SUPPLEMENT ARTICLE

Blood Chemistry Measurements and D-Dimer Levels Associated with Fatal and Nonfatal Outcomes in Humans Infected with Sudan Ebola Virus

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Blood samples from patients infected with the Sudan species of Ebola virus (EBOV), obtained during an outbreak of disease in Uganda in 2000, were tested for a panel of analytes to evaluate their clinical condition and to compare values obtained for patients with fatal and nonfatal cases and for uninfected (hospitalized control) patients. Liver function tests showed higher levels of aspartate aminotransferase (AST) in blood samples from patients with fatal cases than in samples from patients with nonfatal cases, whereas alanine aminotransferase levels were comparable and only slightly increased in all patients, suggesting that increased blood AST levels are due to a greater degree of injury in tissues other than the liver. Significantly higher levels of amylase, urea nitrogen, and creatinine suggest that acute pancreatitis and renal dysfunction develop in fatal cases, whereas reduced albumin and calcium levels may be linked to these conditions or to liver damage. D-Dimer levels in blood specimens were drastically increased in patients with fatal and nonfatal infections but were 4 times higher in patients with fatal cases than in patients who survived (180,000 vs. 44,000 ng/mL), during the most acute period of the infection (6–8 days after onset). These results indicate that disseminated intravascular coagulation is an early and important component of EBOV disease. This study has identified levels of analytes with prognostic value, which can also be used to target therapeutic interventions, and expands on the findings of prior blood tests conducted on this group of patients.

The filoviruses, Ebola virus (EBOV) and Marburg virus, cause a severe, often fatal, hemorrhagic fever syndrome in humans [1]. Pathological studies have characterized the disease in human and nonhuman primates as a widespread/pantropic infection, with the liver and spleen being the principal target organs and exhibiting obvious pathology [2–12]. For EBOV infections in humans, the vast majority of recognized cases have been caused by Zaire EBOV (ZEBOV) and Sudan EBOV (SEBOV), with case fatality rates of ~90% and 55%, respectively [1]. Injury to the liver occurs as a result of extensive infection of hepatocytes and Kupffer cells and can be monitored by liver function assays, as has been demonstrated in a handful of human cases [7, 8, 13, 14] and a large number of experimentally infected monkeys [5, 6, 15–18]. During the acute phase of filovirus disease, serum enzyme levels reflective of liver functions are increased, most notably aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT), with AST levels typically higher than ALT levels.

Hemorrhagic manifestations are characteristic of severe and fatal filovirus infections, but bleeding is believed to be a consequence of disseminated intravascular coagulation (DIC). Although infection of endothelial cells lining blood vessels does occur, experimental infection of nonhuman primates with ZEBOV showed no direct correlation between infection and damage to the endothelium [18]. DIC, a life-threatening coagulopathy

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stemming from the activation of the procoagulant and fibrinolytic systems, can lead to hemorrhage, diffuse thrombosis, and associated end-organ damage and shock [19]. From early pathological examinations to more recent studies of experimentally infected nonhuman primates, DIC has been implicated as an important factor in the pathogenesis of filovirus diseases and is likely to be an important component of shock syndrome associated with fatal infections [2, 7, 9, 13, 18, 20]. This condition is reflected by increased concentrations of D-dimers (DDi; a product of plasmin digestion of cross-linked fibrin) in the blood, and assays targeting this fibrin degradation product are very reliable in the diagnosis of DIC [19]. Quantitative assays have been used to monitor the development of DIC in nonhuman primates experimentally infected with of ZEBOV [9, 20]; increased concentrations of DDi can appear as early as the second day after infection and increase to severely high levels as the disease progresses.

