Monitoring of clinical and laboratory data in two cases of imported Lassa fever

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Abstract

During 2000, four cases of fatal Lassa fever were imported from Africa to Europe. In two patients, consecutive serum samples were available for monitoring of virus load and cytokine levels in addition to standard laboratory data. Both patients had non-specific early clinical symptoms including high fever. Patient 1 developed multi-organ failure and died of hemorrhagic shock on day 15 of illness, while patient 2 died of respiratory failure due to aspiration without hemorrhage on day 16. Ribavirin was administered to both patients beginning only on day 11. High serum aspartate aminotransferase and lactate dehydrogenase (LDH) levels were remarkable in both patients. Patient 1 had an initial virus load of $10^6$ S RNA copies/ml as measured by real-time RT-PCR. Viremia increased steadily and reached a plateau of approximately $10^8$–$10^9$ copies/ml 4 days before death, while IFN-$\gamma$ and TNF-$\alpha$ rose to extremely high levels only shortly before death. In contrast, in patient 2 the virus load decreased from $10^7$ to $10^6$ copies/ml during the late stage of illness which was paralleled by a decrease in the IFN-$\gamma$ and TNF-$\alpha$ levels. The IL-10 level increased when specific IgM and IgG appeared. These data suggest that a high virus load and high levels of pro-inflammatory cytokines in the late stage of Lassa fever play an important role in the pathogenesis of hemorrhage, multi-organ failure, and shock in Lassa fever. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

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1. Introduction

Lassa fever is the most frequent hemorrhagic fever observed in Africa. It is caused by Lassa virus, an arenavirus transmitted to humans by contact with feces or urine of the African rodent *Mastomys natalensis*. In addition, the blood of infected rodents contains high virus titers and may be a source of transmission during peri-domestic rodent hunting in rural West Africa. Transmission between humans has been reported as a result of exposure to blood, sexual contact, and breast feeding. Lassa fever is highly endemic in Guinea, Sierra Leone, Liberia and Nigeria where it had been first described in the village of Lassa. However, no cases of Lassa fever have been confirmed by virus isolation in countries south of Liberia or north of Nigeria.

In January 2000, Lassa fever caused the death of a 22-year-old German student who returned to Germany from Ivory Coast. The Lassa virus was isolated and found to be a new strain (Lassa AV), distinct from known strains of Nigeria, Sierra Leone, Guinea, and Liberia. In March 2000, a 50-year-old British peacekeeper working in rural Sierra Leone was evacuated by air ambulance to the United Kingdom where he subsequently died. The clinical diagno
sis of Lassa fever was confirmed in London. In April 2000, a Nigerian male died after being airlifted from Nigeria to Germany for medical treatment due to neurological disease. He was diagnosed with Lassa fever in Hamburg. In July 2000, a 48-year-old physician working in Sierra Leone returned to The Netherlands, was diagnosed with Lassa fever and died 16 days after onset of fever.

Follow-up specimens were obtained from the student beginning on the 6th day of illness and from the physician beginning on the 9th day of illness. Various laboratory parameters of coagulation, hematology, and clinical chemistry were available on these patients from day 6 onward. In addition, Lassa virus RNA levels as well as IFN-γ, TNF-α, and IL-10 levels were monitored using real-time transcription polymerase chain reaction (RT-PCR) and ELISA, respectively. Only a few Lassa fever patients have been treated in a setting where all of these tests are available. Therefore, our data represent the first comprehensive panel of these laboratory data in Lassa fever and may lead to a better understanding of the pathophysiology of the disease.

2. Materials and methods

2.1. Virus isolation

Vero E6 cells grown in 25-ml flasks were inoculated with a series of dilutions (10^{-1}–10^{-5}) of fresh patient serum, which arrived 8–24 h after venipuncture. In addition, Lassa virus was isolated from the patients’ serum samples which had been shipped on dry ice and had been stored aliquoted at −70 °C. The growth of virus was demonstrated by immunofluorescence, using mouse monoclonal antibody (mAb) 2F1 directed to the Lassa virus nucleocapsid.

