Cytokine and Chemokine Expression in Humans Infected with Sudan Ebola Virus

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The size and duration of the 2000 outbreak of Sudan Ebola virus (SEBOV) infection in Uganda made it possible to collect serial serum samples from 87 patients (53 survivors and 34 nonsurvivors). Surprisingly, the levels of tumor necrosis factor–α and interferon (IFN)–γ, which had been found to be increased in patients with fatal Zaire Ebola virus infection, were not increased in any of the patients with SEBOV infection. The levels of interleukin (IL)–1β, IFN-γ–inducible protein–10, and RANTES (regulated on activation, normally T cell–expressed and –secreted) were higher in samples from all patients with SEBOV infection than in control samples from healthy hospital staff members, but their levels did not differ between those who survived and those who did not. The levels of IFN-α were significantly higher in surviving patients with SEBOV infection, whereas the levels of IL-6, IL-8, IL-10, and macrophage inflammatory protein–1β were higher in patients with fatal SEBOV infections.

Ebola virus (EBOV) causes the most severe form of viral hemorrhagic fever in humans and nonhuman primates. The requirement of maximum-containment laboratories for EBOV research and the remoteness and lack of proper facilities of the outbreak locations have made opportunities to study the pathogenesis of EBOV infections limited. Although the pathogenesis of this disease is poorly understood, fatalities are predominantly associated with uncontrolled viremia, lack of an effective immune response (low levels or no virus-specific antibodies, antibodies that are produced are non-neutralizing, and no cellular infiltrates at sites of infection), hemorrhage, and circulatory shock [1, 2]. The limited tissue damage that does occur in the blood vessels would not appear to be sufficient to explain the circulatory shock, indicating that soluble mediators certainly play an important role in disease progression.

The human epidemics have been associated with infection by 2 species of EBOV: Zaire (ZEBOV) and Sudan (SEBOV). Most of the information available for human infections has been obtained from studies done during ZEBOV infection outbreaks. SEBOV was associated with outbreaks in 1976 and 1979 in the southern Sudan towns of Nzara and Maridi; in 2000 in Gulu, Uganda; and in 2004 in Yambio, Sudan [3–5]. Although the diseases caused by the 2 species of EBOV are very similar, the lower fatality rate (∼50% for SEBOV infection vs. ∼80% for ZEBOV infection) and the slightly longer progression to death (8 days for SEBOV infection vs. 6 days for ZEBOV infection) indicate that their pathogenesis may be different. In previous studies, fatal human infections with ZEBOV were associated with an increase in the circulating levels of tumor necrosis factor (TNF)–α, interferon (IFN)–α, interleukin (IL)–2, IL-10, [6], and IFN-γ [6–8]. The lack of substantial viral damage to the endothelium has led many to speculate that the high levels of these inflammatory cytokines lead to the development of circulatory shock seen in fatal EBOV infections [9–11].

In October 2000, the third and largest outbreak of SEBOV infection occurred in the Gulu district of Uganda [4]. During the course of the outbreak (August
samples were inactivated by being stored in a liquid nitrogen freezer. Before thawing, the serum samples were thawed and separated into multiple aliquots. Initial sample processing was performed in a laminar-flow biosafety cabinet made available in the laboratory from the hospital. Samples were stored in a liquid nitrogen freezer. Before thawing, the serum samples were inactivated by γ irradiation (5 × 10⁸ rad) on dry ice. This dosage was determined to have little or no effect on the detection or biological activity of highly labile cytokines such as IFN-γ and TNF-α [12].

Cytokine and chemokine assays. With the exception of IFN-α (described below), cytokine and chemokine levels in serum samples were measured using a flow-cytometric–based assay developed in our laboratory, as described elsewhere [13]. Antibodies against the cytokines and chemokines were each coupled to a single set of carboxylated microspheres, using a 2-step carbodiimide coupling procedure. Microsphere sets were mixed so that they would all be at approximately the same concentration. Two hundred microspheres were analyzed for each test. Samples were analyzed on a Luminex 100 analyzer (Luminex). Serial 3-fold dilutions of human recombinant cytokines and chemokines were run on each plate to produce standard curves. For statistical analyses, samples were grouped into 4-day intervals beginning with the onset of clinical symptoms. Data were analyzed using PRISM analysis software (version 3; GraphPad). Statistical significance was determined by use of a 2-tailed t test.

