidentical genomes, referred to as quasispecies (50). It is unclear whether the observed high HPgV variability developed in the patients we describe de novo after infection or if most or all variants were transmitted from the infecting host; both mechanisms could occur together. However, because viral transmission is typically accompanied by narrowing of the quasispecies spectrum (known as the bottleneck phenomenon), some extent of postinfection evolution is highly likely.

In summary, we detected HPgV sequences in the CSF of 3 patients with encephalitis of unclear origin, and these sequences from CSF differed from those circulating in serum. These findings are compatible with the presence of a separate viral compartment in the CNS. Determining if the pegivirus was responsible for encephalitis or if it was present along with another cause of encephalitis will require further research, including histopathological analysis.

This work was supported by Polish National Science Center (grants 2017/25/B/NZ6/01463 and DEC-2013/11/N/NZ6/00961), the Foundation for Polish Science (grant POMOST/2013–7/2), and Infectious Diseases Hospital Foundation.

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Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 24, No. 10, October 2018


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- Neurologic Complications of Influenza B Virus Infection in Adults, Romania
- Implementation and Initial Analysis of a Laboratory-Based Weekly Biosurveillance System, Provence-Alpes-Côte d’Azur, France
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