2.2. Serology

Serum from patients was screened for antibodies against Lassa virus by indirect immunofluorescence (IIF) using cells infected either with the Josiah strain or with the homologous isolate of patient 1 (Lassa strain AV). IgM and IgG antibodies were detected with anti-μ or anti-γ chain conjugates, respectively. Serum samples from Lassa fever patients in Guinea served as positive controls. Serum samples containing specific IgM antibody were retested after removing the IgG fraction by using protein G-Sepharose Fast-Flow (Pharmacia, Freiburg, Germany) with 0.1 ml protein G-Sepharose-Gel per 0.5 ml serum, diluted 1:10 in phosphate-buffered saline (PBS).

2.3. Detection and quantification of Lassa virus RNA by RT-PCR

Lassa virus RNA was detected by conventional RT-PCR using the Superscript-II/Platinum one-step kit (Life Technologies, Glasgow, UK) and S RNA primers 36E2 (ACCCGGGATCTCATGACATT) and 80F2 (ATATAAFTGATGACTGTGTTCTTCTTGCA).

To quantify the amount of viral RNA, a real-time PCR protocol was established using the LightCycler amplification equipment (Roche, Mannheim, Germany). RT-PCR products were detected using the SybrGreen I dye. The 20-μl reaction mix contained 0.0001% SybrGreen I (Roche, Mannheim, Germany) immobilized at the bottom of the capillary (C. Drosten, unpublished data), 0.6 μl Superscript-II/Platinum mix (Life Technologies), 2 μl RNA, 0.2 μM primers 36E2 and 80F2, and 10 μl of 2× RT-PCR reaction buffer. The reaction was run as follows: reverse transcription at 50 °C for 20 min; initial denaturation at 95 °C for 5 min; amplification for ten cycles at 95 °C for 5 s, 60 °C for 5 s with a temperature touch-down of 1 °C per cycle, and 72 °C for 25 s; followed by 40 cycles at 95 °C for 5 s, 56 °C for 10 s, 72 °C for 25 s, and 82 °C for 5 s (fluorescence read step). The virus load measurement was standardized by using serum of a healthy subject, spiked with ten-fold dilutions of a defined amount of in vitro-transcribed Lassa virus RNA molecules. The >95% limit of detection was 20 S RNA copies/assay corresponding to 1000 copies/ml of serum. RT-PCR products were sequenced using the PCR primers and an automated sequencer.

2.4. Cytokine measurement

Cytokine levels were quantified in sera by ELISA in the BSL-4 facility. The following pairs of mAbs were used for capture and detection (PharMingen, Heidelberg, Germany): TNF-α, mAb-1 and biotinylated mAb-11; IL-10, JES3-9D7 and biotinylated JES3-12G8; IFN-γ, NIB42 and biotinylated 4SB3. Recombinant human cytokines TNF-α, IL-10 and IFN-γ (PharMingen) were used as concentration standards. Immunoplates (Maxisorp; Nunc, Wiesbaden, Germany) were coated with 50 μl capture antibody (1 mg/ml) in 0.1 M NaHCO3-Na2CO3 buffer (pH 9.6) overnight at 4 °C. After blocking with 1% bovine serum albumin (BSA), plates were washed with PBS-0.05%Tween 20 and incubated overnight at 4 °C with 50 μl serum (diluted 1:2) or with dilutions of the reference cytokines. The biotinylated antibodies were used at a concentration 1 mg/ml PBS–Tween 20–0.1% BSA. Plates were developed after incubation with streptavidin–peroxidase complex (1:10,000) (Roche, Mannheim, Germany), using 100 μl tetramethylbenzidine (6 mg/ml in DMSO) (Roth, Karlsruhe, Germany) per well as substrate. Enzyme reactions were stopped with 25 μl 4 N H2SO4/well and product was measured at 450 nm. The sensitivity of the ELISA assays was approximately 12 pg/ml.

2.5. Clinical chemistry, hematology, and coagulation

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), lipase, lactate dehydrogenase (LDH), creatine
kinase (CK), gamma glutamyl-transferase, creatinine, bilirubin, fibrinogen, leukocyte and thrombocyte counts, and partial thromboplastine time (PTT) were measured by autoanalyzers, in part in the BSL-4 facility.