IFN-α assays. Monoclonal and a rabbit polyclonal antibodies against IFN-α were obtained from PBL Biomedical Laboratories and were screened in pairs, both as capture antibodies coupled to microspheres and as biotinylated detector antibodies, to determine which 2 would perform best in the bead assay. In these experiments, mouse monoclonal antibody MMHA-1 was used as the capture antibody, and MMHA-2 was biotinylated and used for detection. This antibody pair was chosen because it gave the highest signal-to-noise ratio and the best sensitivity and was accurate over the widest range of concentrations. Serum samples used in the above cytokine assays were thawed and tested for IFN-α as described above.

Antibody purification. Before the IFN-α antibodies could be biotinylated, they first had to be purified to remove any extraneous proteins. The antibodies were purified on Immobilized Protein A/G affinity columns (Pierce) in accordance with the manufacturer’s recommendations. The antibodies were eluted from the columns in 0.5- to 1.0-mL fractions, absorbance at 280 nm was used to detect the fractions containing the concentrated antibodies, and these fractions were pooled. The pooled fractions were neutralized by dialysis in PBS (pH 7.2), using Slide-A-Lyzer dialysis cassettes (Pierce) with a molecular weight (MW) cutoff of 10,000 kDa.

Antibody labeling. Antibodies were biotinylated using EZ-Link Sulfo-NHS-LC-Biotinylation kits (Pierce). Immediately before using 2 mg of Sulfo-NHS-LC-Biotin (MW, 556) were dissolved in 200 μL of ultrapure water. An ~20-fold molar excess of Sulfo-NHS-LC-Biotin was added to the purified antibody. The tubes were incubated at room temperature for 30–60 min. The excess reagent was removed by dialysis in PBS (pH 7.2), as described above.

RESULTS

In these studies, we limited our analyses to the 254 patients from whom serial serum samples were available. To perform statistical analyses, the samples were grouped into 4-day intervals: interval 1 consisted of samples collected from the onset of symptoms to 3 days after onset, interval 2 consisted of samples collected 4–7 days after onset, interval 3 consisted of samples collected 8–11 days after onset, and interval 4 consisted of samples collected 12–15 days after onset. The number of samples for surviving and nonsurviving patients with EBOV infection in each interval is shown in table 1. In addition to the patient samples, serum samples from 15 uninfected hospital staff members were examined to determine the background or control levels for each cytokine and chemokine.

High levels of TNF-α have also been associated with fatal human ZEBOV infections in the past [6, 7]. There was no evidence of increased production of TNF-α in EBOV-infected patients.

| Table 1. The nos. and intervals of patient samples tested for cytokines and chemokines. |
|---------------------------------|------------------|------------------|------------------|------------------|
| Patient group                  | Interval 1       | Interval 2       | Interval 3       | Interval 4       |
| Survivors                      | 16 (15)          | 53 (42)          | 47 (30)          | 31 (16)          |
| Nonsurvivors                   | 22 (13)          | 59 (17)          | 18 (12)          | 8 (5)            |

NOTE. In addition to the patient samples, serum samples from 15 uninfected hospital staff members were tested to determine baseline levels.
Cytokine Secretion in Humans Infected with SEBOV

Figure 1. Kinetic analysis of proinflammatory cytokines tumor necrosis factor (TNF-α) (A) and interferon (IFN-γ) (B) in surviving and nonsurviving patients with Sudan Ebola virus infection. Control levels are from serum samples collected from hospital staff members and are shown for baseline comparisons only. Samples were grouped into 4-day intervals: interval 1, day of onset of symptoms to day 3; interval 2, days 4–7; interval 3, days 8–11; interval 4, days 12–15. Statistical analyses of comparisons between surviving and nonsurviving patients were performed using a 2-tailed t test.

The serum levels of IFN-γ, another inflammatory cytokine that has been implicated as a potential mediator of the shock seen in fatal ZEBOV infections [6, 7], were not increased during infection with SEBOV. At no point during the study period (from the day of onset of symptoms to day 15 after onset) were the levels of IFN-γ detected in patients with EBOV infection, outside of the range seen in uninfected control subjects, and, as was the case for TNF-α, the levels were undetectable in the majority of patient samples (figure 1B). In addition to TNF-α and IFN-γ, levels of granulocyte-macrophage colony-stimulating factor, IL-4, IL-2, and macrophage inflammatory protein (MIP)–1α in patients with EBOV infection, regardless of the outcome, were similar to those seen in the control serum samples (data not shown).