Biochemical testing of blood specimens from filovirus-infected patients has been lacking because of the hazards and logistical difficulties associated with collecting and processing blood samples in remote regions of Africa where outbreaks usually occur. Even though extensive testing has been conducted on samples from infected nonhuman primates, these findings cannot be assumed to mirror human infections, because the disease in monkeys appears to develop more rapidly and is generally more severe (ZEBOV infection is uniformly fatal) than in humans. In the fall of 2000, an opportunity to analyze human blood specimens presented itself during a large outbreak of SEBOV hemorrhagic fever in the northern region of Uganda [21]. As part of an international effort to control the epidemic and provide medical assistance, a team from the Centers for Disease Control and Prevention (CDC) established a diagnostic laboratory within the facilities of St. Mary's Lacor Hospital outside of the city of Gulu. Blood samples collected for the diagnosis of SEBOV infection were also used to assess other parameters through testing in the field and at the CDC. In an earlier study of blood samples from this group of patients, it was determined that patients with fatal cases of disease had higher virus loads, reduced numbers of T cells (total, CD8+, and activated CD8+), and extremely increased nitric oxide levels [22]. The present study extends these initial findings by determining the blood levels of a panel of general chemistry analytes and quantitation of DDi concentrations during the early, acute, and convalescent phases of disease. The results of this testing and their implications for improved prognosis and treatment of EBOV-infected patients are the focus of this article.

MATERIALS AND METHODS

Human samples. Human blood samples were obtained from confirmed SEBOV-infected and -uninfected patients during the 2000–2001 outbreak in Uganda [22]. SEBOV infections were

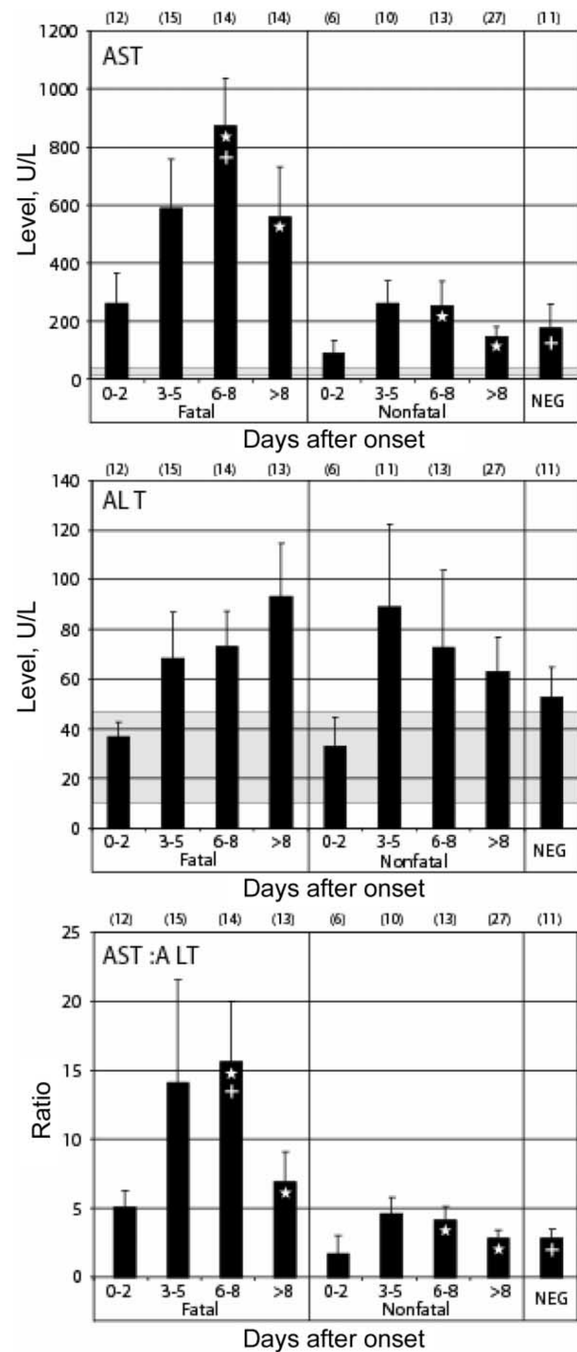

Figure 1. Blood aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) levels. Shown in the top and middle graphs are the mean levels of AST and ALT in patients with fatal or nonfatal disease caused by the Sudan species of Ebola virus, at 0–2, 3–5, 6–8, and >8 days after onset. Nos. of patients sampled (located at the top of graphs in parentheses) and SE bars are shown for each time interval. Levels in hospitalized, uninfected (NEG) patients are also shown. The horizontal gray bar on each graph indicates the normal range for each enzyme. The bottom graph shows the AST:ALT ratio (derived from average values) for each time interval. Statistically significant differences ($P \leqslant .05$) between patients with fatal and nonfatal cases for a given time interval or between infected patients and NEG patients are identified with a white star or plus sign, respectively.