3. Results

3.1. Case reports

Case 1: a 22-year-old female art student (patient 1) from Germany lived for several months in Ivory Coast. In the month prior to her illness, she had traveled to Ghana and Burkina Faso. In Abidjan, Ivory Coast, on January 2, 2000, she had a sudden onset of high fever (39 °C) and flu-like symptoms. She had been vaccinated against yellow fever but had not taken malaria prophylaxis. The presumptive diagnosis of malaria was made at a local hospital and she was given artesunate. She returned to Frankfurt, Germany, on the 6th day of illness and was admitted to the hospital at Schwäbisch Hall with high fever (40 °C) and tinsillitis. Several thick blood smears were negative for malaria. Three days later she was transferred to a hospital specialized in tropical diseases in Würzburg. On admission, a severe pharyngitis and ulcerative tinsillitis were noted, as well as shortness of breath with cough, high fever, and diarrhea. She received ciprofloxacin on an empirical basis. Over the next days she developed a large pleural effusion. Lassa fever was considered and on the 10th day of illness a serum sample was sent to the BSL-4 laboratory of the Bernhard-Nocht-Institute, Hamburg. A positive RT-PCR for Lassa virus was reported on the following day, while PCR was negative for Ebola, Marburg, Rift valley fever, and Crimean-Congo virus RNA. IgG or IgM antibodies to any of the aforementioned hemorrhagic fever viruses, including Lassa, could not be detected. Intravenous ribavirin treatment was started on the 11th day of illness (16 mg/kg every six h). Despite therapy, encephalopathy developed and an increase in serum lipase indicated a pancreatitis. Due to progressive renal dysfunction and hypovolemia, the patient was dialedyzed and received volume expansion. A massive hemorrhage developed which could not be corrected by 19 blood transfusions. The patient experienced seizures and died from hemorrhagic shock on the 14th day after onset of illness. Histological post-mortem examination of the liver showed only rare necrotic cells in the intermediate zone of some lobuli.

Case 2: a 48-year-old male surgeon (patient 2) worked for 5 months at a hospital in rural Sierra Leone. He was healthy until July 10, 2000, when he developed fever and malaise. He had been vaccinated against yellow fever and had not taken malaria prophylaxis. He received artesunate for presumed malaria from a local hospital without subsequent improvement of his symptoms. On July 14, he returned to The Netherlands for scheduled leave to visit his family and was seen at the Leiden University Medical Center on the same day with high fever. On the following day he was admitted to the hospital. He complained of nausea, crampy watery diarrhea, myalgias, arthralgias, and headache. Any recent percutaneous exposure due to his profession or contacts with patients suspected of having hemorrhagic fevers was denied. His temperature was 39.5 °C, blood pressure 120/70, heart rate 80/min, and respirations 12/min. Physical examination revealed a moderately sick male, weight 70 kg, without meningismus, conjunctivitis, pharyngitis, or lymphadenopathy. Chest, abdomen, and extremities were normal except for a faint erythematous rash on the trunk. Thick blood smears were negative for malaria. On suspicion of typhoid fever cefamandol and netilmicin were given. He improved with resolution of headache, nausea, and diarrhea, while his temperature remained elevated at 38.5 °C. Since stool and blood cultures were negative, cefamandol and netilmicin were stopped and doxycycline was started. On day 11, he developed a mild encephalopathy and renal dysfunction. The clinical diagnosis of Lassa fever was made and intravenous ribavirin was started immediately (loading dose: 2000 mg, then 1000 mg qid for 4 days, then 500 mg qid). Serum samples were sent to Hamburg, where Lassa virus RNA was detected by RT-PCR. Subsequently, he developed progressive renal failure and hypoxia with diffuse pulmonary infiltrates. He was transferred to the intensive care unit on day 15. The next day he died of respiratory failure due to aspiration.

3.2. Clinical chemistry

The kinetics of the aminotransferases (AST and ALT) are remarkable. Upon presentation, the serum AST and ALT levels of both patients were already slightly elevated. Over the next days, the levels of both enzymes continued to rise, peaking at days 11 and 12 and maintaining an AST/ALT ratio of 10:1 (Fig. 1). LDH and CK were elevated throughout the clinical course in both patients (Fig. 1). In patient 1, they reached a maximum around day 11 and then deceased, while they steadily increased to extremely high levels in the late stage in patient 2. In the latter patient, these values were accompanied by myoglobinuria (3+) indicating rhabdomyolysis. In patient 1, lipase in serum increased sharply between days 11 and 12, indicating development of pancreatitis (Fig. 1). Renal failure was evidenced in both patients by an increase in creatinine levels in the late stage of the disease. In contrast to patient 2, in patient 1 coagulation parameters were progressively impaired (on day 9: thrombocytes 100,000/µl, fibrinogen 300 mg/dl, PTT 55 s; on day 14: thrombocytes 30 000/µl, fibrinogen 55 mg/dl, PTT 60 s). They did not show substantial restoration upon 19 blood transfusions. Remarkably, patient 2 showed a thrombocytosis during the late stage (600,000/µl on day 14) but PTT and fibrinogen were in the normal range and no hemorrhage was seen throughout the disease.
3.3. Detection of Lassa virus and Lassa virus-specific antibodies

