Lassa Fever Encephalopathy: Lassa Virus in Cerebrospinal Fluid but Not in Serum

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The pathogenesis of neurologic complications of Lassa fever is poorly understood. A Nigerian patient had fever, disorientation, seizures, and blood-brain barrier dysfunction, and Lassa virus was found in cerebrospinal fluid (CSF) but not in serum. The concentration of Lassa virus RNA in CSF corresponded to 1 × 107 pfu/mL, as determined by a quantitative real-time polymerase chain reaction assay. To characterize the Lassa virus in CSF, the 3.5-kb S RNA was sequenced. In the S RNA coding sequences, the CSF strain differed between 20% and 24.6% from all known prototype strains. These data suggest that Lassa virus or specific Lassa virus strains can persist in the central nervous system and thus contribute to neuropathogenesis. Lassa virus infection should be considered in West African patients or in travelers returning from this area who present only with fever and neurologic signs.

Lassa fever is an acute rodent-borne infection endemic in West Africa. It is caused by Lassa virus and is associated with a wide spectrum of clinical manifestations [1, 2]. Flulike and gastrointestinal symptoms are present in most cases. Hemorrhage occurs in a subset of patients and is associated with high mortality. Neurologic complications, such as confusion, tremor, convulsion, and coma, are frequent in critically ill patients, who often die after the onset of these symptoms [1, 3, 4]. Lassa virus has been isolated from serum and cerebrospinal fluid (CSF) of these patients; however, it was not detectable in the CSF of all patients with encephalopathy [3, 5]. In other cases, contamination of the CSF with blood could not be excluded [3]. Therefore, it is still not known whether the encephalopathy during the acute phase is due to infection of the central nervous system or whether indirect effects, such as metabolic changes, play a role. Sensorineural deafness and ataxia are neurologic complications of the convalescence–virus clearance phase and are thought to be caused by immune processes [4, 6]. The presence of Lassa virus in the central nervous system of patients with late appearance of neurologic symptoms has not yet been considered. Here, we report on a patient with Lassa fever encephalopathy from whom the virus was detectable only in CSF during the late phase of infection. The full-length S RNA sequence of the virus in CSF was determined to characterize the pathogen genetically and phylogenetically.

Materials and Methods

Indirect immunofluorescence. Lassa virus strain Josiah was propagated in Vero cells in a biosafety level 4 facility. Cells were spread onto immunofluorescence slides, were air dried, and were acetone fixed. Serum samples were incubated for 2 h with the cells. Antibodies were detected by anti–human IgG or IgM labeled with fluorescein isothiocyanate (Dianova). Virus culture. Vero cells were inoculated with 20 μL of CSF. The cell culture was passaged at 5-day intervals and was examined by indirect immunofluorescence by means of a Lassa virus nucleoprotein (NP)–specific monoclonal antibody and fluorescein isothiocyanate–labeled anti–mouse IgG (Dianova). After 3 passages, virtually all cells were infected and 2.5 × 104 pfu/mL was measured in medium by a modified immunologic focus assay [7]. RNA preparation. RNA was prepared from 140 μL of CSF, serum, or cell culture supernatant by means of the QIAamp viral RNA kit (Qiagen).

Conventional reverse-transcription polymerase chain reaction (RT-PCR). A 340-bp fragment of the S RNA was amplified by use of the Platinum Thermoscript One-Step System (Life Technologies) in a 20-μL assay containing 2 μL of RNA, 0.3 μM primer 36E2, and 0.2 μM primer 80F2 [8] or, alternatively, 0.2 μM primer 80F2-CSF (ATGTAGTGGTGACTTAGATTTTTTGCA; modification to 80F2 underlined). The reaction was run in a thermocycler (model 9600; Perkin Elmer) as follows: 50°C for 30 min; 95°C for 5 min; 10 step-down cycles at 95°C for 5 s, 60°C for 5 s with a 1°C decrease per cycle, and 72°C for 25 s; 40 cycles at 95°C for 5 s, 56°C for 10

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s, and 72°C for 25 s; and a final 5 min at 72°C. The ≥95% detection limit of the PCR was 3 × 10^3 RNA copies/mL of plasma.

**Real-time RT-PCR.** RNA was incubated with 20 pmol of primer 80F2 at 70°C for 10 min. RT was done in a 10-μL assay containing the RNA-primer mix, 10 mM dithiothreitol, 500 μM dNTP, and 100 U of Superscript II reverse transcriptase (Life Technologies) at 45°C for 15 min, 50°C for 15 min, 55°C for 10 min, and 50°C for 20 min. cDNA (1 μL) was amplified in a 10-μL FastStart DNA SYBR Green I assay (Roche Molecular Biochemicals) containing 0.25 μM primers 36E2 and 80F2 and 4 mM MgCl₂. PCR was run in a LightCycler (Roche Molecular Biochemicals) as follows: 95°C for 10 min, followed by cycling at 95°C for 5 s, 52°C for 5 s, and 72°C for 20 s, followed by melting point analysis. Serial 10-fold dilutions of the quantified (2.5 × 10^5 pfu/mL) Vero cell-passaged CSF strain were used as a standard.