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diagnosed in the field by direct detection of virus antigen and RNA in blood samples; in the case of nonfatal cases, seroconversion (specific IgG) was also determined. Samples from negative control (NEG) patients, who were initially suspected to have SEBOV infection and were hospitalized in isolation wards but who tested negative for the virus or antibody, were randomly selected as negative control samples for biochemical testing; no determinations were made as to the causes (if any) of their illnesses. Blood was collected in sodium heparin vacutainer tubes, and either whole blood or plasma was tested. Plasma was isolated by centrifugation at 200–300 *g* for 10 min in a clinical centrifuge, aliquoted into cryovials, stored in a liquid nitrogen freezer, and shipped frozen to the CDC in Atlanta.

Blood chemistry assays. The Piccolo compact clinical chemistry system (Abaxis) was used to generate rapid, multichemistry profiles through the analysis of either heparinized whole blood or plasma added to reagent discs. Tests were performed at the time of blood processing in the field laboratory operated during the outbreak. A disc containing a general chemistry panel of analytes was primarily used in testing, but a disc providing specific liver function panel was also used. Both of these discs provided measurements for AST, ALT, alkaline phosphatase (ALP), amylase (AMY), albumin (ALB), total protein (TP), and total bilirubin (TBIL), but only the general chemistry disc included blood urea nitrogen (BUN), creatinine (CRE), glucose (GLU), calcium (CA^{++}), and cholesterol (CHOL) assays.

DDi assays. A commercial kit (Aserachrom D-Di; Diagnostica Stago) was used to quantitate DDi levels in plasma samples shipped to the CDC. Briefly, the assay is performed using a 96-well plate coated with mouse monoclonal anti-human DDi $F(ab')_2$ fragments. DDi in diluted samples is captured by the immobilized antibody fragments and sandwiched with a rabbit anti–fragment D antibody coupled with peroxidase, and specific binding is colorimetrically detected by the enzymatic oxidation of *o*-phenylenediamine substrate; 200-μL reaction volumes were used throughout the assay. Color development was terminated by the addition of 0.5 volume 1 mol/L HCl to each well after 3 min of incubation at room temperature. Optical density readings were obtained using a Tecan SpectraFluor (492 nm measurement filter; 405 nm reference filter; 3 flashes). Concentrations were calculated from standard curves produced using a reagent with a known DDi level (provided with kit). Patient samples were initially tested at dilutions of 1:21 and 1:42 (following manufacturer recommendations), but further dilutions (to 1:320) were required for specimens with extremely high DDi levels.

Statistical analysis. Differences in test results observed between patients with fatal and nonfatal cases (these patients will hereafter be referred to as "fatal cases" and "nonfatal cases," respectively), as well as between infected and uninfected patients, were analyzed for statistical significance. Differences between mean values for 2 groups were tested using Student's *t* test (2-tailed), under the assumption of equal variances.

RESULTS

A total of 123 blood samples were tested, including 55 and 57 from fatal and nonfatal cases of SEBOV infection, respectively, and 11 from NEG patients. Not all measurements were performed on every blood sample. Test results for AST and ALT are shown in figure 1. Mean values for AST measurements clearly were higher (~900 U/L) in fatal cases, from early in the course of disease through the mean time of death (8 days after onset), than in nonfatal cases (~150 U/L), with statistically significant differences at 6–8 days after onset. It should be noted that the >8-days-after-onset group of fatal infections included patients who were recovering from their SEBOV infections (i.e., showed reduced or low antigen levels and seroconverted) yet nevertheless died (possibly because of disease complications), and the decrease in AST levels during this period reflects this recovery. A statistically significant difference was found in AST levels between fatal and nonfatal cases for the intervals 6–8 days and >8 days after infection, but only the data for fatal cases 6–8 days after onset were significant in comparison with data for NEG patients. In contrast, there were no statistically significant differences in ALT levels between fatal and nonfatal cases and NEG patients or between the different time intervals. Although increased from normal levels ⩾3 days after onset, ALT levels were much lower than AST levels. AST:ALT ratios ranged from ~7 to 12 across the various time intervals for fatal cases and ranged from ~2 to 4 for nonfatal cases and NEG patients. It is apparent from this testing that, in fatal cases, the increase in AST level is more dramatic than that in ALT level and also mirrors the increase in disease severity.