Levels of RANTES, a chemokine involved in chemotaxis of lymphocytes, monocytes, and eosinophils, were significantly higher in patients with SEBOV infection, throughout the study period, than in the control serum samples. The levels were similar in surviving and nonsurviving patients (figure 2A). Similar results were seen for IFN-γ–inducible protein (IP)–10 (figure 2B). IL-1β was undetectable in any of the control serum samples, whereas the level in patients with EBOV infection ranged between 10 pg/mL and 300 pg/mL, beginning at the onset of clinical illness and remaining relatively constant throughout the study period (figure 2C).

The circulating levels of the cytokines IL-6 and IL-10 and of

Figure 2. Kinetic analysis of cytokines and chemokines that are increased by Sudan Ebola virus (EBOV) infection. Control levels are from serum samples collected from hospital staff members and are shown for baseline comparisons only. Interleukin (IL)–1β was not detected in any control serum samples. Samples were grouped into 4-day intervals: interval 1, day of onset of symptoms to day 3; interval 2, days 4–7; interval 3, days 8–11; interval 4, days 12–15. Statistical analyses were performed using a 2-tailed t test. P values are for the comparison of patients with EBOV infection (surviving and nonsurviving) vs. control subjects: *P < .05; ***P < .0005. IP, IFN-γ–inducible protein.
the chemokines MIP-1β and IL-8 were all significantly higher in nonsurviving patients with EBOV infection than in either surviving patients or uninfected control subjects. What is most notable is that levels of all 4 are significantly higher at the onset of illness and, with the exception of IL-8, remain so through the peak of illness, defined as the median time to death (figure 3).

Because IFN-α has been shown to have a protective role in the mouse model of EBOV infections and can delay the time to death in EBOV-infected primate, we extended our bead-based assays to include IFN-α. Aliquots of the samples tested for the cytokines and chemokines discussed above were thawed and tested for the presence of IFN-α. The circulating levels of IFN-α were significantly higher in the serum samples obtained from surviving patients with EBOV infection very early during the clinical disease (interval 1; onset through day 3 after onset). The levels of IFN-α in surviving patients remained high throughout the study period. The levels of IFN-α in nonsurviving patients with EBOV infection were similar to those in uninfected control subjects during interval 1, increased to levels similar to those in surviving patients with EBOV infection during interval 2 (days 4–7 after onset), and decreased back to control levels during interval 3 (days 8–11 after onset) (figure 4).

DISCUSSION

The re-emergence of SEBOV, after a 20-year absence, in August 2000 resulted in the largest EBOV infection epidemic ever recorded. The initial outbreak was centered in the Gulu district of northern Uganda and was subsequently spread by infected individuals to 2 of the country's southern districts. There were a total of 425 cases recorded, of which 224 were fatal infections (case fatality rate, 53%). A field laboratory was established at a local hospital in rural northern Uganda in an attempt to rapidly identify acute SEBOV infection cases. The availability of these samples gave us the rare opportunity to examine cytokine/chemokine expression during the course of human EBOV infections. In this study, we limited our analysis to 87 patients (53 survivors and 34 nonsurvivors) from whom serial samples were available. In addition to the patient samples, 15 samples collected from uninfected hospital staff members were analyzed to obtain control values. In total, 269 samples were used in the cytokine/chemokine analysis reported here.

The ZEBOV and SEBOV species of EBOV cause severe hemorrhagic fever in humans. The infections differ mainly in their progression rate, severity, and mortality rate. The initial symptoms of EBOV infection are nonspecific (i.e., fever and headache) but rapidly develop into increased vascular permeability, hypotension, coagulation disorders, and hemorrhages that often progress to multiorgan failure and death. Fatal EBOV infections have been associated with uncontrolled viremia, a lack of inflammatory cells at the sites of viral infection, a lack of or delayed humoral immune response [7, 14, 15], a significant increase in the numbers of circulating neutrophils, and a de-
levels of IL-6, IL-10, IL-8, and with SEBOV infection, indicating that they did have an inflam-

crease in the numbers of lymphocytes, which is believed to be caused by a massive apoptosis [7, 16–18].

The release of proinflammatory cytokines, chemokines, and nitric oxide has been proposed to be a determining factor of the severity of the hemorrhagic shock and is inversely correlated with survival [10, 18–21]. In this study, we were surprised to find that the levels of TNF-α, IFN-γ, and IL-2 were not increased in the patients with fatal SEBOV infection, compared with those in surviving patients. In fact, the levels detected in both patient groups predominantly fell within the range detected in non–EBOV-infected control subjects. These results are not consistent with the hypothesis that the hemorrhagic shock in patients with SEBOV infection is a direct result of the circulating levels of proinflammatory cytokines, as has previously been suggested. Instead, it is possible that the increased levels of inflammatory cytokines seen in dying patients with ZEBOV infection exacerbates the symptoms, causing a more rapid progression of the infection leading to shock and death; their absence in dying patients with SEBOV infection may partially explain the longer mean time to death observed in these patients.