Virus was detected in serum samples using a fast, one-step RT-PCR protocol and previously described primers [16]. Lassa virus RNA was also detected in a throat washing obtained from patient 1 on day 10, when she was admitted to the second hospital. Surprisingly, Lassa virus could not be detected in a urine specimen obtained on the same day. The RT-PCR products of both patients were sequenced as part of the diagnostic procedure. A comparison with known Lassa virus sequences showed that patient 1 was infected with a new strain (designated Lassa AV), differing in nucleotide sequence from the Sierra Leone and Nigeria strains by approximately 15%. This was confirmed by sequencing the whole S RNA [7]. Consistent with its geographic origin, the virus of patient 2 was closely related to the Josiah strain from Sierra Leone (5% difference). In parallel, virus was detected by culturing, using fresh serum (diluted up to 1:1000) of both patients on day 10 of illness. Lassa virus-specific antibodies were detected by IIF. Patient 1 never had detectable anti-Lassa IgG or IgM antibodies, neither to the Josiah strain nor to the homologous AV strain. In contrast, patient 2 had IgM antibodies on day 13 (see Fig. 5), while both IgM and IgG antibodies to the Josiah strain were present on day 16 (titers 1:512 and 1:256, respectively).

3.4. Virus load and cytokine levels

Virus load and cytokine levels were monitored retrospectively in follow-up serum samples that had been continuously stored frozen at −20 °C. Lassa virus RNA was quantified by real-time RT-PCR (Fig. 3). In patient 1, a concentration of $1 \times 10^6$ RNA molecules/ml serum was found on day 6 of illness. The virus load increased by two orders of magnitudes until day 10 ($2 \times 10^8$ molecules/ml), when the viremia approached a plateau phase. Only a small increase to $10^9$ molecules at days 14 and 15 was observed. In contrast, in patient 2 the virus load was $1.9 \times 10^7$ copies/ml on day 10 and decreased by one order of magnitude during the late stage of illness.

Two pro-inflammatory cytokines (TNF-α and IFN-γ) and one anti-inflammatory cytokine (IL-10) were measured by ELISA in the serum samples (Fig. 3). To circumvent the need of virus inactivation, which might have affected the cytokine levels, all measurements were performed under BSL-4 conditions. Patient 1 showed elevated yet relatively constant serum levels for TNF-α, IFN-γ and IL-10 until day 10, when the TNF-α and IFN-γ levels increased significantly, reaching extremely high levels of 640 pg/ml for IFN-γ and 380 pg/ml for TNF-α on day 14. In contrast, in patient 2 the TNF-α and IFN-γ levels decreased during the course of illness concurrent with a significant increase in the IL-10 level.
4. Discussion

The importation of Lassa fever from Africa to other regions of the world is rare \[17–21\]. However, during 2000 four cases of imported Lassa fever occurred in Europe; two cases in Germany \[7,9\], one case in The Netherlands \[10\], and one additional case in the United Kingdom \[18\].

Lassa fever is classified as a hemorrhagic fever, but clinical diagnosis is difficult because obvious bleeding is often absent even late in the course of illness \[22\]. Furthermore, patients present with a wide range of non-specific clinical symptoms such as high fever, headache, sore throat, and diarrhea. Even in areas endemic for Lassa fever, a presumptive yet erroneous diagnosis of malaria is often made. In travelers returning from Africa, the most common causes of a febrile illness are malaria and typhoid fever. This diagnostic dilemma is well illustrated by both of our cases.