Figure 2 shows the results for BUN and CRE. Similar to AST, levels of the BUN and CRE analytes progressively increased in fatal cases, peaking at 6–8 days after onset at ~85 and 7 mg/dL, respectively. BUN and CRE blood levels in nonfatal cases were much lower (comparable to those in NEG patient samples), increasing to slightly above normal levels and peaking at 3–5 days after onset (~32 and ~2.5 mg/dL, respectively). The differences in BUN and CRE levels between fatal and nonfatal cases were statistically significant at 6–8 days after onset and >8 days after onset. The BUN levels in the samples from fatal cases for these 2 time intervals were statistically different from the NEG patient levels, whereas, for CRE, only the levels in fatal cases at 6–8 days after onset were statistically different from the NEG patient levels. The BUN:CRE ratios in samples from fatal and nonfatal cases were similar for each time interval and were comparable to that in the NEG patient samples.

Figure 3 shows the results of tests for ALP, AMY, ALB, TP,

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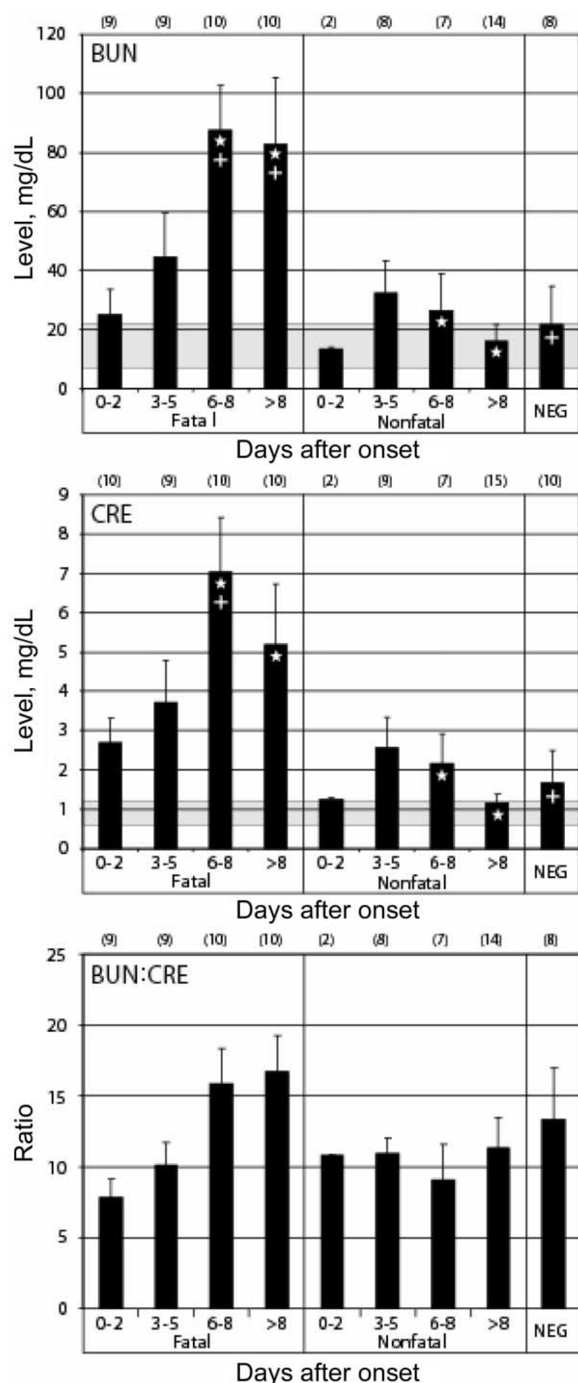

Figure 2. Blood urea nitrogen (BUN) and creatinine (CRE) levels. Shown in the top and middle graphs are the mean levels of BUN and CRE in patients with fatal or nonfatal disease caused by the Sudan species of Ebola virus, at 0–2, 3–5, 6–8, and >8 days after onset. Nos. of patients sampled (located at the top of graphs in parentheses) and SE bars are shown for each time interval. Levels for hospitalized, uninfected (NEG) patients are also shown. The horizontal grey bar on each graph indicates the normal range for each analyte. The bottom graph shows the BUN:CRE ratio (derived from average values) for each time interval. Statistically significant differences ($P \leqslant .05$) between patients with fatal and nonfatal cases for a given time interval or between infected patients and NEG patients are identified with a white star or plus sign, respectively.