**RT-PCR of full-length S RNA.** A protocol suitable for amplification of full-length S RNA of any arenavirus [9] was used. To obtain sufficient material for sequencing, PCR products were gel purified and were reamplified in several preparative PCRs.

**Sequence determination.** PCR products were sequenced on an automated sequencer. A partial sequence of the 3.4-kb PCR product was obtained with 6 primers designed on the basis of known S RNA sequences. Subsequently, 8 Lassa virus strain CSF (virus from CSF of the case patient)–specific primers were designed to confirm and to complete the sequence.

**Phylogenetic analysis.** Phylogenetic analysis was done with the PHYLIP 3.57c program package [10]. Neighbor-joining and maximum likelihood analysis were done by DNADIST-NEIGHBOR and DNAML programs, respectively, by use of a bootstrapped data set (100 replicates) and random input order.

**Results**

**Case report.** A 56-year-old Nigerian man was seen on 21 March 2000 at the Emdee Medical Center at Jos, Nigeria, because of a 2-week history of fever (38.2°C) and diarrhea. Treatment with antibiotics was initiated. On 23 March, he was admitted at the Life Camp Clinic, Abuja, Nigeria. His temperature was 39.6°C; he was drowsy and intermittently disoriented. Flu-like symptoms and diarrhea were absent. His liver enzyme levels were slightly elevated (aspartate aminotransferase, 58.2 U/L; alanine aminotransferase, 80.6 U/L), coagulation was normal, and human immunodeficiency virus serology was negative. On 25 March, he experienced a 30-min episode of generalized seizures without losing consciousness. His temperature decreased, and, on 27 March, he was transferred to the neurology department of the Dr.-Horst-Schmidt-Kliniken in Wiesbaden, Germany. On admission, he was afebrile, was disoriented in time and place, and was somnolent. Meningeal signs were absent. An electroencephalogram showed neither focal nor specific or unspecific epileptic signs, and nuclear magnetic resonance tomography of the brain yielded normal results. CSF findings demonstrated a dysfunction of the blood-brain barrier (cells, 41/μL; glucose, 40 mg/dL; lactate, 32.7 mg/dL; protein, 667 mg/dL; albumin, 334.9 mg/dL; IgG, 159.9 mg/dL; serum albumin, 2910 mg/dL; IgG, 1910 mg/dL; and IgM, 7.54 mg/dL). The IgG index was not elevated (0.7), indicating that IgG in CSF was derived mainly from serum. Blood tests showed normal or borderline values.
Figure 2. Genetic and phylogenetic characterization of Lassa virus in cerebrospinal fluid (CSF). A, Comparison of GP and NP amino acid sequences of Lassa virus from CSF with the most closely related prototype strain from Nigeria (GA391). Residues unique in strain CSF, compared with all known Lassa virus prototype sequences, are boxed. Region in GP that is completely conserved among known strains is underlined. GP residue 253, which corresponds to position 260 in lymphocytic choriomeningitis virus (LCMV), is indicated by an asterisk (*). LCMV position 260 is involved in receptor binding and tissue tropism [12, 13]. GP1–GP2 cleavage site [14] is marked with an arrow. B, Phylogenetic trees showing relationships among Old World arenaviruses, including Lassa CSF (virus strain and GenBank accession no.: LCMV Armstrong, M20869; LCMV WE, M22138; Ippy 188d, U80003; Mobala 3076, AF012530; Mopeia AN21366, M33879; Mopeia AN20410, U80005; Lassa LP, AF181853; Lassa 806321, AF182225; Lassa 806319, AF182224; Lassa 803208, AF182232; Lassa 9608911, AF182272; Lassa 803796, AF182271; Lassa 803201, AF182259; Lassa 803213, AF181854; Lassa GA391, X52400; Lassa CSF, AF333969; Lassa AV, AF246121; and Lassa Josiah, J04324). New World arenavirus Tacaribe TRVL11573, M20304, was used as an outgroup to root the tree. Trees were calculated by maximum likelihood and neighbor-joining (not shown) methods with 4 different data sets: with partial NP gene fragment (positions 1724–2349 of genomic S RNA of Lassa Josiah); with full-length NP gene sequences. Topologies calculated with maximum likelihood and neighbor-joining (not shown) methods with 4 different data sets: with partial NP gene fragment (positions 1724–2349 of genomic S RNA of Lassa Josiah); with full-length NP gene; and with full-length NP + GP gene sequences. Bootstrap support values (%) of maximum likelihood and neighbor-joining methods are indicated at respective branches (maximum likelihood/neighbor-joining; dash indicates that the neighbor-joining method calculated different branching order than did the maximum likelihood method). Clusters IIIa, IIIb, and V have been tentatively designated (at right) by extending the previous classification [11].
(aspartate aminotransferase, 45 U/L; alanine aminotransferase, 20 U/L; amylase, 124 U/L; partial thromboplastin time, 31 s; international normalized ratio, 1.19; and C-reactive protein, 0.8 mg/dL). The patient received methylprednisolone (500 mg/day) and heparin (2 × 7500 U/day) as prophylaxis against thrombosis. Further seizures were not observed, and the patient’s orientation improved. Five days after admission, the patient suddenly died, with signs of pulmonary embolism. The diagnosis of Lassa virus infection firmly was established shortly after the death of the patient. Autopsy was not performed.