In the patients, the AST and ALT were elevated at the uncommon ratio of 10:1. A concomitant elevation of CK and LDH in serum, as well as myoglobin in urine suggests rhabdomyolysis rather than hepatocytolysis as the main cause of these enzyme elevations. This view is also supported by histological post-mortem examination of the liver of patient 1 showing only rare necrotic areas. In view of

Fig. 2. Kinetics of IFN-γ, TNF-α, and IL-10 levels. The concentration in control serum samples of healthy persons was <10 pg/ml for all three parameters tested. The Lassa virus-specific IgM titer is shown for patient 2. Patient 1 did not develop specific IgM and IgG antibodies.

Fig. 3. Measurement of the virus load in consecutive serum samples. The virus load is shown as mean (patient 1, \(n = 3\); patient 2, \(n = 5\)). Bars indicate standard deviation. The correlation coefficient of the standard curve (RNA molecules versus threshold cycle) was \(r = 0.99\).
these data, the suspicion of Lassa fever should arise when a patient presents with high fever following a recent visit to West-Africa, when malaria is ruled out, the fever persists despite antibiotic treatment, and blood cultures are negative. Laboratory data supporting this suspicion are elevated serum levels of CK, LDH, AST, and ALT as well as a high AST/ALT ratio.

In patient 1, the kinetic of the virus load roughly correlates with the clinical course of the disease. The virus load increased dramatically until day 10, when it approached a plateau phase. During this phase the clinical complications developed. In patient 2, the virus load was lower than in patient 1 and declined from day 10 to day 16 of the disease. Although the illness was generally less severe than in patient 1, there was no improvement of the clinical condition evident during this period. Ribavirin was given late in the course of illness in both patients, and at least in patient AV the drug did not obviously alter the clinical outcome, which may happen for various reasons (i.e. organ failure) during Lassa fever. Our longitudinal data on cytokine levels during Lassa fever clearly show that depending on the time of sampling, either early or late during the course of the disease, strongly divergent cytokine level can be found. There is indeed evidence that patients with a viral hemorrhagic shock may die due to a systemic inflammatory response syndrome, a condition which is characterized by expression of high levels of pro-inflammatory cytokines. In patients with Argentine hemorrhagic fever, which is also caused by an arenavirus, increased levels of TNF-α were directly related to severity of illness which was reproduced in a guinea-pig model of arenavirus infection. Recently, arenavirus-induced systemic shock and death was prevented in a mouse model by blocking the receptor for lymphotixin-β, which is a cytokine related to TNF-α. TNF-α can cause thrombocytopenia and it is known to induce endothelial damage via apoptosis. In patient 1, TNF-α reached serum levels that were comparable to concentrations causing endothelial injury in animal models. Ineffective control of virus replication in the early phase of the disease probably resulted in the high virus load. This may have triggered the expression of high levels of pro-inflammatory cytokines in the late phase, thus contributing to the severity of illness and subsequently leading to hemorrhagic shock. The lack of IgM or IgG antibody production in patient 1 may also be the result of a dysregulated and ineffective immune response.

In contrast to patient 1, decreasing levels of TNF-α and IFN-γ were measured during the course of illness in patient 2 simultaneously with a decrease in the virus load. Importantly, this patient did not die from hemorrhagic shock. This strengthens the hypothesis that an imbalance between pro- and anti-inflammatory cytokines plays an important role in Lassa fever hemorrhagic shock. The decrease in pro-inflammatory cytokines is paralleled by an increase in the anti-inflammatory cytokine IL-10 and the appearance of IgM and IgG antibodies. This further indicates an effective and correctly regulated cytokine response. This situation may be similar to that of patients with Ebola fever, where also lethal, mild or even asymptomatic infections can be observed. As had been shown for patients with asymptomatic Ebola virus infection, in whom virus replication was controlled effectively by an initial increase of IL-1β, IL-6 and TNF-α followed by a downregulation to baseline levels, the outcome of Lassa fever may also depend on an early cytokine response. In any case, neutralizing antibodies do not play a role in acute Lassa or Ebola infection, since virus and antibody may simultaneously be found in the patients’ sera for several weeks.

Taken together, the results of this study suggest that dysregulated and ineffective cytokine response, leading to high levels of virus and pro-inflammatory cytokines in the late stage of the disease, is important in the pathogenesis of hemorrhage and shock in Lassa fever. Further studies on both the cellular immune response and cytokine response in patients with Lassa fever are required to deepen our understanding of the pathophysiology of Lassa virus infection.
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