GLU, TBIL, CA^{++}, and CHOL. Of these, only the AMY, ALB, CA^{++}, and CHOL tests showed significant differences between fatal and nonfatal cases. For the AMY and ALB measurements (increased and below normal, respectively), only the values 6–8 days after onset were found to be significantly different. CA^{++} levels in fatal cases were depressed at all time points, decreasing to <5 mg/dL at 6–8 days after onset, and significant differences were found for the intervals 6–8 and >8 days after onset when fatal cases were compared with nonfatal cases and NEG patients. TP and TBIL levels were generally ∼2–3 times higher in fatal cases than in nonfatal cases, but these variances did not reach statistical significance. GLU levels tended to be lower in fatal cases than in nonfatal cases and NEG patients, but the values were not statistically significant. TP, GLU, TBIL, and CHOL measurements showed little or no deviation from normal values, and the only statistical difference noted was in CHOL levels, between fatal and nonfatal cases at 6–8 days after onset. Although the mean ALP levels in fatal cases were increased, reaching ∼3.5 times the maximum normal level at 6–8 days after onset, the peak level fell short of the statistical cutoff in comparison with the levels in nonfatal cases 6–8 days after onset and levels in NEG patients ($P = .092$ and .068, respectively).

Figure 4 shows the results of DDi measurements and clearly indicates increased DDi concentrations in SEBOV-infected patients, with fatal cases having much higher levels during the most acute period (peaking at 6–8 days after onset). Statistical comparisons between fatal and nonfatal cases showed a significant difference only at 6–8 days after onset. However, compared with measurements in samples from NEG patients, the means in samples from fatal cases at 3–5, 6–8, and >8 days after onset all showed significant differences, as did those in samples from nonfatal cases 3–5 days after onset. At peak periods for DDi in fatal (6–8 days after onset) and nonfatal (3–5 days after onset) cases, values were, respectively, ∼450- and ∼180-fold higher than the normal upper limit (400 ng/mL).

DISCUSSION

Our results indicate acute involvement of the liver (hepatobiliary system) in SEBOV infections. These findings are consistent with previous studies of humans and experimentally infected monkeys. Although ALT levels were increased over normal levels after 2 days after onset, this increase was not statistically significant and did not differ appreciably between fatal and nonfatal cases or NEG patients (figure 1). Similar to ALT, only modest increases were seen in levels of other markers of hepatobiliary dysfunction, ALP and TBIL, and gluconeogenesis and CHOL metabolism were largely maintained (figure 3). A similar observation was made after tests performed on a single patient infected with the Cote d'Ivoire species of EBOV [13]. In contrast, AST levels were consistently higher than ALT levels for all cases and were dramatically higher (∼2–3 times