IL-1β, IP-10, and RANTES levels were higher in the patients with SEBOV infection, indicating that they did have an inflammatory response, but the levels were similar among surviving and nonsurviving patients. The levels of IL-6, IL-10, IL-8, and MIP-1β were significantly higher in nonsurvivors, and this was apparent during the first few days of clinical illness. Higher levels of IL-10 have also been seen in patients with fatal ZEBOV infection [6, 22] and could be involved in maintaining the immune suppression. One of the primary sites for IL-6 pro-
duction is the liver; therefore, the high levels of IL-6 seen may reflect the amount of viral replication occurring in the liver [17]. IL-6 is involved in the induction of the acute-phase response, and, therefore, the high levels seen in dying patients may be influencing vascular permeability; it also acts on the pituitary gland to increase corticosteroid production, so, like IL-10, it could be involved in immune suppression. Our results differ from those of a similar study of serial plasma samples collected from patients with fatal and nonfatal ZEBOV infection. In that study, IL-6 levels were significantly higher in survivors than in nonsurvivors, and chemokines, IL-8, and MIP-1β were not detectable in any of their samples [22].

Surviving patients with SEBOV infection had significantly higher levels of IFN-α within the first few days of the onset of clinical illness. To our knowledge, this is the first report showing a potential protective effect of IFN-α in human patients with EBOV infection. Further evidence for the importance of IFN-α in surviving EBOV infection can be found in both the mouse model of ZEBOV infection [23, 24] and the nonhuman primate model in which a single high dose of IFN-α given 18 h after infection was able to delay death 1–2 days [25]. IFN-α is essential for both antiviral defense and the activation of the innate immune response. Two roles of IFN-α are the activation of NK cells and the maturation of dendritic cells (DCs) into cells that present antigen to and activate naïve T cells.

Previous data from human infections have been limited, but there is evidence from previous ZEBOV infection outbreaks that suggests that differences in the innate immune response determine whether the outcome will be favorable. Surviving ZEBOV infection was associated with a very early and transient appearance of proinflammatory cytokines, whereas the appearance of these cytokines was delayed in dying patients, and their levels continued to increase until death. In addition to having increasing concentrations of proinflammatory cytokines, dying patients also had higher concentrations of the anti-inflammatory cytokine IL-10 [6, 7, 22]. This early strong proinflammatory cytokine response facilitates the induction of an adaptive response, and survival has been associated with the appearance of specific IgG during the second week of illness [7, 26].

The initial sites of EBOV infection and replication are the macrophages and DCs [17, 11]. In vitro studies using primary human myeloid DCs (mDCs) and macrophages have shown that infected cells fail to produce IFN-α and that mDCs failed to mature and were incapable of activating T cells [19, 27–29].

The EBOV VP35 and VP24 genes inhibit the production of type I IFN in infected cells and block the response to exogenous IFN [30–32]. It is possible that EBOV has other proteins that are involved in the evasion of the host’s antiviral response, because infection of human liver cells by ZEBOV, Reston EBOV, or Marburg virus all caused the down-regulation of many acute-
phase response, complement, and coagulation-related genes. The down-regulation of the type I IFN response was similar in cells infected with ZEBOV and Marburg virus, but Reston EBOV was impaired in its ability to regulate the IFN response pathway, which could explain its attenuation in humans [33].

The pathogenesis of EBOV infections is multifaceted and complex, and certainly much more work is needed before we can comprehend the mechanisms involved. The results of this study show several differences in circulating cytokine and chemokine expression between patients with ZEBOV infection and patients with SEBOV infection. The amount of information on patients with EBOV infection as a whole is limited, and this is an even greater problem concerning human SEBOV infections. Do the differences presented here indicate differences in pathogenesis between these 2 EBOV species? Currently, there is not enough information to answer this question; therefore, caution should be used before making generalizations.

Although the primate model shares many characteristics of human infections, it does have some major differences. The illness seen in nonhuman primates is more severe than that seen in humans, and the majority of research experiments have used ZEBOV, which causes a much more rapid and uniformly fatal infection than do other EBOV species. The information on SEBOV infections of nonhuman primates is extremely limited, but there is some indication that a small percentage of infected animals can survive and that a specific immune response is detectable before death [34]. Further investigations in nonhuman primates are necessary to confirm the results observed here in human patients and to identify and understand the role of the immune response in the patients’ outcomes.

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