Clinical virology. Lassa virus–specific IgM and IgG antibodies were detected by indirect immunofluorescence in both CSF (IgM titer, 1:10; IgG titer, 1:320) and serum (IgM titer, 1:320; IgG titer, 1:2560) samples obtained on 28 March, when the patient was afebrile and showed solely neurologic signs. RNA was isolated from the CSF and from 4 serum samples taken on 27 March, 28 March, and 1 April. A conventional Lassa virus RT-PCR was done with use of primers [8] binding to the 5′ region of the S RNA. Unexpectedly, Lassa virus RNA was detected in CSF but not in serum samples. The presence of Lassa virus in CSF was confirmed by isolation of the virus in Vero cells (figure 1A). Quantitative RT-PCR experiments showed that the concentration of Lassa virus RNA in the CSF corresponded to 1.2 × 10⁶ pfu/mL, when the titrated Vero cell–passaged CSF isolate was used as a standard. This concentration was 500-fold above the detection limit of the PCR, whereas serum samples yielded negative results. Thus, the CSF:serum concentration gradient of viral RNA was ≥500-fold. PCR inhibitors in the patient’s serum were excluded by spiking it with in vitro–transcribed Lassa virus RNA before real-time PCR. The amplification efficiency was not reduced, compared with a water control (first signal after 18.6 vs. 18.9 cycles), indicating the absence of PCR inhibitors in serum. Because the sequence analysis of Lassa strain CSF (see below) revealed several mutations within the binding site of primer 80F2, this primer was modified accordingly (designated 80F2-CSF). The conventional PCR was repeated with this perfectly matching primer. Again, Lassa virus RNA was detected in CSF but not in serum samples (figure 1B).

Characterization of the virus. The full-length S RNA of the virus in CSF (designated as Lassa virus strain CSF) was amplified directly from CSF [9], rather than after cell culture passage, to obtain the original sequence. A single fragment of ~3.5 kb was amplified (figure 1C) and was sequenced (GenBank accession no. AF333969). A comparison of this S RNA sequence with that of all known Lassa prototype sequences from Nigeria (Lassa LP, 803213, GA391), Ghana–Ivory Coast–Burkina Faso (Lassa AV), and Sierra Leone (Lassa Josiah) revealed a high degree of nucleotide (glycoprotein [GP], 20%–22.8%; NP, 21.5%–24.6%) and amino acid difference (GP, 7.7%–8.6%; NP, 7.9%–11.6%). These differences corresponded to those seen among prototype strains [9, 11]. Several amino acid residues were specific for strain CSF, compared with those for prototype strains (figure 2A, boxed residues). Noteworthy is an aspartate–glutamate change at GP position 250 in a highly conserved region. This change is close to a position (figure 2A, asterisk) that, in lymphocytic choriomeningitis virus, is involved in receptor binding and tissue tropism [12, 13]. Phylogenetic analysis was done with a 600-bp NP sequence known for a large number of Lassa virus isolates [11]. Lassa virus CSF was related most closely to the Nigerian strain 9608911 (figure 2B; partial NP), which was isolated in Jos in 1996 [11]. The strains were 98% and 99.5% identical at the nucleotide and amino acid levels, respectively. Thus, phylogenetic and geographic origins of strain CSF agree. However, maximum likelihood and neighbor-joining analyses calculated different relationships among Lassa virus strains CSF, GA391, and AV. Therefore, full-length NP, GP, and NP plus GP sequences were subjected to maximum likelihood and neighbor-joining analysis. These analyses consistently revealed a sister relationship between Lassa CSF and Lassa GA391 with high bootstrap support values (figure 2B; NP, GP, and NP+GP).

Discussion

Clinical and virologic findings indicate that the patient experienced an uncommon course of Lassa fever. Encephalopathy characterized by disorientation, seizures, and blood-brain barrier dysfunction has been the chief manifestation during the late phase of infection. For this reason, neither in Nigeria nor in Germany was Lassa fever clinically suspected. It was eventually diagnosed by laboratory screening. Several PCR assays demonstrated Lassa virus RNA in CSF but not in serum, a finding that has not yet been reported in Lassa fever patients. Lassa virus was isolated from CSF, and quantification by real-time PCR indicates that the virus load in CSF was magnitudes higher than that in serum. These findings suggest that Lassa virus persisted in the central nervous system, where it may be directly involved in neuropathogenesis or indirectly involved via induction of cytokines, as suggested for influenza virus–associated encephalopathy [15]. The CSF isolate can be regarded as a prototype strain of a phylogenetic cluster (designated as IIIb in figure 2B) on the basis of its considerable genetic differences from all known prototype strains. Thus far, this cluster comprises only a second isolate of which no clinical features have been reported [11]. Therefore, it is speculative whether strains of this cluster are associated with an increased risk of central nervous system manifestations. Finally, this report shows that Lassa fever should be taken into consideration in West African patients or in those returning from this area who present only with fever and neurologic signs.

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References