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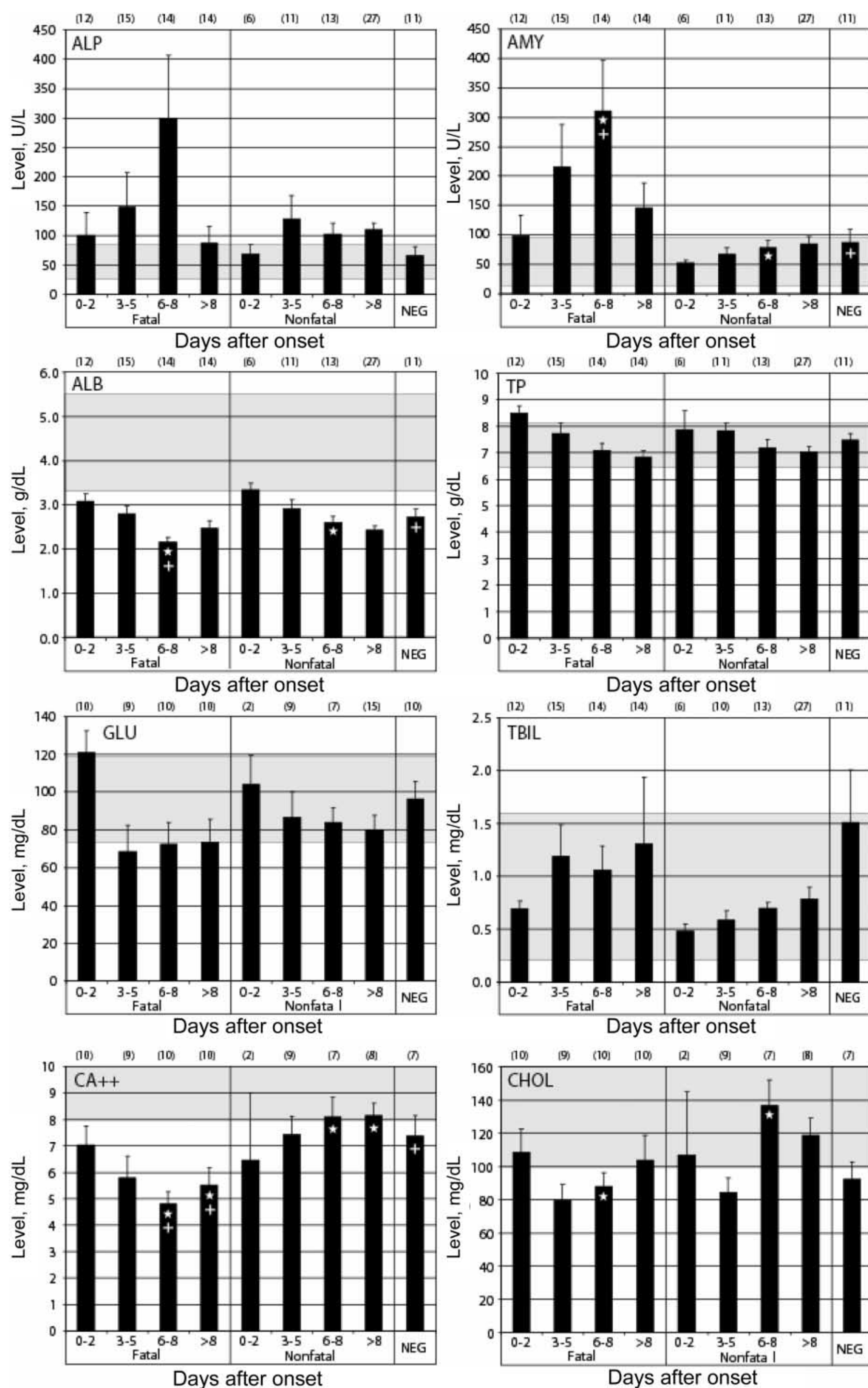

Figure 3. Levels of various blood analytes. Graphs show the mean levels of alkaline phosphatase (ALP), amylase (AMY), albumin (ALB), total protein (TP), glucose (GLU), total bilirubin (TBIL), calcium (CA^{++}), and cholesterol (CHOL) in patients with fatal or nonfatal disease caused by the Sudan species of Ebola virus, at 0–2, 3–5, 6–8, and >8 days after onset. Nos. of patients sampled (located at the top of graphs in parenthesis) and SE bars are shown for each time interval. Levels for hospitalized, uninfected (NEG) patients are also shown. The horizontal gray bar on each graph indicates the normal range for each analyte. Statistically significant differences ($P \leqslant .05$) between patients with fatal and nonfatal cases for a given time interval or between infected patients and NEG patients are identified with a white star or plus sign, respectively.

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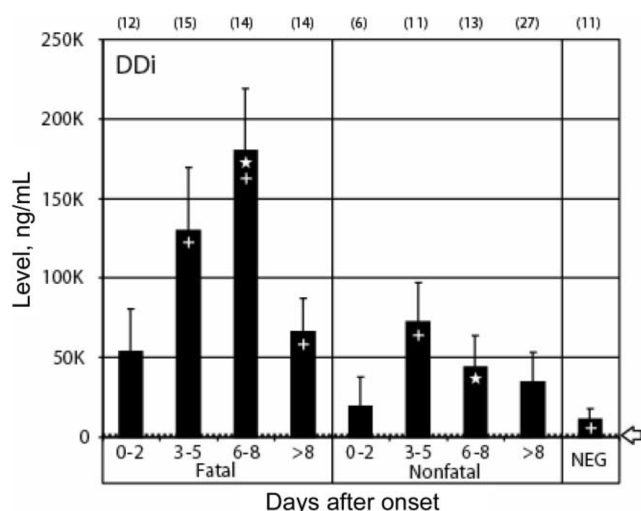

Figure 4. D-Dimer (DDi) levels in plasma samples. Shown in the graph are the mean levels of DDi in patients with fatal or nonfatal disease caused by the Sudan species of Ebola virus, at 0–2, 3–5, 6–8, and >8 days after onset. Nos. of patients sampled (located at the top of graphs in parentheses) and SE bars are shown for each time interval. The upper limit for a "normal" DDi level (⩽400 ng/mL) is shown as a horizontal dashed line with an arrow along the right margin. Statistically significant differences ($P \leqslant .05$) between patients with fatal and nonfatal cases for a given time interval or between infected patients and hospitalized, uninfected (NEG) patients are identified with a white star or plus sign, respectively.

higher from 3 to >8 days after onset) in fatal cases than in nonfatal cases. This difference, as seen in the AST:ALT ratios (figure 1), suggests that tissue damage is not confined to the liver. Because AST is also present in red blood cells, muscle, kidney, and pancreas, it is reasonable to conclude that the increased level of this enzyme in fatal cases is due to its release into the blood from ⩾1 of these tissues/cells. This release could be a direct result of virus infection (and injury) and/or an indirect consequence of septic shock and cell necrosis [22, 23].

Blood AMY levels in fatal cases were significantly increased, peaking at 6–8 days after onset (3 times the normal level), whereas samples from nonfatal cases and from NEG patients were normal (figure 3). Possible causes of this included pancreatitis, ileus or duodenal disease, salivary gland inflammation, and decreased renal clearance. Parotitis was not noted clinically in of any of the patients, although SEBOV has been isolated from saliva [24]. In contrast, the typical symptoms of pancreatitis—fever, epigastric pain, shortness of breath, anorexia, nausea, and vomiting—are characteristic of EBOV infection. The increase in ALP levels might reflect pancreatic inflammation and obstruction of the pancreatic duct. Pancreatitis could be either a cause or a consequence of shock. Measurement of lipase levels and fractionated amylase and bilirubin levels in future studies could help to clarify this aspect of the pathogenesis.

Possible causes for the low ALB levels noted include impaired hepatic ALB production; decreased food intake, perhaps exacerbated by premorbid malnutrition, during acute disease when patients were almost uniformly anorexic (nasogastric or parentaral feedings were not possible); loss of ALB due to increased capillary permeability and third spacing; and urinary loss of ALB. Interestingly, TP levels were generally maintained in the normal range, but concentration of protein by dehydration could influence this measurement; the same observation of low ALB levels and normal TP levels was made in studies of ZEBOV-infected nonhuman primates [18]. Peripheral soft-tissue edema was rarely noted clinically, although significant intra-abdominal third spacing may be difficult to detect. Significant amounts of protein were rarely noted in urine dipstick tests performed on a small subset of patients with SEBOV infection (data not shown), but most urine samples were collected after the acute disease had resolved. Regardless of the cause, the loss of colloidal pressure from hypoalbuminemia likely contributes to the hypotension and shock of EBOV infection. The abnormally low levels of CA^{++} in blood from fatal cases at 6–8 and >8 days after onset mirror the hypoalbuminemia seen during these periods and probably reflect decreased carrying capacity; free calcium was not measured.

SEBOV-infected patients also had impairment of renal function, as demonstrated by elevated BUN and CRE levels (figure 2). As was seen with AST, blood samples from fatal cases had higher BUN and CRE levels than did samples from nonfatal cases (at all corresponding time periods) and NEG patient samples, with a significant increase at the most acute phase of the infection (6–8 days after onset) that extended to >8 days after onset. Perhaps the obvious explanation for this is decreased renal perfusion due to a general reduction in circulating volume resulting from blood loss, third spacing, vomiting, diarrhea, fever, and insensible losses. However, acute renal failure from a direct effect of SEBOV infection on the kidney or the presence of microvascular thrombi cannot be ruled out, especially given the relatively low BUN:CRE ratio (<20:1). Gastrointestinal bleeding and impaired liver function may complicate interpretation of this ratio.

As has been noted with other tests, results of DDi quantitation indicated that this breakdown product of fibrin was increased far beyond normal levels (figure 4). At the peak of infection (6–8 days after onset), fatal cases had ~4- and ~16-fold higher levels than did nonfatal cases and NEG patients, respectively. This finding is, to our knowledge, the first conclusive evidence for the presence of DIC in patients with EBOV infection, and, given the serious consequences of this condition, DIC has to be viewed as a critical factor in mortality. In addition to the heart, lungs, liver, and central nervous system, the kidneys may also be organs at a high risk for forming microvascular thombi in DIC, which could contribute significantly to the renal dysfunction noted in acutely infected patients. The highest con-

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centration of DDi was ~520,000 ng/mL, in a fatal case 5 days after onset, but several nonfatal cases were found to have very high concentrations, in the range of ~240,000–390,000 ng/mL. This observation suggests that DIC by itself does not lead to a fatal outcome in SEBOV-infected patients but acts as a compounding factor in the development of a dangerously imbalanced physiological state. In a therapeutic study of ZEBOV-infected rhesus monkeys [20], high levels of DDi were also detected in a control animal (~140,000 ng/mL on day of death), whereas surviving animals showed a delayed production and lower lever of DDi (peak of ~100,000 ng/mL), which is consistent with our observations in humans.

In an earlier study, we demonstrated that higher virus loads, increased nitric oxide levels, and decreased T cell numbers correlated with fatal SEBOV infections [22]. From our results, we have now shown that abnormal levels of a number of analytes are also associated with fatal outcomes. Table 1 shows the concentrations of these analytes that can be viewed as indicators of increase disease severity and fatal outcome. We arbitrarily selected these values on the basis of our test results (figures 1–4) and believe that they can be useful in disease prognosis and management of patients with SEBOV hemorrhagic fever and infections caused by other filoviruses.

Despite basic differences in their genesis, the shock syndrome associated with fatal EBOV infection is very similar to septic shock in the development of not only DIC but also extensive lymphocyte apoptosis, a proinflammatory cytokine burst, and refractive hypotension [25, 26]. Patients with septic shock can be treated for this condition, and the same should be true for severe EBOV infections. With therapy in mind, the early appearance of DDi in the acute phase (0–2 days after onset) may warrant looking at DIC as the first target for devising and implementing intervention therapies in the treatment of infections caused by EBOV. Given the current inability to eliminate the underlying condition of DIC (EBOV infection), the only therapeutic option is to try to prevent the formation of thrombi. Although controversial, the use of heparin, activated protein C, inhibitors of the tissue factor pathway, antifibrinolytic agents, thrombin inhibitors, or other compounds that have been promising in the treatment of DIC [27] could result in reduced mortality when applied to the treatment of EBOV infections. Preliminary use of a recombinant inhibitor of factor VIIa/tissue factor to treat rhesus monkeys experimentally infected with ZEBOV has resulted in the survival of some animals [20]. The observed azotemia in fatal SEBOV infections (elevated BUN and CRE levels) suggests that renal dysfunction is an important feature of the disease. The dangerous accumulation of toxic compounds in the blood as a result of this condition can be lessened by hemodialysis or peritoneal dialysis. This type of therapy has not been attempted in outbreak situations, largely because of a lack of suitable medical infrastructures, but could be helpful in relieving the burden of acute renal failure.

Table 1. Analyte levels associated with severe disease and fatal outcomes in patients infected with the Sudan species of Ebola virus.

Analyte	Level associated with fatal disease
AST	>400 U/L
BUN	>60 mg/dL
CRE	>5 mg/dL
ALP	>200 U/L
AMY	>150 U/L
ALB	<2.5 g/dL
CA^{++}	<6 mg/dL
DDi	>100,000 ng/mL

NOTE. ALB, albumin; ALP, alkaline phosphatase; AMY, amylase; AST, aspartate aminotransaminase; BUN, blood urea nitrogen; CA^{++}, calcium; CRE, creatinine; DDi, D-dimer.

The 2000 SEBOV outbreak in Uganda, the largest episode of filovirus disease on record, provided a rare opportunity to obtain blood chemistry data and to relate that information to disease progression and outcome. Biochemical assays for a panel of analytes provided important insights into the condition of patients and helped to identify abnormalities associated with fatal outcomes that can be used to gauge disease severity. We have shown that, during the acute phase of fatal infections, injury to the liver is evident (although not dramatically affected), that acute pancreatitis may develop, that renal function is compromised, and that DIC is a prominent feature of the pathological process. These conditions contribute to a very dangerous physiological imbalance and toxicity in severe cases that lead to multiorgan failure, shock, and ultimately death. Therapeutic interventions for some of these conditions are available, and their use should be considered in the management of patients with filovirus hemorrhagic fevers.

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